Utilization of high pressure processing for the production of starch-based hydrogels for innovative applications

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UTILIZATION OF HIGH PRESSURE PROCESSING FOR THE PRODUCTION OF STARCH-BASED HYDROGELS FOR INNOVATIVE APPLICATIONS

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- **Dominique Larrea-Wachtendorff**, Gino Di Nobile, Giovanna Ferrari. (2020). Effects of processing conditions and glycerol concentration on rheological and texture properties of HHP starch-based hydrogels. International Journal of Biological Macromolecules, 159, 590-597. https://doi.org/10.1016/j.ijbiomac.2020.05.120

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- **Larrea-Wachtendorff D.,** Pulgarin M., Ferrari G. (2019). Starch-based hydrogels produced by high hydrostatic pressure (HHP): Evaluation of physical and microbiological stability. Proceedings of 2019 IFT/EFFoST International Nonthermal Processing Workshop & Short courses, November 3 - 6, 2019, Monterrey, Mexico.

- **Larrea-Wachtendorff D.,** Ferrari G. (2019). Starch-based hydrogels produced by high hydrostatic pressure (HHP): Effect of processing time and type of starch. Proceedings of 2nd Innovations in Food Science & Technology International Conference, June 25 - 27, 2019, Amsterdam, Netherlands.

- Di Nobile G., **Larrea-Wachtendorff D.,** Ferrari G. (2018). Rheological and structural characterization of HHP hydrogels based on rice or corn starch in presence of glycerol and lycopene. Proceedings of 32nd EFFoST International Conference, November 6 - 8, 2018, Nantes, France.

- **Larrea-Wachtendorff D.,** Tabilo-Munizaga G., Ferrari G. (2018). Potato starch hydrogels produced by high hydrostatic pressure: A first approach. Proceedings of the IFT-EFFoST 2018 International Nonthermal Processing Workshop and Short Course, September 25 - 27, 2018, Sorrento, Italy (p. 92).

- **Larrea-Wachtendorff D.,** Tabilo-Munizaga G., Ferrari G. (2018). Effect of type and particle size of different native starches on the structural and mechanical properties of hydrogels produced by HHP. Proceedings of the IFT-EFFoST 2018 International Nonthermal Processing Workshop and Short Course, September 25 - 27, 2018, Sorrento, Italy (p. 150).

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Abstract

Nowadays, the development of plant-based systems to replace or reduce the utilization of synthetic materials has been receiving significant attention, in view to fulfil consumers demand natural products in all the industrial areas.

Hydrogels represent a group of polymeric materials, composed of three-dimensional crosslinked polymeric networks, capable to absorb and retain a significant amount of water. They have been listed as “smart structures” whose tailor-made design confers them different functional attributes allowing their use in biomedical, cosmeceutical, pharmaceutical and food applications. Hydrogels can be produced either from natural or synthetic sources. However, those produced from natural sources have gained a great interest in research for the development of novel biomaterials for which a wide range of applications could be envisaged due to their safety, biocompatibility, and biodegradability. In the last decade, among the natural sources to produce hydrogels, starches have been receiving increasing attention as one of the most promising natural biopolymers. Hydrogels are traditionally produced by chemical or physical methods. However, long processing time, high energy consumption, and safety issues related to the synthesis of these products have been identified as important limitations of these methods.

Therefore, to overcome these drawbacks, in this work has been proposed the utilization of high pressure processing (HPP), which has been shown as a promising and suitable technology to obtain the gelatinization of starch suspended in aqueous media in less harsh processing conditions than in conventional methods. HPP treatments consist of subjecting products packaged in flexible containers to a high level of isostatic pressures (100-1000 MPa) in a short period of time (minutes). HPP has been established as a commercially viable food preservation method allowing minimal sensory and nutritional damages of processed products. It is well known that HHP causes the disordering of biopolymers, including proteins and starches, due to the modifications of non-covalent intermolecular interactions such as those inducing pressure-assisted gelatinization. Although the use of HPP has shown evidence in inducing pressure-assisted gelation, there was still a strong need to increase the knowledge on the topic not only to better understand the fundamentals of the gelation process but also to highlight the main factors

affecting the production of starch-based hydrogels by high pressure. Moreover, the structural and mechanical properties of the starch-based hydrogels obtained by HPP as well as their performance should be deeply investigated in view of their future exploitation.

The aim of this PhD thesis work was to propose a complete study of the utilization of HPP technology to produce starch-based hydrogels under optimized processing conditions in view of further innovative applications.

To this purpose, different native starches (rice, corn, wheat, potato, and tapioca), which are the most produced and traded natural biopolymers, have been selected as raw materials. The starches have been completely characterized in terms of physical properties, such as particle size distribution, and chemical composition. Hydrogels were produced under pressure and the effects of type of starch, formulation and processing conditions on physical-chemical characteristics were investigated. Among the properties of high pressure starch-based hydrogels, the mechanical and structural characteristics, the stability at different storage conditions, and the in vitro digestibility have been determined, also when interest compounds were added to the gelling suspension.

All the experiments were carried out in lab-scale HPP units (U111 and U22, Unipress, Poland) and several analytical methods have been used to investigate the properties of the hydrogels, namely proximate analysis (AOAC guidelines), laser scattering, light microscopy, Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), controlled stress and strain rheometer, texturometer, colourimeter, microbiological counts techniques, water activity meter, among others.

The results obtained highlighted the potential use of HPP to produce stable starch-based hydrogels in processing conditions less severe than those utilized in conventional methods. Starch-based HPP hydrogels displayed remarkable mechanical and structural properties even superior to those produced by thermal treatments. Moreover, it was identified different energy requirements of the starches to produce starch-based hydrogels under high-pressure conditions. HPP hydrogels based on corn, rice, wheat and tapioca starch were obtained at 600 MPa for >5 min, while stable potato starch hydrogels were obtained only utilizing the mass fraction with granule size lower than <25 μm and the synergistic effects of pressure and heat treatments at moderate temperature, namely pressure at 600 MPa for 15 min temperature at 50 °C. Rice, wheat and corn HHP-hydrogels showed a cream-like structure while those based on tapioca and potato starch were more compact. With increasing HPP processing time up to 15 min, more stiff tapioca and rice HHP-hydrogels were produced showing higher viscosity, G' values and firmness with respect to those obtained by applying the pressure for reduced residence time. Moreover, a marked influence of starches PSD has been detected. Utilizing starches with small granules size facilitates swelling and gelatinization processes under pressure due to the small particles are more exposed to the action of pressure than bigger particles, but also, being the number of particles per unit volume increased the starch-starch and starch-water interactions are favoured.

Physical and microbiological stability evaluations have shown that a storage temperature of 20 °C granted the best storage condition for all the starch-based HPP hydrogels allowing to reduce evaporation and keep the TPA profiles of products during storage time. The stability analysis allowed also predicting a very good performance of all starch-based hydrogels produced, which are displaying a strength of the network even superior to commercial hydrogels and temperature sensitiveness only at temperatures higher than 40 °C.

In vitro digestion tests demonstrated different behaviours of rice and tapioca hydrogels. Mastication phase was the critical step for rice hydrogels digestion, while the intestinal phases were the critical step for tapioca hydrogels digestion.

The addition of humectants influenced the structural and mechanical properties of starch-based hydrogels as well as the occurrence and extent of gelation by high pressure. However, the optimization of processing conditions to produce stable starch-based HHP hydrogels allowed us to demonstrate that stable hydrogels with rheological and structural properties can be produced also in this case.

In conclusions, the approach of this work, which was considering the analysis of the effects of product and process parameters as a fundamental step to understand the gelation phenomenon, has been demonstrated to be sound. The determination of optimal product formulation, preparation and characterization of the hydrogels obtained with different starches represented a clear advancement of the knowledge on the production of starch-based hydrogels by high pressure processing (HPP) technology. However, further research efforts are needed at a fundamental level to unveil the thermodynamic of the gelation phenomenon under pressure, as well as at processing level analyzing how the utilization of more complex formulation affect the preparation of stable starch-based hydrogels by high pressure processing (HPP). Finally, it will be also necessary to scale-up the process in view of the industrial exploitation of the results towards industrial applications.

Chapter I
State of the art

I.1 Introduction

Human civilization is facing a series of critical issues that threaten our survival. The constantly growing world population and the uncontrolled global climate change have been put at first place, for all the internationals AGENDA's, the necessity to encourage industries and academia to research and develop natural and sustainable products, allowing the decrease of the ecological, social and health imbalances.

The development of plant-based systems to replace or reduce the utilization of synthetic materials has been receiving significant attention, in view to fulfil consumers demand natural products. Hydrogels which represent a group of versatile polymeric structures have been contributed to the development of green materials. In fact, hydrogels produced either from natural or synthetic sources have been listed as "smart structures" whose tailor-made design confers them different functional attributes allowing their use in biomedical, cosmeceutical, pharmaceutical and food applications. In the past years, exponential research and exploitation of hydrogels by the scientific community have been detected, being this phenomenon clearly observable in Figure I.1 by analysing the number of articles on the topic in the last 5 years (2016-2020).

Even though hydrogels have existed for more than 50 years, and great advances have been made in terms of their formulations and applications, the processing conditions and preparation methods have shown important limitations such as processing time and energy consumption, resulting on high production costs, thus limiting their further commercialization (Chirani *et al.*, 2015). The full exploitation of such structures might be achieved by utilization of alternative green technologies for their preparation; whose concept, purpose and general overview of this PhD thesis, have been described in the following sections.

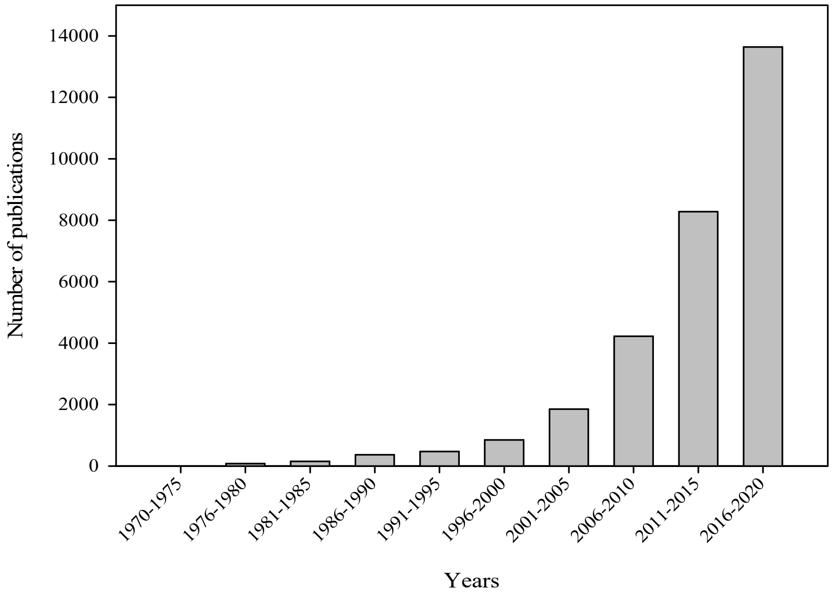


Figure I.1 *Exponential increase of the number of publications referring to the "hydrogel" keyword in the past 50 years (PubMed Data science).*

I.2 Hydrogels

Hydrogels are defined as a hydrophilic three-dimensional polymeric network able to absorb or retain a significant amount of water (Ismail *et al.*, 2013). The swelling properties are due to the thermodynamical affinity of the polymeric material to the water and the polymeric swollen network is mainly formed by chemical or physical bonds or interactions by the simple reaction of one or more monomers, with a corresponding absorption capacity up to 10 g/g (Kabiri *et al.*, 2011). Due to their tuneable characteristics such as water-swollen nature, mechanical characteristics, permeability and biocompatibility, hydrogels can resemble natural living tissue (Peppas *et al.*, 2000) or be tailored for different applications, being an uncountable number of types of hydrogels developed so far.

I.2.1 Classification

Starting from the point of the huge number of structures defined as hydrogels and the no consensus about their classification, the simplest way to classify these structures can be based on literature reviews previously reported on this topic (Peppas *et al.*, 2000; Gulrez *et al.*, 2011; Ahmed, 2015; Buwalda *et al.*, 2014; Chirani *et al.*, 2015). Figure I.2 summarized the classification of hydrogels based on different bases according to literature findings.

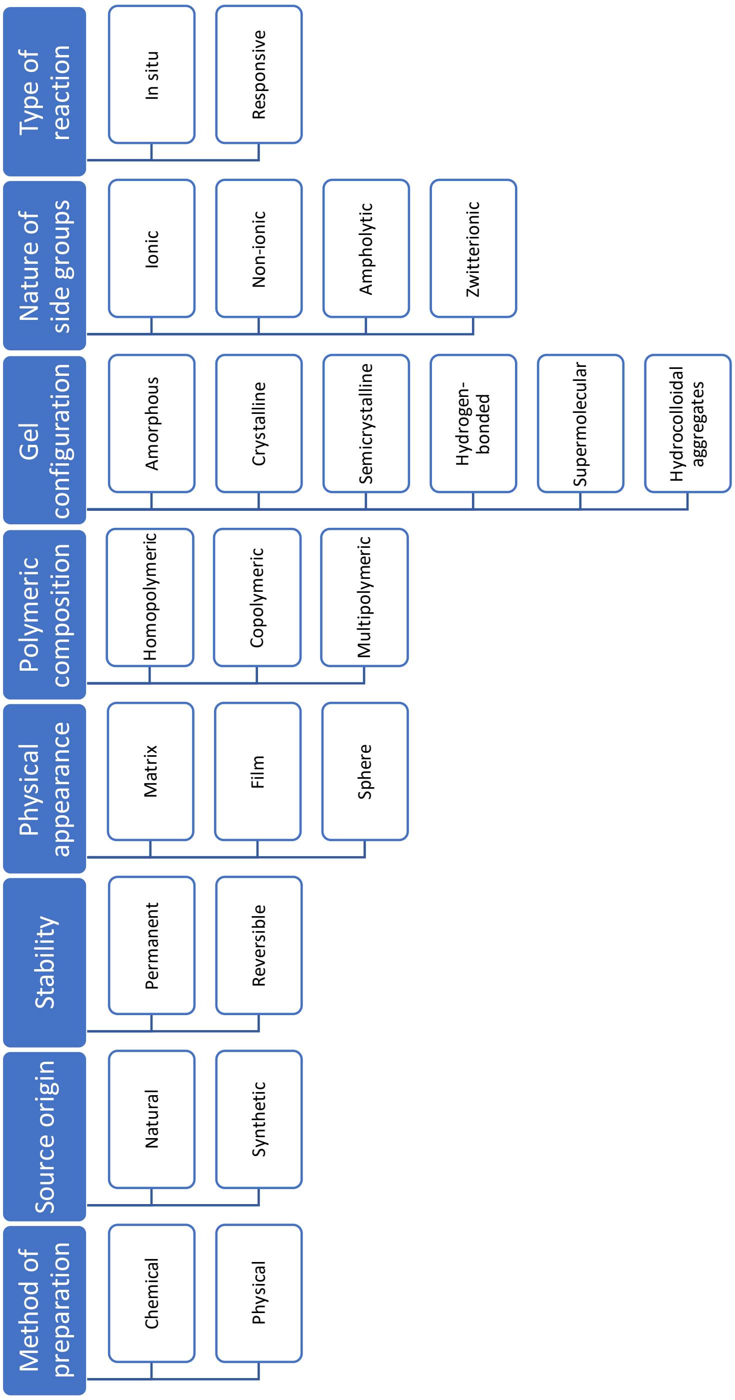


Figure I.2 *Hierarchical classification of hydrogels*

I.2.2 History

At the end of the XIX century, it was reported the first research work mentioning the term "hydrogel" describing just a colloidal gel prepared with organic salts (Van Bemmelen, 1894). However, after a half-century, in 1960, two researchers from the Institute of Macromolecular Chemistry of Prague published the landmark paper on the topic, first defining hydrogels as we know them at nowadays (Buwalda *et al*., 2014) and second, the very first successful application, made of poly (2-hydroxyethyl methacrylate), p-HEMA hydrogels were produced for manufacturing contact lenses (Wichterle and Lim, 1960), which production is still ongoing at industrial scale. After this work, during 10 years were exponentially increased the investigations on the development of this kind of hydrogels for several biological applications (Refojo and Yasuda, 1965; Refojo, 1965; Gasset and Kaufman, 1970; Winter and Simpson, 1969; Rosenberg *et al*., 1960; Dohlman, Refojo and Rose, 1967; Refojo, 1967; Riley, 1970). Throughout the years, the aims, and goals of the number of materials changed and enlarged (Chirani *et al*., 2015). According to Buwalda *et al*., (2014), the history of hydrogels can be divided in three generations, the first generation of hydrogels was related to the preparation of these structures by the utilization of different polymeric sources, the second generation of hydrogels, marked by the preparation of materials capable of a response to specific stimuli such as pH, temperature or concentration of biomolecules to produce gel formation (in-situ) or drug release. The third generation (still ongoing), focused on the production of tailor-design hydrogels with tuneable and trigger stimuli, by crosslinking methods with a special affinity to the human body.

Figure I.3 summarizes the historical advancement of hydrogels research, based on literature findings.

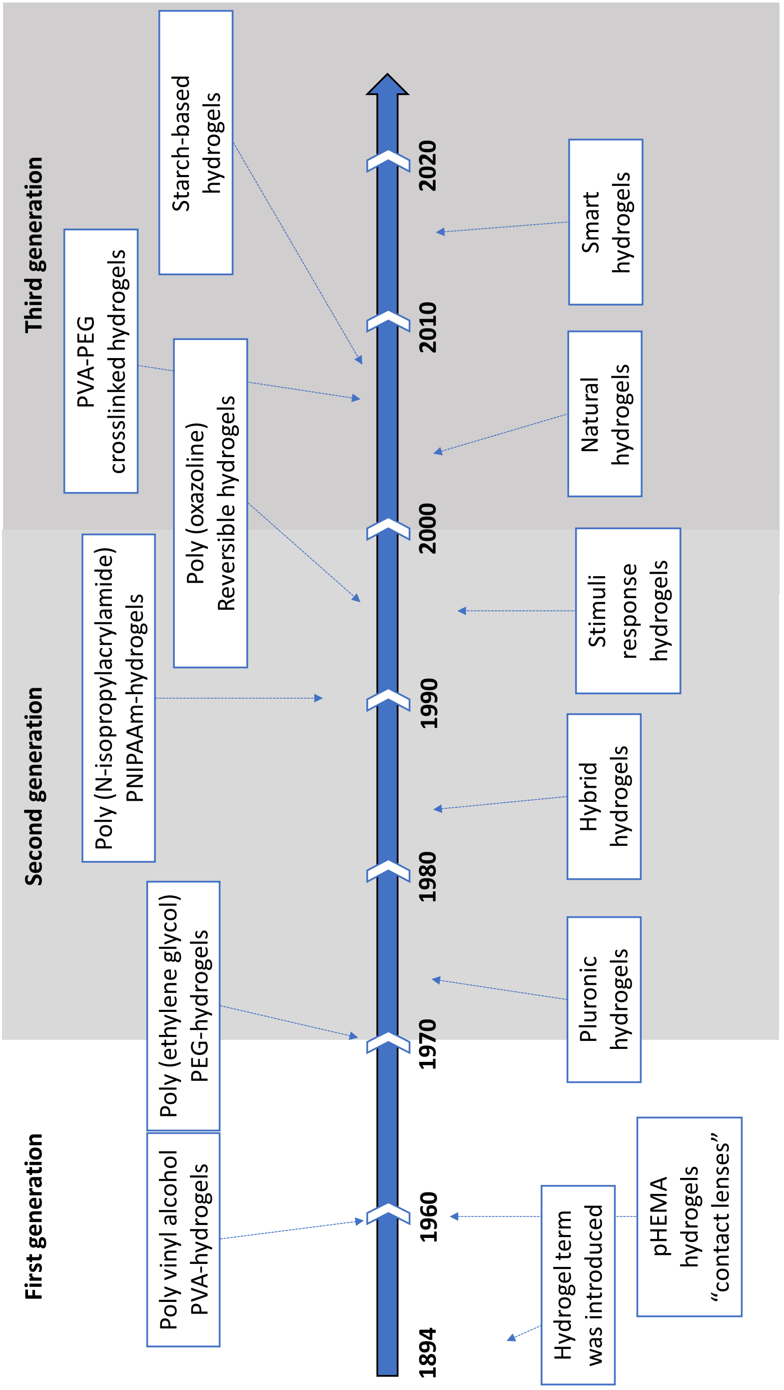


Figure I.3 *Historical overview of hydrogels research.*

I.2.3 Applications

Hydrogels have received considerable attention in the recent decades, their production has been increased exponentially, as well as, their applications. Hydrogels has been produced, patented and commercialized for products such as contact lenses, hygiene products, tissue engineering scaffolds, drug delivery systems, wound dressings, hygienic products, food additives, electric devices, among others (Rodríguez-Rodríguez *et al.*., 2020; Han *et al*., 2017; Caló *et al*., 2015; Ahmed *et al*., 2015; Zhao *et al*., 2013; Singh *et al*., 2010; Saxena *et al*., 2010; Hamidi *et al*., 2018; Park and Kim, 2001; Chen *et al*., 1995).

At nowadays, an innovative category of hydrogels called "natural hydrogels" has been extensively exploited. Natural hydrogels produced from renewable polymeric sources such as gelatine, alginate, cellulose or starch, have been receiving significant attention by the scientific community due to the human body friendly profile of these structures widen the applicability and the future of these biomaterials (Rodríguez-Rodríguez *et al*.., 2020; Ali and Ahmed, 2018; Biduski *et al*., 2018; Guilherme *et al*., 2015).

A summary of the main applications of hydrogels is reported in Table I.1.

Table I.1 *Summary of literature findings and patents of applications of hydrogels produced with different polymeric sources.*

Uses	Polymeric source	Reference, patent or brand
Soft, hard, coloured Contact lens	• poly-2-hydroxyethylmetacrylate • pHEMA-hydrogel • poly (methyl methacrylate) pMMA-hydrogel • poly (hexa-fluoroisopropyl methacrylate) HFIM-hydrogel • acid-co-acrylamide • Silicon	Wichterle and Lim (1960) Maulvi *et al*. (2016) US patents: • 6,861,123 B2 • 3,679,504 • 3,575,946 • 4,472,327 • 3,808,178 European patent EP2365360A2 Gentle 59®
Wound dressing	• Chitosan/Polyethylene glycol fumarate/thymol • Poly (ethylene glycol) derivative (DF-PEG) • Pectin, carboxymethylcellulose and propylene glycol	Koosehgol *et al*. (2017) Zhu *et al*. (2017) Granugel® Intrasite gel ® Purilon Gel ® Aquaflo ™ Woundtab®

	• Sodium carboxymethylcellulose • Polyethylene and propylene glycol • Sulphonated copolymer, carboxymethylcellulose, glycerol •	
Drug delivery	• Carboxymethyl chitosan poloxamer • Dextran aldehyde • Chitosan-g-polyaniline (QCSP) and benzaldehyde poly (ethylene glycol)-co-poly (glycerol sebacate) (PEGS-FA) • poly (itaconic acid-co-N-vinyl-2-pyrrolidone) • Polyurethane • Polyethylene oxide/urethane	Yu *et al*. (2017) Conde *et al*. (2016) Zhao *et al*. (2017) Koetting *et al*. (2016) European Patent EP054718A1 US patents: 5,514,380 8,383,153 B2 7,066,904 Cervidil ®
Tissue engineering	• Methacrylate chondroitin sulphate (CSMA); Acryloyl chloride-poly (ε-caprolactone) poly (ethylene glycol)-poly (ε-caprolactone)-acryloyl chloride (PECDA); AAm; PEGDA.	Liao *et al*. (2017)
	• Poly (propylene fumarate)	European patent 1664168B1
	• Peptides	US Patent 8,039,258
	• PVA + phenylboronate	US Patent 2013/0236971
	• Keratin	US Patent 6,379,690
	• Beta-glucan	US Patent 8,592,574
Hygiene products	• Crosslinked starch-g-polyacrylate	Masuda (1994)
	• Acrylate	Sannino *et al*. (2009)
	• Carboxylic and acid anhydrides	US Patent 32,649

I.2.4 Preparation

In simple words, the process, which leads to the formation of a hydrogel, is called polymerization which consists of a series of intermolecular reactions among the segments of polymer chains, causing an increase of their molecular weight and mechanical properties (Borgogna *et al*., 2016).
Typically, hydrogels polymeric networks, either from natural or synthetic sources are formed by changing the medium conditions through chemical or physical stimuli, resulting in two important categories: the physical or chemical hydrogels (Figure I.4). However, in the last decade, another category of these structures, called composite or hybrid hydrogels has been extensively exploited (Zhang and Khademhosseini, 2017).

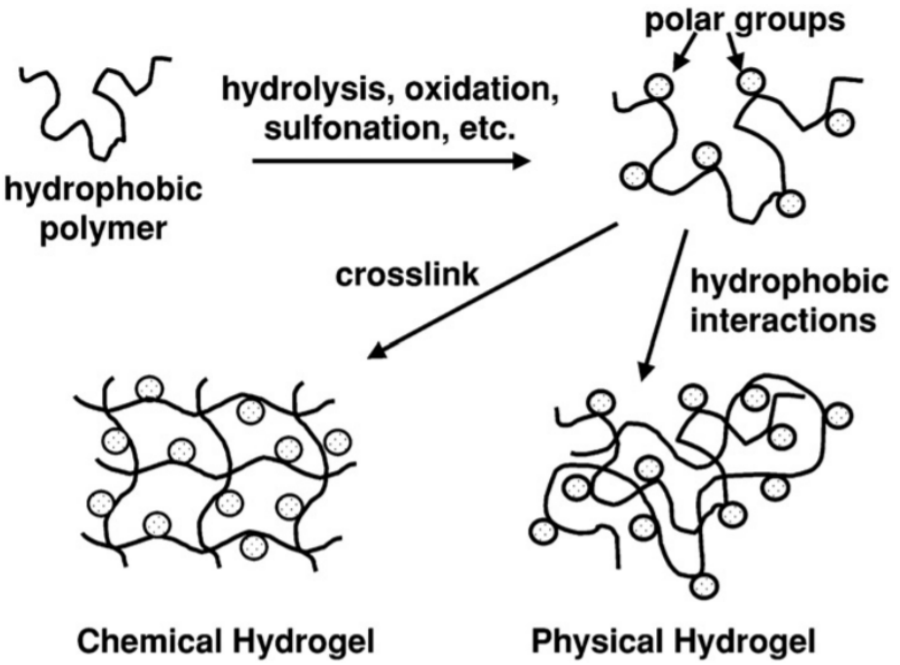


Figure I.4 *Schematic representation of chemical and physical hydrogels preparation (Hoffman, 2002).*

I.2.4.1 Physical hydrogels

Hydrogels obtained from physical methods (physical hydrogels) are considered reversible due to their non-permanent bonds. This hydrophilic polymeric network is typically formed by either physical entanglement of the polymer chains, or for non-covalent interactions, including self-assembly through hydrogen bonds, van der Waals interactions, hydrophobic interactions, ionic forces, among others (Mohammadinejad *et al*., 2019). Due to their characteristics, physical hydrogels are considered as weak gels with poor mechanical properties compared to chemical hydrogels, being this the major disadvantage and the actual challenge of the scientific community. Nevertheless, at nowadays, a growing interest in physical or reversible gels due to the advantages such as ease of production, absence of cross-linking agents, and reversibility has been detected (Ahmed *et al*., 2015). For instance, the reversibility can be tuned and triggered by external stimuli applied to the crosslinking points such as pH, temperature, ionic strength, and/or addition of competing solutes. Additionally, the main advantage of this preparation is the non-utilization of chemical crosslinkers, it which commonly represent ethical constraints to the consumers.

Drug delivery, nutrients encapsulation or edible coatings have been the most remarkable applications of physical hydrogels (Ali and Ahmed, 2018; Kimura et al., 2014). Among the physical methods to produce physical hydrogels, temperature (heating/cooling), freeze thawing, hydrogen bonding, and complex coacervation have been the most reported methods in literature.

I.2.4.1.1 Temperature

The process which leads to the formation of a hydrogel is called gelation and one of the traditional methods to produce a complete gelatinization of a polymeric source is by heating treatments. Reversible hydrogels are formed when solutions of polymers in water, such as starch, gelatine, alginate and carrageenan (in presence of ions), for instance, are heated and subsequently cooled (Gulrez *et al*., 2011). Thermal hydrogel formation is due to helix-formation, association of the helices, and forming junction zones as ascribed by Funami et al. (2007).

I.2.4.1.2 Freeze-thawing

Gelation of polymers by freeze-thawing method consists of producing the physical crosslinking of a polymer in aqueous solution, using freeze and thawing cycles to form polymer microcrystals (Hoffman, 2012). This method is driven by phase separation and cycling, which occurs as the solution freezes and the polymer is rejected from the growing ice crystallites, and the crystalline structure formed increased as freeze-thawing cycles increases (Guan *et al*., 2015). Physical poly (vinyl alcohol) PVA-hydrogels produced by the latter method have demonstrated enhanced mechanical properties, particularly for biomedical applications (Hassan and Peppas, 2000). An example of the freeze-thawing mechanism to produce hybrid hydrogels is illustrated in Figure I.5.

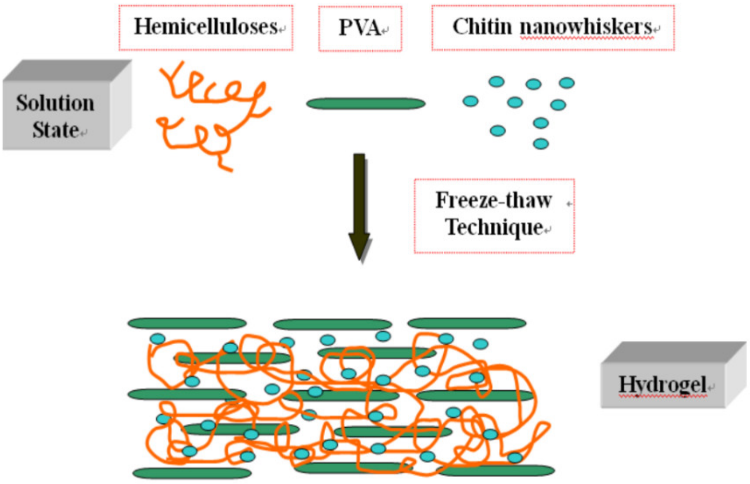


Figure I.5 *Gelation mechanism of hemicelluloses, PVA, and chitin nanowhiskers in a freeze/thaw process (Guan et al., 2015).*

I.2.4.1.3 Complex coacervation

Complex gels can be formed by mixing anionic with cationic polymers (Figure I.6). This method is based on polymers with opposite charges remain together, it may form a physical hydrogel known as an "ionotropic" hydrogel. Calcium alginate coacervated with alginate-poly(lysine) to stabilize the structure is a typical example of physical hydrogels produced by complex coacervation (Lim and Sun, 1980). At nowadays, physical hydrogels from polysaccharides coacervated with proteins, micelles, dendrimers and dendrimers has been produced (Du *et al*., 2015; Water *et al*., 2014; Morfin *et al*., 2011; Li *et al*., 2012; Chiappisi *et al*., 2014; Leisner and Imae, 2003).

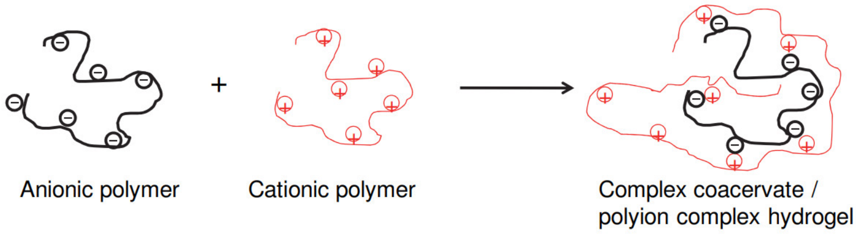


Figure I.6 *Complex coacervation between polymers with different charges (Gulrez et al., 2011).*

I.2.4.1.4 H-bonding

H-bonded hydrogels can be obtained by changing the medium conditions with the presence of a polymeric source with hydrophilic nature. Its ability to H-bonding and stability of the structure depends on the intrinsic properties of the

polymeric source. At nowadays, to improve the mechanical performance of hydrogels, researchers have extensively investigated different reinforced networks including hydrogen bond cross-linked networks (Zhu *et al*., 2017). In fact, H-bonded hydrogels are considered as self-healing structures being the addition of nanomaterials into the hydrogels network a great promise of further exploitation of these novel structures (Talebian *et al*., 2019).
A schematic illustration of a self-assembled hydrogel produced by H-bonding has been shown Figure I.7.

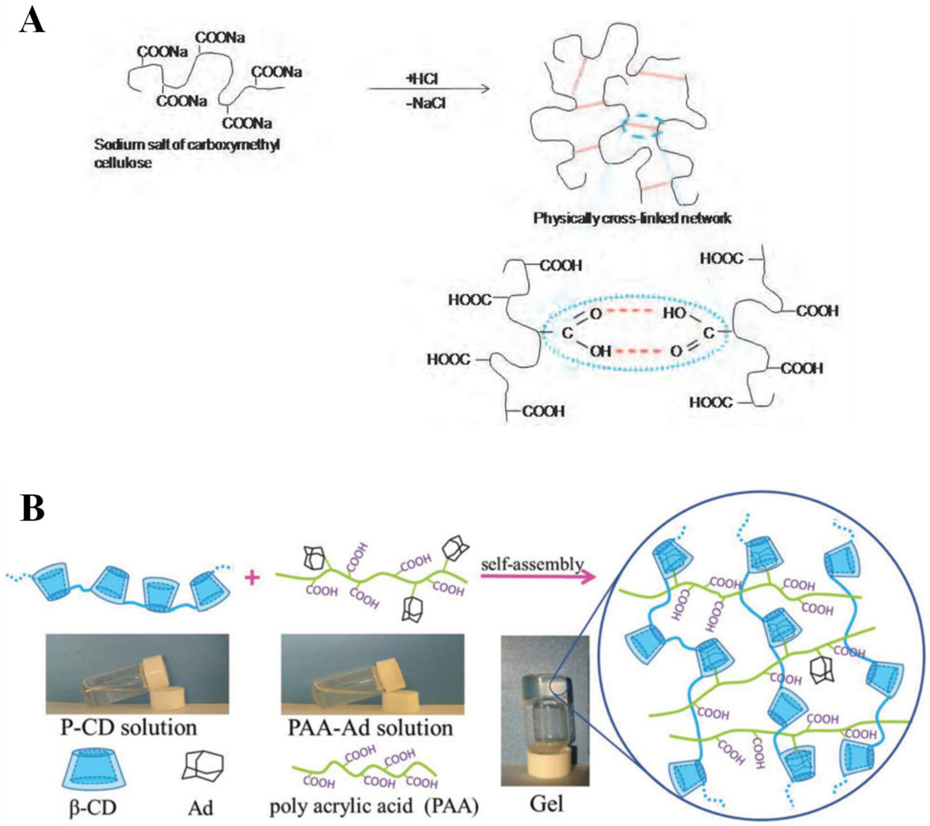


Figure I.7 *Schematic illustration of hydrogel formation (Hou et al., 2019; Gulrez et al., 2011).*

H-bonding preparation consists in the formation of hydrogen bonds between the polymeric source and water, favouring the formation of the gel network. Hydrogen bonds occur when the positive hydrogen atom establishes an electrostatic link with electronegative acceptor atoms such as oxygen, nitrogen, or fluoride, respectively (Desiraju, 2002). An example of H-bonding hydrogel preparation is the production of physical carboxymethyl cellulose (CMC) hydrogels as was reported by Takigami *et al*., (2007) (Figure I.7a), CMC was dispersed into an acid solution to replace the sodium molecules and promote hydrogen bonding, resulting in the formation of an elastic hydrogel. Recently, Hou *et al*., (2019) published a research work about the preparation of hydrogels for wastewater treatment utilizing H-bonding methodology by self-assembly poly-cyclodextrin and poly-adamantane. The authors observed an interconnected three-dimensional porous network, with a good recovery performance and it was confirmed that the host-guest interactions, mainly hydrogen bonds, were the main driving forces of hydrogels performance as selective adsorbents (Figure I.7b).

I.2.4.2 Chemical hydrogels

Chemical or irreversible hydrogels are mainly obtained through covalent cross-linking (Mohammadinejad *et al*., 2019). Chemical cross-linking involves grafting monomers on the backbone of the polymers or the use of cross-linking agent to link two polymers chains (Gulrez *et al*., 2011). Chemical hydrogels can be produced either from natural or synthetic polymers through the reaction of their functional groups with specific crosslinkers (Mohammadinejad *et al*., 2019). In the most succinct sense, chemical hydrogels preparation is based in the presence of a monomer, crosslinker, initiator and unwanted by-products produced, which in some cases a certain level of toxicity was detected (Mohammadinejad *et al*., 2019) (Figure I.8).

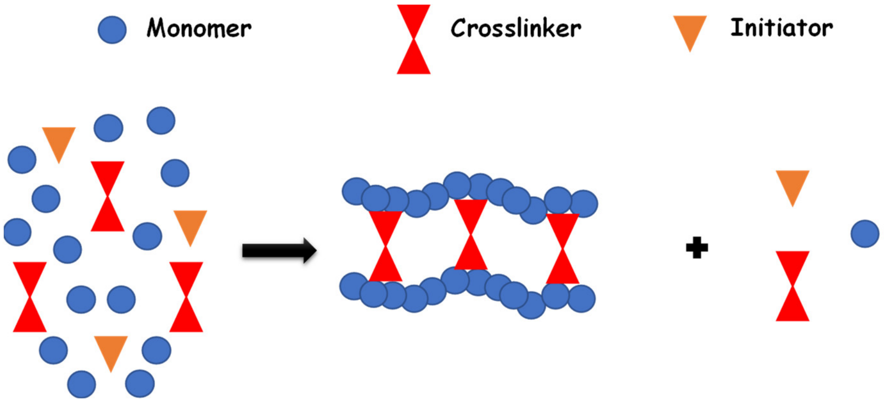


Figure I.8 *Schematic representation of chemical hydrogel preparation by crosslinking method (Adapted from Ahmed, 2015).*

The preparation of chemical hydrogels has been characterized as a more complex and longer process with respect to physical hydrogels production, however permanent hydrogels are more adaptable to the applications desired, present higher stability and better mechanical properties. Although, chemical hydrogels durability and stability are two desirable characteristics, in the last two decades, these positive characteristics have been considered as important

disadvantages, in fact, rigidity, toxicity, the hydrophobic aggregation of the crosslinking agents (high-density clusters), as well as, the gel network defects due to the presence of free chains, have been identified as important drawbacks that reducing the applicability of these structures (Mohammadinejad *et al*., 2019; Hoffman, 2012).
The following section summarizes the chemical methods more reported in the literature to produce chemical hydrogels.

I.2.4.2.1 Click chemistry

Click chemistry has been considered one of the most promising methodologies to produce crosslinked hydrogels due to their high specificity, high yield, biorthogonality, and mild reaction conditions (Trombino *et al*., 2019; Jiang *et al*., 2014). Chemical reactions such as Diels Alder, Azide-Alkyn Huisgen Cycloaddition, Thiol-ene Photocoupling, Aldehyde-Hydrazide Coupling have been categorized as click chemistry methodologies to ease production of functional hydrogels (Trombino *et al*., 2019). Click chemistry preparation relies on their high efficiency and specificity, which in the presence of catalysts such as minerals or light, its overall performance can be enhanced (Rostovtsev *et al*., 2002). Injectable hyaluronic poly-ethyleneglycol (HA-PEG) hydrogels, 3D patterned hydrogels and injectable HA-pectin based hydrogels have been recently produced by click chemistry methods for biomedical applications (Yu *et al*., 2015; Fu *et al*., 2017; Gramlich *et al*., 2013).

I.2.4.2.2 Grafting polymerization

This technique that involves the generation of free radicals onto a stronger support surface and then polymerizing monomers directly, as a result, a chain of monomers are covalently bonded to the support (Gulrez *et al*., 2011). Grafting process, where the polymer chains are activated, can be produced by chemical agents or radiation treatment. Figure I.9 illustrates the grafting process from a polymeric material to a grafted network.

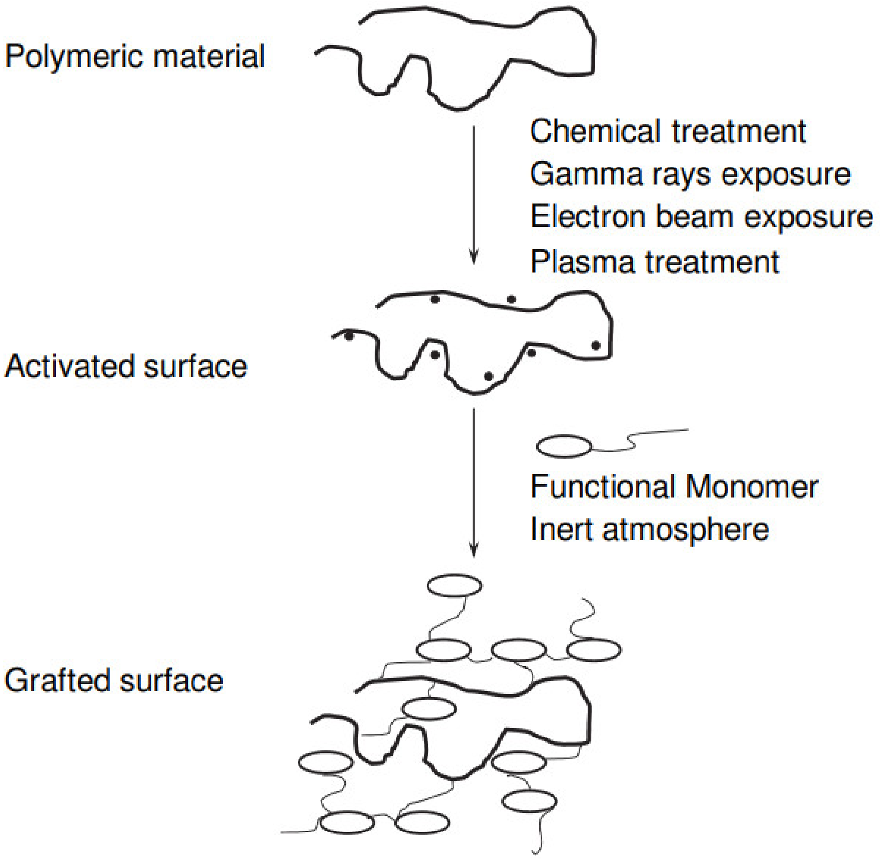


Figure I.9 *Schematic representation of chemical hydrogel formation by grafting methodology (Gulrez et al, 2011).*

Ionizing high energy radiation, like gamma rays and electron beams, has been used as an initiator to prepare the hydrogels (Ajji *et al*., 2008; Karadag *et al*., 2001). The irradiation of an aqueous polymer solution produces the radical formation on the polymer chains and resulting in the formation of macro-radicals (Ahmed, 2015). Recombination of the macro-radicals on different chains results in the formation of covalent bonds, in consequence, a cross-linked structure is formed. Examples of polymers crosslinked by the radiation method are poly (vinyl alcohol), poly (ethylene glycol), and poly (acrylic acid). The major advantage of the radiation initiation over the chemical initiation is the production of relatively pure and initiator-free hydrogels (Ahmed, 2015).

I.2.4.2.3 Chemical crosslinkers

This technique involves the introduction of new molecules by crosslinker agent action into the polymeric chains to produce a crosslinked network (Figure I.10). Typically, for this kind of preparation, the main target groups present in the polymeric sources are OH, COOH, NH_2 with crosslinkers such as aldehyde, epichlorohydrin, glutaraldehyde, bis-epoxide, among others (Trombino *et al*., 2019; Hoffman, 2012).

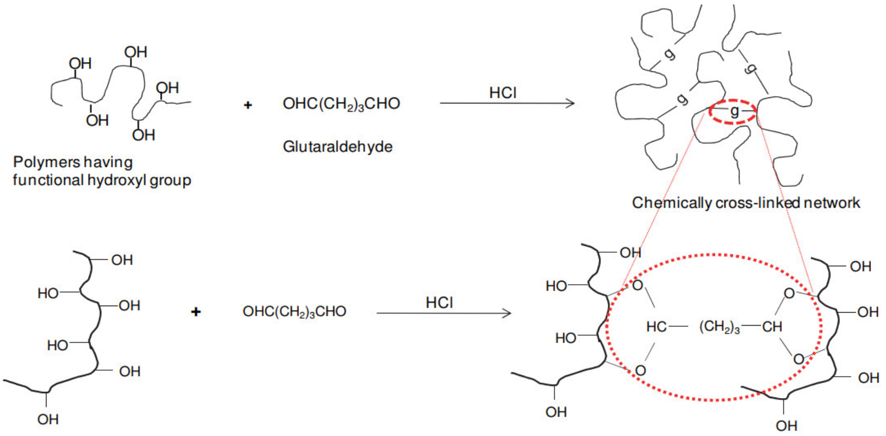


Figure I.10 *Schematic representation of chemical hydrogel formed by crosslinker method (Gulrez et al, 2011).*

I.2.4.3 Composite hydrogels

In the last decade, the preparation of hybrid or composite hydrogels by different crosslinking approaches or by addition of nanomaterials, inorganic fillers or nanofibers in the matrix, as well as, the combination of polymeric sources has been proposed as an alternative approach to overcoming the major limitations of conventional physical or chemical hydrogels (Buwalda *et al.*, 2017). Its main objective is to provide a synergistic advantage by mixing components and preparation methodologies reducing the intrinsic limitations of conventional hydrogels (Mohammadinejad *et al.*, 2019).

For instance, PVA-based hydrogels are one of the first hybrid hydrogels tailored for tissue engineering. Polyvinyl alcohol (PVA) is a hydrophilic polymer with reduced biocompatibility, biodegradability and polar solubility, and in a polymerized form, displays rigidity and poor adhesiveness, as well as, certain levels of cytotoxicity. Due to their disadvantages, structural modifications for desired and targeted applications have been applied (Kumar and Han, 2017). PVA-based hydrogels have been prepared through physical and chemical crosslinking techniques, such as hydrogen bonding, crosslinking agents, irradiation, ionic species, freeze-thawing cycles, high hydrostatic pressure, among others (Lejardi *et al*., 2014; Liu *et al*., 2010; Shi *et al*., 2014; Ino *et al*., 2013; Thankam *et al*., 2013, Kimura *et al*, 2004), and by combination of different polymeric sources such as nanoparticles, natural polymers (starch, alginate) and bioceramics (Kumar and Han, 2017). At nowadays, PVA-based hydrogels are widely utilized for wound dressing, bone tissue, vascular tissue, artificial cornea, cancer cells, among others (Kumar and Han, 2017). However, even though, composite hydrogels have been produced to reduce or replace the multiple disadvantages of the traditional hydrogel preparations, drawbacks related with the synthetic nature of the network or the cost of compounds utilized for their production, as well as, the processing time and high energy requirements are still challenging the industry and scientific academia, driving the attention to a more green and sustainable alternatives to produce these novel structures.

I.2.4.4 Natural or green hydrogels

Through history, world civilization nutrition relied mostly on animal, plants and plant-based materials. The demands for plant-based materials, based on the healthy lifestyles stated by governments policies, have increased along time, being part of the scope and mission of the most recognized industries around the world. Likewise, in order to reduce or replace the utilization of synthetic materials in view to fulfil consumers demand for natural products in all the industrial areas, a new class of biobased hydrogels has emerged. These structures are prepared from natural biopolymeric sources displaying specific structural and mechanical properties. Figure I.11 reported the most relevant green sources of biopolymers for hydrogels synthesis so far.

Hydrogels based on renewable natural polymers such as cellulose, polysaccharides or proteins, which are biocompatible, accessible and inexpensive, have been produced and popularized for biomedical applications in the last 10 years (Mohammadinejad *et al*., 2019). Additionally, this type of polymers can be obtained from the waste products of several industrial elaborations (El-Zawawy and Ibrahim, 2012).

Green hydrogels have been playing an interesting role in several bio-applications due to their tailor-design and tunable properties (Caló and Khutoryanskiy, 2015). Green hydrogels from cellulose/hemicellulose, lignin, starch, pectin, gum, soy and zein, have been produced by various traditional techniques and categorized as the most promising biomaterials for different applications envisaged (Chang *et al*, 2009; Maleki *et al*., 2017; Thakur and Thakur, 2015; Lee *et al*., 2014; Palm and Zacchi, 2004; Thakur, 2013; Naseem *et al*., 2016; Ismail *et al*., 2013; Ali and Ahmed, 2018; Peñaranda and Sabino, 2010; Fariña *et al*., 2019; Tanan *et al*., 2019; Ali and AlArifi, 2009; Ngoenkam *et al*., 2010; Sila *et al*., 2009; Mehrali *et al*., 2019; Seslija *et al*., 2016; Rakshaee *et al*., 2011).

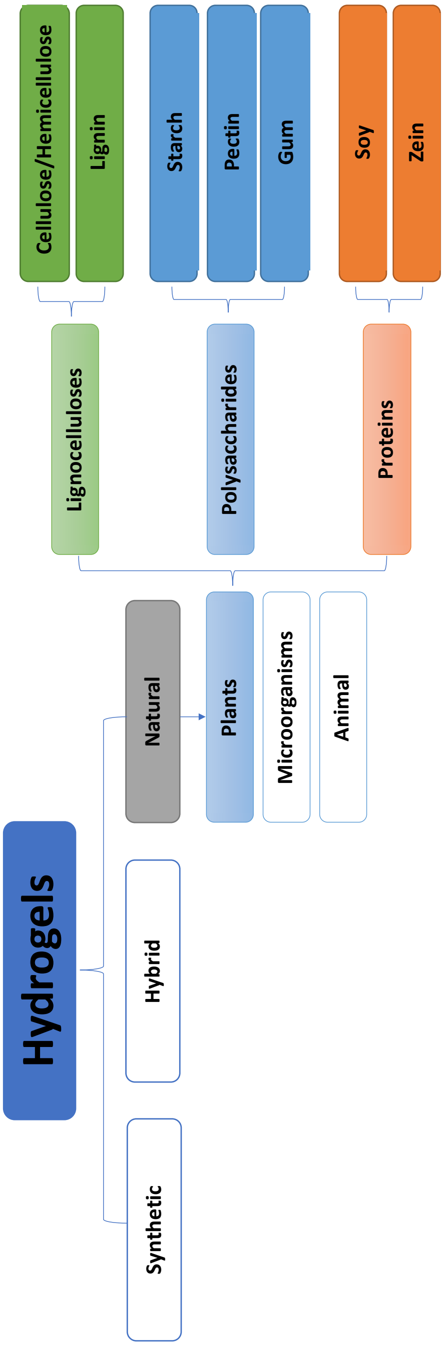


Figure I.11 *Most relevant biobased polymeric sources on green hydrogels production (Thakur and Thakur (2014) and El-Sherbiny and Yacoub (2013)).*

I.2.5 Starch-based hydrogels

Among the green hydrogels, starch-based hydrogels are one of the most promising alternatives to achieve biocompatible materials. Starch-based hydrogels are hydrophilic polymeric networks with high absorption capacity. Given the flexibility of the hydrogels based on starch, the physical, mechanical and their durability can be fine-tuned by change the method intensity, components or the liquid environment.

Starch is one of the most abundant biopolymers in nature, industrially produced by almost all countries around the world. This biopolymer is formed by photosynthetic plants that convert light energy which is stored in a highly condensed manner in granular structures as amylose and amylopectin (Knorr *et al*., 2006). Starch is one the most versatile and economical material of potential use in polymer technology, and in food and non-food applications, representing along with the history an important role in the food industries as a stabilizer, bulking and gelling agent (Pei-Ling et al., 2010).

At worldwide, approximately 60 million of tons per year or starch are extracted from various cereals, tubers and roots crops of which roughly 60% is used in food and 40% is used in pharmaceuticals and non-edible purposes, such as a matrix for fertilizers, seed coatings, paper, cardboard, packaging materials, adhesives, amongst others (Copeland et al., 2009; Burrell, 2003).

Starch exists in form of granules which vary in size, typically from 1 to 100 um diameter, and shape (polygonal, spherical, and lenticular), depending on their origin (Copeland et al., 2009). All the starches have a general structure composed by a head, tail, ring and hilum (Figure I.12) and are mainly composed by amylose and amylopectin reaching up 99% on a dry basis, along with a minor amount of lipids, proteins and minerals (Yang et al., 2017). The amylose and amylopectin ratio depends on the source of starch and this ranges from 15:85 to 35:65, except for some variations like the waxy starches or high amylose starches (Liu, 2005). Although lipids and minerals are minor components by weight, they can play a significant role in some properties of starch (Copeland *et al*., 2009) and during gelatinization (Pei-ling *et al*., 2010). The moisture content of native starch granules is usually between 10 and 20%, for example, 13-15% for wheat starch and up to 20% in potato starch (Yang *et al*., 2017).

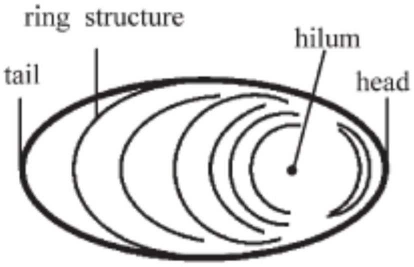


Figure I.12 *Structure of typical starch granule (Pei-Ling et al., 2010).*

Among the starch components, amylose (AM) and amylopectin (AP) content are playing a significant role in the characteristics and properties of the starch, before and after polymerization. In the most graphical sense, starch represents a building where the slabs (floors) is the amylose fraction and the columns (walls) are represented by the amylopectin content. Furthermore, the ratio of AM/AP present in starch granules determines the performance, potential utilization and physical characteristics of the starch-based products, as well as, their crystallinity (Yang *et al*., 2017). Moreover, crystallinity is related to the amylopectin fraction and amorphous relies on the amylose content.

Amylose is a glucose polymer with predominantly α-1,4 glycoside linkages and some α-1,6 glycoside branches (Figure I.13a). Amylose is generally considered a linear chain, with a helical secondary structure (Liu *et al*., 2009). On another hand, amylopectin is a highly branched polymer containing α-1, 4 linkages with α-1, 6 branching at about every 15-30 glucose units of the main chain (Figure I.13b) with a molecular weight of 10^7-10^9 Da and is arranged in double helix configuration (Gallant *et al*., 1997). Amylopectin could form cluster like-structure consisting in three type of chains: A, B and C, which could be determined by X-ray diffraction (Rubens *et al*., 1999). Several studies have investigated and classified starches according to their crystalline structure (Table I.2), being the most used categorization to explain the starch behaviour under different conditions (Pei-ling *et al*., 2010; Katopo *et al*., 2002; Stute *et al*., 1996; Wu and Sarko, 1978a, b).

Table I.2. *X-ray structure of some starches (Pei-ling et al., 2010; Vallons and Arendt, 2009; Stute et al., 1996).*

Starch source	X-ray pattern
taro, corn, wheat, rice, oat, barely, amaranth, sorghum	A
potato, high amylose corn	B
tapioca, lentil, mung bean, lotus root, pea	C

The A-chain is unbranched and linked to the molecule by the reducing the end-group, B-chain is linked to the molecule in the same way as the A-chain one or more A-chains, and C-chain is the one that carries the reducing end-group of the molecule (Liu *et al*., 2009). However, the principal difference of the A, B or C chains instead the spatial configuration and crystallinity, is related with the compactness and the water content inside the structure, being the A-chain a relatively compact structure with a lower content (2/3 water molecules per 1 glucose molecule) and B-chain a structure a less compact with higher water content (3 water molecules per 1 of glucose), C-type is considered a mixture between A and B-chains (Pei-Ling *et al*., 2010; Knorr *et al*., 2006; Bauer and Knorr, 2005).

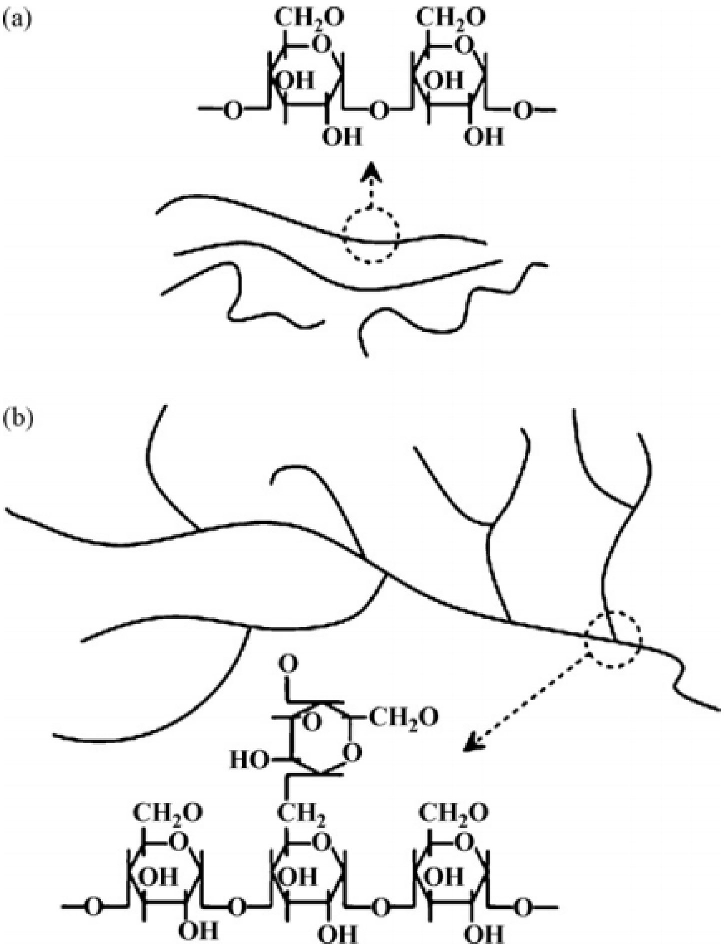


Figure I.13 *Chemical structures and physical schematic representation of (a) amylose and (b) amylopectin (Liu et al. 2009).*

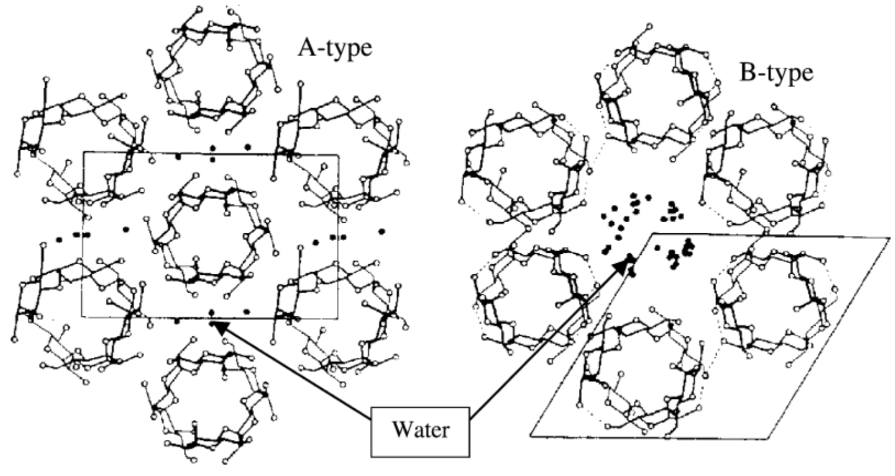


Figure I.14. *Example of A-, and B-type conformations (Tester et al., 2004; Wu and Sarko, 1978a, b).*

Starch in aqueous media, regarding of its intrinsic and extrinsic properties such as botanical origin, physical or chemical properties, can form a three-dimensional network, called hydrogel, which is the main product of its gelatinization. One ease methodology to produce the gelatinization of the components of the starch producing stable starch-based hydrogels, it is by thermal treatments coupled to mechanical stresses (Mohammadinejad *et al*., 2019).

According to Schirmer *et al*. (2015) when starch is heated in excess of water, physicochemical interactions between AM and AP occurs, resulting on different structural changes such as glass transition, gelatinization, melting of the amylose-lipid complex and recrystallization (Figure I.15). In particular, starch gelatinization process by thermal treatments is initiated by the water entering into the granules leading to the hydration of the amorphous region and the disruption of hydrogen bonds (Jenkins and Donald, 1998).

The destabilizing and disruptive stresses over the amorphous region caused by the heated water produce the transmission of this swelling process to the crystalline regions, being mediated by the amylopectin chains that interconnect the amorphous regions with the crystalline layers in the granules (Schirmer *et al*., 2015). Continuing heat transfer results in irreversible changes ascertained with starch granule gelatinization, such changes are mainly related with the melting of starch crystallites, starch solubilization, leaching out of the starch granules and, in consequence, the formation of a network with granules totally destroyed (Cooke and Gidley, 1992).

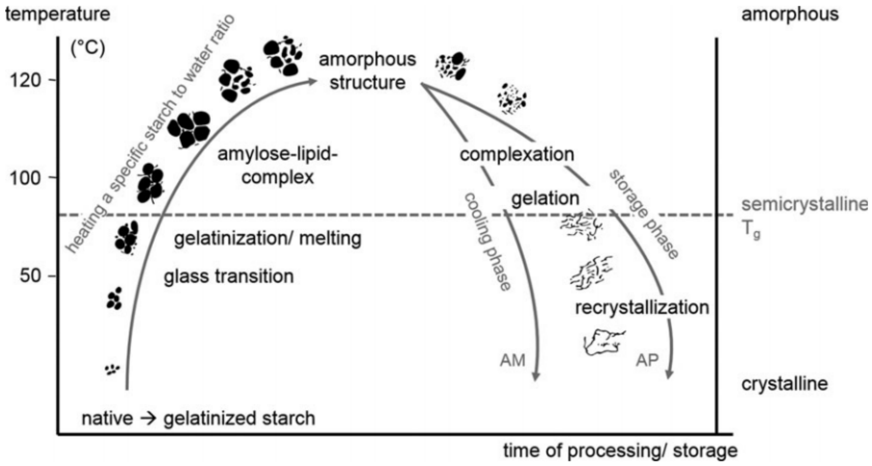


Figure I.15. *Phase diagram showing the phase transitions of starch in aqueous medium when applying a temperature profile (Schirmer et al., 2015).*

Starch-based materials produced by thermal processing have received significant attention in easing the environmental burden of petrochemically derived polymers (Liu *et al*., 2009). Moreover, the versatile characteristics of the different starches and their numerous transitions have been utilized to produce different biodegradable starch-based materials with tailored-design. Starch-based materials can be found in a variety of configuration such as particles, beads, and films (Ismail *et al*., 2013).

Starch-based hydrogels from potato, rice, wheat, corn, tapioca, pea, and lentil starch have been produced by physical or chemical crosslinking for various innovative applications such as sorption of dyes, metal capture from wastewater and agricultural uses, as well as for electrical uses, food preservative, personal care products, tissue engineering, drug delivery, among others (Ismail *et al*., 2013; Hashem *et al*., 2007; Dragan *et al*., 2011; Zheng *et al*., 2010; Chauhan *et al*., 2006; Singh *et al*., 2008; Abd El-Mohdy *et al*., 2011; Guo *et al*., 2005a; Guo *et al*., 2005b; Chen *et al*., 2004; Ngoenkam *et al*., 2010; Liu *et al*., 2011; El-Hag Ali and AlArifi, 2009; Mahkam, 2010; Vittadini *et al*., 2008; Leite *et al*., 2017). Recently, Biduski *et al*., (2018) investigated the different physical characteristics of starch-based hydrogels produced by different gelatinization methods (alkaline or thermal methods) utilizing native or cross-linked rice starch with different amylose content (8%, 20% and 32%), concluding that gelatinization method and starch amylose content has influenced hydrogels characteristics. The alkaline gelation (NaOH 50%, 55 °C, 0.5 h) was the most suitable method to obtain firm and well-structured gels when using cross-linked rice starch (obtained after several processing steps in more than 24 h) with 20 % amylose content.

Among the different methodologies to produce chemically crosslinked starch-based hydrogels, graft copolymerization of the starch presence of a crosslinker or direct crosslinking of polysaccharide, have been the most utilized (Ismail *et al*., 2013). For instance, starch-based hydrogels for agricultural or for the removal of pollutants, as well as, tailored for drug delivery and tissue engineering has been widely synthesized by grafting starch with acrylic acid utilizing persulfates as initiators (Fariña *et al*., 2019; Tanan *et al*., 2019; Mohammadinejad *et al*., 2019; Ismail *et al*., 2013; Athawale and Lele, 1998; Dragan and Apopei, 2011; Pourjavadi *et al*., 2004) or with the addition of methacrylic acid or β-glycerol-phosphate, pH-responsive starch-based hydrogels for ketoprofen delivery and thermo-responsive starch/chitosan hydrogels for chondrocyte delivery cartilage tissue engineering have been produced respectively (Ngoenkam *et al*., 2010; Ali and Alarifi, 2009). Indeed, due to their fine-tuned structured modulated by the cross-linking intensity chemically-crosslinked starch-based hydrogels have been utilized for the delivery of drugs on the intestinal tract (Noh *et al*., 2019; Ozay *et al*., 2019; Solomevich *et al*., 2019; Liu *et al*., 2011; Casadei *et al*., 2008). Other authors have summarized the production of hydrogels (Ali and Ahmed, 2018; Caló and Khutoryanskiy, 2015; Ismail *et al*., 2013) and reported physical or chemical crosslinking and graft polymerization as traditional methods utilized to produce this kind of structures. However, long processing time, high energy consumption, and safety issues related to the synthesis of these products have been identified as important limitations of these gelatinization methods.

I.2.6 Alternative methods to produce starch-based hydrogels

In the last decades has been developed and investigated several non-traditional technologies to produce starch-based hydrogels. The nonthermal technologies which belong to the industrial emerging technologies field have been proposed as an alternative to conventional methods to produce the polymerization of starches. The different nonthermal technologies that are defined as those in which temperature is not the main factor in the process are reported in Table I.3.

Table I.3. *Nonthermal technologies.*

Technology
Pulsed electric fields
High pressure processing
Pulsed light
Ultrasound
Ozone
Cold plasma

In the last decades, alternative production methods to obtain starch-based hydrogels have been investigated to overcome the problems arising from the utilization of the traditional one, among which high pressure processing (HPP) has been extensively investigated.

I.2.6.1 High pressure processing (HPP)

High pressure processing (HPP) is a technology which the products, already sealed in its final package, are introduced into a vessel and subjected to extremes levels of pressure with ranges from 100 to 1000 MPa (Figure I.16). The pressure could be transmitted by water or oil and the temperature of the process oscillates between 0 and 100°C with times from seconds to hours (San Martín *et al*., 2002).

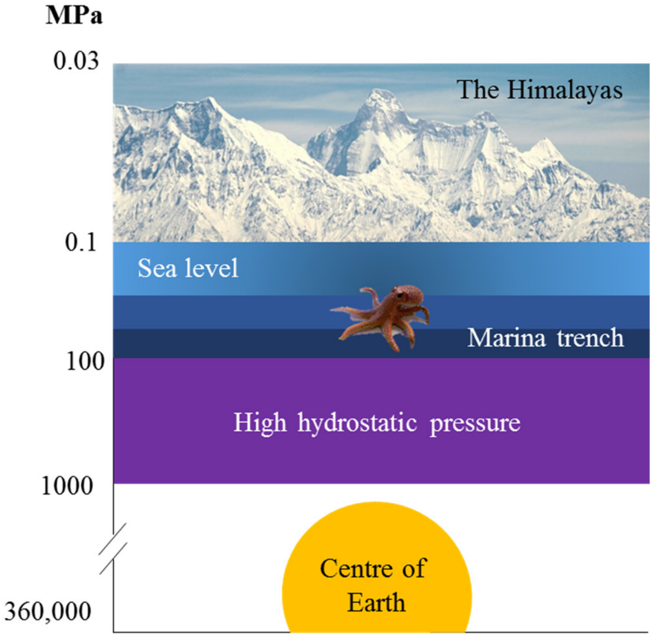


Figure I.16. *Diagram of comparative pressure levels.*

The high pressure process started as a supply process on some chemical or physical process (Rivalain *et al.*, 2010). At industrial scale, the first application was the synthesis of NH_3 (Travis, 1998) and in the last 30 years, this technology was focused towards food systems becoming an industrial reality, in fact, FDA approved its utilization as cold pasteurization, based on their positive effects over shelf life extension of several food matrices (Bermudez-Aguirre and Barbosa-Canovas, 2011). HPP is a well-known non-thermal technology mostly applied for food preservation, causing no or minimal sensory and nutritional damages to processed products. HPP technology has been also proposed for other applications, such as the recovery of bioactive compounds, the enhancement of bioaccessibility and bioavailability of micronutrients, the reduction of food allergenicity, preservation of lipids and salt content reduction (Barba *et al*., 2015), as well as alternative processing method for modification of starch suspensions or for starch suspensions gelatinization to prepare starch-based hydrogels (Blaszczak *et al*., 2015; Blaszczak *et al*., 2005a; Blaszczak *et al*.., 2005b; Blaszczak *et al*., 2007; Buckow *et al*., 2007; Katopo *et al*., 2002; Kawai *et al*., 2012; Li *et al*., 2012; Li *et al*., 2015; Oh *et al*., 2008; Stute *et al*., 1996).

High pressure processing (HPP) can provide the possibility of produce a "clean" sol-gel process on some polymers like starch, with different characteristics and multiples advantages which sets it apart to the gels produced by traditional methods (Buckow *et al*., 2005). The principal advantages of the use of HPP on the hydrogels based on starch production are:

- The non-utilization of a cross-linker
- No polymer destruction
- Less time production
- Low energy waste
- Friendly with the environment
- Allow the addition of bioactive compounds inside the networks
- The natural character of the products

I.2.6.1.1 HPP Equipment

The high pressure processing is typically carried out in batch processing mode and the batch systems can process pre-packaged liquid and solid materials (Martínez-Monteagudo and Balasubramaniam, 2016).

The HPP units (Figure I.17) are composed principally by:

- pressure chamber or vessel which can be horizontal or vertical, closures,
- yoke,
- pressure generator (hydraulic pump and pressure intensifiers),
- systems to monitor and control the pressure, time and temperature (Balasubramaniam *et al*., 2016).

The commercial use of high hydrostatic pressure for food preservation started in Japan in the late 1980s, being almost all very primitive machines (Farkas, 2016). At nowadays, there are 12 suppliers of industrial-scale high pressure equipment around the world (Balasubramaniam *et al*., 2016), being Hiperbaric and Avure technologies the most branded with excellent features towards industrial processing (Table I.4). Indeed, the number of high-pressure units has been growing exponentially at an annual rate, since the beginning of year 2000 (Figure I.18), with important developments on countries such as United States, Mexico, South- Korea, Spain and Japan (Huang *et al*., 2017), and recently in southamerican countries such as Colombia and Chile.

Figure I.17. *HPP commercial unit 300L (Source:* ***www.hiperbaric.com****)*

Table I.4. *Main characteristics and processing cost of the Hiperbaric equipment portfolio (Balasubramaniam et al., 2016).*

Model	55	120	135	300	420	525
Vessel volume (L)	55	120	135	300	420	525
N° of pressure intensifiers	1	2	2-4	4-6	6-8	8-10
Footprint (m^2)	22	37	39	61	56	63
Number of cycles/h	9.7	9.1	8.7	8.1	9.0	9.0
Production in kg/h	321	658	708	1459	2257	2821
Processing cost in €/kg	0.14	0.102	0.117	0.079	0.074	0.071

*Calculations for HHP during 3 min at 600 MPa considering a vessel filling efficiency of 60%, including amortization in 5 years, 300 working days/year, 16/h day, wear parts, and utilities. Exchange rate: 1€ = US$1.15 (Balasubramaniam *et al*., 2016).

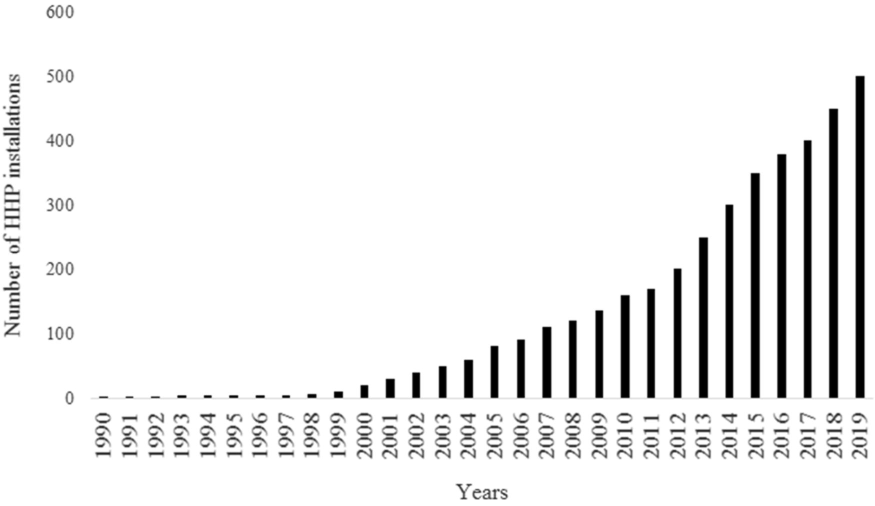


Figure I.18. *Development of the number of HPP units in the world (Hiperbaric, 2019).*

I.2.6.1.2 HPP process

In commercial applications range from 200 to 600 MPa have been mostly utilized, regarding the product and objectives of the process (Mújica-Paz *et al*., 2011). The fundament of the process is based that the food components undergo under static high pressure at certain temperatures, several changes such as destruction or formation of non-covalent bonds, electrovalent bonds, and hydrophobic bonds, deactivate, denature or gelatinize biopolymers, and destroy pathogenic bacteria and microorganisms in the case (Huang *et al*., 2017).

High pressure process is based in two physical principles; Le Chatelier principle, where the application of pressure produces a volume decrease causing an increase in the reaction and interaction of the molecules, and the Pascal principle, where the energy applied in a compression of an incompressible fluid like water, will be divided equally to all the parts inside the vessel. Hence, in a high pressure cycle, the high pressure it is applied uniformly and instantly to the products, regardless of the composition, size and shape (Oey *et al*., 2008).

All industrial or lab-scale process of high pressure processing can be divided into 4 steps (Figure I.19): Product loading: The unprocessed batch, usually under vacuum in a flexible package, is placed in a pressure vessel. Vessel pre-filling: The pressure vessel is filled with the pressure-transmitting liquid.

Pressurizing: The pressure is produced by a hydraulic pump or a piston and isostatically transmitted inside the pressure vessel to the product instantaneously and uniformly. Product unloading: The processed batch goes out of the cylinder after the decompression process.

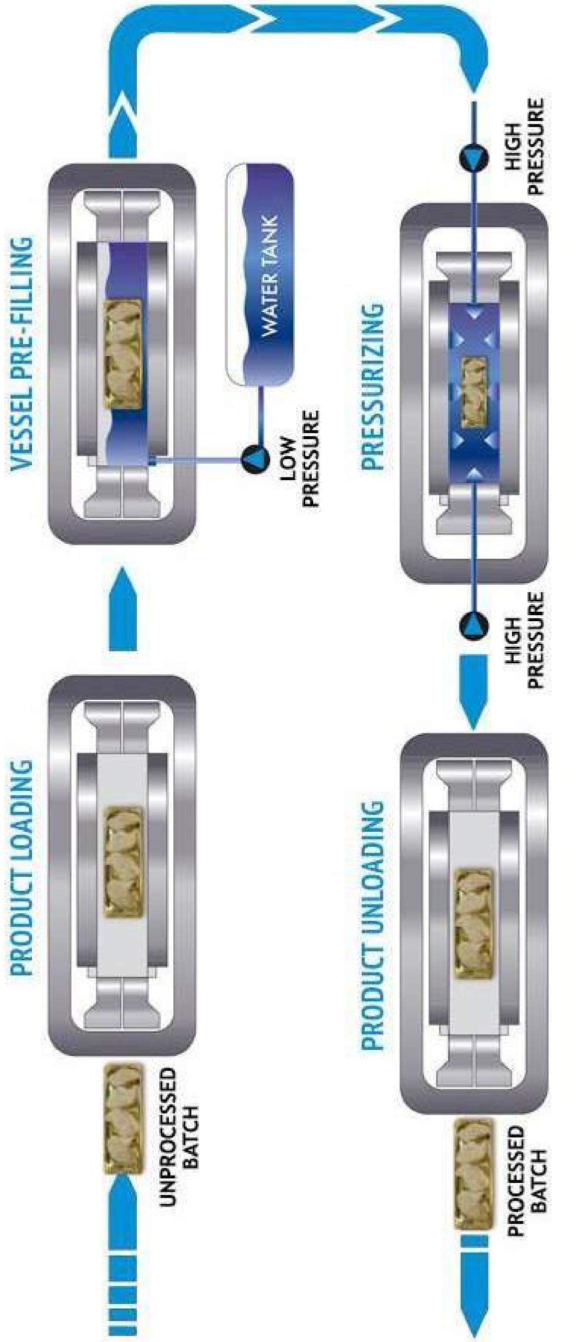


Figure I.19. *Schematic representation of an HPP unit operation (Hiperbaric, 2019).*

I.2.6.1.3 HPP effects on biological macromolecules

The use of pressure as a thermodynamic parameter began at 100 years ago, Regnard, Royer, Hite, Bridgman and Basset were the pioneers in introducing the high pressure process into the biology field (Rivalain *et al.*, 2010).

During the last decade, a great interest of research on biological components under high pressure towards from food processing to potential developments in medicine, cosmetics and pharmaceutical applications has been detected (Rivalain *et al.*, 2010).

Biopolymers, such as protein and starches, under high pressure show changes of their native structure under high hydrostatic pressure different to the changes occurring at high temperatures (Larrea-Wachtendorff *et al.*, 2015; Buckow *et al.*, 2007), representing an opportunity to develop an interesting class of structures and materials. The effects of high pressure on biopolymers could be reversible or irreversible (Knorr *et al.*, 2006) and after pressure, the conformation of these biological components change, and in consequence, their functionality.

In the case of the starches, the HPP produce the gelatinization of this biopolymer producing characteristic rheological and functional properties, being this transformation an interesting field to research (Yang *et al.*, 2017). However, the mechanisms and fundaments of the HPP sol/gel process are still not completely unravelled.

I.2.6.1.4 HPP effects on starch

HPP causes the disordering of biopolymers, including proteins and starches, which induce modifications of non-covalent intermolecular interactions, thus pressure-assisted gelatinization (Balny, 2002). Under high pressure, starches undergo morphological and structural changes, resulting on physical modification of the starch granules or their gelatinization, with intact granules and limited swelling of starch particles remaining after treatment, with different structural properties compared to conventional gelatinization methods (Yang *et al.*, 2017; Li *et al.*, 2012; Buckow *et al.*, 2007; Hibi *et al.*, 1993). Knorr *et al.*, (2006) and Buckow *et al.*, (2007) suggested a gelation mechanism for the starch under high pressure (Figure I.20). This mechanism attempted to describe the gelation process by pressure as a process which the amorphous and crystalline region of the granules is hydrated producing the swelling of the starch granules. The smectic crystalline structure is decomposed by helix-helix dissociation followed by helix coil transition and helix reorganization. Thus, under high pressure, the disintegration of the starch macromolecule is incomplete due to the role of the helix conformations (Pei-Ling *et al.*, 2010). Likewise, is very common the occurrence of differences on the rheology of the starch gels produced by pressure compared to thermal gels (Knorr *et al.*, 2006).

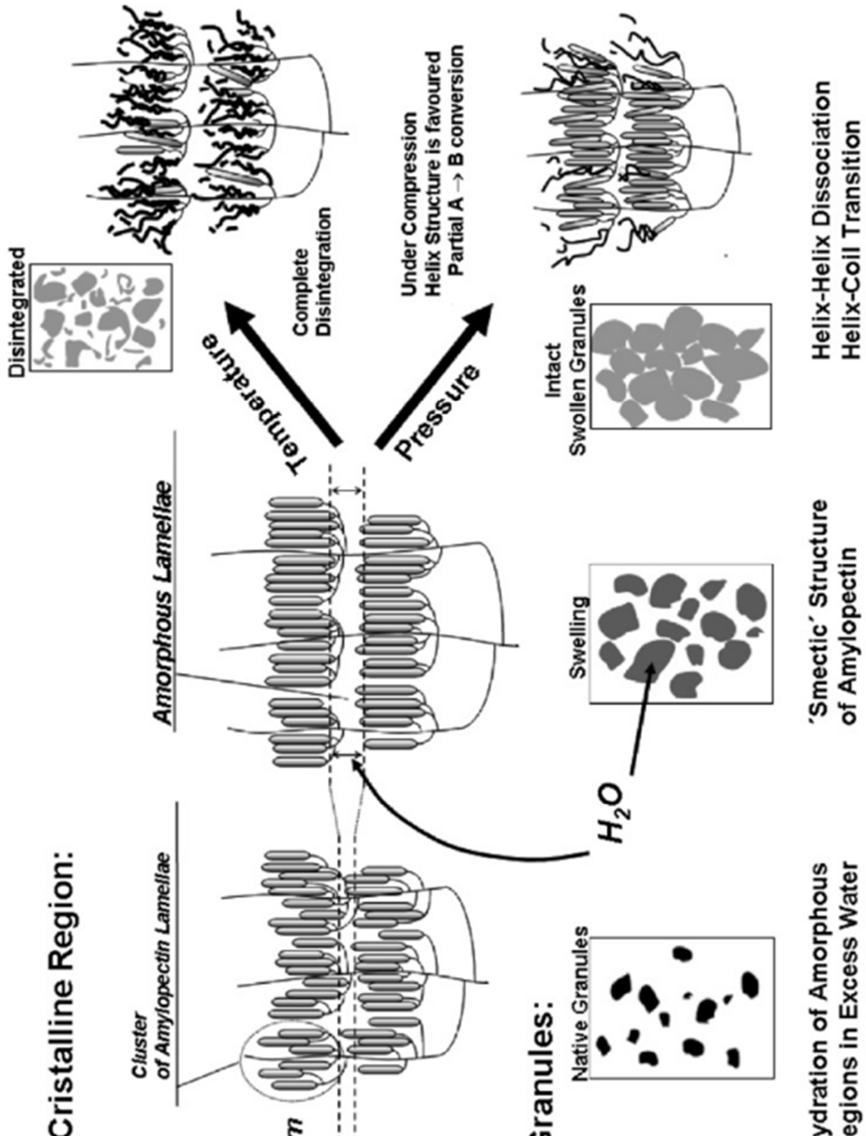


Figure I.20. *Scheme of starch gelatinization (Buckow et al., 2007).*

It is well known that almost all starch suspensions could be gelatinized under high pressure at room temperature. However, it has been shown that HPP assisted gelatinization process, consisting in the hydration of the amorphous and crystalline regions in excess of water under compression forces, is strongly influenced by the type of starch, starch/water ratio, pressure level, temperature and processing time (Bauer and Knorr, 2005; Pei-Ling *et al.*, 2010).

The pressure range in which the sol/gel process occurred is typically for any starch and depends on the crystalline structure (e.g. AM/AP ratio) and the pressure conditions, being expected that with increased treatment pressure the degree of gelatinization increases (Pei-Ling *et al.*, 2010). The starch concentration also plays an important role in the gelation process by pressure, because the good relationship between starch-water is the key step for obtaining stable gels with almost complete swelling granules (Katopo *et al.*, 2002). On another hand, the pressurization temperature showed a direct relationship with the degree of gelatinization and the decreasing time for reach the complete gelation by pressure, in fact, when a starch-water suspension is heated and treated by 200 MPa, starch gelatinization could take place at a lower temperature range than when it is heated at 0.1 MPa (Douzals *et al.*, 1996). With respect with the pressurization time, there are contradictions, because it is well known that all the starch-water samples have to reach an energetic equilibrium to start the gelation process, however, this phenomena does not showed at linear behaviour in function of the time, for instance, Buckow *et al.*, (2007) demonstrated that with treatments from 1 to 20 minutes at 650 MPa, at 5 minutes the gelation and the maximum swelling was completed without any difference with the samples treated for 10, 15 and 20 min.

In the last 30 years, a great deal of research has been performed to gelatinize or modify starch from different origins as can be seen in Table I.5.

Table I.5. *Synthesis of main literature findings on the use of high pressure as a gelation process on starch suspensions.*

Source	Major findings	Reference
Corn, rice and potato starch	Gelatinization was reached by treatments above 500 MPa for 20 min for starch from corn and rice. Potato starch remained unchanged.	Hibi *et al.* (1993)
Corn, waxy corn, amylocorn, waxy rice, potato canna,	The pressure range in which the gelatinization starts and is completed depend on the starch type.	Stute *et al.* (1996)

lotus root, tapioca, taro, chestnut, pea starch.	B-type starch (Potato) was more pressure resistant than A-and C-type starches.	
Normal maize, waxy maize, high amylose maize, tapioca, and rice starches.	All the starches evaluated showed a complete gelatinization at 690 MPa for 5 and 60 min.	Katopo *et al*. (2002)
Potato starch, wheat starch, and tapioca starch	Pressure induced gelatinization was highly sensitive to process conditions. Potato starch was fully gelatinized at 700 MPa for 15 min at 50°C.	Bauer and Knorr (2005)
Potato starch	Potato starch only showed a complete gelatinization treated at 1000 MPa for 1 h at room temperature.	Kawai *et al*. (2007)
Corn starch	Corn showed a complete gelatinization at 650 MPa for 20 min at 40°C.	Buckow *et al*. (2007)
Normal rice, waxy rice, normal corn, waxy corn, tapioca, and potato starch	Waxy rice, corn and tapioca starches were fully gelatinized at 600 MPa for 30 min. Potato starch was less affected by the pressure treatment.	Oh *et al*. (2008)
Rice and waxy rice starch	At 600 MPa at >10 min, rice starches showed a complete gelatinization. Waxy rice gels showed a higher viscosity.	Oh *et al*. (2008)

Rice starch	Rice starch was completely gelatinized at 600 MPa for 30 min.	Li *et al*. (2012)
Red azuki bean starch	At 600 MPa red azuki bean starch was fully gelatinized.	Li *et al*. (2015)
Pea starch	HPP treatments at 600 MPa for 15 min at 25°C caused a complete gelatinization of the pea starch suspensions.	Leite *et al*. (2017)
Corn and quinoa	At 600 MPa for 5 min and 25°C, Quinoa and corn starch were gelatinized.	Li and Zhu (2018) Li *et al*. (2019)
Sweet potato flour	Sweet potato gels were formed at 600 MPa for 15 min at 25°C.	Cui and Zhu (2019)
Quinoa flour	HPP induced gelatinization of quinoa flour at 600 MPa for 1 h at 25°C.	Zhu and Li (2019)

Data reported in Table I.5 demonstrated the great of research about the application of HPP on starch suspensions to produce the physical modification of the starch as a pre-treatment or to induce their gelatinization. Even though, many efforts have been devoted to unravelling the role of chemical-physical characteristics of starches and HPP processing variables on gelatinization process, only a few investigations were carried out to individuate the potential use of such biopolymers as can be seen in the data reported in Table I.6.

Table I.6. *Literature findings of high pressure as an alternative method to produce starch-based hydrogels.*

Source	Process condition	Major findings	Reference
Potato and corn starch suspensions.	300 and 700 MPa 5 and 25 min at 25 °C	-HHP-hydrogels were produced for drug delivery (theophylline). -HHP-hydrogel from corn resulted in a gel exhibiting highly viscosity and sustained drug release. Authors claimed that the different pressure sensitivities permit the use of HPP as a selective non-conventional means	Szepes *et al*. (2008)

		of starch modification and well promote their application in drug formulation and development.	
Corn, waxy corn, amaranth and sorghum starch.	650 MPa 9 min 30°C Autoclaving	-Hydrogels were produced as potential carriers of theophylline. -Independent of the treatment, waxy corn HPP-hydrogel (amylopectin starch matrix) showed the better-controlled theophylline release profile. Authors claimed that the differences in the matrix morphology, porosity and the characteristics of the gels are governed by source origin.	Blaszczak *et al.* (2015)
Polyvinil(alcohol) DNA Heparine	980 MPa for 10 min at 37°C	PVA-nano Hydrogels crosslinked with DNA or heparin were produced by HPP treatment for biomedical applications.	Kimura *et al.* (2004; 2007; 2010; 2011; 2014)

Tapioca	600 MPa 10, 20 and 30 min 30, 50 and 80°C	Starch-based hydrogels from tapioca were produced by HPP process, with good mechanical and structural properties.	Vittadini *et al.* (2008)

Chapter I

Chapter II
Objectives of the work

From the literature study on high pressure processing (HPP) technology, previously schematised in Tables I.5 – I.6, it has been shown that to exposure starch suspensions to high pressure levels for short periods of time can produce either the modification or gelatinization of the system, thus allowing first, a clean and efficient production of starch-based hydrogels and second, to overcoming the major limitations of conventional gelation methods such as long duration of the process, high energy requirements and use of hazardous materials.

Although the use of HPP has shown evidence in inducing pressure-assisted gelation, there are still a series of "drawback to overcome".

In particular, even though many efforts have been devoted to unravelling the role of chemical-physical characteristics of starches and HPP processing variables on gelatinization process, however, only a few investigations were carried out to individuate the potential use of such biopolymers. Indeed, most of the studies were either focused on achieving the HPP gelatinization of several starches or to utilize HPP as pretreatment due to the modification of the microstructure of the starches, however, no studies on the evaluation of structural and mechanical properties of the hydrogels obtained at different processing conditions have been reported.

Additionally, and based on the reported results, which are generally obtained at lab-scale with several purposes, are in some cases contradictory due to the lack of standardization of the raw materials and processing conditions utilized. Moreover, the gelatinization phenomenon by HPP is a chemical-physical process governed by several factors and thermodynamic events whose concepts and fundamentals are still at nowadays unravelled.

Thus, there is a strong need to increase the knowledge on the topic not only to better understand the fundamentals of the gelation process but also to highlight the main factors affecting the production of starch-based hydrogels by high pressure. Moreover, the structural and mechanical properties of the starch-based hydrogels obtained by HPP as well as their performance should be deeply investigated in view of their future exploitation.

Therefore, the main objective of this Ph.D. thesis was to investigate the use of high pressure processing technology (HPP) for a standardized production of starch-based hydrogels in view of the future exploitation of these structures for innovative applications, as well as, a complete understanding of the factors involved in the hydrogel formation by pressure and their structural and mechanical properties have been also investigated. The factors considered to achieve the proposed objective of this PhD thesis are shown in the following scheme.

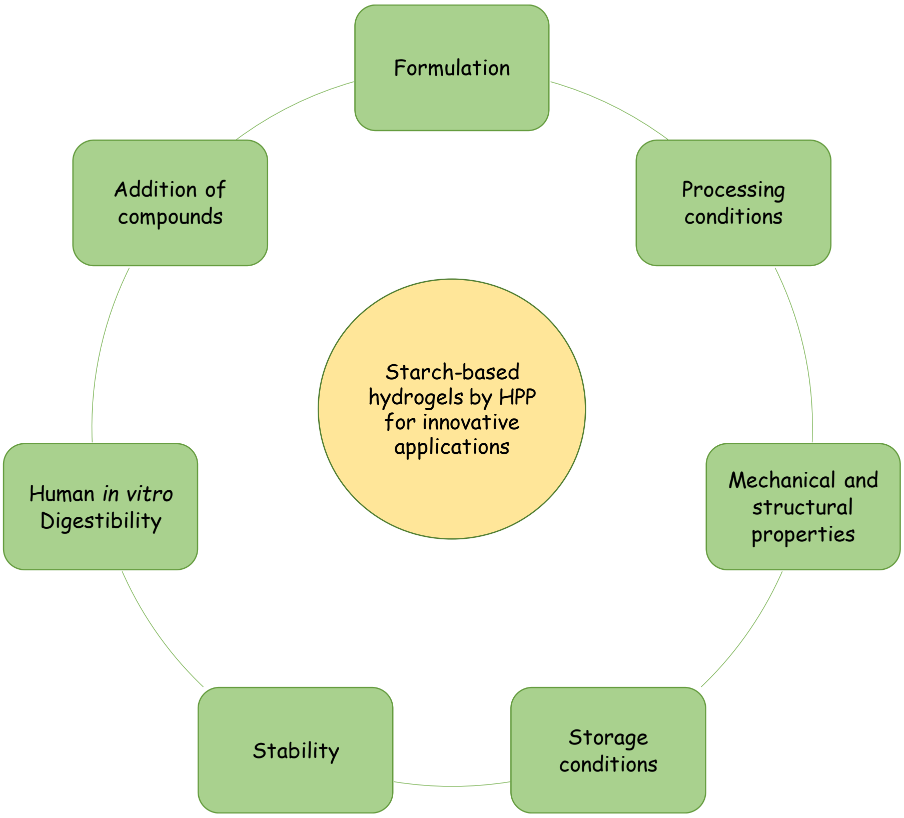


Figure II.1. *Schematic work plan of the PhD thesis investigation.*

As reported in Figure II.1, this thesis work was focused on several factors, and for the sake of achieving the attempted goals has been structured on 3 different sections:

- **Section I - “Starch-based hydrogels produced by high pressure processing: Preparation and characterization” (Chapter IV - V)**: in this section was evaluated the possibility to utilize HPP technology to produce hydrogels based on several starches identifying the process and products parameters and the structural and mechanical properties of the products obtained.
  In particular, in **Chapter IV** the pioneer production of potato HPP hydrogels identifying the most suitable conditions to obtain stable hydrogels focusing on product formulation namely starch-water concentration, particle size distribution and HPP processing conditions, as well as, a complete characterization of the structural and mechanical properties of the products obtained are presented and discussed.
  In **Chapter V**, the production of starch-based HPP hydrogels evaluating the influence of the type of starch (rice, corn, wheat, tapioca) and processing time on gel formation and physical, texture and rheological characteristics of hydrogels was performed.
  Results of Chapter IV-V allowed to produce starch-based HPP hydrogels in standardized conditions and to discriminate them in the function of their processing conditions and characteristics in view of further utilization. In particular, two different types of hydrogels the creamy-like structures and the compact rubbery structures have been identified.
- **Section II - “Starch-based hydrogels produced by high pressure processing: Physical stability and *in vitro* digestibility” (Chapters VI-VII)**: in this section, the profiling of the starch-based hydrogels in view of further innovative applications was attempted.
  In **Chapter VI**, the physical stability of the starch-based HPP hydrogels evaluated through long term and accelerated methodologies has been reported and discussed.
  Microbiological counts, organoleptic evaluation, swelling stability and texture were assessed for 90 days. Rheological measurements simulating stress conditions namely temperature changes and deformation have been performed in order to predict the physical stability of the starch-based hydrogels.
  In **Chapter VII**, the simulated human digestibility behaviour of the starch-based hydrogels in view of future oral applications was addressed. In this chapter, a complete in vitro digestibility model mimicking the human digestive system was implemented and tailored designed.

- **Section III - “Starch-based hydrogels produced by high pressure processing: Effects of compounds addition” (Chapter VIII)**: in this chapter the influence of compounds addition namely glycerol which is humectant agent on gel formation and on the characteristics of the hydrogels formed at different operating conditions were investigated. Moreover, a general consideration in terms of glycerol concentration/processing conditions to obtain functional and stable rice or corn HPP hydrogels are reported.
- **General conclusion and future perspective (Chapter IX):** the major conclusion regarding the starch-based hydrogels are discussed. Finally, future perspectives of these new novel biomaterials are presented in the frame of further innovative applications of the starch-based HPP hydrogels.

Chapter III
Materials & Methods

III.1 Raw materials

In this thesis work, five different starches have been selected as raw materials, and completely characterized and investigated as a natural source to produce hydrogels by the utilization of high pressure processing (HPP). In the following tables (Table III.1 and Table III.2) main characteristics and relevance of the selected starches are listed.

Table III.1 *Chemical and physical characteristics of the selected starches to be utilized as raw materials in this Ph.D. investigation.*

			Chemical composition (%)					Particle size distribution		
Starch	Provider	Code	Water	Fat	Protein	Ash	Amylose	Dv_{10} (µm)	Dv_{50} (µm)	Dv_{90} (µm)
Rice	Sigma-Aldrich (Italy)	S7260	7.2	1.2	0.5	0.3	17.7	2.9	14.6	62.2
Corn		S4126	8.3	1.7	0.34	0.6	21.2	8.12	14.9	23.4
Potato		S4251	13.5	0.8	0.05	0.3	25.8	19.5	45.5	83.8
Wheat		S5127	7.8	0.5	0.21	0.4	25.5	10.7	20.2	32.0
Tapioca	Rudolf sizing Amidos (Brazil)	-	9.6	0.6	-	0.6	20.3	7.0	12.7	19.2

Starches chemical composition was determined according to the methods 925.10, 922.06, 923.03, ascribed by the AOAC (2005) official guidelines for moisture, fat, protein and ash content on flour and starches, respectively. Amylose content was determined by an enzymatic rapid assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland). On the other hand, starches particle size distribution was determined by dynamic light scattering (DLS), using a Malvern Mastersizer 2000 instrument (Malvern Instruments Ltd., Worcestershire, UK).

Table III.2 *World production, articles published about, and price of the selected starches to be utilized as raw materials in this Ph.D. investigation.*

Starch	World production as a crop (million tons)[1]	N° Articles published[2]	Price (USD/ton)[3]	World starch production ranking[4]
Rice	769	9,045	600	5
Corn	1134	11,389	335	1
Potato	388	10,180	1943	3
Wheat	771	13,324	500	4
Tapioca	291	4,848	300	2

[1] Food and Agriculture Organization of the United Nations (FAO), World statistics, 2017
[2] Isi web of knowledge database, 2019.
[3] International Starch Institute, International Market Place, Seller prices, 2019.
[4] Evaluation of the community policy for starch and starch products, Report 2010.

In this PhD thesis work, the selection of these starches as raw materials arises, firstly, from their economic importance, being the most produced and traded biopolymers for food and non-food applications at a global scale. Secondly, the scientific relevance because of the versatility of these biopolymers which relies on their physical and chemical composition resulting in several research articles using them for numerous scientific and innovative developments. Thirdly, from their availability and industrial importance, starch after cellulose is the most abundant biopolymer in the world, cultivated all the yearlong, and the selection of these starches was because are the five most-produced starches at worldwide, due to its high importance on several industrial areas, such as food, pharmaceutical, cosmeceutical, paper, plastic, among others.

III.3 Experimental procedure

III.3.1 Samples preparation

For each experimentation performed in this PhD investigation, each starch powder (wet basis) was suspended in double-distilled water and dissolved under gentle mixing conditions. Always, to ensure sample homogeneity and avoid particles settling, starch suspensions were prepared immediately before treatments.

III.3.1.1 Evaluation of potato starch as a source for hydrogels production via HPP.

In this investigation, the effects of suspension formulation and processing conditions to obtain potato starch-based hydrogels by HHP were studied through a cascade methodology approach.

Potato starch was suspended in distilled water at different concentrations (10%, 20% and 30% (w/w)) and the suspension was formed under gentle mixing.

In order to test the effects of PSD on HPP hydrogels formation, samples of potato starch with different mean particle size were separated utilizing laboratory scale sieves with different openings (Endecotts LTD, London, England) coupled to a multidimensional high performance sieving machine (Pbi international, IG/3/Export, Milano, Italy).

Sieving was carried out on samples of native potato starch of 15 g each and sieving time was set at 2 min. The different fractions from sieving were collected as function of the particles diameter (dp) as follows: dp < 25 μm, 25 μm < dp < 36 μm, 36 μm < dp < 50 μm, 50 μm < dp < 63 μm, 63 μm < dp < 90 μm and dp > 90 μm. Only two fractions of potato starch were utilized for HHP hydrogel preparation: Medium granules (36 μm < dp < 50 μm) and small granules (dp < 25 μm), with weight fractions of 41% and 7%, respectively.

For the sake of comparison, also a sample of unsieved potato starch was used in the experiments. Each potato starch fraction was suspended in distilled water at 20% w/w and immediately subjected to HPP-treatments.

To evaluate the effects of combined HPP and thermal treatments at moderate temperature on hydrogel formation only potato starch suspensions (20% w/w) with small granule sizes (<25 μm) were used.

III.3.1.2 Evaluation of corn, wheat, rice and tapioca starch as sources for hydrogels production via HPP.

Wheat, rice or tapioca starch were suspended at a starch-water concentration of 20% (w/w) and thoroughly mixed, before the HPP-treatments.

III.3.1.3 Evaluation of the addition of compounds to the gelling suspension of starch-based HPP hydrogels.

Starch suspensions with a concentration of 20% (w/w) were obtained by dissolving rice or corn starch powder in distilled water by gentle mixing at room temperature (25 °C). Certain amounts of glycerol (Steinheim, Germany) were added to the suspensions in order to obtain two final concentrations, namely 5 and 10% (w/w). Starch suspensions without glycerol were also prepared. In order to avoid starch particle settling, starch-water-glycerol suspensions were prepared immediately before HHP treatments.

III.3.2 Samples processing

III.3.2.1 Thermal treatments (control samples)

In this PhD thesis, for the sake of comparison, thermal gels were produced as control samples. A rheometer TA instruments AR 2000 (TA instruments, New Castle, DE, USA) equipped with a starch cell and a starch pasting rotor (545,751.001) was used to produce gels by thermal treatments. The following processing parameters were used:
Step 1- temperature increase (in the temperature range 25–79 °C): Ramp rate: 2 °C/min, shear rate: 16 s^{-1});
step 2 - holding (constant temperature of 79 °C): holding time 5 min, shear rate: 10 s^{-1}) and (iii) step 3 - temperature decrease (in the temperature range 79–25 °C), ramp rate: 2 °C/min, shear rate: 0 s^{-1}.
Thermal gel samples produced at least in triplicate were kept at 25 °C until its further experimentation.

III.3.2.2 High pressure processing (HPP) treatments

For each test, 3 or 10 g of starch suspensions (w/w), with or without the addition of other compounds (in function of the features of the HP units, further described), were introduced in flexible containers made of a polymer/aluminium/polymer film (OPP30-A19-LDPE70), deaerated and sealed. Flexible pouches were thoroughly mixed and introduced in the vessel of the high pressure units. Pressurization, decompression rates, and the temperature of the pressure vessel were always set before the pressurizing process. In all cases to reach the set temperature, pouches containing the samples were kept in the vessel for 1 min before starting the compression phase.
All the HPP processing of samples was done at least in triplicate.

III.3.2.2.1 Evaluation of potato starch as a source for hydrogels production via HPP.

Plastic pouches containing 3 g of starch suspensions were thoroughly mixed and introduced in the high pressure multivessel unit U-111 (UNIPRESS-Polish Academy of Sciences, Warsaw, Poland). In this investigation, based on our experimental results (data not shown) and previous observations reported by Rubens *et al.* (1999) we utilized a pressure level of 600 MPa for 15 min, commonly used in industrial productions, which are suitable for an economically attractive industrial process, being the challenge of this investigation the successful production of potato starch hydrogels with the right formulation.

Pressurization and decompression rates were set at 8 MPa/s, while the temperature of the pressure vessel was set at 25 °C for all the experiments carried out to evaluate the effects of water concentration in the starch suspension and particle size distribution on gel formation, while in combined HP-thermal treatments the temperature of the vessels was set at 40 °C and 50 °C, respectively. HPP-treated samples were stored at 25 °C before further analyses.

III.3.3.2.2 Evaluation of corn, wheat, rice and tapioca starch as sources for hydrogels production via HPP.

In each test, flexible bags containing 10 g of starch suspensions were gently mixed and loaded in the pressure vessel of the HPP unit U-22 (Institute of High Pressure Physics, Polish Academy of Sciences, Unipress Equipment Division, Poland). HPP treatments were carried out at 600 MPa for 5 and 15 min at 25 °C with a compression rate of 8.4 MPa/s and a decompression time of < 15 s. All HPP-treated samples were stored at room temperature until further analysis.

III.3.3.2.3 Evaluation of the addition of compounds to the gelling suspension of starch-based HPP hydrogels.

Experiments were carried out utilizing the multivessel HHP system U111 (Unipress, Poland). Starch/water/glycerol samples (3g) packed in flexible pouches were thoroughly mixed and treated at pressure levels of 500 and 600 MPa at room temperature for 5, 10, 15 and 20 minutes. The pressurization rate was always set at 4.4 MPa/s, while the depressurization rate was 9.2 MPa/s. The temperature increase due to pressure build-up was 2-3 °C for all the experiments. HPP-treated samples were kept at 25°C until further analysis.

III.4 Experimental apparatus

The experimental campaign of this PhD investigation was performed in two different high-pressure systems which belong to the ProdAl facilities (Italy) and are specifically design for lab-scale high pressure operations. The main features and characteristics of the HPP units utilized are reported in detail in the following section.

III.4.1 High pressure processing unit U22

The system U22 (UNIPRESS-Polish Academy of Sciences, Warsaw, Poland) is a laboratory-scale unit for high-pressure research in chemistry, biology and food material sciences. This high- pressure unit holds a unique high pressure vessel with a maximum processing volume of 50 mL and can be operated in a wide pressure range between 0 and 1400 MPa (± 15 MPa) under thermal controlled conditions (0 – 120 °C). The temperature in the HPP vessel is set-up and controlled by an electronic temperature control unit (TCU) connected to the main unit with K-type thermocouples cables. The vessel is provided with electrical heaters to increase the temperature during treatment while the cooling of the vessel is obtained with compressed air. A control panel allows to set-up the operating pressure, the ramp rate by controlling the pump frequency, the processing time, as well as to control the opening and the closure of the HP vessel. Decompression process is controlled by an electronic valve and automatically set at rates of less than 15 seconds per processing operation. The pressure medium is Plexol (Bis (2-ethylhexyl) sebacate from Sigma-Aldrich (Italy) and the estimated temperature increase due to pressure build-up is 2-3 °C/ 100 MPa.

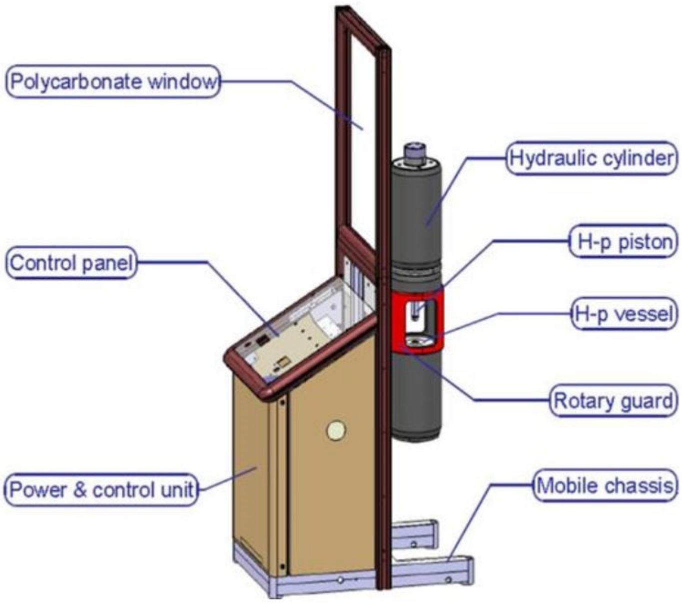


Figure III.1 Schematic representation of the main components of the experimental apparatus, HPP system U22 (Institute of High Pressure Physics, Polish Academy of Science, Unipress Equipment Division, Poland).

III.4.2 Multivessel high pressure unit U-111

The laboratory scale multivessel U111 HP unit (UNIPRESS-Polish Academy of Sciences, Warsaw, Poland), possess five high pressure vessels with a maximum processing volume of 9 mL. Experimental trials can be carried out in the HP system at pressures up to 700 MPa (± 2 MPa) under thermal controlled conditions (-40 – 100 °C).

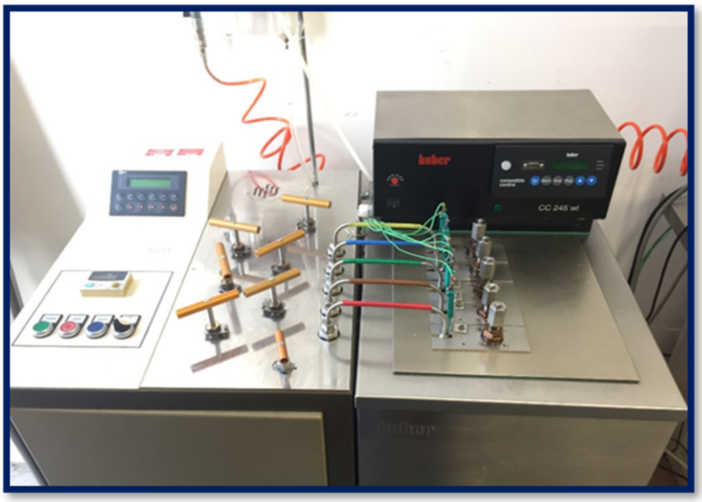


Figure III.2 Experimental apparatus, HPP multivessel system U111 (Institute of High Pressure Physics, Polish Academy of Science, Unipress Equipment Division, Poland).

The five high pressure reactors made of a highly conductive material with CuBe alloy and working in parallel, are immersed in a thermostatic bath (Huber CC245 wl, Offenburg, Germany) which ensures the internal equilibration of temperature during HP treatment. Each reactor is equipped with a manual cut-off valve and a K-type thermocouple, placed at the bottom of each reactor and able to measure the temperature of the liquid in contact with the samples.

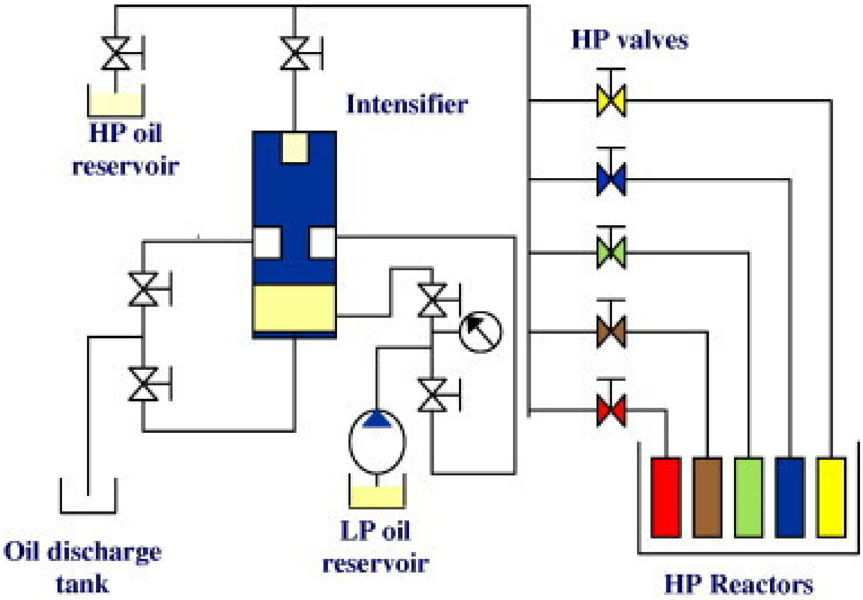


Figure III.3 Schematic view of the high pressure multivessel system U111.

The pressurization system consists of a hydraulic low-pressure pump connected to a high pressure intensifier, while a high pressure manual valve is used to release the pressure. The compression rate can be changed in the range between 2.5 and 25 MPa/s. A silicon oil (Huber thermofluid M40.165/220.10, Offenburg, Germany) is used as a pressurization medium and heating fluid of the thermostatic bath. Due to the technical features of the high pressure system, equipped with a control unit, which allows setting up the pressure level, set up, and control the pressurization ramp, start the filling of the intensifier and reactors, as well as to measure and control the pressure and temperature levels in the reactors. A data acquisition system, U111-DAS, records the pressure as well as the temperature measured in the reactors and that of the thermostatic bath.

III.5 Analytical determinations

III.5.1 Optical measurements

Samples were observed using an optical inverted microscope Nikon Eclipse (TE 2000S, Nikon instruments Europe B.V., Amsterdam, The Netherlands) equipped with a polarization filter, with a 10×, 20× and 40x objective, coupled to a DS Camera Control Unit (DS-5M-L1, Nikon Instruments Europe B.V, Amsterdam, The Netherlands) for image acquisition and analysis.

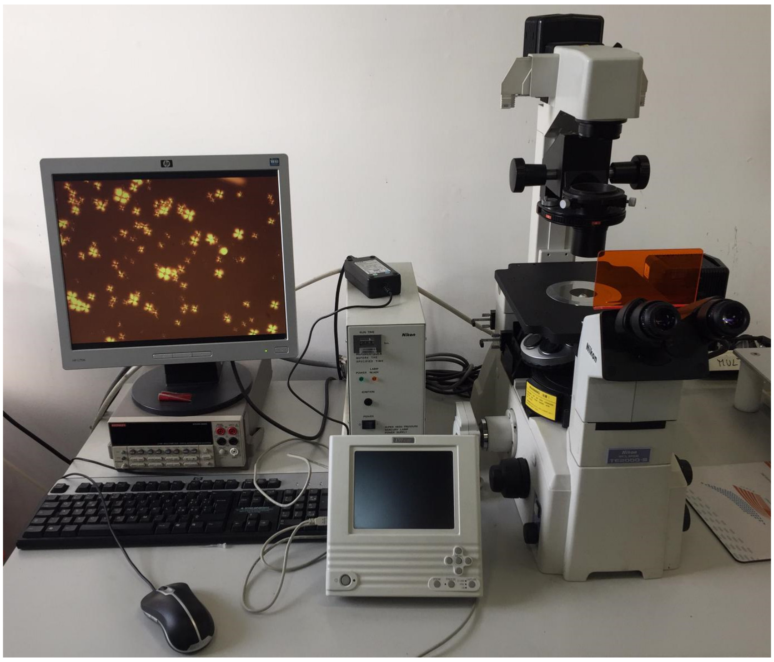


Figure III.4 Optical microscope (Nikon Eclipse TE 2000-S) coupled to a camera and computer control unit.

Before the observation, a small amount of sample was spotted on a microscope slide and covered with a cover glass. For the visual observation of starch microstructure and determination of granules optical size, the light microscope with the photographing option in the DS Camera Control Unit was used. The degree of gelatinization of samples was detected measuring the loss of the optical birefringence of starch granules under polarized light (20×), calculated according to the equation 1, developed by Buckow *et al*. (2007).

$$DG = \left(1 - \frac{NB}{N}\right) \times 100, \quad (1)$$

where: NB is the number of granules with birefringence and N the number of total granules.

The percentage of swelling increment of the granules in all samples was calculated according to equation 2:

$$\text{Swelling (\%)} = \frac{\textit{size after HPP treatment } (\mu m) - \textit{initial size } (\mu m)}{\textit{initial size } (\mu m)} \times 100 \quad (2)$$

The determination of granules optical size was performed from the images on the screen of the DS Camera Control Unit and utilizing the equation 3, developed to determine the perimeter of the ellipse, fitting with potato starch granules shape.

$$p \sim \frac{2\Pi\sqrt{(a^2 + b^2)}}{2}, \quad (3)$$

where: p is the approximated perimeter of an ellipse, a is the radius of the major axis and b the radius of the minor axis. For all the optical analyses, a statistically sufficient number of starch granules were considered (225).

III.5.2 Water holding capacity (WHC)

Water holding capacity (WHC) on the samples was determined according to equation 4:

$$\text{WHC (\%)} = \frac{\textit{weight of structured part (g)} - \textit{weight of dry starch (g)}}{\textit{weight of dry starch (g)}} \times 100 \quad (4)$$

All the WHC determinations were performed at least in triplicate on different samples.

III.5.3 Percentage of structured part

The % of the structured part or hydrogel formed in the samples was calculated using equation 5:

$$\text{Structured part (\%)} = \frac{\textit{Weight of structured part (g)}}{\textit{Total weight of sample (g)}} \times 100 \quad (5)$$

Percentages of structured part were calculated at least in triplicate on different samples.

III.5.4 Efficiency index (EI)

An efficiency index, defined by the authors as an empirical parameter to assess the extent of gelation under HPP conditions, was evaluated according to equation 6 as follows:

$$\text{Efficiency index} = \frac{\textit{Amount of hydrogel formed (g)}}{\textit{Initial weight of starch suspension (g)}} \quad (6)$$

where the amount of hydrogel formed is the quantity of the structured hydrogel formed (i.e. the hydrogel without free water obtained after HPP treatment), and the initial weight of the starch suspension (weight of the sample before HPP treatment). EI evaluations were determined at least in triplicate on different samples.

III.5.5 Swelling power (SP)

The swelling power was determined according to the method reported by Leach *et al*. (1959), slightly modified. 1 g of HPP hydrogel was centrifuged at 3200 rpm for 10 min, the supernatant was removed, and the pellet was weighed and dried for 6 h at 105 °C. The SP of samples was evaluated according to equation 7 as follows:

$$\text{Swelling power (g/g)} = \frac{\textit{Weight of the pellet (g)}}{\textit{Total weight of the sample (dry basis) (g)}} \quad (7)$$

SP evaluations were determined at least in triplicate on different samples.

III.5.6 Fourier Transform Infrared Spectroscopy (FT-IR) measurements

In order to study the structural characteristics of the samples, several FTIR spectra were obtained with an FT/IR-4100 spectrometer (Jasco Corporation, Kyoto, Japan).

Figure III.5 FTIR spectrometer (FT/IR-4100, Jasco Corporation, Kyoto, Japan) (A) coupled to an attenuated total reflectance (ATR) device (B).

Of samples, 0.3 mL was spotted at the centre of an IR transparent ZnSe optical disc of the ATR device. For each measurement, 128 scans were collected at 4 cm−1 resolution. The FTIR spectra of the samples were determined at wavelengths ranging from 1200–950 cm−1. Six samples from each experimental condition were used for each spectra measurement. The resultant starch-averaged spectrum was smoothed with a fifteen-point under adaptivesmoothing function to remove the possible noises, and then, baseline modification and normalized function were applied utilizing the manufacturer software Spectra Manager Version 2 (Jasco Corporation, Kyoto, Japan)**.**

III.5.7 Thermal Properties

The thermal properties of native and treated samples during this PhD investigation were evaluated in a differential scanning calorimeter (DSC) instrument (DSC 204 Phoenix, Netzsch, Wittelsbacherstraße, Germany).
Prior to experiments, DSC was calibrated for temperature and enthalpy using indium as a standard (T_m: 156.6 °C and ΔH_m: 28.45 J/g). For the analysis, 20 ± 0.01 mg of HHP-treated samples were placed in a 25 µL aluminium pan. Pans were hermetically sealed, and an empty pan was used as a reference. DSC measurements were carried out through an isothermal phase (25 °C for 3 min) and then scanned at a dynamic phase at 5 °C/min from 25 to 90 °C.

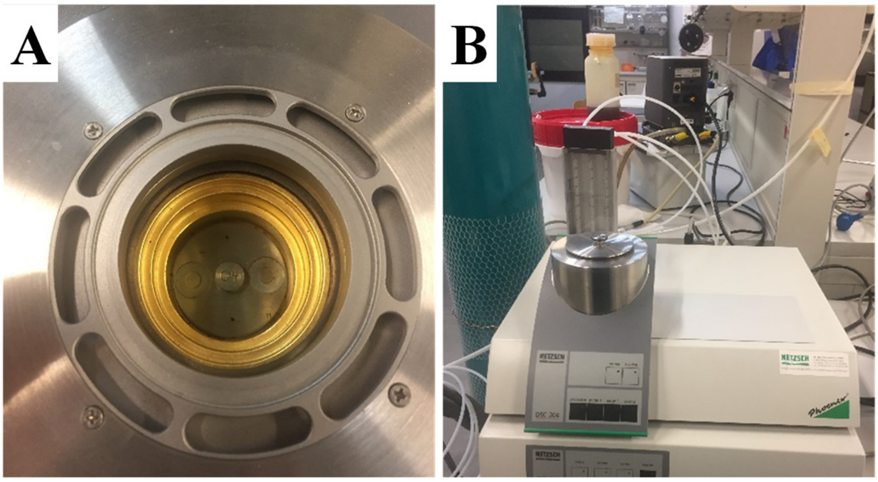


Figure III.6 Differential Scanning Calorimeter (DSC 204 Phoenix, Netzsch, Wittelsbacherstraße, Germany) (A) coupled with an oven thermally controlled by a water-air system with sample and reference option (B).

Denaturation temperature (T_d) and denaturation enthalpy (ΔH_d) were estimated by measuring the area under the DSC transition curve with the manufacturer software Proteus Analysis Software (Version 4.2/3, Netzsch, Wittelsbacherstraße, Germany). All the DSC measurements were done in triplicate.
% of gelatinization was calculated using equation 8 reported by Blaszczak *et al*. (2007)

$$\%\ \text{Gel} = \frac{\Delta H_{ns} - \Delta H_{ts}}{\Delta H_{ns}} \qquad (8)$$

where ΔH_{ns} and ΔH_{ts} were the gelatinization enthalpies of native and treated samples, respectively.

III.5.8 Rheological Measurements

Rheological determinations of the samples were carried out in a controlled stress and strain rheometer (AR2000, TA instruments, New Castle, DE, USA), thermally regulated by a Peltier plate and a circulating water bath (DC10-Haake K10, Karlsruhe, Germany) (Figure III.7).

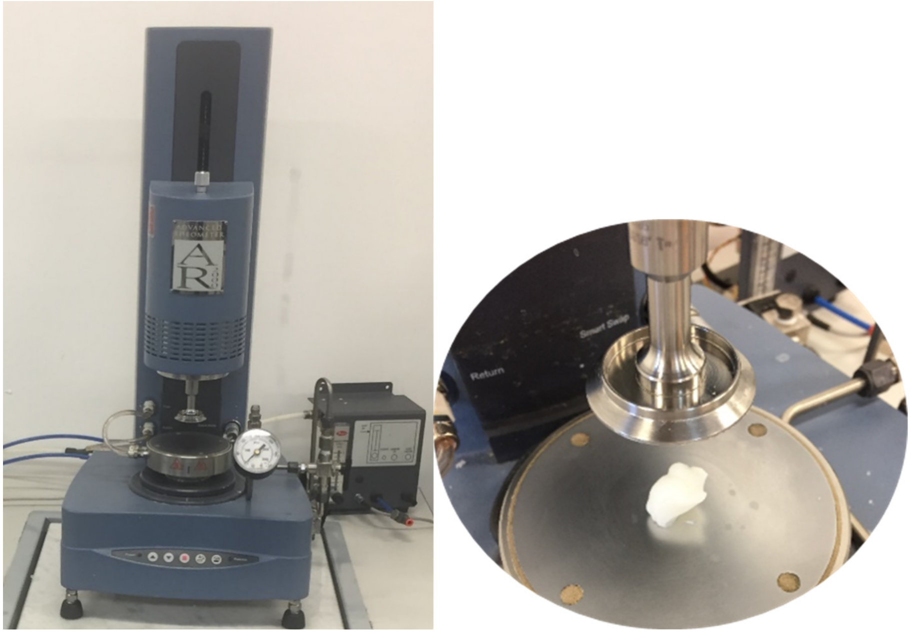


Figure III.7 Rheometer equipped with a plate-cone geometry.

The instrument was fitted with a plate-cone geometry (40 mm diameter, 2°) with a fixed gap of 52 μm. For the analysis, 1.0 g of the sample was put on the centre of the rheometer plate and kept at fixed measurement temperature (25 °C) for 2 min to allow stress relaxation and temperature equilibration. The experiments were carried out in triplicate. A new sample was used for each determination. Different types of tests were carried out to determine the rheological behaviour of the samples, as described in detail in the following sections. The same geometry described above was used in all the measurements.

III.5.8.1 Steady-state flow

To characterize the system the steady-state flow mode was used. Steady-state flow tests were carried out in all the samples at 25 °C. The apparent viscosity (η) and shear stress (γ) of the samples were determined in the range of shear rates between 0.1 and 100 s−1. The equilibration time of the samples was set at 120 s.

III.5.8.2 Oscillatory rheology

Oscillatory rheological measurements (dynamic rheology) were performed to complete the information on the viscoelastic properties of the samples produced in this investigation. This oscillatory mode makes it possible to describe the viscoelastic behaviour of the samples under different stress conditions such as different frequency, oscillatory and temperature range. The different types of tests carried out to determine the viscoelastic behaviour of the samples, as described in detail in the following sections.

III.5.8.2.1 Frequency sweep test

Mechanical properties were obtained from frequency sweep tests recorded at 25 °C. A continuous oscillation at fixed controlled strain (3%) was utilized. The amplitude of deformation was kept constant while the frequency was changed in a selected range (0.1 to 100 rad/s). The equilibration time of the samples was set at 120 s. The storage modulus G’ and loss modulus G” as a function of frequency of the samples were recorded.

III.5.8.2.2 Stress sweep test

The structural strength of the samples was obtained from stress sweep tests recorded at 25 °C. At a continuous frequency value (1 Hz) oscillation stress was increased from 0.01 to 1000 Pa and the storage modulus G’ and loss modulus G” as a function of the linear viscoelastic response of samples were recorded. The equilibration time of the samples was set at 120 s.

III.5.8.2.3 Temperature sweep test

In order to predict the physical stability of samples, a temperature sweep test was performed. The storage modulus G’ response under different temperatures range (25 to 80°C) was monitored at a temperature rate of 1°C/min. Frequency and oscillation stress were kept constant at 1 Hz and 1 Pa, respectively.

III.5.9 Colour measurements

Colour parameters, namely lightness (L*), redness (a*) and yellowness (b*), of samples, were determined by the colourimeter CR-400 (Konica Minolta Inc., Tokyo, Japan). The colour difference (ΔEab*) was calculated according to equation 9, as reported by Bodart *et al.* (2008).

$$\Delta E_{ab}{}^* = \sqrt{(\Delta_L{}^*)^2 + (\Delta_a{}^*)^2 + (\Delta_b{}^*)^2} \qquad (9)$$

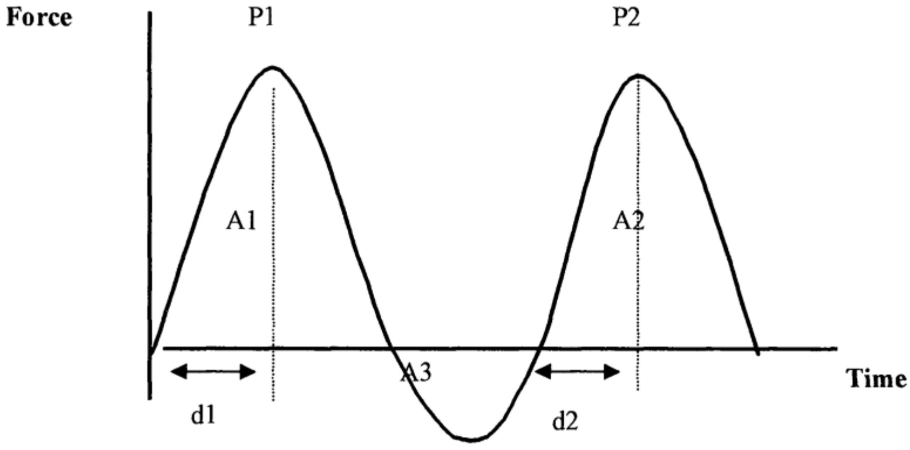
The whiteness index (WI) was calculated according to Equation 10 reported by Kaur *et al*. (2013).

$$WI = 100 - \sqrt{(100 - L^*)^2 + (a^*)^2 + (b^*)^2} \qquad (10)$$

At least ten measurements were done on each sample.

III.5.10 Texture profile analysis (TPA)

Texture profile analysis (TPA) of samples was performed by a TA.XT2 texture analyser (Stable Micro Systems, Surrey, UK) equipped with a load cell of 5 kg, connected to a microcomputer.
6 g of hydrogel samples were loaded into a cylindrical cell (24 mm height and 25 mm ID) and tests were carried out with a cylindrical probe (10 mm diameter). Penetration test of the sample, at a rate of 1 mm/s, was stopped when 50% of the height was reached and repeated two times to generate force-time curves. Firmness, springiness, chewiness, cohesiveness, and gumminess of hydrogels were calculated from the penetration data recorded (Figure III.9). All the tests were carried out at least in triplicate.



Primary parameters
P1 = Peak height 1 = TPA hardness
A1 = Area 1
P2 = Peak height 2
A2 = Area 2
A3 = Area 3 = TPA adhesiveness

Secondary parameters
TPA springiness = d2/d1
TPA cohesiveness = A2/A1
TPA gumminess = P1 x (A2/A1)
TPA chewiness = P1 x (A2/A1) x (d2/d1)

Figure III.9 Force-time curve output produced by instrumental TPA and interpretations.

III.5.11 Human *in vitro* digestibility measurements

To evaluate the digestibility of starch-based hydrogels throughout human gastrointestinal tract, an in vitro human gastrointestinal digestion model consisting of three phases, oral, gastric and small intestine was used in this study, based on literature findings (Lopez-Pena *et al* 2016; Mun *et al*, 2015; Margareth, 2011; Lyliam and Lopez-Pena, 2016; Dartois, 2011; Hu, 2014; Mun, 2015).

III.5.11.1 Oral phase

In order to determine the effects of the oral phase on the digestion of starch-based HPP hydrogels, a complete simulation of the mastication and swallowing processes was implemented.

10 g of the samples were mixed in a 1:1 ratio to simulated saliva fluid (SSF), composed of a mixture of α-amylase 2 g/L, sodium chloride 0.117 g/L, potassium chloride 0.149 g/L, sodium bicarbonate 2.1 g/L, dissolved in double-distilled water at 37 ºC.

Rice starch hydrogels were subjected to a physical disruption process in a controlled homogenizer (Stomacher 400, Steward, England) at 200 rpm for 1 minute, simulating the mastication and swallowing process of the oral phase, according to the method reported by Mun *et al*. (2016). Based on considerations regarding the appearance of tapioca hydrogels samples, prior to the oral phase digestion step, a size reduction step emulating higher mastication forces was applied according to Jalabert-Malbos & Mishellany-Dutour (2007). The authors stated that for rubber-like food, during mastication particles reach sizes smaller than 2.3 mm before swallowing. Furthermore, the samples were cut into 2.3 mm pieces with a bistoury, and then, the oral digestion phase was performed following the procedure above-mentioned for rice starch hydrogels.

III.5.11.2 Gastric phase

The entire sample from the oral digestion step was mixed (1:1 ratio) to a simulating gastric fluid (SGF), consisting of a mixture of 2 g sodium chloride, 7 mL of hydrochloric acid 37% and 3.2 g of pepsin, dissolved in 1 L of double-distilled water at 37 ºC, adjusted to a pH of 1.4. The pH of the resulting mixture was adjusted to 2.5 with NaOH 0.25 N, then the mixture was placed under constant mixing at 100 rpm for 120 minutes at 37 ºC (Orbital incubator SI50 system, Bibby Sterilin LTD, UK).

III.5.11.3 Intestinal phase

Prior to the intestinal phase step, the pH of the gastric mixture was adjusted to 6 with NaOH 0.25 N. 10 mL of simulated gastric fluid (SIF) consisting of bile salts, 25 g/L, calcium chloride. 0.2 g/L, sodium chloride, 1.75 g/L and pancreatin, 5 g/L, were added to the gastric mixture with adjusted pH. The resulting mixture was mixed at 100 rpm for 120 min at 37 ºC.

III.5.11.4 Disintegration extent

To quantify the physical disintegration of starch-based hydrogels in each simulated digestion phase, an empirical evaluation was performed. Based on the different phases present in the suspension, the separation of the solids, that is the structure of hydrogels remaining not hydrolysed after each digestion step, was performed by separation due to gravity using a sieve with a mesh of 600 µm (Endecotts LDT, London, England). This procedure was used after each in vitro digestion phase. The percentage of physical disintegration of the starch-based hydrogels was calculated according to equation 11:

$$\% D_s = \frac{(W_o - W_s)}{W_o} \times 100, \quad (11)$$

where Wo is the weight of the total sample and Ws is the weight of the solids remaining on the sieve. All the determinations were carried out in triplicate.

III.5.11.5 Determination of total starch content in suspension

Evaluate the amount of hydrolysed starch in each phase of the in vitro gastrointestinal digestion, it was necessary to determine the total amount of non-hydrolysed starch in the suspensions. Total starch (TS) content in the suspensions was determined by a commercial kit (Megazyme K-TSHK, Wicklow Ireland). An aliquot of 2µL of the liquid suspension was taken after the oral phase, gastric phase, 20 and 120 minutes of the intestinal phase weighted, and the weight of the sample was recorded. Immediately after, the sample was mixed with 10 mL of ethanol 80% and heated at 80-85 ºC for 5 minutes to inhibit the enzymatic activity. The mixture was then centrifuged at 4000 rpm for10 minutes and the supernatant was discarded. This step was repeated twice in order to eliminate the sugars remaining in the sample.
The precipitated sample was suspended in 2 mL of NaOH 1.7 M and stirred on a vortex mixer until complete dilution. 8 mL of sodium acetate buffer (600 Mm) containing 5 mM of calcium chloride, 0.1 mL of thermostable α-amylase and 0.1 mL of amyloglucosidase were added to the mixture, which was then incubated in a water bath (PBI international N. 54270, Milan Italy) for 30 minutes at 50 ºC. The solution was then removed from the water bath and

cooled at room temperature. 2 ml of the sample was loaded in a microfuge tube and centrifuged at 13.000 rpm for 5 minutes. A 0.1 mL aliquot was then transferred into a Falcon tube and 3 mL of GOPOD reagent were added. The resulting mixture was incubated for 20 minutes at 50 ºC. The absorbance of the solution was measured at 510 nm against a blank consisting of 0.1 mL of sodium acetate buffer 100 mM and 3 mL of GOPOD reagent.
The percentage of total starch in the sample was calculated according to equation 12, which was provided by the kit manufacturer.

$$TS\,\% = \Delta A \times F \times \frac{EV}{0.2} \times \frac{100}{W} \times \frac{1}{1000} \times \frac{162}{180} \quad (12)$$

where:
ΔA is the absorbance of sample solution read against reagent blank;
F is a factor to convert absorbance values to mg glucose (100 mg glucose divided by the GOPOD absorbance value obtained for 100 mg of glucose);
EV is the sample extraction volume (equal to 10.4 mL in our case);
0.2 mL is the volume of sample analyzed.
100/W is the conversion factor to 100 mg of sample and W is the sample weight (in mg);
162/180 is the conversion factor from free glucose, determined in the sample, to anhydroglucose, occurring in starch.
All the determinations were performed at least in triplicate.

III.5.11.6 Hydrolysation extent

In the human body, starch is hydrolysed by the enzymatic action of α-Amylase present in saliva and intestinal fluids. After starch hydrolysis, molecules of glucose, maltose and maltotriose form which are absorbed through the walls of the small intestine (Kingman, 1992). The percentage of hydrolysed starch in the different digestion phases was calculated according to equation 13.

$$\%H_s = \frac{S_o - (R_s \times X_{so} + V_s \times TS)}{S_o} \times 100, \quad (13)$$

where:
S0 is the initial amount of starch in the sample (g);
Rs is the weight of remaining structure after each phase (g);
XS0 is the initial concentration of starch in the sample (in % w/w);
Vs is the total volume of suspension (in mL);
TS is the total starch content in the suspension (in g/mL).
All the determinations were performed in triplicate.

III.5.11.7 Determination of nutritional fractions

Starch can be classified by its digestibility in the human body, more specifically in the small intestine. For nutritional purposes, starch is classified into rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS), depending on the rate and degree of its assimilation (Kingman, 1992).

The values of RDS, SDS and RS fractions in each sample were obtained according to equation 14, equation 15, and equation 16:

$$\mathrm{RDS}\ (\%) = \frac{H_{20}}{TS} \times 100, \quad (14)$$

$$\mathrm{SDS}\ (\%) = \frac{H_{120} - H_{20}}{TS} \times 100, \quad (15)$$

$$\mathrm{RDS}\ (\%) = \frac{TS - H_{120}}{TS} \times 100, \quad (16)$$

where, H_{20} and H_{120} are the amounts of starch hydrolysed within 20 and 120 min of the intestinal digestion step, respectively, and TS is the initial weight of starch in the sample.

III.5.12 Microbiological analysis

All samples were analysed for the number of mesophilic aerobic microorganisms and yeasts and moulds. Each bag containing 10 gr of starch-based hydrogel was aseptically opened and homogenised with 90 mL chilled maximum recovery diluent (Oxoid, Basingstoke, Hampshire, England) in an aseptic stomacher bag using a homogenisation system (Seward Laboratory, London, UK) at 260 rpm up to complete samples homogenisation. Further decimal dilutions were prepared with the same diluent and plated on appropriate media. In order to enumerate aerobic mesophilic microorganisms, 1.0 mL of each dilution was pour-plated in Plate Count Agar (PCA, Merck, Darmstadt, Germany) and incubated at 30°C for 72 h. To count the yeasts and moulds, 1.0 ml of each dilution was spread-plated on Dichloran Rose Bengal Chloramphenicol medium agar (DRBC, Oxoid, Basingstoke, Hampshire, England) and then incubated for 3-5 days at 25°C.

The microbial colonies obtained were identified by standard methods. All microbiological data were transformed into the number of colony-forming units (log CFU/g). Detection limit was always 10 CFU/g. No colonies detection an arbitrary value of 1 log CFU/g was assigned.

III.6 Statistical analysis

All experiments and analysis of results obtained in this investigation were performed at least in triplicate and mean values and standard deviations (SD) of experimental data were calculated. Statistically significant differences ($p \leq 0.05$) among the averages were evaluated using one-way analysis of variance (ANOVA), and Fisher least significant difference (LSD) test using Statgraphics Centurion XVI Statistical Software (Statistical Graphics Corp., Herdon, USA).

Section I

Starch-based hydrogels produced by high pressure processing: Preparation and characterization

Chapter IV - *Potato starch hydrogels produced by high pressure processing (HPP): A first approach*

Chapter V - *Starch-based hydrogels produced by high pressure processing (HPP): Effect of type of starch and processing time*

Chapter IV
Potato starch hydrogels produced by high pressure processing (HPP): A first approach

Abstract - Starch-based hydrogels have received considerable interest due to their safe nature, biodegradability, and biocompatibility. The aim of this study was to verify the possibility of producing natural hydrogels based on potato starch by high pressure processing (HPP), identifying suitable processing conditions allowing to obtain stable hydrogels, as well as to characterize structural and mechanical properties of these products. Sieved (small size granules and medium size granules) and unsieved potato starch samples were used to prepare aqueous suspensions of different concentrations (10 - 30% w/w) which were processed at 600 MPa for 15 min at different temperatures (25, 40, 50 °C). Products obtained were characterized by different techniques (light and polarized microscopy, Fourier transform infrared spectroscopy (FTIR), rheology and differential scanning calorimetry (DSC)). Results obtained so far demonstrated that potato starch suspensions (20% starch-water concentration (w/w)) with granules mean size smaller than 25 μm treated at 600 MPa for 15 min and 50 °C showed a complete gelatinization and gel-like appearance. Potato HPP hydrogels were characterized as a highly structured profile (G' >> G''). Moreover, their FTIR spectra, similarly to FTIR profiles of thermal gels, presented three absorption bands in the characteristic starch-gel region (950-1200 cm-1), whose intensity increased with decreasing the particle size and increasing the processing temperature. In conclusion, potato starch hydrogels produced by HPP in well-defined processing conditions exhibited excellent mechanical properties, which can be tailored according to the requirements of the different applications envisaged.

Chapter IV

IV.1 Introduction

Hydrogels represent a group of materials, composed by three-dimensional crosslinked networks of hydrophilic/hydrophobic polymers, capable to absorb and retain a significant amount of water (Biduski *et al*., 2018).

They have been listed as "smart structures" whose tailor-made design confers them different functional attributes to be used in the design, synthesis and self-assembly of novel biomaterials and drug delivery systems (Mahinroosta *et al*., 2018; McClements, 2017). Since their first application in the production of contact lenses, reported by Wichterle and Lim (1960), nowadays hydrogels are widely used in several applications such as tissue engineering, controlled drug delivery, agriculture, bioactives protection, water purification, among others (McClements, 2017; Nieuwenhove *et al*., 2017; Garcia-Astrain and Avérous, 2018; Xiao *et al*., 2017; Qi *et al*., 2017).

The production of hydrogels using natural or synthetic polymers has been extensively investigated and, in general, involves intermolecular interactions, covalent and non-covalent interactions induced by physical or chemical treatments, being the crosslinking method and the graft polymerization the most traditional processes (Ali and Ahmed, 2018; Mun *et al*., 2015; Caló and Khutoryanskiy, 2015). Hydrogels produced from natural ingredients such as alginate, gelatine, cellulose, chitosan, and starch (Biduski *et al*., 2018; Chan *et al*., 2009; Gattás-Asfura *et al*., 2005; Li *et al*., 2008; Moura e al., 2007; Yamazaki *et al*., 2009; Varaprasad *et al*., 2010; Peng and Chen, 2011; Chang *et al*., 2010; Zhou *et al*., 2007), have received increasing attention in the last years, due to their safety, biocompatibility and biodegradability (Ismail *et al*., 2013). The utilization of starches has been receiving increasing attention due to the abundance, availability, cost-effectiveness, manageability, high swelling capacity, and the versatility of this kind of materials (Ismail *et al*., 2013). Rice, potato, corn, and other starches, in fact, have been successfully utilized to produce starch-based hydrogels (Biduski *et al*., 2018; Xiao, 2013; Yoshimura *et al*., 2006).

In general, the production of starch-based hydrogels involves chemical cross-linking or physical gelatinization to form a stable three-dimensional network (Biduski *et al*., 2018). By heating a water suspension with a certain starch concentration, gelatinization occurs, being the main structural change occurring to starch granules (Schirmer *et al*., 2015). Structural changes take place at defined process conditions, according to the characteristics of the starch source (Jenkins and Donald, 1998). Gelatinization is a phase transition process from an ordered to a disordered status (Szepes *et al*., 2008). Initially, fast swelling of starch granules is observed, due to particles hydration, while during heating amylose leaching and granules disintegration are likely to occur. Finally, during the retrogradation stage, hydrogels form due to the recrystallization and reorganization of the polysaccharide structure (Ismail *et* *al*., 2013). Several techniques, including differential scanning calorimetry (DSC), Fourier transform infrared spectroscopy (FTIR), optical microscopy and rheology have been used to detect phase transition, structural changes and mechanical characteristics of starches during and after the gelatinization step (Schirmer *et al*., 2015; Carlstedt *et al*., 2015; Xie *et al*., 2009; Van Soest *et al*., 1995).

Starch gelatinization can be also achieved by applying innovative technologies, such as high pressure processing (HPP), at more suitable processing conditions than those of traditional methods (Blaszczak *et al*., 2015; Kawai *et al*., 2012). HPP, the most diffused non-thermal technology at industrial level, is mainly used for microbial inactivation in liquid, solid and particulate foods. It is well known that HPP causes the disordering of biopolymers, including proteins and starches, due to the modifications of non-covalent intermolecular interactions such as those inducing pressure-assisted gelatinization (Balny, 2002). Under high pressure, starches undergo morphological and structural changes, exhibiting different gelatinization extent, with intact granules and limited swelling of starch particles remaining after treatment, and different rheology compared to thermally treated starches (Buckow *et al.,* 2009; Hibi *et al*., 1993; Li *et al*., 2012). In the last 20 years, many studies have been carried out to evaluate the modifications and gelatinization of starches from different plant sources induced by HPP (Yang *et al*., 2017). It has been shown that the effects of HPP on starches depend on the type of starch, the water concentration in the suspension and processing conditions, namely pressure level, temperature and processing time (Pei-Ling *et al*., 2010; Bauer and Knorr, 2005). It has been observed that, at room temperature, HPP treatments at 600 MPa cause the complete gelatinization of starches (wheat, rice, beans, corn, quinoa, tapioca) in aqueous suspensions (Kawai *et al*., 2012; Li *et al*., 2012; Stute *et al*., 1996; Katopo *et al*., 2002; Buckow *et al*., 2007; Oh *et al*., 2008; Li *et al*., 2015).

As far as potato starch is concerned, it has been demonstrated that this starch shows high resistance to pressure-induced gelation, thus very high-pressure levels are required to achieve the complete gelatinization of potato starch-water suspensions (Kawai *et al*., 2012; 2007a; 2007b). However, playing potato starch a very important role in the food industry as coating, blending, bulking, thickening agent and, more recently, as gel-hardener, a deeper understanding of its behaviour during HPP assisted gelation would be of utmost importance for future industrial application of HPP hydrogels (Vasanthan *et al*., 1999). Muhr and Blanshard (1982), proved that to obtain the complete gelatinization of potato starch aqueous suspensions (12.8% w/w) at 23 °C pressures of about 800 MPa were needed. Bauer and Knorr (2005) achieved the complete gelatinization of 5% (w/w) potato starch aqueous suspensions at processing conditions of 700 MPa for 15 min only by applying a simultaneous thermal treatment at moderate temperature (50 °C).

Other authors demonstrated that HPP treatments of potato starch suspensions at 1000 MPa and different treatment times were effective to achieve gelation only with holding times well above one hour (Kawai *et al*., 2007a; 2007b).

To the best of our knowledge, while many efforts have been devoted to understanding and achieve the HPP gelatinization of potato starch, no studies on the evaluation of structural and mechanical properties of the hydrogels obtained at different processing conditions have been reported in the literature. Thus, the aim of this study was verifying the possibility of producing potato starch HPP-hydrogels, identifying the most suitable processing conditions to obtain stable hydrogels, and determine structural and mechanical properties of the products obtained.

This investigation provides information allowing to forecast future further applications of this kind of materials.

IV.2 "Short" Materials and Methods

IV.2.1 Raw materials and samples preparation

Potato starch powder was utilized in this experimental work. The chemical and physical characteristics of potato starch, as well as, the provider was reported in § III.1 and Table III.1. Samples preparation carried out in this work were accurately described in § III.3.1.

In this investigation, the effects of suspension formulation and processing conditions to obtain potato starch-based hydrogels by HPP were studied through cascade methodology approach. All the operations related to preparing samples to evaluate the effects of starch-water concentration, particle size distribution and combined HPP and thermal treatments were accurately described in § III.3.1.1.

IV.2.2 Samples processing

HPP treatments of samples upon the different operations framed in this work were reported specifically in samples processing section (§ III.3.2) for control samples (§ III.3.2.1) and HHP-treated samples (§ III.3.2.2.1), respectively. The experimental apparatus utilized on HPP treatments of samples was the HP unit U-111 described in § III.4.2. All the experiments were carried out at least in triplicate.

IV.2.3 Analytical determinations

Control and HPP-treated samples were qualy-quantitatively analysed in terms of optical measurements, structural, thermal and rheological properties, as previously described in § III.5.1, § III.5.6, § III.5.7 and § III.5.8, respectively.

IV.3 Results and discussion

IV.3.1 Effect of starch-water concentration on gel formation and structural properties of potato starch suspensions by HPP.

The results of gel formation of native and pressurized potato starch suspensions with different starch-water concentrations are reported in Table IV.1.

Table IV.1 *Optical and physical parameters of untreated and HPP treated (600 MPa for 15 min at 25 °C) potato starch suspensions at different starch concentration.*

	Starch-water concentration		
	10% w/w	**20% w/w**	**30% w/w**
Samples	Average granule size (μm)		
Native	50.9 ± 24.4^{a}		
HPP-treated	59.9 ± 32.4^{Ab}	62.5 ± 20.9^{Ab}	56.7 ± 27.1^{Aa}
HPP-treated	Swelling %		
	17.7	22.8	11.4
	% of Gelatinization		
	18.5 ± 8.2^{A}	16.6 ± 7.5^{A}	12.6 ± 6.9^{A}
	WHC		
	70.1 ± 2.3^{A}	71.2 ± 2.6^{A}	48.2 ± 6.3^{B}
	% of structured part in the sample*		
	33.5 ± 2.6^{B}	69.9 ± 6.3^{A}	58.3 ± 7.1^{A}

All values are means of 225 (granules) determinations ± SD.
(a,b) Values in the same row with different superscripts differ significantly ($p<0.05$; LSD).
(A,B) Values in the same column with different superscripts differ significantly ($p<0.05$; LSD).
*The % of structured part refers to weight of structure formed after HPP without free water against total weight of the sample before HPP treatment (Eq.5, § III.5.3).

Starch suspensions with concentrations in the range 10 - 30 % (w/w) were used to investigate gel formation under high pressure conditions according to the literature (Kawai *et al*., 2012; 2007a; 2007b). At the HPP treatment conditions utilized in this work, namely 600 MPa for 15 min, a slight increase of particle size was observed, due to starch granules hydration as well as the partial gelatinization of starch suspensions. However, these results were independent of starch concentration ($p>0.05$) (Table IV.1). This is in agreement with experimental findings reported previously in the literature, underlining the baroresistance of potato starch under certain pressure conditions due to the chemical structure of this material (B-type starch) (Bauer and Knorr, 2005; Stute *et al*., 1996; Katopo *et al*., 2002; Oh *et al*., 2008; Muhr *et al*., 1982; Blaszczak *et al*., 2005b).

The independence of the extent of gelatinization on starch concentration observed in this study is slightly in agreement with the results reported by Kawai *et al*., (2012; 2007a; 2007b). The authors reported that with increasing the water contents of the starch suspension the degree of gelatinization was increasing. However, it should be emphasized that the processing conditions utilized in their work were by far more severe (1000 MPa for 1 h to 66 h and 40 °C). In our case, it can be assumed that a holding time of 15 min at a pressure level of 600 MPa was not sufficient to produce statistically significant differences among the potato starch suspensions at different water concentrations as far as % of gelatinization is concerned. Although the visual observation of samples could allow concluding that at lower starch concentrations more extensive gelation was occurring, however, analysing the standard deviations there is no evidence of inhomogeneity among all partially gelatinized samples. Water holding capacity of starch granules (WHC) and % of the structured part in the sample could be considered as an indirect measurement of the amount of structured material formed under pressure. Analysing the data reported in Table IV.1 it can be concluded that water holding capacity of granules (WHC) and percentage of gel-structure of samples with 20% (w/w) starch-water concentration were higher ($p<0.05$), demonstrating that suspensions with this starch-water content were more prone to gelation at the processing conditions tested.

The effects of starch concentrations on structural properties of samples were evaluated also by FTIR. Fourier transform infrared spectroscopy (FTIR) is a vibrational technique, which provides useful information on the conformational structure of food components (Larrea-Wachtendorff *et al*., 2015) and starch gel structures characterization (Van Soest *et al*., 1994; 1995; Warren *et al*., 2016; Flores-Morales *et al*., 2012; Wilson *et al*., 1988).

In Figure IV.1 have reported FTIR spectra of HPP-treated samples with different starch concentration (10-30%). Spectra of thermal gels (control) with the same starch concentrations were also shown (red lines) as a reference (pattern of well-structured gels). In the wavelength range 950 - 1200 cm-1, it is possible identifying bands characteristic of starch gelation (Rubens *et al*., 1999). According to Rubens *et al*., (1999), the bands of the infrared spectra in this range of wavelengths mainly account for C-O stretching of the ring and linkage of (C-O-C) and COH groups. Spectra variations due to pressure gelation can be determined individuating changes of intensity, changes of bandwidth and frequency shifts, being the change of intensity, the main effect occurring during gelation. In this study, the changes of intensity of peaks in the sensitive starch region (wavelength range 950 - 1200 cm-1) were determined.

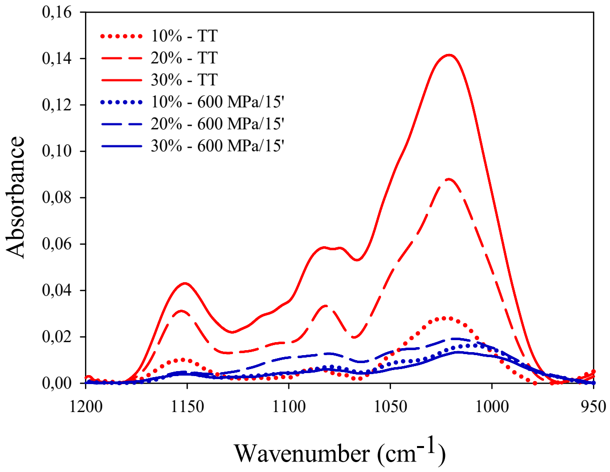


Figure IV.1 *FT-IR spectra of potato starch suspensions at different starch concentration (10-30% w/w) treated by high pressure (600 MPa for 15 min at 25°C) and thermal treatments.*

According to data reported in Figure IV.1, all spectra showed the characteristic absorption peaks at 1022, 1080 and 1150 cm-1 (Van Soest *et al.*, 1994), related to the absorption bands of amorphous and crystalline regions and to the amount of ordered structures (Van Soest *et al.*, 1995; 1994; Wilson *et al.*, 1988). These absorptions peaks have been identified as indicators of starch gelation (Lizuka and Aishima, 1999) among which the amplitude of the peak at 1022 cm-1 accounts for amorphous structures (Warren *et al.*, 2016). Thermal gels (control) showed high absorption peaks in the considered range, highlighting highly structured profiles, regardless of the starch concentration. In the starch sensitive region (Figure IV.1) higher absorptions, corresponding to higher starch concentrations, were evidenced. Similar results were reported by Van Soest *et al.*, (1995) in a study on the influence of water content on the deconvoluted spectra of potato starch suspensions at different water concentrations. The authors demonstrated that with increasing water concentration an increased intensity of the peak at 1022 cm-1 can be detected (overlapping the band at 994 cm-1).

Pressurized potato starch samples showed lower absorbance values in the sensitive region (950 - 1200 cm-1) as a result of the partial formation of a gel

structure. Slightly higher absorption values of the amorphous and ordered structures were detected in samples with 20% (w/w) starch content with respect to samples with different starch concentrations. This demonstrated that HPP treatment of starch suspensions at 20% (w/w) starch-water concentration was more effective and allowed the formation of samples with a more structured profile. Although through FTIR spectra the influence of water concentration on the structural behaviour of HPP-treated starch suspensions could not be assessed, the results of FTIR analyses enabled highlighting the extent of gelation and as such, they were in good agreement with the results of optical and physical parameters measurements of both untreated and HPP-treated starch suspensions.
Further analyses described in the following, were carried out on samples with 20% (w/w) starch-water concentration.

IV.3.2 Effect of particle size distribution on gel formation and structural properties of potato starch suspensions treated by HPP

The results of gel formation of native and pressurized potato starch samples with different mean particle size are reported in Table IV.2.

Table IV.2 *Swelling behaviour and % of gelation of potato starch suspensions (20% w/w) of different particle size, after pressure (600 MPa for 15 min at 25 °C) and thermal treatments, determined from optical measurements.*

	Mean size		
	<25 µm	**50 µm**	**Unsieved**
Samples	Average granule size (µm)		
Native	28.0 ± 8.6^{Cb}	70.0 ± 6.3^{Ab}	50.9 ± 24.4^{Bb}
HPP-treated	48.9 ± 14.0^{Ca}	80.4 ± 13.2^{Aa}	62.5 ± 20.9^{Ba}
HPP-treated	Swelling %		
	74.6	14.9	22.8
	% of Gelatinization		
	30.2 ± 4.3^{A}	13.8 ± 0.8^{B}	16.6 ± 7.5^{B}

All values are means of 225 (granules) determinations ± SD.
(A,C) Values in the same row with different superscripts differ significantly ($p<0.05$; LSD).
(a,c) Values in the same column with different superscripts differ significantly ($p<0.05$; LSD).

Based on previous results, starch gelatinization by HPP depended on granules particle size (data not shown). It is well known that the gelatinization of starches with small granules size, such as rice, wheat, corn and pea starch, takes place more easily at the processing conditions utilized in this investigation, namely 600 MPa for 15 min (Stute *et al.*, 1996; Oh *et al.*, 2008). However, as already discussed in Section IV.3.1, potato starch is characterized by a significant pressure resistance to gelatinization, which has been mainly attributed by other authors to its chemical spatial conformation (B-type) (Stute *et al.*, 1996; Kawai 2007a,b; 2012) without considering other possible influencing factors, such as granules size. To confirm this hypothesis, the effects of particle size distribution on structural properties of potato starch suspensions and on gel formation were evaluated.

Data reported in Table IV.2 clearly showed that with smaller starch granules (<25 µm) gel formation at HPP treatment conditions investigated was facilitated ($p<0.05$). In particular, considering the swelling ratio, which is the difference between starch granules size before and after HPP treatment, it was possible to observe an increase of size of 74.6% for small granules (<25 µm) while this increase was 14.9% and 22.8% only for medium granules size (50-63 µm) and for the unsieved starch granules, respectively. Although to the best of our knowledge these findings were never reported in the literature for potato suspensions treated by HPP, this behaviour could be explained taking into account that small particles move during HPP treatment and they are more exposed to the action of pressure than bigger particles. Moreover, being the number of particles per unit volume increased when the particle size decreases, starch-starch and starch-water interactions in suspensions of small starch granules are much higher in the space available, thus a stronger network can be formed. Ahmed (2018) reported that a higher effect of HPP-treatments (>450 MPa) was detected on tapioca starch suspensions of small granules size rather than on those of bigger size. At 600 MPa, a size increment of 329% of the smaller granules size portion Dv10 (from 1.56 µm to 6.69 µm) was detected, while an increase of only 55% and 15% were detected on the granule size portion (Dv50) and on the overall granule size distribution (Dv90). The authors explained these findings considering the ability of small granules to binding more water, due to the higher surface area which may lead to more efficient hydration and, consequently, a higher swelling (Douzals *et al.*, 1996). Similar observations were reported by other authors on barley, wheat and potato starch suspensions gelatinized by thermal treatments (Jiang *et al.*, 2015; Nurul *et al.*, 1999; Nguyen *et al.*, 1998).

In Figure IV.2 are reported FTIR spectra of thermal and pressurized potato starch samples of different mean particle size. Thermal gels showed a characteristic spectrum of well-structured gels, being the absorption peaks in the region of interest more pronounced with increasing the size of the granules. Samples obtained at 25 °C after pressurization at 600 MPa for 15 min showed an opposite trend. When granules of smaller size (<25 µm) were present in the suspension better structure profiles were observed while poorer structured samples were obtained with bigger starch granules size (36-50 µm) and unsieved potato starch, confirming that the smaller the granules size the higher the effectiveness of HPP treatments on starch gelation, thus reinforcing the results of Table IV.2. Observing the absorption peak at 1022 cm^{-1}, which, according to Warren *et al.*, (2016) is influenced by the water content of the starch suspension, the influence of particle size distribution clearly appeared on both thermal and pressurized samples. Thus, it can be concluded that the utilization of starch with small size positively affect potato starch gelation by HPP as well as the structural properties of the samples obtained. Further experiments presented in the following have been carried out on suspensions of small granules size (<25 µm) at 20% (w/w) starch concentration.

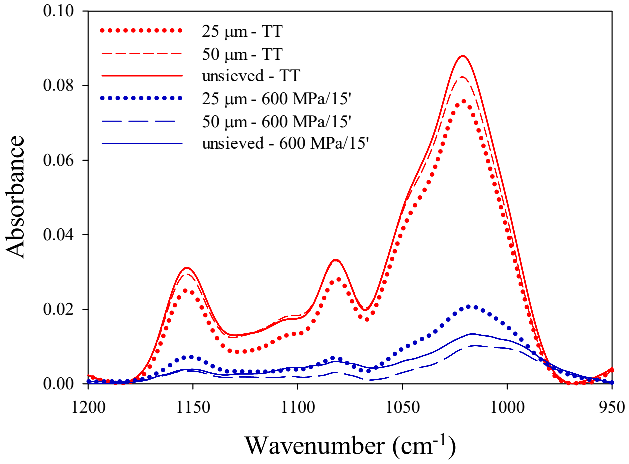


Figure IV.2 *FT-IR spectra of potato starch suspensions (20% w/w) of different particle size treated by high pressure (600 MPa for 15 min at 25°C) and thermal treatments.*

IV.3.3. Effect of combined HPP-thermal treatments on gel formation, structural and mechanical properties of potato starch suspensions

According to the results presented so far, at the HPP treatment conditions investigated, namely 600 MPa for 15 at 25 °C, it was not possible to induce the complete gelatinization on potato the starch suspensions utilized. Thus, other experiments were carried out at higher processing temperatures (40 °C and 50 °C) to evaluate the influence of moderate heating on HPP gel formation, structural and mechanical properties of products, in a hurdle approach.

Potato starch suspensions (starch concentration of 20% (w/w) and granules size <25 µm) were treated at 600 MPa for 15 min at 40 °C and 50 °C. The changes of birefringence of the treated samples were reported in Figure IV.3.

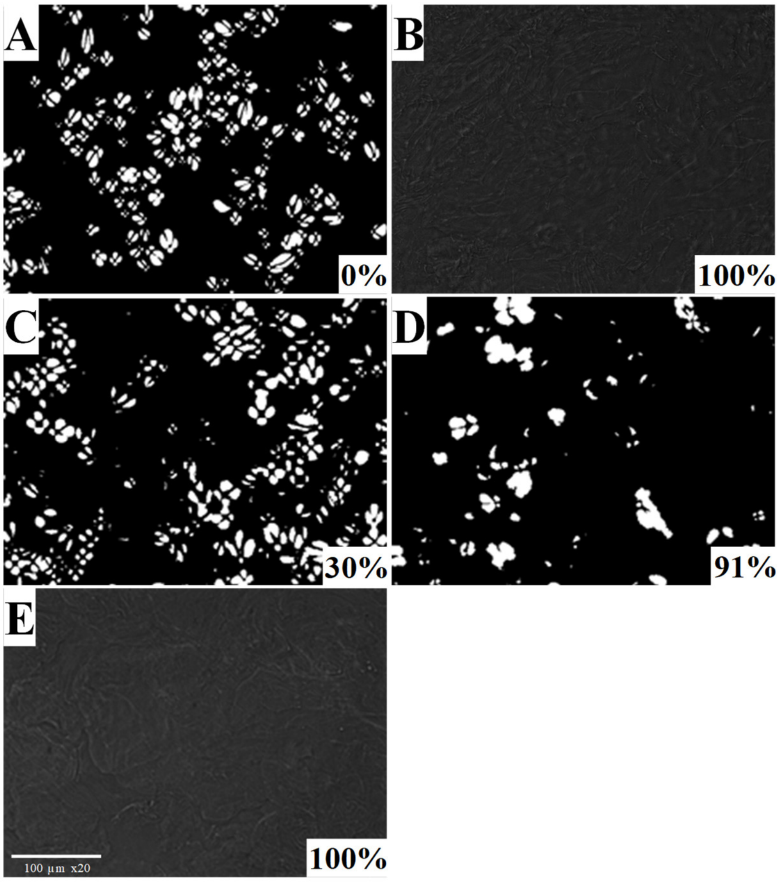


Figure IV.3 *Polarized light micrographs of untreated potato starch suspensions (25 µm, 20% w/w) (A), thermal treated (B), pressure treated at 600 MPa for 15 min at different processing temperatures, namely 25 °C (C), 40 °C (D) and 50 °C (E), respectively. The % of gelatinization of samples, determined as a function of birefringence loss, are also indicated in the pictures.*

Under polarized light, native starch granules show birefringence in the typical form of a "maltese cross" (Li *et al*., 2012). Normal birefringence and no-birefringence were clearly observed on untreated and thermal treated potato starch suspensions, respectively. These patterns were used for comparison (Figure IV.3 a, b). Data of Figure IV.3 demonstrated that the loss of birefringence was increasing with increasing the treatment temperature. Birefringence of potato starch granules was reduced after HPP treatments at 25 °C (Figure IV.3c), while it was almost completely lost after treatment at 40

°C (91%) (Figure 3d). Furthermore, potato starch suspensions HPP treated at 600 MPa for 15 min at 50 °C showed a complete loss of birefringence (Figure IV.3e), accounting for a complete gelatinization and hydrogel formation. Our results were in good agreement with those of other authors (Hibi *et al.*, 1993; Kawai *et al.*, 2012; 2007a; 2007b). Although many research efforts have been made to study HPP potato starch gelatinization, only a few papers reported results on gel formation at economically feasible processing conditions (Hibi *et al.*, 1993; Stute *et al.*, 1996; Katopo *et al.*, 2002; Muhr *et al.*, 1982; Rubens *et al.*, 1999; Nezhad *et al.*, 1998). Bauer and Knorr (2005) reported that HPP induced the complete gelatinization of potato starch suspensions (5% w/w) at 700 MPa for 15 min and 50 °C, and Kawai *et al.*, (2012; 2007a; 2007b) reported that the complete gelation of potato starch suspensions (20% w/w) occurred at 800 MPa and 40 °C for processing time longer than 60 min. This study demonstrated that the complete gelatinization of potato starch suspension can be obtained at lower pressure levels, provided that a combination of pressure and moderate heating treatment (at 50 °C) was applied. With increasing the treatment temperature, the molecular motions are favoured, and the simultaneous application of heat and pressure had a synergetic effect on the gelatinization process (Kawai *et al.*, 2012).

In order to obtain information on the degree of gelatinization of potato starch suspensions obtained at the above-mentioned processing conditions, thermal analysis by DSC was performed. Gelatinization enthalpy (ΔH_{gel}) and degree of gelatinization (D_g) of each HPP treated sample are shown in Figure IV.4. According to data of Figure IV.4, ΔH_{gel} decreases with increasing the processing temperature. Consequently, the increase of D_g measured confirmed the results of microscopy tests (Figure IV.3). However, higher D_g values shown in Figure IV.4 allowed us concluding that the birefringence loss method underestimates the extent of gelatinization as already discussed by Kawai *et al.*, (2012). The authors hypothesized that very small granules of potato starch can keep their birefringence, thus the underestimation of the degree of gelatinization through microscopy technique is likely to occur. Although the determination of the degree of gelatinization is strongly dependent on the testing method, the aim of this study was to confirm that HPP induces the complete gelatinization of potato starch suspensions at 50°C, which was clearly confirmed by both methods utilized.

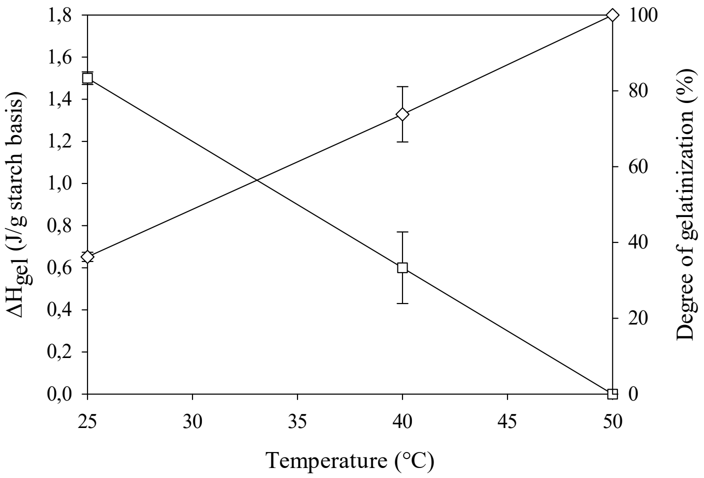


Figure IV.4 *ΔHgel (J/g starch, open square) and degree of gelatinization (%, open diamond) as a function of HPP processing temperatures of potato starch suspensions (25 μm, 20% w/w) treated at 600 MPa for 15 min.*

Data reported in Figure IV.5 demonstrated the effects of processing temperatures (25 °C, 40 °C and 50 °C) on structural properties of HPP hydrogels. The spectrum of a thermal gel, representing a highly structured material, was also depicted in Figure IV.5.

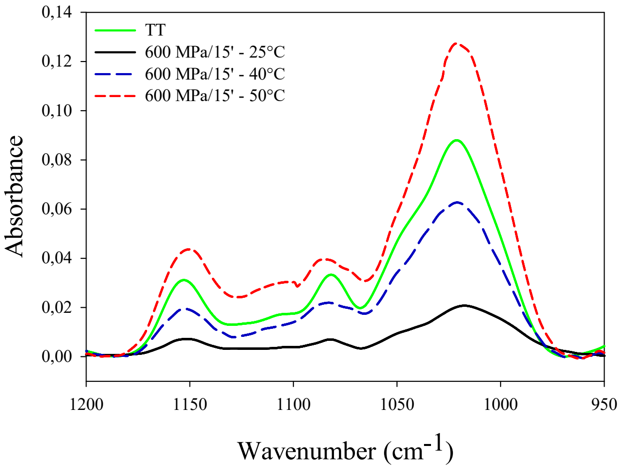


Figure IV.5 *FT-IR spectra of potato starch suspension (20% w/w) treated by HPP at different processing temperatures.*

The intensity of all peaks in the sensitive region (950 - 1200 cm^{-1}) tended to increase with increasing the processing temperature. According to Van Soest *et al.*, (1995), alterations in this wavelength region are expected as gelation causes damages in the crystalline region and, consequently, the increase of the amorphous structures. HPP treated samples at the processing temperature of 50 °C showed the highest absorption peaks in the sensitive region, accounting for a highly structured profile, even higher than the spectrum of thermal gel (TT). It can be concluded that combined high pressure and thermal treatments produced a synergistic effect on hydrogels structure, resulting in stronger and more dense networks due to strong inter and intra-molecular interactions, clearly highlighted in FTIR spectra.

The effects of processing conditions on mechanical properties of hydrogels were assessed by rheological measurements. Viscoelastic properties of thermal and HPP hydrogels (600 MPa for 15 min at 25 °C, 40 °C and 50 °C) are shown in Figure IV.6.

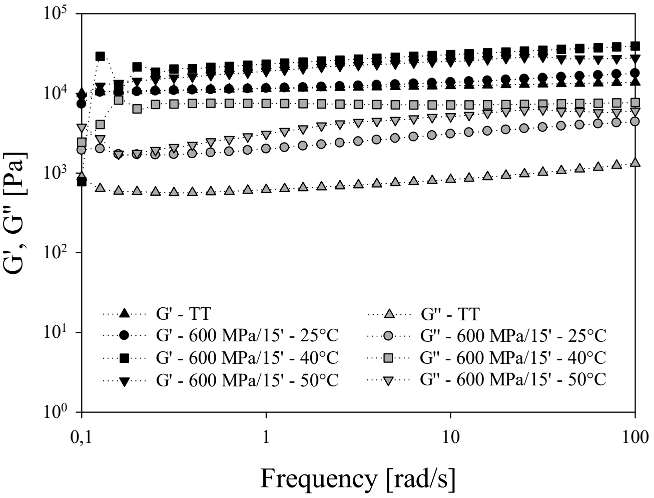


Figure IV.6 *Rheology of potato starch suspension (25 µm, 20% w/w) subjected to thermal and high pressure treatments at 600 MPa for 15 min at 25 °C, 40 °C and 50 °C.*

The mechanical spectrum, also known as the frequency sweep test, is also utilized to classify products, materials, or gels (Nezhad *et al.*, 2018; Torres *et al.*, 2018; Galkowska *et al.*, 2014). Standard rheological characterization of the viscoelastic properties of complex fluids and gels is traditionally carried out in the frequency domain where gels are subjected to deformation forces and the stress response is measured (Douglas, 2018).

The mechanical behaviour of samples was obtained as a function of G' (elastic) and G" (viscous) moduli in the frequency sweep range 0.1-100 [rad/s] at 25 °C. Viscoelastic properties of HPP-treated samples at different temperatures (25 °C, 40 °C and 50 °C) are reported in Figure IV.6. Thermal gel (control) curves were also added for comparison. Frequency sweep curves of all samples showed the typical trend of gel structures, with G' greater than G'', indicating a predominance of elastic over viscous properties (Torres *et* *al*., 2018; Galkowska *et al*., 2014). Moreover, G' and G'' values of fully gelatinized samples (600 MPa for 15 at 50 °C and control) were higher than those of partially gelatinized samples (600 MPa for 15 at 25 °C and 40 °C), in agreement with the conclusions reported in a recent paper on fully HPP-gelatinized and not gelatinized quinoa starch (Ahmed *et al*., 2018).

One of the main objectives of determining the viscoelastic properties of gels by frequency sweep tests was evaluating the strength of the gels, which is a function of the position of G' and G'' curves (Nurul *et al*., 1995), and the dependence of G' and G'' in the frequency range considered (Lapasin, 2016). The position of G' and G'' curves reported in Figure IV.6 clearly indicated that fully gelatinized samples showed mechanical characteristics typical of strong gels. Moreover, hydrogels formed by the synergistic high pressure-thermal treatment at moderate temperature (600 MPa for 15 min at 50 °C) showed a weak frequency-dependence, which indicated a superior gel strength profile.

According to Douglas (2018), "strong gels" are characterized by an elastic response to shear deformations due to the well-structured network that stores the energy when stress forces are applied to the material. HPP-thermal hydrogels obtained at 600 MPa for 15 min at 50 °C showed the characteristics of strong gels described above, as also confirmed by the other properties measured, namely flow behaviour and structural properties.

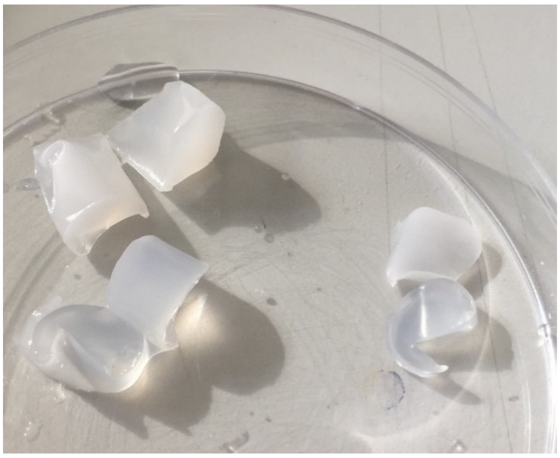


Figure IV.7 *Picture of the potato starch hydrogels obtained in this investigation at fixed conditions (starch-water concentration of 20% w/w, mean size of <25 µm and processed at 600 MPa for 15 min at 50°C).*

IV.4 Conclusions

Although in the literature several papers described that HPP is a suitable technology to obtain potato starch hydrogels, starting from suspensions at different starch concentrations, and illustrated the most appropriate processing conditions, to the best of our knowledge no data have been published on the characterization of the products obtained. In this study, the effects of starch concentration in the suspension (10-30% w/w), particle size distribution (<25 µm, 50 µm and unsieved), and HPP processing temperature (25, 40 and 50 °C) on potato starch gel formation were studied and structural and mechanical properties of the products obtained were investigated.

The analysis of the results allowed concluding that high pressure processing was effective to produce potato starch-based hydrogels at economically feasible conditions, with processing times significantly lower than those used in conventional gelation methods. At starch concentration of 20% w/w, the utilization of granules with small size (<25 µm) positively affected gel formation by high pressure, and a hurdle approach was necessary, that is coupling high pressure with moderate heating at low temperature (50 °C), to obtain a stable hydrogel with excellent structural and mechanical properties, even superior to those of thermal gels.

More work is needed to further characterize these hydrogels through measurements of the stability of these structures, the determination of the optimal storage conditions as well as the investigation of the effects of adding others compounds on product properties in view of their applications in different industrial sectors.

Chapter V

Starch-based hydrogels produced by high pressure processing (HPP): Effect of type of starch and processing time

Abstract - In this work, the effects of type of starch (rice, corn, wheat and tapioca) and HPP processing time (5 and 15 min) at 600 MPa on gelatinization and physical characteristics of hydrogels obtained were evaluated.

At the pressure level utilized, regardless of the processing time, a complete gelatinization of starches occurred, and the hydrogels produced displayed a non-Newtonian behaviour, with G'>G''. HPP processing was more effective to produce hydrogels based on tapioca and rice starch. Moreover, HPP hydrogels made of rice, wheat and corn starch showed a cream-like structure while a more compact structure was characterizing tapioca starch hydrogels. With increasing HPP processing time up to 15 min, tapioca, and rice HPP hydrogels showed higher viscosity and firmness, that is an overall structural reinforcement. However, the increase of time negatively influenced the lightness and whiteness of rice, wheat, and corn HPP hydrogels, as a consequence of the higher amount of water absorbed in starch granules.

These results allowed to conclude that both the type of starch and processing time are playing an important role in the formation of starch-based HPP hydrogels and influenced their physical characteristics, and this must be taken into account in view of the exploitation of possible future applications of these materials.

Chapter V

V.1 Introduction

Starches, after cellulose, represent by far the most abundant biopolymers on earth, as well as one the most versatile and economical material of potential use in polymer technology, and in food and non-food applications. Among staple foods rich in starch, rice, wheat, corn and tapioca (or cassava) are the most abundantly produced and traded in the world (FAO, 2017). However, often these crops present defects which impede their utilization in food processing lines and are discarded. These defected crops contain starches which can be recuperated for further utilization in view of reducing food wastes, setting up recovering strategies to reduce the environmental burdens and finding alternative applications of starches which, through the implementation of eco-efficiency processes, could increase the sustainability of crops production.

In the last decades, the utilization of starch-based products, such as biodegradable packages, has been increasing as an alternative to replacing or reducing the use of petroleum derivate plastics (Liu *et al*., 2009). Indeed, one of the most promising applications of this natural biopolymer is the development of starch-based hydrogels, which are hydrophilic 3D polymeric networks able to absorb and retain a significant amount of water. The most important characteristics of starch-based hydrogels are safety, biocompatibility and biodegradability, which encourage their extensive use in several applications (García-Astrain and Avérous, 2018; Ismail *et al*., 2013; Mahinroosta *et al*., 2018; McClements, 2017; Mun *et al*., 2015; Nieuwenhove *et al*., 2017; Qi *et al*., 2017; Xiao *et al*., 2017).

By applying physical or chemical stresses to starches, water penetrates in starch granules causing the formation of a strong and stable network, namely the hydrogel structure (Biduski *et al*., 2018). Biduski *et al*. (2018) investigated the different physical characteristics of starch-based hydrogels produced by different gelatinization methods (alkaline or thermal methods) utilizing native or cross-linked rice starch with different amylose content (8%, 20% and 32%), concluding that gelatinization method and starch amylose content have influenced hydrogels characteristics. The alkaline gelation (NaOH 50%, 55 °C, 0.5 h) was the most suitable method to obtain firm and well-structured gels when using cross-linked rice starch (obtained after several processing steps in more than 24 h) with 20 % amylose content.

Other authors have summarized the production of hydrogels (Ali and Ahmed, 2018; Caló and Khutoryanskiy, 2015; Ismail *et al*., 2013) and reported physical or chemical crosslinking and graft polymerization as traditional methods utilized to produce this kind of structures. However, long processing time, high energy consumption, and safety issues related to the synthesis of these products have been identified as important limitations of these gelatinization methods.

In the last decades, alternative production methods to obtain starch-based hydrogels have been investigated to overcome the problems arising from the utilization of the traditional one, among which high pressure processing (HPP). The latter is a well-known non-thermal technology mostly applied for food preservation, causing no or minimal sensory and nutritional damages to processed products. HPP technology has been also proposed for other applications, such as the recovery of bioactive compounds, the enhancement of bioaccessibility and bioavailability of micronutrients, the reduction of food allergenicity, preservation of lipids and salt content reduction (Barba *et al.*, 2015), as well as alternative processing method for modification of starch suspensions or for starch suspensions gelatinization to prepare starch-based hydrogels (Blaszczak *et al.*, 2015; Blaszczak *et al*, 2005a; Blaszczak *et al.*, 2005b; Blaszczak *et al.*, 2007; Buckow *et al.*, 2007; Katopo *et al.*, 2002; Kawai *et al.*, 2012; Li *et al.*, 2012; Li *et al.*, 2015; Oh *et al.*, 2008; Stute *et al.*, 1996). It is well known that HPP causes the disordering of biopolymers, including proteins and starches, which induce modifications of non-covalent intermolecular interactions, thus pressure-assisted gelatinization (Balny, 2002). Under high pressure, starches undergo morphological and structural changes, exhibiting different gelatinization extent, with intact granules and limited swelling of starch particles remaining after treatment, and different structural properties compared to thermally treated starches (Li *et al.*, 2012; Buckow *et al.*, 2007; Hibi *et al.*, 1993). Almost all starch suspensions could be gelatinized under high pressure at room temperature. However, it has been shown that HPP assisted gelatinization process, consisting in the hydration of the amorphous and crystalline regions in excess of water under compression forces, is strongly influenced by the type of starch, starch/water ratio, pressure level, temperature and processing time (Bauer and Knorr, 2005; Pei-Ling *et al.*, 2010).

In the last 20 years, many efforts have been devoted to unravelling the role of chemical-physical characteristics of starches and HPP processing variables on gelatinization process, however, only a few investigations were carried out to individuate the potential use of such biopolymers (Blaszczak *et al.*, 2015; Szepes *et al.*, 1998). Therefore, more research efforts are needed to identify the production conditions and the physical characteristics of HPP starch-based hydrogels which could help to forecast their industrial exploitation.

The aim of the present study was evaluating the influence of the type of starch and processing time on gel formation and physical characteristics of hydrogels obtained by high pressure processing in view of their future innovative applications.

V.2 "Short" Materials and Methods

V.2.1 Raw materials and samples preparation

Rice, wheat, corn, tapioca starch powders were utilized in this experimental work. Chemical and physical characteristics of raw materials were accurately reported in § III.1 and Table III.1. Samples preparation carried out in this work were described in § III.3.1. Briefly, rice, wheat, corn, or tapioca starch in a wet basis were suspended at a starch-water concentration of 20% (w/w) and thoroughly mixed, before the HPP-treatments.

V.2.2 Samples processing

HPP treatments of samples upon the different operations framed in this work were reported in § III.3.2.2, and for the evaluation of corn, wheat, rice and tapioca starch as sources for hydrogels production via HPP (§ III.3.2.2.1). Briefly, HPP treatments were carried out at 600 MPa for 5 and 15 min at 25 °C in an HP unit U-22 described in § III.4.1. All the experiments were carried out at least in triplicate.

V.2.3 Samples characterisation

Starch-based HPP hydrogels samples were characterised by optical measurements (degree of gelatinization), efficiency index, swelling power, rheological and texture properties, as previously described in § III.5.1 (Eq.1), § III.5.4, § III.5.5, § III.5.8; § III.5.10, respectively.

Macroscopic analysis of samples was performed by a digital camera (Sony Corp, Japan) in angular mode. Original pictures without editing and filtering were reported.

Colour parameters were determined by the methodology described in § III.4.9. Results reported in Table V.2 was calculated by equation 9 and equation 10, as reported on § III.5.9.

Data reported in Table V.3, to account the colour differences among HPP hydrogels obtained with different starches at the two processing times (5 min and 15 min) were evaluated according to the modified forms of equation 9 (§ III.5.9) as follows:

$$\Delta E_{600/5} = \surd(L_{starch1} - L_{starch2})^2 + (a_{starch1} - a_{starch2})^2 + (L_{starch1} - L_{starch2})^2$$

$$\Delta E_{600/15} = \surd(L_{starch1} - L_{starch2})^2 + (a_{starch1} - a_{starch2})^2 + (L_{starch1} - L_{starch2})^2$$

At least ten measurements were done on each sample.

V.3 Results and discussion

V.3.1 Gel formation

In Figure V.1 the polarized micrographs are reported for starch suspensions untreated and pressurized at 600 MPa and two processing times (5 min and 15 min).

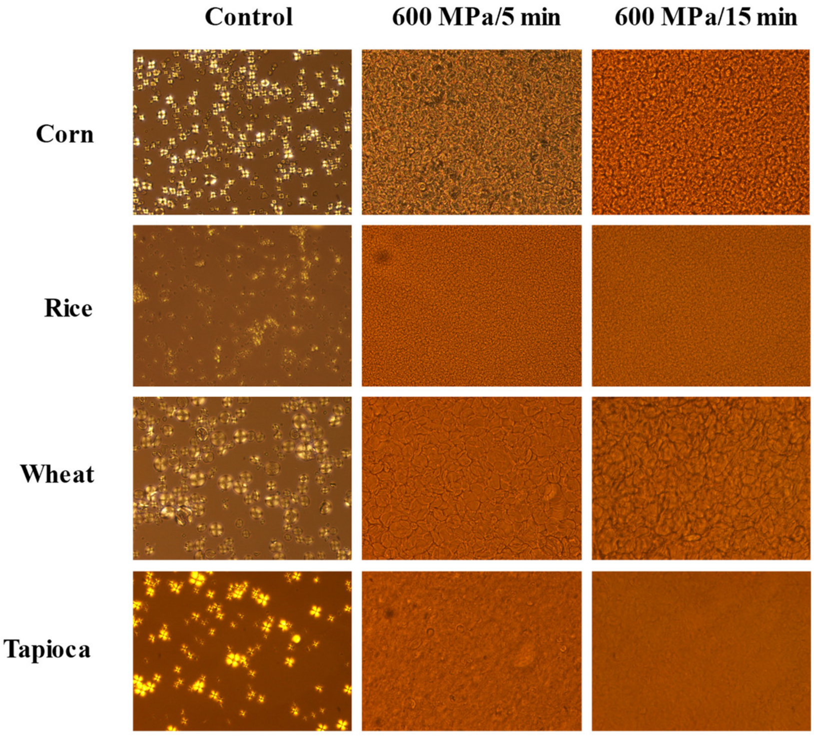


Figure V.1 *Birefringence of untreated and HPP treated suspensions of corn, rice, wheat, and tapioca starch.*

Under polarized light, native starch granules show birefringence, in the typical “maltese cross” form, while a complete loss of birefringence of starches after gel formation is detected (Li *et al*., 2012). This visual observation can be easily accounted for a gelatinization index of starch samples. As expected, untreated starch granules (control) displayed complete birefringence (Figure V.1, left side), while after pressure treatments at 600 MPa and regardless of processing time, all polarized micrographs showed a complete loss of birefringence, indicating the complete gelatinization of starch suspensions. This is in agreement with data reported by other authors, which were obtained utilizing rice, corn, wheat and tapioca starch suspensions pressurized at similar processing conditions (Bauer and Knorr, 2005; Buckow *et al.*, 2009; Buckow *et al.*, 2007; Hibi *et al.*, 1993; Katopo *et al.*, 2002; Li and Zhu, 2018; Oh *et al.*, 2008; Stute *et al.*, 1996).

Although these results were evidencing the complete gelatinization of starches after HPP treatment at the processing conditions utilized, other physical parameters, such as swelling power and efficiency index, were determined to confirm the effectiveness of HPP to produce starch-based hydrogels.

In Table V.1 the efficiency index (EI) and swelling power (SP) of HPP hydrogels produced with a different type of starch and HPP processing conditions are reported.

Table V.1 *Efficiency index and swelling power of corn, rice, wheat and tapioca starch HPP hydrogels obtained at 600 MPa and different processing times.*

Treatments			Gel formation parameters	
Pressure (MPa)	Time (min)	Starch	Efficiency index	Swelling power ($g/g_{dry\ starch}$)
600	5	Corn	0.82 ± 0.01^{c}	5.62 ± 0.10^{bcd}
	15		0.81 ± 0.01^{c}	5.38 ± 0.33^{cde}
	5	Rice	1.00 ± 0.00^{a}	6.03 ± 0.41^{bc}
	15		1.00 ± 0.00^{a}	6.45 ± 0.15^{b}
	5	Tapioca	0.91 ± 0.00^{b}	5.86 ± 0.19^{bcd}
	15		0.93 ± 0.00^{b}	7.39 ± 0.75^{a}
	5	Wheat	0.75 ± 0.04^{d}	4.59 ± 0.45^{e}
	15		0.73 ± 0.01^{d}	5.16 ± 0.44^{de}

$^{a-e}$Different letters in the same column indicate significant differences (LSD, $p<0.05$) for each treatment.

From data reported in Table V.1 it can be clearly observed that for corn, rice and wheat HPP hydrogels, the values of EI and SP were independent on processing time ($p>0.05$), while tapioca starch hydrogels showed higher swelling power values with increasing the processing time ($p<0.05$). Moreover, a strong dependence of the two indexes on the type of starch ($p<0.05$) was detected in all cases. Due to the different extent of swelling, tapioca and rice starch showed higher SP and EI values than corn and wheat starch, being this difference more pronounced at a processing time of 15 min. Similar behaviour was described in the study of Stute *et al.*, (1996). The authors observed that some types of starch granules are characterized by limited swelling, such as corn and wheat, while in others, such as tapioca starch granules, an extensive swelling is likely to occur during HPP treatments. Our results suggested that the gelatinization parameters under high pressure are more affected by the type of starch than by HPP processing time, as already reported by other authors (Blaszczak *et al*, 2005; Blaszczak *et al.*, 2005; Buckow *et al.*, 2007; Oh *et al.*, 2008).

V.3.2 Macroscopic analysis

The pictures of the HPP hydrogels based on the different starches produced in this work are reported in Figure V.2.

It can be clearly noticed that independently on processing time, the starch-based hydrogels showed very good homogeneity and structural integrity. As expected, the type of starch influenced the macrostructure of the hydrogels produced by HPP, whatever was the processing time. Corn, rice and wheat starch HPP hydrogels presented a cream-like appearance while those based on tapioca starch were characterized by a rubber-like structure. Similar observations were reported by Katopo *et al.* (2002). The authors, utilizing suspensions at 25% of starch and processing conditions of 600 MPa and 5 min, obtained spreadable adhesive hydrogels with corn and rice starch, and less adhesive hard gels with tapioca starch.

The effects of pressure on starches are strongly influenced by their crystalline structure, determined by X-ray diffraction (Katopo *et al.*, 2002; Stute *et al.*, 1996). Tapioca has a C-type spatial configuration (a mixture of A- and B-pattern), whereas rice, corn and wheat present an A-type spatial configuration. In this latter case, less room is available for water absorption with respect to B-pattern (Wu and Sarko 1978a, b). The same could be assumed also for C-pattern, in agreement with the SP data reported in Table V.1. This is also confirmed by visual observation of corn, rice, and wheat hydrogels, which appear lighter in comparison to tapioca hydrogels, being this more pronounced at lower processing times.

It can also be hypothesized that the compact structure of tapioca HPP hydrogels reflects a stronger starch profile. Due to better water-starch interactions and/or a better assembly of starch components (Vittadini *et al.*, 2008), more water is entrapped during gel formation, thus a highly structured gel form (Ahmed *et al.*, 2014). Moreover, Ahmed *et al.*, (2014) underlined that it is still not clear why after HPP processing hydrogels with different structures are formed. The visual observation of the macrostructure of starch-based hydrogels produced by HPP in this investigation allowed to conclude that hydrogels formation under HPP treatments is strongly influenced by the type of starch and this must be taken into account to forecast future applications of these structures.

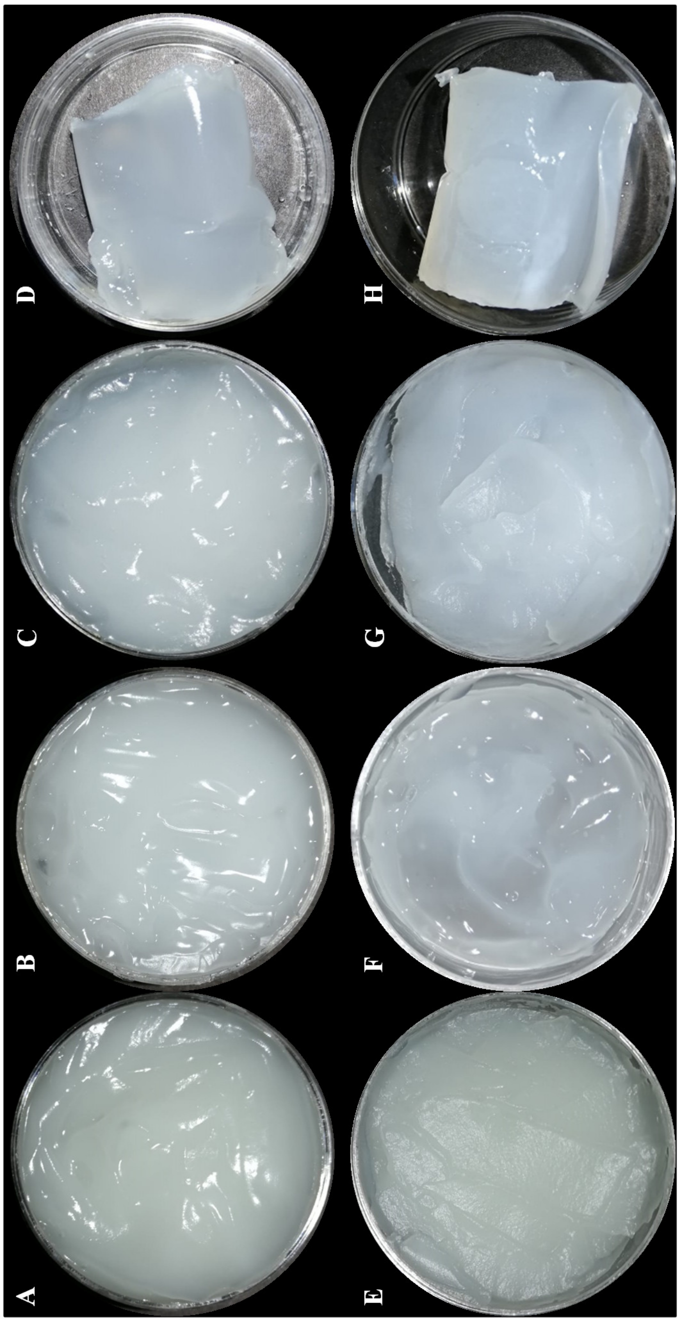


Figure V.2 *Physical appearance of HPP hydrogels based on corn (A, E), rice (B, F), wheat (C, G) and tapioca (D, H) starch at 600 MPa for 5 min (A-D) and 15 min (E-H).*

V.3.3 Colour measurements

Colour is an important sensory attribute determining consumer acceptance of products (Torres *et al*., 2019). In Table V.2 the colour parameters of HPP hydrogels based on different starches and obtained at different HPP processing conditions are reported.

Colour parameters of starch-based HPP hydrogels were influenced by the type of starch and processing time ($p<0.05$). All HPP hydrogels obtained in this study showed positive values of L*, in the range 45 to 66, indicating that the white components were dominant in all cases. Corn HPP hydrogels were the brighter ones, followed by wheat, rice and tapioca HPP hydrogels ($p<0.05$). With increasing the processing time to 15 min, L* values of HPP hydrogels produced with corn, wheat and rice starch decreased of 18%, 20% and 20% ($p<0.05$), respectively. An opposite trend was detected for tapioca HPP hydrogels with an increase of lightness of 9% ($p<0.05$). Although there is no evidence of similar data measured on this kind of structures, the darker colour observed for corn, rice, and wheat HPP hydrogels with respect to tapioca HPP hydrogels at processing conditions of 600 MPa for 15 min, can be attributed to the greater amount of water entrapped in these structures with increasing the processing time (i.e. lower amounts of superficial water are available to reflect light). As expected, WI values evidenced showed a similar trend.

HPP hydrogels produced with corn, wheat, and rice starch evidenced negative values of the parameter a*, in the range between -2.11 to -0.07, indicating a slight greenness of these samples, being corn the greener, followed by wheat and rice starch ($p<0.05$). Tapioca HPP hydrogels, instead, showed positive values of a*, i.e. a more pronounced redness ($p<0.05$). At processing times of 15 min, a* values were significantly increased in all samples ($p<0.05$), with increments up to +85% for rice, +55% for wheat, +28% for tapioca and +6% for corn HPP hydrogels.

The parameter b* accounts for the tendency to yellow of samples. HPP hydrogels produced with corn and tapioca showed a higher yellowness, in the range from 0.9 to 4.5, having tapioca HPP hydrogel the higher value of the yellow components. On the contrary, rice and wheat HPP hydrogels showed negative b* values (higher blueness) with values in the range from -4.17 to -2.02., being wheat starch-based HPP hydrogel the more blue. With increasing HPP processing times up to 15 min, the yellowness of all hydrogels significantly increased ($p<0.05$), with an increment of +200% in corn and 10% in tapioca HPP hydrogels and a blueness reduction in rice (- 43%) and wheat HPP hydrogels (- 21%).

Table V.2 *Colour parameters of corn, rice, wheat, and tapioca HPP hydrogels as a function of processing conditions*

Treatment			Colour parameter				
Pressure (MPa)	Time (min)	Starch	L*	a*	b*	WI	ΔE
600	5	Corn	66.70^{a} (0.02)	-2.11^{g} (0.01)	0.86^{d} (0.00)	66.66^{a} (0.02)	11.94^{b} (0.05)
	15		54.89^{d} (0.04)	-1.98^{f} (0.02)	2.62^{b} (0.02)	54.90^{d} (0.05)	
	5	Rice	56.94^{c} (0.04)	-0.47^{e} (0.02)	-3.56^{g} (0.01)	56.91^{c} (0.04)	11.29^{c} (0.22)
	15		45.60^{e} (0.23)	-0.07^{c} (0.06)	-2.02^{e} (0.06)	45.76^{g} (0.23)	
	5	Tapioca	45.36^{f} (0.05)	1.69^{b} (0.02)	2.35^{c} (0.02)	45.31^{h} (0.05)	4.82^{d} (0.11)
	15		49.27^{d} (0.41)	2.17^{a} (0.05)	4.51^{a} (0.05)	49.66^{e} (0.08)	
	5	Wheat	60.75^{b} (0.01)	-0.42^{e} (0.02)	-4.17^{h} (0.02)	60.72^{b} (0.01)	12.64^{a} (0.06)
	15		48.29^{e} (0.10)	-0.19^{d} (0.03)	-2.71^{f} (0.03)	48.20^{f} (0.05)	

$^{a\text{-}e}$Different letters in the same column indicate significant differences (LSD, $p<0.05$). In round brackets has been reported the standard deviations.

In order to better understand whether the effects of the processing time and type of starch on colour characteristics of HPP hydrogels could be detected by the human eye, the values of the parameter ΔE were calculated. Values of ΔE > 3 indicate differences perceived by the human eye (Torres *et al.*, 2019). For the sake of comparison, ΔE was evaluated at first by comparing hydrogel samples obtained at different processing time with the same starch (data of Table V.2) and then comparing the values of ΔE of samples produced with different types of starches with the same processing time (data of Table V.3).

Table V.3 *Colour differences (ΔE) among HPP hydrogels as a function of the type of starch.*

		ΔE values		
		600MPa/5 min		
Processing Time (min)	Starches	Rice	Tapioca	Wheat
600 MPa 5 min	Corn	10.8^{d}	21.7^{a}	8.0^{e}
	Rice	-	13.2^{c}	3.9^{f}
	Tapioca	-	-	16.9^{b}
		600MPa/15 min		
		Rice	Tapioca	Wheat
600 MPa 15 min	Corn	10.4^{a}	7.0^{e}	8.7^{b}
	Rice	-	8.0^{c}	2.5^{f}
	Tapioca	-	-	7.7^{d}

$^{a\text{-}e}$Different letters indicate significant differences between the starches (LSD, $p<0.05$) for each processing time.

Processing time strongly influenced the colour perception of starch-based HPP hydrogels ($p<0.05$) (Table V.2). The values of the parameter ΔE confirmed that with increasing HPP processing time the colour differences are clearly perceptible by the human eye ($\Delta E > 5$). Higher net colour changes (ΔE) were detected for wheat, corn and rice HPP hydrogels ($\Delta E>11$). Analysing the data reported in Table V.3 referring to samples obtained with different starches and at the same HPP processing time, it is worth noting that rice and wheat HPP hydrogels presented similar colour characteristics with lower ΔE values ($p<0.05$) than corn and tapioca HPP hydrogels and no colour differences can be detected by human eye ($\Delta E < 3$) in all samples obtained at 600 MPa for 15 min. These results are coherent with the outcomes of visual observation and could be explained considering the structural differences of the various HPP hydrogels, which influence their colour profiles.

V.3.4 Rheology

To further investigate the effects of HPP processing time and type of starch on hydrogels structure, the mechanical characteristics of HPP hydrogels were evaluated by rheology assessments. The results of the measurements of flow behaviour and viscoelastic properties of HPP hydrogels as a function of different types of starch and HPP processing time at a given pressure level are reported in Figure V.3 and Figure V.4.

Figure V.3 shows the viscosity as a function of shear rate of starch-based HPP hydrogels formed at 600 MPa and 5 or 15 min. All curves are typical of gel-like structures, with a behaviour of a non-Newtonian material, independently of the type of starch and HPP processing time. This rheological behaviour was already found for starch-based gels (Jiang *et al.*, 2015: Xie *et al.*, 2009). Generally, starch-based gels show shear-dependent flow behaviour, flowing at high shear forces, which cause irreversible changes in the network, or fractures, and, consequently, the reorganization of the molecules and a reduced intermolecular resistance to flow (Nguyen *et al.*, 1998; Nurul *et al.*, 1999).

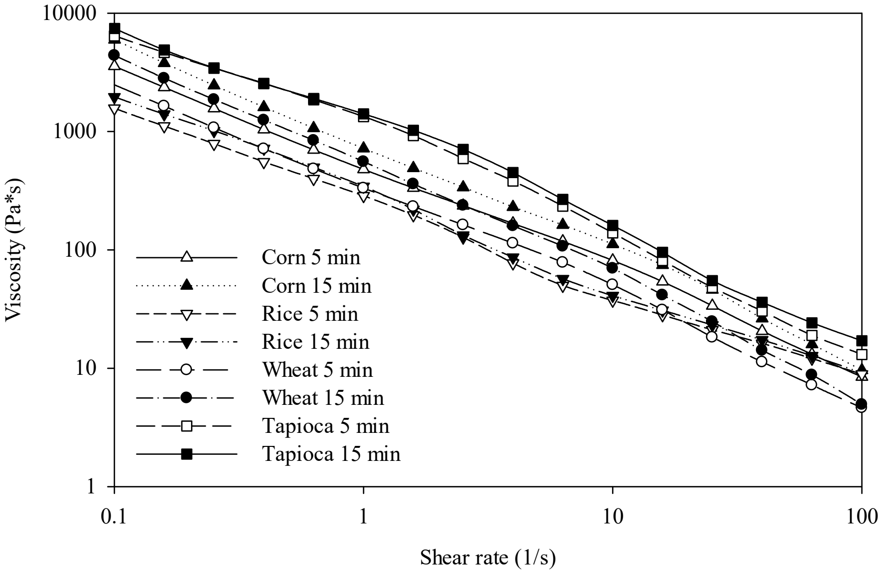


Figure V.3 *Flow behaviour of corn, rice, wheat, and tapioca starch HPP hydrogels obtained at different HPP processing times.*

Despite the non-Newtonian behaviour of starch-based HPP hydrogels, a marked difference in the flow profiles as a function of the type of starch and HPP processing time was observed. Indeed, at low and high shear forces (0.1, 100 s-1), all HPP hydrogels have different initial and final values of viscosity, being tapioca the one offering the highest resistance to flow, followed by corn, wheat, and rice ($p<0.05$). The different shape of the viscosity curves confirmed the different flow behaviour of starch-based HPP hydrogels. In the entire range of shear rate applied on tapioca HPP hydrogels, higher viscosity values were measured confirming their high resistance to flow due to the strong gummy structure (Galkowska *et al.*, 2014), whereas the viscosity values of HPP hydrogels based on corn, rice and wheat starch were much lower confirming the low resistance to flow of these spreadable materials and their weak structure. These differences were more pronounced at intermediate shear rates (1-10s^{-1}). In addition, HPP hydrogels obtained at 600 MPa and 15 min showed higher flow profiles than those produced at 600 MPa for 5 min, indicating that with increasing the HPP processing times stronger structures can be obtained (higher viscosity values).

The mechanical spectra of starch-based HPP hydrogels produced with different starches and HPP processing time are shown in Figure V.4. G’ (elastic) and G’’ (viscous) moduli were recorded for all samples at 25 °C in the frequency sweep range between 0.1 and 100 (Hz).

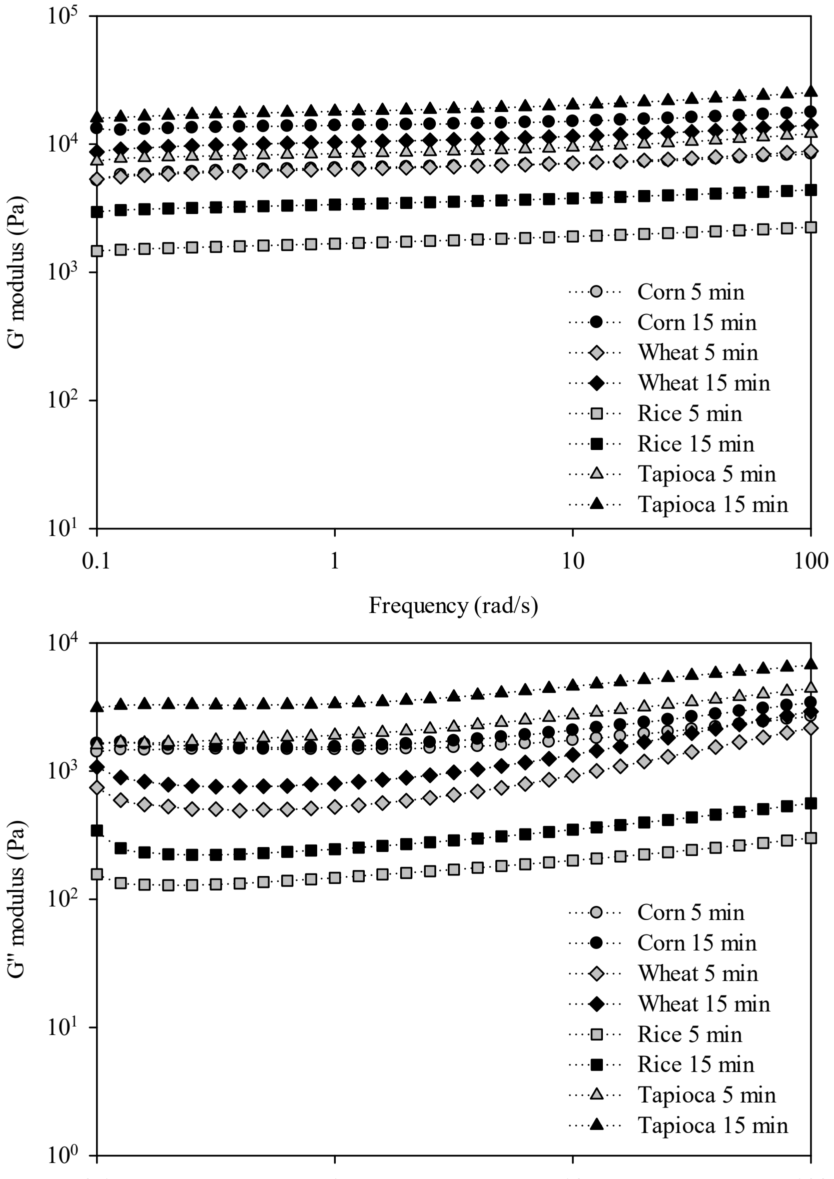


Figure V.4 *Mechanical spectra of corn, rice, wheat and tapioca starch HPP hydrogels obtained at different HPP processing times (top graph: storage modulus, G’ (Pa); bottom graph: loss modulus, G’’ (Pa)).*

From Figure V.4 it is worth noting that all starch-based HPP hydrogels produced in this investigation are characterized by mechanical profiles typical of a strong gel structure, with elastic response one order of magnitude higher than viscous response (G' > G''). Both moduli are almost independent on frequency, confirming that starch molecules form under pressure a stable and well-structured crosslinked continuous gel network (Fradinho *et al.*, 2019; Lapasin, 2016).

From a quantitative point of view, the type of starch and processing time influenced the mechanical properties of all samples. As expected, tapioca HPP hydrogels showed stronger mechanical profiles in the entire frequency range investigated with respect to rice, wheat and corn HPP hydrogels, evidencing a stronger gel network. With increasing HPP processing times (600 MPa for 15 min) the G' values of all HPP hydrogels increased, as a consequence of the increased stability of the internal structure. In gels obtained with physical methods, polymer chains are physically cross-linked by hydrogen bonds, and crystalline segments and hydrophobic interactions cooperate to producing molecular connections of different strength, stability and spatial distribution (Lapasin, 2016).

V.3.5 Texture profile analysis (TPA)

Texture parameters of HPP hydrogels are reported in Figure V.5, where the effects of type of starch and HPP processing times on texture parameters of starch-based HPP hydrogels can be recognized.

Tapioca HPP hydrogels had a different texture profile than corn, rice and wheat HPP hydrogels, with higher firmness ($p<0.05$) and negligible adhesiveness ($p<0.05$). These differences were even more remarkable when an HPP processing time of 15 min was used, confirming that in this case, a structural reinforcement of hydrogels occurred. This is in agreement with the previous discussion on rheology measurements. Despite the small values of firmness of "creamy" corn, wheat and rice HPP hydrogels, with increasing HPP processing time to 15 min a slight but not negligible increment of this parameter was observed for rice gels ($p<0.05$), while for corn and wheat gels no statistically significant differences were detected.

Cohesiveness is related to the strength of internal bonds in gels (Gokhale *et al.*, 2019). Cohesiveness values of rice, tapioca, and wheat HPP hydrogels were independent on processing time ($p>0.05$), while the values of cohesiveness of corn HPP hydrogels increased with increasing HPP processing time up to the same values of rice HPP hydrogels. All HPP hydrogels showed excellent cohesiveness (0.6-0.8), in agreement with the findings of other authors, which measured the values of this parameter on gel-matrices proposed for innovative food and non-food applications (Amjadi *et al.*, 2018; Froelich *et al.*, 2017; Gokhale *et al.*, 2019; Liu *et al.*, 2019; Tugcu-Demiröz *et al.*, 2013).

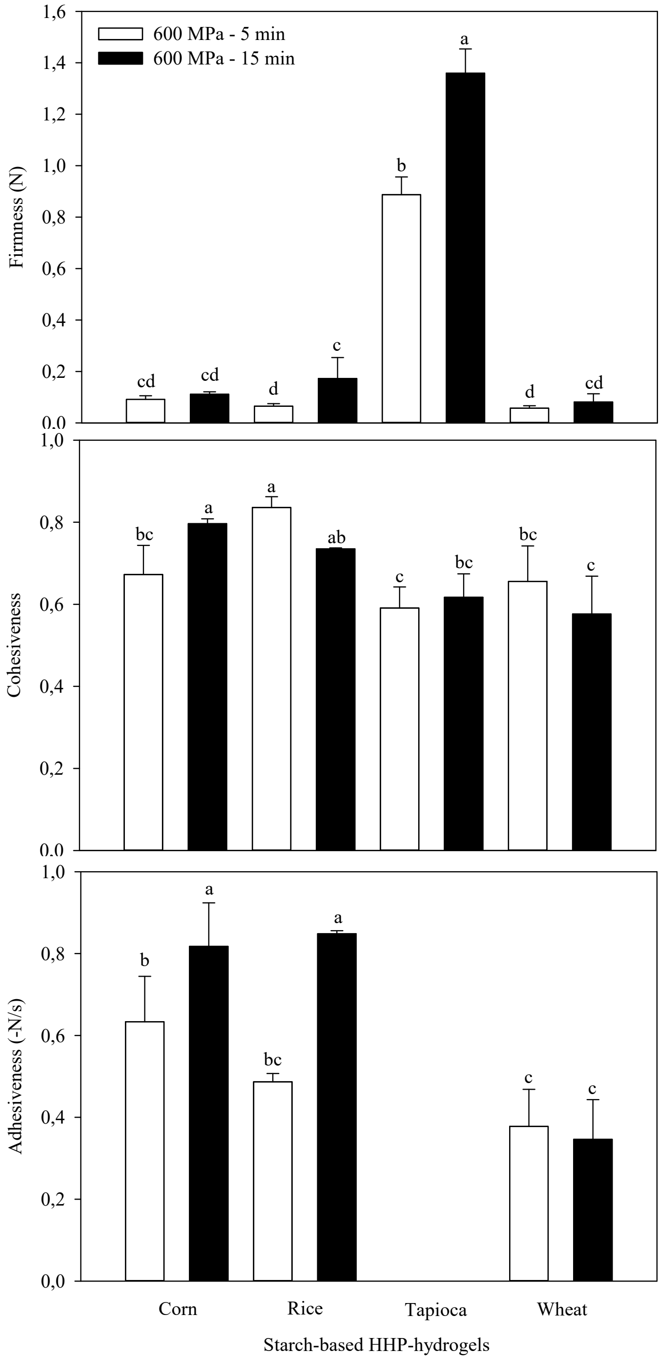


Figure V.5 *Textural parameters of corn, rice, wheat and tapioca HPP hydrogels starch obtained at different HPP processing times (Bars marked with different letters indicate significant differences ($p < 0.05$, LSD Fisher)).*

Adhesiveness, which is defined as the force required to remove the probe from the material, can be related to the force necessary to remove a bolus adhering to the palate during mastication (Bourne, 2002), a cosmetic product from the skin, or a pharmaceutical product from the mucosa (Lucero *et al*., 2016). With increasing HPP processing time from 5 min to 15 min, the adhesiveness of corn and rice HPP hydrogels increased from 0.63 and 0.49 [N*s] to 0.81 to 0.85 [N*s] ($p<0.05$), respectively. Wheat HPP hydrogels presented good adhesiveness (0.37 [N*s]), which, however, was unaffected by HPP processing times ($p>0.05$). Adhesiveness values of the spreadable HPP hydrogels were in good agreement with those found for skin photoprotection starch-based emulsions and for drug delivery systems for topical use (Lucero *et al*., 2016; Marto *et al*., 2016). Finally, gumminess and chewiness were evaluated from firmness values. Gumminess is the energy required to disintegrate a semisolid food until it is ready for swallowing and chewiness is the work needed to reduce the consistency of foods to make them suitable for swallowing (Bourne, 2002). The values of these parameters confirm that two types of gels can be obtained by HPP processing with the starches utilized in this work: easily swallowing corn, rice and wheat HPP hydrogels and firm tapioca HPP hydrogels, for which more chewing force is needed before it can be ready for swallowing.

In conclusion, texture and rheology results are coherent and demonstrated that HPP processing time and type of starch are important parameters determining the structural and mechanical behaviour of starch-based HPP hydrogels. Their key role in HPP hydrogels formation should be taken into consideration if it is desired to designing products based on these novel structures for different applications.

V.4 Conclusions

This investigation demonstrated that HPP is an alternative process to produce stable starch-based hydrogels with lower processing time and energy consumption with respect to traditional chemical and physical methods. However, the production of stable HPP hydrogels was influenced by the type of starch and processing time, both determining the physical properties of these structures as well as their potential application.

Corn, rice, tapioca, and wheat starch were completely gelatinized at the processing conditions investigated, being rice and tapioca, the most suitable starches to produce hydrogels by HPP treatments. HPP hydrogels based on corn, rice and wheat starch evidenced the characteristic “soft” gel structures, with low hardness (N) and viscosity (η) and high adhesiveness (-N/s), whiteness (WI) and lightness (L^*). Due to their good spreadability, these hydrogels are very similar to creams. HPP hydrogels based on tapioca, instead, presented a gummy or rubbery compact structure resistant to flow, with high hardness (N) and viscosity (η) and translucid colour. The mechanical properties of all hydrogels were improved with increasing the processing time at 600 MPa from 5 min to 15 min, whereas the increase of time negatively affected the colour of the hydrogels, particularly lightness (L*) and whiteness (WI).

Notwithstanding the results of this research could allow to forecast new potential applications of starch-based HPP hydrogels, further investigations are needed to assess the physical and microbiological stability of these structures as well as their performance during shelf life.

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Section II

Starch-based hydrogels produced by high pressure processing: Stability and *in vitro* digestibility

Chapter VI – *Evaluation of physical stability of starch-based hydrogels produced by high pressure processing*

Chapter VII – *Evaluation of in vitro digestibility of starch-based HPP hydrogels*

Chapter VI

Evaluation of the physical stability of starch-based hydrogels produced by high pressure processing (HPP)

Abstract Starch-based hydrogels have been extensively investigated as suitable natural structures for innovative applications. Recently, we have developed natural starch-based hydrogels by high pressure processing (HPP) in more suitable conditions than conventional methods.

The aim of this work was to evaluate their physical stability using conventional (long term) and accelerated methodologies in view of their further exploitation. To this purpose, rice, wheat, corn, and tapioca HPP hydrogels, packaged in flexible pouches, were stored at 20 °C for 90 days. Organoleptic properties, swelling stability, textural properties and microbiological counts were measured every 4 weeks. Rheological measurements and temperature cycling tests were also carried out to predict the physical stability of starch-based hydrogels in accelerated conditions.

The stability of starch-based hydrogels was strongly influenced by microbiological contamination. Rice HPP hydrogels did not show any modifications of microbial count during storage (<1 log CFU/g), whereas wheat HPP hydrogels showed a microbial load up to 3 log CFU/g after the third month of storage. Corn and tapioca HPP hydrogels showed a microbial load of 4 log CFU/g after the second month of storage and of 3 log CFU/g after the first month of storage, respectively. All the samples evidenced important organoleptic modifications with a marked reduction of the swelling stability, firmness, and adhesiveness.

On another hand, the accelerated tests predicted a very good physical stability of the starch-based HPP hydrogels, which showed good strength of the network, even superior to that of commercial hydrogels (Carbopol). Textural

properties of samples were influenced by temperature cycling test with a reduction of firmness values (tapioca > rice > corn > wheat).

Moreover, high dependence of G' modulus under T° was observed for tapioca starch hydrogels, whereas viscoelastic alterations of corn, rice and wheat hydrogels were observed only at temperatures higher than 40 °C.

In conclusion, accelerated methods could provide, in a short period compared to conventional methods, useful information on the physical stability of gel structures. Further investigations considering microbiological stability should be performed to draw more relevant conclusions from accelerated methodologies.

VI.1 Introduction

Hydrogels constitute a group of polymeric materials which are composed by three-dimensional crosslinked networks of hydrophilic/hydrophobic polymers capable to absorb and retain a significant amount of water (Biduski *et al.* 2018).

In the last years, hydrogels have been catalogued as "smart structures" owing to their tailor-made design capacity which confers different functional attributes to be used in the design, synthesis and self-assembly of novel biomaterials and drug delivery systems (Mahinroosta *et al.*, 2018; McClements, 2017).

Currently, hydrogels produced from renewable sources have been receiving significant attention from the scientific community due to their human body-friendly profile (Mohammadinejad *et al.*, 2019). Their utilization has been increasing as an alternative to replacing or reducing the use of petroleum derivates (Liu et al. 2009). Among the natural hydrogels, starch-based hydrogels are one of the most promising alternatives to achieve polymeric biomaterials (Ismail, Irani, y Ahmad 2013). Indeed, biocompatibility, hydrophilicity and biodegradability have been highlighted as remarkable characteristics of these structures which encourage their extensive use in several applications (García-Astrain and Avérous, 2018; Ismail *et al.*, 2013; Mahinroosta *et al.*, 2018; McClements, 2017; Mun *et al.*, 2015; Qi *et al.*, 2017; Van Nieuwenhove *et al.*, 2017; Xiao *et al.*, 2017).

Moreover, given the flexibility of starch-based hydrogels, the physical, mechanical and their durability can be fine-tuned by change the method intensity, components or the liquid environment. Even though being starch one of the most abundant biopolymers in nature, and industrially produced by almost all the countries around the world, the commercialization of the starch-based hydrogels has been limited by the high production costs mainly related to the long processing time and high energy consumption.

Recently, we have developed natural starch-based hydrogels by the utilization of a technology called high pressure processing (HPP). HPP, which is a nonthermal technology widely utilized in the food industry as pasteurization method enabling to minimize nutritional and sensorial properties losses in processed products, has been proposed to physically gelatinize or modify different types of starch suspensions overcoming the major limitations of conventional gelation methods, such as long duration of the process, high-energy requirements and use of hazardous materials (Błaszczak *et al*., 2015, Blaszczak *et al*., 2007; Błaszczak *et al*., 2005; Buckow *et al*., 2007; Katopo *et al*., 2002; Kawai *et al*., 2012; Larrea-Wachtendorff *et al*., 2019; Li *et al*. 2012, Li *et al*., 2015; Oh *et al*., 2008; Stute *et al*., 1996).

Physical hydrogels are either formed by physical entanglement of the polymer chains, or other non-covalent interactions, giving the reversibility profile to these structures, which is indeed an advantage for being utilized in biomedical or food related applications (Mohammadinejad *et al*., 2019). However, due to their non-permanent bonds, physical hydrogels are considered as weak gels with poor mechanical properties compared to chemical hydrogels, hindering their utilization in applications where a level of mechanical properties and certain stability is desired (Mohammadinejad *et al*., 2019).

The physical stability evaluation has been highlighted as a key feature for the applicability of pioneering gels, in order to satisfy several requirements such as structural and mechanical behaviour before their potential commercialization, it which has been characterized by long-term stability evaluation based on industrial protocols (International Conference on Harmonization, ICH) or by under accelerated stability conditions allowing to foresee the storage of samples at different stresses in short periods of time (Almeida y Bahia 2006).

To the best of our knowledge, while many efforts have been devoted to understanding and

achieving the HPP gelatinization of several starches, no studies on the evaluation of the physical stability of the hydrogels obtained by high pressure processing (HPP) have been reported. Thus, the aim of this work was to evaluate their physical stability using conventional and accelerated methodologies in view of the further exploitation of these novel structures. Moreover, this knowledge will provide baseline information for additional improvements in the processing and design of these novel structures in the future.

VI.2 “Short” Materials and Methods

VI.2.1 Raw materials, samples preparation and processing

Briefly, rice, wheat, corn, or tapioca starch in a wet basis were suspended at a starch-water concentration of 20% (w/w) and thoroughly mixed, before the HPP-treatments. HPP treatments were carried out at 600 MPa for 15 min at 25 °C in an HP unit U-22 described in § III.4.1. All the experiments were carried out at least in triplicate.

VI.2.2 Samples characterisation

Starch-based HPP hydrogels samples were characterised by long term and accelerated stability methodologies. Microbiological analysis, organoleptic evaluation, swelling stability (selling power and Aw), and texture properties were assessed up to 90 days.

All samples were analysed for the number of mesophilic aerobic microorganisms and yeasts and moulds by Plate Count Agar (PCA, Merck, Darmstadt, Germany) and Dichloran Rose Bengal Chloramphenicol medium agar (DRBC, Oxoid, Basingstoke, Hampshire, England) as was described in § III.5.12.

Organoleptic analysis of samples was performed by collecting data from visual observation and by a digital camera (Sony Corp, Japan) in angular mode. Original pictures without editing and filtering were reported.

Swelling power was determined as the methodology reported in § III.5.5. The water activity of samples was determined using a water activity metre (Novasin TH 500, Heythuysen, The Netherlands).

Texture parameters were assessed every 4 weeks based on the methodology accurately reported in § III.5.10.

Accelerated methodologies were evaluated through rheological and temperature cycles measurements. Oscillatory rheology namely temperature and stress tests were performed as previously described in § III.5.8.2.2-3. The cycling test was performed according to the method reported by Almeida and Bahia (2006). Samples were subjected to several freeze-thaw cycles for 7 days. In each cycle, the starch-based HPP hydrogels were incubated at 4°C for a period of 24 h and then changed to an incubator set 40° C for 24 h. Before TPA measurements all samples were stored at 20° C for 24 h.

VI.3. Results and discussion

VI.3.1. Long term stability

VI.3.1.1 Microbiological measurements

Growth curves of aerobic mesophilic microorganisms and yeasts and moulds on starch-based HPP hydrogels stored for 90 days at 20°C are shown in Figure VI.1.

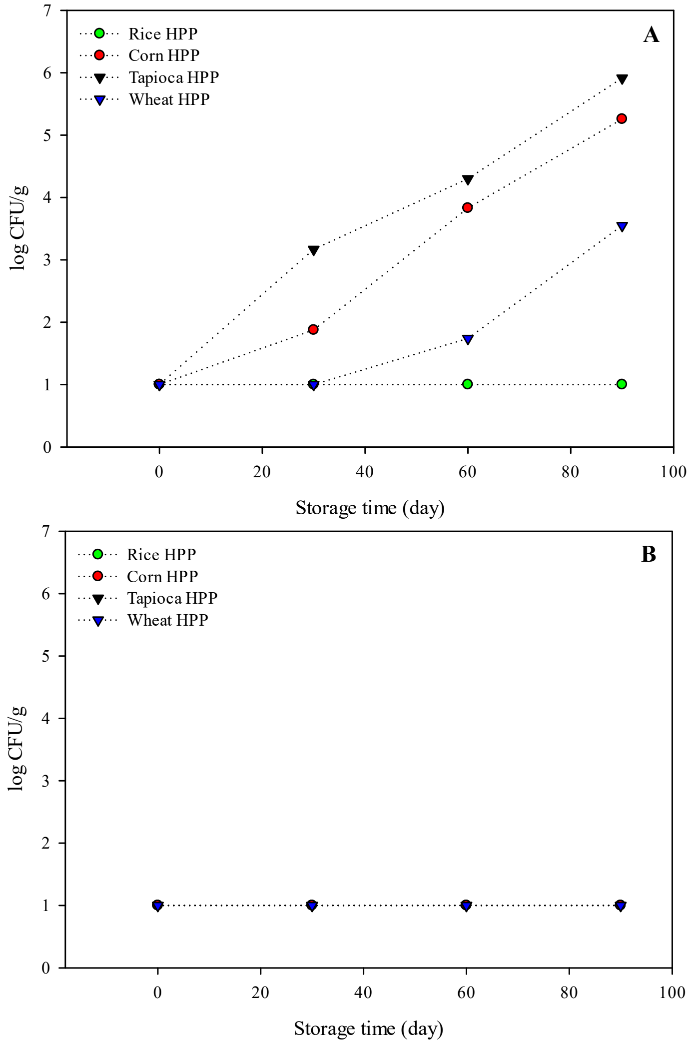

Figure VI.1 *Growth curve of total count (A) and yeasts and moulds (B) in starch-based HPP hydrogels during storage period. Symbols are means of three measurements.*

Initial microbial counts determined in this study were within the undetectable levels <1 log CFU/g in all the starch-based hydrogels evaluated. However, different microbial behaviour of these structures throughout the storage period has been detected. From data reported in Figure VI.1a, it is clearly observed that the microbial counts of rice HPP hydrogels remained unchanged during the storage period. On the contrary, the counts for mesophilic groups on tapioca and corn HPP hydrogels increased exponentially at the beginning of the storage period, reaching microbial counts of >5 log CFU/g at the end of the controlled storage (90-d). A delayed growth was observed for wheat HPP hydrogels being increased their microbial population after the first month of storage. Although microbial loads were below the detection limit immediately after HPP treatments (0 d), aerobic mesophilic microorganisms' growth during controlled storage at 20°C, demonstrating the characteristic sublethal cell injury effect caused by HPP treatment on different microorganisms (Carlez *et al*., 1994; Yi *et al*., 2013).

Even though, HPP utilized elevated pressures to inactivate harmful pathogens and spoilage microorganisms in their vegetative state (Daryaei *et al*., 2016), the microbiological inactivation by HPP is affected by the microorganisms' type (bacteria, yeasts, and moulds), form (vegetative cells or spores, gram-positive or negative), genus, species, strain, and growth phase (Balci and Wilbey, 1999; Mañas and Pagán, 2005).

From our results, and taking account that no yeast and moulds were detected during the whole storage period for all the starch-based hydrogels evaluated (Figure VI.1b), demonstrating a complete inactivation by the processing conditions utilized (Reyes *et al*., 2015), corn, wheat and tapioca starch-based HPP hydrogels were likely dominated by bacterial cells in their sporulated forms regarding their characteristic recovering trend throughout the storage period (20° C) and samples characteristics. Bacterial spores are extremely resistant life-forms triggered by stress scenario such as high pressure conditions being considered as the Achilles heel of this process due to their persistence and return to active growth after processing (Daryaei *et al*., 2016; Modugno *et al*., 2020; Nguyen Thi Minh *et al*., 2010).

VI.3.1.2 Organoleptic evaluation

The organoleptic evaluation of starch-based HPP hydrogels during the storage period are reported in Table VI.1 and Figure VI.2.

Table VI.1. *Influence of storage time on the organoleptic properties of starch-based HPP hydrogels.*

HHP-hydrogel	Appearance	30 days	60 days	90 days
Rice	Homogeneous Brilliant	N.M	N.M	More liquid
Corn	Homogeneous Opaque	N.M	Yellowish	More liquid and yellow
Tapioca	Homogeneous Compact	-Syneresis -Phase separation	Broken structure	
Wheat	Homogeneous opaque	N.M	N.M	Some lumps

N.M: No modifications at a glance.

A strong influence of the storage period on the appearance of the corn, wheat, and tapioca HPP hydrogels have been detected. As expected, a direct relationship between organoleptic changes and microbiological spoilage for all the samples evaluated has been identified. As microbiological counts increased more organoleptic changes were observed. Indeed, tapioca HHP-hydrogels evidenced syneresis and phase separation since the first month of storage whereas those hydrogels based on rice starch no modifications were observed during the whole storage period, being in agreement with the results of microbial growth discussed previously. Figure VI.2 reports the pictures of the physical appearance of the starch-based HPP hydrogels at the beginning and at the end of the storage period. It can be seen at glance, that corn, rice, and wheat starch HPP hydrogels presented a cream-like appearance while those based on tapioca starch were characterized by a rubber-like structure. After 90 days of storage at 20 °C, all hydrogels presented changes on their homogeneity and structural integrity at different extents, being more marked those changes on tapioca HPP hydrogels as was previously discussed.

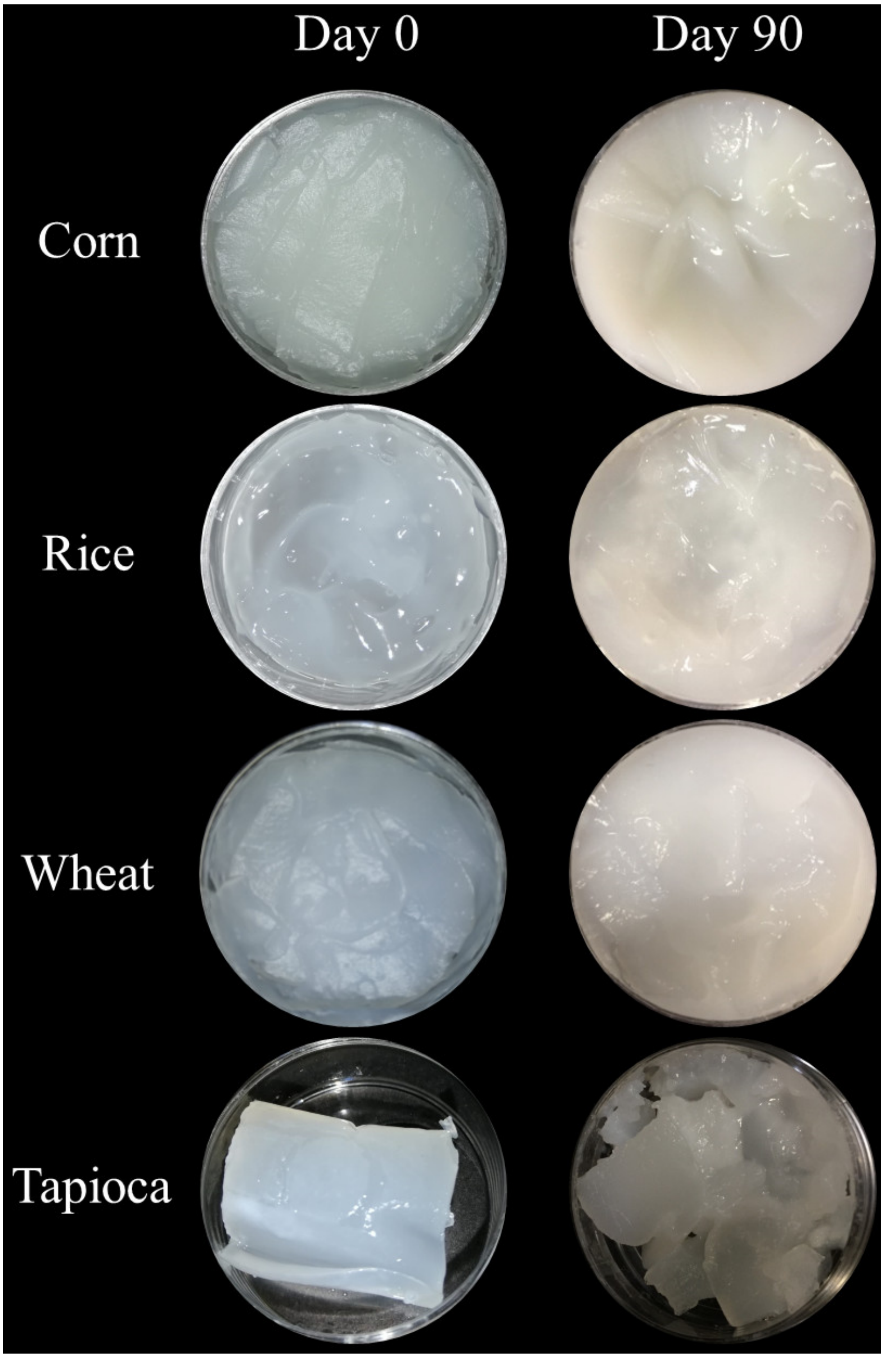

Figure VI.2. *Influence of storage time on the appearance of starch-based HPP hydrogels at the beginning and end of the storage period.*

VI.3.1.3 Swelling stability

In order to determine the swelling stability of starch-based hydrogels parameters such as water activity (Aw) and swelling power (SP) were assessed every 4 weeks. To highlight the evolution of these parameters during the storage period their variability (Δ values) against initial values (0 day) was determined. Figure VI.3 shows the evolution of Aw and SP values of corn, rice, wheat, and tapioca HPP hydrogels through controlled storage (20 °C).

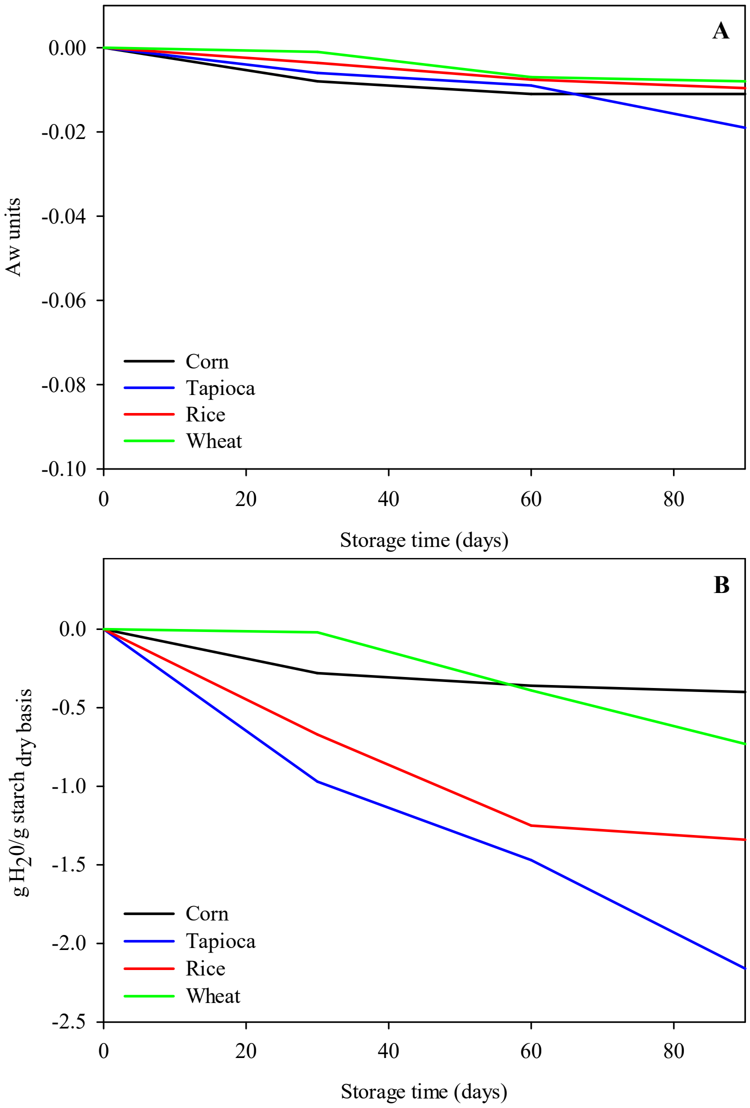

Figure VI.3. *Influence of storage time on the Aw (A) and swelling power values (B) of starch-based HPP hydrogels.*

Water is the most important component in starch-based hydrogels that influences their quality, textural properties, stability (Chung and Lai 2004), as well as their processing (Kawai *et al.*, 2007a, b; 2012). Water status describes the physicochemical state of water molecules in association or interaction with water or other molecules (Chung and Lai, 2004), which dynamic aspects can be measured by the water activity (Aw) parameter. Aw is and direct indicator of the hydrodynamic equilibrium of water in some complex matrix such as foods (Scott, 1957).

Figure VI.3a shows the changes in Aw values of rice, wheat, corn and tapioca HPP hydrogels during storage time. All samples evaluated presented similar initial Aw values ($p>0.05$) within the range of 0.974-0.969 (data not shown). However, significant reductions of Aw values were observed as storage time increased ($p<0.05$), demonstrating that the storage period affected the hydrodynamic equilibrium inside the hydrogels network driving water outwards (syneresis), which is always expected in gels with higher Aw values as ascribed Chung and Lai (Chung and Lai 2004). The authors observed in gelatine gels a significant reduction of Aw values from 0.968 to 0.914 after 15 days of storage time, due to the interaction of the water molecules with the solutes and macromolecules, as well as, with the environment that influences a continuous water redistribution, which is in agreement with the results obtained in this investigation despite the differences of the polymeric source and storage conditions.

Different hydrodynamic aspects can be identified on the different decreasing trends observed in each starch-based HPP hydrogel during the storage period. Rice and wheat HPP hydrogels showed a constant decreasing of Aw values whereas those HPP hydrogels based on corn and tapioca starch were characterized by an abrupt reduction. These differences can be explained by the different microbial counts observed in Figure VI.1 for each starch-based HPP hydrogel in function of storage time.

Swelling power (SP) indicates the capacity of the starches to bind water. In hydrogels, the water binding capacity of the starches determines their structural and mechanical properties (Hibi *et al.*, 1993; Stute *et al.*, 1996), but also it stabilize them against effects such as syneresis (Kaur *et al.*, 2011). SP is the ratio of the sedimented gel (after centrifugation forces) to the dry weight of starch. From the data reported in Figure VI.3b, it is clearly observed that SP values decreased as storage time increased, however the reduction path of each starch-based hydrogel was characteristic. Indeed, tapioca HPP hydrogels evidenced the higher reduction of its binding capacity (-2.2 g H2O/g dry starch) at the end period of the storage (90 days) due to syneresis phenomena caused by microbiological spoilage and the rupture of the structure as can be seen in Figure VI.2. Rice HPP hydrogels showed a similar reduction of the SP values during the storage time, while wheat and corn displayed higher swelling stability.

From the results reported in Figure VI.3, it can be concluded that the swelling stability of starch-based HPP hydrogels has been affected by the storage period. In particular, intrinsic factors such as starch type regarding composition and the different interactions, but also extrinsic factors such as the microbiological populations, determined the equilibrium state of water inside the hydrogels network.

VI.3.1.4 TPA measurements

Texture parameters of rice, wheat, corn, and tapioca HPP hydrogels throughout the storage period were assessed monthly. Based on our previous results, firmness and adhesiveness are the parameters that efficiently discriminate the characteristics of starch-based based HPP hydrogels. From the original data obtained from the Texturograms, firmness and adhesiveness values were analyzed in function of their variability during storage time.

Figure VI.4 reports the changes in the firmness and adhesiveness values of the starch-based HPP hydrogels throughout the storage period.

Firmness has been ascribed as an index that gives the overall information of the internal and external forces of a gel structure. Rice, corn, wheat, and tapioca HPP hydrogels presented initial firmness values (day 0) of 0.17 N, 0.11 N, 0.1 N and 1.36 N, respectively (data not shown). A marked decrease of firmness values of all starch-based HPP hydrogels throughout storage period can be observed in Figure VI.4, being tapioca HPP hydrogel the one showing the highest reduction of this parameter (>70%), whereas rice, corn and wheat HPP hydrogels, at the end of the storage period (90 days), evidenced a firmness reduction of 58%, 36% and 30%, respectively. This is in agreement with the results previously discussed, confirming the influence of the storage time on the structural conformation of the starch-based HPP hydrogels and the effects observed can be linked with intrinsic and extrinsic factors, such as starch composition, microbiological spoilage and level of structuring.

Adhesiveness, which is defined as the force required to remove the probe from the material, can be related to the force necessary to remove a bolus adhering to the palate during mastication (Bourne 2002), a cosmetic product from the skin, or a pharmaceutical product from the mucosa (Lucero *et al.*, 2016). Based on previous results (data not shown), adhesiveness is a characteristic parameter of creamy starch-based HPP hydrogels.

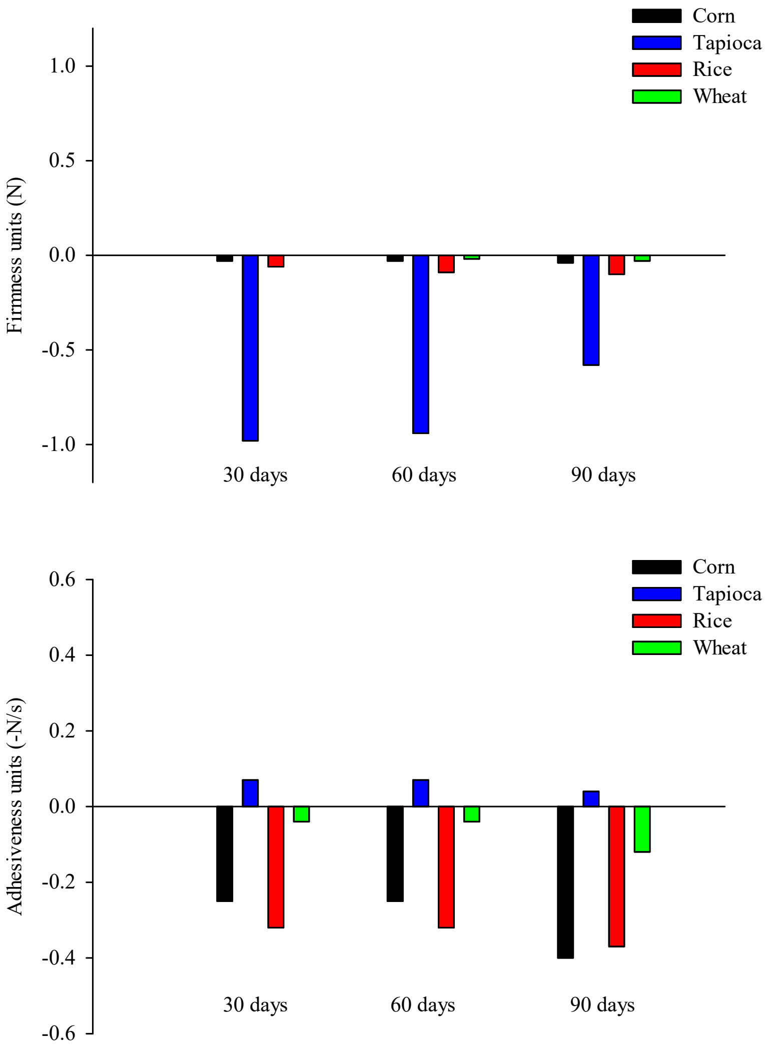

Figure VI.4. *Influence of storage time on the firmness and adhesiveness values of starch-based HPP hydrogels.*

Rice, corn, wheat, and tapioca HPP hydrogels presented initial adhesiveness values of -0.85 N*s, -0.82 N*s, -0.44 N*s and 0 N*s, respectively (data not shown). From Figure VI.4 it is clearly observed that the storage time caused several changes on the adhesiveness of the samples evaluated. As increased the storage time the adhesiveness values of rice, corn and wheat HPP hydrogels decreased. In starch gels, adhesiveness depends on the adhesive forces and viscoelasticity properties of the network (Pycia *et al*., 2015), whose were clearly affected by storage time in the case of the samples evaluated in this study.

On the contrary, tapioca HPP hydrogels evidenced a slight increment of the adhesiveness values with respect to the initial values. This result can be explained by the rupture of the structure evidenced at the first month of the storage period causing the leaching of starch components such as water, amylopectin and amylose, whose are mainly responsible for adhesive forces in starch gels (Pycia *et al.*, 2015).

Remarkably, the results of Figure VI.4 are in agreement with the previous results above-reported, indicating that the physical stability of the samples evaluated by long term methodologies has been influenced either by microbiological spoilage, starch origin and the structural properties of the hydrogels (as stiffer the hydrogels the structural deterioration was more marked).

VI.3.2. Accelerated stability

In accelerated stability tests, a product is stressed at elevated stress conditions and the amount of stress input required to cause product failure is determined. This information is utilized to the predict the capability of the product to remain within physical, chemical, and microbiological characteristics possessed at the time of its packaging, as well as to design specific container systems (Bajaj *et al.*, 2012). Parameters such as temperature, moisture, gravity, pH, package, and mechanical stress have been set as stress conditions during accelerated stability testing of gels and pharmaceutical products (Almeida and Bahia 2006; Kommanaboyina and Rhodes 1999).

VI.3.2.1 Cycling temperature stress testing

The cycling test is a commonly utilized method in pharmaceutical sciences which provides information about the products instabilities not available from isothermal tests (Carstensen and Rhodes, 1986). Cyclic temperatures are designed based on the characteristics of the products mimicking extreme storage conditions. In this study, starch-based HPP hydrogels were packaged and stored at 4°C and 40°C and the temperature was changed every 24 h for 7 days (Almeida and Bahia, 2006).

Results of the effects of the cycling test on the texture parameters of starch-based HPP hydrogels are shown in Table VI.2.

Table VI.2. *Influence of the cycling conditions on the firmness and adhesiveness of the starch-based HPP hydrogels.*

HPP-Hydrogel	Time (days)	Firmness (N)	Adhesiveness (N*s)
Corn	0	0.11±0.01^{a}	0.82 ± 0.11^{a}
	7	0.06±0.01^{b}	0.36 ± 0.05^{b}
Tapioca	0	1.36±0.09^{a}	-
	7	0.24±0.07^{b}	0.01 ± 0.01^{a}
Rice	0	0.17±0.08^{a}	0.85 ± 0.01^{a}
	7	0.06±0.01^{b}	0.50 ± 0.02^{b}
Wheat	0	0.11±0.01^{a}	0.44 ± 0.05^{a}
	7	0.06±0.00^{b}	0.25 ± 0.02^{b}

$^{a-d}$Different letters in the same column indicate significant differences for each starch evaluated separately (LSD, $p<0.05$).

Textural properties of all the starch-based HPP hydrogels were significantly affected by the different conditions of the cycling test at different extents. Tapioca HPP hydrogels evidenced a sharp decrease in its firmness values (87.5%) and the appearance of adhesiveness after the cycling test. Rice, corn and wheat HPP hydrogels showed a reduction of firmness and adhesiveness values ($p<0.05$), being those texture changes more marked in rice HPP hydrogels. These results demonstrate the influence of temperature cycles on hydrogels samples, and remarkably, were similar to the ones observed after the storage period by long term methodologies, highlighting the efficacy of the cycling test to determine temperature effects on this type of structures in a short period of time.

VI.3.2.2 Rheology

Rheological tests have been proposed to predict the stability of products and provide useful information in a short period of time to improve formulations or certain instabilities of gels structures (Almeida and Bahia 2006). Oscillatory measurements, such as temperature and stress sweep tests have been used to determine physical stability of emulsions, oleogels, hydrogels and pharmaceutical products (Almeida and Bahia 2006; Rahman and Doolaanea, 2017; Taherian *et al.*, 2008).

VI.3.2.2.1 Temperature sweep test

In order to predict the thermal stability of the starch-based HPP hydrogels produced in this investigation a temperature sweep from 25 °C to 60 °C at a heating rate of 1 °C/min has been performed using a small amplitude oscillatory shear testing. For the sake of comparison, thermal gels after storage for 7 days at 20 °C were also investigated. The results of Figure VI.5 report the measured elastic response (G') of corn, rice, tapioca, and wheat hydrogels as a function of the dynamic temperature ramp.

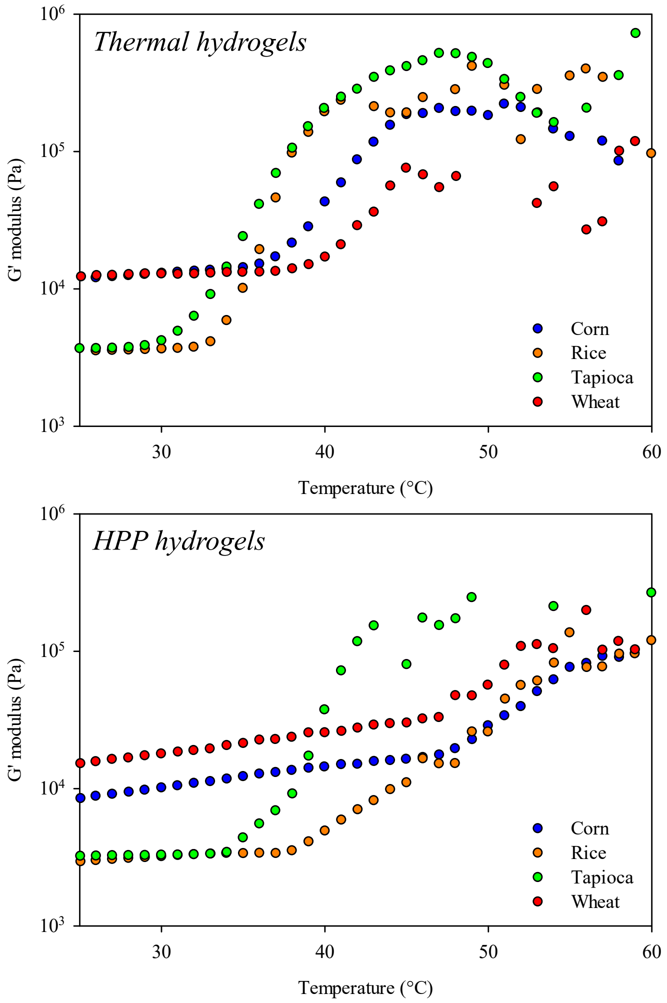

Figure VI.5. *Influence of temperature on the elastic modulus (G') of starch-based hydrogels produced by either thermal or HPP treatments.*

From Figure VI.5 it is clearly observed that temperature increment influenced the rheological properties of the starch-based HPP hydrogels at different extents. Corn and wheat HPP hydrogels displayed a less G' dependence on temperature than tapioca and rice HPP hydrogels. Indeed, elastic instabilities were evidenced over 45°C in corn and wheat HPP hydrogels whereas for rice and tapioca HPP hydrogels at 38°C and 33°C, From Figure VI.5, it can be observed that the elastic moduli of corn and wheat HPP hydrogels displayed a weaker dependence on temperature than pressurized tapioca and rice samples. In particular, viscoelastic instabilities were evidenced over 47°C in corn and wheat HPP hydrogels, whereas for rice and tapioca HPP hydrogels at 39°C and 35°C, respectively. Moreover, the inflexion points of G' values which correspond to the temperature that affects the gel-like profile of the hydrogels were always observed at higher temperatures (± 6.5 °C average) in hydrogels produced by high-pressure processing than those thermally obtained, demonstrating superior thermal stability.

Traditionally, physical gels exhibited a high dependence of the G' modulus on increasing temperature, being observed significant decreasing of the elastic domains mainly related to the loss of interconnectivity of the network constituents (Almeida and Bahia, 2006). Interestingly, an opposite behaviour can be observed in Figure 5 since the elastic domains (G') of starch-based hydrogels increased (inflexion points) as temperature increased. This trend can be attributed to the retrogradation phenomena typically observed in starch gels produced by thermal treatments and, to a less extent, in HPP-treated starch (Doona et al., 2006; Hu *et al*., 2011; Yang *et al*., 2017).

Starch retrogradation is a process where the gelatinized starch recrystallizes during storage, resulting in rheological changes such as increment in viscosity and elasticity (Fang *et al*., 2019; BeMiller and Whistler, 2009).

Moreover, some studies demonstrated that differently from thermal gelatinization, HPP produces a characteristic retrogradation phenomenon due to different water dynamics (absence of stirring) and almost intact starch granules, thus with a less quantity of leached amylose prone to be retrograded (Doona *et al*., 2006; Hu *et al*., 2011; Yang *et al*., 2017).

BeMiller (2019) stated that amylose molecules undergo retrogradation with a much more rapid rate than amylopectin, being the rate and the extent of retrogradation dependent on several factors such as amylose content, structures of the amylose and amylopectin molecules and botanical source, which is in agreement with the differences observed in the thermostability of hydrogels produced by HHP and thermal treatments (Figure VI.5), and with the results observed in the conventional methodologies such as organoleptic evaluation (§ VI.3.1.2) where the retrogradation effects upon storage have been identified.

Nevertheless, more research should be performed about HHP-induced retrogradation on formed starch-based hydrogels upon storage which is still unknown. However, the results obtained in this investigation provide valuable information not only to predict the physical stability of these novel structures under different environmental conditions, such as temperature changes, but also allowing to set the proper storage conditions range in view of further applications.

VI.3.2.2.2 Stress sweep test

To predict the network stability of the starch-based HPP hydrogels, a rheological stress test was performed. For the sake of comparison, a curve with the analysis of commercial physical hydrogel namely Carbopol was also added. LVR revealed the range of stress that the polymer network would remain intact and interconnected (Rahman and Doolaanea, 2017). The linear viscoelasticity range (LVR) of the starch-based hydrogels through the storage time was tested using stress sweep test, and the results obtained in function of elastic modulus (G') and viscous modulus (G'') deflection are depicted in Figure VI.6.

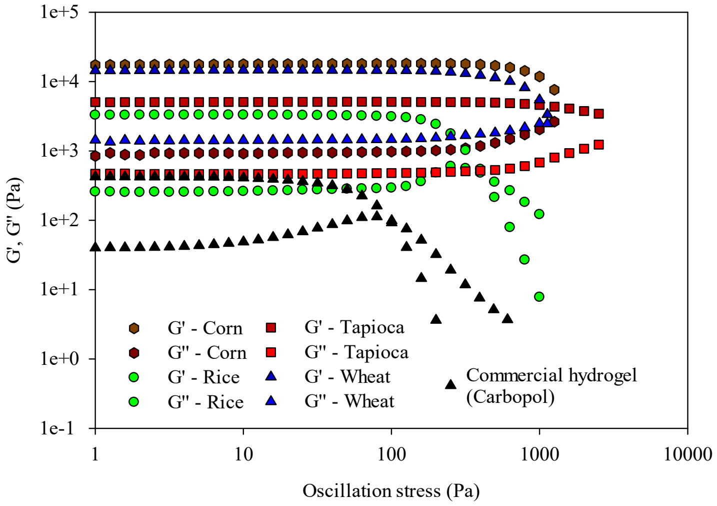

Figure VI.6. *Influence of stress on the viscoelastic behaviour of starch-based HPP hydrogels.*

From data depicted in Figure VI.6, it can be observed the different viscoelastic trend of all the hydrogels evaluated, highlighting the superior viscoelastic behaviour of the starch-based hydrogels over the commercial hydrogel (Carbopol). In particular, a higher position of the elastic (G') and viscous modulus (G''), as well as an extended LVR further the LVR of the commercial hydrogel was observed.

This result predicts a good physical stability of the starch-based hydrogels produced in this investigation regarding the superior network strength (tapioca>corn>wheat>rice>carbopol) detected. Moreover, it can be assumed that stronger starch-starch and starch-water interactions are present in these novel biomaterials, allowing to hypothesize a good performance in view of their future exploitation.

VI.4 Conclusions

Starch-based hydrogels produced by HPP are polymeric structures suitable for innovative applications. In this chapter, preliminary results of the physical stability of starch-based HPP hydrogels were obtained.

Based on the results of long term and accelerated methodologies, it was found that the physical stability of starch-based hydrogels was strongly influenced by the microbial load, starch source and the structural characteristics of the HPP hydrogels.

Moreover, accelerated methods predicted a very good physical stability of the hydrogels, which showed very good strength of the network, even superior to that of commercial hydrogels (Carbopol ®). High dependence of G' modulus under T° was observed for tapioca HPP hydrogels, where viscoelastic alterations of corn, rice and wheat hydrogels were observed only at temperatures higher than 39°C.

In conclusion, accelerated methodologies provided in a short period, a useful tool to determine the stability of starch-based HPP hydrogels under different stresses (T°, deformation). However, the microbiological stability of HPP hydrogels is a matter of concerns and must be further investigated.

Chapter VII

Evaluation of *in vitro* digestibility of starch-based HPP hydrogels

Abstract – The aim of this work was to evaluate the digestibility of HPP hydrogels based on different starches in an in vitro system simulating human digestion in view of future applications. Rice and tapioca starches were suspended in distilled water and subsequently treated at 600 MPa for 15 min at 25°C. In vitro digestion tests were carried out simulating the oral, gastric and intestinal phases of the human digestion and hydrogels disintegration, the total hydrolysed starch and nutritional fractions were determined. Experimental results demonstrated that hydrogels based on rice and tapioca starch are characterized by different behaviours during the in vitro digestion process. Rice starch hydrogels showed a more pronounced disintegration and a higher extent of starch hydrolysis than tapioca starch hydrogels, being this trend more evident in HPP hydrogels ($p<0.05$). Mastication and intestinal phases were identified as the critical steps for rice and tapioca hydrogels digestion, respectively. However, both starch-based hydrogels were almost completely digested at the end of the in vitro digestion phases, with values of hydrolysed starch higher than 94% and 88% for rice and tapioca starch, respectively, irrespective of the production method.

In conclusion, in vitro digestibility of starch-based HPP hydrogels was strongly influenced by the type of starch and the preliminary information obtained in this study could be considered the baseline for future oral applications of these structures.

VII.1 Introduction

Starch, throughout the history, represents an essential component of human nutrition and is widely used in the food industry as a coating, blending, bulking and thickening agent and, more recently as a gel-hardener (Larrea-

Wachtendorff *et al*., 2019; Papathanasiou *et al*., 2015). Moreover, starches, are one of the most abundant biopolymers on earth, as well as one the most versatile and economical material of potential use in polymer technology, and in food and non-food applications representing by far an interesting raw material for several preparations, due to their low-cost, availability and safe nature.

At nowadays, one of the big challenges of the industries and scientific community has been the development of plant-based systems to replace or reduce the utilization of synthetic materials in view to fulfil consumers demand for natural products, as well as, implementing strategies to reduce the environmental burdens and finding alternative applications of renewable sources like starch, through the implementation of eco-efficiency processes, could increase the sustainability of crops.

One of the most promising applications of this natural biopolymer is the development of starch-based hydrogels, which are hydrophilic 3D polymeric networks able to absorb and retain a significant amount of water. The most important characteristics of starch-based hydrogels are safety, biocompatibility and biodegradability, which encourage their extensive use in several applications (García-Astrain and Avérous, 2018; Ismail *et al*., 2013; Mahinroosta *et al*., 2018; McClements, 2017; Mun *et al*., 2015; Nieuwenhove *et al*., 2017; Qi *et al*., 2017; Xiao *et al*., 2017).

By applying physical or chemical stresses to starches, water penetrates in starch granules causing the formation of a strong and stable network, namely the hydrogel structure (Biduski *et al*., 2018). Moreover, the versatile character of the different starches and their numerous transitions regarding the processing method, have been utilized to produce starch-based materials with tailored design.

Several starch-based hydrogels have been synthesised from potato, rice, wheat, corn, tapioca, pea, and lentil starch for several innovative applications such as sorption of dyes, metal capture from wastewater and agricultural uses, as well as for electrical uses, food preservative, personal care products, tissue engineering, drug delivery, among others (Ismail *et al*., 2013; Hashem *et al*., 2007; Dragan *et al*., 2011; Zheng *et al*., 2010; Chauhan *et al*., 2006; Singh *et al*., 2008; Abd El-Mohdy *et al*., 2011; Guo *et al*., 2005; Guo *et al*., 2005; Cheng *et al*., 2004; Schoeack *et al*., 2002; Tsuji *et al*., 1990; Weisman *et al*., 1986; Viyoch *et al*., 2010; Liu *et al*., 2011; El-Hag Ali and AlArifi, 2009; Mahkam, 2010; Vittadini *et al*., 2008; Leite *et al*., 2017).

Among the different methodologies to produce starch-based hydrogels, thermal processing, graft copolymerization of starches in presence of a crosslinker or by direct crosslinking of polysaccharide, have been the most utilized techniques (Ismail *et al*., 2013). For instance, starch-based hydrogels for agricultural or for the removal of pollutants, as well as, tailored for drug delivery and tissue engineering has been widely synthesized by grafting starch with acrylic acid utilizing persulfates as initiators (Fariña *et al*., 2019; Tanan *et al*., 2019; Mohammadinejad *et al*., 2019; Ismail *et al*., 2013; Athawale and Lele, 1998; Dragan and Apopel, 2001; Pourjavadi *et al*., 2004) or with the addition of methacrylic acid or β-glycerol-phosphate, pH-responsive starch-based hydrogels for ketoprofen delivery and thermo-responsive starch/chitosan hydrogels for chondrocyte delivery cartilage tissue engineering have been produced respectively (Ngoenkam *et al*., 2010; Ali and Alarifi, 2009). Indeed, due to their fine-tuned structured modulated by the cross-linking intensity chemically-crosslinked starch-based hydrogels have been utilized for the delivery of drugs on the intestinal tract (Noh *et al*., 2019; Ozay *et al*., 2019; Solomevich *et al*., 2019; Liu *et al*., 2011; Casadei *et al*., 2008).

In order to respond to the increasing demand of energy-saving processes with reduced environmental impact, a high number of investigations were dealing with the use of alternative non-conventional methods, such as high pressure processing (HPP), to produce hydrogels. These methods could allow overcoming the major limitations of conventional gelation methods, such as long duration of the process, high-energy requirements and use of hazardous materials.

HPP is a well-known non-thermal technology mostly applied for food preservation, causing no or minimal sensory and nutritional damages to processed products, as well as, an alternative processing method for modification of starch suspensions or for starch suspensions gelatinization to prepare starch-based hydrogels (Blaszczak *et al*., 2015; Blaszczak *et al*, 2005a; Blaszczak *et al*., 2005b; Blaszczak *et al*., 2007; Buckow *et al*., 2007; Katopo *et al*., 2002; Kawai *et al*., 2012; Li *et al*., 2012; Li *et al*., 2015; Oh *et al*., 2008; Stute *et al*., 1996). It is well known that HPP causes the disordering of biopolymers, including proteins and starches, which induce modifications of non-covalent intermolecular interactions, thus pressure-assisted gelatinization (Balny, 2002). Under high pressure, starches undergo morphological and structural changes, exhibiting different gelatinization extent, with intact granules and limited swelling of starch particles remaining after treatment, and different structural properties compared to thermally treated starches (Li *et al*., 2012; Buckow *et al*., 2007; Hibi *et al*., 1993). Almost all starch suspensions could be gelatinized under high pressure at room temperature. However, it has been shown that HPP assisted gelatinization process, consisting in the hydration of the amorphous and crystalline regions in excess of water under compression forces, is strongly influenced by the type of starch, starch/water ratio, pressure level, temperature and processing time (Bauer and Knorr, 2005; Pei-Ling *et al*., 2010).

In the last 10 years, a great deal of research have been devoted to understanding the effects of HPP treatments on the digestibility of several starches such as amaranth, quinoa, wheat, waxy wheat, rice, waxy rice, tapioca, corn, waxy corn, buckwheat, sorghum (Lindsberger-Martin *et al*., 2012; Tian *et al*., 2014; Zeng *et al*., 2014; Papathanasiou *et al*., 2015; Liu *et* *al.*, 2016a, b; Hu *et al.*, 2017; Zeng *et al.*, 2018; Okur *et al.*, 2019), highlighting that HPP treatments modify the overall digestibility of such biopolymers, however, no studies were carried out to individuate the potential use of such biopolymers considering all the conditions of the digestive system such as oral, gastric and intestinal phase.

Therefore, the aim of the present study was to evaluate the digestibility behaviour of starch-based HPP hydrogels produced from rice and tapioca starch by an in vitro assessment in view of further oral applications of these newly emerged structures. Physical disintegration, hydrolysis extent and nutritional quality have been measured in order to determine the complete digestibility behaviour of these structures throughout the different simulated human digestive system stages.

VII.2 “Short” Materials and Methods

VII.2.1 Raw materials, samples preparation and processing

Briefly, rice and tapioca HPP hydrogels obtained at 600 MPa for 15 min and by thermal treatments were utilized in this experimental work. Samples preparation and processing were previously reported in § III.3.1, § III.3.2.1-2, and § III.3.2.2.1. All the experiments were carried out at least in triplicate.

VII.2.2 Samples characterisation

In order to evaluate the digestibility of hydrogels samples throughout the human gastrointestinal tract, an in vitro digestion model consisting of three phases, oral, gastric and small intestinal was implemented in this study, based on literature findings as was accurately described in sections § III.5.11, and § III.5.11.1-3. In vitro digestibility of starch-based hydrogels samples was characterised by disintegration extent, hydrolysation extent and nutritional fractions, based on methodologies described in § III.5.11.1-6.

All the experiments were carried out at least in triplicate.

VII.3 Results and discussion

VII.3.1 In vitro digestibility of starch-based hydrogels

In order to forecast the potential utilization of starch-based HPP hydrogels for oral applications, a complete study of their digestion behaviour of rice and tapioca hydrogels through an in vitro system simulating the human digestive system has been carried out. Moreover, for the sake of comparison, starch-based hydrogels produced by thermal treatment were also analyzed.

When performing an in vitro analysis simulating the oral, gastric and intestinal phases of the human digestion, it is of utmost importance to specifically individuate a series of parameters to quantify and qualify, in the most representative manner, the digestion behaviour of this structures throughout the different steps of the human digestive system. Considering the structural characteristics of the starch-based hydrogels, physical disintegration extent, total hydrolysed starch and different nutritional fractions were determined.

VII.3.1.1 Disintegration extent

In order to quantify the total digestibility of the two starch-based hydrogels produced in this work, their physical disintegration in the different in vitro human digestion phases was determined. The disintegration extent was measured determining the fraction of hydrogel remaining unchanged after each phase of the *in vitro* model. The remaining structure of hydrogels was considered as the part of hydrogel which was not hydrolysed or destroyed in the different conditions of the *in vitro* model. The effects of each *in vitro* phase on the disintegration extent of starch-based hydrogels produced by HPP and thermal treatments are shown in Figure VII.1.

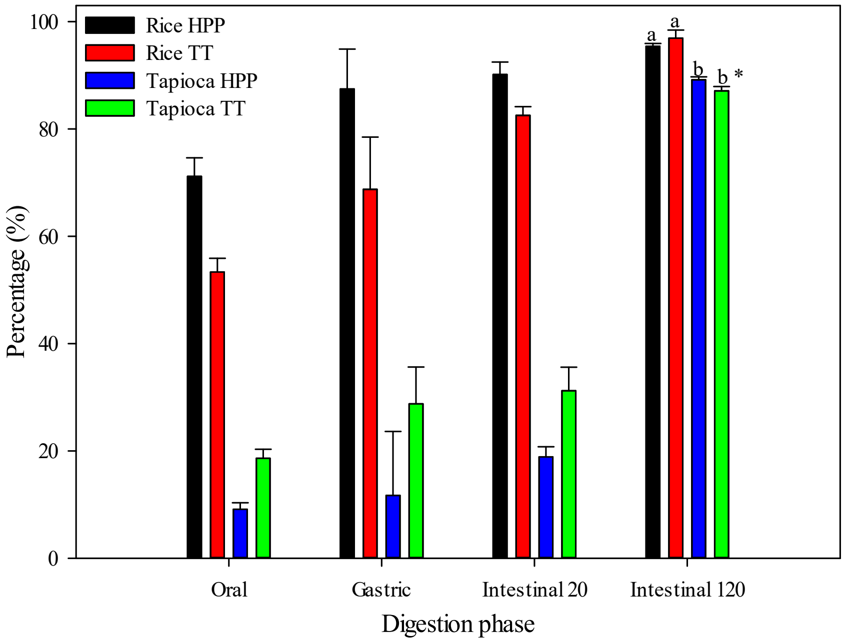

Figure VII.1 *Disintegration extent (%) of rice and tapioca hydrogels produced by high-pressure processing (HPP) and thermal treatment (TT) throughout the different phases of the in vitro GIT model.* $*^{(a\text{-}b)}$ *Different letters in superscripts indicate significant differences.*

From Figure VII.1 it is clearly noticed that the physical disintegration of starch-based hydrogels produced by HPP and thermal treatment was influenced by the different conditions of the in vitro digestive system (p<0.05). Moreover, rice starch hydrogels showed higher disintegration values than those produced with tapioca starch in all *in vitro* phases (p<0.05). In the oral phase, rice hydrogels reached up to 71% disintegration, while tapioca hydrogels were affected by mastication and swallowing conditions of the oral phase only slightly, reaching 20% of disintegration only. During the gastric phase, rice hydrogels were almost completely disintegrated (87%), while tapioca hydrogels were disintegrated up to 90% in the intestinal phase. These results can be explained by the different structural characteristics of rice and tapioca hydrogels as can be seen in Figure VII.2.

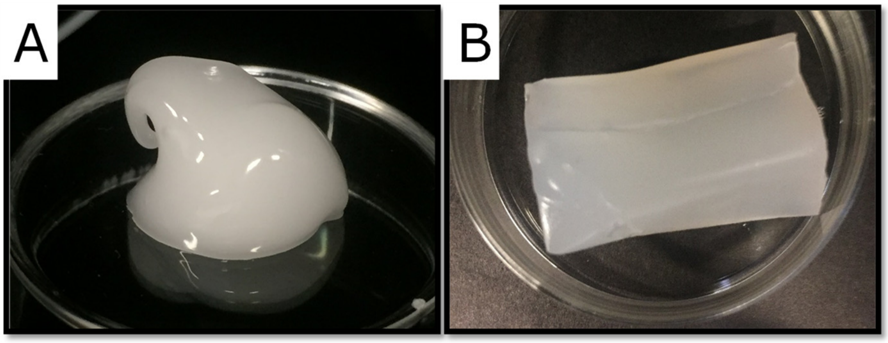

Figure VII.2 *Visual appearance of rice (A) and tapioca starch (B) HPP hydrogels obtained at 600 MPa for 15 min.*

Based on previous determinations (§V.3.5), tapioca hydrogels produced by HPP have firmness values almost ten times higher than rice hydrogels, highlighting the role of the network strength as a barrier to the structure disintegration produced by mastication and swallowing processes. In the oral phase, the effectiveness of shear forces applied during the mastication and swallowing processes in determining the extent of structure disruption is governed by the physical properties of the food (Mosca and Chen, 2017). Our results allowed us to determine that the cream-like structure of rice hydrogels was considerably affected by mastication while the compact rubbery structure of tapioca hydrogels was able to resist chewing and swallowing processes. Nevertheless, the latter structure was practically completely disintegrated in the final stage of the *in vitro* digestion, demonstrating the important role of the pancreatin present in the intestinal phase over highly structured matrices such as tapioca starch hydrogels (Dekkers *et al*., 2016). Based on the results, it was identified the oral phase as the key step for rice hydrogels digestion, whereas the intestinal phase and its inherent conditions were the critical step for tapioca starch hydrogels digestion.

Moreover, rice HPP hydrogels presented a higher disintegration extent than those produced by thermal treatment (TT) in all the *in vitro* digestion steps prior to the intestinal phase (p<0.05). This result is explained due to that Rice

TT hydrogels displayed a highly structured profile differently from those produced by HPP, thus clarifying the differences observed on the early stages of the simulated digestion of HPP and TT rice starch hydrogels. However, the disintegration values of rice HPP hydrogels were greater than 95% at the final stage of the intestinal phase, similar to the values measured for rice TT hydrogels ($p>0.05$), demonstrating that for those kinds of structures a synergetic effect of mastication and swallowing processes and the enzymatic action in the intestinal digestion phase is likely to occur, regardless the processing method.

On another hand, tapioca TT hydrogels showed higher disintegration compared to that produced by HPP in the early phases of the digestion process, with a difference of 10% in the oral phase, and of 24% in the gastric phase. These differences are explained considering the physical characteristics of these structures, having tapioca HPP hydrogels a higher structured profile with respect to those produced by TT (data not shown). Despite these differences in the early stages of digestion, the disintegration of tapioca HPP hydrogels and tapioca TT hydrogels reached a value of 85% at the end of the intestinal phase ($p<0.05$), highlighting that for the starch-based hydrogels produced in this investigation either by high-pressure processing or thermal treatments were characterized by a good digestibility.

VII.3.1.2 Degree of hydrolysis

In humans, starches and their derivatives are digested in several stages (Lehman and Robin, 2007). In the mouth, in contact with saliva α-amylase, the starch polymeric chains, is cleaved into shorter oligosaccharides. Once entering the gut, the partially digested material is further hydrolysed by pancreatic enzymes. The reaction rate of this hydrolysis is governed by the degree of polysaccharide branching, the presence of pores, as well as, by the structure surrounding the starch granules (Park & Rollings, 1994). Rice starch hydrogels produced either by HPP or thermal treatment evidenced higher hydrolysis extents in all the steps of the in vitro human digestion system compared to those based on tapioca starch ($p<0.05$), mainly due to physical and steric hindrance effects, as can be seen in the results of the degree of hydrolysis reported in Figure VII.3.

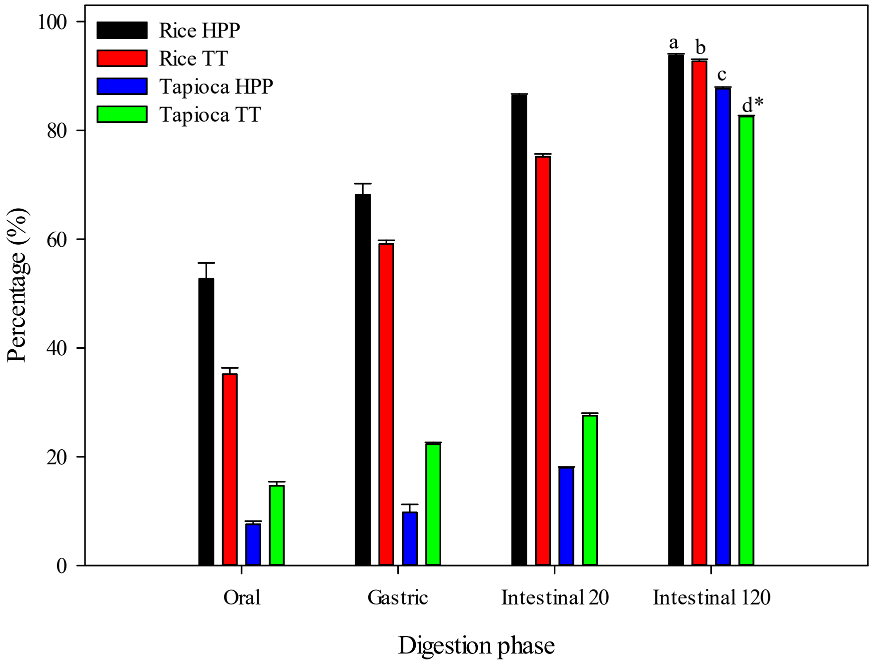

Figure VII.3 *Degree of hydrolysis (%) of rice and tapioca hydrogels produced by high-pressure processing (HPP) and thermal treatment (TT) throughout the different phases of the in vitro digestion GIT model.* $^{*(a-b)}$ *Different letters in superscripts indicate significant differences.*

According to Watcharatewinkul *et al.* (2010), starch digestion is a complex process which involves the accessibility of hydrolytic enzymes in the starch granules. Thus, a marked dependence of digestion on the physical characteristics of the structures surrounding the starch granules can be assumed for the interpretation of our results, which demonstrated that the reduction of hydrogel structural integrity gives rise to higher digestion effectiveness. Zhang and Hamaker (2009) observed that starches from cereal sources, such as rice starch, contain peripheral pores and channels, which enable α-amylase penetration, the hydrolysis reaction resulting as an inside-out mechanism. On the contrary, starches from roots or tubers, such as potato or tapioca starches, are digested by exocorrosion, starting from the surface of the starch (Gallant *et al.*, 1997). These different hydrolysis mechanisms can explain the higher digestibility of rice starch hydrogels compared to tapioca starch hydrogels and the different steps characterizing the enzymatic action during the hydrolysis reaction over these structures.

Rice and tapioca HPP hydrogels, at the final stages of the digestion phases, showed higher hydrolysation extents than those produced by thermal treatments ($p<0.05$). The different amount of hydrolysed starch in rice TT hydrogels and rice HPP hydrogels was evident since the oral phase. The two values of hydrolysed starch differed of 17% demonstrating that the physical structure of rice HPP hydrogels was more affected by the physical stresses exerted in the mouth during the oral digestion phase compared to rice TT hydrogel, but also due to the swelling of starch granules during HPP, the pores of rice starch granules could expand, this facilitating the entrance of α-amylase enzymes in the particles to making an inside-out hydrolysis (Zhang *et al.*, 2006). Even though HPP and TT rice starch hydrogels structures did not show strong differences in the following digestion phases, HPP structure always showed a higher extent of hydrolysis up to 120 minutes of intestinal digestion. This difference could be attributed to starch retrogradation occurring in thermally treated structures. In fact, the reorganization of the crystalline fraction of starch due to retrogradation makes the structure more resistant to enzymatic attacks (Papathanasiou *et al.*, 2015).

A different behaviour was observed for tapioca starch hydrogels structures. In this case, the percentage of hydrolysed starch in the early phases of digestion was higher in the thermally treated tapioca starch hydrogels than in tapioca HPP hydrogels. The difference between hydrolysed starch fractions during oral, gastric and beginning of intestinal digestion phases was always between 7% and 11%. Due to the more structured profile of tapioca HPP hydrogels, the enzymes can enter the structure less easily, this hindering the enzymatic reaction rate. Tapioca TT hydrogels were less structured, thus opposing less resistance to physical damages. Therefore, the enzymatic attack to this structure was facilitated and the extent of hydrolysis incremented. This behaviour changed at the end of the intestinal digestion phase, where the hydrolysis difference of both structures dropped to 5%. This suggests that HPP tapioca starch hydrogels were more prone to enzymes action due to the swelling of starch granules (Yang, 2017), while due to starch retrogradation, phenomena commonly observed in starch-based TT hydrogels the effectiveness of hydrolysis was reduced.

In conclusion, the starch-based HPP hydrogels produced in this investigation were better and more extensively hydrolysed in the *in vitro* human digestion simulating system than the starch-based TT hydrogels, highlighting the good digestibility of starch-based HPP hydrogels as well as their potential future utilization in products for oral applications.

VII.3.1.3 Nutritional quality

Starch can be classified by its digestibility rate in the human body. For nutritional purposes, starch is generally classified into rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS), depending on the rate and degree of its assimilation (Kingman, 1992). RDS is the fraction of starch responsible of the fast increase of glycaemic index in the bloodstream, while SDS keeps a constant glucose release, helping to control the glucose level in the blood, the RS is the remaining fraction of starch that is not hydrolysed in the small intestine, having different beneficial effects on microorganisms present in the large intestine, and thus, on human health (Lehmann and Robin, 2007).

Figure VII.4 reports the different nutritional fractions of rice and tapioca hydrogels produced by HPP and thermal treatment.

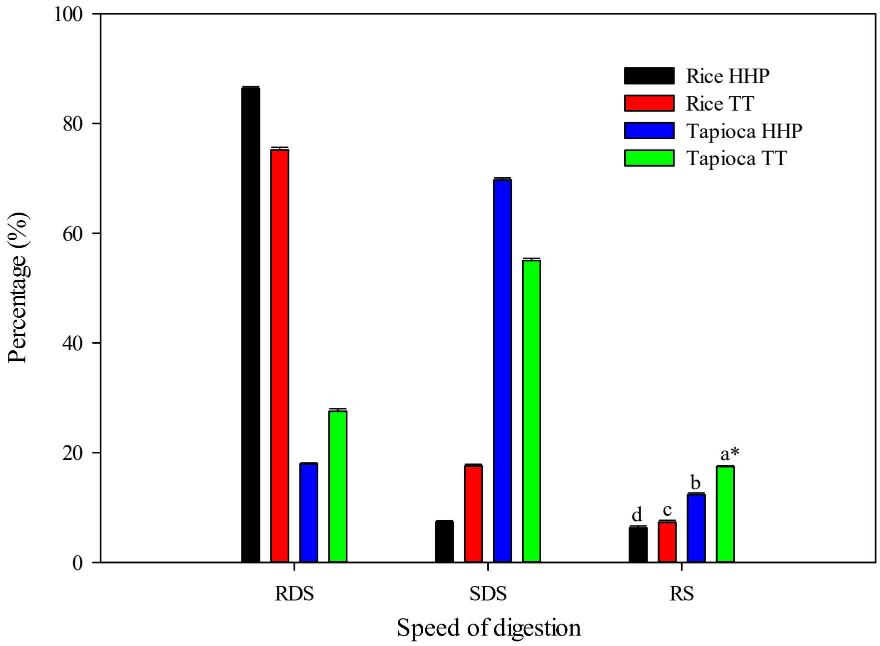

Figure VII.4 *RDS, SDS, and RS fractions in starch-based hydrogels produced by high-pressure processing (HPP) and thermal treatment.* $*^{(a\text{-}d)}$ *Different superscripts indicate significant differences.*

Rice starch hydrogels presented higher values of RDS than SDS and RS, whereas tapioca starch hydrogels showed higher values of SDS than RDS and RS, both regardless of the process used ($p<0.05$). This different nutritional profile observed for both hydrogels agreed with the results previously discussed for hydrolysis extent results (§ VII.3.1.2). The difference of RDS and SDS fractions between the two starches was due to higher rate of hydrolysis of rice hydrogels observed in the early digestion stages, which increased the concentration of released glucose in the first 20 minutes of the intestinal phase. On the contrary, tapioca hydrogels were characterized by a lower disintegration and hydrolysis rate, which kept most of the starch intact up to the second intestinal digestion phase, thus increasing the SDS. These results are in agreement with those reported in previous papers (Zhang *et al.*, 2006; Conway and Hood, 1976). Moreover, the difference of RDS value between rice and tapioca starch hydrogels could be also due to the physical characteristics of the granules, as already discussed previously. Starch granules from cereals, such as rice and corn, present numerous pinholes on the surface and pores penetrating inside the granules (Dreher *et al*., 1984). Due to these characteristics, the entrance of the enzymes in the granules and the exit of the sugars after hydrolysis was facilitated, and, consequently, the absorption of the latter troughs the intestine was facilitated.

VII.4 Conclusions

In this work, a complete study of the digestibility behaviour of different starch-based hydrogels produced by high-pressure processing technology compared with thermally produced in view of further exploitation of these novel natural structures. It was performed through the set-up of a tailored in vitro digestion model mimicking the human digestive system by simulating the conditions of the oral, gastric and intestinal phase.

It was observed that the starch-based hydrogels produced by HPP showed excellent digestibility behaviour even superior to those produced by thermal treatments, highlighting their potential in further oral applications. In particular, the results obtained so far demonstrated that the forces of mastication and swallowing in the oral phase and the enzymatic activity in the intestinal phase were the critical steps for the digestion of rice starch and tapioca starch hydrogels, respectively.

The different digestibility behaviour observed in both hydrogels determined their nutritional quality allowing to hypothesize the future applications of these novel natural structures.

However, additional research should be performed to determine the digestibility of starch-based hydrogels loaded with additional ingredients of interest in the gelling solution, as well as, the preservation of bioactivity and bioavailability of these ingredients, contributing to forecast the future exploitation of starch-based HPP hydrogels.

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Section III

Starch-based hydrogels produced by high pressure processing: Addition of compounds

Chapter VIII – *Effects of processing conditions and glycerol concentration on rheological and texture properties of starch-based HPP hydrogels*

Chapter VIII

Effects of processing conditions and glycerol concentration on rheological and texture properties of starch-based hydrogels produced by high pressure processing (HPP)

Abstract - In this work, the effects of processing conditions (pressure levels of 500 and 600 MPa and holding time of 5, 10, 15 and 20 min) and the addition of glycerol as humectant agent in corn and rice starch suspensions (at concentration of 5 and 10% w/w) on rheological and texture properties of HPP hydrogels were evaluated.

Experimental results demonstrated that at 600 MPa a homogenous gelation of the different formulations tested was occurring and stable hydrogels were formed. However, the presence of glycerol at high concentration (10%) in the starch suspensions was playing an antagonistic role with respect to the occurrence of gelation, being longer processing times necessary to obtain stable hydrogels. Viscosity and G' values of the two starch-based HPP hydrogels were decreasing with increasing glycerol concentration in the formulation, particularly for corn starch HPP hydrogels. At all processing conditions investigated, rice starch suspensions in the presence of the humectant were more prone to gelation under pressure and the hydrogels formed had better texture properties than those based on corn starch. A glycerol concentration of 5% in both starch suspensions was identified as the critical value allowing to obtaining stable HPP hydrogels with good rheological and texture properties.

Chapter VIII

VIII.1 Introduction

The interest to develop novel materials based on biodegradable polymers to replace or reduce the use of petroleum-based synthetic polymers has been increasing remarkably in recent years (Li *et al.*, 2009).

Among these new materials, in the last decade, starch-based hydrogels gained attention especially in the biomedical, pharmaceuticals and cosmeceutical sectors and were successfully developed due to their safe utilization, biocompatibility, hydrophilicity and biodegradability (Ismail *et al.*, 2013).

Starch-based hydrogels are defined as hydrophilic polymeric networks able to absorb and retain significant amounts of water. Under the action of chemical or physical stresses, water is able to enter in the starch granules, which, consequently, form strong and stable interconnected structures (Biduski *et al.*, 2018).

In order to respond to the increasing demand of energy-saving processes with reduced environmental impact, a high number of investigations were dealing with the use of alternative non-conventional methods, such as high pressure processing (HPP), to produce hydrogels. These methods could allow overcoming the major limitations of conventional gelation methods, such as long duration of the process, high-energy requirements and use of hazardous materials.

High pressure processing (HPP), already introduced in the food industry as non-thermal pasteurization method enabling to minimize nutritional and sensorial properties losses in processed products, has been proposed to gelatinize or modify different types of starch suspensions to produce hydrogels (Blaszczak *et al.*, 2015; Blaszczak *et al.*, 2007; Blaszczak *et al.*, 2005; Buckow *et al.*, 2007; Katopo *et al.*, 2002; Kawai *et al.*, 2012; Li *et al.*, 2015; Li *et al.*, 2012; Oh *et al.*, 2008; Stute *et al.*, 1996).

HPP assisted gelation can be described as the hydration process, in excess of water, of the amorphous and crystalline regions of starch under the action of compression forces. Almost all starch suspensions could be gelatinized under high pressure at room temperature. However, several factors play a significant role in determining the occurrence and extent of hydrogel formation, such as type of starch, starch/water ratio, pressure level, temperature and processing time (Pei-Ling *et al.*, 2010; Bauer and Knorr, 2005), or the addition of other compounds in the starch suspensions, such as humectants and rheology modifiers.

At present, limited information on structural properties of starch-based hydrogels obtained with HPP is available and deep rheological characterization of these polymeric networks are necessary in order to exploit their potential applications. The aim of this work was to evaluate the ability of rice starch and corn starch suspensions to form hydrogels under HPP and determine the effects of different pressure level and processing times. Moreover, the influence of the concentration of a humectant on gelation and on the characteristics of the hydrogels formed at different operating conditions was also studied. Glycerol, which is a colourless, odourless and viscous liquid with high solubility in different polar liquids (Tabasum *et al.*, 2019), was used as humectant due to its extensive use in the pharmaceutical and cosmeceutical industry to control and modify consistency and mechanical properties of the products (Simoes *et al.*, 2019).

VIII.2 "Short" Materials and Methods

VIII.2.1 Raw materials and samples preparation

Rice and corn starch powders were utilized in this experimental work. Chemical and physical characteristics of raw materials, as well as, the methods utilized for their determination were reported in § III.1 and Table III.1.

Samples preparation carried out in this work has been reported in § III.3.1. and in particular for this work in § III.3.1.3.

Briefly, rice and corn starch-water suspensions with a concentration of 20% (w/w) were obtained by dissolving starch powders in distilled water by gentle mixing at room temperature (25 °C). Defined amounts of glycerol were added to the suspensions in order to obtain two final concentrations, namely 5 and 10% (w/w). Starch suspensions without glycerol were also prepared. In order to avoid starch particle settling, starch-water-glycerol suspensions were prepared immediately before HHP treatments.

VIII.2.2 Samples processing

HPP treatments of samples upon the different operations framed in this work were reported specifically in samples processing section (§ III.3.2) for control samples (§ III.3.2.1) and HHP-treated samples (§ III.3.3.3), respectively. The experimental apparatus utilized on HPP treatments of samples was the HP unit U-111 described in § III.4.2. All the experiments were carried out at least in triplicate.

VIII.2.3 Analytical determinations

Control and HPP-treated samples were characterised by rheological measurements namely steady state flow behaviour, strain sweep tests and frequency sweep test, accurately described in section § III.5.8. Texture profile analysis of samples was performed according to the methodology reported in § III.5.9.

VIII.3 Results and discussion

VIII.3.1 Flow measurement tests

The effects of HPP processing conditions on gelation and flow behaviour of starch-glycerol suspensions were assessed by flow measurements in steady state conditions. Tests were performed to discriminate among the different HPP processing conditions utilized and, in particular, to individuate the minimum pressure level to be applied to induce starch gelation.
In Figure VIII.1 and Figure VIII.2, the data of viscosity as a function of shear rate of corn and rice starch HPP hydrogels with different glycerol concentrations and processed at different conditions were reported. The curves reported were only those of starch suspensions forming hydrogels at the processing conditions tested. It can be clearly noticed that both HPP processing conditions and glycerol concentration in the initial suspension influenced the occurrence of gelation of starch suspensions under pressure and the flow properties of hydrogels formed. According to the results of the flow measurement tests, a pressure level of 600 MPa was the most appropriate to obtain stable hydrogels in presence of glycerol for both starches. At 500 MPa, hydrogels were formed only in absence of glycerol and at processing times longer than 10 min in the case of rice starch and longer than 20 min in the case of corn starch. However, in both cases, the rheological properties of the hydrogels were far from those desired, the hydrogels showing low viscosity and high weakness. Moreover, the presence of glycerol at high concentration (10%) in the starch suspensions was damping the gelation under pressure. With increasing glycerol concentration in the starch suspension up to 10%, rice starch HPP hydrogels were formed at 600 MPa and at all processing times utilized in this work, while corn starch hydrogels were only obtained at 600 MPa and 20 min. These results demonstrated that rice starch was able to undergo gelation under pressure better than corn starch and this can be attributed to the different physicochemical properties of the two starches, which determine their susceptibility to pressure-induced gelation, as reported by Rubens *et al.*, (1999). The presence of glycerol in the starch suspensions had an antagonistic effect on gelation under pressure. Glycerol, indeed, acts as anti-plasticizer during thermal gelatinization, due to the increment of molecular weight and viscosity of the cosolvent (Chen *et al.*, 2017; Onuchic and Lacaz-Vieira, 1985). Thus, more energy is required to move water molecules into the starch granules and complete the gelatinization process. The increased viscosity of the liquid phase in the starch suspension could make difficult the migration of water inside the starch granules and the formation of the gel network under pressure. Moreover, it has been proved that glycerol has a baroprotective effect on biological membranes (at pressure levels up to 800 MPa) due to the association of this compound with proteins or phosphate groups (Onuchic and Lacaz-Vieira, 1985). Being both proteins

and phosphate groups commonly present also in starch granules (Xu *et al.*, 2017), this could explain why the presence of glycerol was hindering the gelation of starch suspensions at the HPP processing conditions tested in this work.

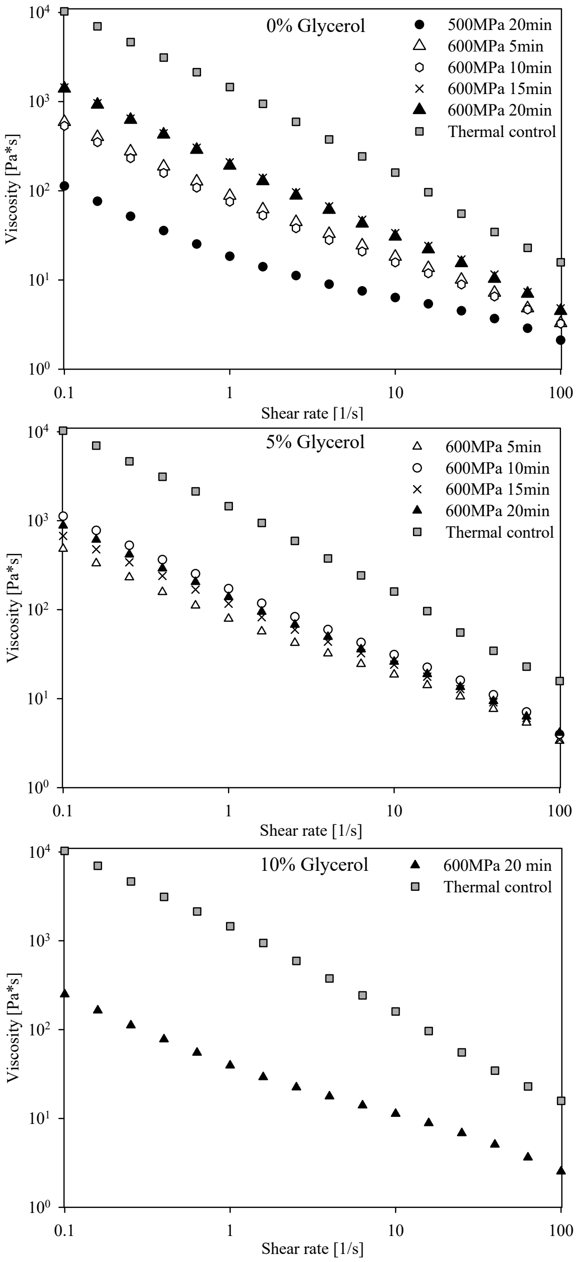

Figure VIII.1 *Viscosity of corn starch HPP hydrogels, obtained at different HPP processing conditions and glycerol concentrations, as a function of shear rate. Data of thermally formed hydrogels are also reported.*

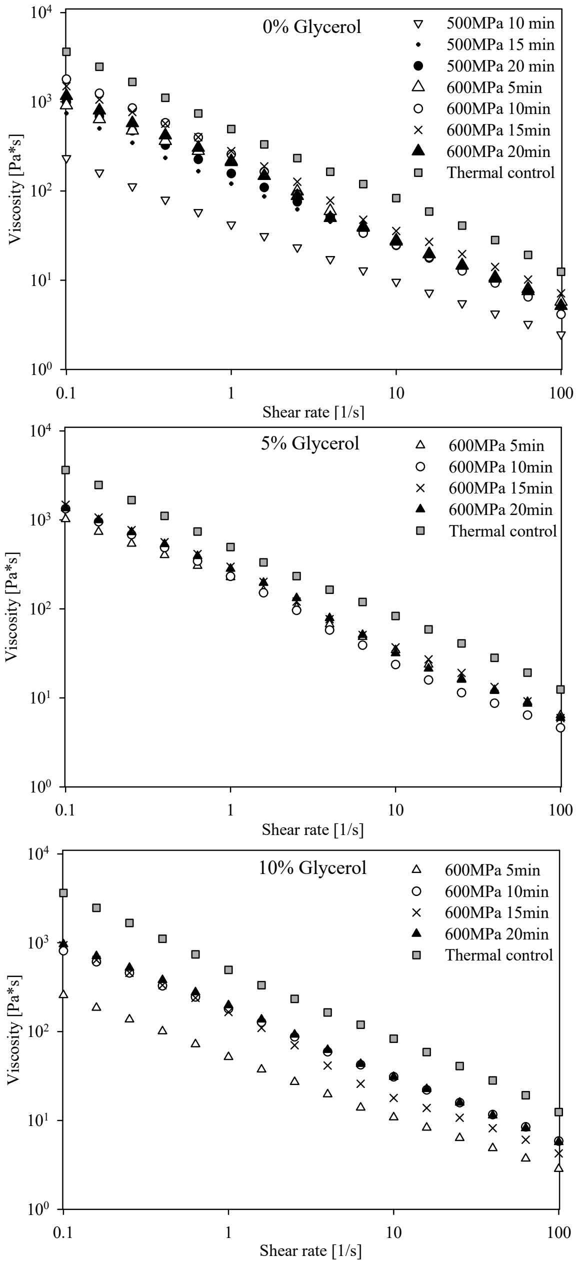

Figure VIII.2 *Viscosity of rice starch HPP hydrogels, obtained at different HPP processing conditions and glycerol concentrations, as a function of shear rate. Data of thermally formed hydrogels are also reported.*

All starch-based HPP hydrogels showed a typical gel-like behaviour (non-Newtonian shear-thinning fluid), exhibiting a reduction of the viscosity with increasing the shear rate. Such behaviour is typical of starch-based gels (Jiang *et al.*, 2015; Galkowska *et al.*, 2014; Xie *et al.*, 2009). With increasing the stress applied all polysaccharide chains were oriented towards the direction of the stress, allowing a better sliding and, thus, a reduced viscosity is measured. As already discussed, starch-based HPP hydrogels are characterized by a non-Newtonian shear-thinning behaviour. However, a marked difference in their flow profiles as a function of HPP processing conditions and glycerol concentration was observed. Indeed, in the entire range of shear rate applied, all starch-based HPP hydrogels have different initial and final values of viscosity, the highest viscosity profiles having been measured at more severe HPP conditions and no glycerol or 5% glycerol concentration in the suspension. HPP treatments at higher pressure levels and longer processing times increased the strength of hydrogel structures, confirming the role played by glycerol described above.

VIII.3.2 Strain sweep tests

Rheological properties of viscoelastic materials are usually strain-independent up to a critical value of the strain. Beyond this value, the rheological behaviour observed is non-linear, and the storage modulus G' declines. Therefore, measuring the dependence of strain amplitude on storage, G', and loss moduli, G", can be considered a sound method to characterize the viscoelastic behaviour of HPP and thermally formed hydrogels. Indeed, strain sweep test allowed establishing the extent of the Linear Viscoelastic Region (LVR) of the material. In this region, the dynamic parameters are independent on the strain, and, consequently, it is possible to compare the rheological characteristics of the various hydrogels obtained at different operating conditions.

Strain sweep tests were carried out on starch-glycerol suspensions (5% glycerol) processed at 500 MPa and 600 MPa for 5, 10, 15, and 20 minutes to individuate the LVR of starch-glycerol hydrogels and select a proper value of the strain to be used in frequency sweep tests. Results demonstrated that the critical strain was equal to 3% for all samples. Below this threshold value, the structure of the hydrogels remained intact, and the hydrogels behaved like a solid (data not shown).

VIII.3.3 Frequency sweep tests

Once the LVR of hydrogels was individuated, rheological properties of hydrogels were further characterized through frequency sweep tests at strain value (%) below the critical one.

In Figure VIII.3 and Figure VIII.4, the values of G' and G" moduli as a function of frequency for corn and rice HPP hydrogels are reported. Data refer only to the gel-forming suspensions at the processing conditions tested. It can be observed that the rheological properties of both starch-based HPP hydrogels depended on processing conditions and glycerol concentration. It can be clearly noticed that all samples exhibited mechanical profiles typical of physical gels (Lapasin, 2016), with elastic response G' prevailing on viscous response G" (G' > G"). A continuous gel network was, thus, formed between starch/water/glycerol molecules. Moreover, from data of Figure VIII.3, it can be observed that the values of G' slightly increased with frequency, and the shape of G' curves depended on processing conditions. As the treatment time was extended, the dependence of G' on frequency became linear and higher values of the elastic modulus were detected. Similarly, the values of the G" modulus increased with increasing frequency and processing time. Differently from thermal treatments, pressure affected mainly the volume of the system due to compression effects (Balny, 2002). With increasing the pressure level and the processing time, the molecular interactions in the starch suspensions were favoured and the resulting hydrogels structures became stronger.

In this study, it was demonstrated that glycerol played an antagonist effect on gelation phenomenon. At 600 MPa a concentration of glycerol of 10% significantly influenced the values of G' and G" moduli, and a clear dependence of these parameters on the treatment time was observed. At this glycerol concentration, the values of rheological modulus G' was increasing up to two orders of magnitude when the treatment time was increased from 5 to 15 minutes. G' and G" values of hydrogels obtained with treatment times equal to or higher than 15 minutes were less influenced by glycerol concentration. However, for a concentration of glycerol of 5%, the values of G' did not change significantly with treatment time, while the G" values were constant in the treatment time range investigated. G' and G" of corn starch HPP hydrogels without glycerol showed similar trends. At a pressure of 600 MPa, low glycerol concentrations (≤ 5%) did not cause any rheological changes in corn starch hydrogels. However, at 500 MPa gelation occurred only in absence of glycerol and at a treatment time of 20 minutes (data not shown). In these conditions, the increase of the treatment time influenced the G' and G "moduli, whose values increased of up to three orders of magnitude (data not shown).

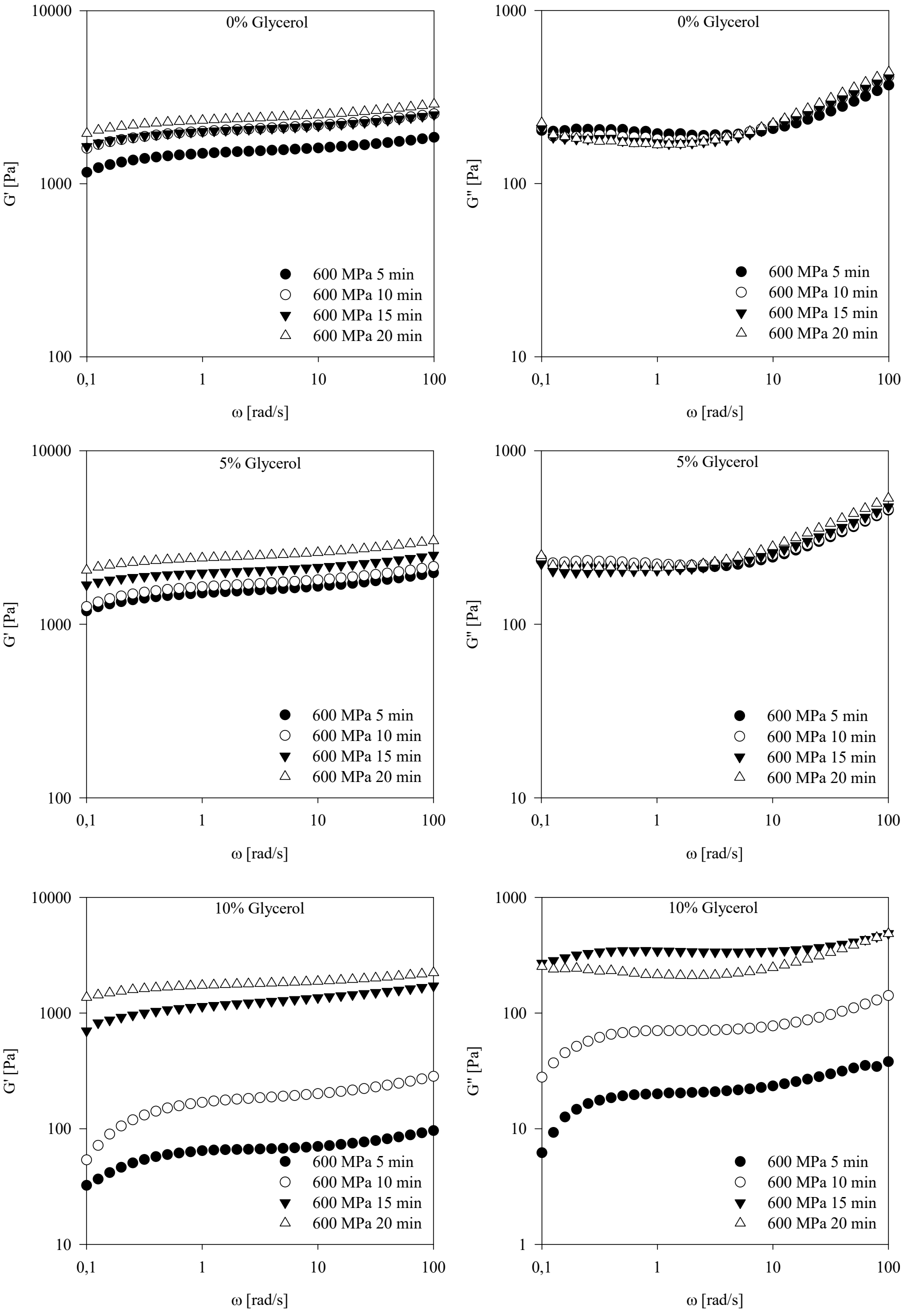

Figure VIII.3 *G' and G'' moduli of corn HPP hydrogels, obtained at different HPP processing conditions and glycerol concentrations, as a function of frequency.*

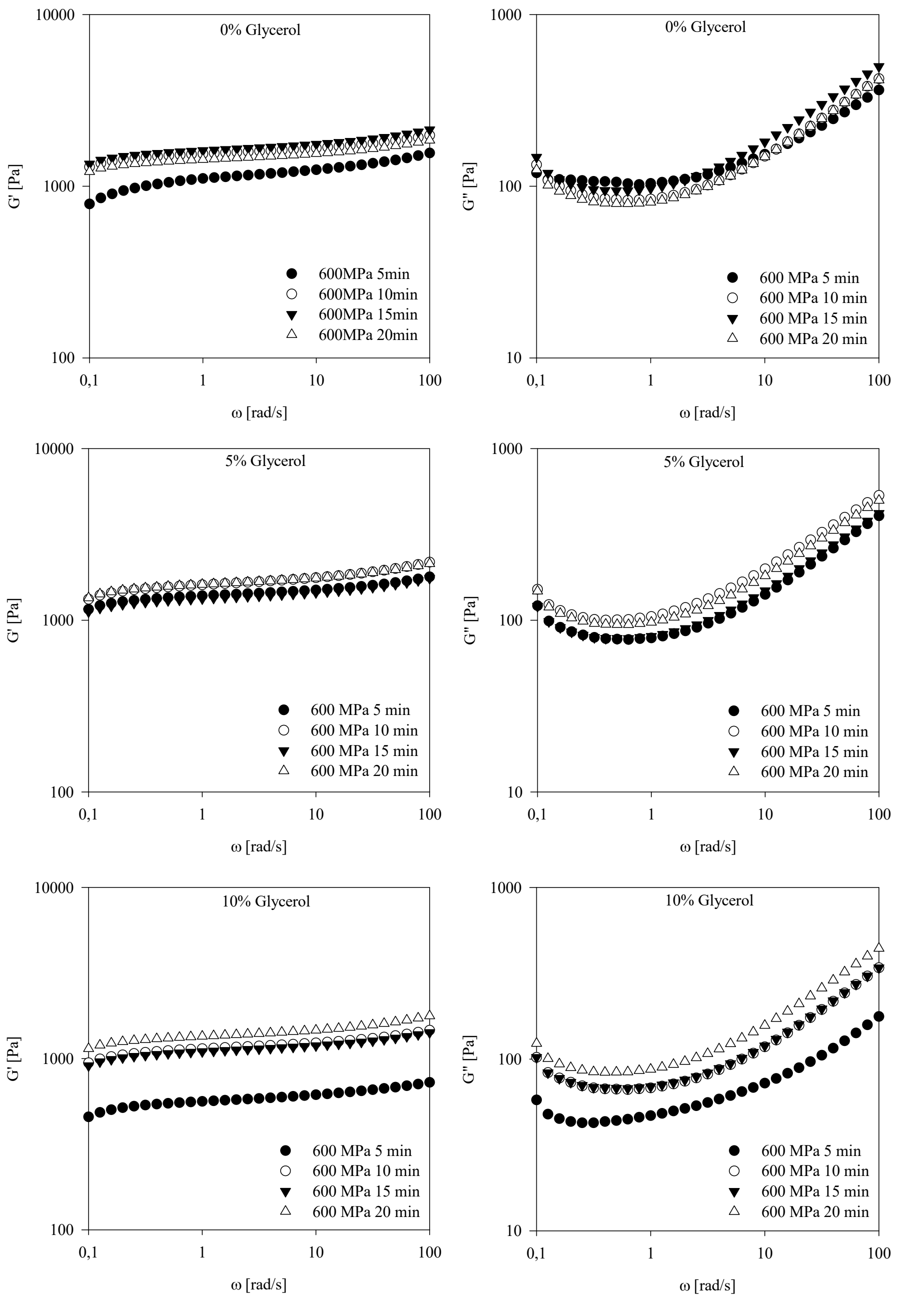

Figure VIII.4 *G' and G'' moduli of rice HPP hydrogels, obtained at different HPP processing conditions and glycerol concentrations, as a function of frequency.*

The influence of glycerol on the rheological characteristics of starch-based HPP hydrogels was highlighted in this study. Indeed, a reduction of glycerol concentration from 10% to 5% in the starch suspension caused the increase of one to two orders of magnitude of all rheological parameters of HPP hydrogels. Stronger gel structures were obtained, whose viscoelastic properties were similar to those of glycerol-free HPP hydrogels.

In agreement with previous observations (Douglas, 2018; Lapasin, 2016), the values of G' of rice starch HPP hydrogels depended on frequency only slightly and increased with increasing the treatment time or with decreasing glycerol concentration, as shown in Figure VIII.4. Moreover, similarly to corn starch HPP hydrogels, rice starch HPP hydrogels were highly structured and the values of their storage and loss moduli were affected by the treatment time only slightly. Indeed, significant differences on G' values were only observed for samples with 10% of glycerol treated at 600 MPa, demonstrating that the antagonistic effects of glycerol concentration were less pronounced in rice starch suspensions than in corn starch suspensions. Elasticity and viscosity of rice starch HPP hydrogels were improved at lower concentration of glycerol in the starch suspensions.

The different behaviour of rice and corn starch hydrogels in the oscillatory regime might be explained taking into account the different physical properties of the two starches. Rice starch granules have a smaller size than corn starch granules, thus a higher surface area was exposed to the action of high pressure. This was leading to the formation of stable, texturized hydrogels, and counteracted the antagonistic role of glycerol concentration on gelation phenomenon. Rice starch suspensions underwent gelation at 500 MPa, even in presence of glycerol at 5% concentration.

VIII.3.4 Texture profile analysis

Texture of corn and rice starch HPP hydrogels, obtained at different processing conditions and glycerol concentration, was evaluated through texture profile analysis (TPA). Data reported refer only to starch suspensions forming strong gels, being some properties, such as firmness, of HPP hydrogels, obtained at 500 MPa below the minimum detection limit of the instrument (< 0.045 N).

The results of TPA of corn and rice starch HPP hydrogels, obtained at 600 MPa and 5 and 20 min at glycerol concentration of 0, 5% and 10%, are reported in Figure VIII.5 and Figure VIII.6.

Firmness and adhesiveness were the only two parameters enabling to discriminate among the different formulations of HPP hydrogels, while the values cohesiveness and springiness were the same for all samples. This indicated that rice and corn starch HPP hydrogels were able to withstand compression forces and regain the initial size and shape after TPA tests, evidencing good integrity and high strength, whatever were the processing time and glycerol concentration (Bourne, 2002).

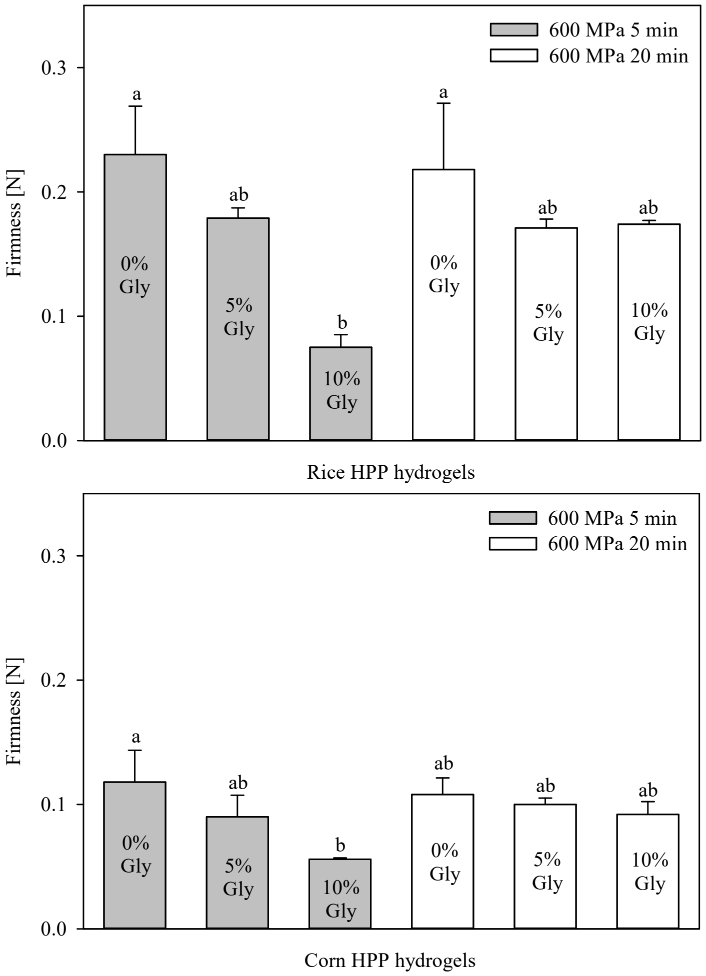

Figure VIII.5 *Firmness of rice and corn HPP hydrogels, obtained at different HPP processing conditions and different glycerol concentrations.*
$^{a-c}$*Bars marked with different letters indicate significant differences (Tukey, p<0.05).*

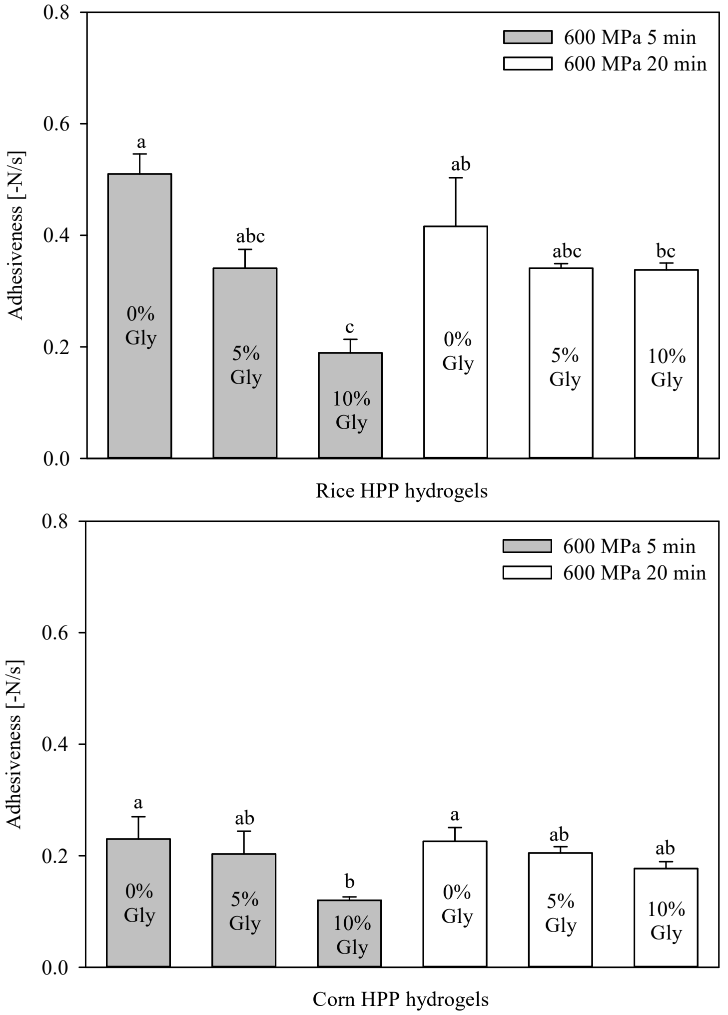

Figure VIII.6 *Adhesiveness of rice and corn HPP hydrogels, obtained at different HPP processing conditions and different glycerol concentrations.* $^{a\text{-}c}$*Bars marked with different letters indicate significant differences (Tukey,* $p<0.05$*).*

From data reported in Figure VIII.5 and Figure VIII.6, it can be observed that rice starch HPP hydrogels were characterized by higher firmness and adhesiveness than corn starch hydrogels, and this is in agreement with the discussion reported previously. However, HPP processing conditions and glycerol concentration affected in a similar manner the texture properties of both starch-based HPP hydrogels. Firmness, which is related to the resistance of gels to deformation forces (Bourne, 2002), and adhesiveness, which accounts for gels intrinsic cohesive forces, of rice and corn starch HPP-hydrogels were not influenced by HPP processing conditions (glycerol-free samples) but decreased with increasing glycerol concentration in the suspensions ($p<0.05$). This was more evident for samples with higher glycerol concentration (10%) and obtained with lower processing time (5 min). Although glycerol is used as a humectant in the formulation of pharmaceutical and cosmeceutical products, increasing its concentration in starch suspensions was inducing the formation of hydrogels with weak texture properties

VIII.4 Conclusions

In this chapter, the effects of processing conditions on gelation of corn and rice starch under HPP were investigated also in presence of a humectant in the suspensions. Samples processed at 600 MPa were able to form hydrogels, whatever was the processing time, differently from what observed at 500 MPa. In this latter case, gelation was observed only for rice starch suspensions processed with longer treatment times. The hydrogels formed showed a pseudoplastic behaviour, which was influenced by glycerol concentration in the suspensions. Glycerol was playing a strong antagonistic action towards gelation, whatever were the processing pressures and treatment times utilized. The inhibition of starch gelation under pressure was observed in certain experimental conditions, namely for rice starch at glycerol concentration equal or higher than 10% and for corn starch at 500 MPa at all the processing times tested. At the latter pressure level, rice starch suspensions were able to form gels, particularly at glycerol concentrations lower than 10%. The antagonist role of glycerol was not always counteracted by HPP longer treatment times.

The values of texture parameters of rice starch HPP hydrogels were higher than those of corn starch hydrogels. Rice starch hydrogels exhibited higher firmness and adhesiveness than corn starch hydrogels, while both had similar resistance to compression and, consequently, comparable values of cohesiveness and springiness.

The mechanical properties of hydrogels based on both starches were affected by processing conditions as well as glycerol concentration. Indeed, when rice starch and corn starch suspensions at 5% glycerol concentration were treated at 500 MPa, even if gelation occurred, the mechanical properties of the hydrogels could not be measured being their values below the minimum detectable ones. Texture parameters had lower values when HPP treatments were carried out with shorter times, indicating that with increasing the processing time the hydrogels formed were firmer and stiffer.

Chapter IX
General conclusion and future perspectives

Results shown in Chapter IV-VIII, have highlighted the potentiality of the starch-based HPP hydrogels as novel biomaterials that can be considered as potential carriers for several innovative applications. Moreover, from this PhD thesis, as the first work in the field, it emerges that high pressure processing was an effective technique to produce starch-based hydrogels by standardized processing conditions and with significantly lower processing time and energy consumption compared to the conventional methods.

It was possible to produce at fixed HPP conditions, hydrogels based on potato, corn, rice, wheat and tapioca starch identifying the main factors involved in their production under high pressure ensuring good mechanical and structural properties.

The type of starch and the processing conditions influenced the final characteristics of the hydrogels produced by HPP and in consequence their potential utilization in different fields as well as their physical stability and digestibility.

The addition of compounds, like common humectant agents to functionalized starch-based HPP hydrogels, influenced the hydrogels characteristics, playing an antagonistic action over the gelation under high pressure, however, the further production of HPP hydrogels based on rice and corn starch, considering HPP conditions and formulation aspects to ensure good rheological and textural characteristics was successfully achieved. In conclusions, the approach of this work, which was considering the analysis of the effects of product and process parameters as a fundamental step to understand the gelation phenomenon, has been demonstrated to be sound.

The determination of optimal product formulation, preparation and characterization of the hydrogels obtained with different starches represented a clear advancement of the knowledge on the production of starch-based hydrogels by high pressure processing (HPP) technology.

However, further research efforts are needed at a fundamental level to unveil the thermodynamic of the gelation phenomenon under pressure, as well as at processing level analysing how the utilization of more complex formulation affect the preparation of stable starch-based hydrogels by high pressure processing (HPP).

Finally, it will be also necessary to scale-up the process in view of the industrial exploitation of the results.

Hopefully, the approach and the results obtained in this Ph.D. thesis will represent a useful baseline for opening up new scenarios of these novel biomaterials taking account the “green process” and “sustainability” concept to be accomplished.

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