University of Naples “Federico II”
Department of Agricultural Sciences

Ph.D. Course in Food Science (XXXIII cycle)

Development of antioxidant/antimicrobial active packaging for food preservation

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Preface

The present thesis describes the study and research activities carried out during the PhD course "Food Science" - XXXIII cycle, belonging to the Department of "Agriculture Science - DIA" of University of Napoli Federico II, attended in 2018-2021. The thesis is framed within the original experimental work accomplished by the author under the supervision of Professor Elena Torrieri, from DIA, and the co-supervision of Ing. Vincenzo Scognamiglio, from the "Icimendue" company, and of Professor Valerie Guillard, from the University of Montpellier. In fact, the research fellowship has been supported by European Commission and Italy Research Ministry (MIUR) in the frame of the program "*Programma Operativo Nazionale (PON) Ricerca e Innovazione 2014-2020- Asse I- "Capitale Umano", Azione I.1 "Dottorati Innovativi con caratterizzazione industriale"*. The program required the exploitation of the research activity in collaboration with a company and a foreign university. Thus, part of the activities (6 months) was carried out at Icimendue, Srl, a company working in the food packaging sector, and part was carried out at the INRAE istitute of Montpellier (10 months).
The research highlights came to fruition as four articles in scientific journals and two more articles are under review. Where use is made of the co-work of others, it has been clearly stated in the text. Two works are under preparation for future submission.

// Abstract

Changes in consumer demand, industrial production trends (such as fresh, tasty, and convenient food products), retailing practices (such as transregional and transnational long-distance sales of food), and customer lifestyles (such as a fast-paced lifestyle resulting in less time spent shopping for fresh food at the market and cooking) are the main forces driving the evolution of novel and innovative packaging techniques that maintain and monitor food safety and quality, extend shelf-life, and reduce the environmental impact of food packaging. For this purpose, the aim of the PhD project was to develop biopolymer-based active packaging to extend the shelf-life of perishable foods. Three film forming technologies were investigated: casting, rod coater coating, and extrusion.

The results of the first part of the work, related to the production of active film by casting technology, showed that among tested biopolymers, blends based on chitosan and sodium caseinate and whey protein, gelatin and inulin were good substrates for dispersing rosemary essential oil and lactic acid bacteria producing bacteriocin, respectively. On the contrary, the only biopolymer able to properly include an antioxidant polyphenol, the gallic acid, selected on the base of the antioxidant capacity, was the sodium caseinate. The addition of the active compound allowed to improve the functional properties (antioxidant or antimicrobial) of the film, without significantly affecting its physical properties. In addition, in the case of lactic acid bacteria, an improvement in the mechanical properties of the films were also observed. The active film based on chitosan, sodium caseinate and rosemary essential oil were also applied to meat burgers and it showed promising results in terms of shelf-life extension, due to reduction of lipid oxidation. The second part of the work was aimed to develop active films by using the rod coater technology. Thus, the active biopolymer solution investigated in the first part of the work were also used as a coating for commercial packaging films. Results showed the biopolymer solution can be properly used as coating on PLA or PBS films with no need to modify the film surface, thanks to its hydrophilic nature. The realized films were used to make packages for two food matrices (hazelnut-based creams and grated Grana

Padano cheese) and the results showed that although there are some protective effects on oxidation kinetics, the amount of active substance conveyed using this active film technique is very low and many layers would have to be made to obtain significant results. However, the technology proved to be very flexible and with possible strengths to be explored further.

The third possibility studied for the development of active films was to include the active substance in the production phase of the packaging film. As a case study, a matrix based on Poly (3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) was investigated, in which gallic acid enriched with a chemical base, previously selected for its antioxidant and oxygen-absorbing properties, was added during the dry mixing phase. The results of release tests in food simulants and the antioxidant activity of the film confirmed that the films can be successfully applied to fatty food products to extend their shelf-life. However, the addition of the active substance has a negative effect on the structure of PHBV-based films, increasing their porosity and reducing their oxygen barrier properties. Mathematical modelling approach was used to design the active packaging by quantifying the oxygen scavenger capacity and the kinetic of adsorption of the active compound. The model was used to predict the changes of oxygen in a packaging headspace once the active compound was included in the material. Some limitations of the model were highlighted due to the film structure modification induced by the active compound which negatively affected the gas transport diffusivity. Thus, production technology must be optimized to properly apply the active film as food packaging.

Introduction and objectives

Nowaday, one major issue is to develop a sustainable packaging with low environmental impact by using a bio-based and/or biodegradble materials able to maintain a long shelf life of the food product and consequently reducing food losses and waste. Indeed, the utilization of renewable sources on the development of this kind of packaging guarantees a reduced environmental impact compared to conventional packaging (Reichert *et al*., 2020; Sivakanthan, Rajendran, Gamage, Madhujith, & Mani, 2020; Bhardwaj, Alam, & Talwar, 2019).

Currently, biopolymers, commonly called bioplastic or bio-based plastic, represent about one percent of the about 335 million tonnes of plastic produced annually. But as demand is rising and with more sophisticated biopolymers, applications, and products emerging, the market is continuously growing. In 2018, global production capacities of bioplastics amounted to about 2.11 million tonnes with almost 65 percent (1.2 million tonnes) of the volume destined for the packaging market – the biggest market segment within the bioplastics industry (https://www.european-bioplastics.org/market/).

Biopolymers can find large application in the food packaging sector. The development of biopolymer packaging systems is one of the most fashionable trends to improve the food market. Biopolymers obtained from agricultural commodities and/or food waste products have emerged as an option about their film-forming capacity. Moreover, due its biodegradable nature, it does not produce residues and do not cause any negative effect on the environment; therefore, they can be considered a sustainable technology.

Among the most studied biopolymers for packaging applications are poly (lactic acid) (PLA), poly(hydroxyalkanoate) (PHA), starch, cellulose derivatives, protein obtained from plants (corn zein, wheat gluten, soy or sunflower) as well as proteins obtained from animal sources (gelatin, keratin, caseinates or whey) (Arrieta, Sessini, & Peponi, 2017). As food grade materials, polysaccharides and proteins has been extensively studied to develop edible coating and film. It can be applied to food products, extending product shelf life through reduction of the transfer of gas, moisture, lipids, aromas, and other constituents. Several studies have been published on edible coatings applied to different

food such as fruits, vegetables, cheese and meat-all with the aim of increasing shelf life and guaranteeing food quality (Koushesh Saba & Sogvar, 2016; Azeredo & Waldron, 2016; Salinas-Roca, Soliva-Fortuny, Welti-Chanes, & Martín-Belloso, 2016; Hashemi *et al*., 2017; Echegoyen & Nerín, 2015; Yang *et al*., 2018; Caetano, Hessel, Tondo, Flôres, & Cladera-Olivera, 2017) .

However, the performance of biopolymers film is still very far from the performance achieved with synthetic polymers. Nevertheless, packaging structures based on biopolymers have interesting properties, such as the CO2/O2 permeability ratio close to one, which, for fruit and vegetables, is as important as the gas permeability factors (Mahajan, Oliveira, Montanez, & Frias, 2007; Mensitieri *et al*., 2011). The need of new technologies to improve the performance and the application of biopolymer film is urgent. Today due to its expansiveness in creating new products, the food industry has created completely new demands on the packaging market. Innovatives packaging solution (active, smart and intelligent) extends the shelf life of food products and better propose new techniques for storage and refrigeration chains enable longer transportation. Packaging of the new generation affects a product and thus controls its quality (Man, 2015; Robertson, 2009; Baker, 2012; Wyrwa & Barska, 2017; Majid, Ahmad Nayik, Mohammad Dar, & Nanda, 2018; Yildirim *et al*., 2018).

Active packaging is an innovative approach to maintain or prolong the shelf-life of food products and at the sime time ensuring their quality, safety, and integrity. As defined in the European regulation (EC) No 450/2009, active packaging comprises packaging systems that interact with the food in such a way as to "deliberately incorporate components that would release or absorb substances into or from the pack- aged food or the environment surrounding the food" (European Parliament, 2004). Active packaging systems can be divided into active scavenging systems (absorbers) and active-releasing systems (emitters). Different realization technique such as casting, coating and extrusion can be used for a realization of active food packaging materials in which the active compound can be combined with a biopolymer to be activated into a realization phase (Ozdemir & Floros, 2004; Lagarón, López-Rubio, & José Fabra, 2016).

Many studies have evaluated the incorporation of natural substances for replacing synthetic chemicals (Siripatrawan and Harte, 2010). In this context, essential oils and polyphenols are interesting for their potential use as natural preservatives (Bonilla and Sobral, 2016). Extracts of aromatic plants (rosemary, clove, etc.) have in their constitution several active compounds. The main groups are composed of terpenes and terpenoids and the others of aromatic and aliphatic constituents, all characterized by low molecular weight. These phenolic compounds are part of the secondary metabolites of plants, contributing for their defense against ultraviolet (UV) radiation (antioxidant activity) and against pathogens, parasites and predators (antimicrobial activities). Several studied demonstrate that phenols has antioxidants activity, thanks to their capacity to release hydrogen molecules captured by free radicals, this one responsible for the alteration of the quality of food product (de Moraes Crizel *et al*., 2018; Joanne Kam *et al*., 2018).
One of the major food degradation is oxydation, responsible for alternation of the food stucture, producing of off-flavors, discoloration and lost in nutritional quality and safety caused by the formation of secondary, potentially toxic compounds (lipid and protein oxydation), thus making foods unsuitable for consumption (Gómez-Estaca, López-de-Dicastillo, Hernández-Muñoz, Catalá, & Gavara, 2014; Hellwig, 2019). These oxydation reactions strongly depend on extrinsec parameters (temperature, light, gas composition) and can be limited by using an active packaging as antioxydant release or oxygen absorber to keep an atmosphere free of oxygen (Vermeiren, Devlieghere, Van Beest, De Kruijf, & Debevere, 1999). The inclusion of natural extracts with antioxidant activity within the packaging material can protect food from lipid oxidation.

The main aim of the PhD research project was to develop new active films based on biopolymers to extend the shelf life of perishable food. In particular, the work was focus on the study of four main biopolymers, chitosan, sodium caseinate, whey protein and polyhydroxy-co-3-butyrate-co-3-valerate (PHBV), and on active film based on natural compounds (antioxidant and antimicrobial). Moreover, for the development of the active

films, different techniques available for the production of the film were investigated: casting, rod coater coating and extrusion.

In the first part of the work, the effect of different active compounds on the chemico physical properties of biopolymer films made by casting were investigated. Thus, different biopolimer films enriched in active compounds were optimized in terms of composition and charaterized to understand the role of the active compounds on the struxcture and functional properties of the film. The efficacy of the developed film were tested in vitro and in real condition at contact with food. In particular, the following biopolymer system were studied: antioxidant film based on chitosan and sodium caseinate enricched with rosemary essential oil (Chapter 3); antimicrobial active packaging based on whey protein and acid lactic bacteria producing bacteriocine (Chapter 4).

The second part of the work was focused on the application of the active biopolymer solutions previous optimizes as coating on commercial film to realize active film. The antioxidant film forming solution enriched with rosemary essential oil or gallic acid were deposited as monolayer on commercial sealable flexible films. The industrial meyer rod coater tecnique, corrently used in the film converter companies, was applied for a deposition of the active coating. The realized film was characterized and used for a shelf life study on hazelnut paste and grated grana padano cheese (Chapter 5). This part of the work was performed in collaboration with the Icimendue company in Caserta (NA).

The last part of the work was focused on the developing of a active film by using a microcompounding and thermoforming tecnology. Thus, the active compound were mixed with PHBV biopolymer before processing. Among several polyphenols, it was chosed to work with gallic acid, which can also act as oxygen scavenger in alkaline environment. Thus, the objective of the work was to study the oxygen scavenger capacity of the gallic acid to properly describe it by mathematical model. Moreover, antioxidant and oxygen scavenger properties of the realized film (PHBV+gallic acid) were evaluated on different food simulants and results were compared to a theoretical mathematical

model (Chapter 6). This part of the work was performed in collaboration with the INRAE istitute of Montpellier (France).

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Chapter 1 – Biopolymers for sustainable food packaging

Abstract

As the IV generation of packaging, biopolymers, with the advantages of biodegradability, process ability, combination possibilities and no pollution to food, have become the leading food packaging materials. Biopolymers can be directly extracted from biomass, synthesized from bioderived monomers and produced directly by microorganisms which are all abundant and renewable. The raw materials used to produce biopolymers are low-cost, some even coming from agricultural industrial waste.

This review summarized focus on the main aspect in the development of a biobased polymer food packaging taking in account different aspect such as: (i) objective of cycle economy; (ii) the classification of biopolymers from different sources; (iii) the film realization technique; (iv) the chemical-physical properties of a packaging materials; (v) focus on the most used biopolymer such as chitosan, caseinate and polyhydroxybutyrate (PHB) polymer.

1.1. Introduction

Most of the materials used in food packaging applications are derived from fossil or biobased polymers such as PE (polyethylene), PP (Polyprolpylene), PET (Polyethylene terephthalate) and bioPE, bioPP, bioPET (González & Alvarez Igarzabal, 2013). A recent study reported that about 6300 tons of plastic waste was generated in 2015, of which about 79% was accumulated in the natural environment (Geyer, Jambeck, & Law, 2017). The waste stream represented by food and non-food packaging has been subject to specific legislation at European level for years and is constantly monitored by member states. In January 2018, the European Commission proposed a European strategy for plastics in a circular economy. According to the Commission, this proposal represents an ambitious step towards greater efficiency in the use of plastics and a shift from a linear to a circular system. As stated by the European Commission, the circular economy enables

the pursuit of a development model capable of establishing a new type of relationship between production and consumption, a real step change in the integration of environmental and economic policies, based on the life cycle of products and focused on the recovery of every single valuable raw material. In this mechanism, materials of biological origin are considered to re-enter the cycle of the biosphere, while materials of synthetic origin are designed to remain within a flow and, once obsolete, can then return as basic components of a new product. The circular economy is also an important component of the transition to sustainable development: it is based on renewable energy sources and aims to minimise, track and eliminate the use of environmentally harmful substances, reduce waste and wastefulness as much as possible, and facilitate the reconversion of products. The circular economy is an economy in which nothing becomes waste in the classical sense of the term, but everything is put back into the system, in a virtuous circle, in various ways, starting with those that involve less dispersion of value and greater efficiency (Stahel, 2016; Ellen MacArthur Foundation, 2017) (fig. 1.1). In this context, member states promote the use of bio-based materials. Among bio-based materials, biopolymers as a starting point for materials and packaging is one of the most innovative and competitive challenges. Interest in these materials has grown a great deal in recent times. In particular, food packaging industries increase their interests to the development of biopolymers packaging in response to consumer demand for recyclable, biodegradable and compostable packaging materials with a low environmental impact.

Figure 1.1. Circular economy cycle, (https://www.european-bioplastics.org).

1.2. Definition and classification

Biopolymers belong to the category of bio-based materials, i.e. organic materials in which the carbon comes exclusively from renewable biological resources. More specifically, a biopolymer is a polymeric material extracted directly or produced indirectly from biomass.

Biopolymers can be classified according to the origin and method of production as below:

- agro-polymers, such as polysaccharides and proteins, e.g. chitosan, cellulose, casein and gelatin, obtained from biomass fragmentation processes;
- polymer obtained from the chemical synthesis of synthetic monomers such as poly-ε-caprolactone (PCL), or from chemical synthesiis using renewable biobased monomers such as polylactic acid (PLA);
- biopolymers synthesised by microorganisms, such as polyhydroxyalkaloates (PHA) and polyhydroxy butyrates (PHB).

(Zhong, Godwin, Jin, & Xiao; 2020 Napper & Thompson, 2019).

Biopolymers can be considered (i) bio-based and biodegradable, (ii) bio-based and non biodegradable but also petroleum based and biodegradable (fig. 1.2). (Balaji, Pakalapati, Khalid, Walvekar, & Siddiqui, 2017; Jha & Kumar, 2019).

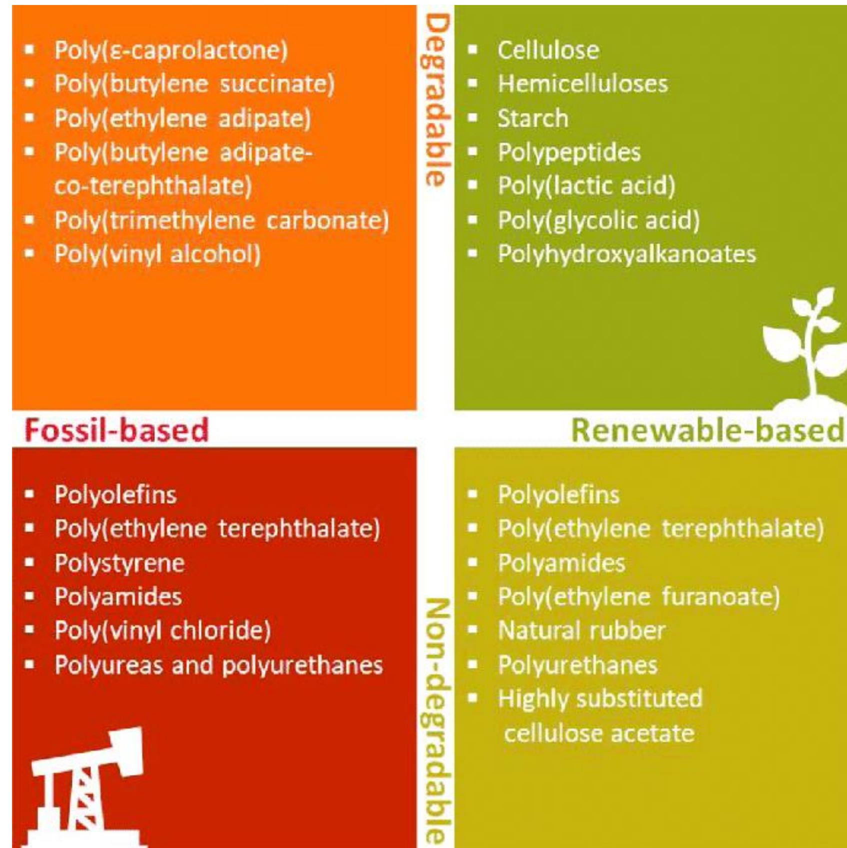

Figure 1.2. Schematic rapresentation bio-based and fossil based biodegrable and non polymers for food packaging application.

1.2.1. Biopolymer materials composition

Biopolymer's materials are mainly produces starting from polysaccharides or protein of different nature (plant, animal or microorganism). Polysaccharides such as pectin, carrageenan, alginate, starch, gum and xanthan have been recently used as a sustainable material in coatings and edible films formulation (Lagarón, López-Rubio, & José Fabra, 2016; Espitia, Du, Avena-Bustillos, Soares, & McHugh, 2014). Polysaccharides are

available in nature, not toxic and show good barrier properties to carbon dioxide and oxygen. They can be classified on function of the origin as:

- animal derived polysaccharides: chitosan and chitin;
- plant origin polysaccharides: methylcellulose, hydroxypropyl methylcellulose and carboxymethyl cellulose, pectine, starch, arabic gum;
- marine origin polysaccharides: alginates, carrageenan;
- microbial polysaccharides: pullulan, gellan, xanthan gum;

Because of the polar nature and tightly packed network structure of polysaccharides, they are good carbon dioxide and oxygen barriers, but they are poor vapor barriers. So, their application for food packaging is quite limited. Several strategies, such as addition of particles of inorganic water-resistant, combination with hydrophobic lipids, polymer cross-linking and manufacture film's multi-layered, can be used to enhance barrier properties and water resistance (Alves, Costa, & Coelhoso, 2010).

Proteins can be found naturally, as fibrous proteins (bonded to each other on parallel) or globular proteins (rolled over their selves) (Enujiugha & Oyinloye, 2018). For edible film application lactic serum, caseinate, collagen, zeine and Soy protein are the most used. Protein films show s good mechanical properties due to their unique structure (Yai, 2008a; Bourtoom, 2009). Whey protein and casein protein are the most constituents of milk proteins (Cow's milk contains about 33 g of protein per liter). Into details casein protein represent 80% of milk protein and it contains α, β, and κ-casein components. Casein based films remain stable for a range of pH, temperatures and salt concentrations.

Microbial biopolymers are produced by microorganisms during fermentation. Microorganism-based biopolymers are considered non-toxic, biodegradable and biocompatible food packaging materials. According to structure and chemical composition it can be categorized into:

- polyamides including poly (γ- glutamic acid), poly (ε- L -lysine) and multi- L -arginyl- poly (L -aspartic acid);
- polysaccharide-based biopolymers such as alginate, dextran, xanthan, gellan gum and hyaluronic acid;

- polyesters such as polyhydroxyalkanoates (PHA);

(Rodríguez-Carmona & Villaverde, 2010; Mokhtarzadeh, Alibakhshi, Hejazi, Omidi, & Ezzati Nazhad Dolatabadi, 2016)

Biocompatibility, safety and biodegradability make PHA-based and PHB polymers an ideal candidate for a food packaging application. These materials are attractive candidates shows good mechanical properties and are considerate good vehicles for active substances delivery.

1.3 Manufacturing of biomaterials, film, and packaging

The manufacturing of biopolymers can be done by various methods such as:

- solvent cast technology
- twin sceew granulation
- extrusion

1.3.1. Solvent cast technology

Nowadays, the solvent cast technology is becoming increasingly attractive for the production of films with extremely high-quality requirements. This process has been used for thermolabile polymers that cannot be transformed by thermal molding. Ingredients are dissolved into the film forming solution (polymer, plasticiziers,etc) using a solvent like water, alcohol or a mixture of water and alcohol, or other solvents. Then film forming solution mix is cast in a thin layer onto a continuous roll, moved into an oven or convection chamber to drive off the solvent at a fixed relative humidity (RH) an temperature condition. Dried film are removed from the line by a blade and collected (fig. 1.3). The production process for the film realization is conducted at low thermal and mechanical stress. As a result, degradation or adverse side reactions are insignificant (Ebnesajjad, 2012;Siemann, 2005). This technique allows to obtain an almost perfectly isotropic material (i.e. with equal mechanical characteristics in both orthogonal directions), since it is produced without tension or orientation of the macromolecules. The cast film can be processed in-line with an optical coating design.

The key elements of solvent casting technology are:

- the polymer must be soluble in a water or volatile solvents
- a stable solution with a minimum of solid content and viscosity should be formed (a dilute solution is difficult to work with and would produce very thin film that is prone to defects and holes)
- a formation of a homogenous film and a release from the support must be possible

To obatin the properties mentioned above, different adjuctments can be adopt such as: (i) co-solvent systems; (ii) dissolution at overpressure, (iii) use of special molecular weight distributions of polymers or co-polymers, (IV) additives such as plasticizers, (V) release agents. If soluble solid or liquid compounds such as plasticizers are used in the process, the solubility and stability in solution and in film formation process need to be take into account.

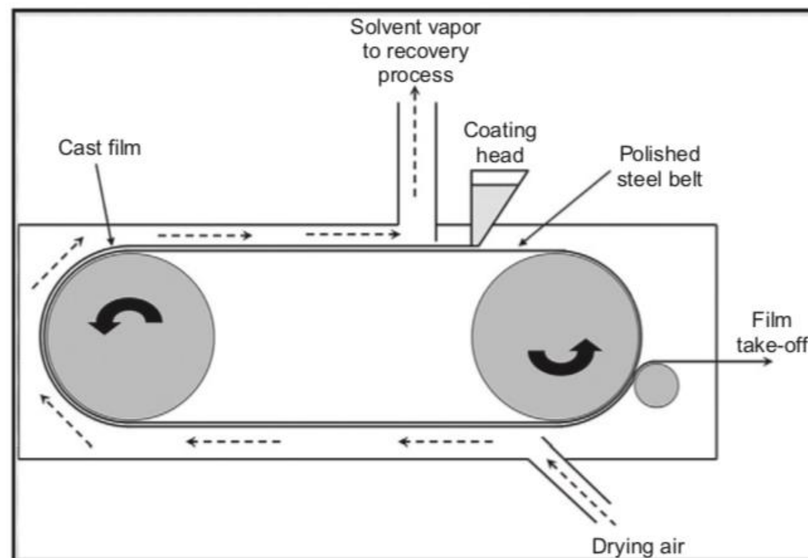

Figure 1.3. Schematic of belt film casting process.

A possibile disavantage can be in the phase of the scaling up of this film realization technique, from a lab scale to a industrial plants, several problems can be encountered such as the air entrapment of the solution, the thickness, humidity and temperature control (Jeya Jeevahan *et al.,* 2020).

The homogeneity of the temperature on the complete surface of the roll during the process is essential to avoid changing in viscosity of the film forming solution with consequent differents in thickness (Ebnesajjad, 2012)

1.3.2. Twin screw granulation

Twin-screw granulation presents a great potential and offers many advantages relative to conventional granulation processes (Bandari *et al.*, 2020).The application of this tecnique is wide used in the pharmaceutical industry to compound drugs into tablets, capsules and powder formulation (Psimadas, Georgoulias, Valotassiou, & Loudos, 2012; Kreimer, Aigner, Lepek, & Khinast, 2018; Crawford *et al.,* 2020). The mixing effect of twin screw extruder relies on the shear generated between the walls of the barrel and the screw. The shear mixing of twin screw extruders is more efficient and the mix (polymer and additive) is subjected to less thermal history (Kittikunakorn, Liu, & Zhang, 2020)**.** The twin-screw extruders can be used to mix fillers and additives with the polymer in a continuous manner, so that the compound will perform as required and achieve the desired properties (Hietala & Oksman, 2018). Typically this process is performed in the molten state with a goal to achieve a homogeneous blend, and is a crucial step in the polymer development process. In the development of the pellets for a finally realization of a film, a compounding it's important for the homogenious mixing of the polymer with the additives such as for a conseguently uniform distribuition in all the realized packaging (Seem *et al.,* 2015).

However a critical aspect could be the particle size of the single powder components that can adversely affects the physical properties of the composite or final film. The particle size must be checked and should be uniformly controlled before the compounding process (Sohn, Ryu, Yun, Zhu, & Cha, 2019). Into details the equipment consists of two co-rotating screws enclosed in a barrel and screw elements loaded onto a shaft rod. The kneading zone within the barrel can be adjusted in any location, as needed to optimize the mixing process, to obtain suitable granules with a uniform distribuition of the active inside the granules (fig. 1.4). Each section of the screw can be thermo controlled during the process using air or water. Finally a cutting machine, allows to cut the realized couled

materials into pellets (fig. 1.5).The granules obtained can either be used directly for further processing or subjected to size reduction (milling) to obtain the desired size for the granules (Li, Pradhan, & Wassgren, 2019).

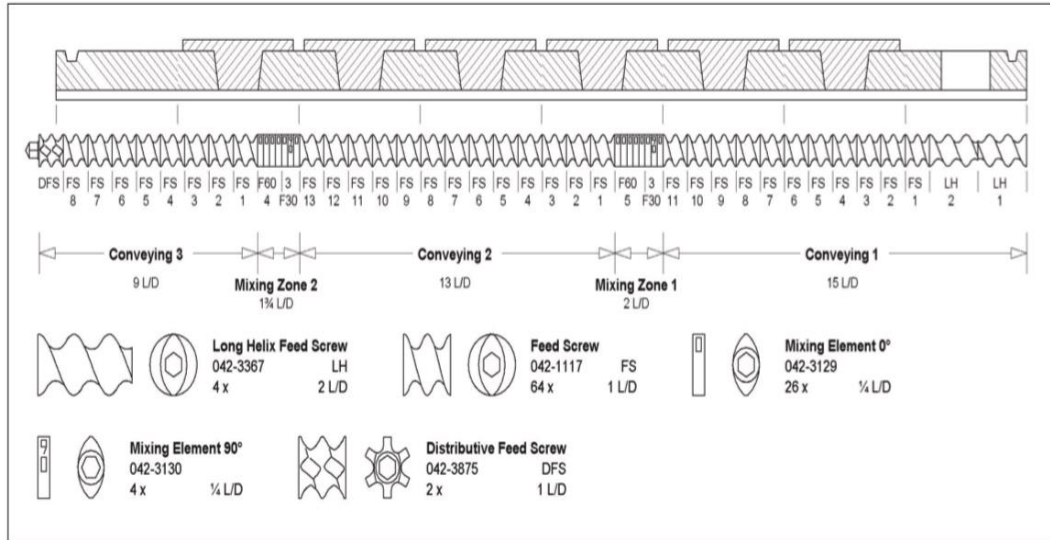

Figure 1.4. Screw configuration for dry granulation.

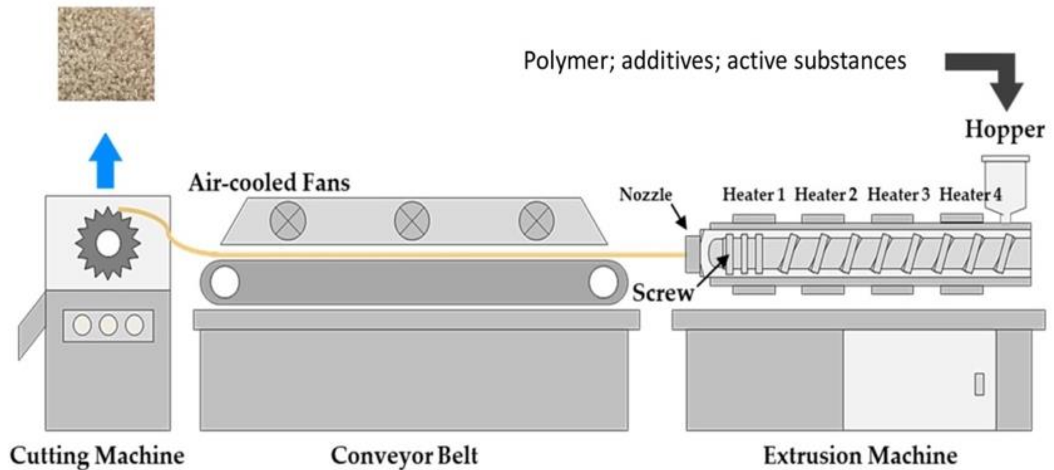

Figure 1.5. Compounding process divided in extrusion machine, conveyor belt, cutting machine.

The advantage of twin-screw extruder:

- economical
- industrially-scalable
- continuous process
- solvent free process
- can be adapted for various active compound (drugs, polyphenols etc) with different physicochemical properties

Factors affecting the quality of twin-screw extruder

- manufactoring plant: operator, RU and temperature
- formulation variables : chemico: physical properties of the mixing powder
- equipment variables: screw configuration and screw elements geometry; extruder and feeder type
- process variables: screw speed, feed rate, torque/shear and process temperature

1.3.3. Extrusion molding

This is the most widely used technique for producing films and foils. The extruder, the fundamental equipment to carry out this technique, consists of an electrically heated hollow steel cylinder, in which a worm screw rotates; in reality, double screw extruders are also widely used for plastic polymers, in which two screws rotate in the same or opposite direction. The profile of the screw and the temperatures of the different zones of the extruder are different depending on the polymer to be processed. The internal diameter of the extruders used for commercial film production is variable (generally from 9 to 15 cm) and the length is related to the diameter; length/diameter ratios are critical for different uses. Generally, the polymer (in pellet or powder form), and other ingredients (additives, pigments, adjuvants) are fed into the extruder through a feed hopper; the combined action of heat and mechanical stress melts and amalgamates the raw materials and the movement of the screw causes the film to be ejected from the head (die) of the

extruder at a temperature very close to the melting temperature. The die can be: (i) a straight slit such as flat head and flat film; (ii) a circular slit, to produce tubular film through a circular channel head (fig. 1.6) (Riley, 2012).

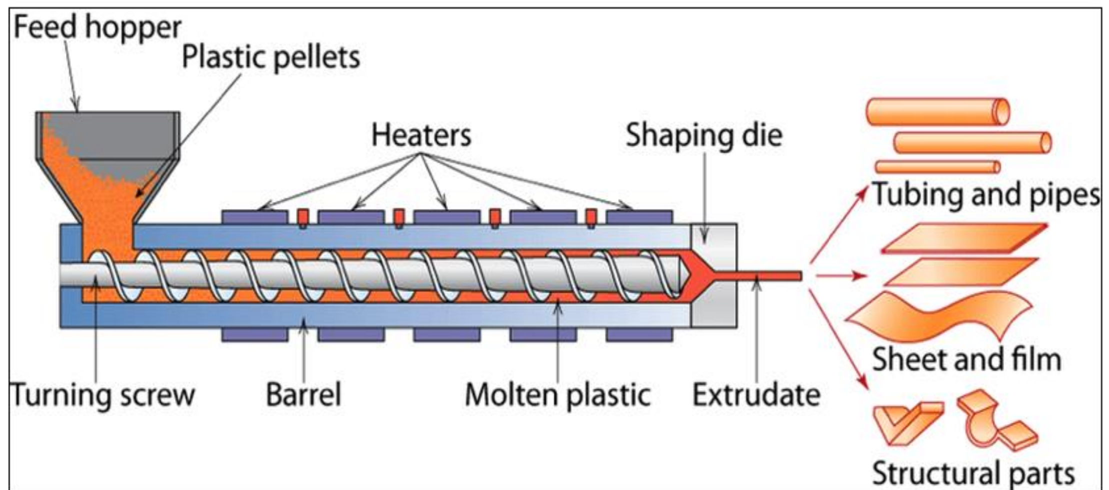

Figure 1.6. Extrusion mold tecnique.

1.4. Physical and chemical properties of packaging materials

Biodegradable and edible biofilms must have a number of specific functional requirements (barrier effect against moisture, gases and solutes, liposolubility or water solubility, color and appearance, rheological and mechanical properties, non-toxicity, etc.). These properties depend on the type of material used, the process of formation and application (Yai, 2008b).

The physico-chemical properties of packaging materials refer to their general characteristics in relation to external agents, such as heat, gravity, etc., and do not involve a change in chemical structure. Physical and chemical properties include: surface, thermal, mechanical, barrier and optical properties.

1.4.1. Surface properties

Surface properties refer to the properties at the interface between two different materials or between a material and a contact phase. Knowledge of the surface properties of a

material is essential for technological operations such as adhesion and printing, for the optimisation of functional characteristics such as water and oil repellency, gloss and for evaluating surface properties before and after specific treatments to improve performance.

1.4.2. Wettability

Wettability is an important characteristic for polymer materials, and in some applications a wettable surface is necessary such as depostion of coating, ink, adhesives etc in the industrial application for mono and multilayer realization on food packaging. Moreover the the wettability affect the mecchanibility of the flexible film on the industrial plants. The wettability consist of the interaction between liquid and a polymer surface and determined by the surface energy of the polymer and the liquid. The surface energy of the liquid is often referred to as its surface tension (with dimensions force per unit length or energy per unit area). Wetting angle can be measured using a micorscope. Today commercial instruments are used s substitute such as a video camera. This method are know as sessile drop method in which a drop are deposite on the surface of a film and visualized by camera, with an elaboration of his wettting angle using a software base on the equation (1, 2). The drop generally must be enough to be measured by the instrument and at the same time small to esclude the gravity effect (0.5-1ml). The geometrical aoect of the drop are sferic in absence of any external influences and change in contact with the solid surface (film surface) his forming angle Θ (fig. 1.7). Surface with a low water wetting contact angles are considered more hydrophilic and surface with hight water wetting /contact angles, hydrofobic (Kondyurin & Bilek, 2015).

$$tan\theta = \frac{\sqrt{2rh-h^2}}{r-h}\ for\Theta > 90° \quad (1)$$

$$tan\theta = \frac{2rh}{r^2-h^2}\ for\Theta < 90° \quad (2)$$

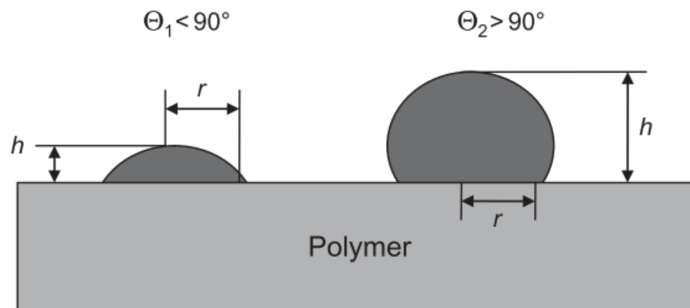

Figure 1.7. Schematic rappresentation of a liquid drop on a film surface with a dimension of a drop (r= radius, h= high) used for a calculation of his wetting angle.

1.4.3. Thermal properties

The thermal properties of a material are those that describe its behaviour in response to thermal stress, during a heat exchange process or as a result of a temperature change. The main thermal properties are

- thermal conductivity: ability of a given substance to transmit heat by conduction;
- thermal capacity: the amount of heat that must be supplied to a body in order to obtain a unit rise in its temperature;
- thermal expansion: a physical phenomenon occurring in a material that undergoes a change in length, surface area or volume for a given temperature change at constant pressure;
- transition temperatures: the melting temperature and the glass transition temperature are considered transition temperatures. The melting temperature (Tm) is the temperature at which, for a given value of pressure, a crystalline solid passes from the solid state to the liquid state. The glass transition temperature (Tg) is characteristic of amorphous or semi-crystalline materials, which soften upon heating and then gradually transition to the liquid state, without manifesting a well-defined melting temperature. Below the glass transition temperature the materials have a behaviour defined as glassy; whereas, above the Tg they show a rubbery behaviour.

1.4.4. Mechanical properties

The behaviour of materials subjected to an external force defines the mechanical properties of the polymer itself. These properties strictly depend on intrinsic characteristics of the material such as molecular weight, crystalline or amorphous morphology, the presence of branching in the structure, production processes, i.e. possible orientation, addition of plasticisers, possible mixing between different polymers and external variables such as temperature, pressure, intensity and type of applied stress. The forces applied to materials can be of different types and the materials, in turn, have a different ability to resist the various types of forces. Depending on the direction of the force, static forces can be defined as those applied in a constant manner and for discrete times, and dynamic forces as those that are exhausted in a short time, as a result of an impact, fall or vibration. Depending on the origin of the forces, they can be distinguished into internal and external forces. If, on the other hand, we consider the direction of application, they can be classified as: tensile forces (two forces of equal intensity are directed along the geometric axis of the body and tend to lengthen it) and compressive forces (the two forces are directed along the axis of the body and tend to shorten it). Knowledge of the mechanical performance of a packaging, or packaging material, is always of fundamental importance in assessing its suitability for a given use.

They include:

- tensile strength: the ability of a material to resist applied forces.
- yield strength: the ability to resist slow deformation (creep resistance) under a constant load.
- vibration resistance: ability to withstand repeated compressive and tensile stress.

The tensile strength test is generally conducted with an instrument called a dynamometer. The dynamometer pulls, with a slow and constant movement, a specimen of the material constrained at the ends to a pair of clamps, fixed respectively at the base of the instrument and on a movable crossbar. While the sample is being pulled, the instrument measures

the force it is exerting and the elongation of the sample. The results can be plotted in a force/length graph, but this would be influenced by the size and thickness of the specimen. To ensure that the values of a test are independent of the geometry of the specimen, the stress and strain are measured. Stress (σ) is the ratio of the force exerted to the area of application. Exerted stress, or strain, is the ratio of the force exerted to the application surface. Deformation is defined by the ratio of the change in length to the original dimension. To describe the behaviour of the specimen under tensile stress, the stress-strain curve is used (fig. 1.8).

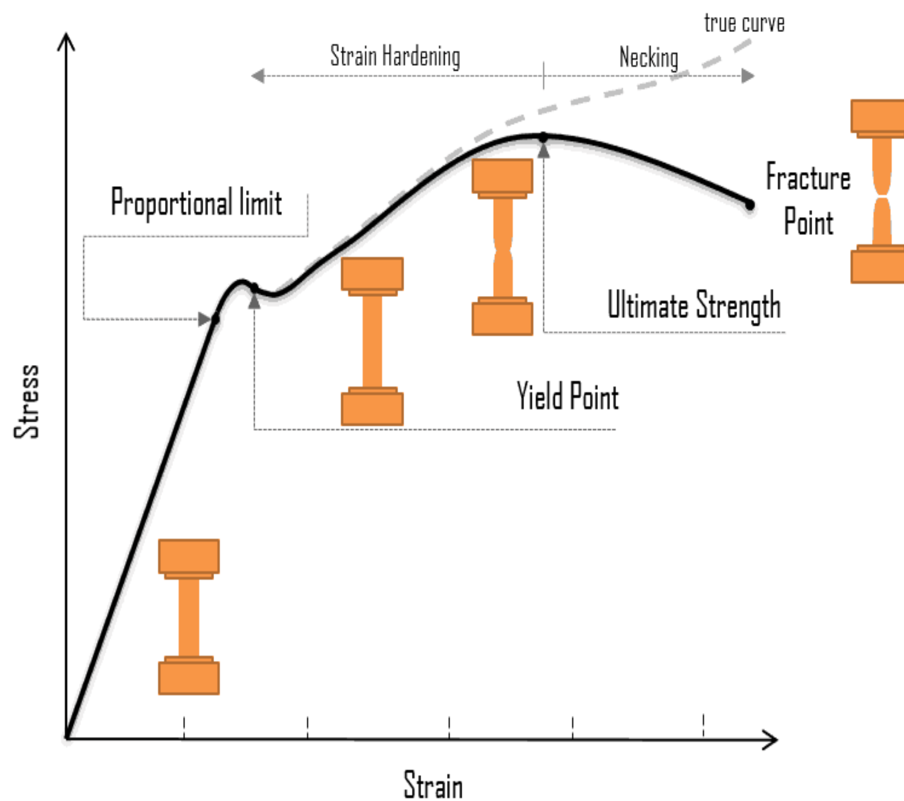

Figure 1.8. Behaviour of a tensile specimen in a stress-strain curve

The time-dependent resistance to deformation can be related to the commercial life of the package. In fact, the commercial life of a product can be limited due to the low yield strength of the packaging.

Dynamic stress is the most common cause of damage to packaged products, corresponding to vibrations and impacts and affecting all products undergoing transport. Many foodstuffs can be sensitive to this type of stress; for example, emulsions, which can become destabilised, or more fragile products which can become damaged.

1.4.5. Permeability properties

There are many applications in the area of food packaging that use mass transfer phenomena. Example include selecting a packaging material to predict the extend product shelf-life, and to control the in-package atmosphere for protection and preservation of food products. Permeation, absorption and diffusion are typical mass transfer phenomena occurring in food packaging systems. Permeation is the ability of permeants to penetrate and pass right through an entire material in response to a difference in partial pressure. This property of the packaging material may also be referred to as the "permeance". To convert the permeance (which is evidently dependent on the thickness of the film) into an intensive property, it is multiplied by the film thickness to derive the permeability (P) of the film. The mass transfer of a solute from a solution thorough a (polymeric) material is a useful way to determine mass transfer coefficients experimentally, because it requires simple permeation apparatus consisting of the high and low concentration solution in chamber divided by the test film material. Diffusion is the movement of a molecules in a medium caused by concentration differences acting as a driving force. Diffusivity (D) is a measure of how well the compound diffuses in the medium. Absorption and its counterpart desorption measure the affinity of a given substance for two media with which it comes into contact. The affinity of a substance for a material can be expressed using the solubility (S) or partition (K) coefficient. The permeability, solubility, and diffusivity are characteristic value for a migration component through a particular medium. These parameter are therefore essential in simulating the mass transfer profile. The mass transfer rates of molecules through a package material or through a membrane are often described as irreversible process. A generalized thermodynamic driving force is required to induce movement of the molecules, which for the movement of gases and solute is the gradient in the chemical potential of the migration species. For most packaging and membrane

applications the area through which transfer occurs is large compared to the to the thickness, so that one-dimensional flow is consider. The linear coefficient linking the flux (for unit cross-section) to the driving force can be consider as a resistance of the package or membrane material to the passage of the given species. With the appropriate substitutions and assumptions, the gradient in chemical potential is related to the concentration gradient of the migration species. The permeation of a molecule is its movement from the region where its concentration is high (C1) to the region where the concentration is lower (C2). Under steady state conditions, a gas or vapour will diffuse through a polymer at a constant rate if a constant pressure difference is maintained across the polymer. Events occurring within the material are examined first where diffusion is the dominant factor. Diffusion obeys Fick' law, and Fick's first law can be expressed as:

$$J_d = -D \frac{\delta C}{\delta x} \tag{3}$$

where J_d, D, C and x are the flux per unit cross-section, the diffusivity, the concentration of the solute, and the distance across which the molecules has to travel, respectively. Fick's second law can be used to analyse unsteady state diffusion with time t:

$$\frac{\delta C}{\delta t} = -D \frac{\delta^2 C}{\delta x^2} \tag{4}$$

When the steady state of diffusion has been reached, J is constant and eq. (3) can be integrated across the total thickness of the polymer, L, and between the two concentrations, assuming D to be constant and independent of C. After integrating equation (3) for the case where C1 and C2 remain constant, the flux of the molecules in the steady state is given by equation:

$$J_d = \frac{Q}{A*t} = D\left(\frac{C1 - C2}{L}\right) \quad (5)$$

Where Q is the amount of diffused moving substance, A is the cross sectional diffusion area, and L is the thickness of the package or membrane. The diffusivity, D, has units of $m^2\ s^{-1}$ and flux has units of mol $m^{-2}\ s^{-1}$ or kg $m^{-2}\ s^{-1}$:

$$D = \frac{J_{d*}J}{\Delta C} = \frac{Q*L}{A*t*\Delta C} \quad (6)$$

Before gas diffuse through the packaging material from C1 to C2 it must first dissolve into material. The sorption of a gas component into a packaging material generally has a linear relationship to the partial pressure of the gas as show in Henry's law under conditions where the gas concentration is lower than its saturation concentration or maximum solubility:

$$p = \sigma X_s \quad (7)$$

Where p and X_s are the partial pressure of the gas in the atmosphere and molar fraction of gas in the packaging material respectively, and σ is the Henry's law constant in Pa. If the permeable gas molecule has an affinity to the packaging material matrix, or is immobilized in the micro voids of the matrix polymer at a relatively low pressure, the sorption behaviour follow a logarithmic non linear relationship, which is expressed as a Langmuir type sorption. Following equation show the linear relationship between the concentration at the surface of the packaging material and the partial pressure of the gas:

$$C_s = H^{-1}\, p_1 \quad (8)$$

Where p1 is the partial pressure of the gas on the high concentration (C1) side. Since the driving force for gas penetration through a packaging material is the difference in gas concentrations or partial pressure between the two sides of the packaging material, the gas flux J of both permeation and diffusion can use partial pressure term instead of the concentration gradient.

In the mass transfer situation, the concentration can be substituted for the partial pressure p and the solubility S in:

$$Q = \frac{D * S\,(C\,1 - C\,2)At}{L} \qquad (9)$$

The product D*S is referred to as the permeability coefficient or constant and is represented by the symbol P. Thus:

$$\frac{Q}{t} = \frac{P}{L} A\ * (\ \Delta p) \qquad (10)$$

The term P/L is called the permeability or permeance (Han and Scanlon *et al.*, 2005).

1.4.6. Optical properties

Optical properties describe the behaviour of a material when exposed to electromagnetic radiation and in particular to visible light. The study and evaluation of the interactions between electromagnetic radiation and matter, with specific reference to packaging materials, are conducted to ascertain their nature or to objectively and measurably describe an aesthetic characteristic, such as opacity, gloss and colour.Opacity can be defined as the percentage of transmitted light which, when passing through the material, deviates from the incident beam due to diffusion and refraction by an angle greater than 2.5°.Colour was assessed using a colorimeter. The colorimeter is a light-sensitive instrument that measures the amount of colour that is absorbed by a substance or object. The subjective perception of a single colour may change depending on the background or

source illuminating the object, while colorimeters have sensitivities corresponding to those of the human eye, but always make measurements with the same light source and illumination system. The colorimeter uses the Lab colour space model to quantify colour and express it numerically. The colour space is a method of expressing the colour of an object or light source using a certain notation, such as numbers. Figure 1.9 shows the Lab colour space, which is currently one of the most widely used colour spaces for colour measuring. Three parameters are evaluated in this colour space: L (lightness), the values of a* and b* (a* positive towards red and a* negative towards green; b* positive towards yellow and b* negative towards blue) define the chromaticity of the colour (Frear, 2017).

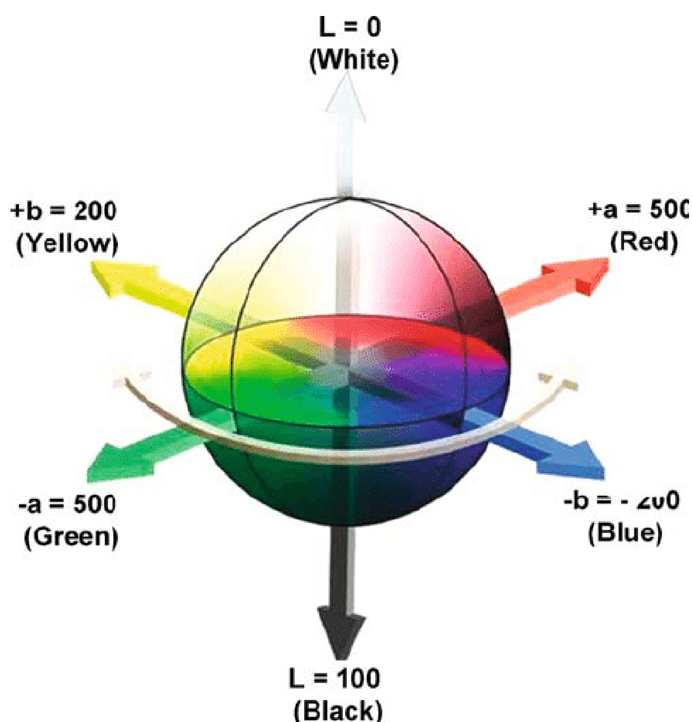

Figure 1.9. CIELAB Lab colour space shart.

1.5. Structure and properties of chitosan-based biopolymer

Chitosan (CH) is a linear polysaccharide of (1-4)-2-amino-2-deoxy-beta-D-glucopyranose and (1-4)-2-acetamide-2-deoxy-beta-D-glucopyranose units. This biopolymer is typically obtained by the diacetylation under alkaline conditions of chitin,

a polysaccharide constituting the exoskeleton of animals, especially crustaceans, molluscs and insects. This diacetylation reaction can be carried out using two techniques: the first is an enzymatic reaction triggered by the catabolic activity of the chitin deacetylase present in the cells of the dimorphic fungus *Mucor rouxii*, and the second involves washing the chitin with highly concentrated alkaline solutions. The chemical structure of chitosan is very similar to that of cellulose. It's glucosamine content is called degree of diacetylation (DD) and is a very important property that influences the solubility, biodegradability, viscosity as well as the emulsifying capacity of the polymer. Another fundamental property is the molecular weight as it influences the solubilisation capacity of the molecule itself, on the basis of this property we can classified chitosan in three fundamental classes:

- hight molecular weight chitosan (MW 2000000;)
- medium molecular weight chitosan (MW 750000;)
- low molecular weight chitosan with (MW 70000;)(Kou, Peters, & Mucalo, 2021)

Chitosan has intrinsic antioxidant and antimicrobial properties, which are influenced by it's molecular weight, the degree of diacetylation, it's concentration, the target organism and the conditions of the medium in which it is applied, in particular the pH. Antimicrobial activity is determined by the presence of positive charges of the protonated amine group (NH_2) which interfere with the negatively charged residues of macromolecules present on the surface of microbial cells, thus inhibiting the growth not only of Gram-positive and Gram-negative bacteria but also of yeasts and moulds. Antioxidant activity is determined by the presence of chromophore groups in the structure of the molecule itself (Abd El-Hack *et al.,* 2020). Chitosan films have been successfully used as a packaging material to improve the quality preservation of a wide variety of foodstuffs. Compared to other bio-based food packaging materials, chitosan has the advantage of incorporating functional substances such as minerals, vitamins, polyphenols and antimicrobial agents (Serrano-león *et al.,* 2018; Rambabu, Bharath, Banat, Show, &

Cocoletzi, 2019; Sun *et al.,* 2017; Priyadarshi, Sauraj, Kumar, & Negi, 2018). Such films have selective gas permeability (CO_2 and O_2) and good mechanical properties (Wang, Qian, & Ding, 2018) (table 1.1, 1.2).

Table 1.1. Water vapour permeability (WVP) of several polysaccharide-based films. Reproduced with permission from (Cazón, Velazquez, Ramírez, & Vázquez, 2017).

Film composition	WVP (g/m s Pa)*	Temperature (°C)	Relative humidity (%)	References
Cellulose derivatives (methylcellulose and hydroxypropyl methylcellulose mixture) with polyethylene glycol and stearic/palmitic fatty with a beeswax surface coat	6.615×10^{-13}–1.632×10^{-11}	25	65–97	Kester and Fennema (1989)
Methylcellulose mixtures with ethanol	0.51×10^{-10}–1.08×10^{-10}	25	52	Nazan Turhan and Şahbaz (2004)
Methylcellulose mixtures with/without nanocellulose	5.44×10^{-11}–7.29×10^{-11}	25	60	Khan et al. (2010)
Hydroxypopyl methylcellulose mixtures with/without silver nanoparticles	1.54×10^{-10}–2.22×10^{-10}	25	70.5–71.9	de Moura et al. (2012)
Agar mixtures with/without silver nanoparticles	1.67×10^{-9}–1.97×10^{-9}	25	50	Rhim et al. (2013)
Chitosan	1.05×10^{-10}	22	58	Abugoch et al. (2011)
Chitosan and starch mixtures	1.18×10^{-10}–1.55×10^{-10}	25	100	Santacruz et al. (2015)
Chitosan mixtures with different molecular weight and different solvents	0.32×10^{-9}–0.51×10^{-9}	25	50	Park et al. (2002)
Chitosan mixtures with different solvents and pH	1.77×10^{-9}–2.26×10^{-8}	25	50	Kim et al. (2006)
Chitosan-tapioca starch mixtures	2.8×10^{-10}–6.7×10^{-10}	32	65	Vásconez et al. (2009)
Tapioca starch mixtures	12.1×10^{-10}	32	65	Vásconez et al. (2009)
Cassava starch mixtures with glycerol	1.8×10^{-10}–2.8×10^{-10}	25	70	Seligra et al. (2016)
Tara gum mixtures with sorbitol and glycerol	0.52×10^{-10}–0.69×10^{-10}	25	75	Ma, hu, wang, et al. (2016)
Tara gum mixtures with glycerol and with/without chitosan	9.91×10^{-11}-1.28×10^{-10}	25	53	Antoniou et al. (2015)
Brean gum with glycerol and beeswax mixtures	2.5×10^{-11}–4.75×10^{-11}	32	75.1	Spotti et al. (2016)
Starch mixtures with glycerol and xylitol as plasticizers	1.94×10^{-11}–1.77×10^{-10}	20	52.9	Muscat et al. (2012)
Alginate mixtures with/without polyethylene glycol	6.5×10^{-10}–0.93×10^{-9}	29–25	76–50	Olivas and Barbosa-Cánovas (2008) Rhim (2004)
Agar with/without silver nanoparticules	1.67×10^{-9}–1.97×10^{-9}	25	50	Rhim et al. (2013)
Quinoa and chitosan mixtures	2.61×10^{-10}	22	58	Abugoch et al. (2011)

*in some data, the units were normalised

Table 1.2. Tensile properties and elongation at break of several polysaccharide-based films. Reproduced with permission from (Cazón, Velazquez, Ramírez, & Vázquez, 2017)

Film composition	Tensile strength (N/mm^2)	Elongation (%)	Temperature (°C)	Relative humidity (%)	References
Methylcellulose and ethanol mixtures with/without polyethylene glycol	25–33	29–14	25	52	Nazan Turhan and Şahbaz (2004)
Hydroxypopyl methylcellulose mixtures with/without silver nanoparticles	51.0–28.3	NR	24	NR	de Moura et al. (2012)
Chitosan	22.2–39.6	13–73.6	22	30–60	Abugoch et al. (2011) Shen and Kamdem (2015)
Chitosan mixtures with different molecular weight and different solvents	6.7–150.2	4.1–117.8	25	50	Park et al. (2002)
Chitosan mixtures with different solvents and pH	0.56–19.2	22–494.8	25	50	Kim et al. (2006)
Quinoa-chitosan mixtures	2.3–8.3	273–117.4	22	60	Abugoch et al. (2011)
Starch mixtures with glycerol and xylitol as plasticizers	5.06–44.3	2.4–70.7	25	53	Muscat et al. (2012)
Potato starch with/without glycerol	30–68	3–5	NR	NR	(Sessini et al., 2016)
Tara gum mixtures with sorbitol and glycerol as plasticizers and with/without oleic acid	26.8–57.4	8.5–2.7	NR	NR	Ma, hu, wang, et al. (2016)
Tara gum mixtures with glycerol and with/without chitosan	58.44–22.71	44–46	25	53	Antoniou et al. (2015)
Brean gum with glycerol and with/without beeswax mixtures	1.64–7.58	8.22–4.85	NR	NR	Spotti et al. (2016)
Pectin mixtures with/without chitosan nanoparticles	58.5–26.07	2.91–0.94	27	50	Lorevice et al. (2016)
Alginate mixtures with sorbitol or glycerol as plasticizers	65.9–64.7	2.5–2.8	25	56	Olivas and Barbosa-Cánovas (2008)
Alginate mixtures with sorbitol or glycerol as plasticizers	18.4–24.1	6.6–7.9	25	98	Olivas and Barbosa-Cánovas (2008)
Sodium alginate mixture	33.6–75.8	3.4–14.0	25	50	Rhim (2004)
Agar with/without silver nanoparticles	51.5–46.38	33.02–33.64	25	50	Rhim et al. (2013)

NR= data not reported

1.6. Structure and properties of casein-based biopolymers

Casein is the most abundant protein fraction in milk, in which it takes on the structure of a phosphorylated heteroprotein aggregate consisting of a complex of associated proteins and calcium phosphate. This phosphorylated heteroprotein aggregate is known as a micelle, which in turn is made up of sub-micelles that interact with each other through numerous hydrogen bonds, hydrophobic interactions and electrostatic interactions, resulting in a stable molecular structure. It's protein fraction, approximately 93% of it's dry mass, is composed of four components synthesised by single genes, called αs1, αs2, β and κ, which differ in their primary structure, type and degree of post-translational modification. The rest of the micellar solids consist of inorganic material, collectively referred to as colloidal calcium phosphate or micellar calcium phosphate. The arrangement of caseins within the micellar aggregate varies according to the κ-casein content of the submicelles: those rich in κ-casein congregate on the surface of the micelle, those poor or totally lacking in κ-casein are positioned in the innermost areas. The absence of a tertiary structure and the chemical bonds that hold the individual submicelles together make the molecule itself extremely resistant to heating, but very susceptible to pH variations and changes in ionic concentrations: the serum casein content can be increased by adding phosphate or acid or by removing colloidal calcium phosphate (Głąb & Boratyński, 2017; "Milk Proteins - From Struct. to Biol. Prop. Heal. Asp.," 2016). In the food packaging development research areas, this biopolymer is mostly used in it's saline form, i.e. as sodium caseinate, because of it's good mechanical properties (when enriched with plasticizend) and, above all, it's barrier properties against oxygen and carbon dioxide. However, it has a high permeability and sensitivity to water vapour. Casein melt at high temperature, are not dissolved completely in water, are tasteless and can be enricched in active compounds(Valentino *et al.,* 2020; Picchio *et al.,* 2018; Ranadheera, Liyanaarachchi, Chandrapala, Dissanayake, & Vasiljevic, 2016). Therefore, sodium caseinate (SC) solutions, as in the case of chitosan (CH), are usually added with plasticisers such as glycerol to optimise the water vapour barrier properties and

mecchanical properties (Sheng *et al.* 2008;Chevalier, Assezat, Prochazka, & Oulahal, 2018) (table 1.3).

Table 1.3 Protein based Biopolymer films, properties reported. Reproduced with permission from (Chen *et al.,* 2019).

Films	Plasticizers	Opacity (A.nm)	Mechanical Properties (TS in MPa)	Thermal Properties	Water Vaper Permeability	References
Wheat gluten						
Gliadins	Gly 35%	~34	%E = ~390 TS = ~7	NR	~7 × 10^{11} [(gm)/(m^2 s Pa)]	[58]
Glutenins	Gly 35%	~101	%E = ~250 TS = ~1	NR	~4 × 10^{11} [(g m)/(m^2 s Pa)]	
Other Sources						
Zein	Gly 40%	NR	%E = ~118 TS = ~4	T_g = ~30 °C	~4 (g mm/m^2 h kPa)	[59]
Kafirin	Gly 40%	NR	%E = ~24 TS = ~1	T_g = ~30 °C	~8 (g mm/m^2 h kPa)	
Avenin	Gly 40%	NR	%E = ~40 TS = ~4	T_g = ~28 °C	~3 (g mm/m^2 h kPa)	
Milk						
Casein	Gly 50%	NR	%E = ~65 TS = ~2.5	NR	~7 (g mm/m2 h kPa)	[60]
Whey fraction						
WPI	Gly 40%	NR	%E = ~33 TS = ~0.9	T_g = ~50 °C	~8 (g mm/m^2 d kPa)	[61]
WPC	Gly 40%	NR	%E = ~18 TS = ~0.7	T_g = ~43 °C	~10 (g mm/m^2 d kPa)	

5

Abrevetation: WPI= isolated whey protein; WPC = whey protein concentrate; Gly= glycerol; NR= data not determinates.

1.7. Structure and properties of Polyhydroxy-co-3-butyrate-co-3-valerate (PHBV)

A PHBV is an aliphatic co-polyester produced through bacterial fermentation of sugar and lipids. It is accumulated in intracellular granules by a wide variety of Gram-positive and Gram-negative organisms (Siracusa, Rocculi, Romani, & Rosa, 2008). The molecular weight of PHBV differs depending on the organism, conditions of growth and method of extraction, and can change from 50 000 to over a million. (PHBV) is: (i) considered 100% biodegradable in composting, backyard or landfill conditions, (ii) shown good barrier and mechanical properties (iii) good proceassability using extrusion or injection process (in function of the synthesis, can be show low thermal stability and mecchanical properties), (iiii) and can used as film carrier of active compounds (Zhao, Ji, Kurt, Cornish, & Vodovotz, 2019; Berthet *et al.,* 2015; Requena, Vargas, & Chiralt, 2017a) (table 1.4). Different authors report the development of active food packaging with antioxidant and antimicrobial activity (Requena, Vargas, & Chiralt, 2017b; Arrieta, García, López, Fiori, & Pep oni, 2019;Figueroa-Lopez, Vicente, Reis, Torres-Giner, & Lagaron, 2019).

Table 1.4. Thermal and mechanical properties of commercial polyhydroxyalkanoates. Reproduced with permission from (Bugnicourt, Cinelli, Lazzeri, & Alvarez, 2014).

Product	Thermal properties			Mechanical Properties		
	T_m [°C]	X_{cr} [%]	T_g [°C]	E [GPa]	σ [MPa]	ε [%]
Biomer P209	–	30–40	–	0.84–1.20	15–20	600–1200
Biomer P226	–	60–70	–	1.14–1.90	24–27	6–9
Biomer P240	–	60–70	–	≈1.85	28	≈11
BIOCYCLE 1000	170–175	–	117	2.2 (Flex)	32	4.0
BIOCYCLE 18BC-1	165–170	–	117	2.4 (Flex)	25	2.2
BIOCYCLE 189C-1	165–170	–	121	2.6 (Flex)	30	2.2
BIOCYCLE 189D-1	165–170	–	125	3.8 (Flex)	36	2.0
ENMAT Y1000	170-176	–	–	2.8–3.5	39	2
Mirel P4001	–	–	110	1.90 (Flex)	20	5
Mirel P4010	–	–	–	1.45 (Flex)	10	10
Mirel P5001	–	–	–	0.30–0.32	20	404–463
Mirel P5004	170	–	–	0.3–0.4	25–30	400–500
Mirel M2100	170	–	–	–	–	–
Mirel M2200	165–170	30–60	–	–	–	–
Mirel M4100	160–170	40–60	–	–	–	–
	164–166	27–45	–	–	–	–

Abbbrevation: Tm= melting temperature; Xcr= crystallinity degree; Tg= glass transition temperature; E= Young's modulus; σ tensile strength; ε elongation at break;

1.8. Conclusions

In view of the severe environmental pollution caused by plastic food packaging, there is a considerable interest in edible and biodegradable films made from renewable and natural polymers. Chitosan, caseinate and PHBV, a natural, non-toxic, biodegradable polymer, available commercially, has been employed in a variety of applications in food industry as a new edible packaging material to control the food quality; it can form films, which may find application in a variety of packaging needs. Blending of chitosan with other natural polymers such as caseinate gives films and coatings with good properties, chitosan addition in edible films leads to good film forming and mechanical properties, no toxicity, biodegradability, relative more hydrophobic nature that could provide higher moisture barrier and water resistance. PHBV based film shows good barrier and mechanical properties and good proceassability using extrusion or injection process (in function of the synthesis, can be show low thermal stability and mecchanical properties). Different film realization technique can be used for a processing of biopolymeric materials.

However, only few authors use the twin screw extrusion tecnique to enrriche polymers with active molecules. And only few or less, the combination of antioxidant and oxygen scavenger properties of gallic acid into a biopolimer film. For this porpouse the twin screw extrusion tecnique was used to realize PHBV pellet enricched in gallic acid (activated with chemical base) to develop an active food packaging with antioxidant and oxygen scavenger activity (Chapter 6).

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Chapter 2– Review on active Packaging for food packaging application

Abstract

The demand for natural antioxidant/antimicrobial active packaging is increasing due to its unquestionable advantages compared with the addition of antioxidants or antimicrobial additives directly to the food. Therefore, the search for active compounds perceived as natural, namely those that naturally occur in herbs and spices, and to add lactic bacteria producing bacteriocins is a field attracting great interest. In line with this, in the last few years, natural antioxidants such as α-tocopherol, caffeic acid, catechin, quercetin, carvacrol and plant extracts (e.g. rosemary extract) and also bacteria recognized as food grade (GRAS) have been incorporated into food packaging. On the other hand, consumers and the food industry are also interested in active biodegradable/compostable packaging and edible films to reduce environmental impact, minimize food loss, and minimize contaminants from industrial production and reutilization by-products. The present review focuses on the natural antioxidants and lactic bacteria already applied in active food packaging and the release mechanism. Furthermore, on the methods used determine the antioxidants in active films and food matrices or food simulants.

2.1. Introduction

The primary functions of a food package are to contain the product, to protect it against physical and environmental damage and to provide information. However, in recent decades the concept of active packaging has advanced in science to satisfy the demand for safe and high quality food products. Active systems are the future direction for development of food packaging and their commercial success should be expected in the coming years. A active packaging is defined as a system in which the food, the package and the environment positively interact to maintain the safety and quality of the products and to prolong its shelf life (Suppakul, Miltz, Sonneveld, & Bigger, 2003; López-Rubio *et al.,* 2004; Wyrwa & Barska, 2017). According to Regulation (EC) No. 450/2009 (European Commission, 2009), active materials and articles are those intended to extend

the shelf life or to maintain or improve the condition of packaged food. They are designed to deliberately incorporate components that would release or absorb substances into or from the packaged food or the environment surrounding the food. Active packaging is an excellent solution for a wide range of applications in the food industry. The most important advantage resulting from their use is reduction in loss of food products due to extension of their shelf life. Active packaging technology positively employs the mass transport phenomena that take place in polymeric materials such sorption and migration. The active packaging could absorb O_2, CO_2, water vapor or food related chemicals from the food or the environment within the packaging surrounding the food (sorption phenomenon); or it could release substances into the food or the environment surrounding the food such as preservatives, antioxidants, and flavorings (migration phenomenon). The active substances can be added to a polymer matrix in different way to be released into a food during a shelf life. The addition of an independent device, such as a sachet, pad or label containing the active agent is not well accepted by consumers. This has led to the use of packaging materials with active agents incorporated in the packaging material itself (Gómez-Estaca, López-de-Dicastillo, Hernández-Muñoz, Catalá, & Gavara, 2014).

Any active agent deliberately added to a packaging system to have a technological effect on the food through its release into the food product or by immobilization on the surface packaging wall should be an authorized food preservative according to the Regulation (EC) No. 1333/2008 on food additives (European Parliament and the Concil of the European Union, 2008; Frear, 2017;Han & goleman, daniel; boyatzis, Richard; Mckee, 2012).

2.2. Absorbing/scavenging packaging system

Absorbing and scavenging systems remove undesired compounds present in the food or in the package headspace such as O_2, CO_2, ethylene, excessive water, and other specific compounds.

Like oxygen, moisture content is considered a critical factor determining the stability of many food products (Escobedo-Avellaneda *et al.,* 2020). The presence of water in the headspace, either permeated from the external environment through the packaging

material or produced by the food in situ, can cause microbial growth, structural changes, loss of characteristic flavours, colour and many functional properties. The absorbing systems are mainly moisture absorber pads used to absorb the drip from packed meat and fish.

Ethylene absorbers are compounds that absorb and act against ethylene, a plant hormone that strongly influences aerobic respiration and fruit ripening. They are inserted directly into plastic materials or into sachets, promoting the removal of ethylene by blocking all ripening-related phenomena: respiration, transpiration, hydrolysis of pectins and carbohydrates. Ethylene removal is achieved by reaction of the hormone with potassium permanganate immobilised on different minerals (silica gel, activated carbon etc.) or by physical adsorption through the use of differently activated ceramic materials, introduced into the packaging in form of small sacchets, or incorporated into the polymer matrix of the packaging material (Majid, Ahmad Nayik, Mohammad Dar, & Nanda, 2018).

The scavenger systems such as O_2 scavengers are those that scavenge or capture residual O_2 inside the packaging (from the environment surrounding the foodstuff or from the foodstuff itself) (Cichello, 2015). Exposure to O_2 may result in microbiological growth on the food or oxidation reactions, both chemical and enzymatic, in the development of off flavours (aromas and odours), in the degradation of pigments and therefore in the loss of nutritional characteristics. There are a variety of commercially available formats, e.g. microperforated sachets containing the active substrate powder that is then inserted into the package, or, the scavenger can be incorporated directly into the packaging material closure accessories or labels.The most widely used existing oxygen scavenger technology is based on the iron oxidation reaction that is activated by moisture in the food product so that the reduced iron is irreversibly oxidised into a stable iron oxide trihydrate complex (Yildirim *et al.,* 2018). The same author demonstrated that by using this technology, oxygen could be removed before it was otherwise available for degradative reactions in the food product. Figure 2.1 shows the typical structure of active multilayer films containing scavengers. An oxygen-absorbing substance, embedded in a layer that is very permeable to oxygen, is used to absorb the oxygen present in the inner package. The entry

of oxygen from the environment to the absorbing layer is limited by a highly gas-impermeable barrier layer. An inner layer, next to the oxygen-absorbing layer, is used to minimise any migration of the oxygen-absorbing substance into the food (Ozdemir & Floros, 2004). To reduce the chemical substances used for oxygen scavenger pads, in the recent years the trend increase to find out natural alternatives such as polyphenols. In this context, Gallic acid (2,3,4-trihydroxybenzoic acid) (GA) a naturally plant phenol present in different sources such as nuts, green tea red wine, also in aok bark and gallnuts as catechin derivatives and hydrolyzable tannins (Luzi *et al.,* 2019; Campo, Pinelli, & Romani, 2016) can be a potential substitute as oxygen scavenger when activated with a chemical base to integrate directly into a food packaging. Gallic acid can act as oxygen absorber when activated with an alkaline base at high relative humidity. In humid environment the base would deprotonate the gallic acid and activate the oxygen absorption. Into details, gallic acid, is a hydrochinoid compound, that in alkaline environment, is subjected to an autoxidation of the initial idrochinone chemical structure to a final gallic acid anion with an open ring, though different intermediare oxidation products. It was showed that from one mole of gallic acid through this autoxidation mecchanism, five mole of O_2 can be absorbed (Wanner, 2010). Indeed, (Ahn, Gaikwad, & Lee, 2016; Pant, Sängerlaub, & Müller, 2017; Singh, Singh, Kumar, & Gaikwad, 2020) already showed that GA combined to alkaline molecule (sodium carbonate, sodium hydroxide or potassium chloride) have a strong oxygen absorption capacity when incroporated in low density polyethylene film; bio-based mulitlayer film and chitosan film respectively.

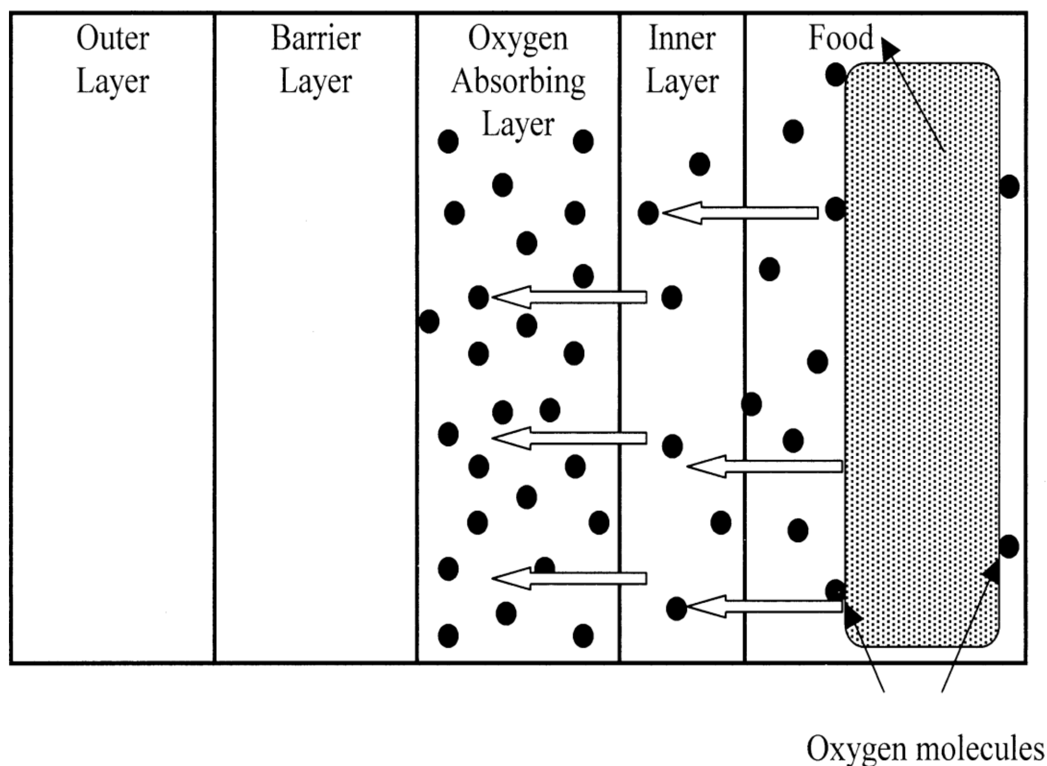


Figure 2.1. Typical structure of a multilayer active film with oxygen absorbers

2.3. Migration system of active compounds

The active agent is incorporated into the matrix of the packaging material and is able to absorb unwanted compounds from the headspace or release antioxidant compounds to the food or the headspace surrounding it. They are developed by incorporating the active compounds into the polymer matrix or onto the surface of the polymer film. The antioxidants that are added to the packaging system can be synthetic or natural antioxidants and come from various sources, both plant and animal, and are distributed throughout the food/packaging/environmental system. The nature of the substances used can be very different, in terms of volatility and solubility, so different release kinetics can be assumed, closely related to the function and form of the system, as antioxidant activity can take place both in the packaging material in direct contact with the surface of the food and in the headspace of the package. The transfer of the active substance from the packaging to the food can take place in different ways:

- Modulated migration, in the case of soluble, non-volatile substances, the mass transfer of the active substance proceeds fairly quickly and is dominated by migration controlled by the diffusion coefficient, but direct food contact is required for the substance to transfer into the food (fig. 2.2).

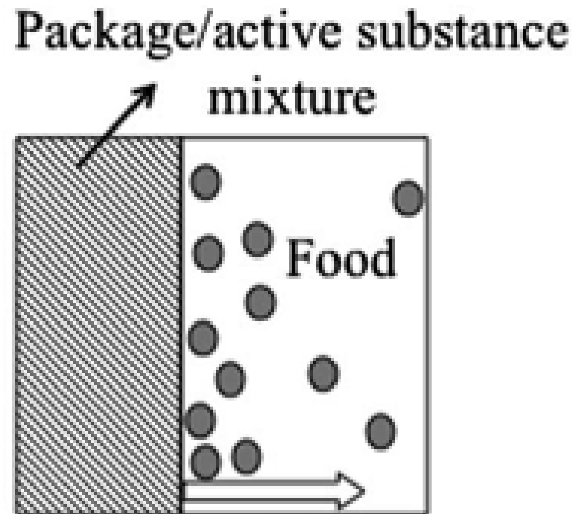


Figure 2.2. Schematic rapresentation of active substances migration from packaging system

A schematic rapresentation of active substances migration from packaging system

- Spontaneous migration, in the case of volatile substances that are released from the packaging to the headspace and does not require contact between the material and the food due to the possibility of the substance transferring in the gas phase. The concentrations of the antioxidant in the headspace depend on temperature, solubility in the food and the rate of release into the medium, i.e. the chemical interactions that occur with the packaging material. However, this migration phenomenon can affect both sides of the packaging, towards the environment and towards the food;

- Non-migration system, in which the antioxidant agent is not released through contact as it is covalently bound to the polymer structure or grafted into its chain (fig. 2.3). These materials deliberately influences the condition of the food without intentional migration.

This category of packaging is thus similar to the releasing system with the difference that the active substance is not released into the food but it stays grafted or "immobilized" on the surface of the packaging where it performs its function. Then, any migration into food is non-intentional.

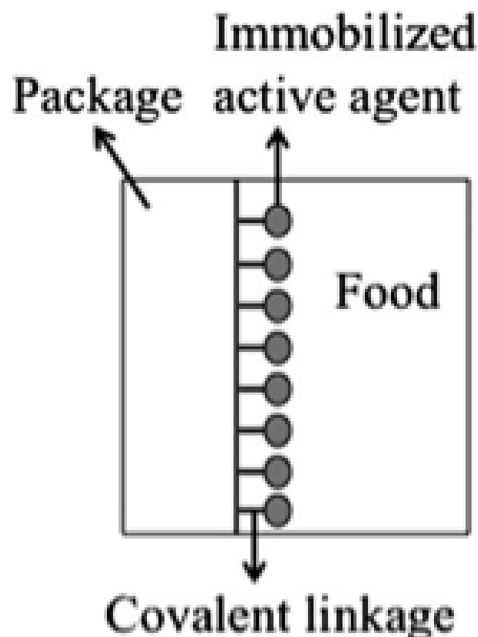


Figure 2.3. Schematic rapresentation of active substances migration from packaging system immobilez on the surface

2.4. Antimicrobial compounds in food packaging application

Antimicrobial packaging is an active packaging concept, and can be considered as a solution to the deterioration of food quality due to microbial growth, which leads to discolouration, off-flavour development, structural changes and loss of nutritional value. Therefore, this packaging system is considered a solution to food contamination, reducing the risk of growth of spoilage and pathogenic microorganisms, prolonging shelf-life and maintaining food quality and safety. Consumer preference for natural food additives and concern about the safety of chemical preservatives has prompted the food industry to look for natural substances. In this regard, there is a place for natural antimicrobial agents that can be used in food products, such as plant extracts, essential oils, enzymes, peptides,

chitosan, bacteriocins, bacteriophages and lactic acid bacteria (Irkin & Esmer, 2015). Moreover, the use of natural antimicrobials is considered safer and is more favorably accepted by consumers than the use of synthetic substances.

2.4.1. Lactic acid bacteria producing bacteriocin

Lactic acid bacteria are considered food-grade organisms and most of them and their metabolites are recognized as GRAS organisms. Lactic acid bacteria are very common microorganisms in nature and are naturally present in raw materials as well as added to foods as starter and/or protective cultures, finding great use in the preparation of fermented foods such as meat, milk and vegetable derivatives. A general definition of lactic acid bacteria is the following: "gram-positive bacteria with a bastoncellar, coccic or cocco-bastoncellar morphology, catalase negative, non sporigenous, lacking cytochromes, aerotolerant anaerobes, nutritionally demanding, acid-tolerant and with a strictly fermentative metabolism". They can be classified into two distinct groups based on the main products of glucose fermentation:

- Homofermentants: ferment glucose by producing lactic acid exclusively (example, *Lactobacillus, Streptococcus, Lactococcus, Enterococcus, Vagococcus, Pedicoccus, and Tetragenococcus*);
- Heterofermentants: ferment glucose with production of lactic acid, acetic acid, and CO_2 (example, *Lactobacillus, Carnobacterium, Leuconostoc, and Oenococcus).*

The genus Lactobacillus includes gram-positive, rod-shaped, anaerobic oxygen-tolerant, catalase-negative, non-spore-forming, homofermenting and heterofermenting microorganisms. They can grow in a temperature range of 5°C to 53°C with optimum values of 30-40°C. They are aciduric, with an optimal growth pH of 5.5-5.8.

Based on the presence or absence of the enzymes responsible for the type of homo- or hetero-fermentation of sugars, species belonging to the genus *Lactobacillus* are divided into three physiological groups:

- Group I: homo-fermenting lactobacilli: species that ferment hexoses to lactic acid and are incapable of fermenting pentoses and gluconate belong to this group. The group

includes the most acidifying (2.7% lactic acid) and thermophilic (optimal growth temperature between 40 and 52°C) species.

- Group II: facultative heterofermentative lactobacilli: the species of this group ferment hexoses almost exclusively to lactic acid or, in the limited presence of glucose, produce lactate, acetate, ethanol or formic acid; they are able to ferment pentoses to lactate and acetate by means of a phosphochetolase inducible by the presence of pentoses.
- Group III: obligate heterofermenting lactobacilli: lactobacilli belonging to this group ferment hexoses to lactate, acetate, CO_2, while pentoses are fermented to lactate and acetate (Khalisanni Khalid, 2011;Ayivi *et al.,* 2020)

To improve food quality and safety, lactic acid bacteria represent a main tool for bio-preservation of food products due to their ability to produce metabolic products with strong antimicrobial effect against pathogenic microorganisms. Inhibition of pathogenic bacteria can be due to competition for nutrients, lowering of pH, production of lactic acid, acetic acid, and production of antimicrobial substances such as bacteriocins.

2.4.2 Bacteriocins

A large number of Gram (+) bacteria and Gram (-) bacteria produce during their growth substances of protein nature (proteins or polypeptides) possessing antimicrobial activities, called bacteriocins. Although, bacteriocins can be classified as antibiotics, they are not. The main difference between bacteriocins and antibiotics is that bacteriocins are synthesized on ribosomes and have a relatively narrow spectrum of antimicrobial activity. Antibiotics, on the other hand, have a broader spectrum of activity and are secondary metabolites (Zacharof & Lovitt, 2012).

The term "bacteriocin" was coined in by Jacob *et al.,* (1953) and stands for primary or modified products of ribosomal synthesis of bacteria, released into the extracellular environment, which may have a relatively narrow spectrum of bactericidal activity (characterized by the inclusion of at least some strains belonging to the same species as the producing bacterium) and against which the producing strain has some specific mechanism of self-protection (Jack, Tagg, & Ev, 1995)**.** Bacteriocins are found in almost

all bacterial species examined to date, and within a species several types of bacteriocins are produced. They can be classified into two main groups:

- Bacteriocins produced by Gram-negative bacteria that interact through the formation of ion channels at the level of the cytoplasmic membrane and show, once penetrated into the sensitive cell, a marked nuclease activity; they adhere to target cells thanks to the presence of specific receptor units present at the level of the outer membrane of the sensitive cells involved (Bruno & Montville, 1993);
- Bacteriocins produced by Gram-positive bacteria closely resemble antimicrobial peptides from eukaryotes. They are cationic, amphipathic, membrane-permeable peptides, approximately 2-6 KDa in size (Heng *et al.,* 2007). They are "membrane active" they operate directly at the membrane level, they do not show any specific absorption, even if we should not exclude a priori the possibility of a preferential route of absorption for those bacteriocins characterized by a more limited spectrum of action (R. Yang, Johnson, & Ray, 1992).

2.4.3. Mechanisms of action of bacteriocins

Bacteriocins have distinct mechanisms of action and can be divided into those that promote a bactericidal effect, with or without cell lysis, or bacteriostatic effect, by inhibiting cell growth (da Silva Sabo *et al.,* 2014). Most of the bacteriocins produced by LAB, especially those that inhibit Gram-positive bacteria, exert their antibacterial effect by targeting the cell envelope (Cotter, Ross, & Hill, 2013). Other bacteriocins use lipid II as a carrier molecule to facilitate the formation of pores on the cell membrane, resulting in a change in cytoplasmic membrane potential and, thus, cell death (Machaidze & Seelig, 2003). Some bacteriocins can kill their target cells through inhibition of gene expression and protein production (Vincent & Morero, 2009).

2.4.4. Applications of bacteriocins

Nowadays, bacteriocins can be applied in the food field as bio-preservatives to control and contain the unwanted microbial population responsible for intoxication and deterioration of the food matrix. Bacteriocins have found wide application as food additives in meats, dairy products, fish products, alcoholic beverages, salads and

vegetables (Terms, 2020). Several studies have shown that bacteriocins can be applied for food biopreservation in three main approaches:

i. use of the bacteriocin-producing strain in the food matrix;

ii. use of purified or partially purified bacteriocins as a food additive;

iii. application of bacteriocins in the packaging film matrix.

Bacteriocin-producing strains can be included in the starter/protective culture of a fermented food. In fermented foods, LAB starter are able to produce bacteriocins in situ and consequently show their antimicrobial activity towards pathogenic or spoilage-causing bacteria. This has been mainly documented for fermented meats, fermented vegetables, olives and dairy products (Leroy & De Vuyst, 2004;Casaburi, Di Martino, Ferranti, Picariello, & Villani, 2016; Giello, La Storia, De Filippis, Ercolini, & Villani, 2018; Aymerich *et al.,* 2000)**.** Bacteriocins have been used in antimicrobial food packaging. The presence of bacteriocins in packaging materials was found to be advantageous because the packaging not only performs a protective function towards food products, but also ensures the control of pathogenic microorganisms in food. In addition, the packaging system protects bacteriocins from interacting with food components such as lipids and enzymes, reducing the risk of bacteriocin inactivation, and their release onto the food surface can be controlled. Cao-Hoang, Chaine, Grégoire, & Waché (2010) introduced nisin into a sodium caseinate film applied to a semi-soft cheese, and observed the antimicrobial activity of the film against harmless *Listeria*. The antimicrobial efficacy of the film varied when *Listeria innocuous* was inoculated deeply. Santiago-Silva *et al*., (2009) incorporate pediocin ALTA 2351 into a cellulose acetate film. This film was applied to sliced cooked ham and evaluated for its antimicrobial efficacy against *Listeria innocua* ATCC 33090 and *Salmonella Typhi ATCC 6539*. Gilamas, Zinoviadou, Biliaderis, & Koutsoumanis (2010) made edible films using sodium caseinate and made it active by directly adding *Lactobacillus sakei* cells to the film-forming solution before casting or by spraying the solution containing *Lactobacillus sakei* cells on the surface of the preformed film. Films embedded with bacterial culture reduced the *Listeria monocytogenes* population by 3 logarithmic cycles compared with the control film

(without *Lactobacillus sakei* cells), whereas in the case of films sprayed with bacterial culture reduced the pathogen population by 3.6 logarithmic cycles compared with the control. Concha-Meyer, Schöbitz, Brito, & Fuentes (2011) developed alginate-based active films realized by solvent casting tecnique enricched in two lactic acid bacteria (*Carnobacterium maltaromaticum*) isolated from smoked salmon and vacuum-packed meat, and nisin to evaluate the growth inhibition of *Listeria monocytogenes* on pieces of smoked salmon stored at 4°C for 28 days. Both types of films applied to the salmon showed a bacteriostatic effect against the pathogen for a period of 28 days. Boelter & Brandelli (2016) used two hydrocolloids, sodium caseinate (CS) and methylcellulose (MC) to produce biopolymer films admixed with two strains of lactic acid bacteria, *Lactobacillus acidophilus* and *Lactobacillus reuteri*. The cell viability of *Lactobacillus acidophilus* was higher than that of *Lactobacillus reuteri* in both polymeric matrices; consequently. Comparing the two hydrocolloid matrices, it appears that sodium caseinate films represent a more favorable environment for LAB survival, since bacteriocin production is higher in proteins. Other authors have found it appropriate to encapsulate the bacteriocins before adding them to the filmogenic solution to protect them from the manufacturing process of the packaging materials so that they are available and can be released during food contact. Boelter & Brandelli, (2016) developed an edible film of gelatin and casein nanocomposites containing halloysite, reinforcements for bio-based materials, and liposomes encapsulated with nisin and used to release the active component into food products. These films also showed antimicrobial activity against *Listeria monocytogenes, Clostridium perfringens,* and *Bacillus cereus*.

Meira, Zehetmeyer, Werner, & Brandelli, (2017) made edible films by casting starch-based incorporated with two bacteriocins, nisin and pediocin, with the addition of halloysite alone. Samples with pediocin demonstrated a halo of inhibition around the film against *Listeria monocytogenes*, whereas against *Clostridium perfringens* the area of inhibition was only around the perimeter of the film. Samples with nisin exhibited a halo of inhibition around the film against both *Listeria monocytogenes* and *Clostridium perfringens*. Bekhit *et al.,* (2018) made bioactive films based on hydroxypropyl

methylcellulose (HPMC) and corn starch added *Lactococcus lactis ATCC 11454*. Free lactic acid bacteria or previously encapsulated in alginate microspheres and pectins were added directly to the filmogenic solution and films were obtained by casting. Differences of 1 logarithmic cycle were observed between free and encapsulated lactic acid bacteria during the storage period in both matrices. HPMC and starch films containing encapsulated lactic acid bacteria showed complete growth inhibition of *Listeria monocytogenes CIP 82110* during the first 5 days of storage at 5°C and a 5 logarithmic cycle reduction after 12 days.

2.5. Antioxidant compounds in food packaging application

The definition of antioxidant, which most frequently appears in literature articles, considers the antioxidant to be any species which, being in much lower concentrations than a given oxidisable substrate, able to slow down or inhibit the oxidative process leading to the degradation of that particular substrate (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002). In addition to the biological context, the concept of antioxidant can also cover all those compounds and/or materials that need to be defended and preserved from photo-oxidative phenomena. The addition of such compounds is essential for the polymer to maintain his mechanical properties following exposure to aggressive agents such as atmospheric oxygen and his radicals, and UV radiation. A classification of the various antioxidant compounds can be made on the basis of their mechanism of action, namely:

- primary or chain-breaking antioxidants, i.e. the substance acting as a radical scavenger by giving up a hydrogen atom or one or more electrons;
- secondary or preventive antioxidants, i.e. they delay the kinetics of the radical initiation process;

Antioxidants have become an indispensable group of food additives, mainly due to their unique properties of prolonging the shelf life of food products, without having a negative effect on their sensory or nutritional qualities. Obviously, antioxidants for food use must meet certain requirements: (i) being inexpensive; (ii) non-toxic (iii) effective even at low

concentrations (to stabilize a fat between 100 to 200 mg of antioxidant/kg of fat is normally used; how many less are needed against browning in fruit juices); (IV) highly stable and capable of surviving processing steps, (V) odour, taste or colour of their own; (VI) easy to incorporate and have good solubility in the product (Fereidoon Shahidi, Janitha, & Wanasundara, 1992). Antioxidants act at different levels in the oxidative sequence involving lipid molecules and are able to decrease the oxygen concentration, intercept singlet oxygen, prevent the start of the first oxidation phase by eliminating initial radicals such as hydroxyl radicals, and are also able to decompose the primary oxidation products into non-radical species and interrupt the oxidation chain reaction in order to prevent the continuous extraction of hydrogen from substrates. Hydroperoxides are the main products of lipid oxidation but being unstable molecules they decompose easily and impart unpleasant tastes and odours to food products.

Antioxidants can often be combined with substances with antimicrobial activity, and together they are able to perform a synergistic action in controlling the proliferation of aerobic, pathogenic and mould micro-organisms on the surface of the food. Again, the use of natural antimicrobials is generally considered to be safer and more readily accepted by the consumer than the use of synthetic substances. Among the various natural antimicrobial agents is nisin, a hydrophobic protein produced by Lactococcus lactis that is considered GRAS (Generally Recognized As Safe). This polypeptide, incorporated into the packaging material, has the ability to inhibit the growth of gram-positive bacteria (Sung *et al.,* 2013).

2.5.1. Essential oil

In the last years, the demand for replacing synthetic chemicals with natural compounds it's increased. More attention are showed to the use of natural antioxidants, such as polyphenols, tocopherol, plant extracts and essential oils (EO) for inclusion in packaging materials, due to consumer concerns about their health and the possible migration of toxic substances into food (Sánchez-González, Vargas, González-Martínez, Chiralt, & Cháfer, 2011;Ju *et al.,* 2019). In this context, EO are interesting for their potential use as natural preservatives (Atarés & Chiralt, 2016). Extracts of aromatic plants (rosemary, clove, etc.)

have in their constitution several active compounds. The main group are composed of terpenes and terpenoids and the others of aromatic and aliphatic constituents, all characterized by low molecular weight. These phenolic compounds are part of the secondary metabolites of plants, contributing for their defense against ultraviolet (UV) radiation (antioxidant activity) and against pathogens, parasites and predators (antimicrobial activities). Phenols has antioxidant activity, thanks to their capacity to release hydrogen molecules captured by free radicals, this one responsible for the alteration of the quality of food product (Sharma, Barkauskaite, Jaiswal, & Jaiswal, 2020). The antioxidant activities of the essential oil containing this compound have been widely demonstrated (Nieto, Ros, & Castillo, 2018). *Rosmarinus officinalis L.* (rosemary), a member of the Lamiaceae family, is an attractive evergreen shrub with pine needle-like leaves that grows wild in most Mediterranean countries (Rezanejad *at al.,* 2019). Moreover rosemary are classified as generally recognized as safe (GRAS)(Aguilar at al., 2008). Many compounds have been isolated from rosemary oil such as flavones, diterpenes, steroids, and triterpenes.The antioxidant activities of rosemary extracts are mainly due to the high content of many polyphenols including and rosmarinic acid and carnosic acid (fig. 2.4).

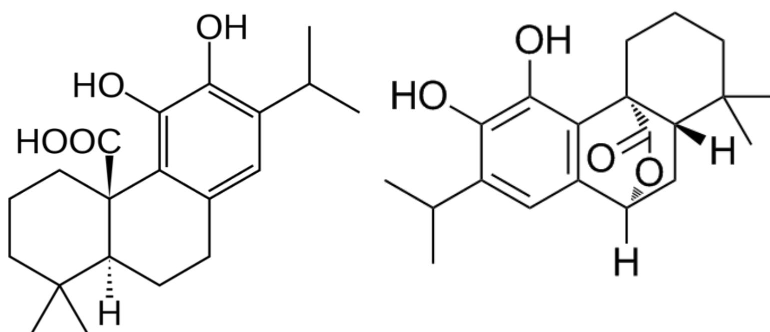


Figure 2.4. Molecular structure of carnosic acid and carnosol acid

Carnosic acid and carnosol act as potent scavenger of peroxyl radicals. The effectiveness of these two diterpenes is greater than that of the most commonly used natural antioxidants such as BHA, BHT and propyl gallate (Aruoma, 1999;Halliwell, Murcia,

Chirico, & Aruoma, 1995). Carnosic acid and carnosol are able to inhibite lipid peroxidation by 88-100 % and 38-39 %, respectively, under oxidative stress conditions. The flavonoids contained in the extract have a synergistic effect with the diterpenes in their antioxidant activity. Carnosic acid, however, is the compound most commonly present and the antioxidant activity is due to it's particular structure. Carnosic acid has two phenolic groups on the aromatic ring which contribute to the termination of the radical reaction. The mechanism involves capturing the species formed by lipid peroxidation, thanks to the hydroxyls present on the ring, to then form quinones. These oxidation products also act as antioxidant agents over time (Wijeratne & Cuppett, 2007). These reactions lead to the termination of radical reactions, limiting rancidity and preserving the characteristics of the product during storage. A wide variety of EOs, from different plants such as basil (*Ocimum basilicum L.*), chamomile flowers (*Matricaria camomilla L.*), cardamom seeds (*Elettaria cardamomum (L.) Maton*) and rosemary (*Rosmarinus officinalis L.*), can be incorporated into polymer matrices derived from biopolymers (table 1). S. Y. Yang, Cao, Kim, Beak, & Song, (2018) report an antioxidant effect of Foxtail millet starch film enriched into clove leaf oil, showing an oxygen scavanger activities of 40.81% and 70.59% of DPPH and Abts radical respectivly. Hashemi & Mousavi Khaneghah, (2017) show the antioxidant properities of novel basil seed gum enriched in oregano essential oil. Results shows an increasing up to 36.84 g of Abts/kg of dry film and 0.71g of DPPH/kg of dry film. The EC 50 of whey protein enriched in different essential oil are reported by (Ribeiro-Santos *et al.*, 2018). Results show an EC 50 of the active film of 0.43 mg/ml. the antioxidant activity of corn starch film enriched in *Bunium persicum* and *Zataria multifora* essential oils are reported by (Aminzare *et al.*, 2017), showing an % of inhibition of DPPH radical of 80% and 71% respectivly. Salarbashi *et al.*, (2014) report IC 50 of soybean polysaccharide film enriched in *zataria multiflora Boiss* and *mentha pulegium* essential oils. Results show an higher antioxidant effect when *mentha pulegium* are used with an IC50 of 8.86 mg/ml. Alternatively, packaging materials can be coated, on the surface that will come into contact with the food, with a lacquer on which the active substance is allowed to adsorb.

For volatile or easily perishable substances, the microencapsulation technique has also been tested: a suitable quantity of active ingredient is enclosed in cyclodextrin or synthetic resin capsules and then added to the polymer during production or deposited by lacquering on the surface of the film (Kadoya, 2012). Furthermore, the presence of antioxidants in plastic films can contribute to the stabilisation of the polymer itself and thus protect the films from degradation. In general, both when incorporated internally and deposited on the packaging, they remain stable for a necessary time and then are released in a controlled manner. There are numerous studies characterizing films produced from biocompatible materials enriched with essential oils, such as the work of (Ojagh, Rezaei, Razavi, & Hosseini, 2010) who highlighted the effects of incorporating cinnamon essential oil into chitosan matrices (table 2.1). Mohsenabadi, Rajaei, Tabatabaei, & Mohsenifar, (2018) studied the effect of encapsulated rsoemary oil into a starch HPMC polymer film, for food packaging application. de Moraes Crizel *et al*., (2018); Ghadermazi, Keramat, & Goli,(2016) using diffent type of essential oil in different polymer matrix for food packaging application.

Table 2.1 Antioxidant activiies of different bipolymer enriched with essential oils.

Film composition	oil type	antiox test	results	how results are reportet	year	References
Foxtail Millet Starch Film	Clove Leaf Oil	DPPH and ABTS	40.81 and 70.59%, r espectively.	% of scavenging act.	2018	S. Y. Yang, Cao, Kim, Beak, & Song, 2018
novel basil-seed gum	oregano essential oil	DPPH; ABTS,	4.56-36.84 g/kg of the film (Abts);0.14–0.71 g/kg (DPPH)	g/kg film	2017	Hashemi & Mousavi Khaneghah, 2017
whey protein blend	C. cassia EO, 34.0% C. zeylanicum EO, 15.0% R. officinalis EO and 0.0% O. basilicum EO.	DPPH	EC50 = 0.43 mg mL−1	mg mL−1	2017	Ribeiro-Santos et al., 2018
Corn Starch	Bunium persicum BPEO, and Zataria multiflora Essential Oils ZMEO	DPPH, Film dissolved in water and then in contact with DPPH for 30', ABTS	80% and 71% in highest concentration for ZMEO and BPEO,	I%	2017	Aminzare et al., 2017
soybean polysaccharide	Zataria multiflora Boiss and Mentha pulegium essential oils	DPPH	IC 50 = 4189 mg/l Zataria and IC0 = 9 mg/ml Mentha p.,	IC50	2014	Salarbashi et al., 2014)

I% = inhibition percentuage; IC50= inhibition concentration of 50 % of Radical;

2.5.2. Phenolic compound

Phenolic compounds that have the ability to interfere with the oxidation process as free radical terminators can be added to the reaction medium (Han & goleman, daniel; boyatzis, Richard; Mckee, 2012). Polyphenols have been extensively studied and it has been confirmed that these molecules have a number of beneficial activities for human health, all of which can be attributed to their antioxidant activity. A number of molecules belong to this family, all of which have the common feature of having several aromatic rings and several hydroxyl functionalities. Flavonoids and phenolic acids can be identified on the basis of their molecular structure. Flavonoids contain, attached to their 2-phenylchroman ring structure, hydroxyl and/or semiquinone functionalities responsible for a variety of biological activities including anti-inflammatory, antibacterial, immunostimulant, anti-allergic and antioxidant activities. Phenolic acids, on the other hand, can be subdivided into hydroxybenzoic acid derivatives (gallic acid, catechol,

vanillic acid) and cinnamic acid derivatives (sinapic acid, caffeic acid) (Panche, Diwan, & Chandra, 2016).

Gallic acid, or 3,4,5-trihydroxybenzoic acid, is an organic acid found in fruit and vegetables with the chemical formula $C_6H_2(OH)_3COOH$. Together with Propyl gallate (propyl 3,4,5-trihydroxybenzoate; PG) a synthetic phenolic antioxidant formed by propanol and gallic acid condensation, its a powerful antioxidant widely used in the pharmaceutical and food industries. The antioxidant activity of the two phenolic acids is strongly dependent on their chemical structure, and is attributable to their free radical scavenging capacity(fig. 2.5). This function depends on the number of electron donors of hydroxyl and methoxyl substitutions, and it has also been observed that gallic acid with three hydroxyl groups and one carboxyl group is more active than its ester, with three hydroxyl groups and one carboxylic acid ester group (Asnaashari, Farhoosh, & Sharif, 2014; F. Shahidi, 2000).Gallic acid and proyl gallate have been used to improve the oxidative stability of fat and oils in the preparation of food stabilized thermecally. One specif application is to prevent lipid oxidation of marine oils, which are considered valuable sources of polyunsaturated fatty acids (PUFA), such as ω-3, rich in eicosapentenoic acid (EPA) and docosahexenoic acid (DHA). The antioxidant action of these molecules in interrupting lipid oxidation takes place in a reaction that occurs much more rapidly with lipid radicals than with PUFAs.

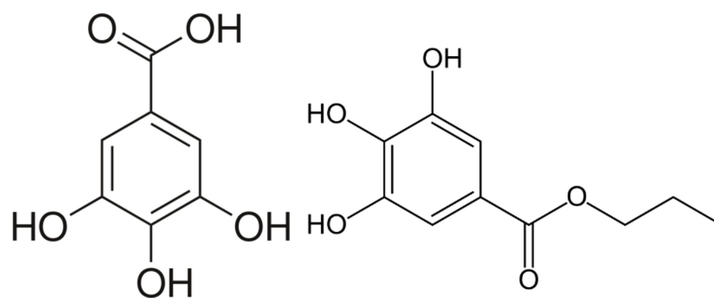


Figure 2.5. Representation of the chemical structure of (a) gallic acid and (b) propyl gallate.

PG are authorized also into food packaging development as polymerisation aid and as antoxidant (additives). The therm"polymerisation aid' means a substance that starting the polymerisation process and/or controls the formation of the macromolecular structure; For"Additive" it means a substance intentionally added to the plastic material to achieve a physical or chemical effect during processing of the plastic material or in the finished material or article; it is intended to be present in the finished material or article. For propyl gallate the european regulation fixed a total specific migration limit of 30 mg/Kg (related to the sum of the substances) expressed in mg of substance per kg of foodstuff (European Parliament and the Concil of the European Union, 2008; European Commission, 2008). Different authors realized active packaging with gallic acid and PG as active molecules. From the work of (López-De-Dicastillo, Gómez-Estaca, Catalá, Gavara, & Hernández-Muñoz, 2012) evaluations emerge regarding the antioxidant activity of packaging materials, consisting of a biodegradable polymeric matrix of poly(lactic acid) (PLA), in which a Chilean spice, merkén, has been incorporated. The most identified antioxidant compounds in the spice are gallic acid and catechin. Wu *et al*., (2016) compared the antioxidant activity of native chitosan films with that of chitosan-gallic acid films and found that the latter exhibited much higher scavenging activity against DPPH and ABTS radicals. The degree of antioxidant power of these films is proportional to the amount of gallic acid grafted onto the chitosan chain. Xie, Hu, Wang, & Zeng, (2014) developed a method to graft gallic acid onto the chitosan chain in aqueous solution in the presence of carbodiimide and hydroxybenzotriazole. The results, show that GA-g-CS grafts have a high antioxidant. The antioxidant capacities are correlated with increasing concentrations of gallic acid grafted onto chitosan. Gim, Hong, Kim, & Lee, (2017) evaluated the effects of gallic acid grafted chitosan on oxidative stability in bulk oil at two different temperatures. The work showed that surface-grafted chitosan films with gallic acid have excellent antioxidant activity based on the scavenging efficiency of the DPPH radical. Wang *et al*., (2020) realized a multilayer packaging system of dextran and gelatin with the innerlayer enrriched in propyl gallate to prolong the release of PG during the shelf life of food. the results show an improvement of the realse and also of the barrier properties

of the realized packaging. Rui *et al*., (2017) in their work prepared, characterised and compared active films of chitosan (CS), gallic acid-chitosan (GA-CS), gallic acid-glycerol-chitosan (GA-g-CS). show that incorporation or conjugation of gallic acid significantly enhanced the antioxidant activity of the CS-based films, dependent on the increase in gallic acid concentration. Furthermore, the DPPH/ABTS radical scavenging activity of gallic acid-glycerol-chitosan films is higher than that of gallic acid-chitosan between gallic acid-chitosan and gallic acid-glycerol-chitosan films could be attributed to the different structure and solubility in water: the compact network structure of gallic acid-chitosan films limits the mobility of DPPH/ABTS free radicals and reduces the efficiency of resonance-stabilised phenoxy radical formation. For this porpouse in this thesis work, gallic acid and also propyl gallate was disperdet into a film forming solution in combination with glycerol for a higher antioxidant properties at low concentrationfilms when the same amount of gallic acid is incorporated or conjugated. The differences

2.6. Effect of active compounds on dry film properties

Consumer concerns of food quality and safety lead to the development of active packaging, which is an innovative packaging performing some functions in the preservation of the food other than providing a barrier property. Active compounds and ingredients can be incorporated into packaging materials to provide several functions that do not exist in conventional packaging. However the addition of active compounds such as polyphenols, can affect the chemico physical properties of the realized active film (table 2.2). Generally the addiction of polyphenols into biopolymer film can affect the water vapor barrier and the flexibility of the film. Into details S. Y. Yang *et al.*, (2018) show that the addition of clove oil reducing the tensile strenght, film solubility, WVTR and increased the moisture content and elongation at break of foxtail millet starch film. (Liu, Meng, Liu, Kan, & Jin, 2017;Liu, Liu, *et al.*, 2017) report the effect of protocatechuic and gallic acid on chitosan film. Results show that the active compounds improving the WVTR values, reducing the eleongation at break and moisture content of the active film comparing to a control ones. Dashipour *et al.*, (2015) report the effect of zataria multiflora on carbossymethyl cellulose respectively. The results show a reduction of the tensile strenght and moisture content and film solubility, but an opposite behavior on the barrier properties (WVTR) and on the elongation at break. The effect of yerba mate and fruit extract on cassava starch and pectin film respectively are reported by (Jaramillo, González Seligra, Goyanes, Bernal, & Famá, 2015 ;Eça, Machado, Hubinger, & Menegalli, 2015). Results show that WVTR and tensile strength are reduced; elongation at break increased in both polymer matrix cassava starch and pectin respectively.

Table 2.2. Effect of active compounds on chemico -physical properties of biopolymer film.

Polymers	Active compound	Sample	Film thickness (mm)	Moisture content (%)	Film solubility (%)	WVTR (gm-1 s-1 Pa-1)	Tensile strenght (Mpa)	young modules (Mpa)	Elongation at break (%)	References
Foxtail Millet Starch Film	Clove oil	control	0.081	6.57	42.01	3.55 10^-9	6.78	x	66.26	So-Young Yang ET AL.,2018
		active	0.085 to 0.095	7.25 - 8.43	38.08 - 33.60	3.53 - 3.49 10^-9	5.65 - 4.00		84.11- 99.48	
chitosan	protocatechuic acid	control	0.061	26.22	19.22	12.69 10^-11	40.84	x	62.8	JunLiu et al., 2017
		active	0.065-0.083	17.58-13.40	22.5-31.38	7.08 -9.90 10^-11	49.59 - 26.71		35.10 -7.86	
chitosan	Gallic acid	control	0.0983	16.81	24.99	10.88 10–11	27.14	103.18	26.3	Liyun Rui et al., 2017
		active (incorporating ga)	0.1007-0.1060	14.55-14.19	25.68-26.95	10.15-9.57 10^–11	28.46-31.04	150.32 -209.28	18.72-15.13	
		active (conjugated ga)	0.0747-0.0783	14.93-15.16	28.5-26.48	10.32-11.42 10^–11	28.15-23.69	159.45-168.13	17.85-14.09	
Basil seed gum	Oregano essential oil	control	0.06	17.58	x	4.33 10^-11	x	x		Hashemi et al., 2017
		active		17.62-17.92		4.33-3.69 10^-11				
Carboxymethyl cellulose	Zataria multiflora	control	0.04	23.54	x	2.98 10^–10	17.75	x	26.86	Dashipour A, et al 2015
		active	0.05-0.09	19.94-15.64	79.84-67.20	3.38-5.79 10^–10	58.84-16.96		30.44-20.77	
Cassava starch	yerba mate	control	x	37.90	35.20	8.8 10^-10	0.51	1.90	107	Carolina Medina Jaramillo et al 2014
		active	x	30.8-27.8	32-29,8	7.3-4.5 10^-10	0.31-0.28	0.69-0.42	156-183	
						g mm h^-1 m^2 kPa				
Pectin	acerola, cashew apple, papaya, pequi, and strawberry	control	0.08	27.00	65.40	0.16	12.10	x	8.90	Kaliana S. Eça et al., 2015
		active	0.06	32.00	70.20	0.14	7.00		11.3	
Cassava starch	oregano essential oil pumpkin residue extract	control	0.16	28.10	18.40	0.40	x	x		dos Santos Caetano et al., 2017
		active	0.17	21.30	21.90	0.94				
Gelatin	durian leaf waste	control		x		6.29 10^-4	x	x		Wai-Yee Joanne Kam 2018
		active				4.1-7.47 10^-4				
Chitosan	Olive pomace 10-30% flour, microparticle	control	0.12	18.28	32.61	0.85	16.76	69.11	23.05	Tainara de Moraes Crizel et al., 2018
		active (flour)	0.22-0.31	18.87- 16.47	31.93- 44.50	1.32 -2.23	7.58 -2.89	40.42-12.39	17.57-13.31	
		active (microparticle)	0.14	18.97 - 15.04	31.31-26.16	0.96 -1.09	22.40-6.51	35.90 -16.28	33.01-20.93	
Potato starch	Green tea extract	control	0.05	17.07	28.36	9.58 g m^-2 day^-1	1.66	0.19	15.90	Nisa et al., 2015
		active	0.143	15.13	24.8	7.17 g m^-2 day^-1	1.95	0.24	11.17	

X=data not determinate

2.7. Analytical methods for the determination of Antioxidant Capacity (AOC)

In selecting the most appropriate method of analysing antioxidant capacity, it is essential to match the source of the radical and the characteristics of the system with the reaction mechanisms of the antioxidant molecule. Very often, to identify the antioxidant capacity of real samples such as food samples, two terms are mainly used, sometimes incorrectly used as synonyms:

- antioxidant capacity (AOC), this term refers to the measurement of the moles of a given free radical that are inhibited and/or removed from a given solution without considering the antioxidant activity of each individual compound present in the solution. It therefore considers the sum of all contributions from the individual antioxidants present in the sample and their synergistic effects;
- antioxidant activity (AOA), referring to the capacity of a particular antioxidant molecule to block or counteract various radical mechanisms, and therefore corresponds to the constant kinetics of a single antioxidant compound against a specific free radical (Ghiselli, Serafini, Natella, & Scaccini, 2000)

The in vitro analytical methods that have been used to assess antioxidant capacity are:

- DPPH;
- ABTS-TEAC.

It is important to emphasise that there is no universal method capable of accurately and quantitatively measuring antioxidant capacity, as each method is strictly dependent on a particular principle of antioxidant capacity (measurement of scavenging activity, measurement of oxidative stress, etc.) and each method responds specifically to a single type of radical species (Prior, Wu, & Schaich, 2005).

2.7.1. ABTS-TEAC method

The TEAC assay was first reported in the literature by Miller and Rice-Evans and is based on the assessment of the scavenging capacity of antioxidants against the cationic radical ABTS-+ (acronym for 2,2'-azinobis (3-eylbenzothiazoline-6-sulfonic acid) (fig. 2.6).

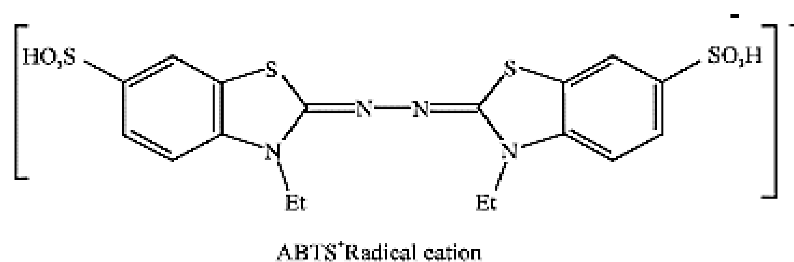
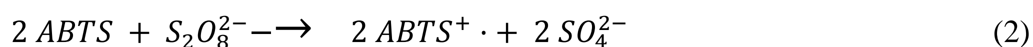


Figure 2.6. Molecular structure of ABTS+ radical

The cation radical is generated by an oxidation reaction with the colourless compound ABTS. The more common and readily available sodium or potassium persulphate ($K_2S_2O_8$ or $Na_2S_2O_8$) was used. The use of sodium or potassium persulphate as oxidising agents involves the generation of a radical solution of ABTS-+, in phosphate buffer at pH 7.4, according to the following reaction (2):



$$2\,ABTS\ +\ S_2O_8^{2-} \longrightarrow\ 2\,ABTS^{+}\cdot +\ 2\,SO_4^{2-} \qquad (2)$$

The solution must not contain an excess of persulphate as, being a strong oxidising agent, it could itself react with the antioxidant molecule and thus invalidate the analysis. This chemical generation of the radical cation takes a long time, about 16/18 hours, in the dark, to reach completeness and present an absorbance value that is stable over time. The solution thus generated has an intense blue-green colouration, with absorption maxima located at wavelengths of 734 nm. Following the addition of one or more antioxidants to the reaction environment, these have the ability to yield one or more hydrogen atoms to the cationic radical, causing a gradual decrease in the initial absorbance value evaluated at λmax. The determination of the antioxidant capacity (AOC) of a generic antioxidant compound is obtained by means of a calibration line in which the loss of the absorbance signal is related to the concentration of antioxidant added to the cell. The method is also called trolox equivalent antioxidant capacity (TEAC), since it reports the AOC values of the various samples as trolox equivalents, which corresponds to the millimolar concentration of a trolox solution that has the same antioxidant capacity as a 1 mM solution of the antioxidant compound to be investigated. Trolox is therefore used as a reference to be able to compare the AOC values of individual antioxidant compounds and to define a trend in antioxidant capacity between the various molecules.
The ABTS method is therefore easy to use, which is why it is widely used for the determination of antioxidant capacity in food samples. Furthermore, due to its ability to be soluble in both aqueous and organic solvents, it is also effective for determining the AOC of hydrophilic and lipophilic compounds (Miller & Rice-Evans, 1997; Ilyasov, Beloborodov, & Selivanova, 2018).

2.7.2. DPPH method

This is a currently used method based on the use of the stable diphenylpicrylhydrazyl radical (DPPH) for measuring the reducing capacity of antioxidants. The DPPH molecule (2,2'-Diphenyl-1- picrylhydrazyl), shown in Figure 2.7, is an organic nitrogen radical that

is stable by virtue of the delocalisation of the odd electron, located on the nitrogen atom, over the entire molecular complex. This delocalisation is responsible for the characteristic deep purple colour of the solution. The radical is commercially available and does not have to be generated prior to testing as is the case with ABTS-+.

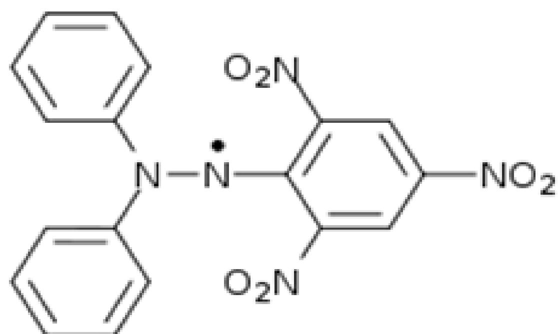


Figure2.7. Molecular structure of the DPPH- radical.

The presence of nitro groups in the molecule is essential if the compound is to absorb radiation in the UV-Vis range, and can therefore be monitored spectrophotometrically. When an antioxidant compound, capable of donating a hydrogen atom, is added to the DPPH- solution, it gives rise to its reduced form accompanied by gradual decolourisation of the solution, which can be monitored over time spectrophotometrically at the wavelength of the absorption maximum (λmax = 516 nm) (fig. 2.8).

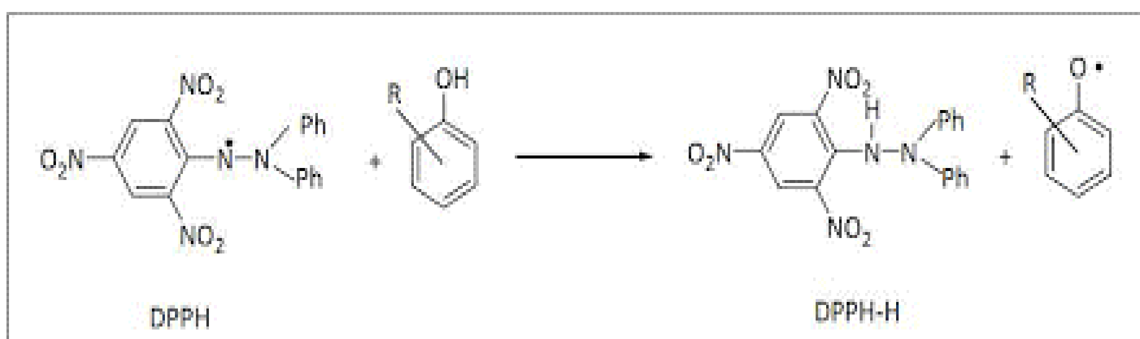


Figure 2.8. Reaction mechanism of DPPH

The deep purple colour of the DPPH- solution can be lost either through a mechanism of antioxidant action of the Hydrogen Atom Transfer (HAT) type, i.e. by the transfer of a hydrogen atom, or through reduction reactions involving the transfer of a single electron (SET, Single Electron Transfer). The determining factor in the reaction is steric accessibility to the radical site: it follows that molecules with less steric clutter, which have better access to the unpaired electron, have higher AOC values than real ones. One parameter used to assess and measure the decolouration kinetics of DPPH is the 'efficient

concentration' or EC50 value (otherwise known as the IC50 value), which describes the concentration of antioxidant required to ensure a 50% decay of the initial absorbance value (Molyneux, 2004).The DPPH assay can be used with both alcoholic and hydroalcoholic solutions of methanol and ethanol as they do not interfere with the reaction, thus allowing the analysis of many antioxidant compounds, both hydrophilic and lipophilic (Kedare & Singh, 2011).

2.7.2.1. The EC50 parameter (the "efficient concentration" value)

Recently, the term "efficient concentration" or EC50 value has become established for the interpretation of the results of the DPPH method. This parameter was introduced in by Brand-Williams *et al*., (1995) and has been widely used by many scientists to present their results. It is defined as the substrate concentration that causes a 50% loss of DPPH activity, or rather, a loss of the intended purple colouration of the DPPH solution. Consequently, the lower the value of this parameter, the more effective the antioxidant capacity, as less of the compound is needed to cause the same decrease in DPPH-concentration. It can therefore be a test of biological activity, allowing us to validate the use of the substrate as an antioxidant in any biological system. In practice, the EC50 value should represent the concentration of the substrate present in the reaction medium, in a cuvette, in the absence of the DPPH radical, knowing the initial concentration of this radical. The commonly used approach to interpreting the data is to work in terms of the percentage reduction of DPPH, referred to as "inhibition" and defined by the equation (3)

$$\%\ I = \frac{(Ao - Ac)}{Ao} * 100 \qquad (3)$$

where A_0 is the initial absorbance at 515nm in the absence of the substrate and Ac is the absorbance value at the same wavelength in the presence of the sample. Reaction kinetics were plotted for each type of antioxidant sample tested, using graphs in which the % inhibition as a function of substrate concentration, is plotted. In this way we can obtain calibration lines for each sample or pure compound which, thanks to linear interpolation, allow us to trace the value of the EC50 parameter (Mishra, Ojha, & Chaudhury, 2012 ;Chen, Bertin, & Froldi, 2013).

2.8. Manufacturing of active film

There are mainly three approaches for developing antioxidant packaging systems:

(i) Independent antioxidant devices: The very first active packaging system that were commercialized (even still widely used today) were the inclusion of an independent device i.e. sachet, label, membranes or pad containing the agent apart from the food product to a conventional packaging system. These independent devices contain oxygen scavengers (Baldino, Cardea, & Reverchon, 2017; Fang, Zhao, Warner, & Johnson, 2017). Fine powders of ferrous and iron oxide are the most common oxygen scavengers, however, sulphites, ligands, catechols, polyphenols, ascorbic acid and some enzymes such as glucose oxidase have also been utilized (Baldino *et al.*, 2017; Fang *et al.*, 2017). To prevent the premature action of oxygen scavengers, specialized mechanisms can be devised to initiate scavenging reaction. For instance, to initiate oxygen removal, the presence of humid conditions is required for iron-based scavengers (Lopez Rubio *et al.*, 2004). Extensive reviews have been published on the uses and applications of oxygen scavenging packaging (Brody, Bugusu, Han, Sand, & McHugh, 2008; Dey & Neogi, 2019; Rooney, 2005).

(ii) Antioxidant packaging materials: active antioxidant agent is incorporated within the containers in which product is packed or into the packaging film walls, employing its activity by absorbing undesirable agents/compounds around the product from the headspace, or by releasing antioxidant compounds into the headspace surrounding the food products or directly to the food (Fang *et al.*, 2017). The development procedure of antioxidant packaging material mainly depends upon the characteristics of the antioxidant (especially its mechanism of action and heat resistance) and the polymer type. If the mechanism of action of a material is based on the principal of migration of antioxidant compounds into the food product, the antioxidant compounds released should not only adhere to concerned regulations in terms of their maximum allowable limit but should also be permitted as food additives (Gómez-Estaca *et al.*, 2014). When manufacturing an antioxidant packaging material, from a technological point of view, the antioxidant compound (or the reactive substances that produces that compound) is closely mixed with the polymer, either

(iii) by polymer melting and inclusion and mixing of the antioxidant agent into the melt by using extrusion technology. This technique is preferred since most of the

conventional packaging structures are either partially or entirely manufactured by extrusion technologies. However, a critical drawback that needs to be considered while using extrusion technology is the degradation of bioactive compounds by severe thermo-mechanical treatment.

(iv) by immobilizing the antioxidant agent on the surface of the film (Fang *et al*., 2017).

(v) by dissolving both (agent and polymer) into an appropriate solvent followed by applying solution to a substrate by coating technology. Although manufacturing of active packaging films through casting is widely used during development of films, however, it cannot be considered as a standard process. Nonetheless, dispersions and solutions are utilized in the manufacturing of polymeric coating on the surface of film through conventional printing technologies i.e. gravure and flexo. Furthermore, the formulated coating film should be able to fulfill the following requirements: i) the validity of coating material for direct contact with food and good adherence to the substrate of film, ii) the packaging requirements of food products especially related to their functionality, and iii) maintaining an effective antioxidant activity by adjusting the release of antioxidant agent (Gómez-Estaca *et al*., 2014). Adherence majorly depends upon the compatibility between the coating polymers and the substrate and can be promoted by chemical methods i.e. primers, physical methods (UV irradiation, corona discharge etc.) or a combination of both just before the coating process,

2.9. Conclusions

Nowadays, a particular attention was given to the natural substances incorporated in active food packaging, and in the tests carried out in both packaging and packed foods to evaluate the efficiency of these new materials.

Recently, besides the great improvement in food packaging materials in order to increase food shelf life with the addition of natural antioxidants/antimicrobials, the tendency is to incorporate these compounds in biodegradable/compostable packaging or edible films to decrease the impact of packaging in the environment because of their biodegradability and also to reduce food loss, which, in turn, impacts less on the environment (sustainability). Newer materials are emerging day by day and it is essential to study their properties, as well as their safety and effectiveness, in order to evaluate which food

products are more suitable to be packed by each material. Fort this pourpose the effect of rosemary oil on a blend obtained from chitosan and sodium caseinate film has been studied (chapter 3) , and the effect of lactic bacteria (producing bacteriocines) on whey protein film has been also investigated (chapter 4).

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Chapter 3 – Development of antioxidant film based on chitosan and sodium caseinate

Based on the following projects of publications:

3.1. Physical properties of active biopolymer film based on chitosan, sodium caseinate and rosemary essential oil

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3.2. Effect of chitosan-sodium caseinate film enriched in rosemary essential oil on the oxidation kinetics of fresh chicken breast hamburger

3.3. Selection of polyphenols for food packaging application

The objective of this chapter was to study the effect of rosemary essential oil (REO) on the (i) chemical-physical properties of active biopolymer film based on chitosan and sodium caseinate; (ii) and to investigate the shelf-life extension of chicken meat hamburger during a storage using the realized active film. Finally to select a single antioxidant polyphenols for food packaging application.

3.1: *Physical properties of active biopolymer film based on chitosan, sodium caseinate and rosemary essential oil.*

In the first part of this chapter the effect of REO on chitosan (CH), sodium caseinate (SC) and blend film based on CH/SC film was evaluated in terms of mechanical, barrier and thermo chemical properties. Then a Fourier- transform infrared spectroscopy (FTIR) analysis was conducted to understand if REO can be recognized into the active materials. This technique is used in food packaging companies in the determination of the polymeric composition of plastic materials.

3.2: *Effect of chitosan-sodium caseinate film enriched in rosemary essential oil on the oxidation kinetics of fresh chicken breast hamburger***.**

The active films characterized in the first part of this chapter were evaluated in terms of antioxidant activity using DPPH and Abts radicals test to understand the potential antioxidant effect. The CH/SC/REO film was selected for its antioxidant properties and applied on chicken breast hamburger. Commercial film was used as control. Color, pH, and the oxidation product (malondialdeyde and metmyoglobin) of chicken breast hamburger were investigated during storage (27 days), to quantify the effect of the active film on the quality properties of the selected food.

3.3: *Selection of polyphenols for food packaging application*

The aim of this work was aimed to evaluate the antioxidant properties of quercitin, cathecol, tannic acid, gallic acid, and sinaptic acid. To reach this goal the antioxidant properties of different polyphenols were investigated using DPPH and ABTS methods.

3.1. Physical properties of active biopolymer film based on chitosan, sodium caseinate and rosemary essential oil

3.1.1. Abstract

To develop an active biopolymer film based on chitosan, sodium caseinate, and rosemary essential oil (REO), the objective of this work was to investigate the effect of REO on the structure and physical properties of biopolymer films. Fourier transform infrared spectroscopy was conducted for fingerprinting to identify the REO addition. The thermal behaviour of each film was investigated using a thermogravimetric analysis. Furthermore, the optical properties, surface hydrophobicity, solubility, mechanical properties, and water vapour permeability were investigated. The results showed that the presence of REO changes the structural organisation of the blended sodium caseinate and chitosan film, thereby inducing an increase in the surface hydrophilicity of the film. However, the solubility and water vapour permeability of the film were not affected by the structural modification induced by REO. Moreover, a mild effect of the REO on the mechanical properties of the sodium caseinate/chitosan film was observed. A thermal analysis confirmed that the incorporation of REO did not influence the degradation temperature of the films. Overall this studying has demonstrated that caseinate/chitosan film enriched in REO, related to his thermal and chemico physical properties can be applied for food packaging application.

Keywords: Chitosan, Sodium caseinate, blend film, Rosemary essential oil, mechanical properties; thermal properties; water vapour permeability

3.1.2. Introduction

Biopolymers, coatings, and natural additives are currently considered sustainable alternatives for food packaging applications as they enhance resource efficiency and reduce negative environmental effects associated with packaging wastes after their useful life (Mohamed, El-Sakhawy, & El-Sakhawy, 2020). Biopolymers, such as proteins and polysaccharides, have been extensively studied as potential edible films or coatings for food application (Calva-Estrada, Jiménez-Fernández, & Lugo-Cervantes, 2019; Elsabee & Abdou, 2013; Giancone et al., 2011; Volpe, Cavella, Masi, & Torrieri, 2017; Yousuf, Qadri, & Srivastava, 2018).

Aiming to prolong shelf life, maintain food quality, and reduce the use of synthetic food additives, many studies have evaluated the incorporation of natural substances, such as essential oils, in biopolymer films or coatings in order to develop active films (Perdones, Chiralt, & Vargas, 2016; Ghadermazi, Keramat, & Goli, 2017). Essential oils are mainly composed of terpenes, terpenoids, and aromatic and aliphatic constituents, all of which are characterised by a low molecular weight (Burt, 2004). These phenolic compounds are part of the secondary metabolites of plants and contribute to their protection against ultraviolet (UV) radiation (antioxidant activity) and against pathogens, parasites, and predators (antimicrobial activities). The antioxidant activity of phenols occurs because of their capacity to release hydrogen molecules captured by free radicals, which are responsible for spoiling the quality of food products (Balasundram, Sundram, & Samman, 2006).

When used as active compounds in a film, it is also important to evaluate their effects on the physical and structural properties of the resulting film (Sánchez-González et al., 2010; Torrieri, Cavella, & Masi, 2015). In particular, essential oil incorporation can affect the structure of the polymer matrix, leading to physical changes depending on the specific polymer-oil components interactions. Moreover, the oil composition and specific interactions with the polymer network determine its effectiveness as an active ingredient (Sánchez-González, Vargas, González-Martínez, Chiralt, & Cháfer, 2011). Rosemary essential oil (REO) has been successfully used as an active food packaging compound to increase the antioxidant and antimicrobial properties of gelatin (Yeddes et al., 2019), gelatin-chitosan-pectin (Yeddes et al., 2020) chitosan-montmorillonite bionanocomposite (Pires, de Souza, & Fernando, 2018), and chitosan based biofilms (Abdollahi, Rezaei, & Farzi, 2012). The influence of essential oils on the physical properties of the film has been studied for corn and wheat starch films (Song, Zuo, & Chen, 2018a), chicken meat protein-based films (Sarıcaoglu & Turhan, 2020), and sodium caseinate films (Sani, Marand, Alizadeh, Amiri, & Asdagh, 2020; Alizadeh-Sani, Moghaddas Kia, Ghasempour, & Ehsani, 2020). On the one hand, it has been reported that essential oils can have a detrimental effect on film barrier properties owing to discontinuities in the structure of the film associated with the formation of lipid droplets embedded in the continuum polymer network (Bonilla, Atarés, Vargas, & Chiralt, 2012; Torrieri, Cavella, & Masi, 2015; Dashipour et al., 2015). On the other hand, no effect on the water barrier properties has been reported as a function of the film composition or its structure (Bahram et al., 2014; Chen & Liu, 2016; Hashemi, Mousavi Khaneghah, Ghaderi Ghahfarrokhi, & Eş, 2017; Nisar et al., 2018; Perone, Torrieri, Cavella, & Masi, 2014; Rocca-Smith et al., 2016). In a previous study, a chitosan-and caseinate-based blend film was optimised to improve the mechanical and water vapour barrier properties of chitosan films (Volpe et al., 2017). On this basis, we decided to develop an active film based on a chitosan and caseinate blend by incorporating REO. Therefore, in this study, the effect of oil on the structure and functional properties of the film was studied using a chitosan and caseinate film as a control to investigate the effect of the oil and biopolymer interaction on the matrix structure.

3.1.3. Materials and methods

Materials

Medium molecular weight chitosan (deacetylation degree 75% – 85%), sodium caseinate salt from bovine milk, glacial acetic acid, REO, Tween 80, sodium hydroxide (1 M), tris hydrochloride buffer, and glycerol were purchased from Sigma Aldrich.

Film-making procedure

Film-forming solutions containing chitosan (CH; 1%), sodium caseinate (SC; 4%), and CH (2%)/SC (4%) in a proportion of 1:1 (weight (w)/volume (v)) were obtained as reported by Volpe et al. (2017). Glycerol (GLY) was added as a plasticizer to all the solutions to achieve a GLY/SC, GLY/CH, and GLY/CH/SC weight ratios of 0.1. A mixture of REO and Tween 80 (4:1) was added to the solutions to obtain a final concentration of 1.5% (v/v). The film-forming solutions were homogenised at 15500 rpm for 4 min at 20°C using a rotor-stator homogeniser ultra-turrax R, T 18 (IKA, Italy), and degassed under vacuum for 15 min to prevent pinhole formation. Films were made by casting, in which 10 mL of each solution was poured into a Petri dish (area = 56.7 cm2) immediately after preparation and left to dry at 40 °C and 50% relative humidity (RH) for 16 h in a circulating air system chamber (MMM Medcenter Einrichtungen GmbH, Germany). Then, the dried films were peeled from the Petri dishes and stored at 50% RH until testing. The composition and solid surface density of the films were calculated as reported by Giancone et al. (2011) and are listed in Table 3.1.

Table 3. 1. Composition, solid surface density, Young's modulus (E), tensile strength (TS), and elongation at break (Ɛ %) of chitosan (CH), sodium caseinate (SC), and blend film (CH/SC) enriched with rosemary essential oil (REO). Letters indicate differences between groups ($p < 0.05$) (each group is symbolised by a different biopolymer matrix, with and without REO).

	Film Forming Composition (% w/v)					Mechanical properties		
Sample code	Chitosan	Sodium caseinate	REO:tTween80	Glycerol	solid surface density ρ (mg cm^{-2})	E (Mpa)	TS (Mpa)	Ɛ %
CH	1	0	0	0.1	1.9	n.a.	n.a.	n.a.
CH/REO	1	0	1.5	0.1	4.64	n.a.	n.a.	n.a.
SC	0	4	0	0.4	7.7	381±90 [a]	6±3 [a]	4±2 [a]
SC/REO	0	4	1.5	0.4	10.4	889±235 [b]	12±3 [a]	3±1 [a]
CH/SC	1	2	0	0.3	5.86	232±43 [a]	10±5 [a]	16±6 [b]
CH/SC/REO	1	2	1.5	0.3	8.33	545±219 [b]	11±6 [a]	14±9 [b]

Film characterization

Film thickness

The film thickness (x) was measured using a micrometer model H062 (Metrocontrol Srl, Italy) with a sensitivity of ± 2 µm. Five replications were conducted for each sample, and five measurements were taken at random positions around the film.

Moisture content and solubility

The moisture content (MC) of the films was evaluated by the gravimetric method, in which the films were dried in an oven at 105 °C for 24 h until they achieved a constant weight. The MC values were determined as the fraction of weight lost during drying as compared to the initial weight, and were reported on a wet basis (ASTM, 1994). The following equation was used for the MC (%) calculation:

$$MC\ (\%) = \frac{Mi - Mf}{Mi} * 100, \quad (1)$$

where Mi and Mf are the initial and final masses of the film, respectively.

The film solubility at different pH values was determined according to the method described by Giancone et al. (2011), as follows:

$$FS\ (\%) = \frac{wi - wf}{wi} \times 100, \quad (2)$$

where “w” is the dry matter, and subscripts “i” and “f” correspond to the initial and final dry matter, respectively.

The determinations of MC and solubility were performed in triplicate.

Surface hydrophobicity

The sessile drop method, which is based on the optical contact angle (°), was used to estimate the surface hydrophobicity of the films. Specifically, a 0.2 µL drop of ultrapure water was deposited on the film surface, and the image of the drop was recorded with a digital microscope M.PG-2 (TQC sheen, China). The optical contact angle was calculated as reported by Williams (2014) using the ImageJ free software. The measurements were performed on the upper side of the film, which was in contact with the air during the drying step.

Optical and color properties

The film opacity was determined according to Park and Zhao, (2004) by measuring the absorbance at 600 nm using a V-550 UV/VIS spectrophotometer (Jasco, Japan). The films were cut into rectangular pieces 3 cm high and 0.4 cm wide and placed directly into the cuvette of the spectrophotometer. The results were reported as absorbance divided by film thickness (mm) based on five replicates. Three film specimens were used for each replicate. The colour of the film was evaluated using a portable colourimeter CR-300 (Konica Minolta, Japan). A CIE-Lab (Commission Internationale de l'Eclairage) colour

scale was used to measure the degree of lightness (L*), redness (+a*) or greenness (-a*), and yellowness (+b*) or blueness (-b*) of the films. The total colour difference (ΔE) was calculated as follows (Rhim, Gennadios, Weller, Cezeirat & Hanna, 1988):

$$\Delta E = \sqrt{[(L^*_{film} - L^*_{standard})^2 + (a^*_{film} - a^*_{standard})^2 + (b^*_{film} - b^*_{standard})^2]} \quad (3)$$

where the standard colour parameters used as background values were L* = 96.94, a* = +0.23, and b* = +1.85. Five replicates were used for each sample.

Fourier Transform Infrared (FTIR)

To elucidate the effect of REO on the structure of the CH, SC, and CH/SC films, FTIR-ATR spectrometry was conducted using a spectrometer Avatar 330 (Thermo Scientific, USA). The FTIR-ATR spectra were recorded from 2000 cm-1 to 650 cm-1 via 32 scans at a resolution of 1 cm-1. The data were analysed using OMNIC software (Thermo Fisher Scientific, USA), and were then normalised to ensure the results were reported as arbitrary units (a. u.).

Thermogravimetric Analyzer (TGA)

The thermal stability of the films was investigated using thermo-gravimetric analysis (TGA) with a thermogravimetric analyser (TGA 7/DZ, Perkin Elmer, Japan). CH powder (CH-P), SC powder (SC-P), GLY, and REO were also characterized using a TGA. To investigate the effect of REO, the initial (Ti) final degradation temperatures (Tf), and the maximum decomposition temperature (DTGmax) were determined from the derivative curves (DTG). The final residual weight after 500 °C (Ress500°C) was evaluated from the TGA curves as the percentage of weight loss. The samples were packed in an aluminium pan inside the thermogravimetric balance and heated from room temperature to 500 °C at a rate of 10 °C min-1 under a dry nitrogen atmosphere (20 mL min-1) in order to avoid thermo-oxidative reactions. All analyses were performed in triplicate.

Mechanical properties

The tensile properties of each film were measured according to the standard method ASTM D882-00 (ASTM, 2002) using a universal testing machine (Instron 5900R-4467; Instron, USA). The load cell was 1 kN, and the speed of the mobile crosshead was set to 50 mm min-1. Then, the stress-strain curves were recorded, of which the tensile strength (TS, MPa), elongation at break (ε %), and elastic modulus (E, MPa) were derived according to ASTM (2002). Ten replicates were performed.

Water vapor permeability

The water vapour permeability (WVP) of the films was evaluated using a gravimetric test according to ASTM E 96 (1993) using a payne permeability cup (Carlo Erba, Italy).

Briefly, 9.89 cm2 of the film surface was exposed to vapour transmission by being placed on the top of the permeability cup containing silica gel. All the cups were placed into a desiccator at an aw of 0.85 and stored in a thermostatic incubator KBF240 (Binder, Italy) at 20 °C. The cups were weighed at scheduled times, and the water vapour transmission rate (WVTR) through the film was estimated according to the linear portion of the diagram obtained by plotting the weight increment of the cup as a function of time.
A steady state was assumed to be reached once the regression analysis (R2) based on the last four data points resulted in an R2 ≥ 0.998. The WVP was calculated as:

$$WVP = \frac{WVTR\ x\ L}{\Delta p} \quad (4)$$

where WVTR is the water vapour transmission rate (g m -2 s-1), L is the average film thickness (m), and Δp is the partial water vapour pressure difference between the two sides of the film (Pa). The results were reported as the average of three replicates for each sample.

Data analysis

Results are reported as mean ± standard deviation. Anova analysis has been used to evaluate the effect of film composition on the film matrix. Duncan’s test was carried out to find the source of the significant differences within the samples examined. Significant differences were defined at $p < 0.05$. All statistical analyses were performed using the SPSS software (SPSS Inc. 17.0, Chicago, 2002).

3.1.4. Results and discussions

Thickness, moisture content, solubility and contact angle

The thickness, MC, solubility, and contact angle results of the tested films are listed in Table 3.2. The film thickness changed from 27 ± 9 µm for the CH-based film to 76 ± 12 µm for the CH/SC film enriched with REO. This is consistent with previous studies, which have shown that REO affects the thickness of the film ($p < 0.05$) (Giancone et al., 2011; Nogueira, Fakhouri, & Velasco, 2019; Ortega, Giannuzzi, Arce, & García, 2017). Further, as the interaction of CH and SC into a blend film formed a more complex network (entanglements) than a single polymer, as reported by Volpe et al. (2017), it can be assumed that REO in the blend film interacts with both polymers, thereby increasing the volume of the film.

The addition of REO only influenced the MC of the CH/SC blend film, which increased from 12.1% ± 0.3% to 17% ± 2%. Hence, REO mainly interacts with SC, leaving free bonding sites of CH available to link with water molecules, causing additional structural swelling. Similar behaviour was observed for CH-gelatin blend films containing thyme, pink clove, citronella, nutmeg, or cinnamon essential oils (Haghighi et al., 2019). However, the addition of essential oils to biopolymers does not always result in an improvement in the water-holding capacity of the resulting film. For example, Song, Zuo, and Chen (2018b) reported that the addition of lemon essential oil to wheat-corn starch films reduced the MC.

To test the behaviour of the film in contact with a variety of foodstuffs, ranging from fruit and vegetables to dairy and meat products, the solubility of the film was tested under different pH conditions, as summarized in Table 3.2. Overall, the film composition affected the film solubility, but the presence of REO only had a significant effect on the solubility of the SC film at a pH of 8, at which it increased from 33% ± 3% to 50% ± 9% (Table 2). For all other samples, no REO effect was observed on film solubility, revealing average values of approximately 30% at a pH of 4, an average of 31% for the blend and SC films, and 16% for the CH film at pH values of 6 and 8. Note that the CH and SC films showed higher solubilities at pH values of 4 and 8, respectively. Meanwhile, the combination of SC and CH reduced the solubility of the film at both acidic and basic pH values, allowing the film to be in contact with different food matrices. Similar results were reported by Wu et al. (2014), where gelatin /chitosan film enriched in oregano essential oil shows not effect on the solubility.

The optical contact angle values ranged from 75° to 12° (Table 3.2). The ANOVA showed that the film composition and REO had a significant effect on the contact angle. Specifically, the blend film had the highest contact angle, confirming the less hydrophilic nature of the film owing to the interaction between CS and CH. The presence of REO caused an increase in the surface hydrophilicity of the blend film. This result was also confirmed for the CH and SC films. These can be justified by the discontinuities of the REO droplets, which give rise to a more open film structure (Atarés & Chiralt, 2016; Benavides, Villalobos-Carvajal, & Reyes, 2012).

Table 3. 2. Thickness (x), moisture content (MC), solubility and contact angle of CH, SC and CH/SC films incorporated with REO.

Code Sample	x (µm)	MC%	solubility % pH 4	solubility % pH 6	solubility % pH 8	Contact angle °
CH	27±9 [a]	23±2 [b]	30±8 [a]	18±2 [a]	19±5 [a]	32.7 ± 0.3 [a]
CH/REO	27±11 [a]	21±5 [c]	33±7 [a]	13±4 [a]	16±2 [a]	15 ± 4 [a*]
SC	54±7 [b]	11±1 [a]	31±4 [a]	30±6 [b]	33±3 [b]	25 ± 5 [a]
SC/REO	59±19 [b]	11±1 [a]	25±6 [a]	38±5 [b]	50±9 [b*]	12 ± 2 [a*]
CH/SC	61±13 [c]	12.1±0.3 [a]	27±10 [a]	26±1 [b]	22±2 [a]	75 ± 10 [b]
CH/SC/REO	76±12 [c*]	17±2 [b*]	25±3 [a]	31±12 [b]	20±6 [a]	12 ± 1 [a*]

The lowercase letters indicate differences among samples (CH, SCH, SC/CH) with or without oil ($p<0.05$); * indicates the effect of oil for each sample (CH or SC or CH/SC) ($p<0.05$)

Optical and color properties of the film

Opacity is a fundamental parameter for a packaging film as it influences the appearance of the products. Moreover, light can affect the food quality also since it acts on the rate of oxidation of lipids. The presence of REO increased the opacity of CH/SC film. This result is observed also in control film based on CH and SC ($p<0,05$) (table 3.3). Norajit, Kim, & Ryu, 2010 report the same effect when ginseng extract was added to alginate film. As the film transparancy depends on their internal structure (Sánchez-González, González-Martínez, Chiralt, & Cháfer, 2010), the increased opacity of the film in presence of REO may depends by the interaction of REO with the used polymers (Aguirre *et al.*, 2018) . The colorimetric parameters of films are reported in table 3.3. ANOVA showed that film composition, has a significant effect on color parameters ($p<0,05$). However REO has a significant effect only on color parameters of CH/SC films ($p<0.05$) causing the increase of the values of L* and b* and in turn of ΔE. A similar behavior were observed by Ekthamasut & Akesowan, 2001 when corn oil and sunflower seed oil were added to edible Konjac films.

Table 3.3. Opacity and colorimetric parameters (lightness (L*), redness (+a*) or greenness (-a*), yellowness (+b*) or blueness (-b*), and total colour difference (ΔE)), of chitosan (CH), sodium caseinate (SC), and blend film (CH/SC) films incorporated with rosemary essential oil (REO).

Code Sample	Opacity (Abs/mm)	L	a*	b*	△E
CH	3.84±0.02 [a]	92.24±2 [a]	-0.2±0.9 [b]	2±1 [a]	4±1 [a]
CH/REO	4.3±0.3 [a*]	92.26±2 [a]	-0.2±0.2 [b]	3±2 [a]	4±1 [a]
SC	3.6±0.9 [b]	90.1±0.4 [a]	-1.0±0.1 [a]	5.3±0.8 [b]	6.1±0.6 [b]
SC/REO	7±1 [b]	92.9±0.5 [ab*]	-1.5±0.8 [a]	8.5±0.8 [b*]	6.8±0.9 [b*]
CH/SC	3.1±0.7 [a]	90.4±0.5 [a]	-0.7±0.6 [a]	4.9±0.9 [b]	5.7±0.9 [b]
CH/SC/REO	4.8±0.3 [a*]	93.3±0.5 [b*]	-1.0±0.8 [a]	10±1 [c*]	7.8±0.9 [c*]

Lowercase letters indicate differences between groups ($p<0.05$) (each group is symbolized by different biopolymer matrix, with and without oil); * indicates the effect of oil within the same film ($p<0.05$)

ATR -FTIR

The FTIR-ATR spectra of the CH and CH/REO, SC and SC/REO, CH/SC and CH/SC/REO films are shown in Fig. 3.1. For a better understanding, pure REO was also analysed, as shown in Fig. 3.1. In the CH spectra, the amide I and amide II bands were at 1640 cm-1 and 1560 cm-1, respectively (Kam, Khor, & Lim, 1999). With regard to SC, the absorption peaks in the area between 1631 cm-1 and 1516 – 1532 cm-1 were attributed to amide I and amide II, respectively. For the CH/SC film, the absorption peaks dominated by the amide I band of CH at 1640 cm-1 and 1560 cm-1 decreased to 1635 cm-1 and 1538 cm-1, confirming that there were electrostatic interactions between the amino groups of CH and the negatively charged carboxyl groups of the CS in the complex films (Volpe et al., 2017; Khwaldia, Basta, Aloui, & El-Saied, 2014). Regarding REO, an evident peak at 1744 cm-1 was related to a keto group of camphor, and two peaks at 1214 cm-1 and 984 cm-1 were related to the epoxy ring of 1.8-cineole, as confirmed by Stramarkou, Oikonomopoulou, Missirli, Thanassoulia, and Krokida (2020).

Only one new peak at 1744 cm-1 was identified in the SC/REO film (Fig. 3.1). This peak was not clear in the CH/REO and CH/SC/REO films because of the noise in the wavelength regions. For the CH/SC film, the addition of REO led to a reduction in the peak intensity, suggesting a reduction in the hydrophilic character of the films. This result is in agreement with the findings of Pereda, Aranguren, and Marcovichs (2010).

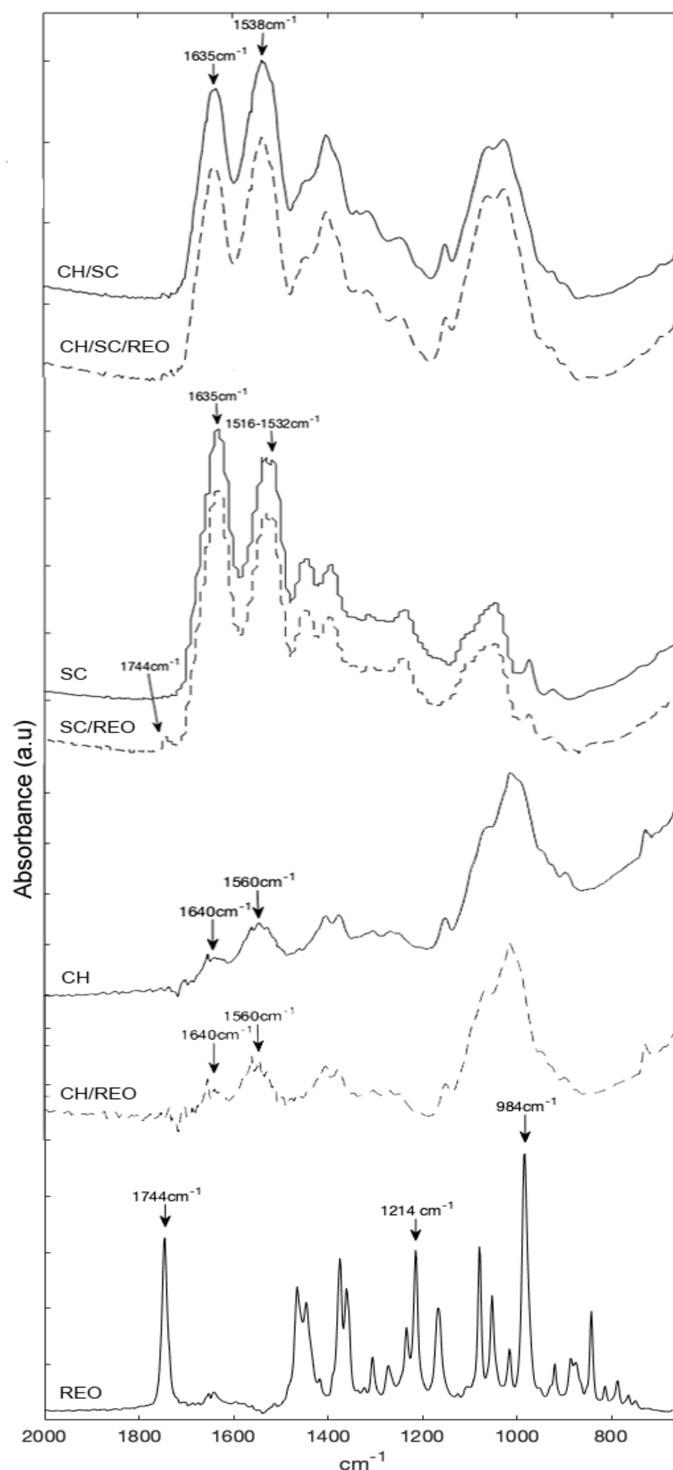
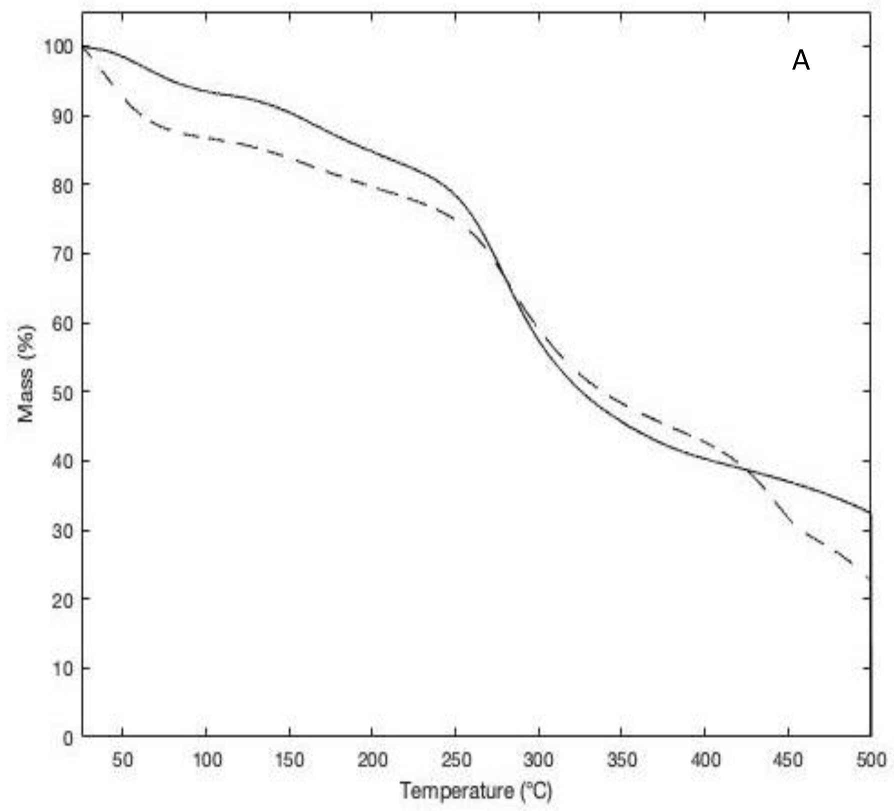


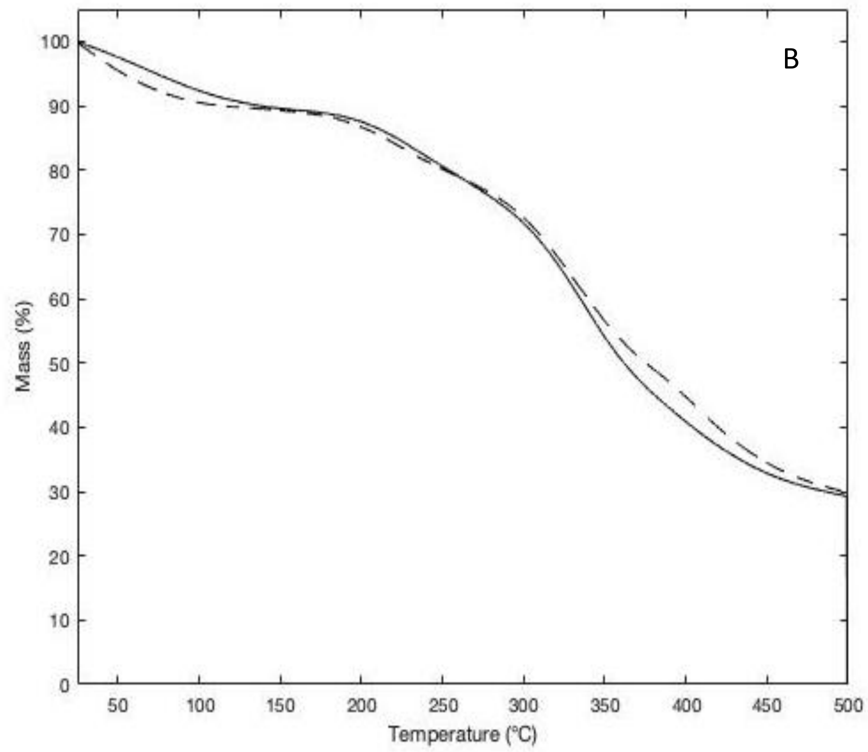
Figure 3.1. Fourier transform infrared spectroscopy – attenuated total reflectance (FTIR-ATR) spectra of (A) chitosan (CH) and CH/rosemary essential oil (REO); (B) sodium caseinate (SC) and SC/REO; (C) CH/SC and CH/SC/REO films; and (D) REO. All films were equilibrated at 50% relative humidity (RH) and 25 °C. Control film and REO are represented with a straight line and films enriched in REO have a dotted line.

Thermal stability

TGA analysis

The TGA analysis allowed us to gain information on the effect of rosemary essential oil addition on the thermal stability of films. Results are shown in table 3.4. Generally, the addiction of REO did not affect the thermal properties of the film. For a better understanding of this behavior, each film component was separately analyzed. Into details, REO shown a low thermal stability compared to the biopolymers and the plasticizer (SC-P, CH-P, and GLY). In fact within the temperature range from 32 ± 3.17°C to 148±1.6 °C it completely volatilizes, so the final residual weight was 0%. SC-P and CH-P exhibited a similar behavior with a DTG_{max} of about 300°C. However the thermal degradation of the SC-P started at lower temperatures and ended at higher temperatures, its residual weight was higher than that of the CH-P. Glycerol also had a single transition, between 100 and 304°C and with a peak at 258°C. The glycerol, at the concentration used for the preparation of the film, does not affect the thermal stability of the films. This behavior was in agreement with (Pereda, Aranguren, Marcovich, & Plata, 2007) which have proven that the thermal properties of sodium caseinate/chitosan film enriched in glycerol up to 28% were not affected by the presence of glycerol. Observing the TGA curves of the film samples (fig.3.2A, 3.2B, 3.2C) it can be assumed that the individual components had no interaction among each other. In fact, the DTG curves (not shown) were characterized by a maximum peak accompanied by shoulders or relative peaks, due to the simultaneous degradation of CH, SC, CH/SC, resulting in overlapping of their individual peaks. This allowed to confirm that REO incorporation into CH, SC and CH/SC films at the explored concentration have not an influence on the T_i and T_f of degradation of the active films. Similar results are reported by (Dong, Xu, Ahmed, Li, & Lin, 2018; Shen & Kamdem, 2015) where essential oil up to 30 % did not affect the thermal stability of the active films. Moreover, for all the films, a residual weight loss approximately of 30 % was observed after 500°C. The results indicated that all the samples were thermally stable in the range 120°C-150°C and thus suitable for industrial applications in this temperature range.


A
Mass (%)
Temperature (°C)


B
Mass (%)
Temperature (°C)

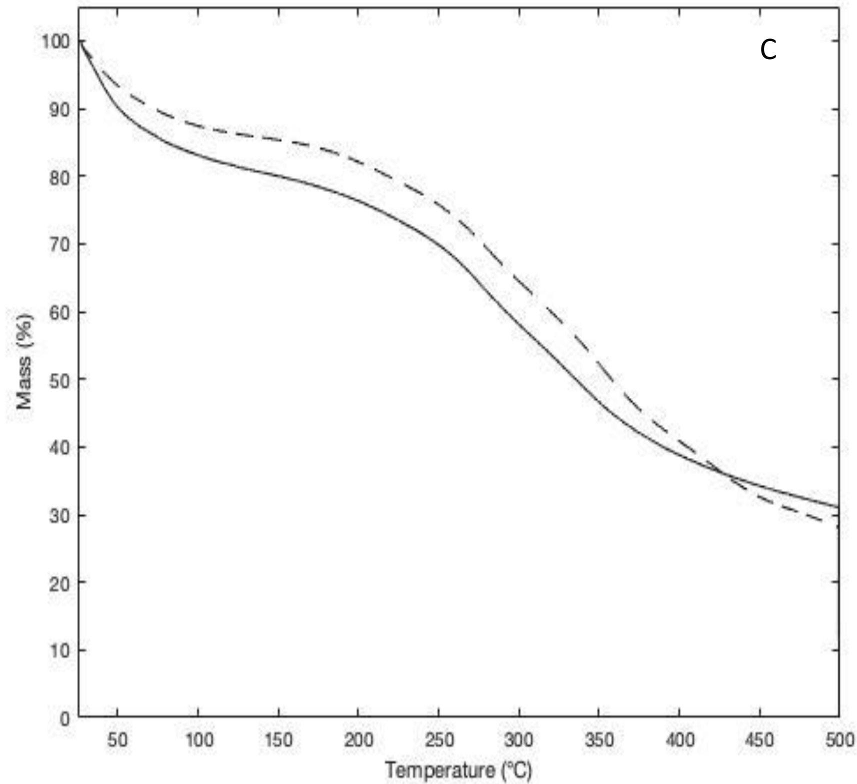


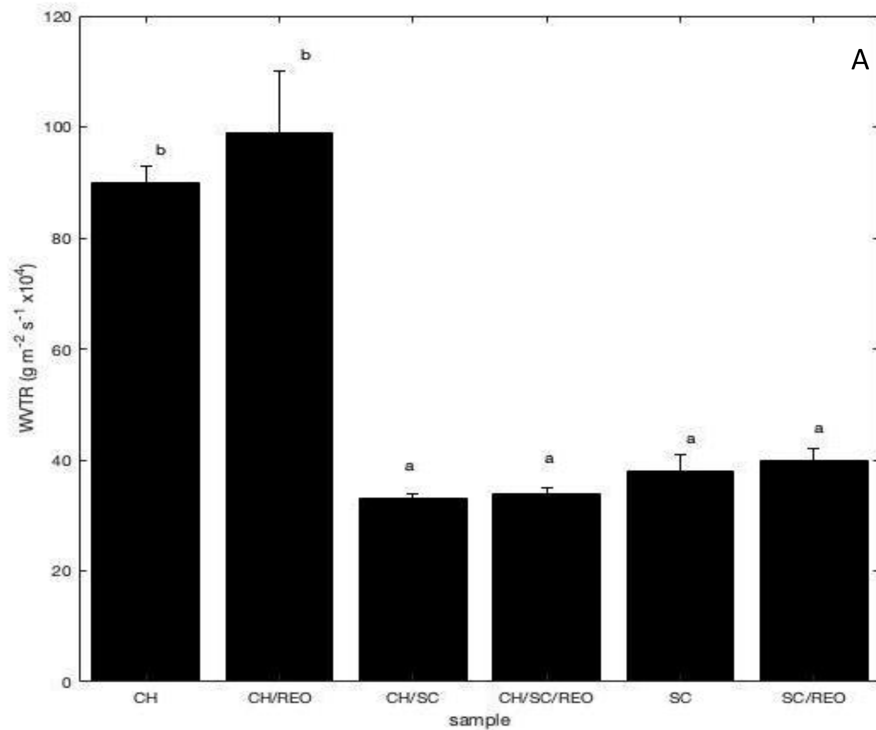
Figure 3.1. TGA (A) curves of CH and CH/REO; (B) SC and SC/REO; (C) CH/SC and CH/SC/REO films obtained at a heating rate of 10 °C/min. Control Films are represented with a straight line and films enriched in REO with a dot line.

Table 3.4. Initial and final decomposition temperature (T_i, T_f), maximum decomposition temperature (DTG_{max}) and percentage of residue at 500°C ($Res_{500°C}$) of films incorporated with REO and their components glycerol (GLY), REO oil (REO) and as chitosan and sodium caseinate powder (SC-P; CH-P).

Code Sample	T_i (°C)	DTG_{max}(°C)	T_f (°C)	Res $_{500°C}$(%)
CH-P	185±10	296±1	408±13	20±8
SC-P	165±5	300±2	475±5	35±1
REO	32±3	93±6	148±2	0±1
GLY	100±1	258±1	304±5	0±0
CH	119±15	282±25	426±16	32±1
CH/REO	121±20	307±35	490±14	23±1
SC	151±1	340±2	494±6	29±0
SC/REO	136±1	329±4	499±1	30±1
CH/SC	149±7	274±0	472±10	33±2
CH/SC/REO	141±2	277±1	477±5	28±1

Water vapor permeability

Barrier to water vapor transmission is one of the most important properties of film for application in food packaging. The WVP and WVTR of the films under investigation are reported in Figures 3.3 A, B. ANOVA showed that the presence of REO does not affect in a significant manner the WVTR and WVP ($p>0.05$) of the films. However, the water vapor barrier properties depend on the nature of biopolymers. In fact, CH showed a higher WVP value of 14.9±0.4 ($g\ m^{-1}\ s^{-1}\ Pa^{-1}\ x10^{-11}$) if compared to the other analyzed films whose WVP is equal to 8.6±0.3 and 8.6±0.1 ($g\ m^{-1}\ s^{-1}\ Pa^{-1}\ x10^{-11}$) respectively for SC and CH/SC films (fig.3.3 A). Concerning the WVTR, to CH/SC film competes the lowest value, equal to 33±1 ($g\ m^{-2}\ s^{-1}\ x10^{-4}$), whereas CH films had the highest value of 99±11 ($g\ m^{-2}\ s^{-1}\ x10^{-4}$) (fig.3.3 B). The inclusion of the REO into the films did not improve the barrier properties of the films. This can be justified considering that, beside the hydrophobic nature of the REO, its presence results in a more open structure of the film (Perone *et al*., 2014; Torrieri *et al*., 2015). Thus, the increment of film hydrophobicity in presence of REO was balanced by the less tied structure which forms in presence of the oil. Similar results were reported by (Ojagh, Rezaei, Razavi, & Hosseini, 2010). As suggested by Atares *et al*. (2016), one cannot assume that WVP of edible film is reduced simply by adding a hydrophobic component in the formulation, since the impact of the lipid addition on the microstructure of the emulsified film is a determining factor in water barrier performance. In turn, the microstructure of the emulsified film is affected by other factors, such as the physical state of the essential oil and its distribution into the polymer matrix. In a previous study we showed that REO droplets has a monomodal particle size distribution in CH solution with a particle diameter between 0,13um to 0,6 um. Moreover, the oil droplets were uniformly distributed along the film thickness, instead their sizes were bigger in the film forming solution. Thus, the film processing condition is of paramount importance since it can affect the microstructure of the emulsified film, as a consequence of possible droplet coalescence during the casting operation.



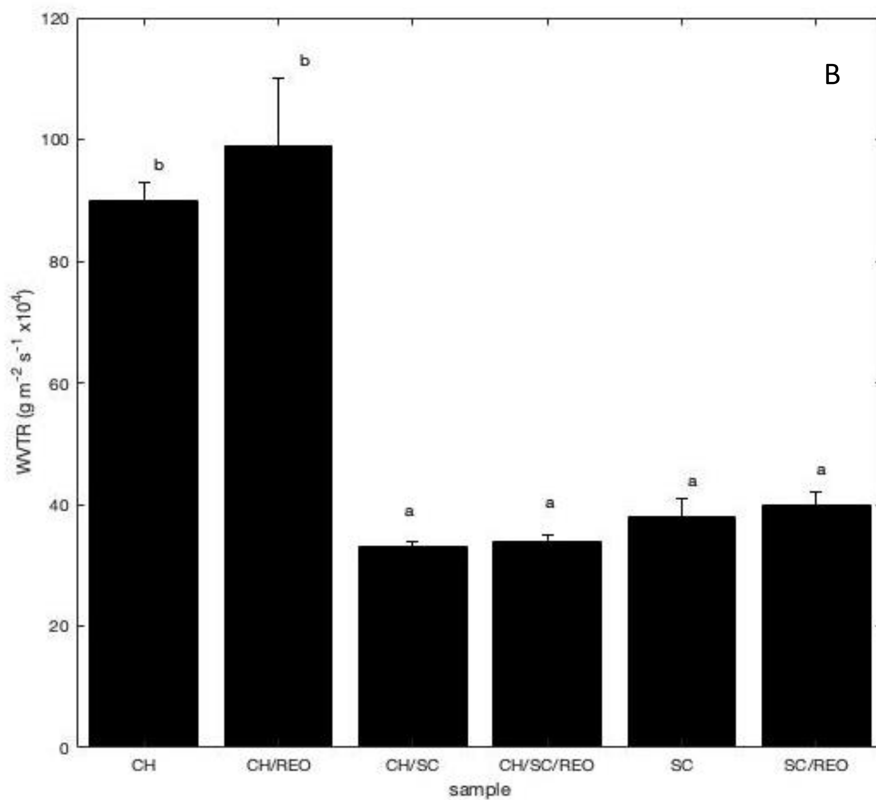


Figure 3.2. Barrier properties: (A) Water vapour permeability (WVP) and (B) water vapour transmission rate (WVTR) of chitosan (CH), sodium caseinate (SC), and blend film (CH/SC) films enriched with rosemary essential oil (REO). Letters indicate differences between groups ($p < 0.05$) (each group is symbolised by a different biopolymer matrix, with and without REO).

Mechanical properties

Elastic modulus (E), tensile strength (TS) and elongation at break (Ɛ %) of films are reported in Figures 3.1. Chitosan films were fragile, and due to their small thickness, it was not possible to submit them to uniaxial elongation test as they cracked while clamping before testing. Therefore, tensile properties of this film were not taken into account in the discussion. REO has a significant effect on the elastic modulus of the CH/SC/REO films leading to an increase in E. Same results for SC/REO film. Instead, REO addition does not affect TS and Ɛ %. These results are in good agreement with (Fabra, Talens, & Chiralt, 2010), which find that the addiction of oleic acid into sodium caseinate films has a mild effect on their mechanical properties. Blend film showed an higher Ɛ % values compared to SC film (fig. 3.1), confirming that the interaction between protein and polysaccharide results into a more flexible structure than film obtained with only protein or polysaccharide (Volpe *et al*., 2017; Pereda *et al*., 2008).

3.1.5. Conclusions

REO addition to CH/SC film modify their structure and this causes some changes of the film properties of interest for industrial applications. REO addition in active film preparation give rise to films having higher thickness with respect to control film. This behavior can be related to the interaction between REO and sodium caseinate which leaves free bonding sites of chitosan available for linking water molecules resulting in a more swelled structure. As a consequence, CH/SC film obtained in presence of REO have higher moisture content, solubility, and surface hydrophilicity if compared to CH/SC film. However, the different structure of the active film did not impact the water barrier properties of the film, which are very similar to that of films prepared without the addition of the essential oil and assumed an average value of the WVP of 9 g m^{-1} s^{-1} Pa^{-1} $x10^{11}$. Thermal properties were also not affected by the presence of REO. Instead, the presence of REO affected the opacity of the film and the elastic modulus that increased in presence of REO. In conclusion, active film based on CH/SC and REO exhibits engineering properties suitable for potential application for food preservation as coating using in contact with food. However, further studies must be conducted to elucidate the effective antioxidant capacity of the films when used in direct contact with a food matrix.

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3.2. Effect of chitosan-sodium caseinate film enriched in rosemary essential oil on the oxidation kinetics of fresh chicken breast hamburger

3.2.1 Abstract

The objective of this work was to evaluate the effect of an antioxidant biopolymer film based on chitosan (CH), sodium caseinate (SC) and rosemary essential oil (REO) on the quality of chicken breast hamburger during the shelf life. Antioxidant activity of CH, SC and CH/SC/REO film were evaluated using DPPH and ABTS radicals and IC50 was determined. Then, films showing best results were used for the shelf-life study. Films were put in contact with chicken breast hamburger before packaging and samples were stored for 27 days at 4°C. During storage, metmyoglobin, malondialdehyde, color and pH were investigated to evaluate how active film can affect the quality index of chicken breast meat. The active film CH/SC/REO show the lowest IC50 of 9.87 mg/g dry film and thus it was chosen as active film for the shelf-life study. CH/SC/REO significantly affect the color parameters a* value. The addition of CH/SC/REO film on chicken breast hamburger affect positively the MDA during the storage. The active film showed a similar protection as the commercial one.

Keywords: active film; antioxidant; biopolymer, meat oxidation, shelf-life, poultry hamburger.

3.2.2. Introduction

Deterioration of packaged food product are complex and unique, depending on intrinsic factor of the food product (e.g., pH, water activity, nutrient content, redox potential, antimicrobial compounds, respiration rate, biological structure, type and load of microorganism) and extrinsic environmental factors (e.g., storage temperature, relative humidity, headspace gas composition) (Quevedo *et al.*, 2013). Poultry makes a substantial contribution to food security and nutrition, providing energy, protein, and essential micro-nutrients to humans. The global poultry sector is expected to continue to grow as demand for meat and eggs is driven by growing populations, rising incomes and urbanisation (Carvalho, Shimokomaki, & Este, 2017; Magdelaine, Spiess, & Valceschini, 2008). For poultry meat, the oxidation of muscle components and discoloration are the most critical attributes used by the consumers to select or rejects a food product affecting the market

value (Mancini & Hunt, 2005; Fletcher, 2019). The high unsaturation degree of the muscle lipids, recognized poultry meat as highly sensitive food product to oxidative reactions (Est, 2015; Min *et al.*, 2008). During processing, storage, and culinary preparation of poultry products, constituents such as lipids, proteins, heme pigments are oxidized causing modifications in their sensory properties and nutritional value. To prolong the shelf life of fresh food products, like chicken breast hamburger, active packaging systems are considered an effective way to reduce alteration process without altering the freshness nature of the product. An active packaging is defined as a system in which the food, the package and the environment positively interact to maintain the safety and quality of the products and to prolong its shelf life (Suppakul, Miltz, Sonneveld, & Bigger, 2003; López-Rubio *et al.*, 2004; Wyrwa & Barska, 2017). According to Regulation (EC) No. 450/2009 (European Commission, 2009), active materials and articles are those intended to extend the shelf life or to maintain or improve the condition of packaged food. They are designed to deliberately incorporate components that would release or absorb substances into or from the packaged food or the environment surrounding the food. For active packaging designed to release active compound, more attention is focused on the use of natural antioxidants, such as polyphenols, tocopherol, plant extracts and essential oils (EO), due to consumer concerns about their health and the possible migration of toxic substances into food (Sánchez-González, Vargas, González-Martínez, Chiralt, & Cháfer, 2011; Ju *et al.*, 2019). In this context, EO are interesting for their potential use as natural preservatives (Atarés & Chiralt, 2016). In a previous study it was showed that chitosan-based film in presence of REO exhibits engineering properties suitable for potential application for food preservation as coating using in contact with food. The REO did not affected the thermal and water vapor barrier propriety of the film, slightly changing the opacity and the mechanical properties. Thus, the objective of this work was to evaluate the antioxidant capacity of the chitosan-based antioxidant film previously developed. There are many methods to measure the efficacy of antioxidant packaging materials (Shahidi & Zhong, 2015). Generally in vitro antioxidant tests using free radical traps are relatively straightforward to perform (Alam, Bristi, & Rafiquzzaman, 2013). Due to the complexity of the oxidation process in food system, it is also important to evaluate their performance when in contact with a food matrix (Shahidi & Zhong, 2015). In case of meat samples, it is common to study the myoglobin oxidation by monitoring the colour of the meat (Robertson, 2009) and lipid oxidation process by evaluating changes in primary or

secondary products (formation of carbonyls, aldehydes, volatiles, malondialdehyde) (Domínguez *et al*., 2019).

Thus, the aim of this work was to study the antioxidant capacity of the chitosan-based film enriched with rosemary essential oil by in vitro test (DPPH and ABTS test) and when in contact with real food, a chicken breast hamburger, by monitoring the quality indices affected by oxidation reaction during storage at 4 °C.

3.2.3. Materials and methods

Materials

Medium molecular weight Chitosan (deacetylation degree 75-85%), sodium caseinate salt from bovine milk, pure acetic acid, rosemary essential oil (REO), tween 80 were purchased from Sigma-Aldrich (Milan, Italy). 2-thiobarbituric acid (CAS 504-17-6), trichloroacetic acid (≥99.0%, CAS 76-03-9), disodium hydrogen phosphate dehydrate (98%, CAS 13472-35-0), sodium dihydrogen phosphate monohydrate (99%, CAS 10049-21-5), and n-Hexane (GLS ≥96%, CAS 110-54-3) were acquired from Carlo Erba reagents s.r.l. (Milan, Italy). High density polyethylene (HDPE) commercial film used as control and to pack the samples.

Film-Making Procedure

Film based on chitosan (CH), sodium caseinate (SC) and blend of chitosan and sodium caseinate (CH/SC) added with 1,5 % (w/v) of REO were prepared as described in chapter 3.1. Table 3.1 show the film samples composition.

Table 3.1. Film sample composition

Film Composition (%w/v)				
Sample code	CH	SC	REO:Tween80	Gly
CH	1	-	-	0.1
CH/REO	1	-	1.5	0.1
SC	-	2	-	0.2
SC/REO	-	2	1.5	0.2
CH/SC	1	2	-	0.3
CH/SC/REO*	1	2	1.5	0.3
HDPE*	-	-	-	-

Abbreviation: CH = chitosan film; CH/REO = chitosan film with 1,5% of REO; SC= sodium caseinate film; SC/REO=sodium caseinate with 1.5% of REO; CH/SC =chitosan/sodium caseinate film; CH/SC/REO= chitosan/sodium caseinate film with 1.5% of REO; Gly= glycerol; REO:Tween 80= rosemary essential oil and emulsifying ; HDPE =high density polyethylene film. * = film used for shelf-life study.

Antioxidant capacity

ABTS-TEAC method

The test is based on UV-VIS spectrophotometric measurement at 734 nm of the absorbance of a solution containing the ABTS+ radical, generated through an oxidation reaction with the oxidising agent potassium persulphate ($K_2S_2O_8$). The addition of the antioxidant to the reaction environment eliminates the radical by the transfer of a hydrogen atom, resulting in a decrease in the initial absorbance value. The extraction of the antioxidants from the film sample was obtained by ethanol (96% (v/v)). 0.2g of film was cut into fragments, and put in contact with 6 g of solvent in a falcon. The falcons, suitably covered with alluminium foil, were placed in the ultrasonic bath (Digital ultrasonic bath mod. DU-45, Carpi, Italy) for 60 min at power 4 to allow effective extraction. A solution of 7.4 mM ABTS- + (5 mL) mixed with 140 mM $K_2S_2O_8$ (88 µL) was prepared, stabilised for 12 h at 4°C and then mixed with ethanol (1:88, v/v). Subsequently, 100 µL of supernatant obtained from the above extraction was added to 1 ml of diluted ABTS - +, incubated in the dark for 10 min and the absorbance was spectrophotometricaly (V-550 Jasco Inc., Tokyo, Japan) recorded at 734nm. For all sample types, the antioxidant activity was evaluated at different extraction times, 1, 24, 48, 72, and 96 h. The results were expressed as mg MTE/g sample using a calibration curve (range of 2 uM TE to 20 uM TE) of trolox, and as % inhibition.

DPPH method

The DPPH method was used to determine the free radical scavenging capacity of the film forming solution (FFS) and casting film based SC and CH/SC enriched with REO.

For the bioactive coating, the methods of Hromiš *et al.* (2014) with a little modification were used. Briefly, 2.4 mL of 30 ppm ethanolic solution of DPPH were mixed with 100 mg of bioactive FFS. Samples were stored for 2.5h and 4 h, 24 h and 48 h at room temperature in dark condition. Then, the samples were centrifugated for 5 min at 10,000 rpm and filtered by using a 20 µm Whatman filter. For all samples, the absorbance was measured at 517 nm using a UV–Vis spectrophotometer (V-550 Jasco Inc., Tokyo, Japan) and pure ethanol as reference. The results were expressed as percentage of DPPH free radical activity.

For the active film, 0.1g of each film samples were placed in flaks containing 2.4 mL of 30 ppm ethanolic solution of DPPH. The mixture was vortexed and incubated in the dark at ambient temperature for 1 h. The UV absorbance was read at 517 nm using a UV–Vis spectrophotometer (V-550 Jasco Inc., Tokyo, Japan) and pure ethanol as reference of the samples. The IC50 value was calculated as the concentration of sample required to scavenge 50% of DPPH free radicals. To estimate the IC50, 5 different dilution in ethanol

were prepared. The results were expressed as inhibition concentration (IC_{50}) mg g^{-1} of dry film (df).

Shelf-life test

Preparation of chicken breast hamburger

In figures 3.1 the preparation of chicken breast hamburger with active film are showed. Briefly, samples of minced chicken meat were purchased from a local supermarket (Portici, Italy). Minced chicken meat (30 g) was put inside of petri dishes (diameter of 8.5 cm). High density polyethylene film (HPDE, commercial film) and CH/SC/REO casting film were placed on the surface of the meat samples. The sample were packed into a Polylactic acid (PLA) bag in presence of oxygen. Sample without film (reported as no film) were packaged in the same condition. Change of the color, pH, metmyoglobin content, and grade of lipid oxidation were analyzed after 4, 7, 11,14, 21, and 27 days of storage at +4°C as described in the following sections.

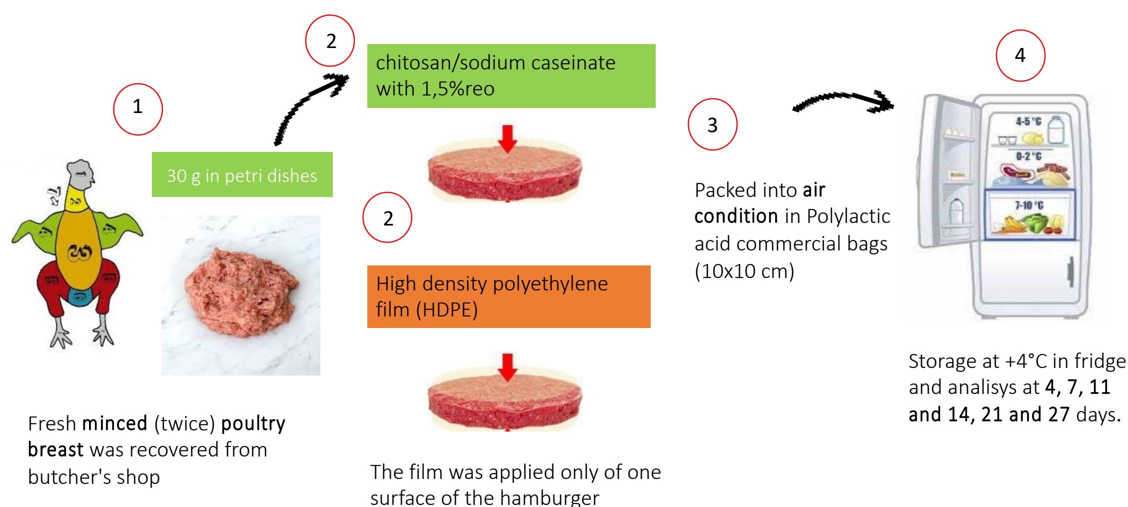

Figure 3.1. Schematic representation of chicken meat preparation using active CH/SC/REO film.

Color and pH evaluation

The color of the meat samples was measured using a colorimeter (Minolta Chroma Meter, CR 300,Osaka, Japan). Color analysis was performed after removing the film from the meat surface and after 15 min of storage at room temperature. Each sample was measured five times. Color was reported as hunter parameters L* (from 0 = black to 100 = white), a* (−a* = greenness to + a* = redness), and b* (−b* = blueness to + b* = yellowness). Values of a* have been normalized respect its initial amount (a_0).

The pH of meat was measured using a pH-meter (FE20/FG2 Mettler Toledo, Schwerzenbach, Switzerland). The electrode of the instrument was applied to the surface of the samples. Each sample was measured five times.

Metmyoglobin evaluation

Metmyoglobin (Metmio) content was determined using the method reported by (Borzi, Torrieri, Wrona, & Nerín, 2019). Briefly, 5 g of meat was added with 25 mL of 40mM iced cold phosphate buffer at pH 6.8. All the samples were kept in a fridge at 4 °C for 60 min. Then, samples were centrifugated at 10.000 xg for 5 minutes and the supernatant was filtered using a paper filter. The absorbance (Abs) of the supernatant was determined using UV–VIS spectrophotometer (UV-550 Jasco, Japan). The percentage of Metmio was calculated according to (Borzi *et al*., 2019).

$$\% \text{ Metmyoglobin} = [-2.51\left(\frac{A572}{A525}\right) + 0.777\left(\frac{A565}{A525}\right) + 0.8\left(\frac{A545}{A525}\right) + 1.098]x100 \qquad (1)$$

Where A_{525}, A_{545}, A_{565}, A_{572} were absorbances for different wavelengths. Values of Metmyoglobin have been normalized respect its initial amount ($Metmio_0$).

Thiobarbituric acid reactive substances (TBARS)

Malondialdehyde is an organic compound which is highly reactive and can be used as a marker of lipid oxidation. To measure the concentration of Malondialdehyde (MDA) present in each of the packed chicken samples, a TBARS assay was carried out according to (Maraschiello, Sa, & Garcı, 1999) with slightly modifications. Briefly,1g of chicken meat was mixed into 10 ml ultrapure water, 5ml of hexane and homogenate (IKA-Labortechnik, Staufen, Germany) at 13500 rpm for 10 min. 3.5 mL of trichloro acetic acid (25 %) was added and stirring at 4 °C for 15 min. Supernatant was obtained by centrifugation at 4000 rpm for 15 min at 4 °C. The supernatant (3.5 mL) was transferred to a test tube, and 1.5 mL of 0.6% aqueous TBA was added. The screw-capped test tube was incubated for 30 min in a water bath at 70 °C. The tubes were cooled, and the TBARS were recorded at 532 nm, against a blank consisting of 10 mL of ultrapure H_2O, 5mL of hexane, 3.5 mL aqueous TCA (25 %), and 1.5 mL TBA (0.6%).

Calibration curves were prepared from MDA standard in the rage of 0.027ug/ml to 1.036ug/ml. The TBARS values were expressed as micrograms of malonaldehyde per kilogram of meat.

Data analysis

Three replications were performed for each test and data was presented as mean values with standard deviation. The mean values were calculated using the results of the treatment replicates and the technical replicates. Anova analysis has been carried out to evaluate the effect of CH/SC/REO film on the quality index of hamburger. Duncan's test was carried out to find the source of the significant differences within the samples examined. Significant differences were defined at $p < 0.05$. All statistical analyses were performed using the SPSS software (SPSS Inc. 17.0, Chicago, 2002).

3.2.4. Results and discussions

Antioxidant activity of film forming solution (FFS) and casting film using DPPH and Abts radicals

Figure 3.1 shows the results of theDPPH test, with the % of radical inhibition on the y-axis and the time on the x-axis, i.e. the hours of contact between the sample and the 30ppm DPPH solution that favours the effective extraction of antioxidants. The percentage of inhibition increases with increasing contact time for all the samples studied. After 2.5 h of incubation in the dark with the 30 ppm DPPH solution, the SC sample shows lower values of % inhibition, whereas higher values are assumed by the sodium caseinate (SC) solution with REO. After 48 h, the FFS showing the lowest % inhibition values is that of sodium caseinate /rosemary essential oil (SC/REO), with a % inhibition value of 34.31±1.52 %. Maximum values of the % inhibition after 48 h of contact were observed for the (CH/SC/REO) sample, reaching values of 90.40±1.03 %. From the results obtained, it can be concluded that the SC/CH/ REO blend solution has a higher inhibition capacity of the DPPH radical than the other types analysed, only after 48 h of contact in the dark with the radical solution.

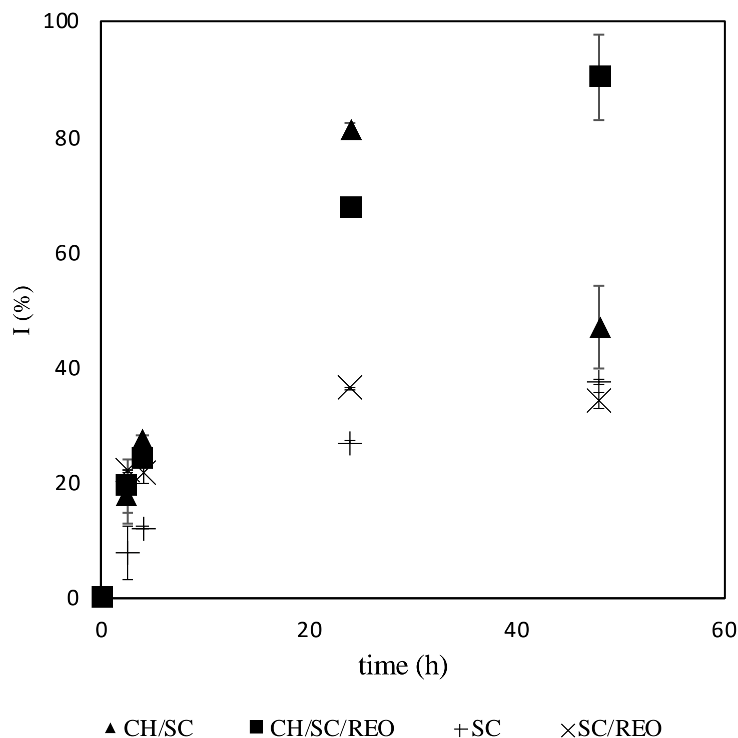

Figure3.2. Evolution of antioxidant activity (I%) of active FFS using DPPH radical

The same data are reported in table 3.2 with the results of Duncan test. Inhibition capacity of the DPPH radical increased significantly (p≤0.05) during the time of analysis with the exception of the CH/SC samples for which there is a reduction after 48h of contact with the radical solution. In particular, after 2.5 hours of contact with the 30 ppm DPPH solution, there is no significant difference (p≥0.05) between between SC/REO and CH/SC. On the other hand, for the same time, there are significant differences (p≤0.05) between CH/SC/REO, SC/REO and SC, samples. After 4 and 24 hours, statistically significant differences (p≤0.05) exist between all samples tested sauf SC/REO and CH/SC samples. After 48 hours the highest value are showed for CH/SC/REO samples with an I% of 90 ±1. CH/SC/REO showed to be the most efficcient samples over the time.

Table 3.2. Antioxidant activity of FFS over the time investigated by DPPH radical.

sample	I (%) DPPH			
	2.5h	4h	24h	48h
SC	7.7±4.6a	12.1±0.3a	26.9±0.1a	37.6±0.5a
SC/REO	22.1±0.1c	21.8±1.92b	36.3±0.4b	34.3a±1.5a
CH/SC	17.7±4.6b	24.4±2.4b	67.7±2.3c	46.9a±7.3a
CH/SC/REO	19.6±0.4c	27.4±0.7c	81.3±1.d	90±1b

Abbreviation: SC= sodium caseinate film; SC/REO = sodium caseinate film enriched with 1.5% of rosemary essential oil; CH/SC= chitosan/ sodium caseinate film; CH/SC/REO= chitosan/sodium caseinate film enriched with 1.5% of rosemary essential oil. Values are given as mean ± standard deviation. Different letters in the same column indicate a significant difference ($p \leq 0.05$) using a Duncan's multiple comparisons test.

The DPPH assay was used to evaluate the antioxidant activity of the films under examination, which were made using the casting technique. The results are shown in Figure 3.3, where the y-axis shows the % of radical inhibition and the x-axis the time, i.e. hours of contact between the sample and the 30ppm DPPH solution that favours the effective extraction of the antioxidants. SC films exhibit low scavenging activity against the DPPH radical at each extraction time. After 2.5h of contact with the 30ppm DPPH solution, the SC sample shows lower values of % inhibition, whereas higher values are assumed by the oil-enriched CH/SC blend. The maximum value that the % inhibition reaches is shown after 24h of contact in the dark for the SC/REO sample, assuming values of 83.86%. Subsequently, after 48h of contact, the % inhibition reaches an equilibrium for the SC/REO samples. On the contrary, for CH/SC/REO and CH/SC samples, a reduction occurs. From the graph, it can also be seen that the SC film after 48h defines an increase in % inhibition. The results show that the antioxidant activity of the cast films increases during the analysis time, reaching a maximum after 24 h, with values between 6% (for SC films) and 80% (for CH/SC, CH/SC/REO and SC/REO films).

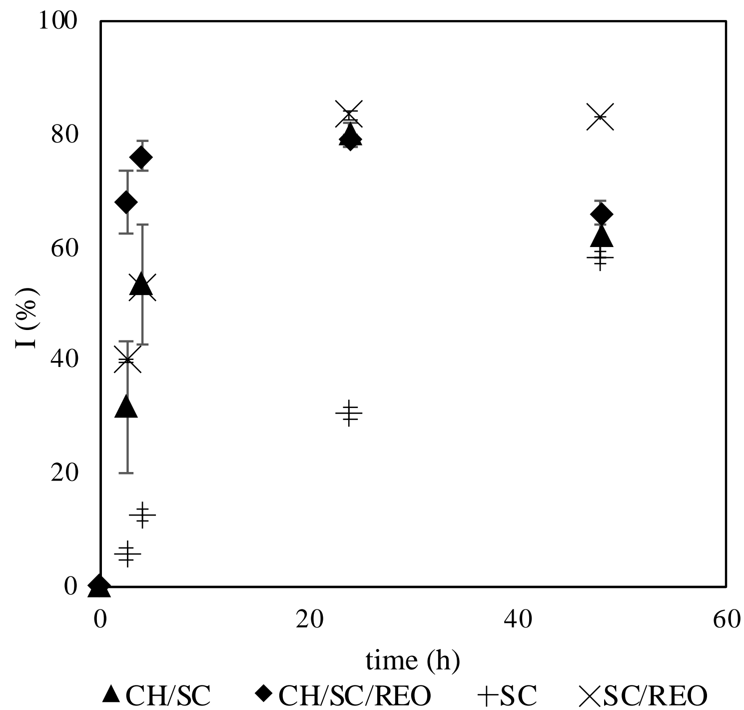

Figure 3.3. Evolution of antioxidant activity (I%) of active casting film using DPPH radical

The same data are reported in table 3.3 with the results of Duncun test. After 2.5 and 4 hours of contact with the DPPH solution, there were no statistically significant differences ($p \geq 0.05$) between the SC/REO and CH/SC samples. In contrast, there are significant differences ($p \leq 0.05$) between SC/REO, CH/SC, SC and CH/SC/REO samples. After 24 hours, significant differences ($p \leq 0.05$) are evident among between samples of SC and CH/SC/REO, CH/SC, SC/REO; conversely, there are no statistically significant differences ($p \geq 0.05$) between samples of CH/SC/REO and CH/SC, SC/REO. After 48 hours, significant differences ($p \leq 0.05$) are evident between samples of SC and CH/SC/REO, SC/REO, on the contrary, for the same time, there are no significant differences ($p \geq 0.05$). Between samples of CH/SC/REO and CH/SC.
From the results obtained, it can be concluded that the inhibition activity of the DPPH radical of SC/REO and CH/SC/REO films are higher than the other films analysed.
Generraly, comparing the results obtained from the film-forming solution with those of the active films made by casting, it can be seen that the dry film have higher antioxidant activities than the FFS exept of SC samples.

Table 3. 3. Antioxidant activity of casting film based over the time investigated by DPPH radical.

sample	I (%) DPPH			
	2.5h	4h	24h	48h
SC	5.7±1.8a	12.8±0.02a	30.5±2.9a	58.2±0.8a
SC/REO	40.0±0.3b	52.8±0.2b	83.6±0.8c	83.2±0.2c
CH/SC	31.8±11.7b	53.6±10.4b	80.3±2.1c	62.2±4.1b
CH/SC/REO	68.1±5.3c	76.3±2.5c	79.2±1.1c	66.1±2.2b

Abbreviation: SC= sodium caseinate film; SC/REO = sodium caseinate film enriched with 1.5% of rosemary essential oil; CH/SC= chitosan/ sodium caseinate film; CH/SC/REO= chitosan/sodium caseinate film enriched with 1.5% of rosemary essential oil. Values are given as mean ± standard deviation. Different letters in the same column indicate a significant difference ($p \leq 0.05$) using a Duncan's multiple comparisons test.

The film matrices were subjected to the ABTS assay in order to evaluate their antioxidant activity in idrophylic matrix. Figure 3.4 shows the results of the ABTS radical scavenging activity of the dry films. SC and SC/CH films show low antioxidant activity against the ABTS radical for all extraction times. After 1 hour of contact, the % inhibition for the SC film assumes lower values, on the contrary, higher values are reached for the CH/SC/REO sample and the same result is obtained after 24 and 48 hours of contact with ethanol. The % inhibition reaches maximum values only after 72 h, with values of 42.61% for the oil-enriched CH/SC film. After 96 and 168 h in each sample, the % inhibition is almost constant, thus reaching equilibrium. Higher values are always found for the CH/SC/REO blend. It can be concluded that the addition of rosemary essential oil significantly increased the scavenging activity of the SCand CH/SC composite films.

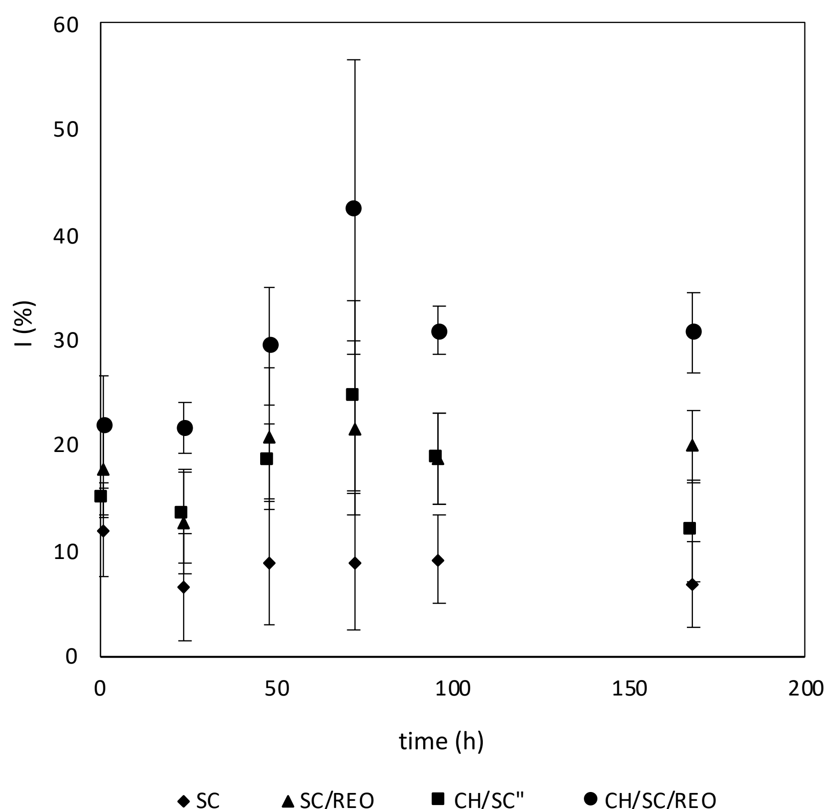

Figure 3.4. Evolution of antioxidant activity (I%) of active casting film using ABTS radical

From the univariate ANOVA statistical analysis we can see that the scavenging activity of the ABTS radical, increases significantly ($p \leq 0.05$) as a function of contact time in each formulation. The results of the test are shown in table 3.4. After 1h of contact, no significant difference ($p \geq 0.05$) is observed between the samples with and without oil, whereas, after 24h, an increase is evident only for the blend. This leads us to deduce that the release kinetics change depending on the formulation. CH/SC film exhibited very similar inhibition of the ABTS radical as SC/REO sample. The loss of the antioxidant effect may be caused by the formation of covalent bonds between the functional groups of the bio-polymer matrix and those present in the rosemary essential oil, which cause the film network to become more compact, leading to a decrease in hydroxyl and amine groups, thereby limiting interactions with the free ABTS radical (Hosseini, Razavi, & Mousavi, 2009). In particular, it can be seen that after 1 hour of contact there are significant differences ($p \leq 0.05$) between the SC and CH/SC/REO samples, on the contrary, there are no significant differences ($p \geq 0.05$) between the CH/SC/REO and SC/REO, CH/SC samples. The same applies between SC/REO and CH/SC, SC samples. After 24 hours there are significant differences ($p \leq 0.05$) between CH/SC/REO and CH/SC, SC/REO, SC samples, in contrast, there are no significant differences ($p \geq 0.05$)

between SC and SC/REO, CH/SC samples. After 48 hours there are significant differences (p≤0.05) between CH/SC/REO and SC samples and also between SC/REO and SC samples, on the contrary, between SC/REO and CH/SC/REO, CH/SC samples there are no significant differences (p≥0.05). The same applies between CH/SC and SC samples. After 72h between CH/SC/REO, and SC samples there are significant differences (p≤0.05), however, between CH/SC and CH/SC/REO, SC samples there are no significant differences (p≥0.05). After 96 hours of contact between samples CH/SC/REO and SC, CH/SC, SC/REO shows significant differences (p≤0.05), on the contrary, between samples CH/SC and SC/REO there are no significant differences (p≥0.05). After 168 hours there are significant differences (p≤0.05) between CH/SC/REO and SC/REO, CH/SC samples, on the contrary, between CH/SC and SC samples there are no significant differences (p≥0.05).

Table 3.4. Antioxidant activity of casting film over the time investigated by Abts radical.

sample	I (%) Abts					
	1h	24h	48h	72h	96h	168h
SC	11.9±4.2a	6.6±5.1a	8.8±5.9a	9.0±6.5a	9.3±4.1a	6.8±4.1a
SC/REO	17.8±4.4ab	12.7±4.9a	20.7±7.6bc	21.7±8.2a	18.9±4.3b	20.0±3.4b
CH/SC	14.9±1.7ab	13.4±4.5a	18.5±3.6ab	24.7±9ab	18.7±4.3b	11.9±4.3a
CH/SC/REO	22.0±4.6b	21.8±2.5b	29.5±5.7c	42.6±14.1b	31.0±2.3c	30.7±3.9c

Abbreviation: SC= sodium caseinate film; SC/REO = sodium caseinate film enriched with 1.5% of rosemary essential oil; CH/SC= chitosan/ sodium caseinate film; CH/SC/REO= chitosan/sodium caseinate film enriched with 1.5% of rosemary essential oil. Values are given as mean ± standard deviation. Different letters in the same column indicate a significant difference (p≤0.05) using a Duncan's multiple comparisons test.

The DPPH assay detects the abilities of the samples to donate hydrogen to the DPPH radical, resulting in bleaching of the DPPH solution. IC50 is reflected in the level of bleaching action and antioxidant activity (Wang *et al.*, 2018). The greater the bleaching action, the higher the antioxidant activity, which is reflected in a lower IC50 (Wang *et al.*, 2018). The antioxidant activity of chitosan (CH), sodium caseinate (SC) and chitosan/sodium caseinate film (CH/SC) with and without rosemary essential oil (REO) are shown in table 3.5. Generally, films enriched in REO showed higher antioxidant activity and respectively lower IC50 value compared to the films without REO. Into details the CH/SC/REO (IC50= 9.87 mg/g df) show a lowest value comparing to the single polymer film. For this reason, a CH/SC/REO film was used for shelf-life study on poultry meat hamburger.

Table 3.5. Antioxidant activity of chitosan, sodium caseinate and blend film enriched in REO.

code sample	IC_{50} (mg/g df)
CH	45.86
CH/REO	40.98
SC	39.55
SC/REO	38.68
CH/SC	18.96
CH/SC/REO	9.87

Abbreviation: CH = chitosan film; CH/REO = chitosan film with 1,5% of REO; SC= sodium caseinate film; SC/REO=sodium caseinate with 1.5% of REO. CH/SC =chitosan/sodium caseinate film; CH/SC/REO= chitosan/sodium caseinate film with 1.5% of REO. df= dry film; IC50= inhibition concentration of 50% of radical.

Meat color and pH determination

Generally, no differences ($p>0.05$) of L* and b* values were observed among samples (table 3.6). Only the parameter a* shown a significantly differences ($p<0.05$) during shelf life. For a better understanding the a* value was normalized, and results are reported in Figures 3.5 Generally, a reduction of a^*/a_0^* for all sample are observed. Meat samples in contact with HDPE film showed a faster decrease of the normalized a* value respect to the samples stores without film or in contact with the active film. In particular, only meat samples stored in contact with the active film showed a slowly variation of color, highlighting a protective effect of the film. Similar results were found by (Al-hijazeen, 2018) in ground chicken meat enriched in oregano essential oil. The reduction of the a* parameter can probably be related to the fact that the longer the meat is stored in the refrigerator, the greater the tendency for oxymyoglobin to oxidize to brownish metmyoglobin (Serrano-león *et al*., 2018). An alternative theory would relate to lipid oxidation, as free radicals can oxidize iron atoms or denature myoglobin molecules, causing meat discoloration (O’Grady, Monahan, & Brunton, 2001).

Table 3.6. Colorimetric indices (L*, a*, b*) of meat samples during storage at 4°C.

time (days)	HDPE	CH/SC/REO	no film
		L	
0	47.02±0.76	50.91±1.70	48.11±1.56
4	48.23±4.23	50.02±1.49	49.86±1.03
7	48.63±4.65	48.36±0.78	51.02±0.76
11	50.38±1.50	49.04±1.28	54.58±0.50
14	50.14±1.00	46.00±2.53	54.88±1.08
21	55.55±2.35	53.90±1.63	62.33±0.66
27	50.44±1.70	52.04±1.93	58.49±0.52
		a*	
0	10.10±0.62	8.00±0.60	7.83±3.79
4	9.79±5.07	6.59±1.05	6.00±1.48
7	6.18±3.86	5.03±0.76	3.67±2.40
11	1.87±0.75	4.46±1.01	2.29±0.63
14	0.97±0.32	6.81±1.31	4.18±3.76
21	1.59±0.62	6.26±1.26	3.15±0.40
27	1.15±0.61	1.48±1.20	1.97±0.60
		b*	
0	10.19±0.67	14.11±1.42	10.90±1.08
4	11.85±3.55	14.27±1.68	14.52±1.73
7	11.94±4.14	12.11±0.94	11.07±1.27
11	13.60±1.12	12.40±1.19	12.54±0.68
14	13.41±1.55	10.43±1.65	15.30±1.41
21	16.32±2.12	14.01±1.15	19.11±0.32
27	15.29±2.29	13.97±1.32	18.38±0.96

Abbrevation: no film= samples without film on the surface of the hamburger; HDPE= high density polyethylene film on the surface of the hamburger; CH/SC/REO= chitosan/sodium caseinate film with 1.5% of REO on the surface of the hamburger. Values are given as mean ± standard deviation.

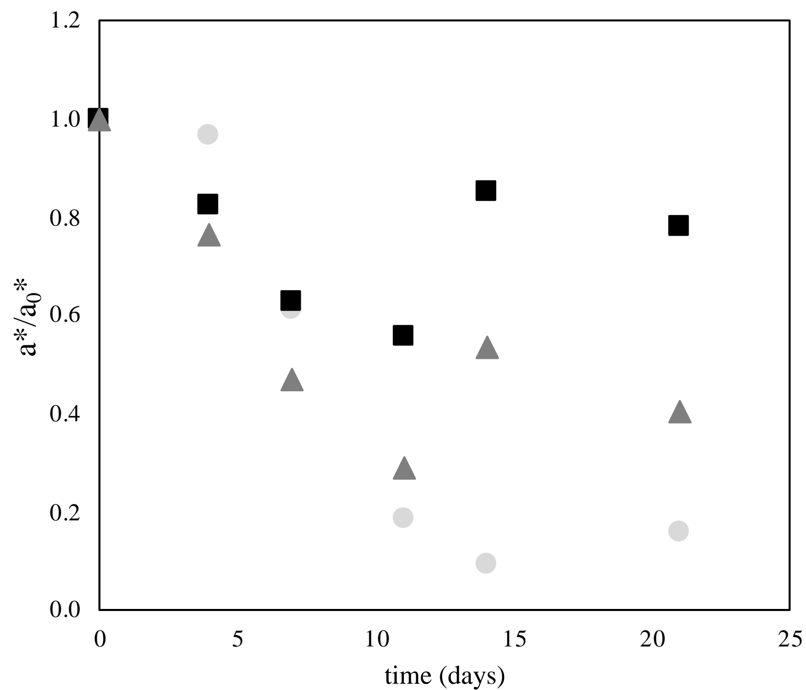

Figure 3.4. Evolution of colorimetric indices a* into fresh breast hamburger stored at 4°C in contact with commercial HDPE (●), CH/SC/REO (■) and no film (▲) over the time. Values expressed as the ratio between colorimetric indices a* at time "t" (a*) and the colorimetric indices a* at time zero (a_o*) of fresh breast hamburger vs storage time.

In Figure 3.5 the pH behavior of the different samples is reported. Generally, CH/SC/REO shows an increasing trend, but with intermediate value between no film and HDPE samples. The average pH of the three sample types (no film, HDPE and CH/SC/REO) was 6.98 ± 0.46. No significant effect is showed for CH/SC/REO samples ($p<0.05$). In general, pH values above 6 correspond to a high water-holding capacity of the meat. However, low pH values (below 5.8-6.0) provide greater microbiological stability and inhibit the growth of spoilage microorganisms (Allen, Fletcher, Northcutt, & Russell, 1998)

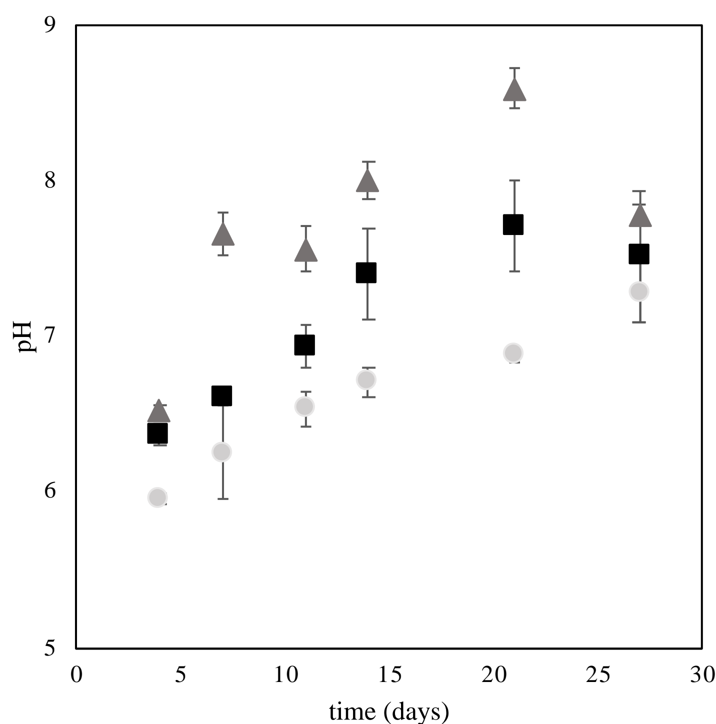

Figure 3.6. pH value of fresh breast hamburger stored at 4°C in contact with commercial HDPE (●), CH/SC/REO (■) and no film (▲) over the time.

Metmyoglobin

The evolution of metmyoglobin (MetMio) during 27 days of storage are shown in Figure 3.7. Generally, metmyoglobin are affected during the storage. Into details, MetMio increased from 34 ± 10 to 40±7% after 4 days, then decrease to 30 ± 13% on day 7 and rise again up to 41 ± 7% at time 21 day for samples with HDPE. MetMio in CH/SC/REO samples, decreased from 39 ±19 % to 29 ±39 % at day 21. The same samples show an increase up to 36 ± 40 % at day 27. Fresh breast hamburger stored with No film samples, showed an increased trend in the value of metmyoglobin from 20 ± 1% to 43± 1 %. Papuc, Goran, Predescu, & Nicorescu, (2017) reported that the susceptibility of myoglobin to auto-oxidation is the main factor in assessing color stability in meat and meat products. In this study, the active film was most effective in controlling myoglobin oxidation, as it contains phenolic compounds capable of reducing lipid radicals. In lipid-containing systems, such as chicken meat, this radical quenching effect prevents subsequent oxidation of myoglobin (Alderton *et al*., 2003). Anova showed that HDPE and CH/SC/REO film are not significant different between each other ($p>0.05$) and significant different with no film samples ($p<0.5$).

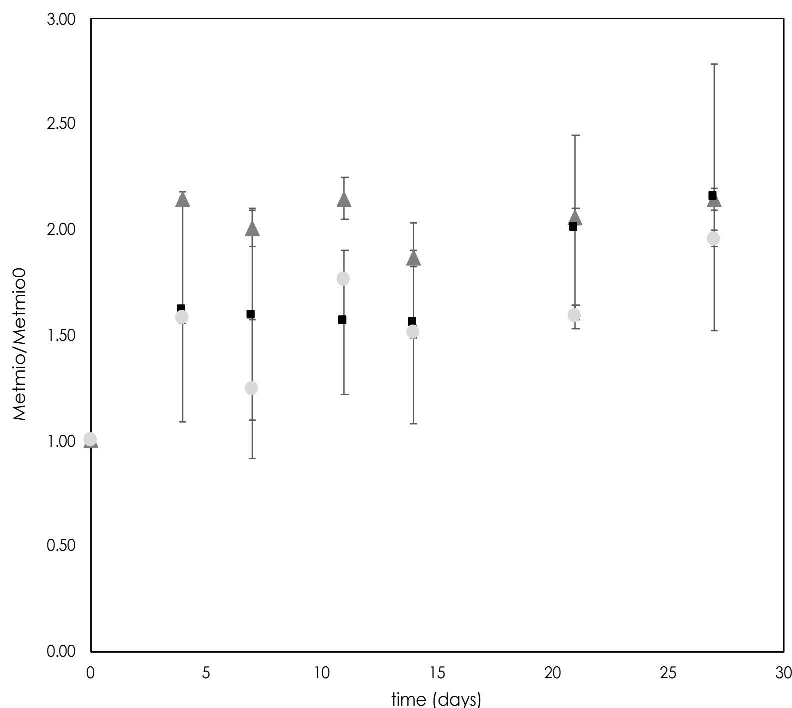

Figure 3.7. Evolution of metmyoglobin into fresh breast hamburger stored at 4°C in contact with commercial HDPE (●), CH/SC/REO (■) and no film (▲) over the time. Values expressed as the ratio between metmioglobin at time "t" (Metmio) and the metmioglobin at time zero ($Metmio_0$) of fresh breast hamburger vs storage time.

Measurement of lipid oxidation

Lipid oxidation of chicken breast meat packed in air and stored at 4°C for 27 days was monitored with the TBARS method and the results are showed in Figure 3.7. Generally, the MDA values for the three different film application varied between from 0.01 to 0.8 mg MDA/kg meat, indicating a very low degree of lipid oxidation. Samples stored up to 11 day with no film assume a significatively increasing ($p<0.05$) of the oxidation trend more than the samples of CH/SC/REO and HDPE (fig. 3.5). Into details the sample with CH/SC/REO film showed a range from 0.0118 ± 0.0001 to 0.0895 ± 0.0111 mg MDA/Kg at time 11 day of storage. This is in according with (Botsoglou, Grigoropoulou, Botsoglou, Govaris, & Papageorgiou, 2003) who reported a threefold reduction in the degree of lipid oxidation (0.6-0.2 MDA/Kg) in turkey meat packaged aerobically, containing 200 mg/kg oregano oil. For the samples stored using HDPE film the values ranging from 0.0315 ± 0.0009 to 0.1870 ± 0.0002 mg MDA/Kg at day 11. The same trend is performed by the samples no film, which showed a higher oxidation from 0.0329 ± 0 to 0.3378 ± 0 compared to the other samples.

The samples with CH/SC/REO film assume an oxidation increase up to 21 day (0.7923 ± 0.0023), and then decrease at day 27 (0.2317 ± 0.0031) (fig.3.8) The same trend is performed by the meat samples with the HDPE film. This results are in agree with (Chouliara, Karatapanis, Savvaidis, & Kontominas, 2007) where observed initial formation of MDA and a reducing during the storage of sea bream filets. According to Sheard *et al*., (2000) the threshold of off-odour perception by consumers corresponds to a TBARS value of 0.5 mg MDA/kg sample for pork patties. Generally, all samples showed a MDA content below 0.5 mg/Kg of MDA up to 11 day of storage and overcome 0.5 mg MDA /Kg of meat after 21 days of storage (fig.3.8). At 21 day of storage, samples stored with HDPE and CH/SC/REO film applied on the surface of hamburger reached a value of 0.6132±0.0024 and 0.7923±0.0023 mg MDA/Kg of meat respectively. CH/SC/REO film reduced at 27 day of storage the value to 0.2317±0.0031 mg MDA/Kg of meat (fig. 3.7).

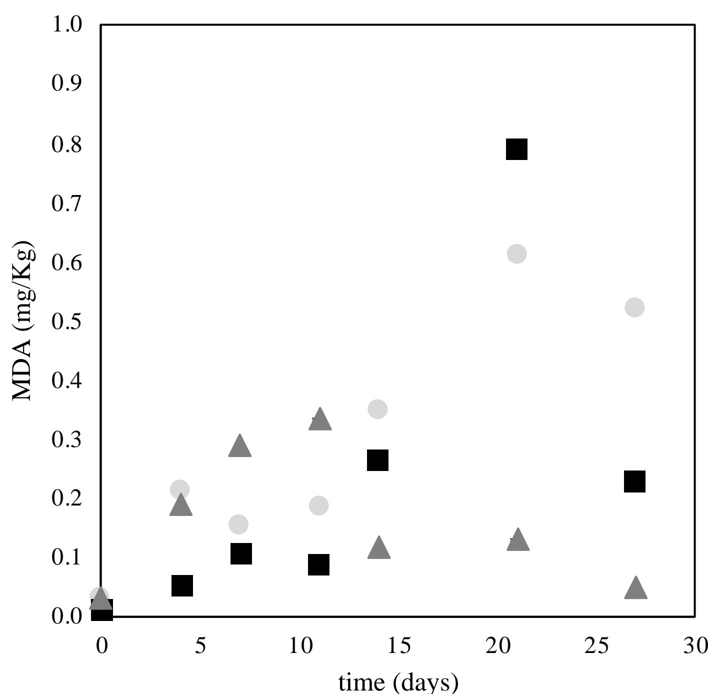

Figure 3.8. Evolution of MDA into fresh breast hamburger stored at 4°C into commercial HDPE (●), CH/SC/REO (■) and no film (▲) from 0 to 27 days of storage and from 0 to 11 days of storage at 4°C.

3.2.5. Conclusions

CH/SC/REO film showed the best antioxidant activity in vitro. The realized active film showed the same protection of the commercial HDPE film. The active blend film on

chicken meat hamburger showed a protective effect on the shelf life up to 11 day of storage at 4°C. In contrast, the same film showed no effect on metmyoglobin, b* and L value. Further microbiological and sensory analysis will be necessary to confirm our results.

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3.3. Selection of polyphenols for food packaging application

3.3.1. Abstract

To develop a future antioxidant packaging, the antioxidant properties of quercitin, cathecol, tannic acid, gallic acid, and sinaptic acid were investigated. For this purpose, the antioxidant properties of different polyphenols were investigated using DPPH and ABTS methods. Results showed that the most effective polyphenol standard was gallic which assumed an IC 50 value of 23.93 ug/mL and 1.74 ug/mL for DPPH and ABTS test respectively.

Keywords: quercitin, cathecol, tannic acid, gallic acid, synaptic acid, active film, antioxidants capacity

3.3.2. Introduction

In recent years, there has been increased interest to design antioxidant food packaging for food preservation. Antioxidants have become an indispensable group of food additives, mainly due to their unique properties of prolonging the shelf-life of food products, without having a negative effect on their sensory or nutritional qualities. Obviously, antioxidants for food use must meet certain requirements: (i) non-toxic; (ii) active at low concentrations; (ii) highly stable and capable of surviving processing steps; (iii) no odor, taste or color ; (iv) easy to incorporate and have good solubility in the product (Shahidi, Janitha, & Wanasundara, 1992). Polyphenols into specific flavonoids and phenolic acids can be identified on the basis of their molecular structure. Flavonoids contain, attached to their 2-phenylchroman ring structure, hydroxyl and/or semiquinone functionalities responsible for a variety of biological activities including anti-inflammatory, antibacterial, immunostimulant, anti-allergic and antioxidant activities. Phenolic acids, on the other hand, can be subdivided into hydroxybenzoic acid derivatives (gallic acid, catechol, vanillic acid) and cinnamic acid derivatives (sinapic acid, caffeic acid) (Cervellati, 2010). To replace synthetic food additives by natural molecules (Shahidi & Ambigaipalan, 2015; Caleja, Barros, Antonio, Oliveira, & Ferreira, 2017), polyphenols,

natural compounds found in plants, are good candidates thanks to their natural antioxydant properties (Sanches-Silva *et al*., 2014). Different authors include antioxidant into a biopolymer film to extend the shelf-life of food (Wang *et al*., 2019; Radi, Firouzi, Akhavan, & Amiri, 2017; Carrizo, Taborda, Nerín, & Bosetti, 2016 u Nisa *et al*., 2015; Licciardello, Wittenauer, Saengerlaub, Reinelt, & Stramm, 2015; Figueroa-Lopez, Vicente, Reis, Torres-Giner, & Lagaron, 2019; Arrieta, García, López, Fiori, & Peponi, 2019; Requena, Vargas, & Chiralt, 2017a). The antioxidant activity can be measured using different analytic method. Generally, the most used one are the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). However, the results of different authors are reported in different way (Muhammad Rehan Khan *et al* 2021, in submission) and difficult to compare if only one test are used. Thus, the objective of this work was to test the antioxidant capacity of several polyphenols by using DPPH and ABTS test to select an active compound to be used in an active film.

3.3.3. Materials and Methods

Materials

Potassium persulphate ($K_2O_8S_2$), DPPH radical (2,2'-Diphenyl-1- picrylhydrazyl), ABTS-+ radical cation (2,2'-azinobis(3-eylbenzothiazoline-6-sulphonic acid), gallic acid, tannic acid, quercitin, catechol and sinaptic acid were purchased from Sigma-Aldrich & Co (Milan, Italy). Absolute ethanol 96% (v/v) purchased from VWR Internationl SrL, (Milan, Italy).

Preparation of polyphenols solution

Single commercial polyphenols were dissolved into ethanol (96%) and opportune concentration were realized for an antioxidant study using DPPH and ABTS radicals. Table 3.1 shows the polyphenol concentration.

Table 3. 1. Polyphenol concentration used into a DPPH and ABTS test.

Samples	DPPH (µg/mL)	ABTS (µg/mL)
gallic acid	40	5
	20	3.75
	13.3	3
sinaptic acid	120	15
	100	5
	80	3
tannic acid	50	5
	20	3.75
	15	3
vanillic acid	5	15
	2.5	5
	1	3.75
catechol	50	50
	30	30
	15	15
quercitin	70	10
	50	7.5
	30	5

Determination of antioxidant capacity

ABTS-TEAC method

The test is based on UV-VIS spectrophotometric measurement at 734 nm of the absorbance of a solution containing the ABTS+ radical, generated through an oxidation reaction with the oxidising agent potassium persulphate ($K_2S_2O_8$). The addition of the antioxidant to the reaction environment eliminates the radical by the transfer of a hydrogen atom, resulting in a decrease in the initial absorbance value. three different diluition of analyzed samples in ehtanol (96%) were prepared (table 3.1). Then, 100 uL of the respective diluition was added to 1 ml of diluted ABTS - + solution, incubated for 10 min into dark condition and spectrophotometricaly (V-550 Jasco Inc., Tokyo, Japan) recorded at 734nm. The results were expressed as mg TE/g sample uisng a calibration curve (range of 2 uM TE to 20 uM TE) of trolox, and as % inhibition.

DPPH method

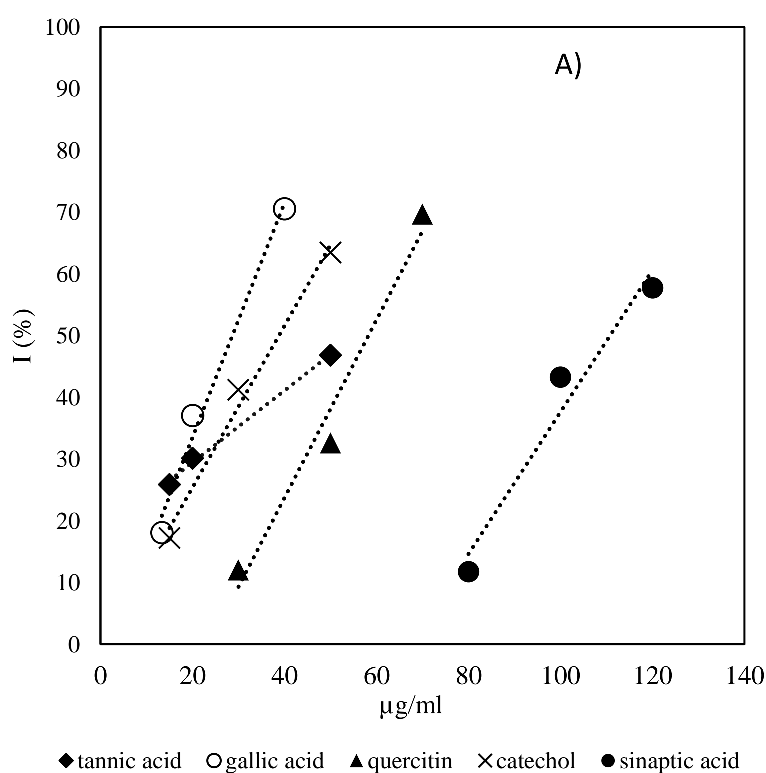
Three different dilutions of analyzed samples in ethanol (96%) were prepared (table 3.1). Then, 2.4 mL of 30 ppm ethanolic solution of DPPH were mixed with 1 mL of sample's dilution. All samples were analyzed after 15 min using a spectrophotometer at 517 nm

against ethanol as reference. The results were expressed as inhibition concentration (IC50) in µg·mL^{-1} (Gülçin, Huyut, Elmastaş, & Aboul-Enein, 2010).

3.3.4. Results and discussion

Antioxidant activity of polyphenol standards by the DPPH and ABTS assay

Figure 3.1A, B, C shows the percentage of inhibition of the DPPH (A, C) and the scavenging capacity against the cationic radical ABTS-+ (B) of the polyphenols investigated as a function of concentration. The inhibition percentage increases with increasing polyphenol concentration, for all investigated substances, from a minimum of approximately 10% inhibition percentage to a maximum of 70% for the DPPH test, and 80% for the ABTS test. Figure 3.2. shows the resuts expressed as uM TE equivalent.



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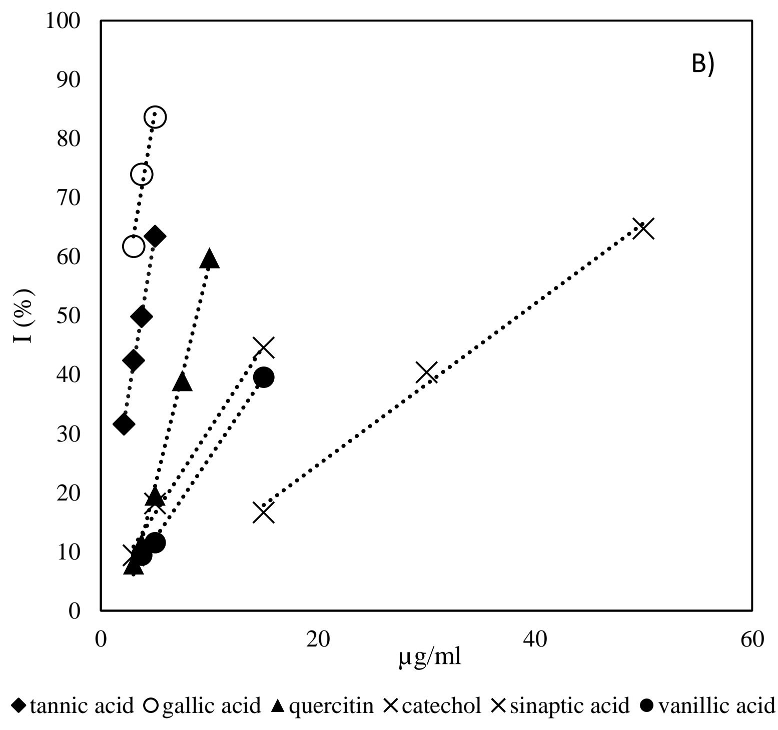


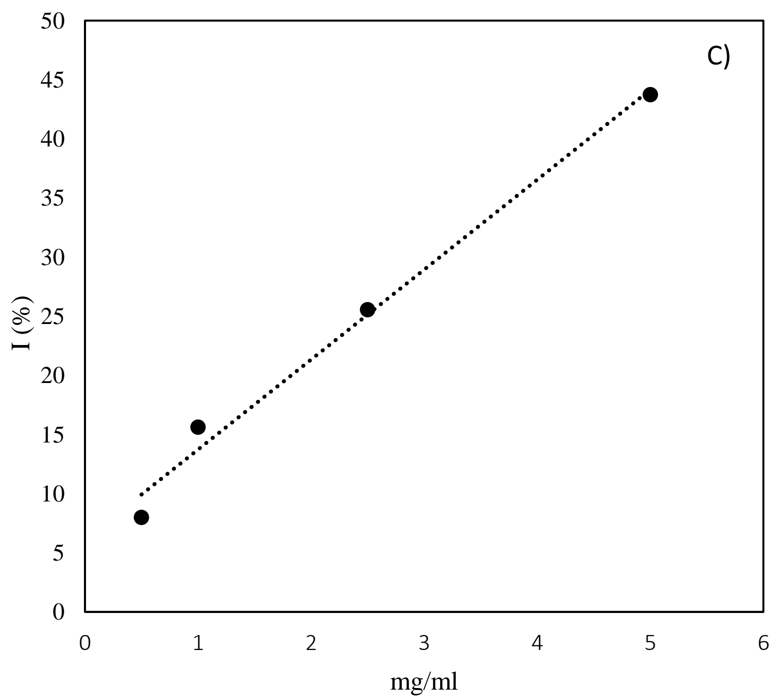
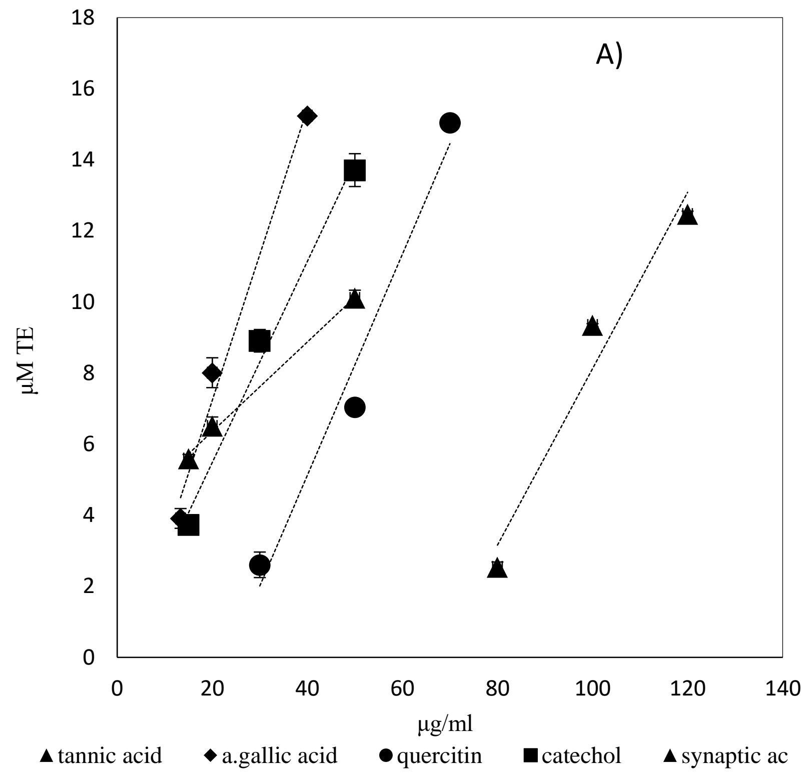
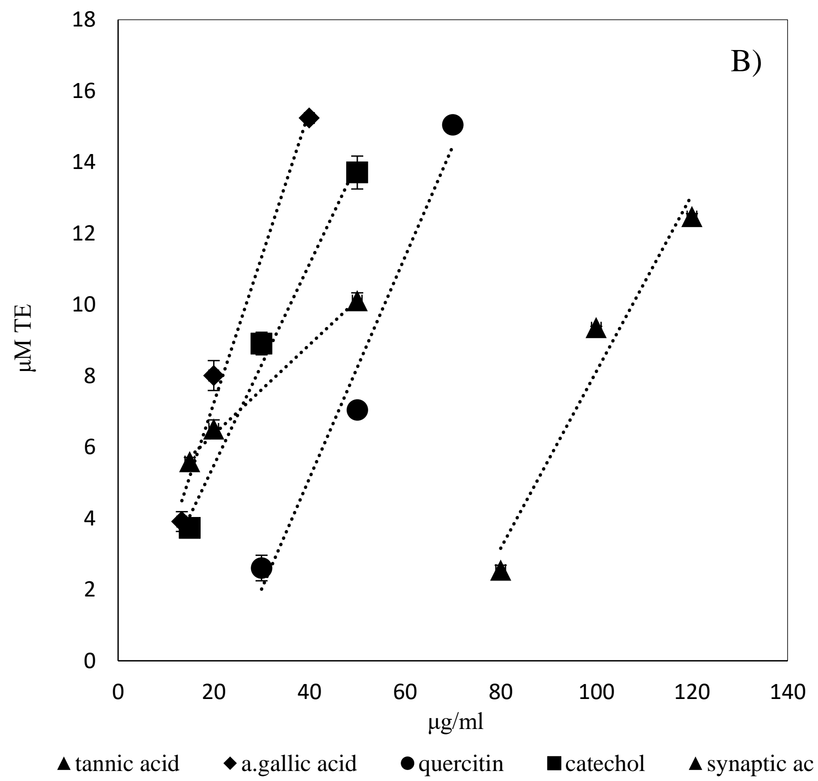


Figure 3.1. Antioxidant activity (I%) of: A) quercitin, catechol, tannic, gallic and synaptic acid using DPPH radical; B) quercitin, catechol, tannic, gallic synaptic acid and vanillic acid using ABTS radical; C) vanillic acid DPPH radical.


A)
µM TE
µg/ml
0
2
4
6
8
10
12
14
16
18
0
20
40
60
80
100
120
140
tannic acid
a.gallic acid
quercitin
catechol
synaptic ac


B)
µM TE
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0
2
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8
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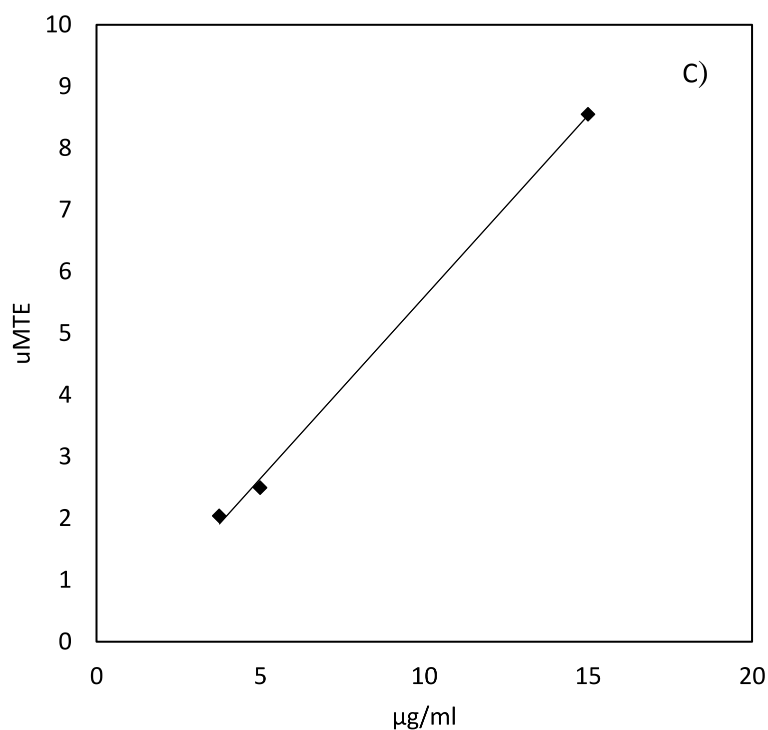

Figure 3.2. Antioxidant activity (μMTE) of: A) quercitin, catechol, tannic, gallic and synaptic acid using DPPH radical; B) quercitin, catechol, tannic, gallic synaptic acid and vanillic acid using ABTS radical; C) vanillic acid DPPH radical.

Table 3.2 shows the average absorbances, expressed in nm, for each concentration of the polyphenol solution examined (μg/ml). The results show that the absorbance values increase significantly ($p \leq 0.05$) as the concentration of the added antioxidant decreases. There are statistically significant differences ($p \leq 0.05$) between all the samples analysed at different concentrations using DPPH and ABTS radicals. The exception is vanillic acid, for which it is noted that there is no significant difference ($p \geq 0.05$) between the concentrations of 5 and 3.75 μg/mL analysed with the ABTS radical. The same applies to the concentrations of 2.5 and 1 μg/mL analysed using the DPPH radical.

Table 3. 2. Absorbance values (abs) of polyphenolic compounds analyzed by the DPPH and ABTS assays

samples	concentration μg/mL	DPPH
		Abs (nm)
gallic acid	40	0.24^{a} ± 0.01
	20	0.53^{b} ± 0.02
	13.3	0.68^{c} ± 0.01
synaptic acid	120	0.34^{a} ± 0.01
	100	0.46^{b} ± 0.01
	80	0.71^{c} ± 0.01
tannic acid	50	0.44^{a} ± 0.01
	20	0.58^{b} ± 0.01
	15	0.62^{c} ± 0.01
vanillic acid	5	0.45^{a} ± 0.02
	2.5	0.59^{b} ± 0.01
	1	0.57^{b} ± 0.01
catechol	50	0.29^{a} ± 0.02
	30	0.47^{b} ± 0.01
	15	0.66^{c} ± 0.01
quercitin	70	0.25^{a} ±0.01
	50	0.56^{b} ± 0.01
	30	0.74^{c} ± 0.01

samples	concentration (μg/mL)	ABTS
		Abs (nm)
gallic acid	5	0.09^{a} ± 0.01
	3.75	0.14^{b} ± 0.01
	3	0.21^{c} ± 0.01
synaptic acid	15	0.32^{a} ± 0.02
	5	0.47^{b} ± 0.02
	3	0.52^{c} ± 0.01
tannic acid	5	0.19^{a} ± 0.01
	3.75	0.26^{b} ± 0.01
	3	0.29^{c} ± 0.01
vanillic acid	15	0.31^{a} ± 0.02
	5	0.46^{b} ± 0.01
	3.75	0.49^{b} ± 0.01
catechol	50	0.29^{a} ± 0.02
	30	0.47^{b} ± 0.01
	15	0.67^{c} ± 0.01
quercitin	10	0.22^{a} ± 0.02
	7.5	0.56^{b} ± 0.01
	5	0.74^{c} ± 0.01

Values are given as mean ± standard deviation. Different letters in the same column indicate a significant difference ($p \leq 0.05$) determinates by Duncan's multiple comparisons test. Abs = absorbance

IC50 determiantion of polyphenols

The evaluation of the antioxidant activity of the individual polyphenol standards was also expressed by means of the IC50 parameter for both DPPH and ABTS radicals and results are reported into table 3.3. The lower the values of the IC50 parameter, the more effective the antioxidant capacity, as less of the compound is needed to bring about the same decrease in the concentration of the radicals used to perform the test. These values are derived from the calibration line obtained for the various pure compounds by plotting the value of the percentage inhibition (% Inhibition) of the DPPH and ABTS radicals as a function of concentration, expressed in μg/mL, as shown in Figure 3.1 A,B,C and 3.2 A,B,C. Using the free DPPH radical, IC50 values were obtained for the antioxidants analysed, expressed in μg/mL. The results, shown in table 3.3, show that vanillic acid was not very effective against the DPPH radical, with an IC50 value of 5768.32 μg/ml. Gallic Acid, on the other hand, proved to be the most active polyphenolic standard, with the lowest values of 23.93 μg/mL. The IC50 parameter was also evaluated using the ABTS radical. From the results obtained, it can be seen that catechol is not very effective against

the ABTS radical, with a high IC50 value of 38.51 µg/ml. Gallic acid, on the other hand, has lower values, and its antiradical activity against the free ABTS radical is very high, reaching an IC50 value of 1.73 µg/ml. It can be concluded that most of the polyphenol standards under investigation are more active against the ABTS radical. This is demonstrated by lower IC50 values than those obtained using the DPPH radical. In addition, gallic acid proved to be the polyphenol standard with the greatest antioxidant capacity, especially against the ABTS radical, compared with the others tested.

Table 3.3. IC 50 determiantion of polyphenols investigated by DPPH and ABTS radicals.

Sample	DPPH	ABTS
	IC50 (µg/mL)	
gallic acid	23.93	1.74
sinaptic acid	110.77	16.80
tannic acid	55.25	3.76
vanillic acid	5768.32	18.84
catechol	38.78	38.51
quercitin	58.24	8.84

3.3.5. Conclusions

In this work, it was found that gallic acid showed the highest antioxidant capacity compared to the other polyphenols analyzed. It was therefore selected as an active substance to be added to biopolymer films chapter 5.1 and chapter 6.

3.3.6. References

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Chapter 4. Physical properties and antimicrobial activity of bioactive film based on whey protein and *Lactobacillus curvatus* 54M16 producer of bacteriocins

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4.1. Abstract

The objective of the work was to study the viability and antimicrobial activity of bacteriocin-producing lactic acid bacteria (LAB) incorporated into whey protein/inulin/gelatine (WP) edible films in presence or absence of nutrient (modified MRS broth). Moreover, the role of the cell on the film structure and properties has been investigated. The results of the work showed that WP-based films were able to ensure a high viability of the bacteriocin-producing strain *L. curvatus* 54M16 during 28 days of storage at 4°C. The addition of nutrient in the film matrix slightly affected the viability of the cells, but it was critical for the antimicrobial activity of the films. Films in presence of nutrient showed a good antimicrobial activity against *L. innocua* C6 as in vitro system as on cooked ham. The presence of LAB has a significant effect on the structure of the film: it reduced the viscosity of the film forming solution and improved the elasticity and the percentage of elongation. Whereas no effect was observed for water vapour transmission rate and solubility. Thus, WP-based films in presence of modified MRS broth can be used as effective delivery and carrier systems for lactic acid bacteria to develop bioactive edible film or coating with antimicrobial properties.

Keywords: Bioactive film; Whey protein; Lactic Acid Bacteria; cell viability; mechanical properties; antilisterial activity.

4.2 Introduction

Packaging plays a critical role in the food supply chain. New packaging technologies designed to improve food safety and quality could help to reduce food loss during distribution chain. Active packaging deliberately incorporates active components intended to release or to absorb substances into, onto or from the packaged food or the environment surrounding the food in order to improve the quality of packaged food or to extend its shelf life (Regulation (EC) No 1935/2004). Among the many types of active packaging, in the last years antimicrobial active packaging is being increasingly experimented with the main goal of preserving the food during storage from microbial contamination and proliferation of pathogenic and spoilage microorganisms (Appendini & Hotchkiss, 2002; Bolívar-monsalve, Ramírez-toro, & Bolívar, 2019; Quintavalla & Vicini, 2002). The most studied antimicrobial food packaging systems have been classified according to their active compound: essential oils, enzymes and bacteriocins, organic acids and their derivatives (Yildirim *et al.*, 2018). Among bacteriocin based active film, nisin-based active packaging is the most studied in food applications (Ercolini *et al.*, 2010; Ferrocino *et al.*, 2013; La Storia *et al.*, 2012; Massani, Morando, Vignolo, & Eisenberg, 2012; Mauriello, De Luca, La Storia, Villani, & Ercolini, 2005) and it is still the only bacteriocin legally approved as food additive to be used by the food industry (Kumariya, Kumari, Rajput, Sood, & Akhtar, 2019). An alternative novel and natural preservation method is the incorporation of viable lactic acid bacteria (LAB) strains into a film or coating matrix for bacteriocin production during food storage (Concha-meyer, Schöbitz, Brito, & Fuentes, 2011; Gialamas, Zinoviadou, Biliaderis, & Koutsoumanis, 2010; Iseppi, Niederh, Anacarso, Messi, & Sabia, 2011; Leonard *et al.*, 2015; Pereira, Soares, Costa, Silva, & Gomes, 2019; Pereira, Soares, Sousa, Madureira, Gomes, Pintado., 2016; Pereira, Soares, Monteiro, Gomes, & Pintado, 2018; Sánchez-gonzález, Iván, Saavedra, & Chiralt, 2013). Protective and probiotic bacterial cultures of LAB have traditionally used in the food production and are considered to meet the specific safety requirements that identify the microorganisms as Generally Recognized As Safe (GRAS, in the US) and with a Qualified Presumption of Safety (QPS, in the EU) (EFSA, 2016).

Edible coating and film can be obtained by using biopolymers, including proteins, polysaccharides or their combination (Bruno, Giancone, Torrieri, Masi, & Moresi, 2008; Giancone *et al.*, 2011; Volpe, Cavella, Masi, & Torrieri, 2017). Among biopolymers, proteins have received considerable interest since it provides a film with distinct and valuable properties. Whey proteins are edible, biodegradable and have interesting mechanical properties. The film-forming properties of this type of protein are useful to

produce transparent, flexible, colourless and odourless films (Calva-Estrada, Jiménez-Fernández, & Lugo-Cervantes, 2019). Gelatin, obtained from the partial hydrolysis of collagen, is a natural water-soluble protein characterized by the absence of an appreciable odour and by a linear structure (Coltelli *et al*., 2016). It has the capacity to form a soft, flexible, and elastic gel that confer a less organized matrix when blended with other protein, such as whey protein (Calva-Estrada *et al*., 2019). Pereira *et al*. (2019) showed that whey protein films were more effective in preserving *Bifidobacterium animalis* subsp. *lactis* BB-12 viability compared to alginate films and the most effective results were obtained with inulin incorporation. Inulin is a dietary fiber, known for its properties as prebiotic and functional ingredient (Shoaib, *et al*., 2016). Soukoulis, Behboudi-Jobbehdar, Yonekura, Parmenter, & Fisk (2014) showed that the cell viability of probiotic strain was higher in film containing inulin for its prebiotic function. Garcia-Argueta *et al*. (2013) showed that edible film based on whey protein, gelatin and inulin assured the survival of *Lactobacillus casei* for 10 days at 25°C. The LAB cells viability and the antimicrobial activity can be affected by the structure and composition of the biopolymer film (Léonard *et al*., 2013; Leonard *et al*., 2015). Moreover, it is also important to know the effects of the incorporated active compound on the physical and structural properties of the films. It can lead to either a positive or a negative effect depending on both the nature of the active compound and of the biopolymers used as matrices (Benbettaïeb, Karbowiak, & Debeaufort, 2019b; Lago *et al*., 2014; Perone, Torrieri, Cavella, & Masi, 2014; Torrieri, Cavella, & Masi, 2015). Thus, the objectives of the present study were to study (i) the viability of *Lactobacillus curvatus* 54M16 cells dispersed into a whey protein/gelatin/inulin film in presence or absence of a modified MRS broth; (ii) the impact of the bacterial cells on physical and structural properties of the films and (iii) the antimicrobial effectiveness of the films against *L. innocua* C6 growth in laboratory media and an actual food model system.

4.3. Materials and methods

Materials

Whey milk proteins isolate (WPI) with a high-purity grade (92%) were provided by A.C.E.F. s.p.a. (Piacenza. Italy). Glycerol was purchased from Merck KGaA EMD Millipore Corporation (Darmstadt, Germany). Gelatin (100% purity; bovine origin; Cameo s.p.a., Desenzano del Garda, Italy) was purchased at the Euroesse supermarket (Portici. Italy). Inulin (Farmalabor Srl., Canosa di Puglia, Italy) was purchased at the

ALMA pharmacy (Portici. Italy). Sodium Acetate and Tris-HCl were purchased by Sigma Chemical Co. (St. Louis. MO. USA). All the culture media were purchased from Oxoid (Rodano, Milano, Italy).

Bacterial strains and growth conditions

Lactobacillus curvatus 54M16 was isolated from traditional fermented sausages and produces the bacteriocins *sak* X, *sak* T_{α}, *sak* T_{β} and *sak* P (Casaburi, Di Martino, Ferranti, Picariello, & Villani, 2016; Giello, La Storia, De Filippis, Ercolini, & Villani, 2018). The strain was stored at -20°C in MRS broth (Oxoid) supplemented with 25% (v/v) sterile glycerol. Before use, the strain was sub-cultured twice in a modified MRS broth (m-MRS) at 30°C. The composition of the m-MRS broth was (g/L of distilled water): 8.0 Lab-Lemco, 10 Peptone, 4.0 Yeast Extract, 20 Dextrose, Tween 80 (1 ml/L). A 10 ml aliquot of reactivated culture was transferred into 1000 ml of MRS broth and incubated for 24 h at 30°C. The culture was centrifuged at 6500 *g* for 20 min at 4°C and the cell pellet was washed twice with phosphate buffer (PBS, Oxoid) and suspended in 15 ml of sterile distilled water or m-MRS broth (about 10 log CFU/ml).

Listeria innocua C6 (Casaburi *et al*., 2016), used as bacteriocin indicator, was grown in Trypticase Soy Broth supplemented with 0.5% yeast extract (TSBY, Oxoid) at 30°C.

Film preparation

The formulation of the film forming solutions (FFS) was reported in Table 4.1. Gelatin and inuline were included in the formulation due to their ability to form gels and to their physicochemical properties that create synergy among the components to enable the formation of three-dimensional networks. Moreover, inuline has also a prebiotic function. Three different films were prepared: a) control film prepared without *Lb. curvatus* 54M16 (WPC); b) bioactive film prepared adding *Lb. curvatus* 54M16 suspended in m-MRS broth (WPM), c) bioactive film prepared adding *Lb. curvatus* 54M16 suspended in distilled water (WPH). Culture of *Lb. curvatus* 54M16 was added to film forming solutions at 4% (v/v) to attain a final concentration of 8.56 and 8.54 Log CFU/mL for WPM and WPH film, respectively.

Glygerol, whey protein, inulin, gelatin and m-MRS broth were dissolved in deionised water under continuous stirring at 50°C for 30 min (WPC). Then, the solution was cooled down to 30°C and enriched with *Lb. curvatus* 54M16 at 4% (v/v) under continuous stirring for 15 min (WPM). WPH samples were obtained as the same WPM sample, but the m-MRS broth was replaced by deionised water. The pH of WPC was 5.85, whereas

the pH of WPH and WPM was 5.95 and 6.01, respectively. Films were obtained by casting: five ml of each FFS were poured into Petri dishes (surface of 56.7 cm^2) and allowed to dry at 30°C and 50% relative humidity (RH) for 24 h in circulating air system chamber (MMM Medcenter Einrichtungen GmbH, Monaco di Baviera, Germany). Dried films were peeled from the Petri dishes and stored at 4°C and 50% RH prior to testing.

Table 4. 1. Film forming composition of bioactive WP-based film

Composition	Film samples[a]		
	WPC	WPM	WPH
Whey Protein isolate (%w/v)	13.0	13.0	13.0
Edible gelatin (%w/v)	6.0	6.0	6.0
Inulin (%w/v)	4.0	4.0	4.0
Glycerol (%w/v)	6.0	6.0	6.0
m-MRS broth (%v/v)	15.0	15.0	0.0
Cells of *Lb. curvatus* 54M16 (%v/v)	0.0	4.0	4.0
Deionized water (%v/v)	56.0	52.0	67.0

a: WPC, control film prepared without *Lb. curvatus* 54M16; WPM, bioactive film prepared adding *Lb. curvatus* 54M16 suspended in m-MRS broth; WPH, bioactive film prepared adding *Lb. curvatus* 54M16 suspended in distilled water.

Rheological properties of FFS

The FFS viscosity was measured as reported in Volpe *et al*. (2017) with small modifications. A strain-controlled rheometer (HAAKE MARS 40 Rheometer, Thermo Fisher Scientific, USA) equipped with coaxial cylinders (30mm o.d. and 26 mm i.d.) has been used. Measurements were performed at 30 °C by increasing the shear rate from 0.1 to 100 s^{-1}. All measurements were replicated three times. Data are reported as steady shear viscosity (η) versus the shear rate (γ). The steady shear viscosity has been calculated with respect to the following equation:

$$\tau = \eta \gamma \quad (1)$$

where τ is the shear stress (Pa), η is the shear rate (s^{-1}) and γ is the viscosity (Pa s).

Viability of *L. curvatus* 54M16 during storage of the film

Viable counts of *L. curvatus* 54M16 were determined in the FFSs just before the casting process, while the viability of the microorganisms was determined in the films after 1, 2, 7, 15 and 28 days of storage at 4°C.

FFS was diluted 1:10 in quarter-strength *Ringers solution* (Oxoid) and aliquots of serial decimal dilutions were poured in duplicate plates of MRS agar. The plates were incubated

in anaerobiosis (AnaeroGen, Oxoid) at 30°C for 48 h. The results were expressed as log CFU/ml.

The films (WPC, WPM and WPH) at each sampling time were placed in a Stomacher bag with 10 ml of *Ringers solution* (Oxoid). The bag containing each film was immersed in a water bath at 40°C for 30 sec for the complete dissolution and release of the cells trapped in the film and then homogenized by stomacher for 5 min. Sequential decimal dilutions were made in Ringer's solution. Each dilution was plated on MRS agar and incubated in anaerobiosis at 30°C for 48 h. The results were expressed as means of log CFU/cm^2.

Film characterization

Scanning electron microscopy

The film surface and the fracture surfaces (transversal area) obtained after immersing film samples in liquid nitrogen (fragile fracture) were observed using a scanning electron microscope (LEO EVO 40, Zeiss, Oberkochen, Germany). The films were mounted on bronze stubs using double-sided tape and then placed on specimen stubs with the cross-section oriented upward and were coated with a thin layer of gold using a DC sputter coater (AGAR B7340, Agar Scientific Ltd, Stansted, UK). Digital images were collected at a tilt angle of 0° to the electron beam using an acceleration voltage of 20 kV. SEM analysis were performed on samples WPC and WPM.

Physiochemical properties

The film surface density (ρ) was calculated as:

$$\rho = \frac{C \cdot V}{A} \quad (2)$$

where C is the blend solution concentration (mg ml^{-1}), V is the volume of the solution poured into the petri dish (ml) and A is the surface area of the petri dish (cm^2).

Film thickness was measured using a micrometer model H062 with sensitivity of ±2μm (Metrocontrol Srl, Casoria, NA, Italy). Five replications were conducted for each sample treatment. Five measurement were taken at random position around the film sample.

The moisture content of the films was evaluated by gravimetric method, maintaining the film at 105°C for a time enough to reach a constant weight. The results are expressed as relative humidity percentage (UR%) calculated as:

$$UR\% = \frac{(p_i - p_f)}{p_i} \cdot 100 \quad (3)$$

Where "p" is the weight of the film (g), "i" and "f" correspond to the initial and final weight of the film. For each sample, three replicates were performed.

The film opacity was determined according to the method described by Siripatrawan & Harte (2010), by measuring the absorbance at 600 nm using the UV-VIS spectrophotometer (Jasco V-550 UV / VIS Spectrophotometer). The films were cut into rectangular pieces 3 cm high and 0.4 cm wide and placed directly into the cuvette of the spectrophotometer. The opacity of the film was calculated with the following equation:

$$T = \frac{Abs600}{x} \quad (4)$$

Where "T" is transparency, "Abs600" is the absorbance value at 600 nm and "x" is the thickness of the film (mm). For each sample five replicates were performed.

The colour of the samples was evaluated using a colorimeter (Minolta CHROMA METER CR-300, Japan). The colour values of L (black/white), a*(redness/greenness) and b* (yellowness/blueness) were measured and averaged from five random positions for each sample, and the total colour difference (ΔE) was calculated according to Odila Pereira *et al*. (2016).

The solubility of the films at different pH was tested with the procedure reported by (Giancone *et al*., 2011). Small pieces of film (20 mg) were dried in an oven at 105 °C for 24 hours and weighed with a value closer to 0.0001 g to determine the initial dry weight of the film. Each piece of film was incubated at 25°C for 24 hours in a falcon containing 10 ml of an acetate solution [0.1 M] (pH 4), distilled water (pH 6), and Tris-HCl (pH 8). At the end of the incubation, the samples were recovered on a Whatman no. Filter. 1. The part of the undissolved film was removed from the filter using 10 ml of distilled water and dried in a vacuum oven at 70 °C and 6.67 kPa for 24 hours and finally weighed. The solubility of the film (FS%) was calculated as follows:

$$FS\% = \frac{w_i/w_f}{w_i} \cdot 100 \quad (5)$$

Where "w" is the dry substance, "i" and "f" correspond to the initial and final dry matter. For each sample, three replicates were performed.

Mechanical analysis

Dynamic mechanical analyses were performed using a DMTA V (Rheometrics, Inc., Piscataway, USA) that applies an oscillatory force at a set frequency to the sample and reports changes in stiffness and damping on rectangular film strips (20 ×7 mm). Each sample was cut with scissors and mounted on grips so that its effective length was 10 mm. All the samples were submitted to a strain sweep test at a given frequency (ω) of 1 rad s^{-1} to determine the linear viscoelastic region. Then, a frequency sweep test was carried out applying a strain amplitude (ε) of 0.005% (within the linear viscoelastic

region) and increasing the frequency from 10^{-2} to 10^{3} rad s^{-1} to monitor the storage modulus (E') that measure of the sample's elastic behaviour, and the tangent delta (tanδ), that is the ratio of the loss storage and the storage modulus. All measurements were conducted in the dynamic mode and the results were reported as average of three replicates.

Tensile tests were carried out by using an Instron Universal Model No 4301 (Instron Engineering Corp., Canton, MA) at room temperature, according to a standard test method (Method D882, ASTM, 2001). The instrument was equipped with a 1kN load cell and the crosshead speed was equal to 50 mm min^{-1}. Films were cut into 25-mm wide and 100-mm long strips. Elastic modulus (E), tensile strength (TS) and elongation at break ($\varepsilon\%$) were calculated. The results were reported as average of seven replications of each sample.

Water vapour permeability

The water vapor permeability (WVP) of the films was evaluated as reported by Volpe *et al*. (2017) using a gravimetric test according to ASTM E 96 (1993) by means of Payne permeability cup (Carlo Erba, Milan, Italy). The Water vapor permeability (WVP) was calculated at 20°C and at 85% of RH, as:

$$\text{WVTR}=\frac{dm}{\text{dt}}\cdot\frac{x}{\text{A}\cdot\Delta\text{p}} \quad (6)$$

Where $\frac{dm}{dt}$ is the slope of the weight curve with respect to time after reaching the steady state, "x" represents the thickness, "A" is the exposed area of the film (9.89 cm^2), "Δp" is the vapor pressure water through the film. The results are reported as the average of three replications of each sample.

Antimicrobial activity

Antimicrobial activity of the films during storage at 4°C was detected against the strain of *L. innocua* C6 as previously described by Mauriello *et al*. (2005), with some modifications. At each sampling time, film pieces (ca 2x2 cm^2) were aseptically cut from each film and placed on TSA plates. After incubation at 4°C for 3 h to allow bacteriocins diffusion, the plates were covered with TSA soft agar (0.75% agar) inoculated with about 10^6 CFU/ml of an overnight culture of the indicator strain. Plates were incubated at 30°C, 10°C and 4°C for 24 and 72 h and 20 days, respectively. After incubation the inhibition zones of the indicator organism around the films were determined and expressed in cm^2. Each value was the mean of two experiments with three replicates each.

The antagonistic activity of the active films was also evaluated by determining *L. innocua* counts, as described by Sánchez-González, Quintero Saavedra, & Chiralt (2014), with some modifications. Overnight culture of *L. innocua* C6 was inoculated (about 10^5 CFU/cm^2) on the surface of solidified TSA plates, which were then covered with active (WPM) and non-active (WPC) films with the same size as the Petri dishes. The counts of *L. curvatus* 54M16 and *L. innocua* C6 were determined after 0, 1, 7, 15 and 28 days of storage at 4°C. At each sampling time the agar covered with the films was withdrawn aseptically from the Petri dishes and transferred in a Stomacher bag with 100 ml of quarter strength Ringer's solution (Oxoid). The content of the bag was first manually ground and then the bag was immersed in a water bath at 40°C for 30 sec for the complete dissolution and release of the cells trapped in the film, and finally homogenized by stomacher for 5 min. Sequential decimal dilutions were made in Ringer's solution. Each dilution was plated on MRS agar incubated in anaerobiosis (AnaeroGen, Oxoid) at 30°C for 48 h and on ALOA (Biolife, Milano, Italy) incubated at 30°C for 48 h. The results were expressed as means of log CFU/cm^2.

Antimicrobial effect of the films during the storage of cooked ham

Cooked ham discs (56.7 cm^2 x 0.5 cm thick) were aseptically cut and transferred into sterile Petri dishes. Overnight culture of *L. innocua* C6 was spread on the surface of ham to obtain a final concentration of approximatively 10^5 CFU/cm^2. The ham discs were covered with the WPC and WPM films. After 0, 1, 7, 15 and 28 days of storage at 4°C, the samples were homogenized with 100 ml of quarter strength Ringer's solution (Oxoid) for 2 min by Stomacher at room temperature. Decimal dilutions of the homogenates were prepared in the same diluent and selective viable counts of Listeria on ALOA (Biolife) were performed. The plates were incubated at 37°C for 48h and the results were expressed as means of log CFU/cm^2.

LAB viability in WPM film covering the cooked ham was also determined during each time as described above.

Data Analysis

All experimental results are reported as mean value ±standard deviation. Data were analysed using variance to study the effect of film composition (WPC, WPM, WPH) on film functional properties. Only for the cell viability and antimicrobial properties of the film, the effect of storage time was also investigated. Five level of storage time were studied (0, 1, 7, 15, 28 days). Duncan's test was carried out to find the source of the

significant differences within the samples examined. Significant differences were defined at $p < 0.05$. All statistical analyses were performed using the SPSS software (SPSS Inc. 17.0, Chicago, 2002).

4.3. Results and discussions

Rheological properties of film forming solution

All solutions had a Newtonian behavior. ANOVA showed that the presence of LAB had a significant effect on viscosity ($p<0.05$), which was equal to 0.11±0.01 (Pa s) for WPC samples and assumed an average value of 0.07±0.01 (Pa s) for WPM or WPH samples. Thus, WPC sample showed higher viscosity values than WPH and WPM samples. Kanmani & Lim (2013) showed that the addition of probiotic bacterial strains in edible film based on pullulan and starch decreased the viscosity and pH of all film-forming solutions. García-argueta *et al*. (2013) studied the effect of inulin, gelatin and LAB on viscosity of WP based film and showed that gelatin and gelatin–LAB interaction decreased the viscosity of the film forming solution. The presence of LAB cell can create discontinuity in the film forming solution that induces a different interaction among whey protein, gelatin and inulin. Since molecular mass affects the dependence of viscosity on the shear rate (Williams & Phillips, 2000), the lower viscosity can be explained by an increment of free volume due to the presence of bacteria cells included in the protein complex.

Viability of *L. curvatus* 54M16 during storage of the film

Ensuring the viability and functionality of microorganisms incorporated in a bio-polymer matrix represent the main conditions to guarantee the effectiveness of active films.

The viability of the producing bacteriocins strain of *Lb. curvatus* 54M16 included in WPM film (prepared by adding nutrients resulting from a modification of the MRS broth (m-MRS)) and in WPH film (prepared without m-MRS) was determined during the storage of the films at 4 °C for 28 days. Viable counts on MRS agar plates are shown in Table 4.2. The *Lb. curvatus* count in WPM film remained stable at level ranging from 7.67 to 7.58 log CFU/ cm^2 during the storage period. In WPH film a significant reduction, though with a very slight decrease (0.47 units after 28 days of storage at 4°C) of the initial population of the strain was observed at 15 days and 28 days of storage, indicating that the presence of nutrients (m-MRS) in the composition of the film influences the viability of the microorganism.

Literature reviews show that similar films based on whey protein, gelatin, inulin and glycerol guarantee the survival of different strains of lactic bacteria during storage and their use as a coating for different foods (Garcia-Argueta *et al*., 2013; 2016; Shoaib *et al*., 2016; Pereira *et al*., 2019). Moreover, protein-based films appear to ensure greater viability of the microorganisms added to the matrix compared to films based on cellulose derivatives (Sánchez-González *et al*., 2013; 2014). The results of this study showed that the optimal formulation of edible films to ensure a high viability of the bacteriocin-producing strain *L. curvatus* 54M16 was 13% whey protein, 4% inulin, 6% gelatin, 6.0% glycerol, 4% of bacterial cells and 15% of m-MRS broth (Table 4.2). Nevertheless, both films, with and without nutrients, can be considered as a good carrier of viable cells, even if the presence of nutrients is critical for the antimicrobial activity of the films, as discussed in paragraph 3.3.4.

Table 4. 2. Viability of *L. curvatus* 54M16 in WP-based edible films during storage at 4°C

Film[a]	Viable cell numbers (Log CFU/cm^2) at days[c]				
	1[b]	2	7	15	28
WPC	<1	<1	<1	<1	<1
WPM	7.67±0.01^{aA}	7.69±0.09^{aA}	7.66±0.04^{aA}	7.51±0.03^{aA}	7.58±0.01^{aA}
WPH	7.59±0.08^{aA}	7.49±0.17^{aA}	7.51±0.01^{bA}	7.41±0.02^{bB}	7.36±0.01^{bBC}

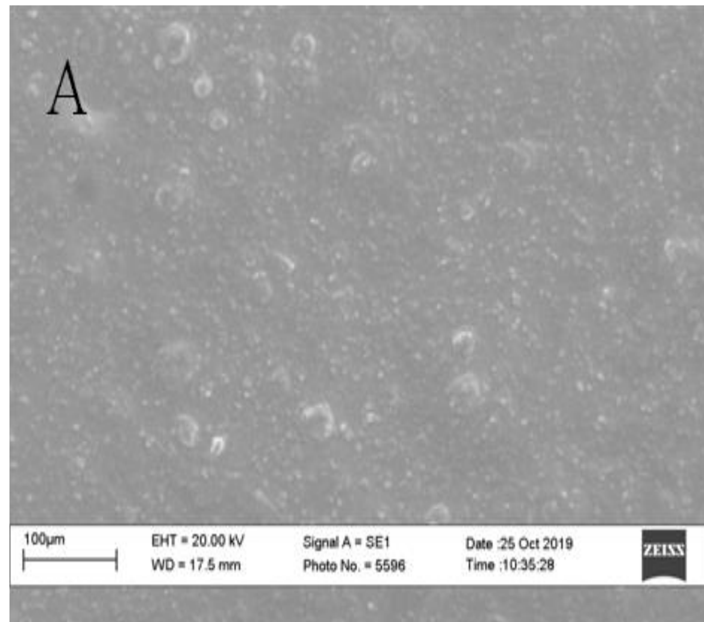
Abbreviation: a: WPC= control film prepared without *Lb. curvatus* 54M16; WPM= film prepared adding *Lb. curvatus* 54M16 and mMRS broth; WPH= film prepared adding *Lb. curvatus* 54M16 and distilled water; b: films immediately after castings at 30°C for 24 h. c: the values are the means ± SD obtained from four plates of three independent trials. Means within a row with the same capital letter are not significantly different (P>0.01); Means within a column with the same lower case letter are not significantly different (P>0.01).

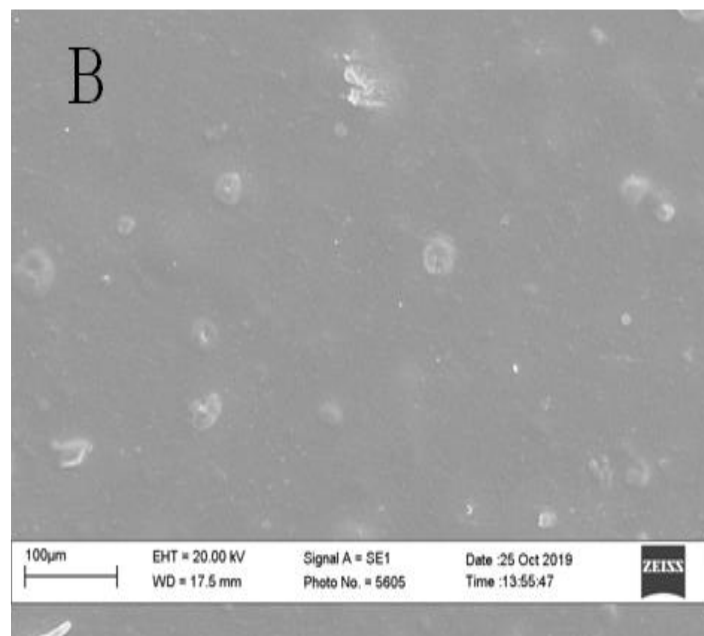
Film characterization

Scanning electron microscopy analysis

Figure 4.1 shows the microstructure of WPC and WPM films. No differences were observed between the cross section of WPC and WPM, since both films were characterized by a compact, homogeneous and uniform structure. Similar results were reported by Odila Pereira *et al*. (2016) who showed how the incorporation of probiotic strains in the edible film did not confer any noticeable modification to the structural conformation of the films. However, it was not possible to distinguish the presence of bacteria cells for WPM films.

It is probable that the bacteria cells were well incorporated into the matrix, so that resulted in a non-discontinuous structure. By looking at the surfaces of the films, it is possible to notice that the surface of WPC resulted rough and uneven due to the formation of steam bubbles during the film casting. On the other hand, the surface of WPM was smooth and compact. The different surface of the films can be explained by the different rheological properties of the FFS. Indeed, the higher viscosity of WPC did not allow to the steam bubble to evaporate, so that the bubbles remain in the matrix.





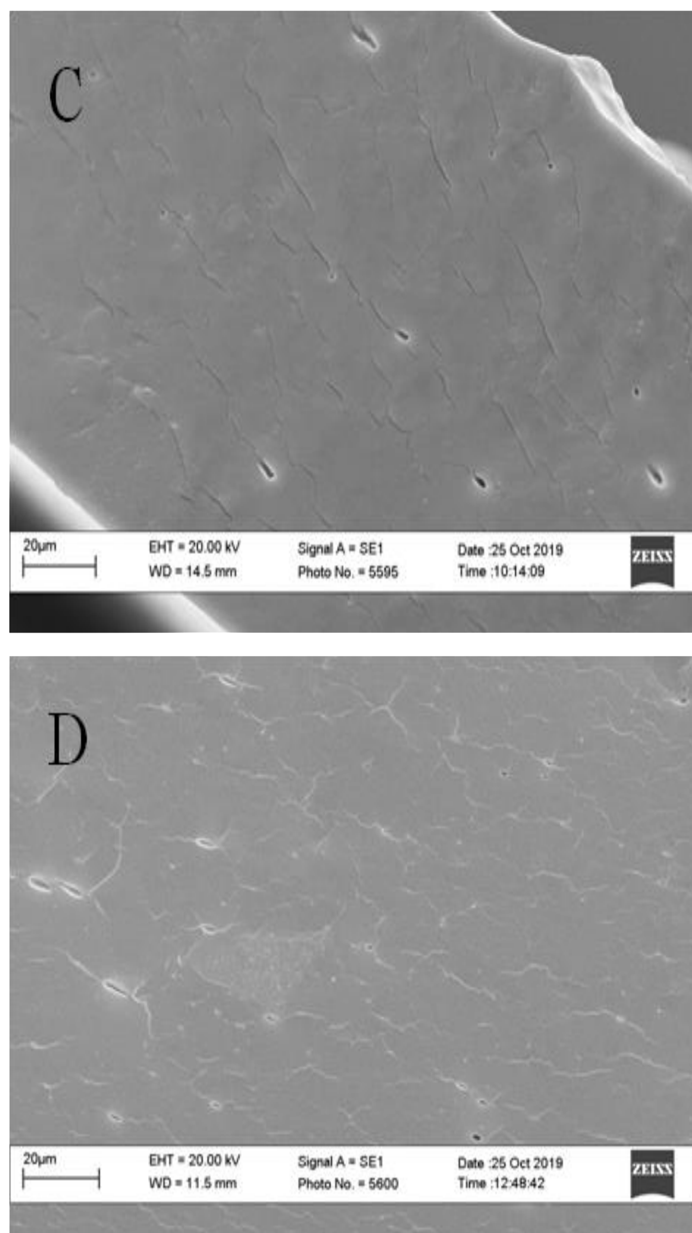


Figure 4.1. SEM micrographs of the WP-based films: A) WPC film surface, B) WPM film surface, C) WPC film cross section; D) WPM film cross section. Magnification 500 x for the surface, 2000x for the cross section.

Physiochemical properties of the film

Film surface density, thickness, moisture content, colour, and opacity of the films are reported in table 4.3. The surface density of both WPC and WPM films was 0.17 g cm^{-2}, while for the WPH film it was 0.15 g cm^{-2}. The thickness of WP-based film ranged from 130±23 µm for WPH film to 162±30 µm for control film (WPC). The lower thickness value of WPH film respect to WPC and WPM film can be related to its lower solid content. However, based on statistical analysis, no significant difference among samples were highlighted ($p > 0.05$). Thus, the presence of the m-MRS broth at 15% or the

presence of the LAB cell did not affect the film thickness.

Moisture content of film was not affected by film formulation ($p>0,05$) (table 4.3). It assumes an average value of 14±1%. Optical properties (opacity and colour) had a direct impact on the visual appearance of the film. All films showed high brightness values, demonstrating that films appeared clear and transparent. The lightness value (L*) of WPC film showed a higher value respect to WPM and WPH film (table 4.3). Statistical analysis highlighted a significant difference among samples. Moreover, from the results of Duncun test, it can be observed that samples WPM and WPH were no statistically different. Thus, the different lightness of the film is due to the presence of the cells. Same results were reported also for the parameters a*. WPC sample showed a higher a* value compared to WPM and WPH film. For b* parameter, a statistical difference between WPH and WPM was observed too ($p<0.05$). In presence of cell and with m-MRS broth the highest b* value is obtained. The total colour difference (□E) showed a significant difference of WPC sample from WPM and WPH, as for L* and a* ($p<0,05$). Same results were obtained for the opacity of the film (table 4.3). Therefore, the addition of lactic acid bacteria cells to the film-forming solution affects the appearance of the films.

Similar results were reported by Bekhit *et al*. (2018), who showed that the disperse bacterial cells, with a different refractive index, decreased the transparency of the film due to the enhancement of the light scattering, the films lightness and whiteness index.

Solubility is an important parameter that measures the water resistance and the integrity of film; therefore, the film solubility at different pH has been calculated. The film solubility ranged between 25% to 30% as function of film composition and pH (data not shown). ANOVA showed that there was no significant difference among samples at single pH values ($p>0.05$) and that the highest solubility values (about 30%) was obtained at pH 6. Therefore, these films in contact with acidic foods (pH 4) or basic foods (pH 8) did not solubilise as much as when in contact with foods that have a pH tending to neutrality. The results can be justified by the relation between solubility and isoeletric point of the proteins. The pH of the solution affects the nature and the distribution of the protein's net charge. Generally, the proteins are more soluble at low (acids) or high (alkaline) pH values because of the excess of charges allowing the water to interact with protein (Pelegrine & Gasparetto, 2005). In any case, the solubility value was not very high. This indicates the high cohesion of the matrix.

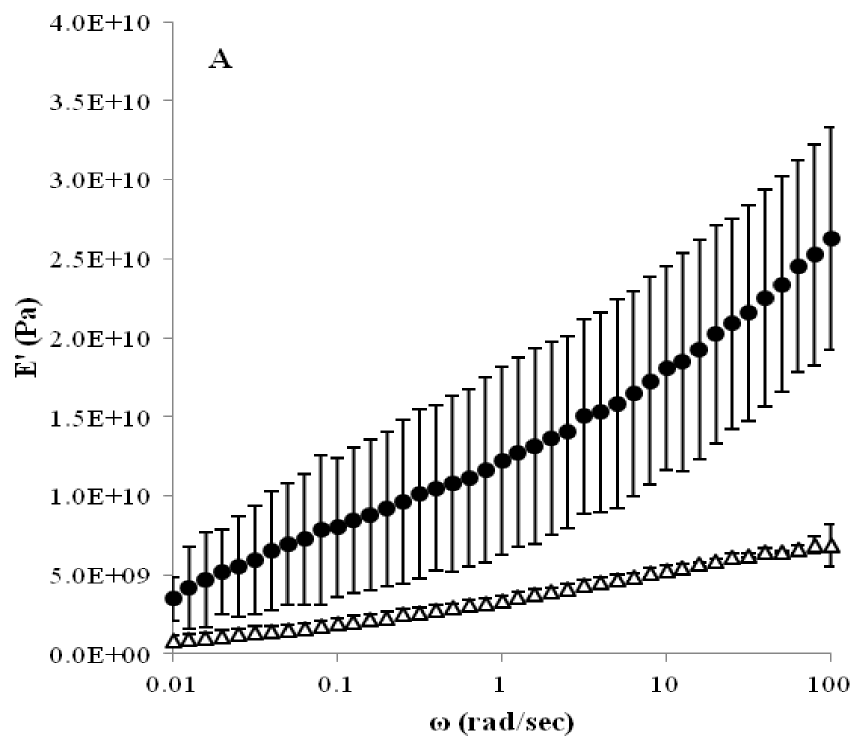
Table 4. 3. Solid surface density (ρ), thickness (Δx), moisture content (MC), colorimetric parameters (L*, a*, b*, ΔE) and opacity of WP-based films

Film	ρ_s (gcm^{-2})	Thickness (μm)	MC%	L	a*	b*	ΔE	Opacity
WPC	0.17	162±30a	15±1a	96,9±0,8^a	0,3±0,2^a	1,3±0,7^a	1±1^a	0,9±0,1^a
WPH	0.15	130±23a	14,8±0,4a	93,6±0,7^b	-0,6±0,2^b	5,9±0,6^b	4±3^b	1,16±0,09^b
WPM	0.17	160±25a	13±2a	93±1^b	-0,9±0,1^b	7,5±0,8^c	6±4^b	1,21±0,09^b

Abbreviation: WPC= control film prepared without *Lb. curvatus* 54M16; WPM= bioactive film prepared adding *Lb. curvatus* 54M16 suspended in m-MRS broth; WPH= bioactive film prepared adding *Lb. curvatus* 54M16 suspended in distilled water. Means within a column with different letters are significantly different ($p < 0.05$).

Mechanical properties and water vapour permeability

Figure 4.2 shows the dependence of the storage modulus (E') and loss tangent (tan δ) on ω for the WPC and WPM films. E' value could be related to how elastic the material is. The loss tangent is an indicator of how efficiently the material loses energy to molecular rearrangements and internal friction. It is calculated as ratio of the loss and the storage modulus and therefore is independent of geometry effects (Menard, 1999). By increasing ω from 10^{-2} to 10^{2} rad s^{-1}, E' increased for both the films; in particular, WPM showed the greatest E' value, which ranged from 3.49×10^{9} Pa to 2.63×10^{10} Pa, whereas WPC started from 7.95×10^{8} Pa until reaching 6.84×10^{9} Pa. Tan δ of WPC decreased from 1.55 to 0.829, whereas WPM had lower values, ranged from 0.839 to 0.343. The results showed that the incorporation of bacterial cells into the matrix influenced the structure of films; WPM film showed more elasticity compared to the WPC film.



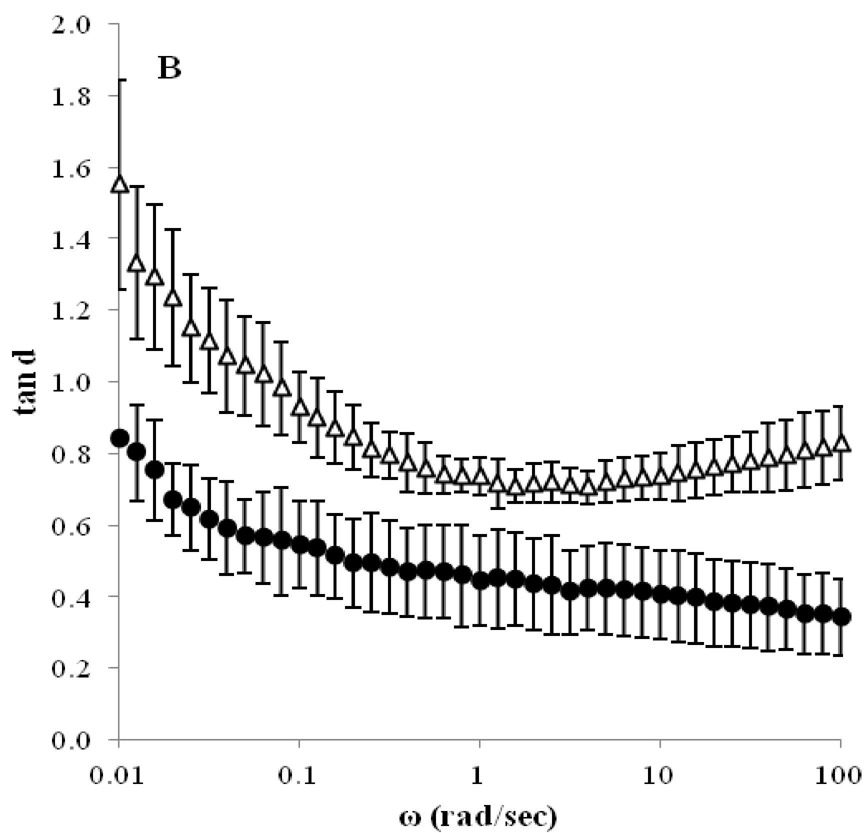


Figure 4.2. Elastic modulus (E0) (A) and loss tangent (tan δ) (B) versus the angular frequency (ω) of the WP-based films: WPC (Δ), control film; WPM (●), film with inoculation of *Lb. curvatus* 54M16 and m-MRS broth.

The tensile properties of the film are reported in table 4.4. WPC film showed a significantly higher elastic modulus values (E > 40%), higher tensile stress values (TS > 30%) and lower percentage of elongation (ε <29%) respect to the films inoculated with strain 56M16, either in presence or in absence of m-MRS broth (WPH and WPM) (P < 0.05), but no significant differences were observed between films inoculated with strain (WPM and WPH) (P > 0.05). The elongation at break of WPC film was of the same order of magnitude as that of whey protein-based film (Fairley, Monahan, Bruce German, & Krochta, 1996; Galietta, Di Gioia, Guilbert, & Cuq, 1998).
The water vapour transmission rate (WVTR) and the water vapour permeability (WVP) values of whey protein-based films are reported in table 4. The WVTR and WVP of WPC film were 55±6 gm^{-2} s^{-1} and 39±6 gm^{-2} s^{-1} Pa^{-1}, respectively. The WVTR and WVP were in the range of other bioactive edible film (Gialamas *et al*., 2010; Soukoulis *et al*., 2014). The presence of the cell or m-MRS broth didn't modify significantly WVP value. Similar results were reported by Bekhit at al. (2018) and Gialamas *et al*. (2010), who reported that the addition of the bioactive culture did not alter significantly the barrier properties of the films.

Table 4. 4. Tensile and water vapor barrier properties of WP-based films.

Film	Tensile properties			WVTR × 10^4	WVP × 10^{11}
	E (MPa)	TS (MPa)	%	gm^{-2} s^{-1}	gm^{-1} $s^{-1}Pa^{-1}$
WPC	289±96^a	6±2^a	16±14^b	55±6^a	39±6^a
WPH	172±51^b	4±1^b	54±19^a	51±4^a	39±15^a
WPM	151±49^b	3.4±0.7^b	44±20a	52±3^a	28±5^a

Abbreviation: EM= elastic modulus; TS= tensile strength; ε %: percentage of elongation; WVTR= water vapour transmission rate; WVP= water vapour permeability; WPC= control film prepared without *Lb. curvatus* 54M16; WPM= bioactive film prepared adding *Lb. curvatus* 54M16 suspended in m-MRS broth; WPH= bioactive film prepared adding *Lb. curvatus* 54M16 suspended in distilled water. Means within a column with different letters are significantly different (p<0.05).

How the incorporation of the cell can affect the structure and functional properties of the film is still unclear. More is known on the incorporation of specific active compound. As well reviewed by Benbettaïeb, Karbowiak, & Debeaufort (2019), organic acid and antimicrobial protein or bacteriocins can have an effect positive or negative on the film structure and in turn of its mechanical and barrier properties as function of the possible interaction between the biopolymer and the antimicrobial agent. As general trend, if new strong interactions, such us covalent bond, are established in the biopolymer matrix, an anti-plasticizer effect is obtained with a positive impact on barrier properties. In fact, a

way to improve the barrier properties of biopolymer film is to improve the cross-link in the structure. On the other hand, when the antimicrobial protein or bacteriocin disrupts the continuous network of the biopolymer matrix, a plasticizer effect is expected.

Acidulant or bacteriocin might exert a plasticizing effect because they are small molecules with hydroxyl groups that replace protein–protein interactions by developing protein-plasticizer hydrogen bonds, and so increase the water-content equilibrium, water vapor permeability, and extensibility that affects the glass-transition temperature of the film (Calva-Estrada *et al*., 2019).

Lactic acid bacteria added in the biopolymer matrix produce in situ bacteriocin that can interact with the film structure. The effect depends by the nature and concentration of compound produced, and by the nature of biopolymer. Bekhit *et al*. (2018) reported that the incorporation of lactic acid bacteria in both HPMC and corn starch films, didn't modify significantly its barrier and mechanical properties. Whereas, for corn starch film, when L. lactis was incorporated in the matrix, an increase of percentage of elongation at break and a notable decrease of elastic modulus was observed. Moreover, García-Argueta *et al*. (2013) studied the effects of different components (whey protein, glycerol, inulin and gelatin) and the addition of probiotic bacteria of lactic acid bacteria (LAB, *Lactobacillus casei*) on edible films; they found that an interaction between LAB and inulin occurred, leading to an increase in film elasticity.

Antimicrobial activity of the films throughout storage

The strain of *L. innocua* C6 was used as indicator organism in all the experiments to detect the antimicrobial activity of the films. The strain showed a similar growth rate but a lower sensitivity to the bacteriocins produced by *L. curvatus* 54M16 when compared with *L. monocytogenes* ATCC 7644 (Casaburi *et al*., 2016). Antimicrobial activity of the films during storage at 4°C for 28 days was determined on plates of *L. innocua* C6 incubated at 30°C, 10°C and 4°C.

The results showed that the presence of nutrients and the temperature at which antagonism was detected were critical for the antimicrobial activity of the films. As shown in table 4.5, no significant decrease in activity was detected during the storage period for WPM films at 30°C, 10°C and 4°C and for WPH films at 4°C; instead, a significant gradually decrease of activity was observed in WPH film during the 28-day period at both 30°C and 10°C (table 4.5), attributable to a lower production of bacteriocins by *L. curvatus* 54M16 in the absence of nutrients. Moreover, control films (WPC) prepared without *Lb. curvatus* 54M16 did not show antimicrobial activity against

the indicator microorganism. A gradual reduction of antimicrobial activity of starch, alginate and D-MRS broth-based films containing cells of two bacteriocin-producing lactic acid bacteria was also observed by Concha-Meyer at al. (2011). The decrease of activity was attributed to a possible death of the bacteria due to a reduction of the nutrients during the storage of the film. These and many other findings from literature reviews confirm that choice of the microorganism and nature of the film matrix represent determining conditions for bacterial vitality and antimicrobial action of the films (Gialamas *et al*., 2010; Sánchez-González *et al*., 2013; 2014).

Moreover, in this study, for both WPM and WPH films, the inhibition areas were significantly wider when the antagonism was detected at 4°C and 10°C (table 4.5, fig. 4.3). This may be due to a better diffusion of the bacteriocins from the film and/or to different growth dynamics of the indicator strain in the presence of bacteriocins at temperatures of 4°C, 10°C and 30°C.

Table 4. 5. Antimicrobial activity of WP-based films containing L. curvatus 54M16 against L. innocua C6 during storage of the films at different temperature

Time (day	Inhibition zones (cm^2)[a]					
	WPM[b]			WPH[c]		
	30[d]	10[e]	4[f]	30	10	4
1	9.0±0.2[Aa]	12.9±0.2[Ba]	13.7±0.2[Ca]	5.3±0.1[Da]	7.84±0.08[Ea]	7.41±0.06[Ea]
7	8.9±0.5[Aa]	12.1±0.3[Ba]	13.5±0.2[Ca]	4.9±0.4[Db]	7.7±0.1[Ea]	7.36±0.05[Ea]
15	9.0±0.1[Aa]	12.9±0.1[Ba]	13.56±0.05[Ca]	4.84±0.05[Db]	6.7±0.1[Ec]	7.65±0.04[Ea]
28	9.1±0.3[Aa]	12.4±0.4[Ba]	13.5±0.1[Ca]	4.6±0.3[Dc]	6.6±0.2[Ec]	7.6±0.1[Ea]

Abbreviation: a: areas of inhibition including the area of the film; b: WPM= film prepared adding *Lb. curvatus* 54M16 and m-MRS broth; c: WPH= film prepared adding *Lb. curvatus* 54M16 and distilled water; d, e and f: plates with indicator strain were incubated at 30°C for 24 h, at 10°C for 72 h and at 4°C for 20 days, respectively. Control film (WPC) prepared without *Lb. curvatus* 54M16 did not show antimicrobial activity against the indicator microorganism. Means within a row with the same capital letter are not significantly different (P>0.01); means within a column with the same lower-case letter are not significantly different (P>0.01).

The antimicrobial activity was also evaluated against *L. innocua* C6 inoculated (about 10^5 CFU/cm^2) on the surface of TSA plates that were then covered with WPM and WPC films and stored at 4°C for 28 days. The results of *L. innocua* counts, reported in table 4.6, showed that the WPM film determined a significant decrease of the microorganisms compared to the control film (WPC). The decrease of *L. innocua* was about 2 log cycles after only 24 hours, reaching values below the detection limits at the end of the storage

period. The results confirmed the bactericidal action of the bacteriocin produced by *L. curvatus* 54M16 (Casaburi *et al*., 2016). On the contrary, the film without *L. curvatus* 54M16 (WPC) did not cause inhibition of *L. innocua* that increased from 5.25 to 6.42 log CFU/cm^2 after 28 days of storage. In a similar in vitro antimicrobial test against a strain of *L. innocua*, Sanchez-Gonzales *et al*., (2014) reported a reduction of the microorganism of 1.5 log cycles for methylcellulose and sodium-caseinate-based films containing cells of bacteriocin-producing *L. acidophilus* and *L. reuteri* compared to the control films, whereas, a 3 log reduction of *L. monocytogenes* was reported by Gialamas *et al*., 2010, for sodium-caseinate films containing cells of bacteriocin-producing *L. sakei*.

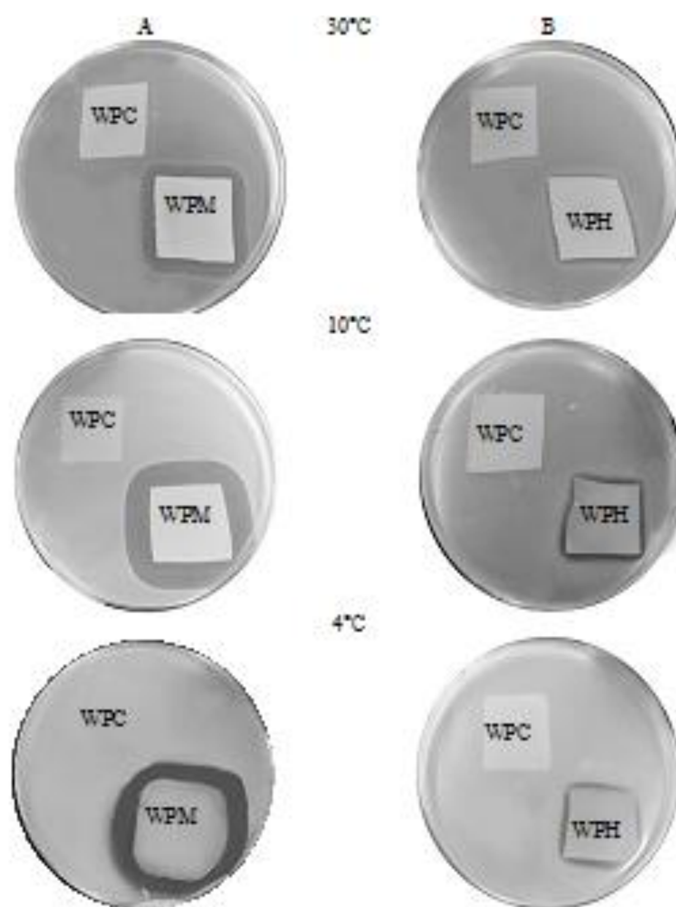


Figure 4.3. Examples of inhibition zones of WP-based films containing *L. curvatus* 54M16 against *L. innocua* C6 after 28 days of storage at 4 °C. A: WPC, control film prepared without *Lb. curvatus* 54M16 and WPM, film prepared adding *Lb. curvatus* 54M16 and m-MRS broth; B: WPC, control film prepared without Lb. *curvatus* 54M16 and WPH, film prepared adding *Lb. curvatus* 54M16 and distilled water; plates with indicator strain were incubated at 30 °C for 24 h, at 10 °C for 72 h and at 4 °C for 20 days.

In our study, *L. curvatus* count remained somewhat stable after the contact of the WPM film with the surface of the medium during the 28 days of storage at 4°C (table 4.6).

However, the bacteriocin-producing strain assured the protective effect of the film throughout the storage period.

Table 4. 6. Antimicrobial activity of WP-based films against L. innocua C6 on TSA during storage at 4°C .

Time	[a]Log CFU/cm^2 of *L. innocua*		[a]Log CFU/cm^2 of *L. curvatus* 54M
(days)	WPM[b]	WPC[c]	WPM[b]
0	5.3±0.2[Aa]	5.25±0.02[Aa]	7.55±0.08
1	3.62±0.04[A]	5.3±0.1[Ba]	7.67±0.03
7	2.13±0.04[A]	6.37±0.03[Bb]	7.7±0.1
15	1.5±0.1[Ad]	6.49±0.02[Bb]	7.77±0.02
28	<1[Ae]	6.42±0.05[Bbc]	7.82±0.05

Abbreviation: a: the values are the means±SD obtained from duplicate plates of two independent trials; b: WPM= film prepared adding *Lb. curvatus* 54M16 and m-MRS broth; c: WPC= control film prepared without *Lb. curvatus* 54M16. Means within a row with the same capital letter are not significantly different (P>0.01); means within a column with the same lower case letter are not significantly different (P>0.01)

Finally, the antimicrobial activity of the WPM film was tested in a food model system consisting of cooked ham. Even in this experiment, *L. curvatus* count remained stable after the contact of the WPM film with the surface of cooked ham during the 28 days of storage at 4°C (data not shown). Counts of *L. innocua* are reported in table 4.7. The films with *L. curvatus* resulted in a reduction of the microorganism by about 2 log cycles compared to the control films where *L. innocua* increased from 5.77 to 6.47 log CFU/cm^2 at the end of storage period. Similar results were reported by Gialamas *et al*. (2010) for NaCas films with *L. sakei* assayed against *L. monocytogenes* on fresh beef. Concha-Meyer at al. (2011) found that alginate films with two bacteriocin-producing lactic acid bacteria had a bacteriostatic effect on *L. monocytogenes* on vacuum packed cold-smoked salmon over a period of 28 days.

In the present work differences in antagonistic activity were observed among WPM film by in vitro and in situ study although the film developed showed promising antilisterial activity.

Table 4.7. *L. innocua* counts of cooked ham covered with WP-based films during storage at 4°C

Time (days)	[a]Log CFU/cm^2 of *L. innocua*	
	WPM[b]	WPC[c]
0	5.8±0.2^{Aa}	5.77±0.05^{Aa}
1	5.40±0.01^{Ab}	5.91±0.01^{Bb}
7	5.39±0.05^{Ab}	5.92±0.02^{Bb}
15	5.1±0.1^{Ac}	6.53±0.09^{Bc}
28	4.62±0.03^{Ae}	6.47±0.03^{Bc}

Abbreviation: a: the values are the means ±SD obtained from duplicate plates of two independent trials; b: WPM= film prepared adding *Lb. curvatus* 54M16 and m-MRS broth; c: WPC= control film prepared without *Lb. curvatus* 54M16. Means within a row with the same capital letter are not significantly different (P>0.05); Means within a column with the same lower-case letter are not significantly different (P>0.05);

4.4. Conclusion

Whey protein/gelatin/inulin films can act as effective carriers of LAB in order to be used as bioactive film. The viability of LAB and its antimicrobial properties depends on the nutrient availability. Films obtained in presence of a modified MRS broth showed good LAB viability and antimicrobial properties during storage time. LAB affected the structure of the film with a positive impact on the mechanical properties. In particular, the presence of LAB decreased the film forming solution viscosity and improved the plasticity of the film. LAB had also an impact on the appearance of the film that resulted opaquer. However, LAB had no effect on barrier properties. The use of the bioactive films against *L. innocua* C6, inoculated on laboratory media and on food model systems, resulted in a significant inhibition of the pathogen compared to the control samples. The above results indicate that whey protein-based films carrying a *L. curvatus* 54M16 strain can be used as an effective and alternative packaging technology for improving food safety. Further investigations will be performed to demonstrate the impact of the bioactive film on food shelf life.

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Chapter 5. – Multilayer biobased packaging using meyer rod coater technology and its application on Hazelnut cream and Grana Padano cheese.

Part 5.1: Design of active multilayer film-based CH/SC/REO and SC/GA coating on commercial support film

This research work has been developed at the "Icimen due" company under the supervision of Dr.

Vincenzo Scognamiglio

5.2. Effect of antioxidant active packaging on shelf life of hazelnut cream

5.3. Developiment of active antioxidant packaging for the preservation of Grana Padano DOP

The objective of this chapter was to develop different multilayer film using a meyer rod coater technology. In the first part of this chapter the deposition of active coating based on CH/SC/REO and SC/GA on commercial film were studied. Then, the film realized were used to pack two food product, hazelnut cream and a shredded cheese, and a shelf life study was performed to evaluate the efficacy of the active film in preserving food quality.

Part 5.1: *Design of active multilayer film based CH/SC/REO and SC/GA coating on commercial support film*
In the first part of this chapter the possibility to deposit an active coating based on chitosan (CH), sodium caseinate (SC) enriched in rosemary essential oil (REO) on commercial support film were studied. The deposition possibility and the single multilayer realization were investigated in term of deposition quantity, wettability, COF, ink and cold and hot sealing properties.

Part 5.2. *Effect of antioxidant active packaging on shelf life of hazelnut cream.*

The research work described in this paper was carried out in order to study the *shelf-life* of hazelnut cream packed with an active film based on polyamide and a coating of sodium caseinate, chitosan and rosemary essential developed in chapter 3. To archieve this goal: (i) active coating previosly study in the chapter 3 was deposited on a nylon commercial film using an industrial approach (rod mayer coater technology); (ii) the antioxidant properties of the realized materials was quantifies using a DPPH radicals; (iii) a shel life study was conducted by storing samples at two temperatures (4°C, 40°C) for maximum 100 days. Commercial film without the active coating was used as control.

Part 5.3. *Active antioxidant packaging for the preservation of Grana Padano DOP*

In this work, the selected polyphenol with highest antioxidant properties (gallic acid) was used to realize an active coating to be used on commercial film. Among different biopolymer, sodium caseinate was chosen as system in which include the gallic acid. The active film realized by using the rod mayer coater technology was characterized in term of antioxidant capacity by DPPH test. Then, to evaluate the effect of the film on the oxidation kinetics of grana Padano cheese, a shelf-life study was conducted by packaging cheese samples with the active film under controlled storage condition (25°C, 40°C). The evolution of the head space gas composition (O_2, CO_2), lipid oxidation (malondialdeide, MDA), volatile organic compounds (VOCs) and colour was studied. Sensory analysis was also performed by triangular test.

5.1. Multilayer biobased packaging using meyer rod coater technology

5.1.1. Introduction

Today the production of an active packaging is mainly focused into the incorporation of the active compound into in the walls of the package (Andrade, Ribeiro-Santos, Costa Bonito, Saraiva, & Sanches-Silva, 2018; Bastarrachea, Wong, Roman, Lin, & Goddard, 2015).

When manufacturing an antioxidant packaging material, from a technological point of view, the antioxidant compound (or the reactive substances that produces that compound) is closely mixed with the polymer, either:

a) by polymer melting and inclusion and mixing of the antioxidant agent into the melt by using extrusion technology. This technique is preferred since most of the conventional packaging structures are either partially or entirely manufactured by extrusion technologies. However, a critical drawback that needs to be considered while using extrusion technology is the degradation of bioactive compounds by severe thermo-mechanical treatment;
b) by immobilizing the antioxidant agent on the surface of the film (Fang *et al.*, 2017);
c) by deposition technique by dissolving active compound into an appropriate solvent or carrier followed by applying the solution to a substrate by coating technology.

Using the deposition technique, a homogeneous film can be obtained by distributing the polymer solution on a suitable flat support and evaporating the solvent. The resulting film should meet three basic requirements:
a) the active coating should have good adhesion to the film substrate and should be valid for food contact;
b) the release of the antioxidant agent must be regulated in order to have an effective antioxidant action;
c) the structure of the actively coated film must meet the functional requirements of food packaging.
Adhesion depends mainly on the compatibility between the substrate and the coating polymers.

Immediately prior to the coating process, the surface of the film substrate is treated by physical methods (corona or flame discharge, UV irradiation), chemical methods (use of primers) or a combination of both (e.g. corona and primer treatment).
Also important to consider that monolayer or multilayer film can show advantages or disadvantages (Busolo & Lagaron, 2015). Normally, if the film has good antioxidant and functional requirements (transparency, good sealing conditions, etc.), a monolayer film can be used. However, the use of monolayer film has the disadvantage that part of the active compound is lost due to its release into the environment with the consequent loss of effectiveness. Therefore, the use of multilayer films would help to minimize the losses due to the migration of the active compound and could also modify the kinetics and release ratios of the antioxidant.

The antioxidants that are added to the packaging system can be of a very different nature, in terms of volatility and solubility, so different release kinetics are to be assumed that are closely related to the function and form of the system, as antioxidant activity can take place both in the packaging material in direct contact with the food surface and in the headspace of the package. Gómez-Estaca, López-de-Dicastillo, Hernández-Muñoz, Catalá, & Gavara, (2014b)

5.1.2. Film surface modification

The common purpose of surface treatment is to modify the layer of a polymer by inserting some functional groups onto the surface of the treatment film in order to improve its barrier properties, wettability, sealability, printability and dye uptake, its resistance to glazing, its adhesion to other materials, or its interaction with a biological environment, while maintaining the desirable bulk properties of the polymer. Surface modification corresponds to a chemical or physical action (acids/oxidants; flame, corona, UV, plasma treatments, etc.) carried out on the surface of the material, which is thereby altered without losing its fundamental properties (Fabbri & Messori, 2017).

5.1.3. Plasma treatment

Not air but selected gases are ionised with electro-magnetic waves to give the plasma state: the contact with the film induces more controlled surface modifications, which determine a better barrier to gases and the formation of active functional groups to which useful substances could be bound for the food or its preservation; the process is also used for the micro-erosion and cleaning effect it exerts on the surfaces, improving adhesion in subsequent coating operations. Accelerated electron treatment. The recent development

of some techniques, developed for other applications, allows today to treat the surfaces of plastic films with the energy of accelerated electrons, generated in special plants that can be an integral part of printing or laminating plants (Ebnesajjad, 2011).

5.1.4. Corona treatment

In corona discharge treatment of polymer films, adhesion usually refers to attractive forces between liquid molecules (ink, adhesive, extradite) and the substrate surface molecules such as PP, PE, and so on, or to the bonding of two substrate surfaces. It is known that increasing the polarity of surface molecules of substrate increases adhesion since inks and adhesives are also polar. Corona treatment also improves autoadhesion (film to film adhesion) (Sun, Zhang, & Wadsworth, 1999)

A high potential difference (3000-9000 V) is maintained between an electrode, as long as the film band, and the insulated cylinder on which the film is run. The air (or more rarely special atmospheres) is ionised by the effect of the electrical discharge; the ozone produced rapidly decomposes into atomic oxygen, which oxidises the surface of the film and partially degrades it, resulting in a surface that is more easily printable and compatible with adhesives (Sun *et al*., 1999).

5.1.5 Flame treatment

The same effect of surface modification of plastics, described for corona treatment, is achieved by exposing the surface to a flame placed in the vicinity of the film which runs continuously on a cylinde (Kadoya, 2012).

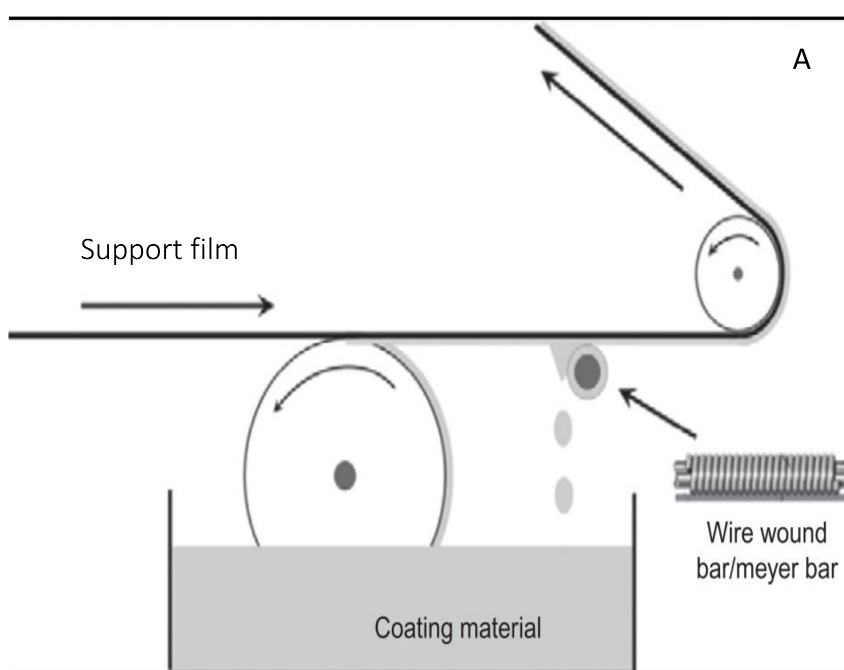
5.1.6. Deposition of active coating on a support film

The deposition of coating is the process by which a thin layer (from a few tenths to a few micrometers thick) of material is applied to the surface of a substrate (support film). The material can either be applied in a molten state or be transferred to the surface to be coated in an appropriate solution, whereupon the solvent evaporates and the coating solidifies (Khwaldia, Basta, Aloui, & El-Saied, 2014). As support film, different commercial flexible films can be used such as polylactic acid (Pla), poly buthylen succinate (PBS), poly propylene (PP), poly ethilene (PE) etc. The are different technique for coating on a support film application such as gravure coating, reverse roll coating, doctor-blade roller coating, Meyer rod coating, reverse gravure roll coating, and other variations of these

methods. The method used in this project are the Meyer rod coating which is used by the Icimendue company as technology.

5.1.6.1 Mayer rod coating

The Mayer rod coating consist of a deposition of a film forming solution on the surface of a support film passing over the bath roller containing the coating solution (fig. 5.1A). The amount of coating removed depends on the diameter of the wire which is wound around the steel rod (table 5.1). The rod coater can be used in series in which the first rod has larger diameter wire and removes most of the excess coating (fig. 5.1B). The second rod, with smaller diameter wire, smoothers the coating and produces the final desired coat weight. For a single layer realization (fig. 5.2A) after a second rod, the coated film are rolled into a evaporation chamber to remove the solvent used into the film forming solution. Furthermore, using between each rod section an evaporation chamber able to evaporate the solvents before the next coating layer it can realized multilayer deposition of coating (fig. 5.2 B).



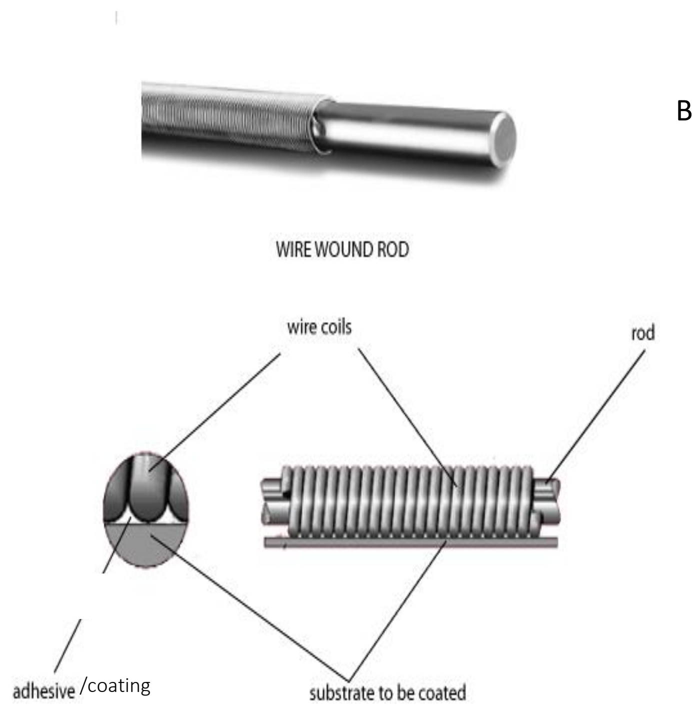


Figure 5.1. (A) Mayer rod coating system in industrial application; (B) single wire wound bar mayer bar

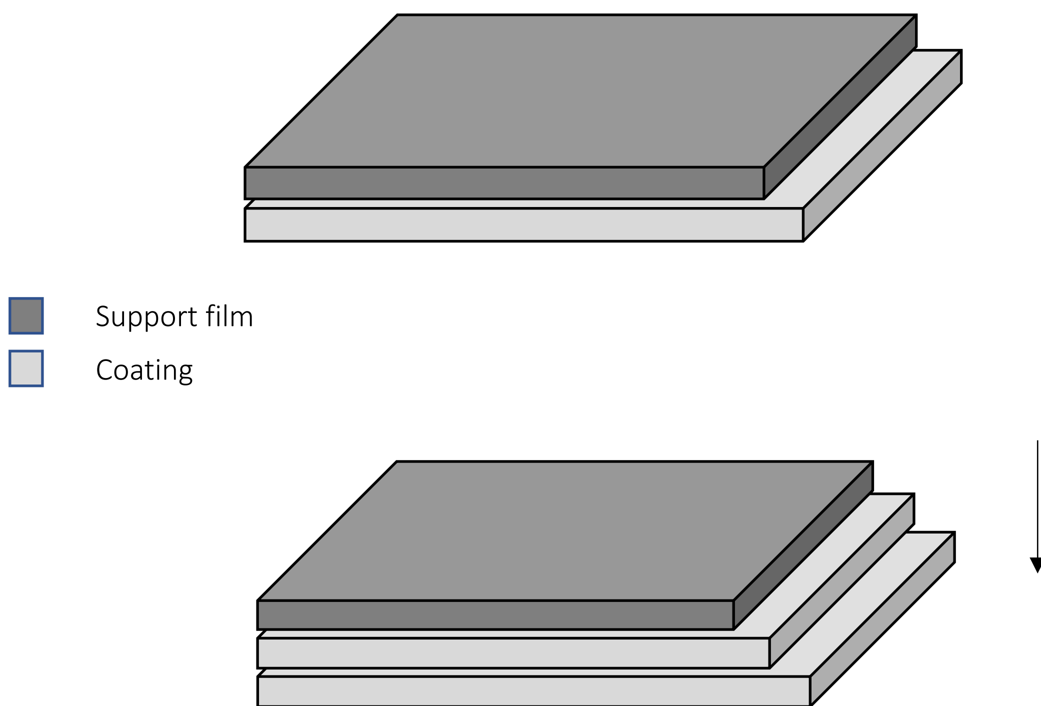


Figure 5.2. Prototype of monolayer (A) and multilayer film (B) using a mayer rod coating

Table 5.1. Theoretical coating amount depends in function of wire rod size (dry grams of coating /m2 of support film) (www.gs-coater.com)

Rod Size	Wet Mils	Thickness Microns	30% Solids	40% Solids	75% Solids	100% Solids
#3	.3	7.6	2.18	2.91	5.47	7.29
#4	.4	10.2	2.91	3.88	6.83	9.11
#5	.5	12.7	3.64	4.85	9.11	12.14
#6	.6	15.2	4.38	5.82	10.93	14.58
#7	.7	17.8	5.09	6.80	12.74	16.99
#8	.8	20.3	5.82	7.78	14.58	19.43
#9	.9	22.9	6.56	8.34	16.40	21.87
#10	1.0	25.4	7.28	9.71	18.21	24.28
#11	1.1	27.9	8.00	10.69	20.04	26.72
#12	1.2	30.5	8.74	11.65	21.87	29.16
#13	1.3	33.0	9.47	12.63	23.71	31.60
#14	1.4	35.6	10.20	13.60	25.51	34.00
#15	1.5	38.1	10.93	14.58	27.32	36.41
#16	1.6	40.6	11.65	15.54	29.14	38.85
#17	1.7	43.2	12.38	16.51	30.99	41.29
#18	1.8	45.7	13.11	17.49	32.78	43.70
#19	1.9	48.3	13.85	18.45	34.61	46.14
#20	2.0	50.8	14.58	19.43	36.41	48.55
#22	2.2	55.9	16.03	21.36	40.07	53.43
#24	2.4	61.0	17.49	23.31	43.72	58.28
#26	2.6	66.0	18.94	25.25	47.38	63.16
#28	2.8	71.1	20.40	27.20	51.04	68.04
#30	3.0	76.2	21.85	29.14	54.65	72.86
#32	3.2	81.3	23.31	31.09	58.28	77.72
#34	3.4	86.4	24.76	33.04	61.62	82.55
#36	3.6	91.4	26.23	34.96	65.55	87.40

Advantages of mayer bar coating method

- fast change overs for coating thickness adjustment;
- precise, consistent, uniform coating thickness control
- longer wear due to utilization of the entire rod surface (rather than constant metering at a single small edge)

Disadvantages of mayer bar coating method

- limitations on coating viscosity,
- rod holders, which provide support to keep the rod from bowing, are often used with smaller diameter metering rods (it need more rod elements to increase the solid on the support film)

The difficulties in using a coating tecnologies are related to the chemical compatibility with the polymers and solvents used, the loss of some volatile substances in the drying phase and the changes induced to the surface properties of the polymer structure (anti-fogging, weldability, processability). However, the coating technology has several advantages related to the possibility of distributing the antioxidant agent in the layer of

the package in direct contact with the food and also, being a non-aggressive heat treatment, minimises the loss of activity of the incorporated thermo-labile molecules.

Scope and approach

The objective of this activity was to develop active film using a Meyer rod coater technology. The deposition functional properties of the active coating solution developed in the chapter 3 were studied to optimize the process parameters and the film structure (mono layer or multilayer). In particular, the deposition of active coating based on CH/SC/REO and SC/GA on commercial films were studied.

5.1.7. Materials and methods

Materials

Medium molecular weight chitosan with 75-85% acetylation degree, sodium caseinate, gallic acid very pure acetic acid, tris hydroxymethylaminomethane hydrochloride (Tris-HCl), rosemary essential oil (REO), Tween 80, glycerol (Merck KGaA EMD Millipore Corporation, Germany); film of polyamide (Nylon), polybuthilene succinate (PBS), Polylactic acid (PLA), with a corona treatment (applied of one side on the film surface) of 38 dyne/cm^2 was gently supplied by (ICIMEN 2, Caserta, Italy) table 5.2.

Table 5.2. Chemico physical properties of support film.

samples	physical properties	units	values	test methods
PLA	thickness	um	15	ASTM D4321
	OTR	cc/m2/day	786	ASTM-F-1927 (85%RH/23°C/24 h)
	WVTR	g/m2/day	440	ASTM-F-1249 (90%RH/38°C/24 h)
	tensile strenght	N/mm2	68.9	ASTM D882
	young modulus	N/mm2	1950	ASTM D882
PBS	thickness	um	50	IO CTR 109
	OTR	cm2/(m2 24h bar)	0.12	ASTM D-3985 (23°C, 50RH)
	WVTR	n.d	n.d	n.d
	young modulus	n.d	n.d	n.d
Polyammide	thickness	um	15	Kolon Method
	OTR	cc/m2/day/atm	32	ASTM D-1434
	WVP	g/m2/day	270	ASTM E-96
	tensile strenght	Kg/mm2	27.5	ASTM D882
	young modulus	Kg/mm2	225.9	ASTM D882

Film-making procedure

Film forming solution (FFS) based on sodium caseinate 4% (SC) and CH/SC in a proportion of 1:1 (v/v) were obtained as reported by (Volpe, Cavella, Masi, & Torrieri, 2017). A mixture of rosemary essential oil (REO) and Tween 80 (4:1) were added to the solutions to obtain a final concentration of 1.5% (v/v). The solution was homogenized at 15500 rpm for 4 minutes at room temperature by using a rotor-stator homogenizer

(Ultraturrax R, T 18 IKA, Milan, Italy), then the solutions were deareated under vacuum for 15 minutes to prevent pinhole formation. Monolayer and multilayer film were produced as schematically described in Figure 5.3. Thus, 2 mL of FFS solution were deposited using a mayer rod coater technique (with a gravure of 24 um of deposition) on the surface of a nylon film and film was dried under controlled condition (50% relative humidity and 40°C) (figure 5. 3).

For multilayer film, the ink deposition and cold seal deposition were realized using a Mayer rod coater 1140/32/26 (R.D. Specialties, Inc., USA) at the same drier condition used for the coating.

Film characterization

Film thickness

Film thickness (x) was measured using a micrometer model H062 with sensitivity of ± 2μm (Metrocontrol Srl, Casoria, Italy). Five replications were carried out for each sample and five measurement were taken at random position around the film samples.

Surface hydrophobicity

The sessile drop method based on the optical contact angle (°) was used to estimate the surface hydrophobicity of the films. A 0,2 μL-droplet of ultrapure water or of the active coating was deposited on the film surface and the image of the drop was recorded with a digital microscope (pocket goniometer M.PG-2, 22140, Chengdu, China). The optical contact angles was calculated as reported by (Williams, 2014) using the ImageJ free software (Stalder, Kulik, Sage, Barbieri, & Hoffmann, 2006). The measurements were performed on the upper side of the film, which was in contact with air during the drying step.

The coefficient of friction (COF)

The coefficient of friction (COF) was measured according to the ASTM D1894 standard on a THWING FP-2260, Albert Instrument (New York, USA).

Seal strength determination

Heat-sealing of the films was conducted at a most used industrial application (food sector) conditions for a commercial used flexible film. The test was conducted on the active materials realized by casting technique. Film samples were cut into strips of 7.62 x 2.54 cm, using a Precision Sample Cutter (Thawing-Albert Instrument Co., Philadelphia,

Penn., U.S.A). Two film strips were placed on top of one another, and an area of 2.54 x 1.5 cm (at the edge of the film) was heat-sealed at 110, 120, or 130 °C for 1 or 3 s of dwell time at 296 kPa pressure, using a thermal heat-sealer Model-12ASL (Sencorp System Inc., Hyannis, Mass., U.S.A). All sealed film samples were conditioned for 48 h under the test conditions prior to determining seal strength as reported in section.

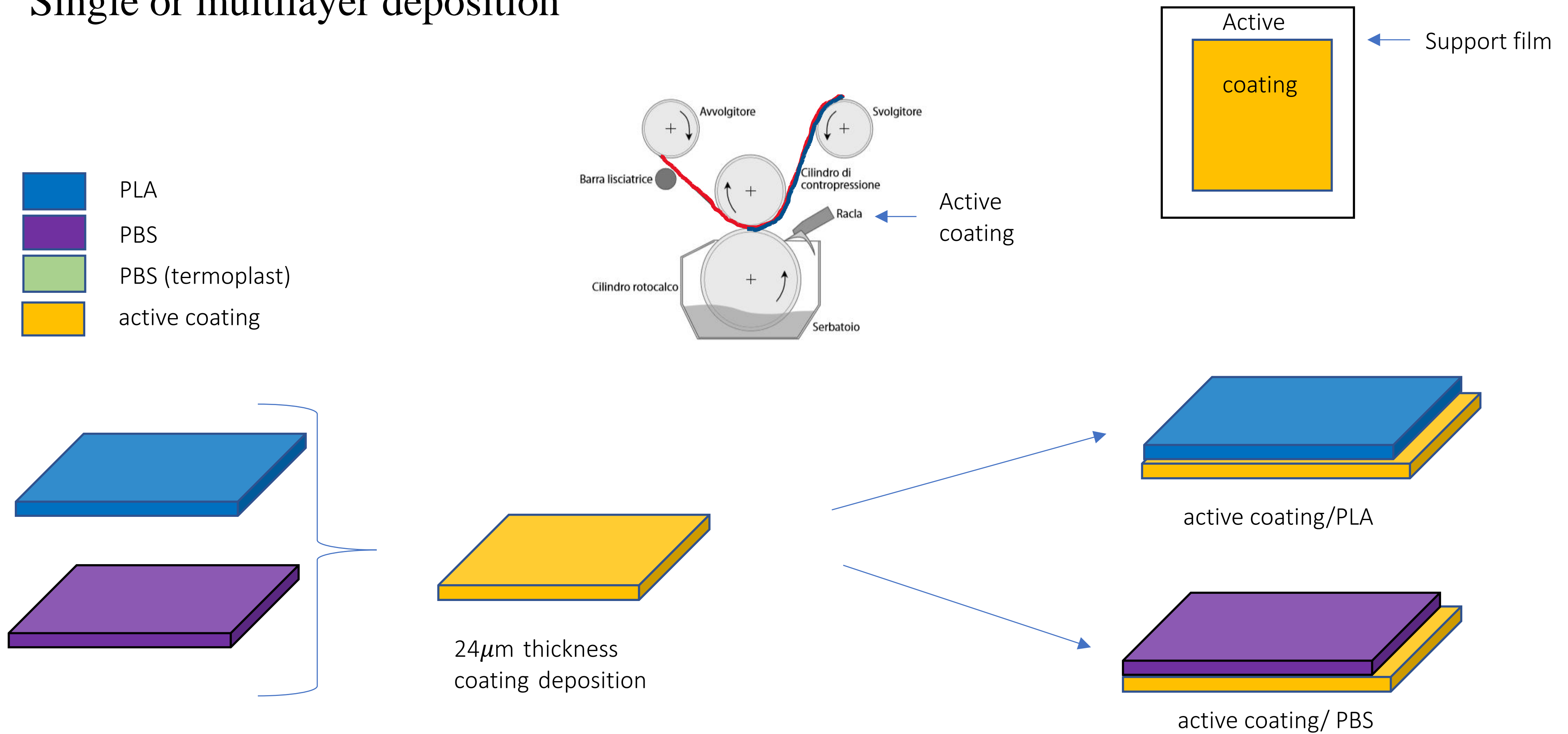


Figure 5.3. Schematic representation of a monolayer and multilayer film realization using a mayer rod coater technique.
Abbrevation: PBS= polybuthylene succinate film; PLA= polylactic acid film

5.1.8. Results

Surface hydrophobicity

In table 5.3 the optical contact angle of SC/REO and CH/SC/REO on PBS and PLA commercial support film are showed. Generally, the contact angles of the active coatings are always lower than the water one, confirming the less hydrophilic nature of the biopolymer coatings respect to the water. These results suggest a potential application of the coating on the surface of the commercial film. Furthermore, the coatings are printable for a packaging design (fig. 5.4) and are resistant to a scratch test (ISO 22557:2019). Scratch testing is carried out to get an insight into materials to determine the resistance to abrasion and wear of modern composite materials and coating.

Table 5. 3. Contact angle determination of Sodium caseinate/rosemary essential oil on commercial bio-based film

Sample code	x (um)	test drop	contact angle (°)
PBS (lab17210)	50	water	77.9±4
		SC/REO	47.4±4
		CH/SC/REO	57.3±3
PBS (termoplast)	15	water	87.5±4
		SC/REO	57.3±4
		CH/SC/REO	60.6±3
PLA	20	water	80.7±3
		SC/REO	54.3±2
		CH/SC/REO	54.9±1

Abbreviation: PBS= polybuthylene succinate film; PLA= polylactic acid film; x= thickness; SC/REO= sodium caseinate film enriched in 1.5% of rosemary essential oil; CH/SC/REO= chitosan/ sodium caseinate film enriched in 1.5% of rosemary essential oil.

Figure 5.4. Ink deposition on CH/SC/REO/PLA and CH/SC/REO/PBS film.

Abbreviation: CH/SC/REO/PLA= chitosan/ sodium caseinate coating enriched in 1.5% of rosemary essential oil on a polylactic acid film.
CH/SC/REO/PBS= chitosan/ sodium caseinate coating enriched in 1.5% of rosemary essential oil on a polybuthylene succinate film.

Heat sealing

The typically used packaging materials, low-density polyethylene and high-density polyethylene, have melting temperatures of 123.6°C and 131.7°C, respectively (Lim *et al.*, 2020). Therefore, the active film supposed undergo sealing at similar or lower temperatures. For the head sealing strength, the casting film did not show any sealing from 120°C up to 160°C, at 0.8s and 400N (fig. 5.5A, 5.5B). This can be justified by the plasticizers, owing to changes in the physical construction and mobility of the polymer matrix that affect the thermal properties (Niknam, Ghanbarzadeh, Ayaseh, & Hamishehkar, 2019; Sanyang, Sapuan, Jawaid, Ishak, & Sahari, 2015).

For this reason, the possibility to apply a delimited coating deposition on a support film was studied and a cold seal was considered as a possible alternative. The head sealing and cold seal delamination of a support film are reported into table 5.4. Generally, the strength

of the hot sealing and a cold seal are higher in the film of PLA compared to Lab17210 related to her chemical composition.

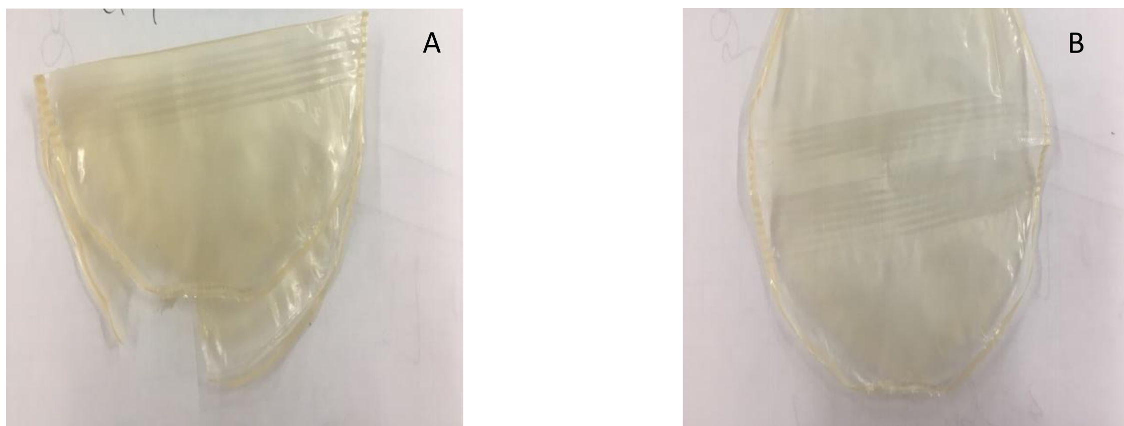


Figure 5. 5. Heat sealing of SC/REO (A) and CH/SC/REO (B) casting film on itself.

Abbrevation: SC/REO= sodium caseinate film enriched in 1.5% of rosemary essential oil; CH/SC/REO= chitosan/ sodium caseinate film enriched in 1.5% of rosemary essential oil .

Table 5. 4. Heat and cold sealing film strenght (N) of PBS and PLA support film.

Sample code	strength (N)
hot sealing	
PBS (lab17210)	29.3±2
PLA	53.5±2
cold seal	
PBS (lab17210)	5.1±1
PLA	6.1±0.5

Abbrevation: PBS= polybuthylene succinate film; PLA= polylactic acid film

Coating deposition

Results of the thickness and coating deposition are reported in table 5.4. Generally, a coating deposition are homogeneous on the surface of the support film as confirmed in previous by the wetting properties of the active coating. Into details the coating showed a higher deposition on the film based on PBS and Lab17210 (0.08 mg/cm^2 dry based) compared to a PLA film deposition (0.05 mg/cm^2 dry based)

Table 5. 2. Thickness, weight and mg/cm^2 of CH/SC/REO coating on different support film.

	x (um) ti	x (um) tf	Weight (g) wi	Weight (g) wf	mg/cm^2 d.b
CH/SC/REO/PLA	32±5	34±3	0.12±0.11	0.13±0.12	0.05±0.01
CH/SC/REOPBS (termoplast)	28±2	35±2	0.10±0.01	0.12±0.01	0.08±0.03
CH/SC/REO/PBS (lab 17210)	52±3	55±3	0.31±0.01	0.33±0.01	0.08± 0.02

Abbrevation: PBS= polybuthylene succinate film; PLA= polylactic acid film; appendix i = initial condition; appendix f= final condition

Moreover, a second layer deposition of CH/SC/REO coating on the first dried layer (after 10 minutes of drying and 1 day). The results showed visibly a not homogeneous distribution of the second layer (fig. 5.6).

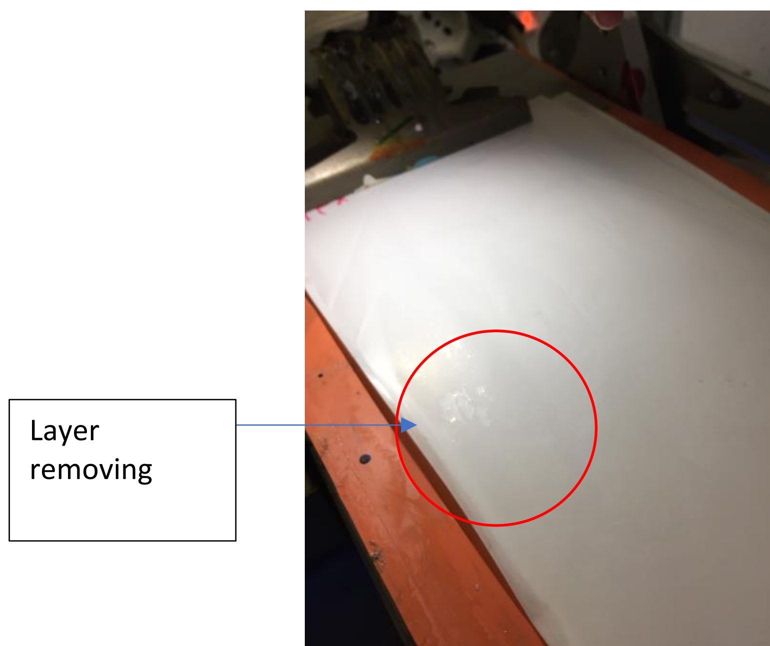


Figure 5.6. Multilayer deposition of CH/SC/REO on support film.

The above studied system reported a limitation into the deposition of multilayer systems using CH/SC/REO coating. For this reason, an other deposition system was studied using as polymer only sodium caseinate (SC) enriched in gallic acid (GA) 5mg/mL on PLA support film. Gallic acid was selected for the good antioxidant properties (previously studied in Chapter 3) and no flavors releasing in contact with food. Into details, the deposition of 11 single layer was evaluated for a future scale up on industrial scale on rotor-coated machines (fig. 5.7). Results showed an increasing thickness from 12± 2 um to 19± 2um. The quantity of coating deposited on the support film was 0.41±0.02 mg/cm^2, higher than the CH/SC/REO on PLA film. The GA incorporated into a realized film was 0.022 g and could be increased thanks to a multilayer depostion and film forming solution optimization.

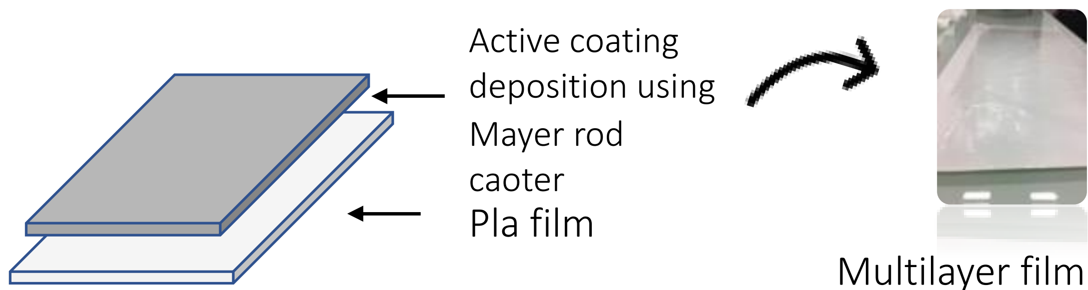


Figure 5.7. Multilayer film (11 layer like an industrial plants) based on SC/GA using a rod mayer technique.

5.1.9. Conclusions

The realization of active coating on a support film, showed to be affected by the coating properties. the CH/SC/REO film could be deposited only with a one layer and showed limitation in a multilayer system. The change in only SC coating enriched in GA, permitted to realize an homogeneous deposition of 11 layer on a support film. The mayer rod coater technique showed as been a promising technique for a active mono and multilayer film realization.

5.1.10. References

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5.2. Effect of antioxidant active packaging on shelf life of hazelnut cream

5.2.1. Abstract

Active monolayer coating based on sodium caseinate enriched in rosemary essential oil (CH/SC/REO) on commercial support film were realized using a rod coater technique. The effect of the active packaging on the quality parameters of hazelnut cream at 2 different storage condition over the time up to 100 day were investigated. Alteration kinetics of hazelnuts cream was studied, and the activation energy (Ea) was calculated by Arrhenius equation. Number of peroxide (NP), free fatty acidity (FFA), malondialdeide (MDA) were used to evaluate the lipid oxidation. Antioxidant performance of the active packaging was demonstrated. Results show a kinetic constant of hazelnut cream at 40°C and 70°C of 1.46 meqO^2/kg/day and 3.7 meqO^2/kg/day respectively and an activation energy of 27.69 kJ/mol K. Number of peroxide was positive affected using an active film during a storage up to 60 day at 40°C. No significantly effect was shown for FFA and MDA value. Active coating based on CH/SC/REO/Nylon can be a promising packaging technology for oxygen sensible food.

Keywords: chitosan, sodium caseinate, blend film, rosemary oil, lipid oxidation, MDA, DPPH test

5.2.2. Introduction

Hazelnuts is the fruit of the hazelnut tree *(Corylus avellana,* like all dried fruit, are a high-energy foodstuff and very high in lipid content such as oleic acid (the main acid) linoleic acid, palmitic acid and stearic acid; these four fatty acids together account for more than 95% of the lipids content (Ciemniewska-Zytkiewicz *et al.*, 2015; Alasalvar, Amaral, Satir, & Shahidi, 2009; Schmitzer, Slatnar, Veberic, Stampar, & Solar, 2011). Because of their composition, one of the changes that can occur in hazelnut cream, is oxidation of the fatty acids (Savage, McNeil, & Dutta, 1997; Alasalvar *et al.*, 2003). Edible oils, fats and fatty foods can undergo deterioration in the course of the time depending on external factors such as heat, light, enzyme and trace metals. As a result of autoxidation especially which occurs with the effect of oxygen in the air, unpleasant taste and smell that are known as the signs of rancidity in oil occur. Autoxidation is an irreversible reaction which goes on spontaneously when it begins, and its rate is directly related to the oil's unsaturation degree. For this purpose essential oil such as rosemary essential oil have been used in food preservation, because they prevent oxidation and microbial contamination (Djenane, Sánchez-Escalante, Beltrán, & Roncalés, 2002; Nieto, Estrada, Jordán, Garrido, & Bañón, 2011). Therefore, rosemary extract could be useful for replacing or even decreasing synthetic antioxidants in foods. As preservatives, rosemary extracts offer several technological advantages and benefits to consumers. Into a previously work (Chapter 3) the effect of rosemary oil on sodium caseinate chitosan film enriched in rosemary essential oil (as casting) on chemico physical properties were investigated. The aim of this work was to apply the active solution of CH/SC/REO on commercial support film using a rod mayer coater technique optimized in collaboration with Icimendue company; to investigate the effect of the active film on the quality parameters of hazelnut cream at different storage condition. To reach this goal: (i) the oxidation kinetic of hazelnut cream was evaluated; (ii) the antioxidant activity of the realized active packaging was teste using DPPH radical; (iii) CH/SC/REO coating was deposited on Polyamide film; (iv) active film was used to wrap hazelnut cream and lipid oxidation was monitored over the time at different storage condition.

5.2.3. Materials and methods

Materials

Medium molecular weight chitosan with 75-85% acetylation degree, sodium caseinate, very pure acetic acid, tris hydroxymethylaminomethane hydrochloride (Tris-HCl), rosemary essential oil (REO), Tween 80, Ammonium thiocyanate (NH_4SCN), ferric chloride (III) (Fe3+Cl3), iron ammonium sulphate (iron II) (Fe2+(NH4)2(SO4)2, 1,3,3-tetraethoxypropane (TEP), 0.1 N hydrochloric acid, NaOH in pellets and 96% ethanol (Sigma-aldrich & co Milano, Italy); glycerol (Merck KGaA EMD Millipore Corporation, Germany); diethyl ether and phenolphthalein, Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), hexane pure acetone (Carlo erba reagents, France); hazelnut paste was supplied packed in 5 kg plastic buckets (Euronut S.p.A. AV); film of polyamide 15 µm with a corona treatment of 38 dyne/cm^2 was purchased by (Icimendue 2, Caserta, Italy).

Active film preparation

Film forming solution (FFS) based on chitosan (1% w/v), sodium caseinate (2% w/v) enriched in 1.5% in rosemary essential oil (CH/SC/REO) in a proportion of 1:1 (v/v) were obtained as reported by (Volpe, Cavella, Masi, & Torrieri, 2017). A mixture of rosemary essential oil (REO) and Tween 80 (4:1) were added to the solutions to obtain a final concentration of 1.5% (v/v). The solution was homogenized at 15500 rpm for 4 minutes at room temperature by using a rotor-stator homogenizer (Ultraturrax R, T 18 IKA, Milan, Italy) then the solutions were deaerated under vacuum for 15 minutes to prevent pinhole formation. 2 ml of FFS solution were deposit using a mayer rod coater technique (with a gravure of 24 um of deposition), on a surface of a nylon film. The film was dried under controlled condition and stored without oxygen before testing to avoid oxydation of the active molecules.

Evaluation of the antioxidant capacity of the active film

For the bioactive multilayer film, the methods (Hromiš *et al*., 2014) with a little modification were used. Briefly, 2.4 mL of 30 ppm ethanolic solution of DPPH were mixed with 100 mg of bioactive film. Samples were stored for 2.5 and 4 h at room temperature in the dark. For all samples, the absorbance was measured at 517 nm using a UV–Vis spectrophotometer (V-550 Jasco Inc., Tokyo, Japan) and pure ethanol as reference. The results were expressed as percentage of DPPH free radical activity (1):

$$I\% = \frac{(Abs_c - Abs_s)}{Abs_c} * 100 \quad (1)$$

where Abs_c is the absorbance of the control and Abs_s is the absorbance of the sample.

Preparation of samples

Storage into active packaging

30 grams of hazelnut cream previosly homogenized was packed in pouches of CH/SC/REO /Nylon (56cm^2) and sealed using a chamber packaging machine (Lavezzini, Milan, Italy). Hazelnut cream samples are stored at 40° for 100 day and at 4°C for 60 days using an incubator (NAPCO series 5400, precision scientific Chicago (USA). To study the alteration kinetic of hazelnuts cream, glass jars was used as hermetically packaging. 40 g of Hazelnut cream was into a glass jar (106 cm^3 in volume) and stored for 21 day at 40°C and 70 °C into a thermostatic chamber (Memmert, Venice, Italy). For all the samples acidity, peroxide number and Tbars analyses were recorded over the time.

Sample analysis

Oxidation status analysis of hazelnut cream

The analyses of the oxidation status of the hazelnut cream were carried out partly on the product as such and partly on the oil mechanically extracted from the paste. A few grams of cream were taken from each sachet and stored in 50 mL falcon containers and centrifuged (HERMLE Z 326 K, Naples, Italy) for 45 min at a 9000 rpm. Oil was separated from the solid part, and a second centrifugation to remove impurities (9000 rpm

for 10 min) was applied. The oil obtained was used for the determination of acidity and number of peroxides.

Titratable acidity of hazelnut paste

Free fatty acid (FFA) content was determined according to the procedure described by AOAC (2000). An aliquot of oil was combined with ethanol: diethyl ether (1:1 v/v), in a ratio of 1:10 w/v. Three to four drops of phenolphthalein were then added, and the resulting solution was titrated with 0.1 NaOH until the phenolphthalein turned pale pink (persistent pale pink coloration for at least 10 seconds). The FFA value (oleic acid g/100g) was calculated using the formula:

$$FFA = \frac{V * N * 28.2}{m} \quad (2)$$

where V is the volume, in mL, of the titrated NaOH solution used, N is the normality of the NaOH solution, m is the weight in grams of the sample analyzed. The titrations were carried out in triplicate.

Number of peroxides by thiocyanate method on hazelnut cream

The method used was developed from studies carried out by (Mihaljević, Katušin-Ražem, & Ražem, 1996) on the use of the ferrous reagent ($Fe2+(NH4)_2(SO4)_2$) as a primary oxidation indicator. 0.3 g of 0.6% ammonium thiocyanate (NH_4SCN) was dissolved in 2 mL of distilled water and made up to 50 mL with acetone. After that, a 0.1% solution of ferrous reagent ($Fe2+(NH4)_2(SO4)_2$) was prepared with ammonium thiocyanate and kept in the dark under continuous stirring for 2 h. Approximately 100 mg of oil to which 900µL of acetone and 2 mL of ferrous reagent in ammonium thiocyanate was added, was used for the preparation of triplicate samples. The blank was made with acetone and ferrous ammonium thiocyanate reagent in a 1:2 ratio. The absorbance of the samples was measured at a wavelength of 485 nm. For the quantification of the number of peroxides, a calibration line was prepared from a stock solution of ferric (III) chloride (Fe3+Cl3) 0.9 mg/mL: 1:10 solution with acetone, in falcon 15. Then a 1:50 diluted solution called D50 (from stock 0.9 mg/mL) was prepared in acetone and the following points of the line were

prepared with a final volume of 3 ml. The formula used to calculate the peroxide number consists of a conversion of the read absorbances into µg Fe^{3+}/ml which in turn is converted into meqO_2/kg oil:

$$F = \frac{Abs - c}{m} \quad (3)$$

$$Np = \frac{F * 3}{2p} * 1000 \quad (4)$$

where F is the µg Fe^{3+}/ml oil, Abs is the absorbance at 485nm, m is the angular coefficient of the calibration line, c is the intercept of the calibration line, Np is the meqO_2/kg oil, p is the mg oil.

Tbars

For the determination of malondialdehyde (MDA), the TBA test (Tbars) was performed as reported by Botsoglou, Fletouris, & Papageorgiou, (1994) with appropriate modifications. Briefly, 1 g of hazelnut cream was added with deionized water and hexane in ration 1:2 v/v ratio. The mixture was treated with ultraturrax (Ultra Turrax®, IKA T18, Milan, Italy) and 2.5 ml trichloroacetic acid (25% TCA) was added and the solution and stored for 15 min in refrigeration. Subsequently, centrifugation (Hermle Z 236K Naples, Italy) at 4000 rpm 5 minutes. After removing the excess with hexane, an aliquot of 3.5 ml of supernatant was added to 1.5 ml thiobarbituric acid (0.6 %) and incubated in a thermostatic bath for 30 minutes at 70 °C. After cooling for 35 seconds in cold water, the samples were diluted 1:5 to meet the linearity parameters of the calibration curve (range from 0.027 to 1.036)

Finally, the absorbance was recorded using a Spectrophotometer (Shimadzu UV-1601 Uv-Visible Spectrophotometer) at 532 nm, against a blank prepared under the same conditions as the sample. Three replicates were conducted for each formulation.

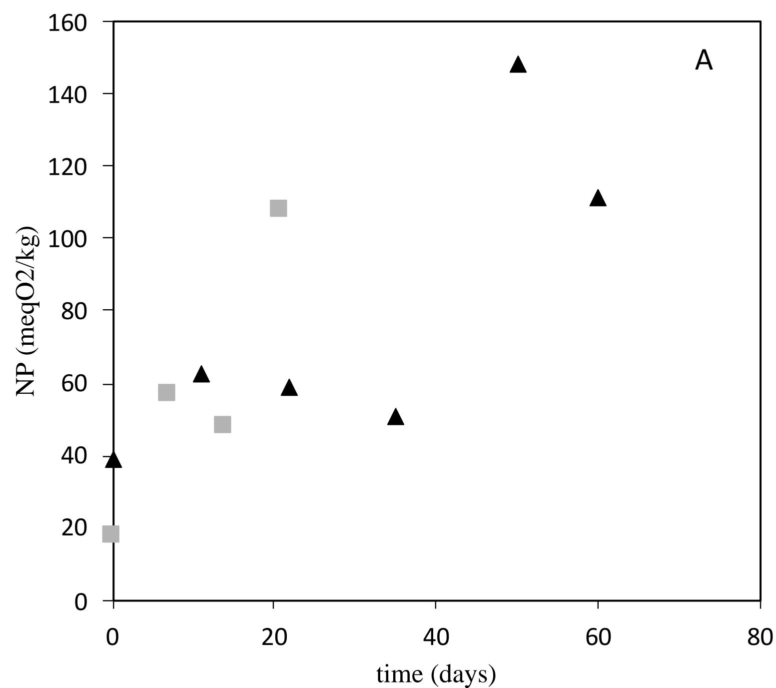
Data Analysis

All the analysis were carried out in triplicate. One-way analysis of variance (ANOVA) and Duncan's multiple comparison test were performed to assess significant differences between the pooled samples. Data were processed using SPSS software for Windows, version 17.0 (SPSS Inc., Chicago, IL, USA).

5.2.4. Results and discussions

Study of kinetics and the effect of temperature on hazelnut cream stored into glass jar.

In the first experimental phase, two indicators of the oxidation status of hazelnut paste stored at 70°C and 40°C were analyzed: the number of peroxides (NP meqO_2/kg) and free fatty acids (FFA oleic acid g/100g) and respective effect of temperature (Arrhenius law) are showed in Figures 5.1A, B; 5.2A, B respectively. Generally, it can be assumed that the oxidation kinetics for both quality indices NP and FFA increased during a storage time. Into details in Figure 5.1A, the number of peroxides increases over time. In both batches, at 70°C and 40°C, the number of peroxides is very high already at time 0 with a value of 17.7±5.9 and 38.9±2.2 meq O_2/kg oil respectively. After that, while for the 70°C batch, the NP reaches a maximum level of 107.4±5.1 after 21 days, in the 40°C cream it grows over time, first slowly for the first 35 days, and then has a significant growth up to 60 days, with a peak at time 50 days of 148.2±19.7 (fig. 5.1A).



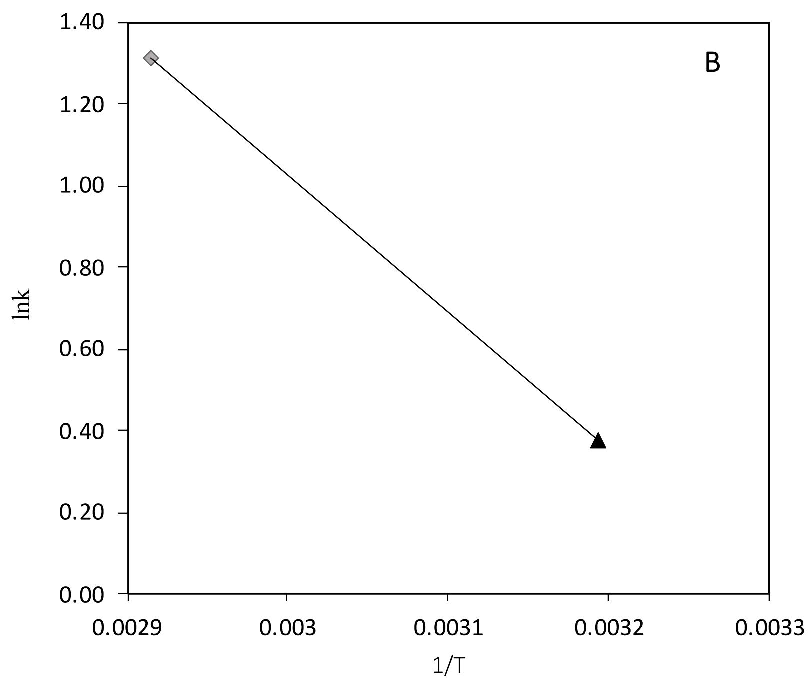


Figure 5.1. (A) Number of peroxides of hazelnut cream packed at 40°C (▲) and 70°C (■). (B) The effect of temperatures (40°C and 70°C) according to Arrhenius' law on the variation in the number of peroxides. Line showed the linear trend.

The variation in food quality, expressed in NP are influenced by temperature, the dependence of which has been studied using the Arrhenius equation (4):

$$K = k_0 e^{\left(-\frac{Ea}{RT}\right)} \quad (4)$$

where: K is the rate constant, k_0 is a pre-exponential factor, constant for not too high temperature variations, Ea is the activation energy, which is also constant for not too high temperature variations, R is the gas constant, T is the absolute temperature

Figure 5.1B shows the linear relationship between the reaction speed and the reciprocal of the absolute temperature, thanks to which it was possible to extrapolate an activation energy for the oxidative phenomenon of the hazelnut cream reported in table 5.1.

Table 5. 1. Parameters for calculating the activation energy (Ea) for number of peroxides

T °C	1/T (K)	k_o meqO^2/kg/day	Ea (kJ/mol K)
70*	0.00291418	3.7	27.69
40*	0.00319336	1.46	

(* stored into glass jar)

The evolution of FFA of hazelnut cream at 40°C and 70°C are reported in Figure 5.2A. During storage an increase of the acidity value are shown. Into details the FFA value increase from 1.71±0.12 at time 0 to 2.19±0.11 g oleic acid/100g at day 21 at 70°C. The increasing trend in time of the free fatty acids value can be due to the exposure of the cream to a higher incubation temperature that accelerated the oxidative enzymatic phenomena. On the other hand, the trend is more constant for hazelnut cream stored at 40°C with an average value of 1.83±0.19 g oleic acid/100g. Figure 5.2B shows the linear relationship between the reaction speed and the reciprocal of the absolute temperature; as was done for the number of peroxides, an activation energy was also calculated for the change in acidity (table 5.2)

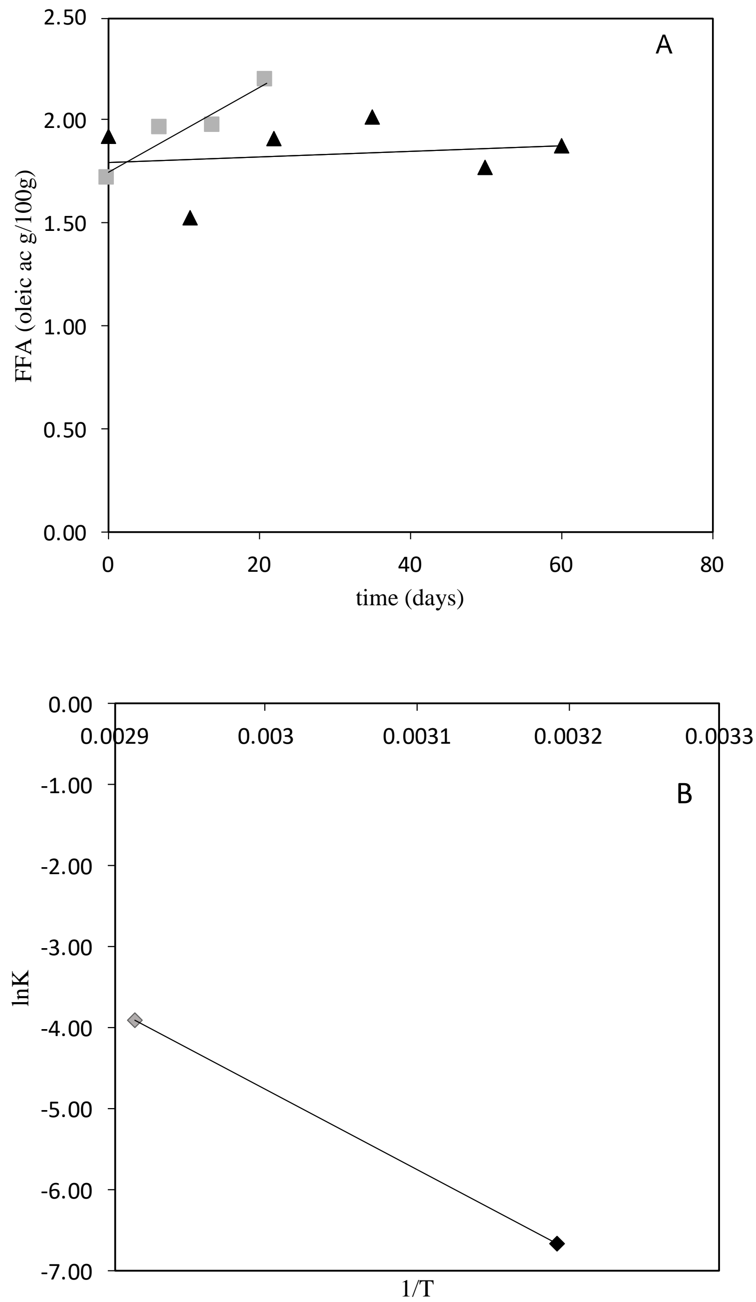


Figure 5.2. (A)Free fatty acid of hazelnut cream packed at 40°C (▲) and 70°C (■) ; (B) the effect of temperatures 40°C and 70°C according to Arrhenius' law on the variation in the number of peroxides. Lines showed the linear trend.

Table 5.2. Parameters for calculating the activation energy (Ea) for free acidity.

T °C	1/T (K)	ko (oleic acid g/100 g/day)	Ea (kJ/mol K)
70*	0.00291418	0.0205	82.13
40*	0.00319336	0.0013	

(* stored into glass jar)

Study of the antioxidant activity of the realized active film

Figure 5.3 shows the results of the DPPH radical scavenging activity CH/SC/REO/Nylon, where the y-axis shows the percentage of radical inhibition and the x-axis shows the time, defined as the hours of contact between the sample and the 30ppm DPPH solution, which favors efficient extraction of the antioxidants. The film shows, after 2.5 h of contact with the radical solution, a % inhibition of 11.4% and after 24 h the inhibition increases reaching a maximum value of 46.5%. The inhibition percentage values remain more or less stable over the following 24 hours (43.62%). By predicting how much rosemary oil is present in each sachet in contact with the hazelnut paste, it was seen that the theoretical amount corresponds to an inhibition of about 45%, the same result as obtained by the in vitro test after 24 hours. It can therefore be assumed that all the antioxidant added to the sachet migrates into the food matrix.

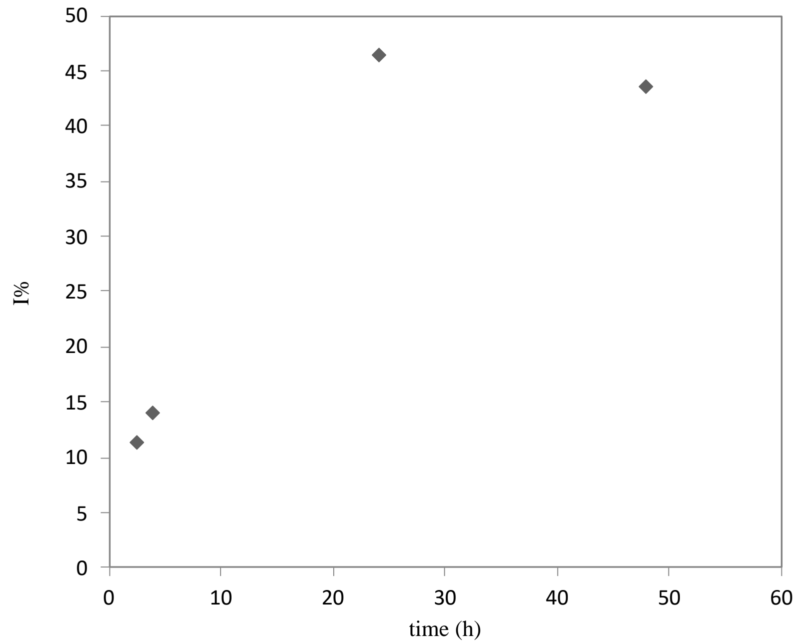


Figure 5. 3. Results of the antioxidant activity of CH/SC/REO/Nylon film as function of the time.

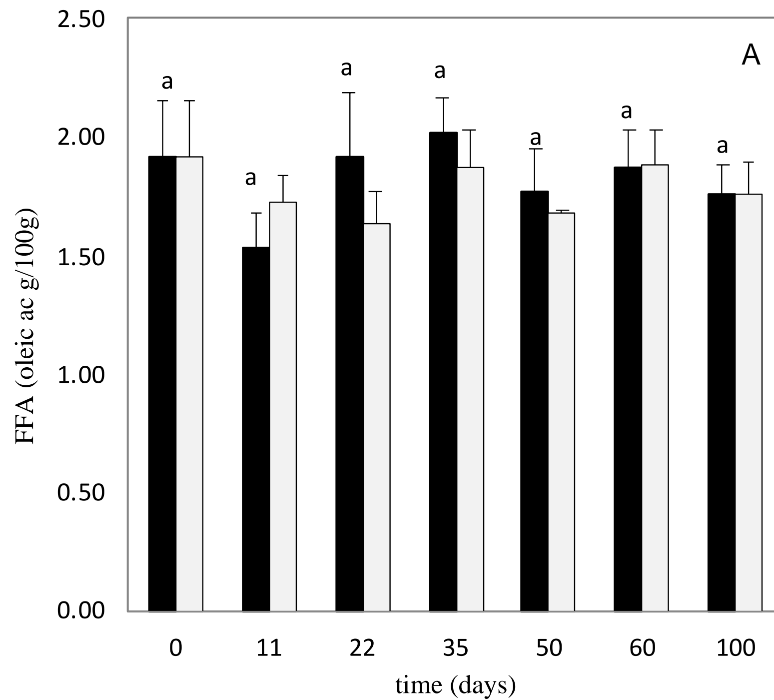
Oxidation status analysis of hazelnut cream stored into active packaging

Free fatty acidity

The FFA evolution of hazelnut cream stored into CH/SC/REO/Nylon film at 40°C and 4 °C are reported in Figures 5.4A, 5.4B respectively. Generally, the free fatty acid (FFA) content of the oil increasing during a storage time and in function of temperature as confirmed by (Yalcin, Toker, & Dogan, 2012). Into details the titration performed with NaOH on hazelnut oil extracted from the cream showed an already very high free fatty acid content at time zero with a value of 1.92±0.24 oleic acid/100 g (fig. 5.4A). During storage at 40°C, the FFA value remained almost constant with a slight increase for the control (2.02±0.14) at time 35 days and a final decrease at time 100 days (1.76±0.14) also for the control. In general, there were no significantly differences ($p > 0.05$) of FFA between active and control packaging, except for a decrease in the index to 1.64±0.11 at time 11, which was not statistically significant according to the ANOVA analysis of variance ($p > 0.05$). The ANOVA analysis did not find a significant effect for either the sample effect (active and control) and the time effect.

It can be concluded that the acidity of the cream at 40°C for 100 days of storage was maintained with an average of 1.83±0.19 for the control and 1.78±0.13 for the active.

At 4°C the FFA value remains almost constant with a mean of 1.79±0.15 for the control and 1.78±0.13 for the active, values very similar to the high starting value at time 0 (fig. 5.4B). Thus, no statistically significant differences ($p>0.05$) are revealed by ANOVA analysis for either the sample effect or the time effect. This can be explained by the storage conditions, which, at refrigeration temperature, slow down the enzymatic oxidation process of lipases. This behavior are confirmed by (Almeida *et al.*, 2019), studies the FFA of palm oil conditioned for 12 months at 4-8°C (FFA values remain constant in the first weeks of storage (0.13±0.01 oleic acid/kg oil), with higher values only for fresh oil at temperatures of 26-32°C (3.06±0.7 in the first three months of storage).



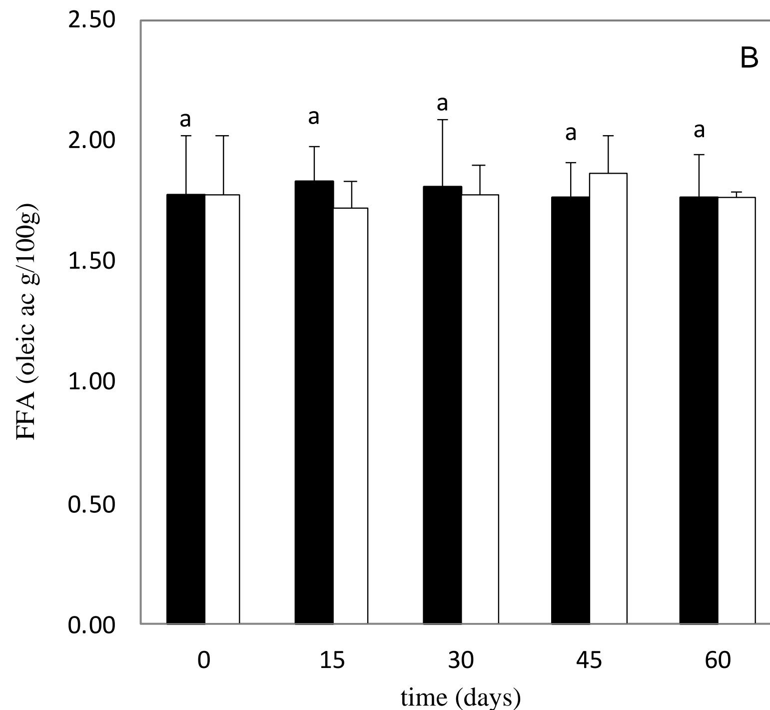
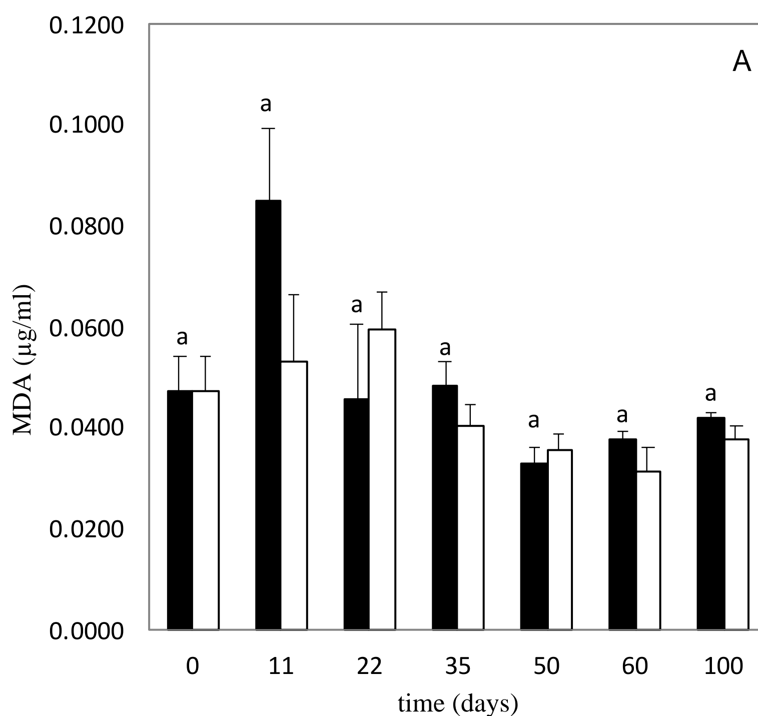


Figure 5.4. (A) Free fatty acidity of hazelnut cream into CH/SC/REO/Nylon film (□) and control (■) stored at 40°C for 100 days and (B) at 4°C for 60 days .

Tbars analisys

Malondialdehyde is a decomposition product of peroxides, and its presence in food is therefore used as an index of secondary oxidation. In Figure 5.5A it can be seen that formation of MDA during the shel-life of hazelnut cream are very low. At time 0 of the batch stored at 40°C there is an MDA value (µg/ml) of 0.0473±0.0068 which remains more or less constant over the 100 days of storage except for a peak after 11 days for the control sample (0.0848±0.0145). According to the ANOVA analysis of variance, there were no statistically significant differences for either the time or sample effect ($p>0.05$). The trend in MDA at 4°C (Figure 5.5 B) is also almost constant with a statistically significant effect at time 45 days where there is a decrease in MDA in the active sample compared to the control (0.047±0.0001 compared to 0.061±0.0004). The minimal formation of malondialdehyde already at time 0 (in both batches), may be due to low % of polyunsaturated fatty acids within the food matrix. Furthermore, according to some

studies carried out by (Chandrasekara & Shahidi, 2011) on the oxidative stability of cashew nut oil stored for 72h at 60°C, the MDA content remains low (from 0.02 ± 0.06 to 0.11 ± 0.01) throughout the storage period and this is due to a higher oxidative stability given by the products of the Maillard reaction released during the roasting phase. Moreover, some paste ingredients can interfere with the formation of malondialdehyde, as demonstrated by the study of (Torre, 2016) on soy-based creams: soy flour possesses antioxidant compounds such as isoflavone glycosides that allow a resistance of the lipid matrix with respect to the oxidative phenomenon, so that low values of MDA (0.1-0.5 µg/ml) were found during storage of the cream at 40°C for 4 weeks.



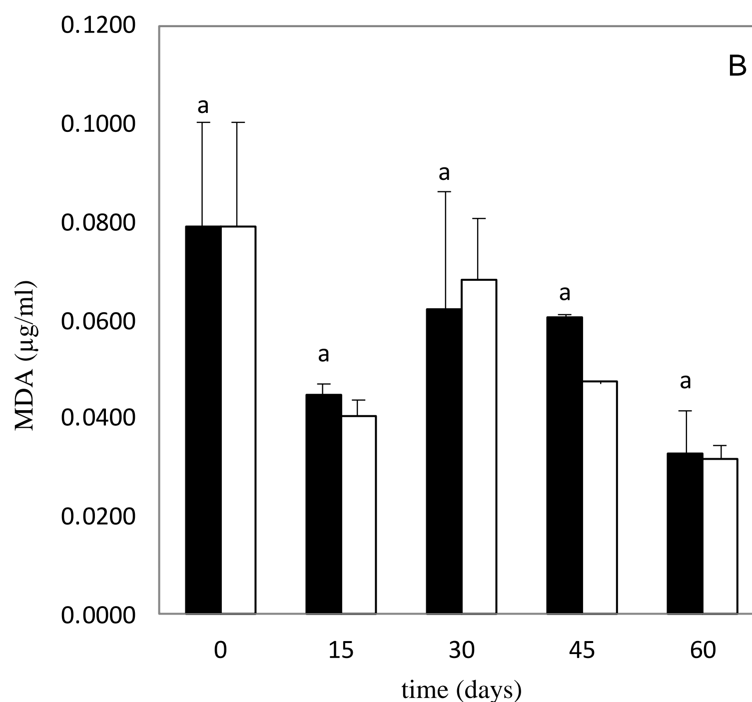
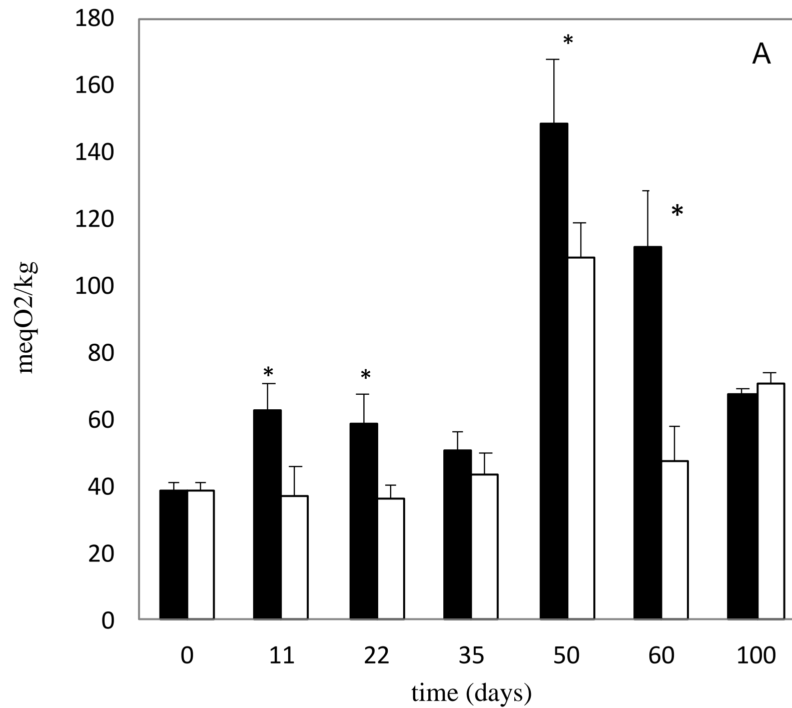


Figure 5.5. Free fatty acidity of hazelnut cream into CH/SC/REO/Nylon film (□) and control (■) stored at 40°C for 100 days (A).and at 4°C for 60 days (B).

Number of peroxides

As can be seen in Figure 5.6A, the number of peroxides of hazelnut paste packaged at 40°C for 100 days presents a high value already at time 0 both for the active and control sample (about 40 meqO2/kg of oil). The number of peroxides for the control sample increases with a maximum peak observed around time 50 (about 150 meqO2/kg of oil), but according to the statistical analysis obtained by means of ANOVA tests, statistically significant differences for the time effect can only be detected at times 11, 22 and 60, marked in the graph with an asterisk. It can therefore be said that the active packaging has a significant effect on the short times, after which the antioxidant effect diminishes. This can be explained by a consumption over time, after 60 days, of the antioxidant dissolved entirely in the food matrix. At 4°C, as shown in Figure 5.6 B, the trend in the number of peroxides is constant for the first 30 days, with no statistically significant differences by ANOVA analysis ($p > 0.05$). The trend is significant after the 45th day

(marked on the graph with the letter b) with a value of 80.95±2.20 for the control and 70.07±12.89 for the active at time 45, and at time 60 days 108.46±9.76 for the control and 101.8±1.8 for the active. Studies carried out by Özcan & Arslan, (2011) on the antioxidant effect of rosemary essential oil on hazelnut oil stored for 14 days at 50°C, showed significant differences between active and control only with an essential oil concentration of 0.50% and only after the tenth day of storage with values of 332.5±13.7 for the active (rosemary essential oil 0.50% added to hazelnut oil) and 413±29 for the control (hazelnut oil without antioxidant). It must be taken into account, however, that (Özcan & Arslan, 2011)added the antioxidant directly into the hazelnut oil extracted from the fruit and not into the coating in contact with the hazelnut paste prior to extraction of the oil from it, as is the case in this work. Probably therefore, in order to obtain a more important effect of the antioxidant, films with a higher concentration of rosemary oil should be designed and its diffusivity within a fatty matrix such as the hazelnut paste should be studied.



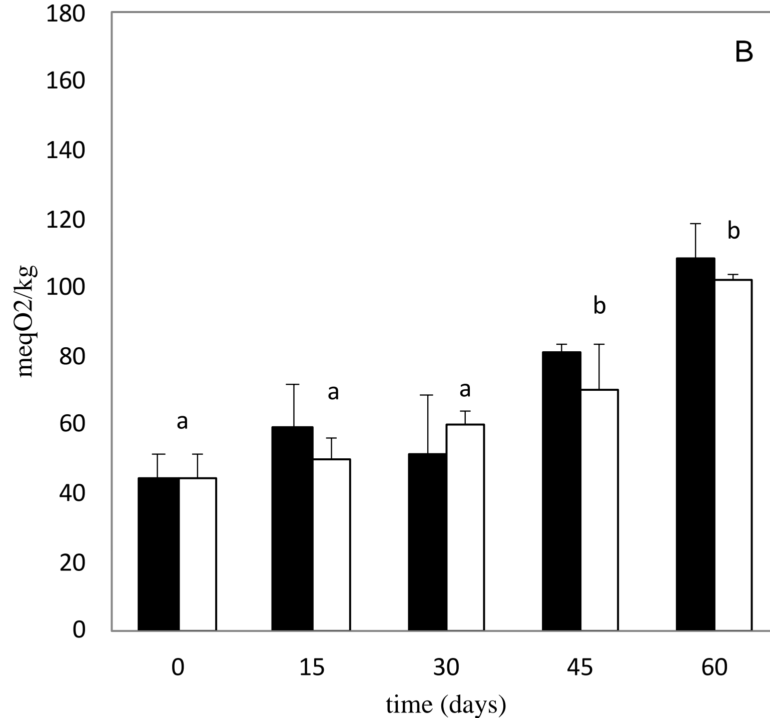


Figure 5.6. Number of peroxides of hazelnut cream into CH/SC/REO/Nylon film (□) and control (■) stored at 40°C for 100 days (A).and at 4°C for 60 days (B).

5.2.5. Conclusions

The oxidation of hazelnut paste can be well described by the peroxide number indicator. From the variation of the peroxide number as a function of time, it can be assumed that the oxidation of hazelnut cream follows pseudo-zero order kinetics. The values of the kinetic constant is 1.46 meq O_2/kg/day and 3.7 meq O_2/kg/day at 40°C and 70°C, respectively, while the activation energy is 27.69 kJ/mol K. The active films based on polyamide and coated with chitosan, caseinate, and rosemary oil showed good antioxidant capacity in vitro by DPPH test. In vivo, they also showed good antioxidant activity to retard oxidation of pastes during short storage times. The results, although preliminary, are promising. Further investigations are needed to fully understand the mechanism of antioxidant release into the food matrix and the effect of rosemary essential oil on the sensory properties of hazelnut cream.

5.2.6. References

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5.3. Development of active antioxidant packaging for the preservation of Grana Padano DOP

5.3.1. Abstract

In order to develop an active monolayer coating on commercial support film based on sodium caseinate enriched in gallic acid, the objective of this work was to investigate the effect the active packaging on the quality parameters of Grana Padano cheese at different storage condition. Antioxidant test on the active film was conducted before and after the shelf life using DPPH radicals. The evolution of the lipid oxidation (Malondialdeide, MDA) was monitored by Thiobarbithuric acid (Tbars). The determination of trace amounts of volatile organic compounds (VOCs) using a headspace solid phase microextraction (HS-SPME) and gas chromatography–mass spectrometry (GC–MS) was conducted. Furthermore, color properties, headspace and sensory analysis were investigated. Results showed that the antioxidant activity of the realized film increased during the contact time of the active film with the radicals. Active film affect positivly the oxygen content at time 7 at 40°C and time 60 day at 25°C. Lipid oxydation are positvly affected at time 7 day into sodium caseinate/ gallic acid/polybuthylene succinate film (SC/GA/PBS) samples stored at 40°C and 14 day into SC/GA/Nylon samples at 25°C. Sensory analisysis descriminate the presence of Gallic acid into a packaging. Volatile organic compounds confirmed the evolution of ketones and aldeide after 14 day at 40°C extending the shelf life from 7 day to 14 day at this storage condition only for SC/GA/PBS samples. Thus, SC/GA/PBS film are a potential candidate as active food packaging for repined Cheese product such as Grana Padano.

Keywords: sodium caseinate, gallic acid, lipid oxidation, volatile organic compounds MDA, coating

5.3.2. Introduction

Grana Padano cheese is an Italian hard cheese with a delicate and characteristic aroma, made from raw bovine milk, partly defatted by creaming, with the addition of natural starter, cooked at 53 °C and matured for at least 12 months (MIPAAF, 2012). Because of their composition, one of the changes that can occur in cheeses, especially mature cheeses, is oxidation of the fatty acids. Lipid oxidation, lead to the formation of molecules such as aldehydes, alcohols and esters which are responsible for off flavors into the product. Oxidation processes in dairy products may also degrade vitamins and b carotene and lead to the formation of cholesterol oxides and conjugated linoleic acid (Shan, Cai, Brooks, & Corke, 2011;Shantha & Decker, 1993).

For this purpose active packaging systems which aims to release antioxidants to protect food against oxidation can be applied for a preservation of the quality properties of cheese.

According to EC Regulation 1935/2004 "*active food contact materials and articles are materials and articles intended to prolong the shelf life or maintain or improve the condition of packaged foodstuffs. They are designed to deliberately incorporate components that release or absorb substances into or from the packaged food or its environment"* (European Commission, 2004). The application of active film or coatings can protect food products from moisture migration, microbial growth on the surface, light-induced chemical changes and oxidation of nutrient (Jalilzadeh, Tunçtürk, & Hesari, 2015).

The shelf-life of cheeses can be extended by adding chemical preservatives such as essential oil and or single polyphenols. The use of natural antioxidant in foods has been widely documented, resulting in shelf life extension for several foods (Carrizo, Taborda, Nerín, & Bosetti, 2016;Yang, Cao, Kim, Beak, & Song, 2018;Priyadarshi, Sauraj, Kumar, & Negi, 2018; Zhang, Liu, Sun, Wang, & Li, 2020). Hence, natural antioxidants like plant

derived essential oils are being incorporated in foods and packaging to reduce lipid oxidation. However the addiction of essential oil can modified the organoleptic aspect of the stored food (Pateiro, Barba, Domínguez, & Sant, 2018; Prasad & Kochhar, 2014; Karabagias, Badeka, & Kontominas, 2011). To reduce a sensory modification of the product related to an antioxidant addiction. The demand for natural products such as single polyphenols increase. In this contest gallic acid is a phenol widely present in various sources such as nuts, tea, and grapes. The three hydroxyl groups are responsible for determining the strong antioxidant capacity of the phenolic compounds (Nenadis, Wang, Tsimidou, & Zhang, 2004; Khan, Khan, & Khan, 2015).
Different study was conducted on fresh cheese. However only few focused on ripened cheese. For this reason, the aim of this work was to investigate the effect of active coating (sodium caseinate enriched in gallic acid) deposited on commercial support film on the quality parameters of Grana Padano cheese at different storage condition. To reach this goal: (i) the solubility of gallic acid was tested in order to incorporate it into polysaccharide and/or protein matrices, such as chitosan (CH), zeins and sodium caseinate (CS); (ii) SC/GA coating was deposited on Polybutylene succinate (PBS) and Polyamide film; (iii) the antioxidant activity of the active film was evaluated at time 0 and after a storage in contact with cheese; (iv) active film was used to wrap Grana Padano cheese and product quality was monitored by chemical, physical and sensory properties at different time.

5.3.3. Materials and methods

Materials

Sodium caseinate (SC), chitosan (CH), zeine, tris hydroxymethylaminomethane chlorohydrate (tris-HCL), gallic acid, DPPH radical (2,2'-Diphenyl-1-picrylhydrazyl) were purchased from Sigma-Aldrich & Co (Milan, Italy). Absolute ethanol 96% (v/v), purchased from VWR Internationl SrL, (Milan, Italy). Glycerol was purchased from Merck KGaA EMD Millipore Corporation, Germany. Grana Padano "Ferrari" was

purchased from Conad (Maddaloni, Caserta, Italy). The polymers used are: Polybutylene Succinate (PBS) 50 um (Icimen 2, Naples, Italy) and Nylon (Polyamide) 15 um (Icimen 2, Naples, Italy). Trichloroacetic acid (TCA) and thiobarbituric acid (TBA) were supplied by Carlo Erba.

Film-making procedure

CH/GA film forming solution (FFS)

Briefly, 0.50 g of gallic acid was dissolved in 60 ml of ethanol; the ethanol solution with the dissolved gallic acid was poured into a beaker fitted with a magnet, and 60 ml of acetylated water was added to this solution drop by drop using a burette. After the acetylated water was added to the solution, chitosan (CH) was dissolved in the solution to give a 2% (w/v) solution. Glycerol was added in an amount equal to 10% of the solids. The chitosan solution was then poured in quantities of 5 ml onto Petri dishes and placed in a climatic chamber at 40 °C and 50% relative humidity overnight. The dry films (cast) were removed from the plates and stored in jars at 50% relative humidity.

Zein/GA FFS

In a Petri dish, 11.8 g of zeins were weighed and 68.5 ml of ethanol and stirred at 200 rpm for 25 minutes. Subsequently, 4.22 g of glycerol was added to the solution and heated to 78.5 °C for 5 minutes. The solution was then cooled to room temperature and 1.47 g of gallic acid was added. The solution was then subjected to ultraturrax for 4 minutes at a power of 4 and was then cast in quantities of 5 ml onto Petri dishes and placed in a climatic chamber at 40 °C and 50% relative humidity overnight. The dry films (cast) were removed from the plates and stored in Jars at 50% relative humidity.

SC/GA FFS

The active coating was realized in according to (Valentino *et al.*, 2020). Briefly, a 4% SC solution was prepared by dissolving the SC powder in an HCl tris buffer at pH 8 by magnetic stirrer for 4 h at room temperature, then 10% of glycerol, referred to solid, was

added. Then, 0.005 g of gallic acid (GA) powder was dissolved into 1 mL of HCI tris buffer using a shaking stirrer (IKA ™ MS 3 Digital Vortex, -Werke GmbH & Co. KG, Staufen, Germany). Then, the GA solution was added to SC solution (100 mL) and homogenized under stirring and light protection, to obtain the solution at 50 μg·mL−1 of GA. The active Film forming solution was prepared as desribed previosly and coated on a support film. Briefly, 2 ml of two different solutions: SC/GA solution were deposit using a mayer rod coater technique (with a gravure of 24um of deposition), on a support of PBS and Nylon film. The film was dried under controlled condition and stored without oxygen before testing to avoid oxydation of the active molecules.

Choice of active solution

For the shelf-life study only SC/GA was choosed as active coating and deposited on a support film of PBS and Polyamide film. Since in the other two formulations (CH and Zeine) gallic acid shows a low dispersion capacity.

Determination of the antioxidant capacity of the realized film

The DPPH method was used to determine the free radical scavenging capacity of SC/GA/Nylon, SC/GA/PBS a casting film of SC/GA.

For SC/GA casting film after 24h of extraction into ethanol, four different dilutions of analyzed samples in ethanol (96%) were prepared. Then, 2.4 mL of 30 ppm ethanolic solution of DPPH were mixed with 1 mL of sample's dilution. The sample was analyzed after 10 min using a spectrophotometer at 517 nm against ethanol as reference. The results were expressed as inhibition concentration (IC50) in $\mu g \cdot mL^{-1}$ (Gülçin, Huyut, Elmastaş, & Aboul-Enein, 2010).

For the bioactive multilayer film, the methods (Hromiš *et al*., 2014) with a little modification were used. Briefly, 2.4 mL of 30 ppm ethanolic solution of DPPH were mixed with 100 mg of bioactive film. Samples were stored for 2.5 and 4 h at room temperature in the dark. For all samples, the absorbance was measured at 517 nm using a UV–Vis spectrophotometer (V-550 Jasco Inc., Tokyo, Japan) and pure ethanol as

reference. The results were expressed as percentage of DPPH free radical activity (1):

$$I\% = \cdot \frac{Absc - Abss}{Absc} * 100 \quad (1)$$

where Absc is the absorbance of the control and Abss is the absorbance of the sample. The assay was carried out in triplicate.

Preparation of samples

25 grams of grated grana padano cheese was packed in pouches of SC/GA/PBS and SC/GA/Nylon and sealed using a chamber packaging machine (Lavezzini, Milan, Italy). Cheese samples packed in PBS were stored at 40 °C and analyses were performed on 0, 7 and 14 day of storage. Samples wrapped into SC/GA/Nylon were stored at room temperature and tested after 0, 14, 30, 45 and 60 days; temperature and humidity, were monitored using a data logger. As control film without coating were used.

Sample analysis

Headspace gas composition

The oxygen fractions within the headspace of the packages were evaluated using the Dansensor instrument (Checkmate 9900 O2/CO2 Ronnedevej, Denmark), which withdraws a volume of approximately 3 * 10-6 ^{m3} of gas for each measurement. Each package was used for a single measurement.

Colorimetric analysis

The color of the film was measured using a colorimeter (Minolta Chroma Meter, CR 300,Osaka, Japan). Results are reported as Hunter parameters L* (from 0 = black to 100 = white), a* (−a* = greenness to + a* = redness), and b* (−b* = blueness to + b* = yellowness). For the colourimetric analysis of cheese, the parameter b* was taken into account. 5 measurements were made on each cheese sample.

Thiobabituric acid (TBARS) analysis

The Thiobabituric acid reactive substances (TBARS) assay was used to study cheese oxidation. TBARS is an analytical method that allows the detection of damage produced by oxidative stress on the product, it allows the assay of one of the end products of decomposition of primary and secondary lipid peroxidation, malondialdehyde (MDA). The analyses were carried out following the method of (Botsoglou, Fletouris, & Papageorgiou, 1994) with appropriate modifications. For this purpose, 5 g of cheese were mixed with 10 ml of aqueous trichloroacetic acid solution (25%) and 10 ml of hexane, which were then homogenised by vortexing for 1 minute. For hazelnut paste 1g of product was added with distilled water and hexane in a 1:2 v/v . The mixture was treated with ultraturrax (Ultra Turrax®, IKA T18, Milan, Italy) and 2.5 ml of 25% trichloroacetic acid (TCA) was added and the solution. Both solution, cheese and hazelnut paste were stored for 15 minutes in the refrigerator. Then centrifuged (HERMLE Z 236K Naples, Italy) at 4000 rpm for 5 minutes. 1 ml of supernatant from cheese was added to 0.430 ml of aqueous thiobarbituric acid solution (TBA) (0.8%) and kept in a silicone bath at 90 °C for 30 minutes. 3.5 ml of supernatant from hazelnut sample was added to 1.5 ml of TBA (0.6 %) incubated in a thermostatic bath for 30 minutes at 70 °C. After cooling for 35 seconds in cold water, the samples were diluted 1:5. The absorbance was recorded using a spectrophotometer (Shimadzu UV-1601 Uv-Visible Spectrophotometer) at 532 nm, against a blank prepared under the same conditions as the sample. Three replicates were conducted for each formulation.

Analysis of volatile organic compounds (VOCs)

Extraction and analysis of VOCs were performed using SPME-GC / MS, following the method in article a with appropriate modifications. The solid phase microextraction device(SPME) equipped with a 50/30 µm thick divinyl benzene/carboxy/polydimethylsiloxane fibre coated with a 2 cm long stationary phase was used. Briefly, 1.5 g of frozen grated cheese was transferred into a 10 ml vial to which 3 ml of deionised water and 15 µl of 2-methyl-3-heptanone was added as an internal

standard (408 mg/L). The vial was sealed and the sample with the standard was subjected to continuous stirring with a magnetic stirrer for 10 minutes at 50 °C. A Supelco Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) fibre was then introduced into the vial and agitation continued for 1 hour at 50 °C.

The SPME fibre was introduced directly into the GC/MS injector, where thermal desorption of the analytes was performed at 250 °C for 10 minutes. A 6890N GC equipped with a 5973 mass detector was used. VOCs were separated on a 30 m × 0.250 mm capillary column coated with a 0.25 μm film of 5% diphenyl l95% dimethylpolysiloxane. Splitless injection was used for the samples. The oven temperature was maintained at 40 °C for 2 minutes and increased from 40 °C to 160 °C at 6 °C/min and from 160 to 210 °C at 10 °C/min, temperature maintained for 10 minutes.

The temperatures of the injection source and the ion source were 250 and 230 °C, respectively. Helium was used as a carrier gas at a flow rate of 1 mL/min. The energy of the ionising electrons was 70 eV and the scanned mass range was 40-450 amu in full scan acquisition mode. Compounds were identified using the NIST Atomic Spectra Database version 1.6 and verified by retention indices. VOCs were calculated using the internal standard method and were expressed as mg/kg cheese.

Sensory analysis: Triangular test

A triangular test was carried out according to UNIU590A2520, 2001. To perform the test, 36 panel members were recruited. The parameters used to discriminate the samples used was odor. The samples analyzed was grana Padano cheese stored for 7 days at 40°c into SC/GA/PBS film. Briefly, 3 g of grana Padano cheese was inserted into a glass tube and coded. To determinates the different between the samples, the panel was informed that only one of the three samples was different and to identify it. The order in which the samples were presented was randomized. Data processing was carried out by comparing the number of correct answers with the value given in a double-entry probability table. In correspondence of the number of panel member who took the test or the number of judgments (number of judges times the number of responses) for the different significance levels, indicates the minimum number of correct answers.

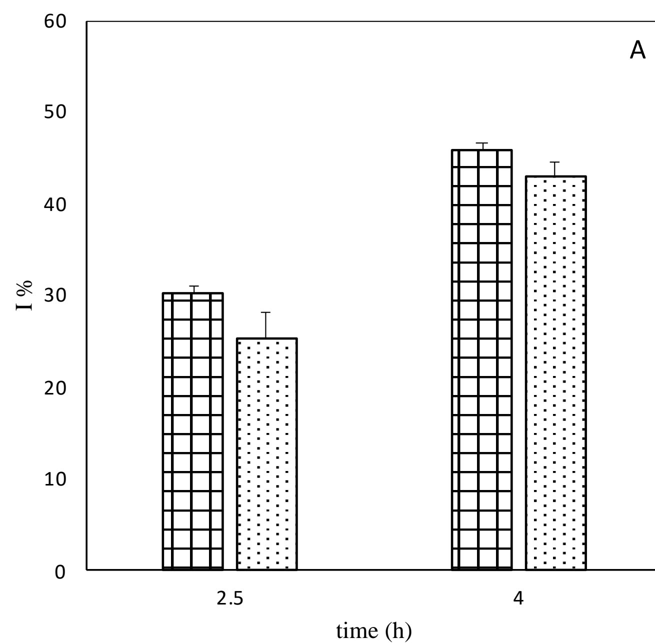
Data Analysis

One-way analysis of variance (ANOVA) and Duncan's multiple comparison test were performed to assess significant differences between the pooled samples. Data were processed using SPSS software for Windows, version 17.0 (SPSS Inc., Chicago, IL, USA).

5.3.4. Results and discussions

Evaluation of antioxidant capacity of caseinate with added gallic acid

In Figure 5.1A and B, the antioxidant capacity of the active films are shown. For a better unsterstading the IC50 of a SC/GA dry film (df) was calculated, and compared to the IC50 of pure GA. The results showed that SC/GA df have an IC50 of 34ug/mL. This value are close to the IC50 of pure GA (Valentino *et al.*, 2020). In fact, we used a quantity of GA in the film to get a 50% of I% and by including the GA in the SC, the antioxidant activity was confirmed. Generally, the antioxidant activities increased during the contact time of the active with the radicals. Into details the I (%) of SC/GA/PBS film increase from 30.19% after 2.5 h to 45.95% after 4 h of contact with a DPPH radicals. In the case of SC/GA/Nylon film the I(%) increased to 25.19 % up to 42.84% after 2.5 h and 4 h respectivly. In addition, the antioxidant capacity of Nylon/SC/GA film was evaluated at days 14 and 30 (fig. 5.1B). Results show a I% of 13.5 ± 0.7% and 10.93 ±0.58% after 14 and 30 day of storage with cheese at room temperature. The analysis carried out by the DPPH assay showed that the percentage of inhibition of the film packets in contact with the radical solution is very low, so it can be deduced that most of the gallic acid added to the protein matrix and subsequently deposited through the coating deposition technique has migrated to the food. The low percentage of gallic acid still present within the coating is due to the gallic acid incorporated within the caseinate protein network.



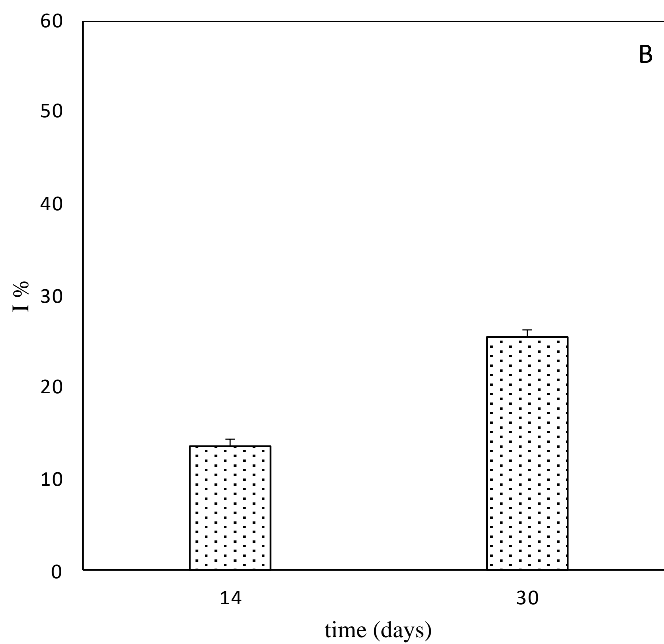
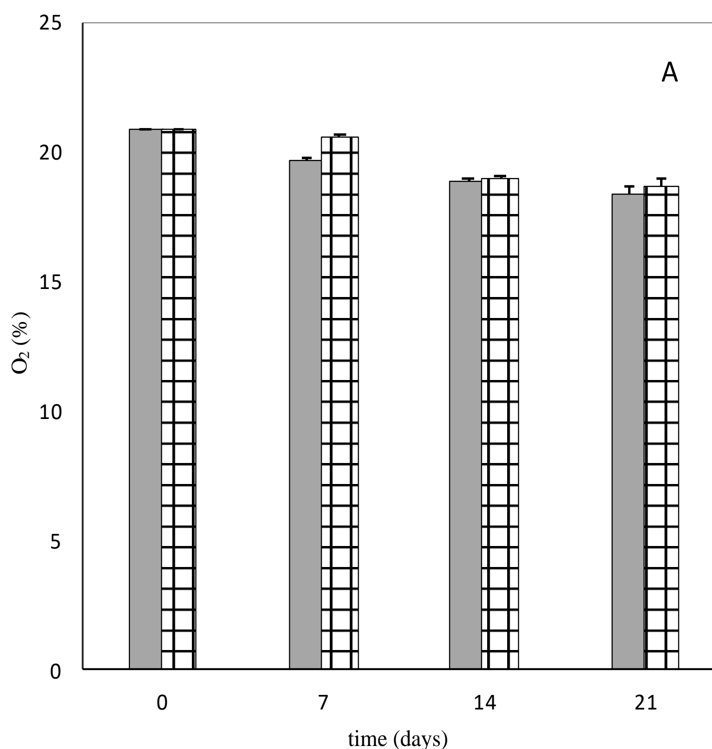


Figure 5.1. Antioxidant activity, inhibition (%) of active film SC/GA/PBS ( ), SC/GA/Nylon ( ) after 2.5h and 4 h (A) of contact with DPPH; antioxidant activity SC/GA/Nylon ( ) after 14 and 30 day of contact with cheese at 25°C (B).

Determination of headspace gaseous composition

The evolution of oxygen into the headspace are showed in Figures 5.2A, 5.2B. Generally, for all the samples stored at different condition, the oxygen concentration remains more or less constant during the first 7 days of storage. At time 7 days SC/GA/PBS showed a significant effect ($p<0.05$). Into details the oxygen content remain stable at 20.6±0.03% comparing to the control sample with 19.7±0.1% After 21 day at 40°C, the oxygen amount decreased from a stating point of 20.9±0.1 % at time 0, up to 18.7±0.1% and 18.4±0.3% for PBS/GA and control film respectivly (fig. 5.2A). Anova shows a significlant efftect ($p<0.05$) after 60 days of storage at 25°C for SC/GA/Nylon samples. Into details the oxygen % decreased up to 15.7±0.1% and 11.2±0.3% for Nylon/GA and control film respectivly (fig. 5.2B). This results are according to Mortensen, Sørensen, & Stapelfeldt, (2002) there is a decrease in oxygen as a function of time.



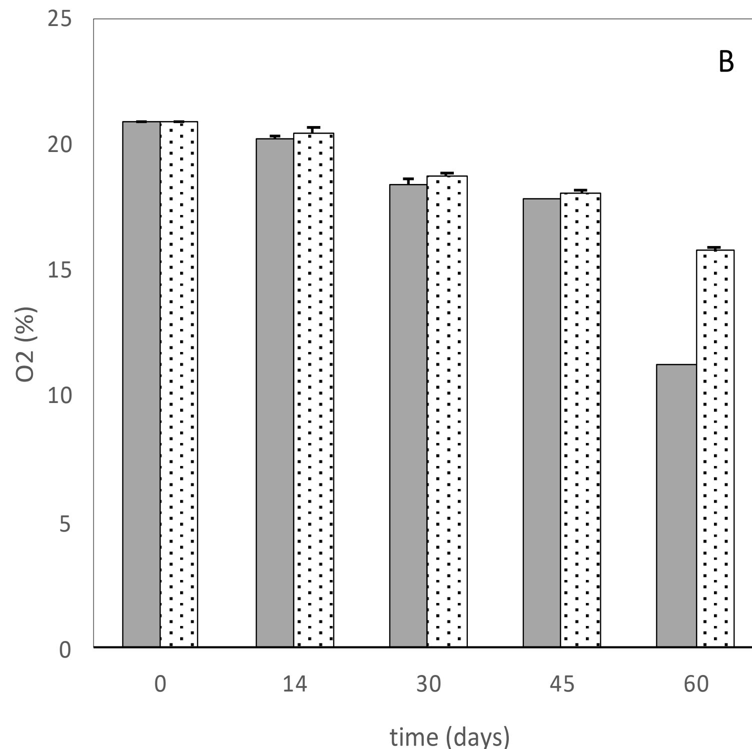
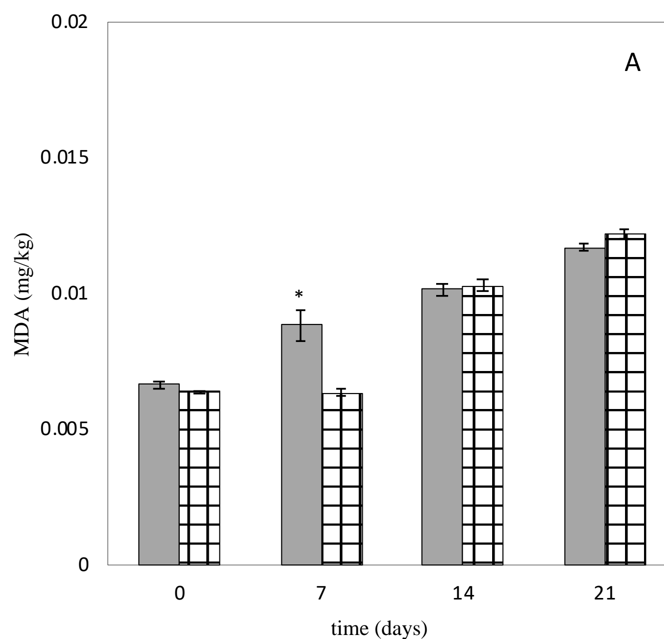


Figure 5.2. Evolution of headspace gas composition during the storage of SC/GA/PBS film (▦) at 40°C (A), and SC/GA/Nylon (⚁) at 25°C with respective control film (■) samples

Lipid oxidation of Grana Padano DOP cheese

The Figures 5.3A, 5.3B, shows the trend in malondialdehyde produced during oxidation of grana padano cheese over the time. Generally for the boths sample stored at 40°C and 25°C the evolution of MDA content increased during the storage time reaching a stable value. Into details the analysis of variance, show a significant effect ($p < 0.05$) only for SC/GAPBS film at time 7 days at 40°C (fig. 5. 3A). Samples of Grana Padano cheese stored into SC/GA/Nylon film shown a significalty effect ($p<0.05$) during the first 14 days of storage at 25°C (fig. 5.3B). This results are in according to Ünalan, Arcan, Korel, & Yemenicioğlu, (2013) the quantity of MDA increases as a function of time until it reaches almost stable values both for samples packed with active and control film.



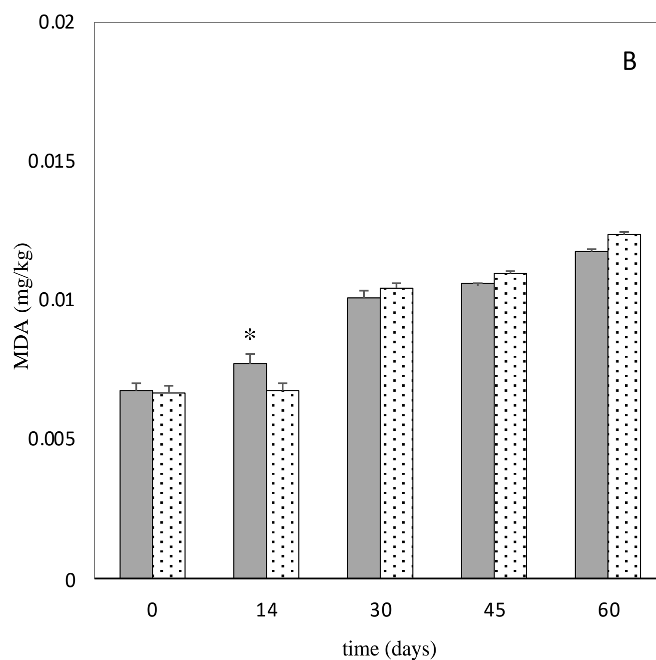
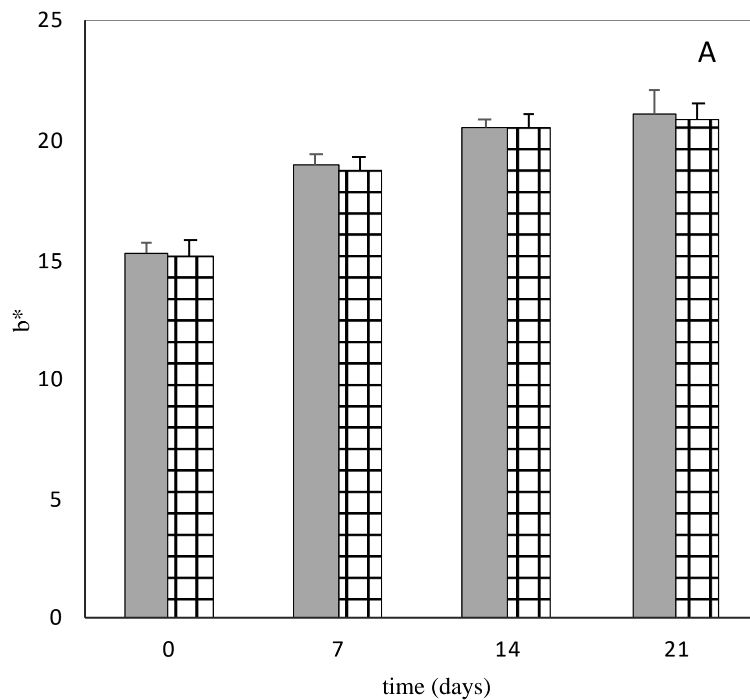


Figure 5.3. Evolution of malondialdehyde content during the storage of SC/GA/PBS film (▦) at 40°C (A), and SC/GA/Nylon (⁘) at 25°C with respective control film (▬)samples

Colorimetric determination

Figures 5.4A, 5.4B shows the change in colorimetric index b* as a function of storage time. The b* parameter increases as a function of time from an initial value of 15.13± 0.64 to a final value of 20.91± 0.60 for grana padano stored into SC/GA/PBS film. Into details the b* parameter increases as a function of time from an initial value of 15.78 ± 0.39 to a final value of 19.10 ± 0.57 for SC/GA/Nylon. According to Kristensen, Hansen, Arndal, Appelgren, & Skibsted, (2001) the parameter b* increases as a function of time, which is favoured by the high temperatures used during storage. The effect of the interaction between time and active film is not significant.



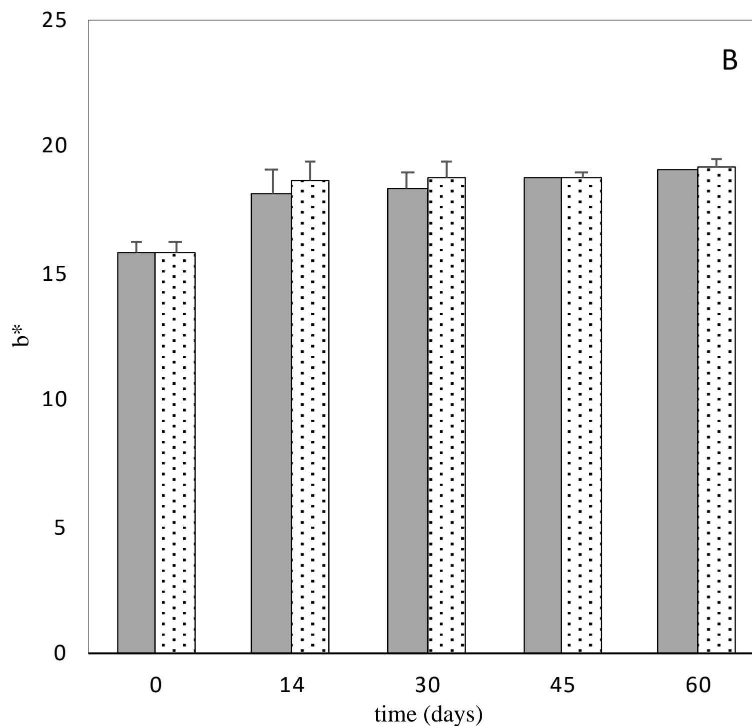


Figure 5.4. Evolution of colorimetric parameter b*content during the storage of SC/GA/PBS film (▦) at 40°C (A), and SC/GA/Nylon ⛶) at 25°C with respective control film (■)samples

Triangular test of Grana Padano DOP cheese

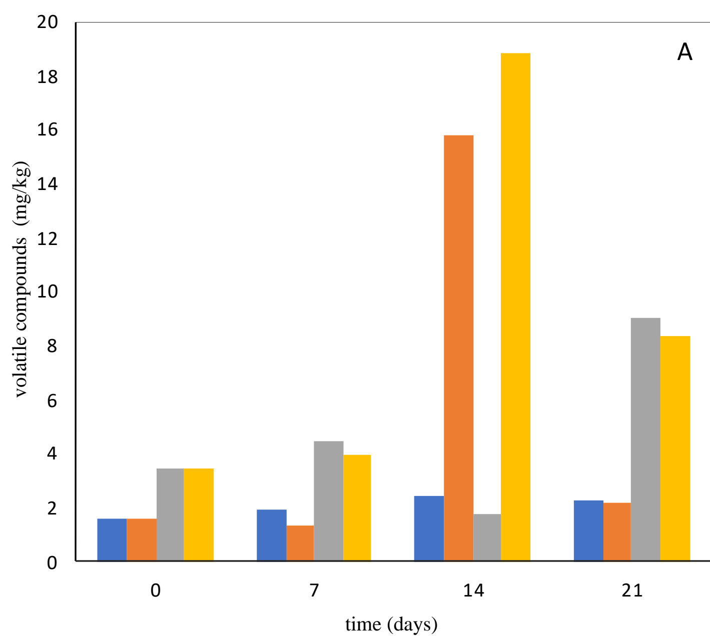
Consumers were presented with samples of Grana Padano PDO packaged in active nylon polymer film and stored at a temperature of 40 °C for a period of 7 days. It turned out that 18 consumers out of 36 panel members were able to discriminate the presented samples. From the processing of the data, the result obtained is significant. The parameter used to discriminate the samples was odour.

Analysis of volatile compounds of Grana Padano DOP cheese

The evolution of volatile compounds of grana Padano cheese during the shelf life at different temperature are reported in figures 5.5 A, 5.5B. Generally, for both storage condition the compounds most commonly formed are 2-heptanone and 2-nonanone; and in less quantities aldehydes, alkenes, alcohols, pyrazines, and free fatty acids. Into details

2-heptanone and 2-nonanone increases at day 14 into SC/GA/PBS film and at time 7 day into a control film respectively (fig. 5.5A).

There is a greater amount of 2-heptanone in the samples packaged with active film than in the samples packaged with control film, while the 2-nonanal content is slightly higher in the samples packaged with control film than in the samples packaged with active film (fig. 5.5A). In addition to ketones, other volatile compounds formed in smaller quantities are aldehydes, alkenes, alcohols, pyrazines and free fatty acids. Into details Samples with SC/GA/Nylon at day 14 shows an significantly increase of the two ketons in both the Grana Padano samples packaged with control film and the Grana Padano samples packaged with active film; there is a greater amount of 2-heptanone in the samples packaged with active film than in the samples packaged with control film, while the 2-nonanal content is slightly higher in the samples packaged with control film than in the samples packaged with active film. The two ketones increase considerably in the Grana Padano samples packed with active film at day 30. They decrease at day 45, due to the formation of other secondary compounds, and then increase at day 60 (fig. 5.5B). Ketones, as well as other compounds produced during oxidation, are characterized by low perception thresholds and are therefore easily perceived by consumers. In fact, during the triangular test, consumers were able to discriminate between samples of Grana Padano packaged with active film and samples packaged with control film.



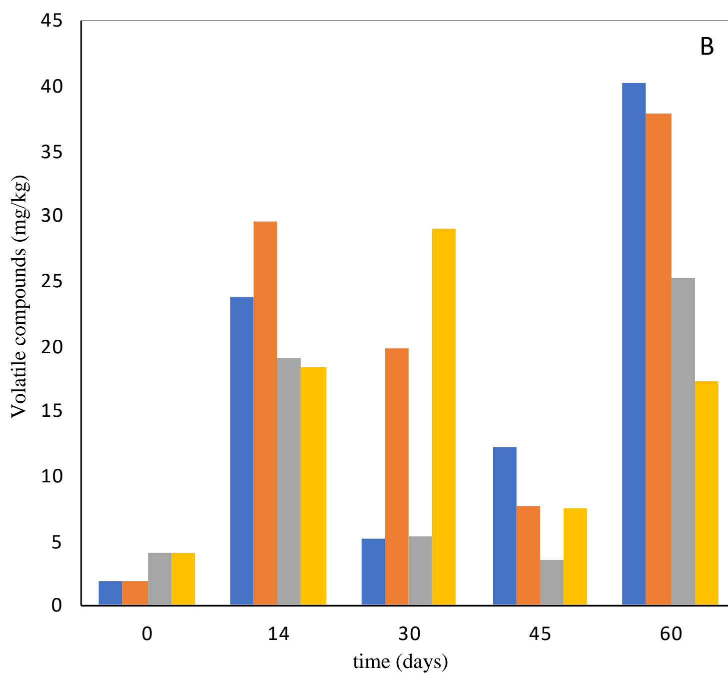


Figure 5.5. Evolution of volatile compounds: control 2-heptanone (▬), active 2-heptanone (▬), control 2-nonanone (▬), active 2-heptanone (▬) by SPME - GC/MS in samples of SC/GA/PBS stored at 40 °C (A) and SC/GA/Nylon at 25°C (B).

5.3.5. Conclusions

In conclusion, the active films developed showed excellent antioxidant capacities in vitro, thus confirming the strong antioxidant capacity of gallic acid. Polymers such as polybutylene succinate (PBS) and nylon have proved to be excellent substrates for functionalization by deposition of a caseinate and gallic acid coating and subsequent solvent evaporation. Analyses carried out in vivo on samples of Grana Padano PDO showed a protective effect of the films with respect to the content of malondialdehyde produced (oxidation index): in particular, the protective effect was showed up to day 7 for samples of Grana Padano PDO packaged in PBS and stored at a temperature of 40 °C and up to day 14 for samples packaged in Nylon and stored at room temperature. Furthermore, significant differences between samples packed with active film and samples packed with control film were found during the triangular test, where the parameter used by consumers to discriminate samples was odor. In contrast, the active films did not show a protective effect with respect to other parameters evaluated, such as color and the change in gas content within the headspace. Therefore, it can be concluded that the functionalization of polymers by deposition of a biopolymer-based coating with incorporation of antioxidants, such as gallic acid, could be a viable packaging technology to improve food preservation. Further experiments can certainly be carried out to better analyze the in vivo effect of the antioxidant film and, from this analysis, deduce the best food application.

5.3.6. References

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Chapter 6. Activated gallic acid as radical and oxygen scavenger in biodegradable packaging film.

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6.1. Abstract

PHBV active packaging based on gallic acid/ sodium carbonate mixture was produced by thermoforming. In contact with acqueous and fatty food simulants, almost 30% of the initial gallic acid was released into food simulants A (10% ethanol), and D1 (50% ehtanol), where it showed a radical inhibition value (I%) reaching 68±0.1 and 77±0.1 respectively. Into a food simulants D2 (isooctane) no release was showed. In addition, the active film displayed O_2 scanvenging properties with a the remaining activated gallic acid displayed_maximal oxygen scavenger capacity of 120 mg O_2/g GA at room temperature, after 10 days of storage. This corresponds to the absorption of 6.2% of oxygen present in a volume of 375 mL. The PHBV active packaging combining both antiradical and oxygen scavenger activities is very promising for food protection. However, some improvements are still needed to enhance its oxygen barrier capacity and to meet the reglementation.

Keywords: active packaging, biodegradable polymer, O2 scavenger, antiradical, O_2 scavenging modelling

6.2. Introduction

Nowadays, one major challenge is to develop a sustainable packaging with low environmental impact, able to preserve food quality and safety. The use of biobased and biodegradable packaging is a way to reduce both the exploitation of fossil resources and the accumulation of plastic waste, thus preventing the environmental and health problems that result from this (Cazón, Velazquez, Ramírez, & Vázquez, 2017; Shen & Kamdem, 2015; Lordanskii *et al.*, 2018; (Guillard *et al.*, 2018; Mohamed, El-Sakhawy, & El-Sakhawy, 2020).

In addition, this biodegradable packaging should also be able to preserve the quality of food and extend its shelf life in order to reduce food waste and prevent food-borne deseases (Angellier-Coussy, Guillard, Guillaume, & Gontard, 2013; Coffigniez, Matar, Gaucel, Gontard, & Guilbert, 2021). Oxidation is one of the major food degradations. It is responsible for structural alterations, producing off-flavors, discoloration and loss of nutritional quality and safety due to the formation of potentially toxic secondary compounds (lipid and protein oxydation), thus making foods unsuitable for consumption (Gómez-Estaca, López-de-Dicastillo, Hernández-Muñoz, Catalá, & Gavara, 2014; Hellwig, 2019). One way to limit these oxidation reactions is the use of active packaging containing antioxidants that can diffuse into the food or act as oxygen absorbers by maintaining an oxygen-free atmosphere (Vermeiren, Devlieghere, Van Beest, De Kruijf, & Debevere, 1999).

To be used in food packaging, antioxidants should meet certain criteria. They have to (i) be safe; (ii) effective at low concentrations and (iii) not modify odor, color and flavor of the product and (iv) above all, should comply with food and packaging regulation into force. Due to their good antioxidant activity, natural phenolic compounds seem to be ideal candidates for integration into a fully biobased and biodegradable system (Sanches-Silva *et al.*, 2014). Diffent studies dealing with the incorporation of phenolic compounds into biobased films to extend the food shelf life have been reported (Wang *et al.*, 2019; Radi, Firouzi, Akhavan, & Amiri, 2017; Carrizo, Taborda, Nerín, & Bosetti, 2016; Nisa *et al.*, 2015; Licciardello, Wittenauer, Saengerlaub, Reinelt, & Stramm, 2015).

Gallic acid (2,3,4-trihydroxybenzoic acid) (GA), a phenolic acid present in different parts of superior plants such as bark, wood, leaf, root and seed (Luzi *et al*., 2019; Campo, Pinelli, & Romani, 2016) has the particularity of having three phenolic hydroxyl groups in the ortho position, which increases its antioxidant activity. By means of electrospining, GA was encapsulated into lentil flour/polyethylene oxide and methylcellulose/polyethylene oxide nanofibers. Due to the release of GA into walnuts, the resulting materials led to the decrease of their peroxide value by half at 40°C for 21 days of storage (Aydogdu, Sumnu, & Sahin, 2019; Aydogdu, Yildiz, *et al*., 2019).

The oxygen scavenging ability of GA was investigated by Ahn, Gaikwad, & Lee, (2016); A. F. Pant, Sängerlaub, & Müller, (2017) and Singh, Singh, Kumar, & Gaikwad, (2020) who showed that GA combined to alkaline molecules (sodium carbonate, sodium hydroxide or potassium chloride) had a strong oxygen absorption capacity when it was incroporated in low density polyethylene film; bio-based mulitlayer film and chitosan film respectively. In the presence of a base, the oxygen scavenger activity of gallic acid is activated by humidity derived from the product or the environnement. Indeed, GA is a weak polyprotic acid with four acidic protons. As a function of the medium pH, different gallate anions can be formed. In the presence of dissolved oxygen, the autooxidation mechanism gives rise to gallate radicals by electron transfer or hydrogen atom transfer. This process leads to the formation of several GA autooxidation intermediates, along with the absorption of oxygen (A. F. Pant, Özkasikci, Fürtauer, & Reinelt, 2019; Wanner, 2010). Accurate determination of the O_2 absorption capacity and absorption rate is a prerequisite to modelling approach of the oxyen diffusion – reaction in material containing GA, which was never carried out up to now. Modelling of such activity is important to design efficient system well targeted to the intended application as food packaging, as it was previously applied on iron based scavenging films for instance (Kombaya-Touckia-Linin *et al*., 2019).

Poly(3-hydroxybutyrate-co-3hydroxyvalerate) communly known as PHBV is a biobased polyester belonging to the wide family of polyhydroxyalkanoate polymers. It displays good barrier properties and its physical properties are similar to some fossil-derived

polymers such as polypropylene. Furthermore, it is non toxic, biocompatible and biodegradable in natural conditions (Berthet *et al.*, 2015; Bossu *et al.*, 2020; Requena, Vargas, & Chiralt, 2017b). To the best of our knowledge, the design of an active packaging based on the PHBV/GA system, which could combine oxygen scavenging (by absorbing atmospheric oxygen) and radical scavenging (by migrating into food) activities, has never been studied before.

In the present work, PHBV film containing 5wt% of activated GA was produced. The antioxidant activity of GA as both radical and oxygen scavenger was deeply investigated and a diffusion-reaction mathematical model was applied to predict the oxygen scavenger activity of the active packaging. This is the first time that such complete experimental and modelling approach was carried out on GA-based material targeting both antioxydant and oxygen scavenger activities.

6.3. Materials and Methods

Materials

Gallic acid monohydrate (GA) and sodium carbonate (Na_2CO_3) were purchased from abcr Gmbh and Geyer (Germany) respectively. Commercial grades of P(3HB-co-3HV) with 3wt% of 3HV in the form of pure uncompounded powder with no additive, was purchased from Natureplast (France). Ultrapure water was obtained from a Millipore Milli-Q system (Millipore, Bedford, MA, USA). 2,2-diphenyl-1-picryl-hydrazyl radical (DPPH), ethanol 96%, methanol 99%, formic acid 96%, acetonitrile 99.9% and isooctane (for synthesis) were purchased from Sigma Aldrich France.

Preparation of the active film: compounding and thermoforming

PHBV, GA and Na_2CO_3 powders were dried at 60 °C for at least 48 h before using. PHBV powder containing 5% (w/w) of GA and 2.5% (w/w) of Na_2CO_3 (weight ratio of 2:1) was melt-blendeded using a co-rotating twin-screw microextruder (model "process 11" thermofisher). The screw speed was set at 200 rpm and the barrel temperature profile to

180°C (from top to bottom). The residence time was 1.5 min. The melt strain was cooled down at room temperature and pelletized (Pelletizer from Thermofischer, Germany). After drying during one night at 60°C, the pellets were transformed into films by means of an hydraulic thermopress (CFM 20T, Pinette Emidecau Industries, Chalon sur Saone cedex, France) at 180°C. Pellets were melt for 1 min at 5 bar, then 1 min at 150 bar. The average thickness of the realized films was about 345± 8 µm and 370±13 µm. The final GA-PHBV films were stored in hermatically box free of oxygen until use.

Determination of the film properties

Evaluation of GA recovery and distribution in the film after thermoforming

From macroscopic point of view

PHBV/GA sheets of 144 cm^2 was divided into four equal parts and GA was extracted from each part, at 25°C, during 18 h using methanol as solvent. The amount of GA was determined thanks to UV quantification in an Aquity UPLC (Waters, Milford, MA) liquid chromatography system, equipped with a photodiode array detector (DAD). The Waters column was 100mm x 2.1mm, HSS T3, with 1.8µm particles size. Solvents used were A (99% H_2O and 1%HCOOH v/v) and B (80% CH_3CN, 19.9% H_2O and 0.1% HCOOH) with a flow rate of 0.55mL/min. The gradient conditions were as follows: from 0 to 5 min, 99% to 60% A; from 5 min to 7 min, 60% to 1% A ; from 7 min to 8 min, 1% A; from 8 min to 9 min, 1% to 99.9% A. The injection volume was 2 µl, DAD was set at 280 nm, and gallic acid was detected at 1.5 min retention time (Rouméas, Billerach, Aouf, Dubreucq, & Fulcrand, 2018). GA was quantified after external calibration with GA pur standard dissolved in methanol.

From microscopic point of view

PHBV/GA films of 1 cm width and 200µm thickness (thinner films were used for this analysis) were observed with a wide-field microscope Eclipse Ni-E (Nikon Instruments Inc, NY, USA) with filter cube UV-2A, exc: 330-380, em: 420-800. The pictures were obtained with the 10X Plan APO objective and a Nikon CMOS DS-Ri2 camera. They were processed with Image J v1.8.0 software.

GA migration into food simulants

Three food simulants were selected to study the GA migration, namely, simulant A (10% ethanol) corresponding to aqueous food, simulant D1 (50% ethanol) and simulant D2 (vegetable oil was replaced by isooctane) simulants assigned for fatty food (European Standard EN 10/2011, European Commission, 2011). Migration studies were conducted in triplicate at 25°C over 10 days in a climatic chamber (Memmert, Germany). Double-sided, total immersion migration tests were performed with 60 cm^2 of films and 100 mL of each simulant (area-to-volume ratio around 6 dm^2/L). A blank test for each simulant was also carried out. Extracts (1 mL) were collected each day and GA concentration in food simulant was quantified by UPLC, as previously mentionned in section 2.3.2.1.

To estimate the corresponding pourcentage of GA diffused in food simulant, the following equation was used:

$$\% \text{ of GA diffused in food simulant } = \frac{C_x \times V_{FS}}{m_f \times \%GA} \qquad (1)$$

With, C_x the mass concentration of GA (mg/L), V_{FS} the volume of food simulant (L), m_f the mass of film (mg) and $\%GA$ the pourcentage of GA included in the PHBV film (5 wt %).

The radical scavenger activity of released GA into food simulants

The DPPH assay consists in measuring the ability of a molecule to reduce the 2,2-diphenyl-1-picryl-hydrazyl radical (DPPH$^{\cdot}$) in methanol, resulting in its bleaching at 517 nm. The scavenging activity of GA against DPPH$^{\cdot}$ was performed spectrometrically at 517 nm and 30°C, according to (Laguna *et al.*, 2020). Solutions containing 100 µL of each food simulant collected at each time (with GA concentrations from 8 to 54 mg/L) and 100 µL of DPPH methanolique solution (40 mg/L) were poured into Humidity cassette microplates (TEC96ft_cell Tecan 96Flat Transparent). The absorbance decay was monitored each 2 minutes until it reached a steady state (15 min). The spontaneous bleaching of DPPH$^{\cdot}$was also measured in absence of antioxidant (blank). All the

determinations were performed in duplicate. The percentage inhibition values (I%) were calculated using the following equation:

$$I\% = \frac{Abs_C - Abs_S}{Abs_C} \times 100 \qquad (2)$$

where Abs_c is the absorbance of pure DPPH and Abs_s is the absorbance of the sample.

Oxygen scavenger properties of active component and active film

The oxygen scavenger capacity of the active mixture composed of GA and Na_2CO_3 in ration 2:1 in both powder form and inclusion in polymer matrix was determined according to DIN 6139 at 23°C and 100% RH. The active mixture (0.3 g of GA and 0.17 g of Na_2CO_3 for powder mixture or 5.8g of film pieces containg 5% of GA and 2.5% of Na_2CO_3 for inclusion polymer matrix) were stored in hermetically closed glass cells (V=514 cm^3) equipped with steel lid. The saturated humidity was assured by distilled water (50 mL) put in a glass bowl at the bottom of the cell. The O_2 depletion in the headspace (initial gas atmosphere: air) during storage was determined non-destructively using a luminescence-based oxygen detection system (PreSens Precision Sensing GmbH, Regensburg, Germany) with an optical sensor spot stuck on the underside of the cell wall. The O_2 partial pressure in the cell was monitored over time and the cell was briefly reopened to regenerate the oxygen at 20.9% when it became zero. The experiment conducted in triplicate, was stopped when the maximum absorption capacity was reached, i.e. when no decrease of oxygen partial pressure has been detected. The O_2 absorption quantity (mg O_2) was calculated from the O_2 partial pressure depletion, using the following equation:

$$m_{O_2} = \frac{P_{O2} \times V_{HS} \times M_{O2}}{R \times T} \qquad (3)$$

Where m_{O_2} is the oxygen content absorbed into the system (g), R=8.314 $Pa.m^3.\ mol^{-1}.K^{-1}$ is the gas ideal constant, T is the temperature (K), V_{HS} is the headspace volume of the cell (m^3) and M_{O2} is the oxygen molar mass ($g.mol^{-1}$). The oxygen absorption capacity of active compound film was calculated in mg of absobed O_2 per gram of gallic acid.

Oxygen permeability of the active film

Active film was cut into circles with 12.5 cm^2 diameter and their oxygen permability ($mol.m^{-1}.s^{-1}.Pa^{-1}$) was measured at 23 °C and 50% RH using an oxygen permeation cell (OTR, PresSens-GmbH, Germany) according to a modified ASTM Standard (2007) procedure. The oxygen partial pressure in the upper chamber was measured using an optical luminescence quenching method (Presens, GmbH).

The oxygen permeability coefficient PO_2 ($mol.m^{-1}.s^{-1}.Pa^{-1}$) was determined as reported in the following equation:

$$PO_2 = \frac{\dot{P} \times l}{A \times P_{atm}} \quad (4)$$

Where $\dot{P}$ ($mol.s^{-1}$) is the slope of the oxygen partial pressure increase in the upper chamber, A (m^2) and l (m) are the surface and the average thickness of the film respectively. P_{atm} is the standard atmosphere pressure. The thickness of the film was determined at five different points of the film using a micrometer (Mitutoyo).

Mathematical models development

Modeling of the apparent diffusion of GA in the PHBV film

Assuming that : (i) the film is a one dimensionnal infinite plane sheet with an homogeneous thickness, (ii) GA is homogeneously distributed in the PHBV film and in food simulant (if GA diffused in food simulant) and (iii) the film does not swell during the process; the estimation of the gallic acid apparent diffusivity (D_{app}) in the PHBV film was made using an analytical solution of the Fick's second law as described by Lajarrige, Gontard, Gaucel, Samson, & Peyron, (2019).

$$\frac{\partial C}{\partial t} = D_{app}\left(\frac{\partial^2 C}{\partial x^2}\right) \quad (5)$$

where: C is the additive concentration, t, the contact time, x, the position in the film and Dapp, the apparent diffusion coefficient. The initial and boudary conditions of this equation are the following ones:

$$C(t = 0, x) = 0 \ \forall \ x \ \mathcal{E} \ [-L, L] \quad (6)$$

$$C(t, x = \pm L) = C_s(t) \ \forall \ t \geq 0 \quad (7)$$

Where C is the GA concentration in food simulant at time t, and L, the film half thickness.

$$\frac{M_t}{M_\infty} = 1 - \sum_{n=0}^{\infty} \frac{2\alpha\,(1+\alpha)}{1+\alpha+\alpha^2 q_n^2}\; exp\left\{-\frac{D q_n^2\, t}{L2}\right\} \quad (8)$$

With

$$\alpha = \frac{1}{K_{P,F}} \frac{V_F}{V_P} \quad (9)$$

where: Mt. is the total amount of GA in food simulant at time t and M∞ is the total amount of GA in food simulant at the steady state, V_P is the polymer volume and V_F the food simulant volume, (qn)n, the positive roots of the equation tanq=−αq and $K_{P,F}$, the partition coefficient of the additive in the polymer/food simulant system.

The numerical simulations were carried out using Matlab® software and its lsqnonlin function to estimate the Dapp. For each model fitting, the quality of fit was estimated through the percentage of Root Mean Square Error (RMSE):

$$RMSE = \frac{1}{M_0}\sqrt{\frac{1}{N}\sum_{i=1}^{N}((M_t)experimental - (M_t)predicted)^2} \text{ x } 100 \quad (10)$$

Where M_0 is the initial mass of GA in the film and M_t is the mass of GA into a food simulant at time t.

Modeling of O_2 absorption by gallic acid

Reaction model for GA powder:

In the presence of oxygen and high humidity, the oxidation of GA is classically described by the following reaction :

$$GA + n\,O_2 \longrightarrow GA_{ox} \quad (11)$$

GA_{ox} are the oxidation product, formed in the reaction assuming that the reaction is irreversible; and n is the stoichiometric factor.

The absorption kinetic was then depicted by an order 2 kinetic, as O_2 absorption depends on both scavenger and O_2 concentrations. As a simplification, partial orders were set to 1, leading to the following system of ODEs:

$$\frac{d[O_2]}{dt} = -nk[GA][O_2]$$

(12)

$$\frac{d[GA]}{dt} = -k[GA][O_2]$$

where $[O_2]$, is the concentration of O_2 in mol m^{-3}, [GA] is the concentration of GA in mol m^-3, n is the apparent stoichiometric coefficient for oxidation of GA by O_2 (%); and k is the kinetic coefficient in m^3 s^{-1} mol^{-1} for GA oxidation.

Reaction-diffusion model of GA included in PHBV film:

A reaction-diffusion system, similar to the one developed by Kombaya-Touckia-Linin *et al.*, (2019), was used to describe the oxygen absorption by GA embedded in the film. The model described the diffusion of O_2 into the polymer matrix using Fick's law of diffusion and the reaction between O_2 and GA acording the equation (12).

It was assumed that : (i) GA was immobile into the polymer matrix; (ii) an homogeneous distribution of GA inside the film structure was achieved; (iii) the polymer was considered as homogeneous material with a single, constant, apparent O_2 diffusivity D_{O2} (m^2 s^{-1}).

The mathematical model for a plane film geometry reduced to the one-dimensional reaction–diffusion system is given in Equation (13), for x ∈]−L/2, L/2[, where L is the thickness of the film:

$$\frac{\partial[O_2]\,(t,x)}{\partial t} = D_{O_2}\frac{\partial^2[O_2]}{\partial x^2} - k\,n[O_2](t,x)[GA](t,x)$$

(13)

$$\frac{\partial[GA]\,(t,x)}{\partial t} = -k\,[O_2](t,x)[GA](t,x)$$

where k and n are the kinetic parameters previously determined for the powder.

The initial GA and O_2 concentrations, supposed uniform in the film were the following ones:

$$[GA](t_0) = \frac{x_{GA}^{f}\,\rho^{f}}{M_{GA}} \tag{14}$$

$[O_2]$ (t_0) =0

Were x_{GA}^{f} represent the mass fraction of GA inside the active film (kilograms of gallic acid per kilogram of active film) ρ^{f} is the apparent density of PHBV (kg.m^{-3}) and and M_{GA} is the molar mass of GA (kg mol^{-1}). It was assumed that $[O_2]$ (t_0) =0.

The boundary conditions are similar to those described by Kombaya-Touckia-Linin *et al.*, (2019).

$$D_{O_2}\frac{\partial[O_2]\,(t,x)}{\partial x} = \frac{\varphi_{L/2}}{A} = \frac{\varphi_{-L/2}}{A} = \frac{k}{RT}\left(P_{O_2,HS} - \frac{[O_2](t,x)}{K_H}\right) at\ x = (-)\frac{L}{2} and \forall t \geq 0$$

(15)

Where T (K) is the temperature , R the ideal gas costant, $P_{O2,HS}$ (Pa) and $P_{O2,His}$ (Pa) are the oxygen partial pressure in the headspace and at the vicinity of the composite surface, respectively.

In equation (15) the external mass transfer coefficient k are reported using Biot number (Bi).

$$k = \frac{2B_i D_{O_2}}{L} \tag{16}$$

For the mass balance of oxygen into headspace it was assumeed that: (i) the film is isolated into a container with a constant headspace volume V_{HS} (m^3) (ii) the gas flow is negligible through the container. Therefore, the variation of the oxygen partial pressure is calculated as below:

$$\frac{\partial P_{O2,HS}}{\partial t} = k\frac{A}{V_{HS}}\left(2P_{O_2,HS} - \frac{[O_2](t,x=L/2)}{K_H} - \frac{[O_2](t,x=L/2)}{K_H}\right) \tag{17}$$

Numerical simulation were perfomed with a biot number ($Bi=10^5$). Equation (12) and (17) were transformed from a partial differential equation system into an ordinary differential equation (ODE) system by a spatial discretization with a second order cenral difference method and mesh of 100 nodes. The resulting ODE system was numericcaly solved using MATLAB (MathWorks).

6.4. Results and discusion

Impact of the thermoforming process on GA recovery and distribution in PHBV film

PHBV/GA sheets were divided into four equal parts and GA contained in each part was quantified after extraction. It was observed that a similar amount of GA (4.25±0.16 g GA /100 g of film) was recovered from each part, indicating a good homogeneity of GA at the macromolecular level. However, the total amount of GA extracted from the film sheet was only 85 ± 3% of the initial amount introduced before the thermoforming process. Thus, the thermal process (3.5 min at 180°C) provoked 15% of GA mass loss, that could be atrributed to thermal degradation (Ahn *et al*., 2016; Santos *et al*., 2012). Indeed, Santos *et al*., (2012) showed that 9% of GA acid was degraded at temperature range between 68°C and 213°C.

The apparent homogeneity of GA in PHBV matrix has been challenged by wide field microscopy analysis. Images of the surface of the PHBV/GA films represented in Figure 6.1 (Figure 6.1B is a close-up of Figure 6.1A) clearly showed that at the microscopic scale, the GA (appearing in yellow color) was not homogeneously distributed in the polymer matrix. Furthermore, the numerous bubbles presented on the surface of the film could be attributed to the sodium carbonate that was not melted after film processing (tf = 850°C). Some studies also reported heterogeneous dispersion of active compounds in polymer films, such as thymol or eugenol in LDPE (Krepker *et al*., 2017; Goñi, Gañán, Strumia, & Martini, 2016) or GA in chitosan (Rui *et al*., 2017; Sun, Wang, Kadouh, & Zhou, 2014; Ahn *et al*., 2016). This heterogeneity depends mainly on the GA concentration and nature of the interactions between the active compound and the

polymer chains (Rui *et al*., 2017). Indeed, GA at low concentrations was able to form hydrogen bounds with polymer matrix; while at high concentrations a part of GA could remain unlinked, forming aggregates.

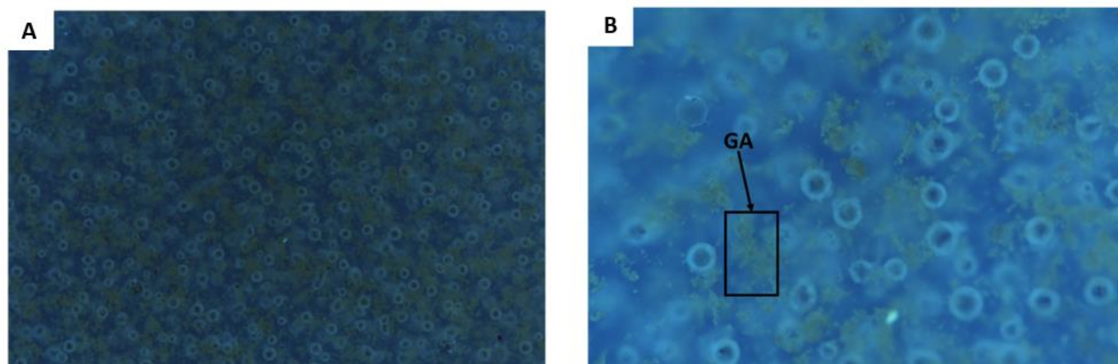

Figure 6.1. Wide-field microscopy of PHBV-GA films of 1 cm width and 200µm thickness, with 10X Plan APO objective. Image 1B is a close-up of image 1A.

The antiradical activity of GA released into food simulants

The concentration and percentage (equation 1) of GA diffused from PHBV film into acqeous food simulant A and fatty food simulant D1 are shown in Figure 6.2. GA was not released into D2 food simulants (data not showed).

GA migration is higher in food simulants D1 (511 mg/L after 5 days, corresponding to 39 % of the initial amount of GA present in the film) than in food simulant A (384 mg/L after 8 day, corresponding to 31% of the initial GA present in the film), likely due to the higher solubility of GA in ethanol compared to water (more than 30 times) (Daneshfar, Ghaziaskar, & Homayoun, 2008; Noubigh, Jeribi, Mgaidi, & Abderrabba, 2012). The same behaviour was observed for the migration of thymol which was proportional to the amount of ethanol in the simulant (Tawakkal, Cran, & Bigger, 2016). Therefore, the maximum release of GA is expected to occur in less polar foodstuffs, such as oil-in-water emulsions (sauces, dressings or high-fat dairy products) and /or alcoholic beverages.

This increase of GA release in ethanol-rich medium was confirmed by the value of adjusted apparent GA diffusivites in PHBV film, which is two time higher when the PHBV sheet is in contact with food simulant D1 ($6.48 x 10^{-14}$ m^2/s) compared to food simulant A ($3.58 x 10^{-14}$ m^2/s). The apparent diffusivity values identified for GA are in the

same order of magnitude than those found in the litterature for other low molecular weight constituents. For example, Rubilar, Cruz, Zuñiga, Khmelinskii, & Vieira, (2017) identified a carvacrol diffusivity between $3.7x10^{-14}$ and $6.1x10^{-14}$ m^2/s from chitosan film into water using a Fickian model. A good fitting was observed between experimental data and model with an average RMSE of 6.5% and 11.5% in food simulant A and D1 respectively.

The released GA into food simulants A and D1displayed a significant inhibition of DPPH radical and as expected, is ascribed to the total amount of GA released into the simulant. As depicted in Figure 6.2, at maximum release, the percentage inhibition value (I%) was 68.7±0.1 and 77.5 ±0.1 in simulant A and D1 respectively. In the case of food represented by simulant D1, the protection of lipids against radical-induced oxidation would be effective. However, the antioxidant activity of GA may not be exploited if the maximum daily intake is not respected.

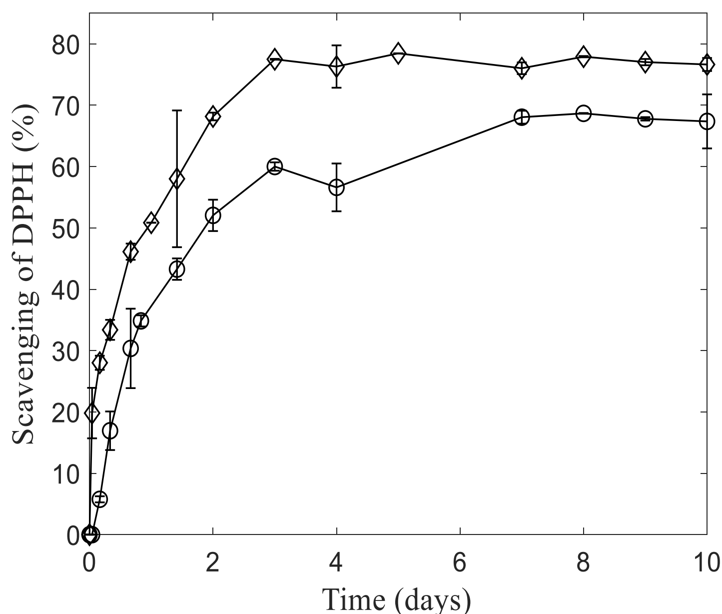

Figure 6.2. Kinetic of DPPH radical scavenging activity after migration of GA in food simulant A, ie 10% ethanol (circle) and D1, ie 50% ethanol (diamond). The error bars represent the standard deviation (n=2)

The amount of GA released in contact with food simulants D1 and A, after 10 days, was about 377 ± 31 mg/L and 344 ± 4 mg/L respectively (Figure 6.3A, 6.3B) (corresponding to around 30 % of the initial GA present in the film), while the amount of GA released in simulant D2 (isooctane) was zero. Altough no Admissible Daily Intake (ADI) was estimated for gallic acid, the regulation established the maximal acceptable ADI at 0.2 mg/kg bw for propyl gallate (a GA ester), corresponding to 14 mg for an adult with an average body weight of 70 kg (FAO, 1976). Assuming that GA would have a similar ADI value, the use of a conventionnal tray with 10g weight (for 150g of food) containing 5% of GA whose 30% diffuses in food after 10 days (simulants A and D1), will lead to the intake of 150 mg of GA for one adult (supposing he consumed all the food), so 10 times higher than acceptable ADI. However, when a conventionnal lid film with 1g weight is used, 15 mg of GA would diffuse into food, which is equivalent to the maximal ADI. Consequently, the development of active material consisting of gallic acid should only be permitted in trays at a concentration lower than 0.5% or in lid film at a concentration lower than 5% for food corresponding to simulants A and D1.

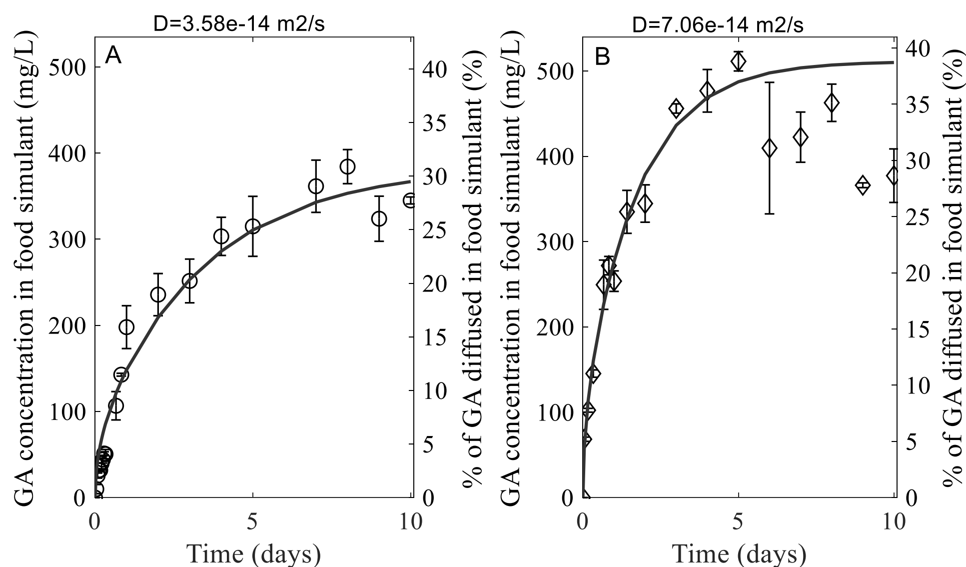

Figure 6.3. The cumulative release of GA from PHBV film into 10% ethanol and 50% ethanol (n = 2), as a function of contact time (o, ◊) and fitted Fick's model (lines). The error bars in the figure represent the standard deviation (SD). A: ethanol 10% (v/v), B: ethanol 50% (v/v).

Oxygen absorption properties of the active PHBV film

Oxygen absorption capacities of GA/Na_2CO_3 powder

The experimental kinetic of O_2 absorption capacity of the GA /Na_2CO_3 powder (2:1) at 23°C and 100% RH and the corresponding, calculated remaining active GA is displayed in Figure 6.4. After 15 days the cell was reopened in order to refill the headspace with oxygen, which is reflected by the two cycles present in the figure. A maximal absorption capacity of 595 mg O_2/g of GA was reached in 30 days. This value is slightly higher than the O_2 absorption capacity of 447 mg O_2 absorbed/ g of GA measured by Pant *et al.*,(2017) using the same mixture composition at 21°C and 100% RH.

The mathematical model showed a good fitting performance to experimental data with a RMSE of 21.9 mg O_2/g of GA. Table 6.1 showed the estimated values of kinetic coefficient k (7.8×10^{-7} $m^3.mol^{-1}.s^{-1}$) and stoichiometric coefficient n (3.57). These parameter values are close to those found by Pant *et al*., (2019) that used the same model on GA/ Na_2CO_3 powder (2:1) with a kinetic coefficient k of 1.496×10^{-6} $m^3.mol^{-1}.s^{-1}$ and a stoichiometric coefficient n of 2.53.

Table 6. 1. Values of parameters used in the oxygen absorption capacity model of PHBV containing 5% of GA and 2.5% of Na_2CO_3 film.

O2 absorption by gallic acid				
Sample	**k ($m^3.mol^{-1}.s^{-1}$)**	**n**	**Conditions**	**Reference**
GA/Na_2CO_3 powder in ratio 2 :1	7.8×10^{-7}	3.87	23°C and 100% HR	This study

O2 barrier properties of PHBV					
Sample	**Permeability ($mol.m^{-1}.s^{-1}.Pa^{-1}$)**	**Diffusivity ($m^2.s^{-1}$)**	**Solubility ($mol.m^{-3}.Pa^{-1}$)**	**Conditions**	
PHBV	1.34×10^{-17}	-	Estimated from S=P×D=1.12×10^{-4}	23°C and 50% RH	This study
PHBV	Not use	1.2×10^{-13}		23°C and 0% RH	Crétois *et al*, 2014
PHB	Not use	1.1×10^{-12}	Estimated from S=P×D=1.22×10^{-5}	24°C and 80% RH	Sanchez-Garcia *et al*, 2008

Oxygen absorption capacities of active film

The kinetic of O_2 absorption capacity of the GA /Na_2CO_3 (2:1) incorporated in PHBV film at 23°C and 100% RH and the calculated remaining active GA in film is reported in Figure 6.4 (the measure was carried out in duplicate). GA in PHBV film reached approximately the same maximal absorption capacity as in powder form, with an average value of 581 mg O_2/g of GA. However, it took three times as long to reach this maximal absorption capacity (around 100 days for the film versus 30 days for the powder). Indeed, in the experiment with activated powder oxygen is directly in contact with GA, while in the PHBV/GA film, it must first be absorbed in the polymer matrix and then diffused into the polymer to reach GA, which consequently slows down its absorption kinetics. The diffusion-reaction mechanism is clearly O_2 diffusion rate-limiting.

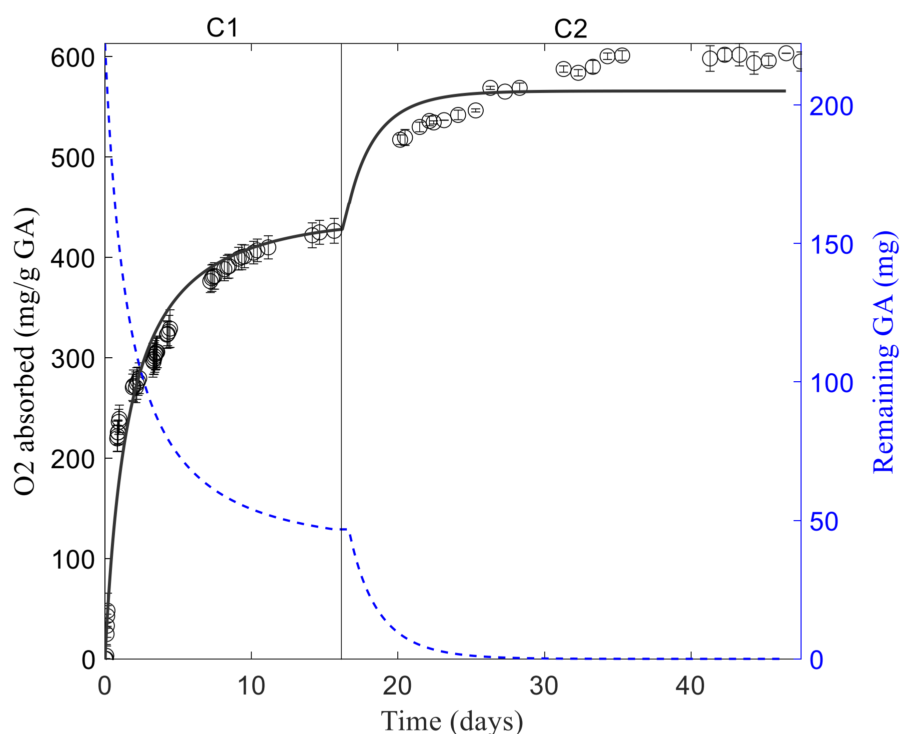

Figure 6.4. Experimental (dots) and predicted (black line) O_2 absorption capacities of gallic acid /Na_2CO_3 powder (2:1) (mg O_2/g of GA) at 23°C and 100% RH and predicted consumption rate of gallic acid /Na_2CO_3 powder (2:1) at the same condition (blue dotted line). Between cycle 1 (C1) and cycle 2 (C2), the jar was reopened to recharge the headspace in oxygen. The error bars represent the standard deviation (n=3)

The mathematical model used to simulate the oxygen absorption by GA present in the PHBV film integrated both : (i) the sorption and diffusion of oxygen in the PHBV film (diffusion part of the model) and (ii) the absorbtion kinetic of GA (reaction part of the model). Assuming that absorption kinetic of GA in its two forms (powder or embedded in film) is similar, the kinetic (k) and stoichiometric (n) coefficients of the GA powder were used, i.e 7.8×10^{-7} $m^3.mol^{-1}.s^{-1}$ and 3.57 respectively. In litterature, the diffusivity value of oxygen D_{O2} in PHBV, or PHB ranges from 1.2×10^{-13} to 1.1×10^{-12} $m^2.s^{-1}$ (Gupta *et al*.,2018; Crétois *et al*., 2014). To estimate the sorption of oxygen S_{O2} (or k_h) in PHBV film, the permeability of the film was measured, its value being $1.34\times10^{-17} \pm 1.66\times10^{-18}$ $mol.m^{-1}.s^{-1}.Pa^{-1}$ (table 6.1) , the S_{O2} was estimated by the relation S=P/D. It ranged from 1.1×10^{-4} to 1.2×10^{-5} $mol.m^{-3}.Pa^{-1}$ depending on the D value considered with the range found in litterature. It was observed that for a same P_{O2} value (here 1.34×10^{-17} $mol.m^{-1}.s^{-1}.Pa^{-1}$) the oxygen absorption kinetic strongly depends on the couple of D_{O2} and S_{O2} used (Figure 6.5). For example, on the first replica, the couple $D_{O2} = 1.2\times10^{-13}$ $m^2.s^{-1}$ and $S_{O2} = 1.1\times10^{-4}$ $mol.m^{-3}.Pa^{-1}$ allowed to reach an absorption capacity of 346 mg O_2/g of GA after 123 days, while the couple $D_{O2} = 1.1\times10^{-12}$ $m^2.s^{-1}$ and $S_{O2} = 1.2\times10^{-5}$ $mol.m^{-3}.Pa^{-1}$ allowed to reach an absorption capacity of 211 mg O_2/g of GA after 123 days (39% less than the previous case) (Figure 6.5). This result is highlighting the importance to well determine both diffusivity and solubility values of oxygen into PHBV to well predict the evolution of oxygen in active packaging headspace.

The model did not fit the experimental data for both replicates (Figure 6.5, $P_{O2}=1.34\times10^{-17}$ $mol.m^{-1}.s^{-1}.Pa^{-1}$), probably because of the modification of gas permeability due to the lack of active compound homogeneity in the PHBV film (confirmed by microscopy analysis-Figure 6.1). The permeability of PHBV film containing exhausted active compounds (5wt% of GA and 2.5 wt% of Na_2CO_3) was measured. Unfortunately, measurement was unexploitable because of exceeded quantification treshold. The excessively high oxygen increase during the permeability measurement confirms the presence of pores in the active PHBV film. These results correlate with Ahn's observations (Ahn *et al*., 2016) that the increase in GA/potassium carbonate from 1% to

20% in LDPE induces a reduction of the intermolecular force between polymer chains, leading to the apparition of pores, and consequently to the increase of gases permeability. The oxygen permeability values of PHBV in litterature usually range from 1 to 7 $\times 10^{-17}$ $mol.m^{-1}.s^{-1}.Pa^{-1}$ (Berthet *et al.*, 2016). In a last trial, the model simulating the oxygen absorption of GA in PHBV film was run with the upper limit of this P_{O2} range, i.e. with the value of 7 $\times 10^{-17}$ $mol.m^{-1}.s^{-1}.Pa^{-1}$, so 7 times higher than that measured in this study (with a S_{O2} range from 1.1×10^{-4} to 1.2×10^{-5} $mol.m^{-3}.Pa^{-1}$ and a D_{O2} range from 5.9×10^{-12} to 6.3×10^{-13} $mol.m^{-3}.Pa^{-1}$). With this new permeability value, the model fited the experimental data for the first replica with the couple D_{O2} $=6.3\times10^{-13}$ $m^2.s^{-1}$ and S_{O2} = 1.1×10^{-4} $mol.m^{-3}.Pa^{-1}$. The same couple of parameters also allowed to well reflect the experimental kinetic of cycle 2 and 3 for the second replica, the delay of 120 mg O_2/ g GA accumulated in cycle 1, being maintained in the two following cycles. Therefore, these observations highlight that further research is needed to better understand and predict the absorption of oxygen by activated GA in polymer matrice.

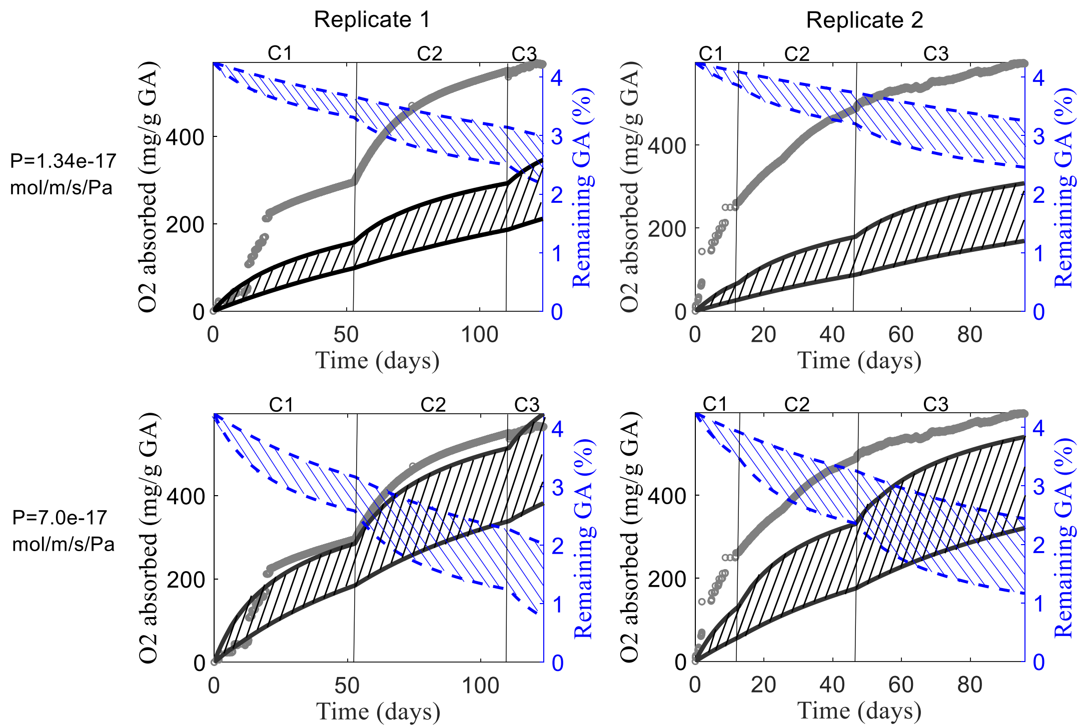

Figure 6.5. Experimental (dots) and predicted (black line) O2 absorption capacities of PHBV/GA(5%)/ Na2CO3(2.5%) film at 21° and 100% RH; and predicted consumption rate of the film at the same condition (blue dotted line) for two replicates (one at right and one at left) and with two different values of O_2 permeability (1.34×10-^{17} mol/m/s/Pa up and 7.0×10-^{17} mol/m/s/Pa down). For each P_{O2} value, two couple of D_{O2} and S_{O2} in PHBV film were tested to represent the predicted absorption capacities (black line) that were joined by cross hatch. Between cycle 1 (C1) and cycle 2 (C2); and between cycle 2 (C2) and cycle 3 (C3), the jar was reopened to recharge the headspace in oxygen.

Experimental (dots) and predicted (black line) O2 absorption capacities of PHBV/GA(5%)/ Na2CO3(2.5%) film at 21° and 100% RH; and predicted consumption rate of the film at the same condition (blue dotted line) for two replicates (one at right and one at left) and with two different values of O2 permeability (1.34×10-17 mol/m/s/Pa up and 7.0×10-17 mol/m/s/Pa down). For each PO2 value, two couple of DO2 and SO2 in

PHBV film were tested to represent the predicted absorption capacities (black line) that were joined by cross hatch. Between cycle 1 (C1) and cycle 2 (C2); and between cycle 2 (C2) and cycle 3 (C3), the jar was reopened to recharge the headspace in oxygen.

6.4. Conclusion and recommendations for the use of PHBV/GA film as food packaging

In this study, active film based on PHBV/activated GA was developped. This film showed a promising capacity as both radical and oxygen scavenger and could be used as biodegradable packaging for different kinds of food.

In the case of contact with aqueous and some fatty food (food simulant A and D1) as meat, fish, or cheese, the GA amount present in the packaging should be lower than 0.5% in trays and could reach 5% in lid filmin the case of a food of 150 ml in an active tray of 357 ml and tray surface of xxx or lid film surface of xxx. After diffusion in food over 10 days, the remaining part of GA in the packaging (55%) could act as oxygen absorber, with an oxygen absorption capacity of 120 mg O_2/g of GA after 10 days, corresponding to 6.2% of oxygen/ g of GA for a volume of 357 mL. This oxygen absorption capacity could limit the oxygen entrance through the packaging and would enhance the benefit of oxygen-free modified atmosphere packaging by strengthening food protection against oxidation.

Since the GA does not diffuse into vegetable oil (food simulant D2, replaced by isooctane in this study), no restriction on the amount of GA in the packaging applied for this type of food application. The protection from oxidation would performed thanks to the oxygen absorption capacity of GA.

However, some improvements are still needed to find the right compromise between the amount of GA needed for effective antioxidat activity and maintaining the structural integrity of the film.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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General conclusions

The development of biopolymer packaging systems is one of the most challenging innovation for food packaging company. Biopolymers obtained from agricultural commodities and/or food waste products have emerged as an option about their film-forming capacity. Moreover, active packaging can play a major role in preserving the quality of food packed with biopolymer based material.

In light of the results discussed in this dissertation, chitosan, sodium caseinate and whey protein showed to be good substrate to produce active film based on rosemary essential oils, gallic acid, or lactic acid bacteria with antimicrobial activity. In all three cases, the active substances have no effect on the chemico-physical properties of the films, except in the case of the lactic acid bacteria, where a positive effect on the mechanical properties was observed. On the other hand, the addition of the active substances guarantees greater functionality in terms of both antioxidant and antimicrobial properties. The limitation in using these biopolymers can be the manufacture technology, i.e. the film were obtained at lab scale by using a casting technology which has several limitation for an industrial application. One possible solution was to apply it as a coating on support film. The meyer rod coater technique can be a promising technique for an active mono and multilayer film realization. The active films developed showed excellent antioxidant capacities in vitro, but in vivo test showed limited results. In the case of hazelnut cream, the application of the active coating based on CH/SC/REO showed only an effect on peroxide reduction over the time and no effect on Malondialdehyde and free fatty acidity values. The active film based on SC/GA coated used to pack Grana Padano cheese showed some promising results in terms of preservation of lipid oxidation. Although high flexible, the meyer rod coater technique showed some limitation in terms of quantity of antioxidant compound that can be carried by the active film.

The results of the last case study showed that active film based on PHBV/activated GA can be realized by extrusion technique. This film showed a promising capacity as both radical and oxygen scavenger and could be used as biodegradable packaging for fatty food products. In fact, gallic acid enriched with a chemical base shown both antioxidant

and oxygen scavenger properties. However, the enrichment gallic acid and sodium carbonate effect on the microstructural part of the materials increasing the porosity and reducing the oxygen barrier properties.

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