UNIVERSITAT POLITÈCNICA DE VALÈNCIA
DEPARTAMENTO DE TECNOLOGÍA DE ALIMENTOS
INSTITUTO UNIVERSITARIO DE INGENIERÍA DE
ALIMENTOS PARA EL DESARROLLO

Development of bioactive edible films and coatings with antioxidant and antimicrobial properties for food use

TESIS DOCTORAL
Presentada por:
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CONSIDERAN: que la memoria titulada **Development of bioactive edible films and coatings with antioxidant and antimicrobial properties for food use** que presenta **Dª. María Jeannine Bonilla Lagos** para aspirar al grado de Doctor por la Universidad Politècnica de València, reúne las condiciones adecuadas para constituir su tesis doctoral, por lo que **AUTORIZAN** a la interesada para su presentación.

Valencia, Abril de 2013

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ABSTRACT

The aim of this work was the preparation and characterization of biodegradable films with antimicrobial and/or antioxidant properties, using chitosan (CH). In order to modulate their properties and enhance their functionality, CH was combinated with other polymer matrices: wheat starch (WS), polyvinyl alcohol (PVA) and polylactic acid (PLA). Moreover, essential oils (EO) of thyme (Th) or basil (B), α-tocopherol (Tp) and citric acid (CA) were incorporated.

The effect of homogenization treatment of the film-forming dispersions (FFD) on CH films with EO at different proportions was evaluated. In the FFD, microfluidization led to a droplet size reduction, a surface charge increase and a viscosity decrease. Furthermore, this treatment intensified all the effects caused by EO addition on the mechanical properties of the films, due to the induced close contact polymer oil. At low EO proportion, microfluidization improved the water vapor barrier properties, although it had no significant impact on the oxygen permeability. These films played a protective role against

oxidation of pork fat. Although EO addition caused an increase in oxygen permeability, lower rates of oxidation were observed in samples covered with CH-EO films (especially at high relative humidity), which is probably due to the specific antioxidant effect of the EO components. Chitosan films effectively controlled the microbial spoilage when applied on minced pork meat, but the incorporation of EO did not improve this antimicrobial activity.

CH was incorporated in WS films in different proportions. As CH proportion was increased, both the viscosity and stability of the FFD were increased. The addition of CH in major proportion resulted. CH and WS appeared to be highly compatible, and homogeneous film structures were observed. The increase of CH proportion gave rise to films with improved mechanical properties, since CH seemed to inhibit starch retrogradation. WS:CH films caused a reduction of the microbial load when applied on minced pork meat samples.

Antioxidant agents were incorporated (essential oil of thyme and basil, citric acid and α-tocopherol) in WS (80%):CH (20%)

blend films. These films showed a heterogeneous microstructure due to the immiscibility of components, mainly those formulated with α-tocopherol, which showed phase separation. This was associated with a rougher surface, lower gloss and, in the case of α-tocopherol, a more intense yellow hue. On the other hand, antioxidant incorporation resulted in a transparency increase and an oxygen permeability decrease. CA caused an increase in the elastic modulus and a decrease in the extensibility of the films.

The incorporation of CH in PVA matrices resulted in highly homogeneous films, which is due to the compatibility of the two polymers. As CH was incorporated, the films became more resistant and rigid, but less extensible. A reduction of the crystallinity degree, an increase in thermal stability and a reduction of the UV light transmission were observed. Moreover, these films showed antimicrobial activity when applied to minced pork meat samples.

CH was incorporated in a PLA matrix by extrusion. CH addition did not affect the thermal behavior of the blend films, nor the crystallinity degree of PLA. Both polymers were incompatible.

The CH particle size reduction minimized its negative impact on the mechanical properties and water vapor permeability. Their application to minced pork meat demonstrated that the antimicrobial properties of the films were improved when CH was incorporated.

RESUMEN

El objetivo de esta tesis fue la obtención y caracterización de films biodegradables con características antimicrobianas y/o antioxidantes a partir de quitosano (CH). Para modular sus propiedades y mejorar su funcionalidad, se estudió la combinación con otras matrices poliméricas: almidón de trigo (WS), polivinil alcohol (PVA) y ácido poliláctico (PLA). Además se incorporaron aceites esenciales (EO) de tomillo (Th) o albahaca (B), α-tocoferol (Tp) y ácido cítrico (CA).

Se evaluó el efecto del tratamiento de homogenización de las dispersiones formadoras de films (FFD) en films de quitosano con EO en diferentes proporciones. La microfluidización dio lugar a una reducción en el tamaño de gota, a una mayor carga superficial de las mismas y una menor viscosidad de las dispersiones. Además, intensificó todos los efectos provocados por la incorporación de EO sobre las propiedades mecánicas de los films debido a potenciación de las interacciones con el polímero. A baja proporción de EO, la microfluidización mejoró las propiedades de barrera al vapor de agua de los films, pero no

tuvo un impacto significativo en la permeabilidad al oxígeno. Los films ejercieron un papel protector frente a la oxidación en grasa de cerdo. La presencia de EO en los films, a pesar de aumentar la permeabilidad al oxígeno, dio lugar a una menor velocidad de oxidación de las muestras (especialmente a alta humedad relativa), probablemente por el efecto antioxidante de los componentes de los EO. Los films de quitosano se mostraron efectivos en el control del deterioro microbiano cuando se aplicaron en carne de cerdo picada, pero la incorporación de los EO no mejoró su actividad antimicrobiana.

Se incorporó CH en films de WS en diferentes proporciones. La adición de CH en cantidades crecientes dio lugar a un aumento en la viscosidad y estabilidad de las FFD. Ambos polímeros mostraron un alto grado de compatibilidad lo que dio lugar a una microestructura homogénea. El aumento de la proporción de CH dio lugar a films con mejores propiedades mecánicas ya que el CH pareció inhibir la retrogradación del almidón. Los films WS:CH dieron lugar a una reducción de la carga microbiana cuando se aplicaron en muestras de carne de cerdo picada.

Se incorporaron diferentes antioxidantes (aceite esencial de tomillo y albahaca, ácido cítrico y α-tocoferol) en films mezcla de WS (80%) y CH (20%). Los films presentaron una microestructura heterogénea por la inmiscibilidad de componentes, principalmente el film con α-tocoferol que presentó separación de fases. Esto llevó asociado una superficie más rugosa, con menor brillo y, en el caso del α-tocoferol, un color más amarillo. No obstante la incorporación de antioxidantes conllevó un aumento de la transparencia y una disminución de la permeabilidad al oxígeno. El CA provocó un aumento en el módulo de elasticidad y un descenso de la extensibilidad de los films.

La incorporación de CH en matrices de PVA dio lugar a films altamente homogéneos, debido a la compatibilidad de ambos polímeros. Se obtuvieron films más resistentes y rígidos, pero menos extensibles. Se observó una reducción del grado de cristalinidad y un aumento de la estabilidad térmica, además de una reducción de la trasmisión de la luz UV. Así mismo, cuando se aplicaron a muestras de carne de cerdo picada presentaron una acción antimicrobiana.

La incorporación de CH en una matriz de PLA mediante extrusión no afectó al comportamiento térmico del PLA ni a su grado de cristalinidad. Ambos polímeros se mostraron incompatibles. La reducción del tamaño de las partículas de CH minimizó el impacto negativo sobre las propiedades mecánicas y de barrera al vapor de agua. En su aplicación a muestras de carne de cerdo picada, la presencia de CH mejoró las propiedades antimicrobianas de los films.

RESUM

L'objectiu d'aquesta tesi va ser l'obtenció i caracterització de films biodegradables amb característiques antimicrobianes y/o antioxidants a partir de quitosano (CH). Per a modular les seues propietats i millorar la seua funcionalitat, es va estudiar la combinació amb altres matrius polimèriques: midó de blat (WS), polivinil alcohol (PVA) i àcid polilàctic (PLA). A més es van incorporar olis essencials (EO) de timó (Th) o alfàbega (B), α-tocoferol (Tp) i àcid cítric (CA). Es va avaluar l'efecte del tractament d'homogeneïtzació de les dispersions formadores de films (FFD) en films de quitosano amb EO en diferents proporcions. La microfluïdització va donar lloc a una reducció en la grandària de gota, a una major càrrega superficial de les mateixes i una menor viscositat de les dispersions. A més, va intensificar tots els efectes provocats per la incorporació d'EO sobre les propietats mecàniques dels films degut a potenciació de les interaccions amb el polímer. A baixa proporció d'EO, la microfluïdització va millorar les propietats de barrera al vapor

d'aigua dels films, però no va tindre un impacte significatiu en la permeabilitat a l'oxigen. Els films van exercir un paper protector enfront de l'oxidació en greix de porc. La presència d'EO en els films, a pesar de augmentar la permeabilitat a l'oxigen, va donar lloc a una menor velocitat d'oxidació de les mostres (especialment a alta humitat relativa), probablement per l'efecte antioxidant dels components dels EO. Els films de quitosano es van mostrar efectius en el control del deteriorament microbià quan es van aplicar en carn de porc picada, però la incorporació dels EO no va millorar la seua activitat antimicrobiana.

Es va incorporar CH en films de WS en diferents proporcions. L'addició de CH en quantitats creixents va donar lloc a un augment en la viscositat i estabilitat de les FFD. Ambdós polímers van mostrar un alt grau de compatibilitat, el que va donar lloc a una microestructura homogènia. L'augment de la proporció de CH va donar lloc a films amb millors propietats mecàniques ja que el CH va parèixer inhibir la retrogradació del midó. Els films WS:CH van donar lloc a una reducció de la càrrega microbiana quan es van aplicar en mostres de carn de porc picada.

Es van incorporar diferents antioxidants (oli essencial de timó i alfàbega, àcid cítric i α-tocoferol) en films mescla de WS (80%) i CH (20%) . Els films van presentar una microestructura heterogènia per la immiscibilitat de components, principalment el film amb α -tocoferol que va presentar separació de fases. Açò va portar associat una superfície més rugosa, amb menor brillantor i, en el cas del α -tocoferol, un color més groc. No obstant això, la incorporació d'antioxidants va comportar un augment de la transparència i una disminució de la permeabilitat a l'oxigen. El CA va provocar un augment en el mòdul d'elasticitat i un descens de l'extensibilitat dels films.

La incorporació de CH en matrius de PVA va donar lloc a films altament homogenis, a causa de la compatibilitat d'ambdós polímers. Es van obtindre films més resistents i rígids, però menys extensibles. Es va observar una reducció del grau de cristalinidad i un augment de l'estabilitat tèrmica, a més d'una reducció de la transmissió de la llum UV. Així mateix, quan es van aplicar a mostres de carn de porc picada van presentar una acció antimicrobiana.

La incorporació de CH en una matriu de PLA per mitjà d'extrusió no va afectar el comportament tèrmic del PLA ni el seu grau de cristalinidad. Ambdós polímers es van mostrar incompatibles. La reducció de la grandària de les partícules de CH va minimitzar l’impacte negatiu sobre les propietats mecàniques i de barrera al vapor d'aigua. En la seua aplicació a mostres de carn de porc picada, la presència de CH va millorar les propietats antimicrobianes dels films.

RESULTS AND DISCUSSION

TABLES

FIGURES

JUSTIFICATION AND INTEREST OF THE WORK

Food packaging, an important discipline in the area of food technology, concerns preservation and protection of all types of foods and their raw materials from oxidative and microbial spoilage (Tharanathan, 2005). Edible films and coatings are thin layers of edible materials applied on food products that can play an important role on their preservation, distribution and marketing (Falguera et al., 2011). These are produced from edible biopolymers and food-grade additives. Film-forming biopolymers can be proteins, polysaccharides or lipids. Edible films and coatings may enhance the quality of food products, functioning as barriers against oils, gases or vapours. Moreover, they can act as carriers of active substances, such as antioxidant, antimicrobial or flavouring compounds, resulting in shelf-life extension and safety improvement (Han and Gennadios, 2005). At present, the consumer demand has shifted to eco-friendly biodegradable materials that come from agro-food industry wastes and renewable low cost natural resources. In this sense, chitosan alone or in combination with other biodegradable biopolymers such as starch, polylactic acid and polyvinyl alcohol were chosen to develop biodegrable films and coatings.

Chitosan is obtained from chitin, which is an important waste of the fishery industry, by deacetylation in the presence of alkali (Sánchez-González et al., 2010). It is a biodegradable cationic polysaccharide, with an excellent film-forming ability. Furthermore, this biopolymer exhibits great potential as antimicrobial packaging material owing to its antimicrobial activity and non-toxicity (No et al., 2001; Tharanathan and Kittur, 2003). The influence of chitosan-based coatings on food lipid oxidation and colour stability has been also evaluated. The antioxidant properties of such coatings is attributed to chitosan's ability to chelate free iron (Bonilla et al., 2012). Nevertheless, chitosan films have relatively high water vapour permeability (Vargas et al., 2009). The functionality of chitosan films can be improved by inclusion of inert materials and/or reactive compounds in the polymer matrix (Rhim et al., 2006), addition of hydrophobic agents (Han and Gennadios, 2005) or blending with other biopolymers (Xu et al., 2005).

Essential oils are aromatic mixtures obtained from plant materials with antimicrobial activity. The latter is commonly related to the chemical structure and concentration of their components, and the interactions among them. Edible films can serve as carriers for essential oils, whose release onto the food surface could control microbial growth.

The shelf life and storage-keeping quality of meat products are influenced by a number of interrelated factors: temperature, oxygen pressure, endogenous enzymes, dehydration, light microbial load, etc. These factors determined the extension and kinetics of detrimental changes, affecting the colour, odour, texture and flavour of meat. Edible coatings containing antimicrobial agents can extend shelf-life at lower active agents concentration than that applied directly onto product surfaces (Lee et al., 2010). In fact, the diffusion rate of the antimicrobial compounds added to the film matrix can be slowed down, and a high content of the active agent remains on the product surface (Sánchez-González et al., 2011).

In this work chitosan was combined with other biodegradable polymers, such as wheat starch, poly-lactic acid and polyvinyl alcohol. Likewise, the effect of the incorporation of essential oils and other antioxidant agents to chitosan or polymer blend films have been analysed through the induced changes in the film properties and the antioxidant/antimicrobial activity of the film in meat products.

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INTRODUCTION

INTRODUCTION

The environmental impact caused by the excessive quantity of non-degradable waste materials is promoting research and efforts to develop new biodegradable packing materials that can be manufactured with the utilization of environmentally friendly raw materials (Avérous et al., 2001). Edible films and coatings have been particularly considered in food preservation, because of their capability for improving global food quality (Chillo et al., 2008). An edible coating or film has been defined as a thin, continuous layer of edible material formed or placed on or between foods or food components (Bravin et al., 2006). Edible films can act as mechanical protection, moisture and gas barriers and, at the same time, can preserve the colour, texture and moisture of the coated product. Coating materials that are currently used include polysaccharides (chitin, starch, cellulose derivatives, gums), proteins (soy, milk, gelatin, corn zein, wheat gluten) and lipids (oils, waxes, resins) (Sanchéz-Gonzáles et al., 2010). The functional properties of edible films are greatly influenced by parameters such as formulation, film forming technology, solvent characteristics, and additives (Gontard et al., 1992). The possibility of incorporating active compounds (antimicrobials, antioxidants, nutraceuticals, flavours, colourants) in polymeric matrices is one of the main advantages of coatings (Sanchéz-Gonzáles et al., 2010).

One of the polysaccharides that can be used to develop edible films and coatings is chitosan, which is a biopolymer derived by deacetylation of chitin, a major component of the shells of crustacean such as crab, shrimp, and crawfish (No et al., 2002). In recent years, applications of chitosan (CH) to the fields of food, medicine, chemical engineering, pharmaceuticals, nutrition, environmental protection and agriculture have received considerable attention (Mohamed et al., 2013). The reasons for CH addition in edible films are the good film forming and mechanical properties, no toxicity, and biodegradability, (Chillo et al., 2008). The potential uses of CH as a food preservative to develop antimicrobial packaging films have been extensively studied (Ouattara et al., 2000; Yang and Lin, 2002). CH has a wide inhibition spectrum for not only Gram-positive and Gram-negative bacteria but also yeasts and moulds (Liu et al., 2004). The mechanism of the antimicrobial activity of CH and its derivatives is unknown. It has been suggested that a positive charge on the NH_3^+ group of the glucosamine monomer at pH<6.3 allows interactions with negatively charged microbial cell membranes that lead to the leakage of intracellular constituents (Liu et al., 2004). Chitosan categorized as GRAS (generally recognized as safe) by U.S. Food and Drug Administration can be considered as additive (FDA, 2012).

Starch, which is one of the most naturally abundant biopolymers, can also be used to prepare biodegradable, edible films and coatings. The starch granule is essentially composed of two main polysaccharides, amylose and amylopectin, and some minor components such as lipids and proteins (Tester et al., 2004). Among the natural polymers, starch has been considered as one of the most promising candidates for future materials because of its attractive combination of price, abundance and thermoplastic behavior, in addition to biodegradability (Galdeano et al., 2009). Starch-based materials have poor mechanical properties, particularly very low extensibility. The main disadvantage of biodegradable starch-based films is their hydrophilic character, which leads to low stability when these materials are submitted to different environmental conditions (Forssell et al., 1999).

Starch and chitosan can act as a matrices containing other natural ingredients with antimicrobial and antioxidant activity, thus giving rise to active films with improved functionality and performance. For instance, polymeric matrices can incorporate essential oils (EO). Natural extracts, such as EO and their constituents, are categorized as flavorings by the European Decision 2002/113/EC. In addition, EOs and their constituents are categorized as Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration (López et al., 2007). The incorporation of EO can lead to an improvement in the water vapor permeability of the film, because of the increment in the hydrophobic compound fraction. The antioxidant properties of these extracts have been maily attributed to phenolic compounds present in their essential oil fractions (Oussalah et al., 2004). Their antimicrobial properties are mainly assigned to a number of small terpenoids and phenolic compounds (thymol, carvacrol, eugenol) (Oussalah et al., 2007). Terpenes have the ability to disrupt and penetrate into lipid structure of the cell wall of bacteria, leading to denaturation of proteins and destruction of cell membrane, cytoplasmic leakage, cell lysis and eventually cell death (Emiroglu et al., 2010). Essential oils also penetrate into mitochondrial membrane, leading to the greater permeability of organelle and the K^+ ion leakage process (Oussalah et al., 2007).

Edible films based on biodegradable materials can be produced by different techniques such as casting, extrusion, thermomolding, injection, sheeting and blowing (Avérous et al., 2001; Gennadios et al., 1993). Casting process uses solvents for the dispersion of film-forming materials, followed by drying to remove the solvent and form a film structure. For this process, the selection of solvents is one of the most important factors. All the ingredients of film-forming materials should be dissolved or homogeneously dispersed in the solvents to produce film forming solutions/dispersions. The film-forming dispersions should be applied to flat surfaces using a sprayer, spreader or dipping roller, and dried to eliminate the solvent, forming a film structure (Han and Gennadios, 2005). Although casting has been extensively used in laboratory studies, because it does not require specific equipment and consumes lesser amounts of raw materials, there is an important disadvantage concerning the industrial scale up of the process. Thus, extrusion seems definitively to have advantages over casting, being a more attractive process to produce films (Galdeano et al., 2009). The extrusion process of edible film production does not use liquid solvents, such as water or alcohol. For the dry process, heat is applied to the film-forming materials to increase the temperature in order to overcome the melting point of the film-forming materials, thus causing their flow. Therefore, the thermoplastic properties (gelatinization, polymer melting, flow profile, etc.) of the film-forming materials should be identified in order to design film-manufacturing processes (Han and Gennadios, 2005). Extrusion has the advantage of being a low cost, continuous and versatile production system (Sothornvit et al., 2007). This technique can be used for large-scale production (Andreuccetti, et al., 2012). When developing biodegradable films by extrusion some synthetic polymers from non-renewable sources such as polyvinyl alcohol (PVA) and poly lactic acid (PLA) are commonly used. PVA is a synthetic, water soluble polymer with excellent film forming, emulsifying, and adhesive properties (Kanatt et al., 2012). It also shows good tensile strength and biodegradability and hence has been used in many biomaterial applications. PVA has also been approved for use in packaging meat and poultry products by the USDA (DeMerlis and Schonek, 2003).

In the family of biodegradable synthetic polymers, poly-lactic acid (PLA) appears one of the most attractive for applications in agriculture and as packaging material due to its bio/hydro-degradability and the biorenewable profile (Fortunati et al., 2012). PLA, a typical linear aliphatic thermoplastic polyester, is known by its high mechanical performance, by satisfying physicochemical properties, the possibility of producing from annually renewable resources and degradability to natural products in a short period of time, in contrast to the conventional plastics. For these reasons, PLA is environmental friendly (Wu et al., 2007).

In the present introduction, in addition to the overview commented on above, two chapters (published articles) are included, which concerns the objectives of the work. In the first the possibilities and limitations of edible films to prevent the detrimental effect of oxygen on food quality are discussed. In the second, the use of antioxidant agents in food is revised, through the analysis of the recent patents in this field.

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EDIBLE FILMS AND COATINGS TO PREVENT THE DETRIMENTAL EFFECT OF OXYGEN ON FOOD QUALITY: POSSIBILITIES AND LIMITATIONS

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Abstract

Oxygen has a deleterious effect on the quality of a wide variety of food products. The application of edible films and coatings to food products represents a new approach to solve this problem. Edible films and coatings can include antioxidant agents in their formulation and at the same time, they represent a barrier to oxygen, which results in a better preservation of quality. The water activity of the product, as well as the ambient relative humidity, determines the antioxidant effect of films and coatings. This paper reviews the latest studies dealing with the effectiveness and application of antioxidant films and coatings.

Keywords: Edible films, oxygen, food oxidation, antioxidant effect, edible coatings, nuts, meat, fish, fruit, vegetables.

1. Introduction

Oxygen is responsible for many degradation processes in foods such as lipid oxidation, micro-organism growth, enzymatic browning and vitamin loss (Ayranci and Tunc, 2003). According to Brown (1992), the most common quality loss of packaged foods is caused by oxygen. The oxidation of fat results in off-flavour, off-colour and nutrient loss (Hong and Krochta, 2006). Oxidative processes cause the degradation of meat proteins, pigments and lipids, which limits the shelf life (Liu *et al.,* 2010). Although some oxygen availability is needed for respiration of living tissues, this process accelerates the consumption of sugars and other compounds, thus increasing ethylene production and causing senescence (Oms-Oliu *et al.,* 2008; Rojas-Graü *et al.,* 2007).

The deleterious effect of oxygen on food is commonly delayed with the application of antioxidant and anti-browning agents, such as ascorbic and citric acids. The inclusion of strongly flavoured antioxidants (e.g. essential oils, N-acetylcysteine, glutathione) in edible films and coatings allow for their encapsulation and can reduce their strong aroma. Additionally, oxidation could be effectively reduced by selecting films and coatings of limited oxygen permeability. According to Kester and Fennema (1986) hydrophilic films and coatings (polysaccharide or protein-based) generally provide a good barrier to oxygen transference. This property is in turn greatly affected by the water availability and temperature.

The present work reviews the latest studies on the antioxidant effect of edible films and coatings and summarizes the application of these on diverse food products (nuts, meat, fish, fruits and vegetables). We will discuss the specific features of each food group in order to better understand the antioxidant mechanisms taking place in the coated product.

2. Oxygen barrier properties of edible films and coatings

The antioxidant effect of stand-alone edible films is strongly linked to their oxygen permeability (OP), which can be measured directly by oxygen permeation tests, which are mostly based on the standard described by ASTM (1988). The measurement of oxygen gas transmission through films involve the flowing of an oxygen gas stream on one side of the film and a nitrogen stream on the other side that carries the transmitted oxygen gas to the analyzer. A coulometric sensor, an infrared sensor, a gas chromatograph or a dedicated oxygen analyser may be used for monitoring (Ayranci and Tunc, 2003).

Films made from proteins and carbohydrates are excellent barriers to oxygen, because of their tightly packed, ordered hydrogen-bonded network structure (Yang and Paulson, 2000). The oxygen permeability of edible materials depends on many factors, such as temperature and relative humidity. Maté and Krochta (1996) measured the OP of whey protein films and β-lactoglobuline plasticised with glycerol and observed that high temperature promoted gas transference across the film in an exponential way.

Oxygen permeability is highly dependent on the relative humidity (Hong and Krochta, 2006). As relative humidity increases, more water molecules interact with the material and the film becomes more plasticized. In these conditions, the mobility and the extensive mass transfer across the film are favoured. For this reason, the antioxidant ability of edible films should always be tested under controlled relative humidity conditions.

Table I.1 shows the oxygen permeability of edible films as compared to common synthetic packaging films. Films prepared with hydrocolloids tend to show higher OP values than those prepared with synthetic polymers. However, the different relative humidity and temperature conditions when performing permeability tests make it difficult to compare most of the reported data.

Table I.1. Oxygen permeability of edible and synthetic films.

Composition		Oxygen permeability mL μm/(m^2 day Pa)	Measurement conditions: RH (%)/T (ºC)	Reference
Polysaccharides	Methylcellulose	0.097	50/24	Miller and Krochta, (1997)
		0.090	52/25	
	Hydroxypropyl methylcellulose	0.272	50/24	
	Amylomaize starch	0.065	100/25	
	Starch	0.0014	57/20	Gaudin *et al.* (1999)
	Starch:sorbitol (4:1)	0.00032	57/20	
	Chitosan	0.0328	50/25	Di Pierro *et al.* (2010)
	Chitosan:whey protein	0.0213	50/25	
Proteins	Zein:PEG + glycerol (2.6:I)	0.039 - 0.090	0/25	Miller and Krochta (1997)
	Gluten:glycerol (2.5:1)	0.0061	0/25	

Table I.1. Oxygen permeability of edible and synthetic films (Cont.).

	Composition	**Oxygen permeability mL μm/(m^2 day Pa)**	**Measurement conditions: RH (%)/T (ºC)**	**Reference**
Proteins	Soy protein isolate:glycerol (2.4:l)	0.0061	0/25	Miller and Krochta (1997)
	WPI:Glycerol (2.3:1)	0.761	50/23	
	WPI:Sorbitol (2.3:1)	0.0043	50/23	
	Collagen	< 0.00004 - 0.0005	0/RT	
		0.023	63/RT	
		0.890	93/RT	
	WPI:Gly (60:40)	0.127	NR	Han and Krochta (2007)
	WPI:Gly (60:40) + antioxidante	0.131	NR	
	WPI:Gly (50:50)	0.087	21/37	Maté and Krochta (1998)
	WPI:Gly (50:50)	0.052	21/29	

Table I.1. Oxygen permeability of edible and synthetic films (Cont.).

	Composition	Oxygen permeability mL μm/(m^2 day Pa)	Measurement conditions: RH (%)/T (ºC)	Reference
Synthetic	PET	0.013	NR	Han and Krochta (2007)
	LDPE	1.73	0/25	Park *et al.* (2010)
	LDPE: Chitosan	1.75	0/25	
	LDPE	1.870	50/23	Miller and Krochta (1997)
	HDPE	0.427	50/23	
	EVOH (70% VOH)	0.012	95/23	
	EVOH (70% VOH)	0.0001	0/23	
	Polyester	0.0156	50/23	
	PE	0.0188	50/25	Hong and Krochta (2006)
	PP	0.0027	50/25	

RH: Relative humidity; RT: room temperature; NR: not reported

3. Antioxidant effect of edible films

The antioxidant efficiency of edible films has been tested using different approaches. In some studies, the film was disintegrated and different tests (such as radical scavenging assays) were performed to the resulting formulation. In these cases, the disintegration procedure depended on the material and its solubility properties. For instance, chitosan films can be dissolved in distilled water (Siripatrawan and Harte, 2010), whereas a more elaborated procedure (freezing, grinding and extraction with methanol) was necessary for alginate films (Norajit *et al.*, 2010). Table I.2 summarizes some of these reported studies that include the following antioxidant tests: 2,2-diphenyl-1-picryhydrazyl radical (DPPH), N-diethyl-p-phenylenediamine (DPD) radical scavenging assay, ferric-reducing antioxidant power (FRAP), 2,2'-azinobis(3-ethylbenzothiazoline- 6-sulphonate) (ABTS) assay and total phenolic content.

Testing the activity of an antioxidant by more than one assay is desirable, because different methods approach this measurement in different ways (Erkan *et al.*, 2008). Very often, radical trapping methods have been applied. These methods - DPPH, DPD, ABTS, FRAP, amongst others (Frankel and Meyer, 2000), measure the ability of an antioxidant agent to intercept free radicals. Additionally, the quantification of specific chemicals of recognised antioxidant activity (such as phenolic compounds) may be performed.

DPPH radical scavenging assay

In this test method, the DPPH radical is reduced by the antioxidant, and the reaction is monitored by measuring the absorbance at 515 nm (Erkan *et al.*, 2008). Antioxidant activity is expressed as the percent inhibition of absorbance and/or as the concentration of the antioxidant required to cause a 50% decrease in initial $DPPH^{\cdot}$ concentration (IC_{50}).

Table I.2. Measurement of the antioxidant capacity of disintegrated edible films.

Film composition	Antioxidant additive	Method of measurement	References
Alginate	Ginseng extract	DPPH radical scavenging and reducing power activity	Norajit *et al.* (2010)
Chitosan	Green tea extract	DPPH radical scavenging and total phenolic content	Siripatrawan and Harte (2010)
Milk protein (calcium caseinate and whey protein isolate)	Oregano and/or pimento essential oils	DPD and total phenolic content	Oussalah *et al.* (2004)
Tuna-skin and bovine-hide gelatin	Oregano and rosemary extracts	FRAP, ABTS	Gómez-Estaca *et al.* (2009a)
Sole skin gelatin/ commercial fish gelatin	Borage extract	FRAP, ABTS, iron chelation activity	Gómez-Estaca *et al.* (2009b)
Squid skin gelatin	Hydrolysates from squid gelatin	FRAP, ABTS	Giménez *et al.* (2009)
Pumpkin oil cake	-	ABTS	Popovic *et al.* (2011)

DPPH: 2,2-diphenyl-1-picryhydrazyl radical; DPD: *N*,*N*-diethyl-*p*-phenylenediamine; FRAP: ferric-reducing antioxidant power; ABTS: 2,2'-azinobis(3-ethylbenzothiazoline- 6-sulphonate)

DPD radical scavenging assay

The capacity of the sample to inhibit the accumulation of oxidative species (able to oxidize DPD) is measured through the absorbance at 515 nm (Oussalah *et al.*, 2004). The sample is subjected to electrolysis, and DPD is added. The oxidative species released during the electrolysis react instantaneously with DPD, producing a red colour. The scavenging percentage is calculated in reference to the optical density of the electrolyzed solution in the absence of sample material. Thus, films able to reduce completely the level of reactive oxidative species will have a 100% scavenging capacity.

FRAP assay

The FRAP assay directly determines the capacity of antioxidant compounds to reduce a ferric tripyridyltriazine complex to the ferrous form at low pH (Frankel and Meyer, 2000). Blue colour results from this reaction, which is spectrophotometrically measured at 593nm. The absorbance is linearly related to the total reducing capacity of electron-donating antioxidants. According to Frankel and Meyer (2000) the main disadvantage of this approach is that the measured reducing capacity does not necessarily imply antioxidant activity. This method does not include a substrate prone to oxidation, hence no information is provided on the protective properties of antioxidants.

ABTS radical-scavenging assay

In this method, modified by Re *et al.* (1999) the radical cation $\mathrm{ABTS}^{\cdot +}$ is produced by oxidation of ABTS with potassium persulphate. The activity of antioxidants to scavenge $\mathrm{ABTS}^{\cdot +}$ is measured by the decrease of its absorbance at 734 nm. The results are reported as TEAC (trolox equivalent antioxidant activity), which is the concentration (mM) of trolox (a vitamin E analogue) required to produce the same activity as 1mM of the test compound.

Total phenolic compounds content

The content of phenolic compounds can be determined following the Folin-Ciocalteu procedure (Erkan *et al.*, 2008; Pastrana-Bonilla *et al.,* 2003). Results are expressed as milligrams of gallic acid equivalent per millilitre of extract.

This first approach entails the disintegration of the film prior to the analysis of the antioxidant capacity. Consequently, the interactions between the matrix and the antioxidant additives taking place in the film are modified. In fact, this type of analysis could be regarded to as a quantification of the antioxidant capacity of the film components, but not so much of the film itself. Therefore, it cannot be considered that this procedure mimic the actual antioxidant effect of the film.

The second approach consists of testing the antioxidant capacity of the stand-alone films. Osés *et al.* (2008) and Atarés *et al.* (2010) performed this measurement by using sunflower oil as a model food system. The oil was placed inside methacrylate cells and the films were secured as cell covers as shown in Figure I.1.

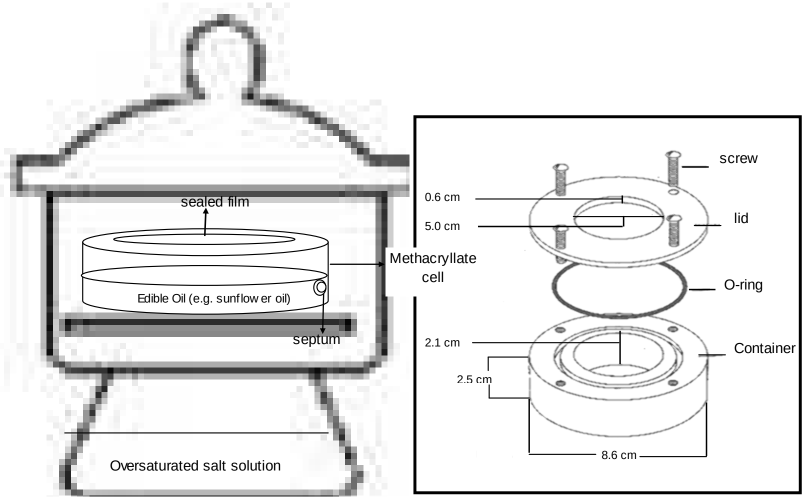

Figure I.1. Methacrylate cell used to test the antioxidant activity of stand-alone films.

Cells covered with aluminium foil and uncovered cells were prepared, and all of them were stored at controlled relative humidity and temperature. The oil was removed from the cell through the septum by means of a syringe and the peroxide value determined throughout storage time. Using this method, Osés *et al.* (2008) corroborated that whey protein isolate films were more effective protectors at 50% RH than they were at 75% RH. Moreover, it is important to point out that at 50% RH edible coatings have almost the same effect as covering with the aluminium foil. Using the same methodology, Atarés *et al.* (2010) tested the antioxidant capacity of sodium caseinate (SC) films incorporated with cinnamon (SC-C) or ginger (SC-G) essential oils. The cells were stored at 33% RH and 40ºC. As shown in Table I.3, all edible films protected the sunflower oil effectively, showing a protective effect against lipid oxidation similar to the aluminium foil. No effects of incorporating the essential oils were detected, despite that they had shown a high TEAC. This was probably due to the reduced moisture content of the films, equilibrated at low RH conditions, which promoted a reduced OP and masked the actual role of antioxidant additives.

Table I.3. Peroxide value (mEqO2/kg) development in sunflower oil unprotected, protected with aluminium foil or with sodium caseinate (SC) films incorporated with cinnamon (SC-C) or ginger (SC-G) essential oils.

Time (days)	SC	SC-C	SC-G	Al foil	Uncovered
0	2.7 (0.7)^{a,x}	2.7 (0.7)^{a,x}	2.7 (0.7)^{a,x}	2.7 (0.7)^{a,x}	2.7 (0.7)^{a,x}
8	4.2 (0.2)^{a,y}	4.6 (0.3)^{ab,y}	3.8 (0.3)^{a,xy}	2.8 (0.4)^{a,x}	6.8 (1.1)^{a,z}
22	7.0 (1.1)^{bc,x}	7.1 (0.7)^{bc,x}	5.6 (1.2)^{b,x}	5 (2)^{ab,x}	57 (4)^{b,y}
30	8.8 (0.2)^{c,xy}	12 (4)^{d,y}	7.2 (1.3)^{c,x}	6.3 (1.4)^{b,x}	96 (4)^{c,z}
41	7.5 (1.4)^{bc,x}	10 (3)^{cd,x}	6.3 (0.6)^{bc,x}	5.5 (0.4)^{ab,x}	168 (2)^{d,y}
50	6.3 (0.8)^{b,x}	6.8 (1.9)^{bc,x}	3.5 (0.3)^{a,x}	5.2 (1.8)^{ab,x}	231 (10)^{e,y}

The same superscript (^{abc} to be compared vertically and ^{xyz} horizontally) means homogeneous group in LSD test.

Another approach for testing the antioxidant activity in stand-alone films was reported by Nerin *et al.* (2008). The test film, enriched with a natural volatile antioxidant, was hanged in the headspace of a glass jar containing a solution of a highly oxidized compound that mimics a real food system, thus avoiding the direct contact with the film. In this way, the antioxidant capacity of the volatile compounds incorporated in the film was tested, and the oxygen barrier effect of the film was not considered. In this way, the antioxidant activity was achieved by scavenging oxygen radicals rather than by protecting from oxygen. This methodology was initially described for polypropylene films. However, it can be potentially used for assessing the antioxidant capacity of edible materials enriched with antioxidants, as long as the film matrix allows for some release of the active additives.

4. Effect of water availability on the antioxidant effect of films and coatings

As commented on above, the antioxidant activity of edible films and coatings is greatly influenced by the water availability, which in turn is affected by both the moisture of the product and the ambient relative humidity. In dry conditions (low moisture products) the network structure of the film or coating is tightly packed and its oxygen permeability is very limited. This mechanism by itself may have positive effects on the preservation of the quality, because of the reduced oxygen availability in the coated product. In some cases, the addition of antioxidants can entail further protection due to the enhancement of the oxygen barrier properties of the film (Ayranci and Tunc, 2003; 2004). However, in these conditions of reduced molecular mobility no chemical activity of the antioxidant agents can be observed and the only antioxidant effect is due to the oxygen barrier effect, as concluded by Atarés *et al*. (2010). On the other hand, in wet systems the coating network is plasticised and mass transference is favoured. In this context, the oxygen permeability of the film or coating is dramatically increased and the specific activity of antioxidant agents could become more relevant.

5. Application of antioxidant films and coatings on food products

In the last decade, many edible materials have been tested as protectors against the deleterious effect of oxygen, both on high moisture (meat, fish, fruit and vegetables) and low moisture products (nuts). Some examples on studies performed on these foodstuffs are given in Tables I.4, I.5 and I.6.

5.1. Application of antioxidant films and coatings on nuts

Nuts are rich in unsaturated fatty acids, which make them very prone to lipid oxidation. Being low moisture products, they should be stored under dry conditions to keep their crispness, which would, in turn, reduce the kinetics of lipid oxidation. Some authors have demonstrated the efficiency of several coatings in prolonging the shelf life of this type of products (Table I.4). The most common indicators of lipid rancidity in nuts are the peroxide value (PV) and hexanal levels. In this sense, Maté *et al.* (1996) measured the PV of nuts coated with whey protein and acetylated monoglycerides, as compared to non-coated nuts. They observed that non-coated samples showed values above the acceptance limit before 20 days of storage, those coated remained below this limit during most of the storage period (70 days). The effect of coating thickness and storage relative humidity was also evaluated. The fact that the increase in coating thickness and the decrease in environmental relative humidity led to a decrease in lipid rancidity pointed out that the mechanism of protection of the coatings relies on its oxygen barrier properties. Moreover, the continuity and homogeneity of the coatings was found to be a critical factor to achieve an effective delay in the oxidative reactions.

Lee *et al.* (2002) tested the effect of vitamin E addition into the formulation of whey protein isolate edible coatings by determining the hexanal content of non-coated and coated roasted peanuts. Vitamin E reduced hexanal levels, although the differences were not significant. As the storage time increased, the hexanal content was significantly correlated with the sensory score for rancid attribute, which corroborated that the hexanal measurement is a good indicator of rancidity in this type of products.

Table I.4. Application of antioxidant edible films to nuts.

Film or coating	Antioxidant compound	Application	Analyses	References
Hydroxypropyl cellulose and carboxymethyl cellulose	α-tocopherol BHA, BHT	Pecans	Sensory analysis Hexanal	Baldwin and Wood (2006)
Native or heat denatured whey protein isolate, glycerol, lecithin, methyl paraben	Vitamin E	Peanuts	Hexanal content	Lee and Krochta (2002)
Whey protein, glycerol, lecithin, methyl paraben	Vitamin E	Peanuts	Sensory evaluation, hexanal content	Lee *et al.* (2002)
Whey protein, glycerol (60:40 and 50:50), distilled acetylated monoglycerides	(none)	Peanuts	Peroxide value, hexanal content	Maté *et al.* (1996)

5.2. Application of antioxidant films and coatings on meat and fish products

Likewise nuts, meat and fish products contain oxidized fat. Additionally, oxygen could have a very negative effect on the colour of meat products, due to the spontaneous oxidation of myoglobin to form metmyoglobin, which imparts brownish colour (Bekhit and Faustman, 2005). For this reason, the application of antioxidant films and coatings to meat products may be beneficial (see examples in Table I.5). In these products, the level of lipid oxidation is frequently evaluated by measuring thiobarbuthuric reactive substances (TBARS), which are expressed as malonaldehyde content. In this way, Gómez-Estaca *et al.* (2007) monitored the malonaldehyde content of cold-smoked sardine during storage at 5ºC, to test the antioxidant efficiency of gelatine coatings incorporated with essential oils. In this case, the incorporation of these plant extracts reduced the lipid oxidation. In other study, Ojagh et al. (2010) developed chitosan coatings enriched with cinnamon oil with the aim of increasing the self life of cold-stored trout fillets. Their results revealed that chitosan coatings were effective in protecting lipids from oxidation. Moreover, these results coincide with the trend previously described by Jeon *et al*., (2002) when applying chitosan-based coatings to herring and cod fillets. In this case, both the inherent antioxidant activity and the oxygen barrier properties of chitosan films may have contributed to the control of lipid oxidation in the fish fillets.

The influence of chitosan-based coatings in terms of lipid oxidation and colour stability has been also examined in meat products such as ground beef. Suman et al. (2010) showed that coating ground beef patties with chitosan reduced TBARS values and improved the surface red colour of patties as compared to non-coated samples. The antioxidant property of chitosan is attributed to its ability to chelate free iron, released by myoglobin degradation during meat storage (Kamil *et al.*, 2002).

Table I.5. Application of antioxidant edible films to meat and fish products

Film or coating	Antioxidant compound	Application	Analyses	References
Milk protein-based films	Oregano and/or pimento essential oils	Beef muscle	TBA	Oussalah *et al*. (2004)
Gelatin-based films with chitosan.	Rosemary or oregano essential oils	Cold-smoked sardine	PV, TBA, total phenol, FRAP method	Gómez-Estaca *et al.* (2007)
Alginate and glycerol coating	Sodium ascorbate and citric acid	Buffalo meat patties	TBA, tyrosine value, sensory quality	Chidanandaiah and Sanyal (2009)
Chitosan coatings	Fish oil, vitamin E	Lingcod fillets	TBA	Duan *et al.* (2010)
Chitosan and gellatin	(none)	Fish patties	Colour, viscoelastic properties, TBA, TVBN	Lopez-Caballero *et al.* (2005)
Soy protein	Ferulic acid	Lard	PV	Ou *et al.* (2005)

5.3. Application of antioxidant films and coatings on fruits and vegetables

Oxygen can also reduce the quality of plant products. Table I.6 shows some examples of antioxidant film and coating applications on some fruits and vegetables. In these formulations, the films and coatings prevent the enzymatic browning, which is caused by the enzyme polyphenol oxidase that in presence of oxygen converts phenolic compounds into dark coloured pigments. Pérez-Gago *et al.* (2006) studied the development of the browning index of coated and non-coated apple slices to test the antioxidant effect of whey protein-beeswax-ascorbic acid coatings. Rather than direct antioxidant addition, the best results in terms of colour preservation were obtained by incorporating the antioxidants into the coatings.

Lin *et al.*, (2011) treated litchi fruit with chitosan edible coatings, which resulted in a significant reduction in the activity of polyphenol oxidase during storage as compared with non-coated samples.

Table I.6. Application of antioxidant edible films to fruits and vegetable products.

Film or coating	**Antioxidant compound**	**Application**	**Analyses**	**References**
Chitosan coatings	Oleoresins: rosemary, onion cranberry, garlic and carvacrol	Butternut squash	Peroxidase and polyphenoloxidase activities	Ponce *et al.* (2008)
Whey protein concentrate and beeswax	Ascorbic acid, cysteine and 4-hexylresorcinol (4-hexyl)	Apple	Weight loss, colour, sensory evaluation	Pérez Gago *et al.* (2006)
Alginate and gellan with glycerol	Ascorbic acid	Papaya	Water loss, respiration rate, ethylene production, firmness, ascorbic acid content	Tapia *et al.* (2008)
Methylcellulose-polyethylene glycol (3g:1ml) stearic acid	Ascorbic acid, citric acid	Mushroom and cauliflower	Water loss, colour, vitamin C, polyphenoloxidase activity, total phenol	Ayranci and Tunc (2003)
Methylcellulose-polyethylene glycol (3g:1ml) stearic acid	Ascorbic acid, citric acid	Apricots and green peppers	Water loss, vitamin C	Ayranci and Tunc (2004)

6. Conclusions

Interactions with oxygen should be limited in order to prevent quality losses in food products. This could be achieved by the application of edible films and coatings, whose action involves two mechanisms: the oxygen barrier effect and the specific activity of the incorporated antioxidant agents. In this sense, the efficiency of films and coatings is greatly affected by the water availability in the film. The increase in the film water content reduces the oxygen barrier effect, but can enhance the chemical action of the antioxidants. For this reason, the moisture content of the foodstuff and the relative humidity in the ambient should be taken into account in order to develop effective films and coatings with antioxidant activity.

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RECENT PATENTS ON THE USE OF ANTIOXIDANT AGENTS IN FOOD

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Abstract

The application of antioxidant ingredients is one of the most common ways to delay and prevent the detrimental effect of oxygen in foods. Some of the most widely used and studied antioxidants are carboxylic acids, tocopherols and thiol-containing compounds. However, consumer trends towards healthier and safer foods, together with the increasing concern for the potential toxicity of some antioxidants are leading research efforts towards the use of antioxidants obtained from natural sources, such as plant phenols, essential oils and chitosan. This paper reviews the latest published studies and issued patents on the use of antioxidants agents in foodstuffs. The properties of the most commonly used antioxidants as well as natural antioxidants are revised. Moreover, examples of recent patents on the application of antioxidants to different foodstuffs (meat, fish, vegetables, fruits and beverages) are given.

Keywords: Carotenoids, chitosan, encapsulation, essential oil, packaging, phenol, tocopherol.

Introduction

To some extent, the occurrence of oxidation processes in some foodstuffs is desirable. For instance, the typical aroma of dry-cured meat products is related to the initiation of lipid oxidation and the subsequent generation of volatiles [1]. However, in most cases the oxidative reactions in food products represent a major detrimental process, resulting in such negative outcomes as the formation of undesirable chemical compounds (aldehydes, ketones, organic acids) yielding rancid odours and flavours [2]. The formation of secondary and potentially toxic compounds decreases the nutritional quality and safety [3]. When the possibility of the undesirable influence of oxidized lipids on the human organism is considered, it seems to be of essential importance to minimize the content of products of lipid oxidation in food.

Antioxidant compounds are receiving more and more attention in research and scientific literature, because they are able to preserve food products by delaying oxidation and, thus, such detrimental processes as fat rancidity and/or colour changes. In industrial processing, mainly synthetic antioxidants are used, in order to prolong the storage stability of food [4]. However, the worldwide concern over safety demands for natural antioxidants, which have the advantage of being more widely accepted by the consumers as these are considered as non-chemical. In addition, they do not require safety tests before being used, although they are more expensive and less effective than synthetic antioxidants [5].

The harmful effect of oxidation on human health has become a serious issue. According to Laguerre et al. [6] research has confirmed that antioxidants supplied by food products are essential for counteracting this oxidative stress. André et al. [3] stated that antioxidants are able to protect biological systems against oxidative damage, thus helping to prevent cardiovascular, neurologic and carcinogenic diseases. One solution to this problem is to supplement the diet with antioxidant compounds that are contained in natural plant sources. The source of these compounds is mainly the plant kingdom, and they are mainly phenolic compounds (flavonoids, phenolic acids and alcohols, stilbenes, tocopherols, tocotrienols), ascorbic acid and carotenoids, which can serve, in some sense, as a type of preventive medicine. In fact, it has been suggested that a high intake of fruits and vegetables could decrease the potential stress caused by reactive oxygen species via a number of mechanisms, including the protection of target molecules (lipids, proteins and nucleic acids) from oxidative damage [7].

In order to evaluate the impact of the use of antioxidants for food applications in terms of issued patents, the evolution in the number of patents from January 2000 until September 2009 was obtained from the Web of Knowledge. This data base obtains the information from the Derwent World Patents Index® and covers over 41 major patent issuing authorities worldwide. The search was performed within the Subject Area Food Science & Technology, and “antioxidant” in the patent title was used as a keyword. The search was refined by selecting the ones within the topic that include the keyword phrases “natural antioxidant” or “plant extract” and the results are also plotted in Fig. (II.1).

The number of patents on antioxidants in foods has increased steadily in the last decade, and this trend can also be detected in the use of natural antioxidants. As regards the number of patents concerning plant extracts, a regular pattern is observed, thus pointing out that the research in this field is in progress.

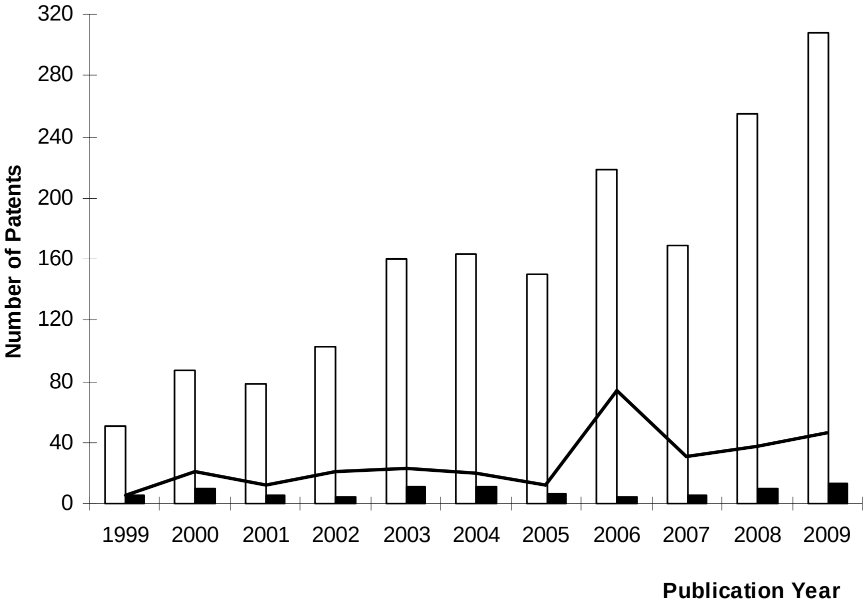


Figure II.1. Natural antioxidant solid line black plant extract.

The uses and applications of antioxidants in foods have been extensively reviewed in the last decade, with emerging highlights on the properties of natural antioxidants. In this sense, Krishnaiah et al. [8] have pointed out the antioxidant potential of extracts from the stems, roots, bark, leaves, fruits and seeds of several important medicinal species such as Diospyros abyssinica, Pistacia lentiscus, Geranium sanguineum L., Sargentodoxa cuneata Rehd. Et Wils, Polyalthia cerasoides (Roxb.) Bedd, Crataeva nurvala Buch-Ham., Acacia auriculiformis A. Cunn, Teucrium polium L., Dracocephalum moldavica L., Urtica dioica L., Ficus microcarpa L. fil., Bidens pilosa Linn. Radiata, Leea indica, the Lamiaceae species, Uncaria tomentosa (Willd.) DC, Salvia officinalis L., Momordica Charantia L., Rheum ribes L., and Pelargonium endlicherianum. Podsedek [9] focused on the content, composition, and antioxidant capacity of the antioxidant compounds present in raw Brassica vegetables such as cabbage, broccoli, cauliflower, Brussels sprouts, and kale. The effects of post-harvest storage, industrial processing and different cooking methods on the stability of bioactive components and antioxidant activity have also been discussed. Moreover, Leopoldini et al. [10] have just published a review article on the molecular basis and working mechanisms of natural polyphenolic compounds (phenolic acids, flavonoids and stilvenes). They have discussed the three main proposed mechanisms through which the antioxidants may play their protective role, reporting some valuable thermodynamic and kinetic data and in this way, this work has contributed to the elucidation of the health benefits of these antioxidants. A brief description of the mechanism involved in the majority of the techniques used for the evaluation of the antioxidant activity of essential oils has been given in a recent review [11]. At the same time, this review presented and compared the antioxidant activities of some essential oils, obtained by means of different in vitro assays. The present paper reviews the most recent patents in the area of antioxidant application in food products. The view of the authors on the future perspectives of this topic is given.

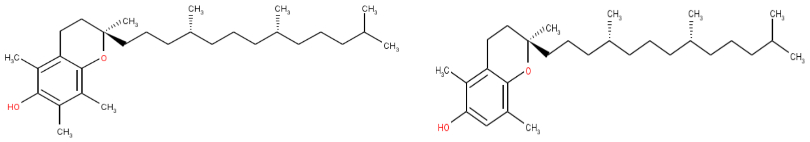
FOOD ANTIOXIDANTS: CHEMISTRY AND NATURAL SOURCES

Many different chemical compounds have shown some antioxidant activity through different mechanisms of action. According to Laguerre et al. [6], these mechanisms result in antioxidant activity of a particular compound, such as UV filtration, singlet oxygen deactivation, peroxidant enzyme inhibition, chelation of transition metals, enzymatic detoxification of reactive oxygen species, and their stabilization through hydrogen radical transfer.

Tocopherols

Tocopherols are amongst the most commercially exploited antioxidants. Figure II.2 shows the chemical structure of this group of compounds, and how the radicals determine the denomination of α, β, γ or δ tocopherols. α, γ and δ-tocopherols are commonly added to food products, and are denoted by the E numbers E-307, E-308 and E-309, respectively.

α-tocopherol β-tocopherol



γ-tocopherol δ-tocopherol

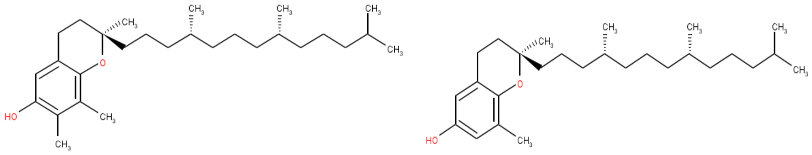


Figure II.2. Chemical structure of α, β, γ and δ tocopherols.

According to Podsedek [9], the predominant reaction responsible for the antioxidant activity of tocopherols is hydrogen atom donation, where a tocopheroxyl radical is formed. Vitamin E (α-tocopherol) is able to disrupt the chain reaction of lipid peroxidation [12], thus preventing free radical damage. Vitamin E works in conjunction with vitamin C, the latter regenerating α-tocopherol from the tocopherol radical formed by the reaction with radical oxygen species [13]. The ability of vitamin E to trap peroxyl radicals and singlet O_2 has been reported by previous studies [14, 15]. According to Leopoldini et al. [10], the radical scavenging ability of vitamin E is due to the OH group. Vitamin E is related to a series of health benefits such as coronary heart disease protection [16]. According to Greaves et al. [17], tocopherols have a potent ability to inhibit lipid peroxidation in vivo by trapping peroxy-radicals. Naguib et al. [18] patented compositions of α, β, γ, and δ-tocopherols showing increased antioxidant potency.

Phenolic Compounds

Phenolic compounds are a large group of antioxidants, widespread in the plant kingdom. Depending on their chemical structure, they are categorised into groups. The most diverse group is that of flavonoids, built upon a flavone skeleton (Fig. II.3).

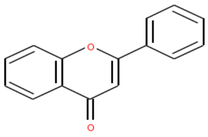


Figure II.3. Flavone (2-phenyl-1,4-benzopyrone) skeleton.

Phenolics are able to scavenge reactive oxygen species due to their electron donating properties [9]. Phenolic acids are also known to show transition metal chelation capacity [6]. Their stability in different systems, as well as the number and location of hydroxyl groups, determine their antioxidant effectiveness. Diverse studies [19, 20] have demonstrated the antioxidant activity of these compounds in vitro, which is higher than that of antioxidant vitamins and carotenoids. According to Leopoldini et al. [10] the planar structure of phenolics, which allows conjugation and electronic delocalisation as well as resonance effects, is directly linked to a good radical-scavenging activity.

The content of polyphenols in vegetables, as well as that of other phytochemicals, is affected by various factors: variety, cultural practices, climatic conditions, maturity at harvest and storage conditions. Colliver et al. [21] patented a process for increasing the flavonoid content of plants by increasing the activity of chalcone synthase and flavonol synthase.

In the last few years, a number of patents have dealt with extraction methods aimed at obtaining phenol-rich extracts from diverse vegetable sources. Cuomo and Rabowskiy [22] invented several methods for extracting antioxidant compounds from olive-based starting materials, including olives, olive pulps, olive oil and wastewater from olive oil manufacturing. Romanczyk et al. [23] claimed cocoa extracts (rich in polyphenols and procyanidins), the methods for preparing such extracts and their uses. Ronzio et al. [24] prepared water-soluble extracts from the seed coats of lentil. These extracts were a rich mixture of tannins (procyanidins and prodciphinidin), flavanone (luteolin), flavonols (e.g. quercetin, kaempferol) and phenolic acids (ferulic acid, protocatechuic acid, caffeic acid), with the ability to quench organic free radicals, to scavenge superoxide, to inhibit the oxidation of both water- and fat- soluble nutrients, and to limit damage caused by oxidants. King and Grabiel [25] patented a relatively inexpensive extraction method to obtain anthocyanins, other flavonoids and related polyphenolic compounds from fruits and vegetables. Nair [26] invented a method for isolating a mixture of anthocyanins, bioflavonoids, and phenolics from an edible berry using adsorbent, regenerable resins for reuse. The extract could be used as a dietary supplement or nutraceutical product.

Resveratrol (trans-3,5,4'-trihydroxystilbene) is a natural product found in grapes, mulberries and peanuts. It is one of the main non-alcoholic components in red wines. Its structure is characterised by two phenolic rings, linked by a double bond (Fig. II.4). The occurrence of multiple OH groups attached to an aromatic ring is directly linked to a good radical-scavenging activity [10].

Resveratrol has proved to be an effective antioxidant in different in vitro assays including: total antioxidant activity, reducing power, $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $DMPD^{\bullet+}$ and $O_2^{\bullet-}$ radical scavenging, hydrogen peroxide scavenging, and metal chelating activities, when compared to standard antioxidant compounds such as BHA, BHT, α-tocopherol, and trolox [27].

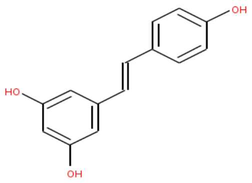


Figure II.4. Chemical structure of resveratrol.

The anthocyanins, anthocyanidins with sugar group(s), are mostly 3-glucosides of the anthocyanidins [28] (Fig. II.5). These contribute greatly to the antioxidant properties of certain colourful foods, such as grapes and cranberries. As pigments, they are almost exclusively responsible for the red, blue and purple colours in fruits. Cyanidin is the most common anthocyanidin, and the 3-glucoside is the most active antioxidant anthocyanin [29].

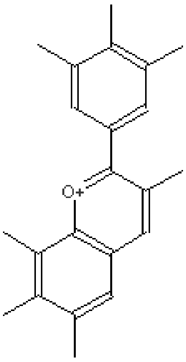


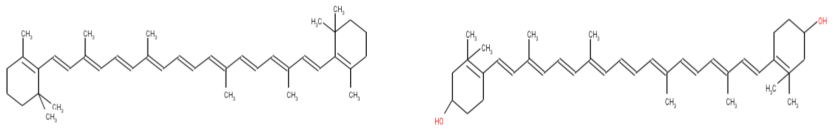
Figure II.5. Chemical structure of anthocyanins.

A method directed at efficient one or two step processes for producing phenol enriched compositions from dried and fresh plant material, particularly anthocyanins and proanthocyanins, was patented by Bailey et al. [30]. The method of preparation includes a novel column purification step using a brominated polystyrene resin.

Carotenoids

This group of compounds comprises both carotens and xanthophylls, which are present in many fruits and vegetables. They are red, orange and yellow pigments, and act as vitamin A precursors. Some examples are β-carotene, cryptoxanthin and lycopene. Lycopene is a bright red carotenoid pigment, naturally found in red fruits. Because of its unsaturated nature, lycopene is considered as a potent antioxidant and an oxygen quencher [31]. Of all the carotenes, it is considered the most efficient at quenching singlet O_2 [32]. Rice-Evans et al. [33] found a correlation between low serum β-carotene levels and high rates of cancer, cardiovascular diseases and a high risk of myocardial infarction among smokers.

Their conjugated double bonds (Fig. II.6) provide carotenoids with a double mechanism of action: they are both quenchers of singlet oxygen and radical scavengers [9]. It is commonly accepted that the presence of additional functional groups, such as carbon-carbon double bonds, is one of the main structural characteristics for good radical-scavenging activity [10].



β–caroten

Cryptoxanthin

Lycopene

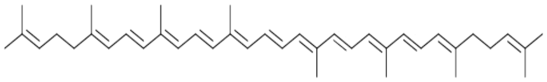


Figure II.6. Chemical structure of some carotenoids: β-carotene, cryptoxanthin and lycopene

Some recent patents have aimed at improving the characteristics of carotenoids. Foss et al. [34] described methods for synthesizing and administering carotenoid compounds with improved antioxidant characteristics. Takeda Chem. Ind. LTD [35] obtained a water-dispersible carotenoid composition comprising a dissolving/suspending agent for the carotenoid and an emulsifier. This composition is useful as an additive for food, pharmaceuticals, cosmetics and feeds. Fullmer and Emmick [36] formulated a stable nanodispersion of one or more carotenoids for use in supplementing aqueous systems, such as foods, beverages and dietary supplements. Hara et al. [37] obtained a microcapsule including a natural high strength carotenoid, hardly undergoing oxidative deterioration, and useful in foods and medicines.

Essential Oils

Essential oils are volatile oils which constitute the aroma and flavour components of organic material [17]. They are used in a variety of products such as incense, aromatherapy oils, perfumes, cosmetics, pharmaceuticals, beverages, and foods. The market for these oils demands a consistently high quality and reliable supplies at competitive prices.

Essential oils from aromatic and medicinal plants have been known to be biologically active, mainly possessing antibacterial, antifungal and antioxidant properties [38]. The main components can represent up to 85% of the total, while the remainder is present as traces. The concentration of the specific compound in the total mix of plant oils can be very variable, depending on factors such as the origin, species and plant organ, climatic conditions and growth, extraction and storage.

Essential oils consist mainly of volatile terpenoids, consisting of linked isoprene units in structures of 10 carbons (monoterpenoids) and 15 carbons (sesquiterpenoids). The oil is composed of at least 100 different chemical compounds classified as aldehydes, phenols, oxides, esters, ketones, alcohols and terpenes [5]. Table II.1 shows the major components of different essential oils with reported antioxidant activity.

Table II.1. Composition of some essential oils.

Essentials oil	**Oregano**	**Sage**	**Basil**	**Cinnamon**
Reference	Fasseas *et al.*, (2007)	Fasseas *et al.*, (2007)	Fasseas *et al.*, (2007)	Simic *et al.*, (2004)
Major components (%)	Thymol (60.9)	Eucalyptol (49.4)	Linalool (28,6)	Cinnamaldehyde (63)
	ρ-cymene (10.5)	Camphor (8,5)	Estragole (21,7)	Limonene (8)
	γ-terpinene (7,6)	α-pinene (5,4)	(E)-Methyl (14.3)	Eugenol (7)
	Carvacrol (5,8)		α-Cadinol (7,1)	Cinnamaldehyde propylene (5.5)
			Eugenol (5.9)	Terpenoid compounds (<1-2)

Essentials oil	**Laurel**	**Rosemary**	**Thyme**
Reference	Jelnar *et al.*, (2010)	Zaouali Y. *et al.*, (2010)	Rota *et al.*, (2008)
Major components (%)	Eucalyptol (40.91)	1.8-Cineole (40.0)	Thymol (57.7)
	α-pinene (5.82)	Camphor (17.9)	p-cymene (18.7)
	β-pinene (4.55)	a-pinene (10.3)	Carvacrol (2,8)
	Sabinene (6.92)	Camphene (6.3)	
	Limonene (2.10)	Carvacrol (0.2)	
	Linalool (1.29)		

Essential oils can be extracted using a number of methods, such as steam distillation, hydrodistillation, organic solvent extraction, microwave assisted distillation, micro-wave hydrodiffusion and gravity, high pressure solvent extraction, supercritical CO_2 extraction, ultrasonic extraction and solvent free microwave extraction. The properties of the essential oils extracted by these methods have been found to vary depending on the method used [39]. The essential oil of R. officinalis has usually been isolated by traditional hydrodistillation, steam distillation or organic solvent extraction. For example, monoterpenes are well known to be vulnerable to chemical changes under steam distillation conditions and even conventional solvent extraction is likely to involve losses of more volatile compounds during the removal of the solvent [40].

Different methods of essential oil extraction have been reported and patented. Some of them generally relate a method for the simultaneous extraction of essential oils and antioxidants from organic material, more particularly organic material from the Lamiaceae family, including rosemary, using solvent blends and which yields a liquid, oily extract containing antioxidants and a liquid extract containing essential oils. The extract containing antioxidants is readily mixed with edible oil to be added to animal feeds and human food. These essential oils are pharmaceutical grade. Generally, methanol extraction is used, but it produces a dry powder that is difficult to dissolve into the preferred carriers, such as edible oils. This patented alternate extraction technology, based on tetrafluoroethane (TFE), uses the vapor pressure of the solvent at room temperature, and permits extraction under mild conditions. Thus, this process minimizes the oxidative decomposition of carnosic acid during the extraction. TFE is substantially apolar and is preferably blended with acetone in the extractions of rosemary. The advantages of TFE show that it is non-flammable, has a low boiling point, is environmentally acceptable (very low toxicity) and easily handled. This study included the solvent blend comprising between about 70% and about 85% tetrafluoroethane, between about 1 % and about 25% acetone, and between about 1 % and about 25% methanol [17].

Some extraction methods produce water soluble antioxidants that can be used in a wide range of food products. Nahas et al. [41]

patented a metal-chelating or sequestering antioxidant composition, derived from edible herbs and spices (mace, thyme, oregano, nutmeg, ginger, cinnamon, clove, basil, marjoram, mustard, savory, laurel and anise) that are useful for incorporating into food, beverages, and nutritional supplements to enhance their stability. In fact, these water soluble antioxidants were disclosed to be useful in fruit juices, processed meat products, such as ham and sausages, processed seafood, butter, margarine, mayonnaise, salad dressings, and essential oils (lemon, lime, grapefruit and orange).

Chitosan

Chitosan is a cationic biopolymer that is considered a secondary antioxidant, since it has the ability to chelate the metal ions involved in the catalysis of an oxidative reaction [42]. Chitosan of differing degrees of N-deacetylation obtained from crab shells showed antioxidant activity, scavenging ability on hydroxyl radicals and chelating ability on ferrous ions. It was more effective as an antioxidant agent when the deacetylation degree increased

[43]. Similar effects were observed in fungal chitosan obtained from shiitake stipes [44].

The origin of the scavenging ability of chitosan is related with the presence of active hydroxyl and amino groups in the polymer chains (Fig. II.7). The hydroxyl groups in the polysaccharide units can react with free radicals and, according to free radical theory, the amino groups in chitosan can react with free radicals to form additional stable macroradicals [45]. As regards the effect of the molecular weight of chitosan on its antioxidant properties, Xing et al., [45] showed that low molecular weight chitosan had a stronger scavenging activity on oxygen and hydroxyl groups than high molecular weight chitosan in an in vitro study. The same effect was observed by Feng et al. [46] by reducing chitosan molecular weight by means of irradiation treatments.

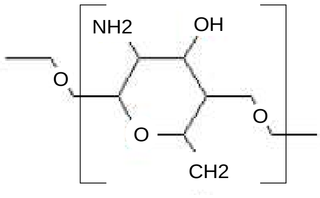


Figure II.7. Chemical structure of chitosan

Synergistic Effects

Numerous studies have reported the synergistic effects found in antioxidant mixtures [47-49]. Indeed, combinations of antioxidants may be more effective at reducing reactive oxygen species than pure compounds, especially if the mixture includes both water-soluble and lipid-soluble antioxidants. If this is the case, the mixture will be capable of quenching free radicals in both aqueous and lipid phases [50].

The application of antioxidants (pure, mixtures and extracts) to food products is described in the following section. The synergistic effect in the antioxidant mixtures is a common phenomenon that takes place in the final products.

PATENTS ON THE APPLICATION OF ANTIOXIDANTS

The number of patents on the application of antioxidants to food products has progressively increased over the last few years. Stuckler [51] formulated a food supplement with advantageous physiological effects containing lecithin, red wine extract and D-alpha-tocopherolacetate. The support material for such supplement would be dairy products, fruit or plant juice, yeast, pectin or olive oil. Fukumoto et al. [52] invented an antioxidant material containing flavonoid aglycon (derived from lemons, limes or sudachis) and vitamin C, a combination in which a synergistic effect is observed. This extract would be added to food products or beverages. Tan et al. [53] reported the invention of a method for extracting the antioxidant compounds (phenolics and flavonoids) from the palm tree. These compounds are potent antioxidants to be applied in foods and edible oils. Ahotupa et al. [54] claimed a food product containing a phenolic compound (hydroxymatairesinol), and found that the administration of this could increase the level of enterolactone, thereby causing cancer prevention.

Meat and Fish Products

Meat, poultry and fish products are very prone to lipid oxidation both because of their high content in unsaturated fatty acids and also due to factors intrinsic to processing and storage conditions. In these products, oxidative reactions can lead to undesirable changes in taste, flavour, and colour. The addition of antioxidants can reduce the rate of lipid oxidation and hydrolysis by sequestering and stabilizing free radicals. In this sense, Montenegro [55] developed a natural antioxidant composition prepared from phenolic extracts of monofloral honey that prevents the oxidation of meat products, especially poultry. The honey extract contained gallic acid, rutin, ferulic acid, salicylic acid, naringenin, kaempferol and a pH of 4.2-5.0. Hadolin Kolar et al. [56] patented a natural mixture for the antioxidative protection of fats and foodstuffs containing fats, such as fish, fresh meat, fresh spiced meat, fresh and cooked sausages, salami, dry cured and cooked cured products, and pastrami. The mixture was prepared with an extract of at least one plant selected from the Labiatae family and green tea extract and comprises carnosic acid, rosmarinic acid, and epigallocatechingallate. Fellenberg Plaza et al. [57] formulated a liquid extract from soapbark tree comprising 1.4-5.4% total phenols. The extract was applied to marinated chicken leg and chicken breast. Results showed that the greater concentration of polyphenols in this extract showed a prooxidant effect after 2 days of refrigeration. Sandoval et al. [58] succeeded in preventing the discoloration of fresh beef slices, which were vacuum packed and refrigerated, by means of the injection of a vegetable protein composition containing antioxidants (alkali metal salt of isoascorbic acid). The vegetable material comprises a soybean protein material (soy flour, soy protein concentrate or soy protein isolate), wheat gluten or zein. Gaynor et al. [59] incorporated ascorbate or erythrobate as antioxidants in the formulation of emulsified casings prepared with cellulose and nisin and applied to meat products (e.g. frankfurter). The casing was stored for two months at between 4 and 37ºC and nisin activity against Listeria monocytogenes was stable during the whole storage period. The results also illustrated the unexpected slow usage rate of the antibiotic and reduced the production cost of the antimicrobial casing to make it economically feasible. The antioxidant activity of chitosan solutions has been tested in a cooked cod model system and results showed a reduction in both the peroxide index and thiobarbituric reactive substances in line with an increase in chitosan concentration and a decrease in its molecular weight [60].

Edible Oils

The development of off-flavours in edible oils represents a serious problem in the food industry. Lipid oxidation, apart from producing rancid odours and flavours, decreases the nutritional quality and the safety of the product. Antioxidant addition allows this deteriorative process to be controlled. Breivik et al. [61] provided a composition comprising marine oil and ascorbic acid and/or an ascorbic acid derivative (such as ascorbyl palmitate) that would slow down lipid oxidation in marine oil. This formulation does not include lecithin, which has to be declared as a potential allergen. Todd [62] reported an activated ascorbic acid and compositions thereof, with the aim of increasing antioxidant activity in fats, oils and fatty products, especially in such materials that are exposed to oxidative stress. Synergistic effects were observed by the incorporation of tea extract.

Antioxidants can also be incorporated in the frying oil. Gertz [63] obtained a water-in-oil emulsion that can be used as an additive for roasting, simmering or frying fat, preventing lipid oxidation. The emulsion contained antioxidants (tocopherol and/or ascorbyl palmitate) in combination with water-soluble carboxylic acids (citric acid) which are present in the aqueous phase. Cholli et al. [64] invented a substitute benzene antioxidant polymer to prevent rancidity. This antioxidant polymer can be blended with or mixed with the packaging material, present as a thin film, or be sprayed on the packaging material to form a coating.

Fruits and Vegetables

In plant products, enzymatic browning represents the main deteriorative process. This is caused by the enzyme polyphenol oxidase which, in the presence of oxygen, converts phenolic compounds into dark coloured pigments. Sardo and Bompeix [65] patented a process for treating fruits and vegetables with a composition containing one or more tocopherol salts and a terpene in an aqueous solution at 40-60ºC. This process was particularly suitable for treating lettuce, apples and pears after harvesting. Paliyath and Murr [66] claimed a composition that may be applied to vegetal produce by spray, drench, dip or a vapour and at either the pre-harvest or post-harvest stage of fruits, vegetables and partially-processed products. This composition comprises diverse antioxidant compounds. Selleck [67] used a flavonoid for the preservation of minimally processed fruits and vegetables. The products, previously cut and peeled, were sprayed or dipped in a flavonoid solution containing some other components/another component, such as ascorbic acid, erythorbic acid or alpha lipoic acid. They can also be preserved by the addition of flavonoid and ascorbic acid into juices (if they are not present). Lee and Ryu [68] patented a tocopherol containing milk rice and a method for its preparation. The method comprised one step where tocopherol was incorporated into the product.

Beverages

The addition of antioxidants to drink products stems from the necessity of increasing their quality and/or prolonging their shelf-life. Additionally, the fortification of these products with vitamins may be the desired goal. Parshall et al. [69] added a tocopherol antioxidant, other than α- tocopherol, to a citrus fruit beverage such as orange juice, in order to preserve the sensory attributes during storage. Cox et al. [70] prepared oil in water dispersions of β-caroten and other carotenoids that are stable against oxidation. These dispersions are especially useful in diluted juice beverages for providing vitamin A fortification and colour, as well as other oil/water, food and beverage products where vitamin A fortification and/or colour are desired. Henry et al. [71] provided a method for fortifying dry beverage mixes. The formulation included a ferric ion reducing agent (ascorbic acid) and/or citric acid, as well as polyphenols and flavonoids which are typically present in these products. Vitamins such as the B vitamins, vitamin A, vitamin C and vitamin E could be added, too. Lahteenmäki [72] presented a drink composition with active agents and a method for composing this drink. Some of the components included are flavonoids, isoflavonoids, vitamin C and carotenoids. Kobayashi et al. [73] claimed that chitosan oligosaccharides can be potentially used as an active ingredient to prevent an oxidative reaction in food or beverage products.

Animal Feeds

Some recent patents on the use of antioxidant products deal with the incorporation of the active substances into animal feeds rather than adding these ingredients directly to meat and fish products. Stevenson-Barry [74] improved the color stability of horse, ostrich, emu, cow, sheep or deer meat by a method that comprises the oral administration of at least one antioxidant (e.g. ascorbic or its metabolites, β- carotene) to the living animal and waiting for the antioxidant to become present in the meat after slaughtering. Saunders et al. [75] presented a method for improving the animal tissue quality, which comprised feeding the animal a diet supplemented with γ-tocopherol in a large enough amount to achieve such an aim. The γ-tocopherol may be fed alone or in combination with other antioxidants, such as α- tocopherol. This method may be carried out both on ruminants and non-ruminants. Kaw et al. [76] improved the carotenoid content in the yolk of eggs by enriching the poultry diets with a carotenoid formulation comprising a carotenoid, a vegetable oil, a surfactant, a chelating agent, an antioxidant, an alkali and a solvent.

CURRENT AND FUTURE DEVELOPMENTS

At present, the most commonly used antioxidants are of a synthetic origin, such as butylated hydroxyanisole (BHA, E- 320), butylated hydroxytoluene (BHT, E-321) and propylgallate (E-310). Some of these synthetic antioxidants have been restricted by legislative rules due to doubts over their toxic and carcinogenic effect. However, they are still broadly used by the food industry. That is the case, for instance, of BHA and BHT. Therefore, there is a growing interest in safer antioxidants for food applications, and a growing trend in consumer preferences for natural antioxidants, all of which have given impetus to the attempts to explore innovative natural sources of antioxidants [27]. In fact, the number of papers dealing with the characterization of the antioxidant activity of natural extracts (obtained from different sources) is rapidly increasing. The most common sources of these new antioxidants are exotic fruits and vegetables as well as aromatic plants. Moreover, some of these antioxidants can be obtained from vegetable waste, such as okara [77], potato peels [78], hazelnut kernels [79], and olive oil mill and wine waste [80, 81]. In this way, important by-products of the food industry have become value-added ingredients, thus simultaneously reducing the amount of industrial waste.

The necessary dose of antioxidant ingredients could be reduced by incorporating them into edible films and coatings [82, 83]. Controlled- release packaging can offer a significant potential for extending the shelf-life of foods by slowly releasing antioxidants over time [84]. Strong flavoured natural antioxidants, such as essential oils, could be incorporated into packaging materials to prevent oxidation and thus find a compromise between the antioxidant role and the pleasant taste and aroma of food. In this sense, Mastromatteo et al. [85] developed zein-based mono and multilayered edible composite films loaded with spelt bran and thymol to control thymol release. For both mono and multilayered films, the thicker the film, the slower the release rate,without the addition of spelt bran. On the contrary, a significant increase in the thymol release rate was detected in line with an increase of the bran concentration for both mono and multilayered films, probably due to the fact that the addition of spelt bran promoted the formation of micro-channels that interconnected the thymol phase, bypassing the zein matrix and leading to an increase in the active compound release rate. Soto-Valdez et al. [86] developed an active packaging consisting of a plastic film that promoted the migration of the active compound (α- tocopherol) toward the packaged product during the storage.

There is a growing interest in the development of encapsulation techniques [87] whose aim is the reduction of the strong aroma and unpleasant taste of some polyphenols, while preserving and improving the stability, bioactive and bioavailability of these compounds. Microencapsulation has also been used to protect essential oils against the environmental factors that contribute to their deterioration, such as oxygen, light, and moisture. Several essential oils have been microencapsulated in various chemical structures and by different processes [88]. These reported studies have pointed out that microencapsulation can preserve essential oil activity without causing its degradation.

In conclusion, the use of antioxidants in food products allows their shelf-life to be extended. The application of these compounds to foodstuffs and supplements could be attained through new technologies, such as microencapsulation and active packaging, which entail additional advantages. Future patents will probably deal with these new advances, placing special emphasis on the incorporation of natural antioxidants.

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Conflict of interest

The authors declare no conflict of interest

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OBJECTIVES

OBJECTIVE

The general objective of this work was to formulate and characterize biodegradable films based on chitosan or blends with other biodegradable polymers, as well as the incorporation of antimicrobial and/or antioxidant components in order to obtain active packaging materials.

SPECIFIC OBJETIVES

- To evaluate the effect of incorporation basil and thyme essential oils and homogenization conditions on properties of chitosan based films.
- To analyze the preservation power of these chitosan films, containing essential oils, on pork meat products: their antimicrobial activity and their impact on other meat quality parameters.

- To characterize the properties of blend films containing chitosan and wheat starch and their potential antimicrobial activity.
- To evaluate the effect of the incorporation antioxidant components on the properties of wheat starch-chitosan blend films and their potential antioxidant capacity.
- To analyze the polymer compatibility and the physical and antimicrobial properties of blend films of chitosan and poly vinyl alcohol.
- To characterize physicochemical and antimicrobial properties of extruded polylactic acid films as affected by addition of chitosan powder with different particle size.

RESULTS AND DISCUSSION

EFFECT OF BASIL AND THYME ESSENTIAL OILS AND HOMOGENIZATION CONDITIONS ON PROPERTIES OF CHITOSAN BASED FILMS.

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Abstract

The influence of essential oil type (basil and thyme), its content and the homogenization treatment on the physical properties of chitosan-based film-forming dispersions and edible films was studied. Two homogenization treatments were applied, without (H1) and with (H2) microfluidization (MF) at 165 MPa. H2 emulsions showed the smallest particles with the highest ζ-potential and the lowest viscosity. Composite films with essential oils were softer, less rigid and more stretchable than pure CH films. MF intensified these changes. H2 films showed micro-cracks due to the weakening of the polymer chain interaction forces when oils are present, which affected their mechanical behaviour. MF increased WVP of pure CH films while oil incorporation was only effective to reduce WVP when they were incorporated at the lowest ratio and when high pressure was used in the homogenization of the film forming dispersion. Gloss was reduced by the essential oil addition, whereas MF tended to yield glossier films.

Keywords: chitosan, essential oils, microfluidization, basil, thyme

1. Introduction

Edible films and coatings are prepared from biopolymers and are able to protect food products extending their shelf-life. The major constituents of these are polysaccharides, proteins and lipids. Chitosan (CH) is a cationic polysaccharide with excellent film-forming properties. It is obtained from chitin by deacetylation in the presence of alkali (Sánchez-González *et al.*, 2010). The combination of this hydrophilic constituent and some lipids could produce films with optimized characteristics (Baldwin *et al.*, 1997). Many lipids have been incorporated to composite films, mainly aiming to reduce the water vapour permeability of these hydrophilic materials. Essential oils (EO) are interesting lipids to be incorporated into the films since these plant extracts exhibit additional characteristics, such as antimicrobial and antioxidant effects (Bakkali *et al.*, 2008;

Miguel, 2010). This would be in line with current research into the reduction of the use of chemical additives in the food industry. As they have a compatible aroma with several kinds of foods, such as meat and fish products or vegetables, basil and thyme oils could be used in the film or coating formulations. Their main components and antioxidant capacity are shown in table III.1. According to Tajkarimi *et al.* (2010), previous studies revealed antimicrobial capacity of basil and thyme against *Bacillus subtilis, Clostridium botulinum, Escherichia coli, Listeria monocytogenes, Salmonella typhimurium and Staphylococcus aureus* and thyme against *Staphylococcus aureus, Listeria monocytogenes, Pseudomonas aruginosa, Salmonella typhymurium* and *Escherichia coli*. Due to the lack of miscibility of components, the preparation of the film forming dispersions requires their emulsification in a solvent (usually aqueous systems) prior to the casting for film formation. Droplet size is a determining factor for emulsion stability, and affects other important properties of the emulsion, including its viscosity. Rotor-stator homogenizers are often used in the food industry, and are able to reach particle sizes in the range of 1 µm. This can be further reduced by applying high pressure homogenizers to the system. Microfluidization (MF) can provide emulsions with very small particles with very narrow particle size distributions due to the fact that the emulsion is submitted to high shear stress in the interaction chamber. According to Vargas *et al.* (2010), there are few studies dealing with the effect of microfluidization on the properties of the film-forming dispersions and the obtained films or coatings.

The aim of this work was to characterize the relevant physical properties of CH edible films containing basil and thyme essential oils and of the corresponding film forming dispersions, in order to evaluate the effect of the oil type, its content and the kind of homogenization treatment on the typical film characteristics.

2. Materials and methods

2.1. Materials

High molecular weight chitosan (CH) (Batch 12913CJ, Sigma-Aldrich Quimica, Madrid,

Spain) solution was used to prepare the film-forming dispersions (FFD) (0.8 Pa s viscosity, at 2% w/w in 2% w/w glacial acetic acid). Glycerol, (Panreac Química, S.A., Castellar del Vallés, Barcelona, Spain) was used as film plasticizer. Basil (B) and thyme (T) essential oils were provided by Herbes del Moli (Alicante, Spain) and $Mg(NO_3)_2$ by Panreac Química, S.A. (Castellar del Vallés, Barcelona, Spain).

2.2. Preparation of the film-forming dispersions and casting of the films

Chitosan (1.0% w/w) was dispersed in an aqueous solution of glacial acetic acid (1% v/w) under magnetic stirring at 40°C. The emulsions were obtained by adding B or T at 0.5 or 1.0% (w/w). Two different homogenization treatments, namely H1 and H2, were used. In method H1 FFD were treated in a rotor-stator homogenizer (Ultraturrax DI 25 basic-Yellowline, Janke & Kunkel, Staufen, Germany) at 21,500 rpm for 4 minutes. Treatment H2 consisted of H1 plus microfluidization (MF) at 165 MPa in a single pass by means of a Microfludizer® M110-P processor. After homogenization, the formulations were degasified at room temperature with a vacuum pump.

The FFD were cast at 5.6 mg solids/cm^2 and were poured onto a framed and leveled polytetrafluorethylene (PTFE) plate (diameter = 15 cm) and dried at room temperature and 60 % RH. The films were peeled off from the casting plates and conditioned for one week at 5ºC and 58%RH in a chamber containing an oversaturated solution of $Mg(NO_3)_2$.

2.3. Characterization of the film-forming dispersions

2.3.1. Particle size distribution

The particle size analyses of the emulsions were carried out using a laser diffractometer (Mastersizer 2000, Malvern

Instruments, Worcestershire, UK) with ultrasound application. The samples were diluted in a sodium acetate buffer solution (175 mM) under the appropriate solvent conditions (pH = 4.8) at 2,000 rpm until an obscuration rate of 10% was obtained. Mie theory was applied considering a refractive index of 1.47 and 1.50 for T and B essential oils, respectively, and 0 absorption in both cases. Three replications per formulation were made. The volume-surface mean diameter ($d_{3,2}$) (Atarés, Bonilla and Chiralt, 2010) as well as the droplet size distribution were determined.

2.3.2. ζ-potential

ζ-potential was measured using a Zetasizer nano-Z (Malvern Instruments, Worcestershire, UK). The electrophoretic mobility of the droplets was transformed into ζ-potential values using the Smoluchowsky model. The samples were diluted to a droplet concentration of 0.02% using a sodium acetate buffer solution (175 mM) at pH 4.8.

2.3.3. Rheological behaviour

The rheological behaviour of FFD was analysed in triplicate at 25ºC using a rotational rheometer (HAAKE Rheostress 1, Thermo Electric Corporation, Karlsruhe, Germany) with a sensor system of coaxial cylinders, type Z34DIN Ti. Samples were left to rest for 5 minutes before the measurements were taken. The shear stress (σ) was obtained as a function of shear rate (γ) between 0 and 300 s^{-1}, taking 3 minutes for each (up and down) cycle. The power law model (Eq. 1) was applied to determine the consistency index (K) and the flow behaviour index (n). Apparent viscosities were calculated at 100 s^{-1}.

$$\sigma = k\,\gamma^{n} \qquad (\mathrm{Eq.\,1})$$

2.4. Characterization of the films

2.4.1. Thickness

Film thickness was determined with a Palmer digital micrometer (Comecta, Barcelona, Spain) to the nearest 0.001 mm. Six samples were considered for WVP tests and four measurements were taken for the tensile tests.

2.4.2. Microstructure

Microstructure was observed by SEM in cross-118 sectioned cryofractured film specimens, using a JEOL JSM-5410 (Japan) electron microscope. The film samples were equilibrated in P_2O_5 to eliminate water, cryofractured by immersion in liquid nitrogen, and then mounted on copper stubs perpendicularly to their surface. After gold coating, the images were captured using an accelerating voltage of 10kV.

2.4.3. Mechanical properties

Mechanical properties were analysed by means of tensile tests (ASTM standard method D882, ASTM, 2001), to obtain the true stress (σ) *vs.* Hencky strain (εH) curves. The mechanical parameters: elastic modulus (EM), tensile strength at break (TS) and elongation percentage at break (%E) were obtained. A universal test Machine (TA.XTplus model, Stable Micro

Systems, Haslemere, England) was used to perform the tests. Rectangular samples (25x100 mm) were cut and stored at 25°C and 58% RH in a cabinet containing $Mg(NO_3)_2$ saturated solution. The moisture content of the films submitted to the mechanical test ranged between 0.09 and 0.15 (wb), with a significant ($p<0.05$) negative effect of both homogenization pressure and oil content. Equilibrated film specimens were mounted in the film-extension grips and stretched at 50 mm min^1 until breakage. Six replicates of each formulation were tested.

2.4.4. Water vapour permeability (WVP)

Water vapour permeability (WVP) was determined gravimetrically at 5ºC and 58-100%, RH gradient, using a modification of the ASTM E96-95 gravimetric method (1995) for hydrophilic films (Mc Hugh *et al.*, 1993). Payne permeability cups of 3.5 cm in diameter (Elcometer SPRL, Hermelle/s Argenteau, Belgium) were filled with 5 ml of distilled water (100% RH). Films were selected on the basis of a lack of defects and three round samples were cut per formulation, and thickness was measured. The films were secured and the cups were placed in pre-equilibrated cabinets fitted with a fan, at 5ºC. The RH of the cabinets was held constant at 58% using oversaturated solutions of $Mg(NO_3)_2$. The shiny side of the films was exposed to the 58% RH atmosphere. The cups were weighed periodically after the steady state had been reached. WVP was calculated with the equations described by Atarés, Bonilla & Chiralt (2010).

2.4.5. Gloss

The gloss of the films was measured with a gloss meter (Multi. Gloss 268, Minolta, Germany) on their shiny side, at an incidence angle of 60º (ASTM D523, 1999). Ten replicates were made in each film. Results were expressed as gloss units, relative to a highly polished surface of black glass standard with a value near to 100.

3. Results and discussion

3.1. Characterization of the film-forming dispersions

3.1.1. Particle size distribution

The droplet size distribution of an emulsion has a great impact on its physicochemical and sensory properties, such as appearance, flavour and texture, and stability (McClements, 2009). Figure III.1 shows the particle size distribution in % volume of the emulsions whose mass ratio CH:EO was 1:1. A similar pattern was found for 1:0.5 ratio. Table III.1 shows the values of mean size $d_{3,2}$ and the mode of size distributions of all the emulsions. In every case, the size distributions were multimodal, probably due to droplet flocculation taking place after the homogenization process. Nevertheless, this aggregation was relevant in terms of volume but not in terms of number, which indicates that only a small part of oil forms great aggregates, probably due to the enough polymer to ensure the complete interfacial coverage of the oil particles, which gives rise to bridging flocculation.

Homogenization treatment H2 led to a significant ($p<0.05$) reduction of the particle sizes, as compared to H1, as can be seen in Figure III.1 for samples containing 1% oil. This can also be observed in Table III.1 through the values of the $d_{3,2}$, regardless of the type of essential oil and its content. This is expected, as reported by McClements (2009) who stated that emulsions that have undergone secondary homogenization (size reduction of the droplets) usually contain smaller droplets than those that have undergone primary homogenization (blending of aqueous and lipid phase to create the emulsion). In these emulsions, $d_{3,2}$ was reduced from 5-9 μm in H1 emulsions to 0.4-1.5μm in H2 emulsions (Table III.2). Similar results were obtained by Vargas *et al* (2010) working with 0.5% chitosan-0.5% oleic acid FFD submitted to H2 homogenization conditions.

Oil content had a significant impact on $d_{3,2}$ and the peak maximum (mode) in size distribution (Table III.2). The higher the oil ratio in the emulsions, the bigger the particles. This effect has been observed previously by Sánchez-González *et al.* (2010) in emulsions of bergamot essential oil in chitosan aqueous systems. This can be explained by the increase in the dispersed phase concentration, which promotes the droplet flocculation rate, as well as the reduction of the ratio between the interfacial stabilizing material and the dispersed phase. The latter can induce the bridging flocculation if there is not enough stabilizing material to cover the complete interfacial area of the smaller droplets (McClements 2005). The type of essential oil significantly affected $d_{3,2}$ ($p < 0.05$). Amongst H1 emulsions, those prepared with thyme oil showed the lowest values while, although H2 emulsions showed very similar values at a determined oil concentration, they showed an opposite tendency. This suggests that thyme oil amphiphilic components could have greater surfactant activity, thus contributing to reduce the droplet size under the softer homogenization conditions. Nevertheless, under more drastic conditions, which promote conformational changes in the polymer and more intense contact among components, this effect was not relevant.

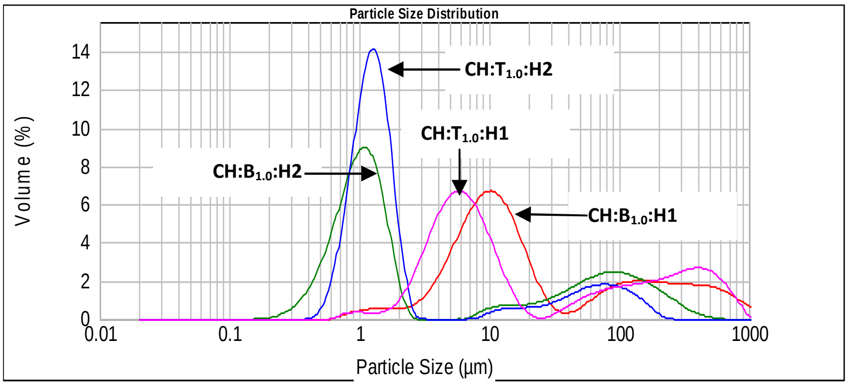

Figure III.1. Particle size distribution in terms of volume of film-formulations with basil (B) or thyme (T) essential oils, at 1% concentration in the film-forming dispersion.

3.1.2. ζ-potential

According to McClements (2005), if the droplet charge is sufficiently high, the emulsion may be stable against aggregation due to electrostatic repulsion. The values of ζ-potential of CH dispersions and all emulsions with 0.5 and 1% oil content are shown in Table III.1, all of which are positive due to the positive charge of the particles. The pH of the FFD was 3.2, regardless of the essential oil concentration and homogenization treatments. At this pH value, the amino groups of chitosan are positively charged (pK_a $NH_3^+/NH_2 \approx 6.5$), which agrees with the obtained values.

In addition, the ζ-potential of the essential oil dispersions in distilled water (in the absence of chitosan) and submitted to both homogenization treatments was obtained. For H1 treatment, the values were -27.8±1.2 mV and -13.4±0.9 mV for basil and thyme oils, respectively. For H2, the ζ-potential values were -31.1±1.1 mV and -10±4 mV for basil and thyme, respectively. This negative charge is associated with the presence of dissociable compounds in the oils and with the adsorption of negative ions on the droplet surface. According to the obtained values, basil oil droplets showed greater surface charge than thyme oil droplets, without significant differences due to the homogenization intensity. This could be attributed to differences in the dissociation degree of ionisable compounds of the different oils. The adsorption of chitosan on the droplets of essential oils results in positively charged dispersed particles. This change in the surface charge of dispersed particles was also found by incorporation of chitosan to oil in water emulsions with different types of surfactants (Hou et al., 2010; Mun, Decker, & McClements, 2006).

The ζ-potential values of pure CH dispersions were the highest (64 mV and 72.3 mV, respectively for H1 and H2 homogenization conditions), and the charge was partially neutralized when adsorbed on the negatively charged oil droplets. It could be assumed that, the more positive the ζ-potential, the greater the amount of adsorbed CH at the droplet interface. All H1 emulsions could be considered as relatively stable due to their relatively high surface net charge and the steric stabilization effect of the adsorbed CH (Roland, Piel, Delattre, & Evrard, 2003).

Microfuidization resulted in a ζ-potential increase ($p < 0.05$) of chitosan dispersions which indicates that homogenization induced conformational changes in the polymer chain, which increased the number of charged groups in the polymer hydration surface. This is also reflected when it is adsorbed on the oil droplets, which also show greater values of ζ-potential than when obtained by the H1 procedure. Therefore, further stabilization of the FFD is attained by H2 treatments, since, additionally to the droplet size reduction, adsorption of chitosan on the oil droplet surface is favoured and the surface charge increases. The effect of the CH:oil ratio and the kind of oil was similar to that commented on for H1 samples: the greater the oil ratio, the greater the surface charge. A slightly lower charge was obtained for thyme than for basil oil.

Table III.1. Average diameter $d_{3,2}$, maximum peak in size distributions and ζ–potential. Averagevalues and standard deviations, in brackets

	Homog.	**CH**	**CH:B$_{0.5}$**	**CH:B$_{1.0}$**	**CH:T$_{0.5}$**	**CH:T$_{1.0}$**
***d$_{3,2}$* (μm)**	**H1**	-	8.7 (0.7)^{x,a}	9.3 (0.7)^{x,b}	5.3 (0.7)^{x,c}	6.6 (0.6)^{x,d}
	H2	-	0.42 (0.02)^{y,a}	1.30 (0.04)^{y,b}	0.47 (0.06)^{y,c}	1.48 (0.03)^{y,d}
Peak (μm)	**H1**	-	2.9 (0.9)^{x,a,b}	4.8 (0.3)^{x,c}	2.5 (0.0)^{x,a}	3.5 (0.0)^{x,b}
	H2	-	0.8 (0.0)^{y,a,b}	1.1 (0.0)^{y,c}	0.9 (0.0)^{y,a}	0.5 (0.0)^{y,b}
ζ (mV)	**H1**	64 (3)^{x,a}	58 (4)^{x,b}	61 (2)^{x,c}	46 (3)^{x,d}	55.0 (1.5)^{x,b}
	H2	72.3 (0.6)^{y,a}	60.3 (0.8)^{y,b}	68.0 (0.9)^{y,c}	62 (2)^{y,d}	64 (3)^{y,b}

The same superscript (^{abc} to be compared horizontally the same homogenization conditions and ^{xy} vertically the same formulation) in LSD test.

3.1.3. Rheological behaviour

The experimental flow curves of FFD between 0 and 300 s^{-1} are shown in Fig. III.2, together with the curves predicted by the Ostwald de Waale model. This equation fitted the experimental data closely. Treatment H2 changed the rheological properties of the FFD, which were shear thinning in H1 treatments (n values ranged between 0.62 and 0.87). The behaviour of the FFD was more Newtonian after microfluidization (MF) in H2 treatments, with n values ranging between 0.94 and 0.98. In the case of pure CH dispersions, an important viscosity decrease is observed (78.5% reduction) when microfluidized, which could be due to the molecular weight reduction caused by MF (Kasaai, Charlet, Paquin, & Arul, 2003) and the conformational change of the chains. Similar results were obtained by Vargas et al. (2011).

Regardless of the homogenization treatment, the incorporation of essential oils had a significant effect on the viscosity. At the lowest content (0.5%), incorporation of both essential oils produced a significant viscosity increase with respect to the pure CH dispersions, in both H1 and H2 dispersions. However, at the highest oil content (1%), viscosity was reduced with respect to the 0.5% oil emulsions, although it was greater than that of the corresponding pure CH dispersion in the H2 conditions. H1 FFD with 1% oil content were less viscous than pure chitosan dispersions. This behaviour can be explained by taking into account two opposite effects: the presence or the increase in concentration of the dispersed phase (oil) tends to increase the viscosity of the system, while the adsorption of the polymer on the droplet surface provokes a decrease of its effective thickening concentration in the aqueous phase. So, viscosity of the FFD was reduced after MF, coherently with the droplet size reduction and due to the increase in the chitosan adsorption on the oil droplet surface, in agreement with the growth of the interfacial area in the system.

The oil type had a significant impact on ηap at 100 s^{-1} ($p < 0.05$) for H1 dispersions, although for microfluidized samples, no notable differences among viscosity of basil and thyme emulsions were detected. For the emulsions submitted to H1, those with thyme oil were the least viscous, which may be due to the fact that they have smaller droplets at a determined oil content.

Analyses of the FFD properties allow us to conclude that CH adsorbs on the thyme and basil droplet surface; the greater the particle size reduction, the greater the CH adsorption. This promoted the emulsion stability, mainly when the samples were submitted to microfluidization. Under these conditions the oil particle size was greatly reduced and the viscosity markedly decreased. No great differences in the behaviour of the FFD of both oils were detected after microfluidization, although for H1 treatments thyme oil gave lower z-potential, smaller particle size and less viscous dispersions than basil oil. Differences could be due to the different surface affinity among CH and the droplet surface of both oils (affected by differences in the opposite charge of droplets and CH macromolecules), affecting the polymer interfacial adsorption, which, in turn, affects the effective droplet size.

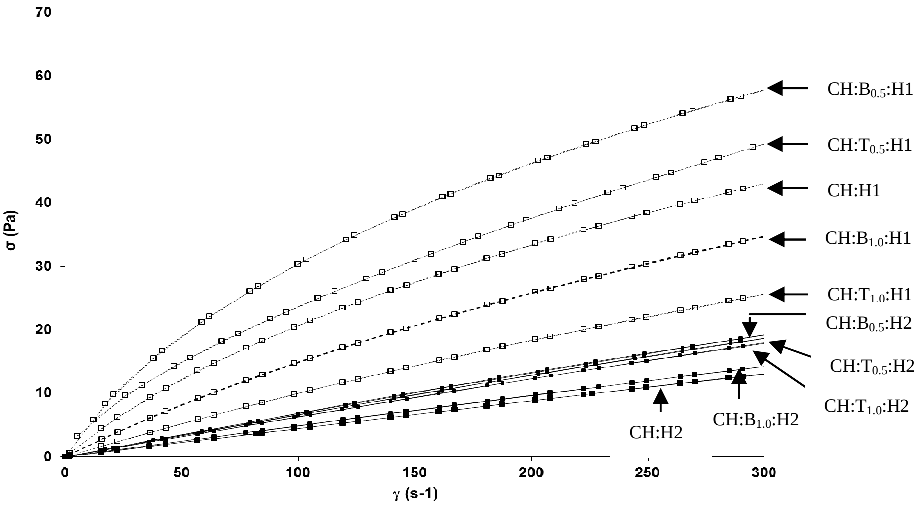

Figure III.2. Typical flow curves, at 25ºC, of the film forming dispersions homogenised in rotor-stator (H1: dashed lines) or additionally microfluidized (H2: continuous lines).

3.2. Characterization of the films

3.2.1. Microstructure

Characteristic SEM images of cross sections of the films are shown in Fig. III.3 (H1treatments) and (H2 treatments). The microstructure was qualitatively analysed, aiming to describe the role of both essential oils and homogenization procedure in the structure of the CH matrix. The films had a fragile appearance, and showed some disruption depending on the sample preparation process. Pure chitosan films were relatively homogeneous, especially those submitted to H2 treatments.

In composite films, the homogenization treatment gave rise to relevant differences in the structure. In films submitted to H1, the polymer matrix showed the presence of oil droplets, which appeared, in some cases, as voids due to the fact that droplets remain in the non-observed part of the film during cryofracture (Fig. III.3). The size of these droplets is in the range of that measured in the H1 emulsions (1-10 μm), for both basil and thyme emulsions. Apparently, H1 emulsions were stable enough not to develop destabilization phenomena (flocculation, coalescence, creaming) during the drying step of the film. Comparing films containing basil and thyme oils at the same concentration, thyme films show a more heterogeneous structure than basil films, especially when 1% oil is present (Fig.III. 3). This could be due to a lower stability of the emulsion during film drying.

Films submitted to H2 showed micro-fractured matrices when essential oils are present (Fig. III.3). In these films, oil droplets were hardly appreciable, since they were smaller and thus more intimately incorporated into the polymer matrix. The particle size reduction taking place during MF led to increased interactions between the polymer matrix and the oils, which led to a less cohesive chitosan matrix and scarcely identifiable oil droplets. Microfluidization blurs the differences between both phases and lipid particles become integrated in the polymer network. Similar results were obtained by Vargas et al. (2010).

When the oil content increases, the greater molecular contact between CH and oil compounds may weaken the polymer chain aggregation forces, making the matrix more open. In fact, for the same surface solid density, the films were significantly thicker when they contained 1% oil (75 ± 2 μm) than in the other cases (52 ± 4 μm).

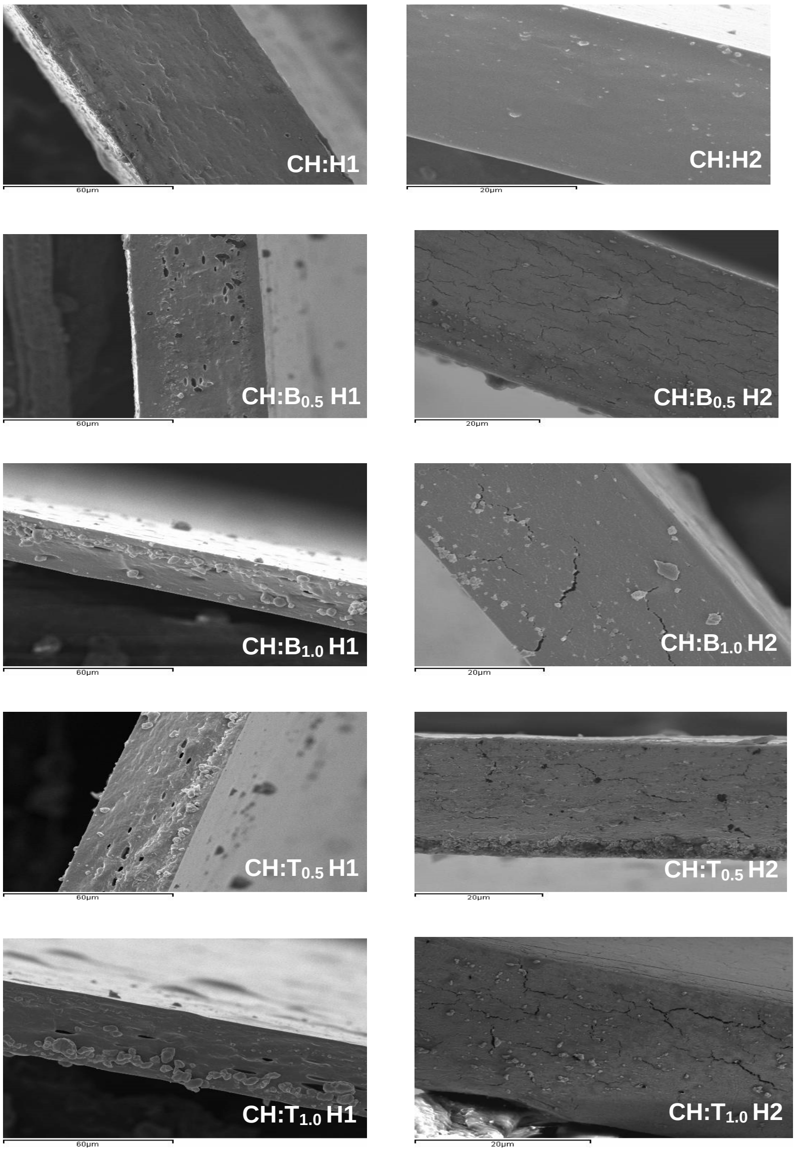

Figure III.3. Scanning electron microscopy micrographs of the cross section of films submitted to H1 and H2 homogenization process.

3.2.2. Mechanical properties

The typical mechanical behaviour of the films is shown in Fig. III.4 in terms of true stress - Hencky strain curves. From these curves, mechanical parameters: elastic modulus (EM) and tensile strength (TS) deformation (%E) at break were obtained (Fig. III.5). It is remarkable that composite films showed a more plastic deformation than pure CH films which made them more stretchable. This can be attributed to the interruption of the polymer chain aggregation in the matrix by the oil presence, which favours the sliding of the chains during film stretching, showing plastic (non elastic) behaviour.

In pure chitosan films, the MF process produced some reduction in rigidity (lower EM, $p < 0.05$). This was previously observed by Vargas et al. (2010) in pure chitosan films submitted to different pressure homogenization treatments (62, 83,124 and 165 MPa) and has been attributed to the reduction in the molecular weight of the polymer during microfluidization. The correlation between the molecular weight of chitosan and the mechanical resistance of chitosan films has been previously reported (Park, Marsh, & Rhim, 2002). Kasaai et al. (2003) investigated the chain scission of chitosan as affected by microfluidization pressure and found that, at 165 MPa pressure, the molecular weight of the chitosan is sufficiently reduced to have a significant impact on the elastic modulus. The rest of the mechanical properties of pure polymer films were not significantly affected by homogenization pressure, although a tendency to increase the film elongation at break, with similar values of tensile strength, was observed, which agrees with the lower resistance to deformation of the film.

Regardless of the homogenization technique, the addition of EO led to a significant decrease in EM and TS, and a marked increase in the stretchability, depending on the oil content. The greater the oil content, the greater the effect. This could be attributed to the discontinuities induced in the chitosan matrix by oil droplets (as previously observed in the micrographs, Fig. III.3), which provoke a loss of the film cohesion and mechanical resistance. However, the interruption of the CH chain aggregations makes the chain displacement during stretching easier, which gives the film a greater ability to be deformed without breaking. This has also been found in previous studies (Fabra, 2010; Vargas et al., 2009). The effect of the oil type was not significant in the mechanical behaviour.

Microfluidization at high pressure intensified the effects described for composite films, giving rise to the least rigid, the softest and most stretchable films ($p < 0.05$). Moreover, the σ vs. ε_H curves (Fig. III.4) reveal the micro-fractures taking place during the first tensile step (curves with small peaks), which are caused by the film fragility associated to the high degree of contact of the oil-CH molecules, which makes the CH chain aggregation forces weaker. In this case, the EM values reported correspond to an apparent value since microstructural failures occurs, as commented in the microstructural analysis. So the homogenization conditions greatly influences the final film structure and thus the mechanical behaviour. In CH films MF did not improve the mechanical characteristics of the films, which became more fragile than H1 films.

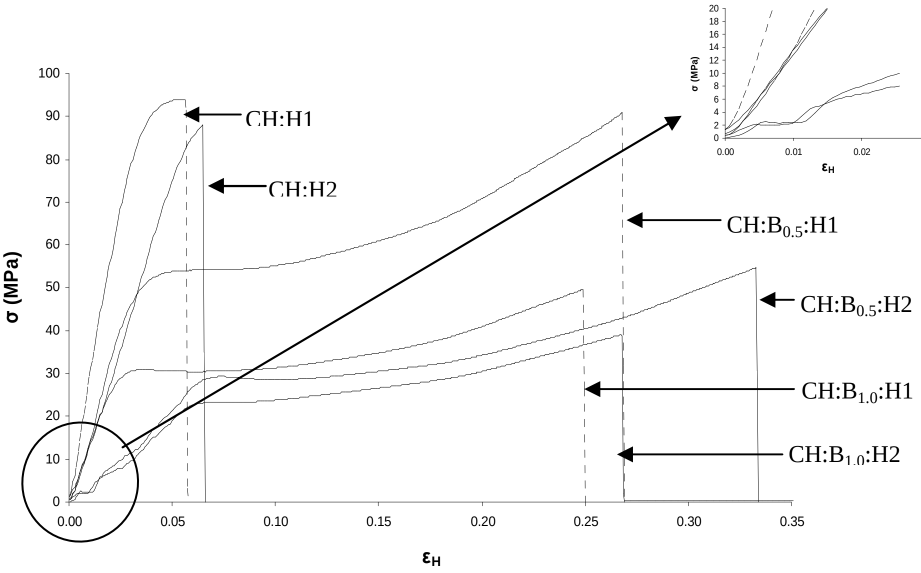

Figure III.4. Typical true stress (σ) *vs.* Hencky strain (ε_H) curves obtained in tensile tests carried out on some composite films submitted to H1 (dotted lines) and H2 (solid lines) homogenization processes.

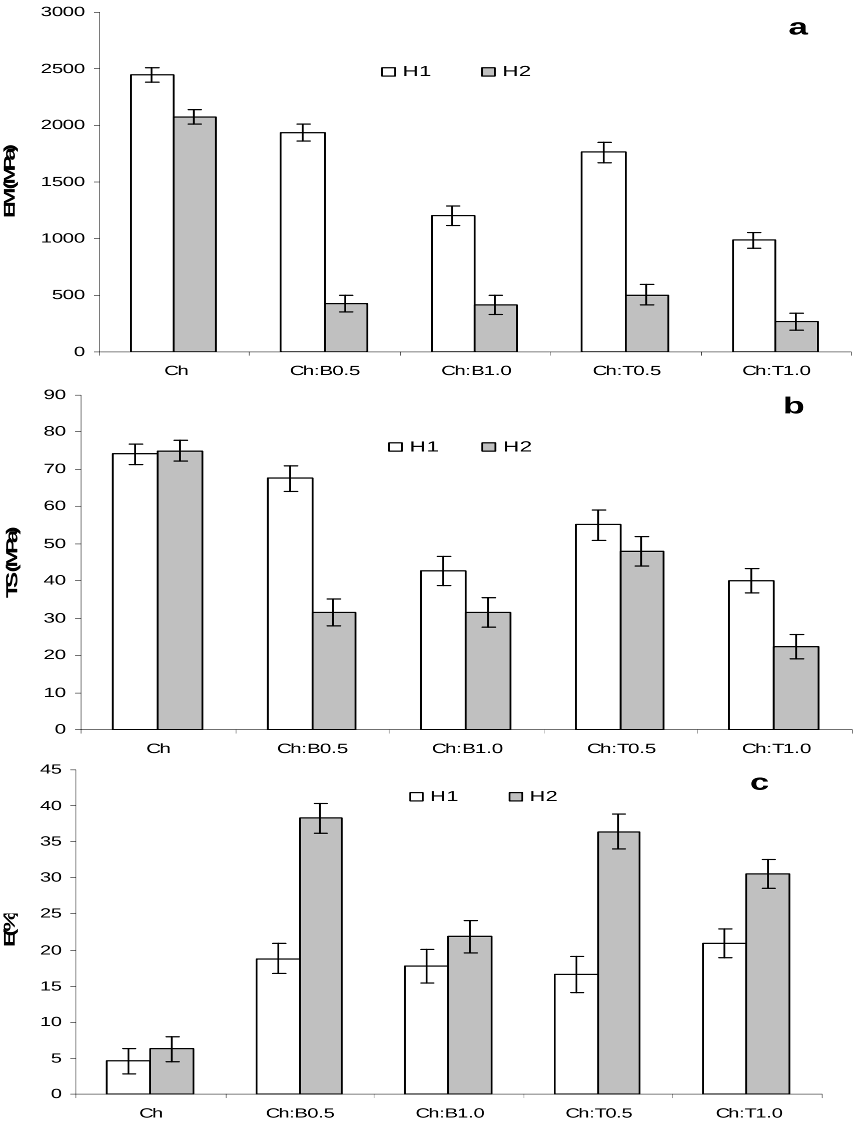

Figure III.5. Mechanical properties of the films: a) Elastic modulus b) tensile strength and c) percentage of elongation (%E) at break. Mean values and 95% LSD intervals.

3.2.3. Water vapour permeability (WVP)

Values of WVP are shown in Table III.2. In pure chitosan films, H2 produced a significant WVP increase ($p < 0.05$), which could be explained by the reduction in the polymer chain length and the subsequent increase in its hydrophilic nature, as reported by Gocho, Shimizub, Taniokac, Chouc, and Nakajimad (2000). These authors studied the effect of molecular weight on the water sorption isotherms of chitosan, and they found that the lower the molecular weight of chitosan, the higher the water affinity.

The oil type did not have a significant effect on WVP ($p > 0.05$), whereas the increase in the oil content seemed to play a key role. Barrier properties of films submitted to H1 were not significantly affected by oil addition when CH oil ratio was 1:0.5 ($p > 0.05$), although a WVP increase was found when the ratio was 1:1 ($p < 0.05$). This could be due to the negative effect of oil incorporation on the cohesion forces of the CH matrix, as commented on above, which enhance transport phenomena trough the film, despite the increase in the hydrophobic character of the matrix when oils were dispersed. Nevertheless, the application of MF led to a significant decrease of the WVP values of the CH matrix when the CH:oil ratio was 1:0.5, but they did not change significantly, with respect of the micro-fluidized pure CH, when the ratio was 1:1. This could be explained by the action of two opposite effects, previously commented: the high contact degree between CH and oil in the micro-fluidized samples, which makes the CH chain aggregation forces weaker, contributing to decrease the cohesion forces of the polymer network (as deduced from the analysis of mechanical properties), and the overall increase in the hydrophobic character of the matrix by the oil incorporation. At the low oil concentration, the effect of the overall increase in the hydrophobic nature of the matrix could predominate over the effect of the loss of matrix cohesion and, then, water vapour transport is limited, whereas did not occur at high oil content, where the two effects seem to balanced.

Although some authors (Perez-Gago & Krochta, 2001) reported that the decrease in particle size of dispersed lipids increases the tortuosity factor for water transfer in the continuous matrix, thus decreasing the mass transfer rate across the film, other factors such as the polymer-lipid interactions, and their effect on the bonding forces of the chains in the network, can play an important role.

3.2.4. Gloss

The gloss of the films was studied because it has a direct influence on the appearance of the coated product. Table III.2 shows the values obtained at 60º incidence angle. Norm ASTM D523 (1999) recommends this geometry when results are comprised between 10 and 70, which was the case for most the obtained results. All values were lower than 30, meaning that all the studied films were only slightly glossy.

The gloss of the films is linked to the morphology of their surface (Sánchez-González et al., 2010) and generally, the smoother the surface, the glossier the film. Hence, composite films are less glossy than pure chitosan films because of the occurrence of discontinuities in the chitosan matrix. These discontinuities increase the roughness of the film surface and decrease the specular reflectance. So, gloss was reduced by the addition of essential oil for both homogenization treatments, showing very small differences among samples. Nevertheless, H2 process tended to yield glossier films, particularly for pure CH and CH:$T_{1.0}$ films, which can be attributed to the particle size reduction and the subsequent surface roughness decrease. Similar results were found by Vargas et al. (2010) in chitosan-oleic acid films submitted to MF.

Table III.2. Water vapour permeability (WVP) ((g s^{-1} m^{-1} Pa^{-1}) x $10^{11)}$ at 58-100%RH gradient and 5ºC and Gloss (60°) of the films. Average values and standard deviations, in brackets.

	Homog.	**CH**	**CH:$B_{0.5}$**	**CH:$B_{1.0}$**	**CH:$T_{0.5}$**	**CH:$T_{1.0}$**
WVP	**H1**	61 (6)^{x,a}	61 (7)^{x,a}	73 (6)^{x,b}	58.9 (0.9)^{x,a}	73 (3)^{x,b}
	H2	83 (7)^{y,a}	42.6 (0.6)^{y,b}	80 (9)^{x,a}	33 (9)^{y,b}	89.6 (0.7)^{y,a}
Gloss	**H1**	14 (4)^{x,c}	7 (2)^{x,a}	9 (3)^{x,b}	9 (2)^{x,ab}	5.2 (1.5)^{x,a}
	H2	20 (5)^{y,c}	11 (3)^{x,a}	9 (2)^{x,b}	7.7 (1.3)^{x,ab}	10 (3)^{y,a}

The same superscript (^{abc}) to be compared horizontally the same homogenization conditions and (^{xy}) vertically the same formulation) in LSD test.

4. Conclusions

Microfluidization at 165 MPa greatly affected the properties of the emulsions and films of chitosan- basil/thyme essential oils. This technique produced a reduction in oil droplet size and viscosity, while promoted the adsorption of chitosan on the oil-water interface. The induced close contact polymer-oil compounds gives rise to more fragile films of increased stretchability due the weaking effect on the CH matrix where chain interaction forces are reduced. This effect was also observed for the films obtained from mild homogenized dispersions, but with less intensity. Water barrier properties of the films were dependent on the oil ratio and the homogenization technique. High-pressure homogenization increased WVP of pure CH films while oil incorporation was only effective to reduce WVP when they were incorporated at the lowest ratio and when high pressure was used in the homogenization of the film-forming dispersion. Gloss was reduced by the essential oil addition, whereas MF tended to yield glossier films.

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PORK MEAT PRODUCT PRESERVATION BY USING CHITOSAN FILMS CONTAINING ESSENTIAL OILS

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Abstract

Edible films based on chitosan were prepared, with and without basil or thyme essential oils, with the aim of assessing their protective ability against lipid oxidation and their antimicrobial activity. Chitosan films had good oxygen barrier properties, which were worsened by essential oil addition, especially when the film equilibrium moisture content increased. Due to the oxygen barrier effect, all the films effectively protected pork fat from oxidation, in comparison to unprotected samples. In spite of the worsening of the oxygen barrier properties, the films with essential oils were more effective than those of pure chitosan, which points to the chemical action of specific antioxidant compounds of the oils. Films were proved to be effective at controlling microbial spoilage of minced pork meat although the incorporation of essential oils did not improve their antimicrobial activity. Films provoked colour changes in minced pork meat during storage, associated with the conversion of myoglobin into metmyoglobin due to the reduction of the oxygen availability.

Keywords: Chitosan, essential oils, microfluidization, edible coating, pork meat, antioxidant, colour, antimicrobial

1. Introduction

Microbial growth is generally responsible for the spoilage in meats and meat products together with biochemical and enzymatic deteriorations (Devlieghere et al., 2004). In fact, bacterial contamination is one of the main factors determining the loss in fresh meat quality, since these products are very prone to be contaminated with microorganisms if they are not properly preserved and handled.

Oxygen has a negative effect on the quality of a wide variety of food products. In the case of the oxidative deterioration of fats and oils, it is responsible for rancid odours and flavours, with a decrease in nutritional quality and safety caused by the formation of secondary, potentially toxic, compounds (Moure et al., 2001). An antioxidant is defined as any substance that, being present at low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits its oxidation (Park et al., 2004).

Many synthetic antioxidants-butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary-butylhydroquinone (TBHQ), propyl gallate (PG)–may be used to retard lipid oxidation (Wanita & Lorenz, 1996), but their utilization is under strict regulation due to their potential health hazards. Therefore, the search for natural antioxidants as alternatives to synthetic ones is of great interest in food preservation (Park et al., 2004).

The most commonly-used strategy with which to extend fresh meat shelf-life is the use of antimicrobial and antioxidant additives of synthetic origin, which are being questioned more and more due to an increasing consumer demand for natural, healthy and safe preservatives. Another approach is to use bioactive ingredients from a natural origin, such as plant essential oils, which have antibacterial properties against food borne pathogens like *Listeria monocytogenes, Salmonella typhimurium, Escherichia coli* and *Staphylococcus aureus* (Burt, 2004). It is reported that essential oils (EO) exhibit their antimicrobial activity by interfering and destabilizing the phospholipid bilayer of the cell membrane, the enzyme systems, and the genetic material of bacteria (Kim et al., 1995).

Thyme essential oil contains more than 60 ingredients, most of which have important antioxidant and antimicrobial properties (Baranauskiene et al., 2003). The most important active compounds of thyme EO are the phenols thymol, rosmarinic acid and carvacrol (Di Pasqua et al., 2006; Shan et al., 2005). Several in vitro studies demonstrated that these compounds exhibit antimicrobial activity against a broad spectrum of gram negative or gram positive bacteria (Burt & Reinders, 2003; Gaysinsky et al., 2005; Singh et al., 2001). Moreover, studies carried out on minced pork meat (Aureli et al., 1992) and feta cheese (Govaris et al., 2011) showed the strong antibacterial activity of thyme EO against *Listeria monocytogenes*.

Basil is a common and popular culinary herb which has been used widely to flavor meat products. There is an enormous variation in the composition of basil essential oils, depending on a great number of factors, such as the geographical origin, and the major constituents are rosmarinic acid, linalool, methyl chavicol, eugenol and methyl cinnamate (Suppakul et al., 2003; Shan et al., 2005). Basil essential oils have been proven to exhibit an antibacterial effect against different bacteria, including common meat spoilage microorganisms such as *Escherichia coli*, *Enerobacter aerogenes*, *E. aglomerans*, *Listeria innocua*, *Listeria monocytogenes* and *Salmonella typhimurium* (Wan et al., 1998).

In order to achieve effective antimicrobial activity, high concentrations of essential oils are generally needed, which might confer inappropriate flavours and odours on the food product (Seydim & Sarikus, 2006). The incorporation of essential oils as natural bactericides in the formulation of edible films appears to be an interesting alternative means to improving coating functionality and performance and reducing the cost of applying essential oils, minimizing at the same time the problems related with their intense aroma (Sánchez-González et al., 2011). Apart from the possibilities of encapsulating essential oils and other active compounds, the use of edible films and coatings has proven to be a useful technique with which to protect food products, thus extending their shelf-life (Vargas et al., 2006; Vargas et al., 2009; Atarés et al., 2011). When the added ingredients exhibit antioxidant properties, the action of such films involves two different mechanisms: the oxygen barrier effect–reducing the oxygen availability in the product and the specific activity of the incorporated antioxidant agents (Bonilla et al., 2010).

Chitosan is an antimicrobial non-toxic biopolymer that has been proven to serve as a matrix to obtain edible films containing essential oils (Sánchez-González et al., 2010; Bonilla et al., 2012). Chitosan is a partially deacetylated polymer of N-acetyl glucosamine, which is obtained after the alkaline deacetylation of the chitin derived from the exoskeletons of crustaceans and arthropods (Park et al., 2004). Chitosan-based films and coatings have been applied to different fish and meat products. For instance, the antibacterial effect of chitosan-oregano essential oil composite films was tested in bologna slices inoculated with L. monocytogenes or E. coli O157:H7 (Zivanovic et al., 2005).

Nevertheless, to the best of our knowledge, neither pure chitosan-based films nor those prepared in combination with thyme or basil essential oils have as yet been applied to fresh minced pork meat.

The aim of this work was to study the antibacterial and antioxidant properties of edible chitosan films containing basil or thyme essential oils. The films were prepared following two different homogenization treatments and with two essential oil concentrations and were assessed as to their oxygen permeability under different conditions. Their protective ability against oxidation and bacterial growth in meat products was evaluated in two cases which can be considered representative of these kinds of products, i.e. pork fat and minced pork meat.

2. Materials and methods

2.1. Reagents

High molecular weight chitosan (Batch 12913CJ/ Batch MKBB0595, Sigma-Aldrich Quimica, Madrid, Spain) was used to prepare the film-forming dispersions (FFD) (0.8 Pa*s viscosity, at 1% w/w in 1% w/w glacial acetic acid). Basil and thyme essential oils were provided by Herbes del Molí (Alicante, Spain). Acetic acid, $Mg(NO_3)_2$, KCl and K_2CO_3 were purchased from Panreac Química, S.A. (Castellar del Vallés, Barcelona, Spain). ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and potassium persulfate were provided by Sigma–Aldrich (Madrid, Spain).

2.2. Preparation of chitosan-based films

High molecular weight chitosan (CH) was dispersed at 1 wt% in an acetic acid solution at 1 % (v/w). To prepare the composite films, basil (B) or thyme (T) essential oils were added to the chitosan solution at 0.5 or 1 wt%. The mixtures were homogenized by means of a rotor-stator (DI25, Yellow Line, IKA®, Germany) at 21,500 rpm for 4 minutes (H1 treatment). H2 homogenization consisted of H1 followed by high-pressure homogenization at 165 MPa in a single pass by means of a

Microfludizer® M110-P processor (Microfludics, Newton, USA). Films were obtained by casting and drying at room temperature and 60% RH. The surface density of solids in the dry films was 56 mg/cm^2. Films were peeled off from the casting plates and conditioned at 58% RH and 10ºC for one week. A Palmer digital micrometer (Comecta, Barcelona, Spain) was used to measure film thickness to the nearest 0.001mm at six random positions.

2.3. Trolox equivalent antioxidant capacity of essential oils

The Trolox Equivalent Antioxidant Capacity (TEAC) of basil and thyme essential oils was determined using a modification of the original TEAC method (Re et al., 1999). The vitamin E analogue Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a standard of antioxidant capacity. ABTS (2,2'-azinobis(3- ethylbenzothiazoline- 6-sulfonic acid) diammonium salt) was dissolved in water at 7 mM and allowed to react with a 2.45 mM potassium persulfate solution (final concentrations) in the dark, for 16 h. During this period, ABTS radical cation (ABTS$^{\bullet +}$), a blue chromophore, was produced. The tests were performed with ethanolic dilutions of the ABTS$^{\bullet +}$ solution, whose absorbance at 734 nm was 0.70 (± 0.02). Essential oils were diluted in ethanol prior to the determination. Dilutions were made so that the addition of a 10 µl-aliquot to 990 µl of ABTS•+ dilution would produce a 20–80% absorbance decrease within 6 min. Absorbance at 734 nm (A734) was registered every minute during the test. For calibration, Trolox standards of between 60 and 500 mg/l were prepared and the same procedure was followed. The TEAC of the essential oils was determined by comparing the corresponding percentage of absorbance reduction to the Trolox concentration–response curve and expressed as the amount of the essential oil which produces the same absorbance reduction as 1mM Trolox solution. All the determinations were carried out three times using a Beckman Coulter DU 730 spectrophotometer and ethanol as the blank.

2.4. Thickness and oxygen permeability of chitosan-based films

Thickness was measured with a Palmer digital micrometer (Comecta, Barcelona, Spain) to the nearest 0.001 mm in six samples per formulation.

The oxygen permeability (OP) tests were conducted following the ASTM D3985 Standard Method (1995). The oxygen barrier performance of the chitosan films was evaluated by measuring the oxygen transference rate with an Ox-Tran 1/50 system (Mocon, Minneapolis, USA). The films were exposed to pure nitrogen and oxygen flows on either side. The tests were performed in continuous mode at 10ºC-58% RH, 40ºC-43% RH and 40ºC-83% RH. OP was calculated by dividing the oxygen transmission rate (OTR) by the difference in oxygen partial pressure between the two sides of the film, and multiplying by the film thickness. Two replicates per formulation were made.

2.5. Protective ability of the films against oxidation of pork fat

Pure pork fat was purchased in a local market and kept in refrigeration until the experiment was started. Inert cups (60 mm diameter, 48.5 mm depth) were totally filled with fat, which was compressed to ensure the absence of air bubbles. Three formulations of edible films, namely CH, $CH:B_{1.0}$ and $CH:T_{1.0}$ were tested (sub-index 1.0 stands for the percentage concentration of essential oils in the emulsions, equal to that of chitosan). All of them were prepared using the H1 homogenization procedure. Film disks were cut and secured to the cups as a lid. The side of the film in contact with the plate during drying was in direct contact with the fat. The cups were stored at 40ºC and at two different levels of relative humidity, namely 43% and 83%. These ambient RH were accomplished with oversaturated solutions of K_2CO_3 and KCl, respectively. After 15, 40 and 60 days of storage, samples of fat were extracted and the extent of their oxidation was evaluated by determining the peroxide value (PV) according to norm UNE 55-023. PV was expressed as mEq of oxygen per kilogram of pork fat. All analyses were performed in duplicate.

2.6. Protective ability of the films in minced pork meat

Pork meat was obtained from a local supermarket. The meat was ground by using a mincer (Severin Elektrogeräte GmbH, Sundern, Germany) and was moulded in Petri dishes to obtain the test samples. The surface of both sides of the samples was coated with the films. Non-coated and coated samples were placed in PET trays (Cubil, Barcelona, Spain) and were stored at 10ºC. Colour measurements of minced pork meat samples were taken at different storage times by using a spectrocolorimeter CM-3600d (Minolta Co, Tokyo, Japan) with a 30 mm diameter window. Immediately before the measurements, the films were peeled off the coated minced pork samples. CIE-$L^*a^*b^*$ coordinates and chromatic parameters, hue (h^*_{ab}) and chrome (C^*_{ab}), were obtained from the reflection spectra of the samples, using D65 illuminant /10º observer (CIE, 1986). The colour of each sample was measured in quintuplicate.

To evaluate the antimicrobial effect of the films, stock cultures of *E. coli* (CECT101) and *L. inoccua* (CECT 910) were supplied by the Spanish Type Culture Collection (CECT, Burjassot, Spain) and kept frozen (−25°C) in Tryptone Soy Broth (TSB, Scharlab, Barcelona, Spain) supplemented with 30% glycerol (Panreac, Barcelona, Spain). The cultures were regenerated by transferring a loopful of each bacterium into 10 mL of TSB or BHI broth and incubated at 37°C overnight. A 10 μl aliquot from each overnight culture was again transferred into 10 mL of TSB or BHI broth and grown at 37°C up to the end of the exponential phase of growth. Subsequently, these appropriately diluted cultures were then used for the inoculation of the minced pork meat samples in order to obtain a target inoculum of 10^3 UFC/mL.

To perform the microbiological analyses, 10g of each coated and non-coated minced meat sample were aseptically obtained and homogenized in a Stomacher with 90 mL of sterile buffered peptone water for 2 min. Aliquots were serially diluted in buffered peptone water and plated out following standard methodologies. In non-inoculated meat samples, the total viable counts were determined in Plate Count Agar plates incubated at 37ºC for 24h. Coliform counts were determined using Violet

Red Bile Agar plates incubated at 37ºC for 48h. In inoculated samples, dilutions and counts were performed by using the specific broth and agar for each bacterium. Every microbial analysis was performed in triplicate and all the culture media were supplied by Sharlau (Sharlab S.L, Barcelona, Spain).

2.7. Statistical analyses

The results were analysed by means of a multifactor analysis of variance with a 95% significance level using Statgraphics®Plus 5.1. (Manugistics Corp., Rockville, Md., USA). Multiple comparisons were performed through 95% Least Significant Difference intervals (LSD).

3. Results and discussion

3.1. Trolox equivalent antioxidant capacity of essential oils

Although the TEAC values obtained for basil and thyme essential oils were quite similar, 1.48 ± 0.04 and 1.31 ± 0.03 mg, respectively, thyme oil shows a slightly higher antioxidant capacity (a lower amount is required to reach the same activity as 1 mM Trolox solution). Lee et al. (2005) investigated the composition of basil and thyme essential oils in order to explain their antioxidant activity, and found that some of their components (eugenol, thymol, carvacrol and 4-allylphenol) exhibited potent antioxidant activity, comparable to other antioxidants, such as BHT and α-tocopherol. In a recent study, Teixeira et al. (2013) evaluated the antioxidant activity of a broad spectrum of essential oils and also reported a higher antioxidant activity for thyme essential oil as compared with basil essential oil, although differences in composition associated to origin and variety can lead to different antioxidant capacity.

When essential oils were incorporated into the chitosan films, they were expected to confer antioxidant capacity. On the other hand, chitosan powder is considered a secondary antioxidant since it has the ability to chelate the metal ions involved in the catalysis of an oxidative reaction (Tharanathan & Kittur, 2003). In fact, chitosan of different N-deacetylation degrees obtained from crab shells has exhibited not only antioxidant activity, but also scavenging ability on hydroxyl radicals and chelating ability on ferrous ions, being a more effective antioxidant agent the higher the degree of deacetylation (Yen et al., 2008).

3.2. Thickness and oxygen permeability of the films

Table IV.1 shows the thickness of the films as affected by both the presence of essential oils and the homogenization treatment. The formulation had a significant effect ($p<0.05$) on the final thickness of the films. Pure chitosan films were the thickest, followed by those with a low proportion of essential oil and, finally, those with the highest content of essential oil were significantly thinner ($p<0.05$). This is explained by the fact that a constant dry mass of FFD were cast in order to obtain the films. Due to the high volatility of the essential oil compounds, these evaporated to some extent during the drying step, which resulted in a lower final surface solid density and in thinner films. Similar results were found by Sánchez-González et al.

(2011) in chitosan films with a variety of volatile plant essential oils.

The homogenization procedure also had an effect on the thickness of the films when they contained essential oils. Those obtained by means of the H2 procedure (rotor-stator plus high pressure homogenization), were significantly thinner than H1 films ($p<0.05$). This difference could be caused by the effect of high pressure homogenization on the integration of both hydrophilic and oily phases. Bonilla et al. (2012) observed the microstructure of chitosan-essential oil edible films elaborated with different homogenization procedures and found that, in H2 films, the oil droplets were hardly appreciable, since they were intimately incorporated into the chitosan matrix.

Due to the reported differences in thickness, both oxygen transference rate (OTR) and oxygen permeability (OP) values are shown in Table IV.1. OP values, being independent of the film thickness, are useful in order to compare the different materials. On the other hand, OTR (volume of oxygen that permeates the film per surface unit and time unit) is dependent on the film thickness and should be checked in order to compare the efficiency of the different films when applied to the meat products. The oxygen barrier properties of the films were tested under different conditions. These were selected with the aim of simulating the behaviour of the films under different conditions when applied to refrigerated meat products or when submitted to extreme conditions which can promote oxidation processes. In this sense, all the films were tested at 10ºC-58%RH to correlate OTR with the colour changes in refrigerated minced pork meat. On the other hand, pure CH films and those containing the highest ratio of EO, obtained by applying the H1 treatment, were additionally tested at 40ºC-43%RH and 40ºC-83%RH to correlate OTR values with the oxidation level of pork fat. Table IV.1 shows the OP values of all the formulations tested at 10ºC-58%RH, where it can be seen that all the films had rather low oxygen permeability values, in the range of those reported by other authors (Di Piero et al., 2011; Casariego et al., 2009; Caner et al., 1998). The good oxygen barrier properties of chitosan films have been previously observed and, in fact, their oxygen permeability has been described as being comparable with those of existing commercial synthetic films, such as polyvinylidene chloride (PVDC) or ethylene vinyl alcohol copolymer films (Casariego et al., 2009). In pure chitosan films, the homogenization procedure had a significant impact on OP ($p<0.05$). The films prepared with H2 procedure were significantly less permeable to oxygen than those prepared with simple homogenization (H1). This is probably due to an improvement in the chitosan chain packaging. Bonilla et al. (2012) studied the microstructure of chitosan-essential oil edible films elaborated with different homogenization procedures and found that pure chitosan H2 films had a more homogeneous structure than those obtained with H1 procedure.

The incorporation of both basil and thyme essential oils slightly affected OP values at 10ºC and 58% RH. When FFD was submitted to H1 treatment, essential oil addition resulted in a small improvement of the oxygen barrier properties of the films, although this did not occur when FFDs were submitted to the more intense H2 treatment, except for thyme oil at the highest ratio, where a significant reduction of OP was observed with regards to the respective chitosan film.

Table IV.2 shows the oxygen transference rate (OTR) and the oxygen permeability (OP) values for H1 treated CH, CH:$B_{1.0}$ and CH:$T_{1.0}$ films, equilibrated at 40ºC-43%RH and 40ºC-83%RH. At 40ºC-43% RH, it was observed that all the films had good oxygen barrier properties, the OP being rather low and in the range of those values obtained at 10ºC-58%. All the film materials behaved similarly and no significant differences were found between the OP values of the different films ($p>0.05$). However, at 40ºC-83%RH, both OTR and OP greatly increased, mainly due to the fact that the film moisture content was higher when equilibrated under much higher relative humidity conditions (80%). The increase in both temperature and film moisture content implied greater molecular mobility in the system, which favours mass transfer properties, thus affecting the oxygen barrier properties of the films, making them more permeable (Gennadios et al., 1993; Maté & Krochta, 1996; Han & Gennadios, 2005; Bonilla et al., 2010). As more and more water molecules become available to interact with the chitosan matrix, the structure is plasticized and mass transfer is favoured in the film. In the films equilibrated at 83% RH, the incorporation of essential oils provoked a significant increase in the OP values, whereas this was observed only as a tendency for films equilibrated at 43% RH. This can be explained by the greater oxygen solubility in the non-polar oil phase which contributes to increase the transfer rate of the oxygen molecules into the plasticized polymer matrix. This effect of the dispersed liquid oil phases on the OP has been previously observed in different film matrices, such as hydroxy-propyl-methylcellulose (Atarés et al., 2011) and sodium caseinate (Fabra et al., 2012), and it is more appreciable when the continuous matrix favours mass transfer due to its high plasticization level.

Table IV.1: Thickness (mm), oxygen transference rate (cm^3 m^{-2} day^{-1}) and Oxygen permeability (OP, cm^3 mm m^{-2} atm^{-1} day^{-1}), at refrigerated conditions and intermediate relative humidity (10°C-58%RH) of all film formulations and both homogenization procedures (rotor-stator: H1; or additionally microfluidized: H2). Average values and standard deviations in brackets.

		Formulation				
		CH	**CH:B$_{0.5}$**	**CH:B$_{1.0}$**	**CH:T$_{0.5}$**	**CH:T$_{1.0}$**
H1	**Thickness**	0.081 (0.004)^{x,d}	0.066 (0.002)^{x,b}	0.054 (0.001)^{x,a}	0.073 (0.002)^{x,c}	0.052 (0.003)^{x,a}
	OP	0.15 (0.04)^{x,bc}	0.094 (0.009)^{x,ab}	0.142 (0.012)^{x,bc}	0.117 (0.010)^{x,c}	0.118 (0.006)^{x,a}
	OTR	4.11 (1.20)^{x,a}	3.15 (0.24)^{x,bc}	5.85 (0.44)^{x,d}	3.54 (0.17)^{x,cd}	5.02 (0.02)^{x,ab}
H2	**Thickness**	0.079 (0.009)^{y,d}	0.051 (0.001)^{y,b}	0.047 (0.001)^{y,a}	0.059 (0.006)^{y,c}	0.043 (0.001)^{y,a}
	OP	0.091 (0.009)^{y,bc}	0.115(0.002)^{x,ab}	0.0938 (0.0008)^{x,bc}	0.149 (0.011)^{x,c}	0.053 (0.002)^{x,a}
	OTR	2.53 (0.05)^{x,a}	4.93 (0.04)^{x,bc}	4.46 (0.03)^{x,d}	5.54 (0.19)^{x,cd}	2.67 (0.16)^{x,ab}

Different letters indicate significant differences ($p<0.05$) due to film formulation (a, b, c, d) or due to homogenization treatment (x, y).

Table IV.2. Oxygen transference rate (cm^3 m^{-2} day^{-1}) and oxygen permeability (cm^3 mm m^{-2} atm^{-1} day^{-1}) of films formulated with pure chitosan (CH), chitosan with basil essential oil 1:1 w/w (CH:B$_{1.0}$) and chitosan with thyme essential oil 1:1 w/w (CH:T$_{1.0}$). Average values and standard deviations in brackets.

	40ºC-43%RH		**40ºC-83%RH**	
Formulation	**OTR (cm^3 m^{-2} day^{-1})**	**OP (cm^3 mm m^{-2} atm^{-1} day^{-1})**	**OTR (cm^3 m^{-2} day^{-1})**	**OP (cm^3 mm m^{-2} atm^{-1} day^{-1})**
CH	3.39 (0.04)^a	0.136 (0.009)^a	119 (4)^a	4.24 (0.04)^a
CH:B$_{1.0}$	5.90 (0.04)^c	0.151 (0.012)^a	312 (5)^c	7.8 (0.3)^b
CH:T$_{1.0}$	5.19 (0.03)^b	0.145 (0.016)^a	280 (13)^b	7.2 (0.5)^b

The same superscript means homogeneous group in LSD test (compare vertically)

3.3. Protective ability of the films against oxidation of pork fat

Figure IV.1a shows the progression of the peroxide values (PV) of pork fat, both uncoated and coated with the three H1 treated films (CH, CH:$B_{1.0}$, CH:$T_{1.0}$), throughout storage time at 40ºC-43%RH. The results of a parallel experiment, performed at 83% RH, are shown in Figure IV.1b. As expected, the sample oxidation levels, as well as the oxidation rate, increased in line with storage time. Likewise, the oxidation progressed more intensely in samples stored at 80% relative humidity, especially in uncoated samples. At 60 storage days, statistical analysis revealed that RH did have a very relevant effect on the final PV of uncoated samples ($p<0.05$), given the positive effect of water availability on fat oxidation (Labuza, 1980). On the contrary, in the case of samples coated with chitosan films, RH did not have a significant effect ($p>0.05$) on the PV values, although slightly higher PV values were found at high RH, which may be linked to the increase in the oxygen permeability of CH films.

In comparison with the uncoated samples, all the edible films had a protective effect against lipid oxidation, which is more appreciable at long storage times when the greater advance of the process has occurred. It is remarkable that, at any control time, PV values were lower in samples coated with films containing essential oils (except at the longest time and 43% relative humidity) which reveals the antioxidant chemical effect of the active compounds of the essential oils, since OTR values of these films were higher, especially at the highest relative humidity. In the case of pure chitosan films, the low PV values may be mainly attributed to their lower OTR values. So, the protective effect of films with essential oils could be attributed to both the oxygen barrier effect and the specific antioxidant action of oil compounds, commented on above. In this sense, it is remarkable that no significant differences in the antioxidant protection of films containing basil or thyme essential oil were observed. Nevertheless, the antioxidant action of the essential oils seemed to disappear at 60 storage days when samples were stored at 43%RH, which could be due to the progressive evaporation of the active compounds in this drier atmosphere or to the progressive oxidation of the antioxidant compounds, causing the films to lose their antioxidant capacity. In this case, pure chitosan films were more effective at preventing fat oxidation. A similar effect was detected by Atarés et al. (2011) when working on HPMC films enriched with ginger essential oil.

Hence, it could be stated that both the oxygen barrier mechanism and the specific chemical action of antioxidant compounds control lipid oxidation, positively affecting the pork fat quality. This chemical action becomes more relevant when the oxygen barrier decreases, because the increase in relative humidity promotes both the plasticization of the polymer network and oxygen transfer.

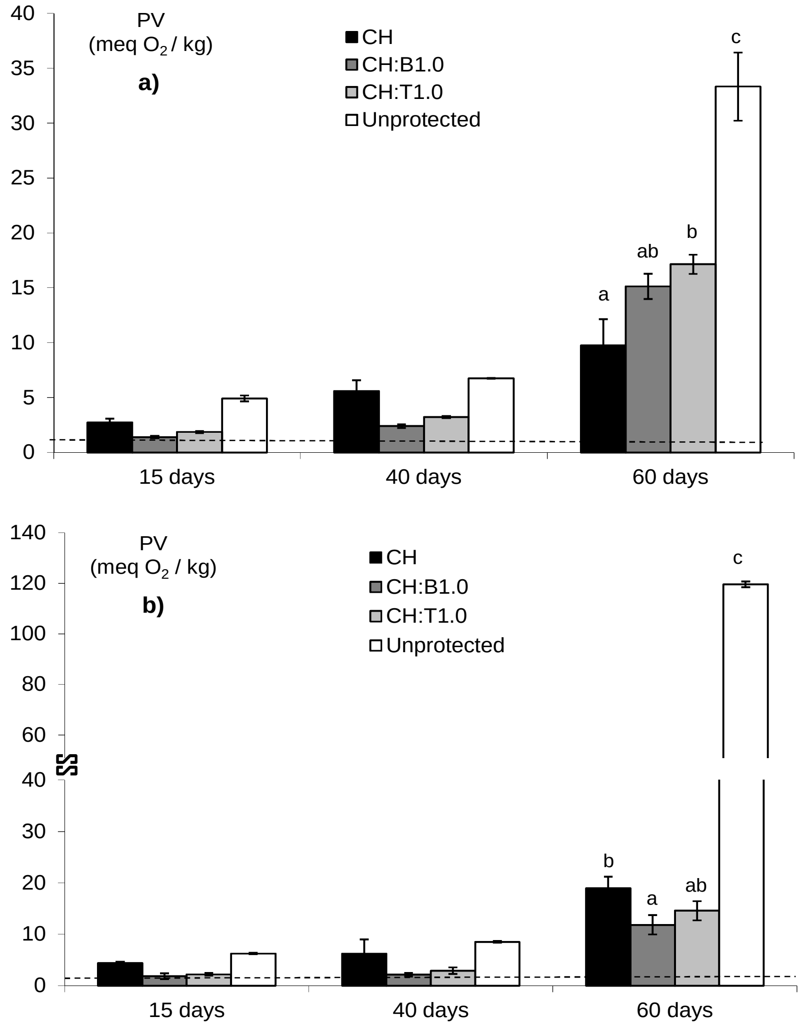

Figure IV.1. Progression of the peroxide value (PV) of fat samples covered with the edible films (CH = chitosan, CH:B = chitosan and basil essential oil 1:1 w/w, CH:T = chitosan and thyme essential oil 1:1 w/w), and those unprotected, over storage at (a) 40ºC and 43%HR and (b) 40ºC and 83%RH. The dotted line represents the initial PV of the fat. Mean values and standard deviation. Different letters (a, b, c) indicate significant differences ($p<0.05$).

3.3. Effect of the films on colour development and microbial spoilage of minced pork meat.

The surface colour of non-coated and coated minced pork meat samples was monitored in terms of CIEL*a*b* colour coordinates obtained from the sample reflection spectra. The samples showed initial values of 49, 65 and 16.3 for luminosity, hue and chrome, respectively. The changes in the colour parameters of non-coated and coated samples during cold storage are reported in Table IV.3. According to ANOVA, storage time was the factor that had the highest influence on colour changes (higher F-ratio values in the LSD test). Although non-coated samples hardly underwent any colour changes during storage, after 7 storage days they did appear with a more vivid (higher chrome values), red (lower hue values) colour. After 2 storage days, the sample coating scarcely affected the colour but led to a slight decrease in hue (redder samples) and chrome (more vivid colour) values. Nevertheless, after 7 storage days, coated samples suffered a significant increase in luminosity and hue (less red colour) and a significant decrease in chrome values (brownish colour development). This can be related with the conversion of myoglobin into metmyoglobin due to the low oxygen pressure induced by coating (Mancini and Hunt, 2005). Neither the film composition nor the homogenization conditions of the film-forming solutions were found due to bring about any significant differences in the colour of the coated samples.

Table IV.3. Luminosity (L*), hue (h^*_{ab}) and chroma (C^*_{ab}) of refrigerated minced pork meat samples coated with all film formulations and homogenization procedures. Average values and standard deviations in brackets. Initial conditions are in the text.

	Parameter	**L^***		**h^*_{ab}**		**C^*_{ab}**	
Treatments	**Storage time (days)**	**2**	**7**	**2**	**7**	**2**	**7**
	Non-coated	47.9 (0.4)	48.4 (1.3)	62.6 (1.3)	57 (3)	16.3 (0.6)	17.86 (1.00)
H1	CH	44.9 (0.2)	51.0 (0.8)	57.1 (0.3)	66.5 (1.4)	19.9 (0.8)	16.1 (0.4)
	CH:$B_{0.5}$	45.00 (0.3)	49.9 (0.4)	55.9 (0.5)	65.0 (0.7)	19.7 (0.3)	16.5 (0.2)
	CH:$B_{1.0}$	46 (0.5)	50.0 (0.6)	57.0 (0.2)	62.5 (0.6)	20.27 (0.14)	15.67 (0.11)
	CH:$T_{0.5}$	44.8 (0.4)	49.2 (1.1)	55.59 (1.02)	63.98 (1.07)	20.4 (0.3)	17.4 (0.3)
	CH:$T_{1.0}$	44.9 (0.3)	49.7 (0.5)	56.0 (0.2)	64 (2)	20.3 (0.5)	16.74 (0.18)
H2	CH	45 (0.8)	50.2 (1.3)	59.6 (0.8)	66.14 (2.09)	17.6 (0.4)	16.8 (0.5)
	CH:$B_{0.5}$	44.5 (0.7)	49.3 (0.8)	59.2 (1.8)	63.3 (0.9)	18.96 (0.71)	16.8 (0.2)
	CH:$B_{1.0}$	45.9 (0.9)	50.8 (0.8)	57.8 (1.9)	63.6 (0.4)	19.45 (0.80)	15.7 (1.2)
	CH:$T_{0.5}$	46.6 (0.4)	50.8 (0.2)	59.5 (1.8)	66.4 (1.9)	19.62 (0.78)	16.1 (0.6)
	CH:$T_{1.0}$	47.6 (0.5)	51.5 (0.7)	61.3 (3.2)	65.8 (1.9)	17.8 (0.4)	16.1 (0.2)

F-ratio values for L^* (homogenization: 24.30, formulation: 8.51, days: 1112.46); h^*_{ab} (homogenization: 34.64, formulation: 4.74, days: 415.23) and C^*_{ab} (homogenization: 47.62, formulation: 5.61, days: 672.09). Rotor-stator: H1. Rotor-stator homogenization plus microfluidization: H2. Chitosan: CH, Basil: B, Thyme: T. Subindex in film formulation stands for chitosan:essential oil ratio.

The effect of different chitosan-based films on the growth and survival of total aerobic mesophiles and coliform microorganisms at 10ºC is shown in Figure IV.2. Chitosan films with essential oils were the most effective at reducing microbial counts and a growth inhibition was observed in both mesophilic and coliform microorganisms through practically the whole storage period. H2 treated films appear to be slightly more effective, which can be attributed to a certain degree of chitosan depolymerization provoked by high pressure (Vargas et al., 2011a) and the subsequent increase in the antimicrobial effect of this polymer, as has been reported by other authors (No et al., 2002). The incorporation of essential oils into the films implied a reduction in the antimicrobial effectiveness, which suggests that the essential oils used are less effective as antimicrobials against the natural microbial load of minced meat than chitosan. The effective reduction in the chitosan ratio in films containing essential oils can explain the decrease in the antimicrobial effectiveness caused by a dilution effect, as reported by Sánchez-González et al. (2011b).

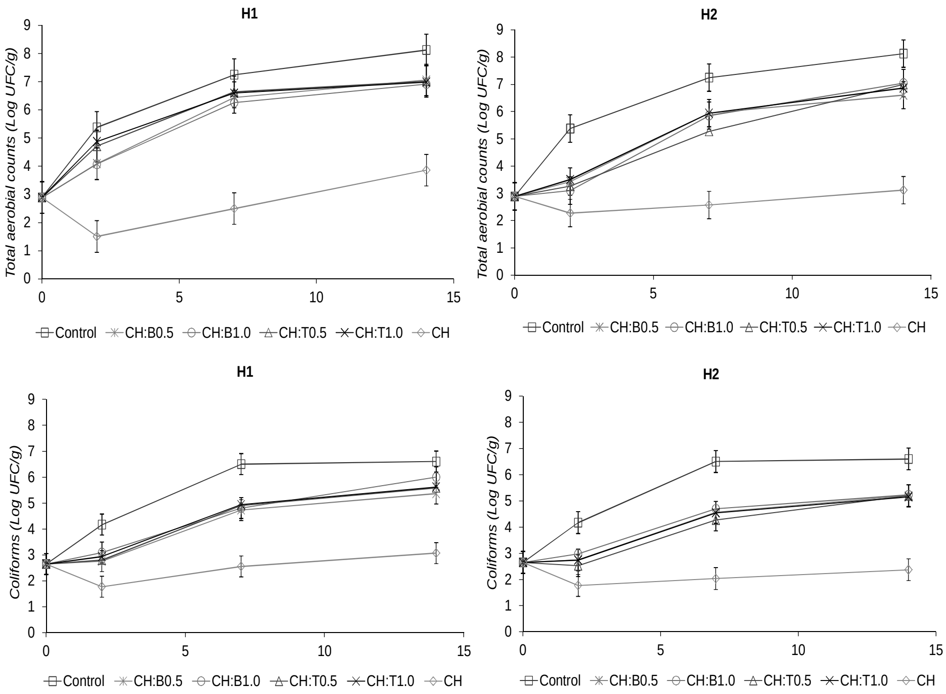

Figure IV.2. Microbial counts of minced pork meat samples coated with chitosan-based edible films. Evolution during storage at 10ºC. Mean values and 95% LSD intervals. Control = non-coated samples.

The effect of chitosan-based films on the growth and survival of *Escherichia coli* and *Listeria inoccua* on inoculated and non-inoculated (control) minced pork meat samples stored at 10° C is shown in Figure IV.3. In the case of these microorganisms, there were no observed differences between the antimicrobial effect of chitosan films with and without essential oils, which suggests that both polymers and oils exhibited a similar degree of effectiveness. At the end of storage, the films led to a significant reduction in microbial load as compared to non-coated samples (about 2 and 4 log CFU/g, for *L. innocua* and *E. coli*, respectively) which was more marked in samples inoculated with *E. coli*. This coincides with the results found when pure chitosan films were applied to pork meat hamburgers (Vargas et al., 2011b) or to intermediate moisture meat products (Rao et al., 2005).

The results obtained reflect the fact that essential oils did not lead to an increase in the antimicrobial effect of the films in minced pork meat, although they can improve the quality preservation through their antioxidant activity. On the other hand, although the total counts of mesophilic and coliform microorganisms are greater when the films contain essential oils, there was no observed negative effect on the microbial control of two potential foodborne microorganisms, such as *L. inoccua* and *E. coli.*

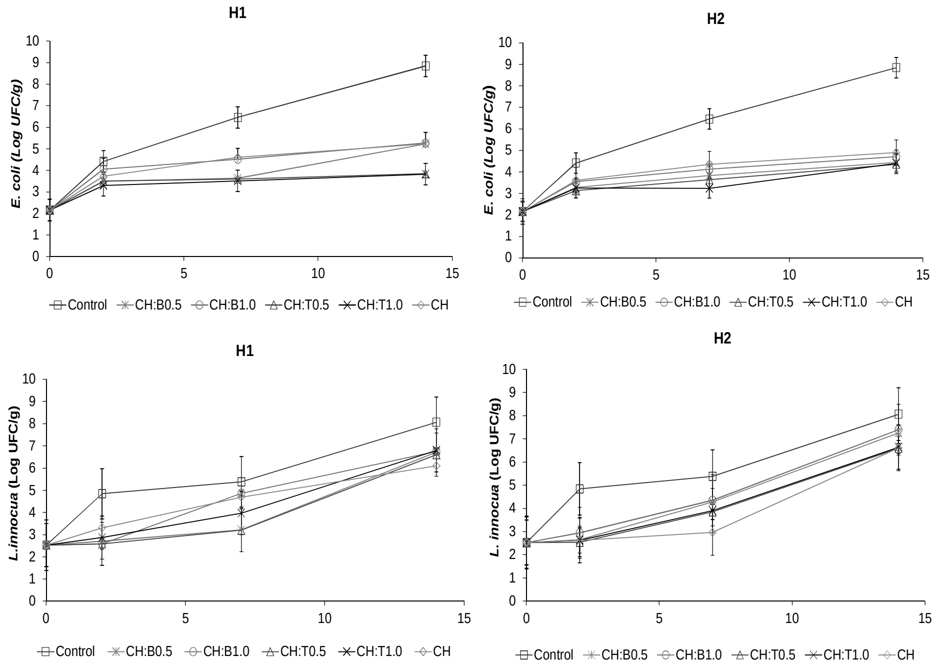

Figure IV.3. Microbial counts of minced pork meat samples inoculated with *L. innocua* or *E. coli* coated with chitosan-based edible films. Evolution during storage at 10ºC. Mean values and 95% LSD intervals. Control = non-coated samples.

4. Conclusions

The addition of essential oils to chitosan films increased their potential antioxidant effects, although it did not improve their antibacterial efficiency in meat products. The improvement in the antioxidant properties of chitosan-based films brought about by adding essential oils occurs in spite of the worsening of their oxygen barrier properties, which points to the specific chemical action of the oil's antioxidant compounds. The reduction in the oxygen availability in minced meat provoked by sample coating leads to the expected changes in colour associated with the conversion of myoglobin into metmyoglobin, but other quality aspects related to food safety are improved. Therefore, chitosan-essential oil films may be formulated in order to be applied to meat products, increasing the product's shelf life and safety.

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PROPERTIES OF WHEAT STARCH FILM-FORMING DISPERSIONS AND FILMS AS AFFECTED BY CHITOSAN ADDITION

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Abstract

In order to evaluate the impact of chitosan on the physical properties of wheat starch–glycerol films, part of the wheat starch was replaced by chitosan, and the effect of composition on the properties of both the films and the film-forming dispersions was studied. The latter became more stable and viscous as the chitosan proportion was increased in the mixture. Both polymers appeared to integrate homogeneously in the film matrix. The combined effect of the glycerol and chitosan proportions affected the mechanical and barrier properties of the films. The tensile strength and elastic modulus of the films were improved as chitosan ratio increased. The oxygen and water vapor permeability slightly increased in line with the amount of chitosan in the blend although the induced differences were very small. Chitosan ratio directly affected the antimicrobial properties of the films, which showed a significant bactericide activity when the chitosan-starch ratio in the film was 50%. Nevertheless, at a starch:chitosan ratio of 80:20, counts of coliforms did not exceed the initial value in the meat after 7 storage days.

Keywords: edible films, wheat starch, chitosan, tensile properties.

1. Introduction

The continuously increasing interest of consumers in the quality, convenience and safety of food has encouraged research into edible films and coatings. The application of edible films permits very diverse objectives, such as the control of moisture loss, gas permeability and microbial growth, the preservation of the structural integrity of the product or the gradual release of encapsulated flavours, antimicrobials and antioxidants into the food (Arvanitoyannis et al., 1996). Biodegradable polymers based on natural polysaccharides, particularly starch, can be produced at low cost and on a large scale (Parra et al., 2004). According to the research of Narayan (2001), starch based materials reduce the utilization of non-renewable resources, as well as the environmental impact of synthetic plastics. Native starch is one of the most naturally abundant biopolymers. The starch granule is essentially composed of two main polysaccharides, amylose and amylopectin, and some minor components such as lipids and proteins. Starch has attracted considerable attention as a biodegradable thermoplastic polymer and biodegradable particulate filler (Angellier et al., 2006). The application of starch-based films in food packaging is promising because of their environmental appeal, low cost, flexibility and transparency (Müller et al., 2009; Bilbao-Sáinz et al., 2010). However, several authors have pointed out that, despite their ease of preparation, starch-based films present some drawbacks, such as their poor mechanical properties which make the addition of plasticizing compounds necessary (Peressini et al. 2003, Bravin et al. 2006). These can also be improved by blending with other polymers.

Chitosan is a cationic polysaccharide that derives from the deacetylation of chitin, a major component of the shells of crustacea such as crab, shrimp, and crawfish (No et al., 2002). It has an excellent film-forming ability with no need for plasticizing compounds (Domard & Domard, 2004; Kittur et al., 1998), and has proven antimicrobial activity (Georgantelis, 2007; Roller et al., 2002; Kendra & Hadwiger, 1984; Sudarshan et al., 1992; Sekiguchi et al., 1994). The exact mechanism for the antibacterial activity is not yet fully understood, but it has been suggested that it involves cell lysis, the breakdown of the cytoplasmic membrane and the chelation of trace metal cations that could be necessary for the microorganism’s growth (Liu et al., 2004).

Chitosan has been previously added to films prepared with starches from different origins, such as potato (Mathew et al., 2006; Shen at a., 2010), tapioca (Vásconez et al., 2009); corn (Xu et al., 2005) and kudzu (Zhong et al., 2010). However, to our knowledge, blends of wheat starch and chitosan have not been used for the preparation of films. The aim of this work was to characterize the most relevant properties of wheat starch-chitosan film-forming dispersions and edible films, in order to evaluate the role of the chitosan addition on these properties.

Likewise, the antimicrobial activity of the films was checked in minced pork meat.

2. Materials and methods

2.1. Reagents and raw materials

High molecular weight chitosan (CH) (practical grade, Batch MKBB0585, degree of deacetylation > 77%), viscosity (1220 cPs), Sigma-Aldrich Quimica, Madrid, Spain) and wheat starch (WS) (food grade, Batch E3748, protein content (0.35% maximum), lipids (0.1% maximum), minerals (0.25% maximum, amylose/amylopectin ratio 25:75), Roquette Laisa S.A., Valencia, Spain) and millipore water were used to prepare the film-forming dispersions (FFD). Glycerol, acetic acid and $Mg(NO_3)_2$ were provided from Panreac Química, S.A. (Castellar del Vallés, Barcelona, Spain).

For the microbiology study, pork meat was purchased in a local supermarket and processed at the laboratory. Tryptone Phosphate Water (peptone buffered water), Violet Red Bile

Agar (VRBA agar), Plate Count Agar (PCA), Tryptic Soy Broth (TSB) and Agar Bacteriological were provided by Scharlau Microbiology.

2.2. Preparation of the film-forming dispersions and casting of the films.

Chitosan (1% w/w) was dispersed in an aqueous solution of glacial acetic acid (1.0% v/w) under magnetic stirring at 40°C for 12h, to obtain a CH solution. Wheat starch was diluted at 1% (w/w) in water and then heated in a water bath for 40 minutes at 95°C to promote starch gelatinization, according to other authors (Jiménez et al., 2012a,b). Glycerol was afterwards added to the wheat starch solution in a starch:glycerol ratio of 1:0.15. The film-forming dispersions (FFD) were obtained by mixing CH and WS solutions at different ratios, where part of the starch was gradually replaced by chitosan, up to 50%. Formulations were named as follows, the subscripts indicating the WS:CH ratio: WS_{100}, WS_{90}:CH_{10}, WS_{80}:CH_{20}, WS_{70}:CH_{30}, WS_{60}:CH_{40}, WS_{50}:CH_{50} and CH_{100}. All film-forming dispersions were prepared by using a rotor-stator homogenizer (Ultraturrax DI 25 basic-Yellowline, Janke & Kunkel, Staufen, Germany) at 21,500 rpm for 4 min at room temperature. After homogenization, the formulations were degassed at 25ºC with a vacuum pump (at 50 mbar, for 1 h). The FFD were cast at 5.6 mg solids/cm^2 in a framed and leveled polytetrafluorethylene (PTFE) plate (15 cm diameter) and dried at room temperature and 60% relative humidity (RH). The films were peeled off from the casting plates and conditioned for at least one week at 5°C-58% RH or 25°C-53% RH in chambers containing saturated solutions of $Mg(NO_3)_2$ prior to all analyses.

2.3 Characterization of the film-forming dispersions

2.3.1. ζ-Potential

ζ-potential was measured in triplicate using a Zetasizer nano-Z (Malvern Instruments, Worcestershire, UK). Formulation WS_{100} was measured without dilution. All formulations with chitosan were diluted to reach a final concentration of chitosan of 0.02% w/w. The electrophoretic mobility of the droplets was transformed into ζ-potential values using the Smoluchowsky model, as described by Kirby & Hasselbrink Jr. (2004).

2.3.2. Rheological behaviour

The rheological behaviour of FFD was analyzed in triplicate at 25°C using a rotational rheometer (HAAKE Rheostress 1, Thermo Electric Corporation, Karlsruhe, Germany) with a sensor system of coaxial cylinders, type Z34DIN Ti. Samples were left to rest for 5 min before the measurements were taken. The shear stress (σ) was obtained as a function of shear rate (γ) between 0 and 150 s^{-1}, taking 5 min for each (up and down) cycle. Experimental data were fitted to the Ostwald de Waale model (Iliuta & Thyrion, 1997) to determine the consistency index (K) and the flow behaviour index (n).

2.4. Characterization of the films

2.4.1. Film thickness

The film thickness was measured using a Palmer digital micrometer (Comecta, Barcelona, Spain) to the nearest 0.001

mm. Six to eight random positions in each film sample were considered.

2.4.2. Microstructure, roughness and gloss

SEM micrographs of the cross-sections of the films were obtained by Scanning Electron Microscopy (SEM), using a JEOL JSM-5410 (Japan) electron microscope. Film specimens were cryofractured by immersion in liquid nitrogen and mounted on copper stubs perpendicularly to their surface. Samples were gold coated and observed, using an accelerating voltage of 10 kV.

The surface of films, previously equilibrated at 33% RH, was analysed by Atomic Force Microscopy (AFM) using a Nanoscope III.a, Scanning Probe Microscope (Digital Instruments, Inc. Santa Barbara, California). The formulations observed were WS_{100}, WS_{80}:CH_{20}, WS_{50}:CH_{50}, CH_{100}. A three-dimensional image of a film surface area (50x50 µm) was obtained in each test, and three images were captured per formulation. Three statistical parameters related with sample roughness (method ASME B46.1, 1995), were calculated, namely the average roughness (Ra: average of the absolute value of the height deviations from a mean surface), the root-meansquare roughness (Rq: root-mean-square average of height deviations taken from the mean data plane) and the Image Surface Area Difference (%ISAD: relative difference between the real and the geometric surface area).

The gloss of the films was measured at 60° incidence angle, according to the ASTM standard D523 (ASTM, 1999) using a flat surface gloss meter (Multi. Gloss 268, Minolta, Germany). Ten replicates were taken per formulation and angle. Results were expressed as gloss units, relative to a highly polished surface of black glass standard with a value near to 100.

2.4.3. Tensile properties

According to ASTM standard method D882 (ASTM, 2001), a Universal Testing Machine (TA.XTplus model, Stable Micro Systems, Haslemere, England) was used to obtain the true stress-Hencky strain curves. From these curves elastic modulus

(EM), tensile strength (TS) and elongation (%E) at break of the films. Rectangular samples (25 x 100 mm) were cut and stored at 25°C, 58% RH, in cabinets containing $Mg(NO_3)_2$ saturated solution (Panreac quimica, S.A., Castellar del Vallés, Barcelona). Equilibrated film specimens were mounted in the film-extension grips and stretched at 50 mm min^{-1} until breakage. The relative humidity and temperature (25°C) of the testing environment were registered with a thermohygrometer (testo 60-H2, Testo GmbH & Co., Lenzkirch, Germany). Measurements were carried out (eight replicates of each formulation) after the film drying and equilibration (7 days) and after 90 days of storage at 25°C-58% RH. Tests in pure starch films were also carried out immediately after 48 h drying.

2.4.4. Equilibrium moisture content and water vapour permeability

The moisture content (MC) of films equilibrated in cabinets containing $Mg(NO_3)_2$ saturated solution, at two temperatures (5 and 25ºC), was obtained gravimetrically. Films were weighed before and after drying in a vacuum oven at 60ºC for 48h (in duplicate). MC (%) was calculated as the percentage of mass of water with respect to the mass of the dry film (DB).

Water vapour permeability (WVP) was determined at two different temperatures (5°C and 25°C) and at 58–100% RH gradient using a modification of the ASTM E96-95 gravimetric method (1995) for flexible films (Mc Hugh et al., 1993). Films were selected based on their lack of bubbles or pinholes, round samples were cut (three per formulation) and six random thickness measurements were taken per sample. Payne permeability cups of 3.5cm in diameter (Elcometer SPRL, Hermelle/s Argenteau, Belgium) were filled with 5 ml distilled water (RH = 100%). Once the films were secured, each cup was placed in a pre-equilibrated cabinet fitted with a fan to provide a strong driving force across the film for water vapour diffusion. The RH of the cabinet was held constant at 53 or 58%, depending on the temperature, using $Mg(NO_3)_2$ over-saturated solution. The shiny side of the films was exposed to the atmosphere at the lowest RH, and the cabinets were stored at the aforementioned temperatures. The cups were weighed periodically (±0.00001g) after steady state was reached. The slope of the weight loss vs. time plot was divided by the exposed film area to calculate the water vapour transmission rate (WVTR) as shown in equation 1. The vapour pressure on the film's inner surface was obtained by means of the method proposed by Mc Hugh et al. (1993) to correct the effect of concentration gradients established in the stagnant air gap inside the cup. WVP was calculated in equation 1.

$$\mathrm{WVTR} = \frac{P \cdot D \cdot \mathrm{Ln}\left[(P - p_2) \big/ (P - p_1) \right]}{R \cdot T \cdot \Delta z} \qquad \text{(Eq.1)}$$

Where P: total pressure (atm); D: diffusivity of water through air at 25 ºC (m^2/s), R: gas law constant ($82.057 \cdot 10^{-3}$ m^3 atm $kmol^{-1}$ K^1); T: absolute temperature (K); Δz: mean height of stagnant air gap (m), considering the initial and final z value; p1: water vapour pressure on the solution surface (atm); p2: corrected water vapour pressure on the film's inner surface in the cup (atm). The WVP was calculated as a function of p2 and the water vapour pressure on the outer (p3) side of the film (Eq. (2)).

$$\mathrm{WVP} = \frac{\mathrm{WVTR}}{(p_2 - p_3)} \cdot \mathrm{thickness} \qquad \text{(Eq.2)}$$

2.4.5 Oxygen permeability (OP)

The oxygen permeability test was conducted according to the ASTM Standard Method D3985-05, 2010. The oxygen barrier performance of the films was evaluated by measuring the oxygen transference rate with an Ox-Tran 1/50 system (Mocon, Minneapolis, USA) at 10ºC or 25°C. One side of the film was exposed to pure nitrogen and the other to pure oxygen flow. The tests were performed in continuous mode at 53% or 58%RH, depending on the temperature. OP was calculated by dividing the oxygen transmission rate by the difference in oxygen partial pressure between the two sides of the film, and multiplying by the average film thickness. Two replicates per formulation were made.

2.4.6. Antimicrobial properties

Four of the film formulations (WS_{100}, WS_{80}:CH_{20}, WS_{50}:CH_{50} and CH_{100}) were tested in their antimicrobial properties using minced pork meat. The meat was ground by using a mincer (Severin Elektrogeräte GmbH, Sundern, Germany) and 10 g of minced meat were moulded (5 cm diameter). The surface of both sides of the minced meat samples was coated with the stand-alone films that were previously prepared by casting, as described in section 2.2. This implies a ratio of 22 g of polymer per kg of meat (0, 4.4, 11 and 22 g CH/Kg meat, depending on the formulation). Non-coated (control) and coated samples were placed in uncovered petri dishes and were stored in duplicate at 10ºC. To perform microbiological analyses, 10 g of each sample were aseptically obtained and homogenized in a Stomacher with 90 mL of sterile buffered peptone water for 2 min. Aliquots were serially diluted in buffered peptone water and plated out following standard methodologies. Total viable and coliform microorganism counts were determined at 0, 2, 7 and 14 days. For the viable counts, Plate Count Agar plates were incubated at

37ºC for 24h. Coliform counts were determined using Violet Red Bile Agar plates incubated at 37ºC for 48h.

2.5. Statistical analysis

The statistical analysis of the data was performed through an analysis of variance (ANOVA) using Statgraphics Plus (Manugistics Corp., Rockville, MD) Fisher's least significant difference (LSD) procedure was used.

3. Results and discussion

3.1. Characterization of the film-forming dispersions

3.1.1. pH and ζ-Potential

Table V.1 shows the average results of ζ-potential of all FFD and the corresponding monomodal ζ-potential distributions are shown in Figure V.1. The ζ-potential of wheat starch FFD was slightly negative, which is due to the adsorption of negative ions from the aqueous medium, since polymer chains do not have ionizable groups. Contrarily, CH_{100} ($pK_a \cong 6.5$, Rinaudo et al., 1999), showed a large positive net charge, because at pH 3.2, the amino group of chitosan is positively charged. As expected, mixing of CH solution with starch solution implied a decrease in the pH value because of the content of acetic acid. It is remarkable that the increasing incorporation of CH solution to the starch did not decrease pH when CH solution was above 20%, and values fluctuate around the pH value of pure CH. This is expected given the weak acid nature of acetic acid whose pH is barely dependent on concentration.

Chitosan incorporation implied a progressive increase in the net charge of the polymer chains of the FFD. Nevertheless, from a WS:CH ratio of 80:20 onwards, ζ-potential increased very slowly when CH ratio increased, even, $WS_{50}:CH_{50}$ FFD did not show a significantly different ζ-potential value compared to that of CH100. These results suggest that a partial neutralization of the CH chain charge occurs in the system as the starch ratio increases, probably due to a certain degree of starch chain adsorption on the CH chains.

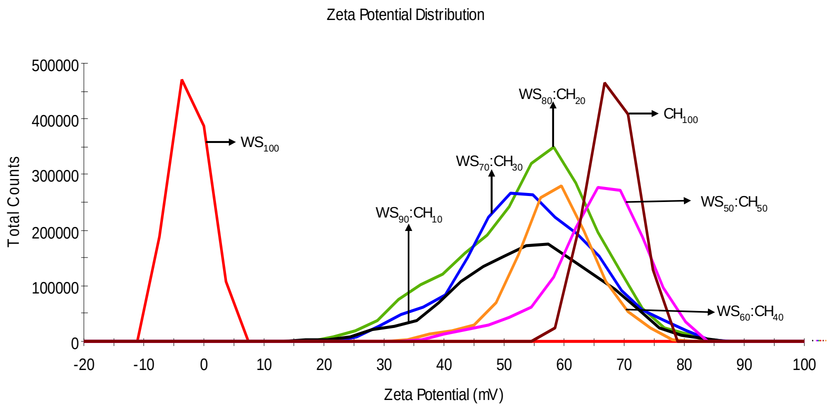

Figure V.1. Typical ζ-potential distributions of pure polymers and CH-WS film-forming dispersions. CH: chitosan. WS: wheat starch. The percentage of each polymer in the film-forming dispersion is indicated as a subscript.

3.1.2. Rheological behaviour

The rheological data were fitted to the Ostwald de Waale model, and the corresponding rheological parameters (K, n) can be found in Table V.1 as well as the apparent viscosity (ηap) at 100^{s-1}. No thixotropic effects were observed from the comparison of the up and down curves. In all cases, the Ostwald de Waale model properly fitted the experimental data between 0 and 150s^{-1} (r^2>0.99). Pure chitosan FFD showed shear-thinning behaviour, as previously reported by other authors (Shepherd et al. 1997; Vargas et al., 2009; Sánchez-González et al., 2010). Moreover, this was the most viscous dispersion, reaching an apparent viscosity of 0.2 Pa·s at $100s^{-1}$. The electroviscous effects in the case of CH contribute to its greater viscosity since chitosan molecules are surrounded by a layer of oriented water molecules, which leads to an increased hydrodynamic volume and so viscosity. On the other hand, WS100 was the least viscous formulation and the apparent viscosity values were in the same range as those found by Che et al. (2008), who studied the rheological properties of Cassava starch dilute aqueous solutions. However, these authors found that 1% cassava starch solutions showed a shear thinning behaviour, unlike the almost newtonian (n very close to 1), shear-thickening behaviour that we observed. According to the research of Dintzis et al. (1996), such differences can be due to structural differences among amylopectins from different botanical sources or with a differing processing history.

The viscosity of WS-CH FFD was gradually increased as the CH content rose. All WS-CH FFD presented shear-thinning behaviour, and the n index decreased to 0.86 as the chitosan content was increased. Chillo et al (2008), prepared mixtures of tapioca starch (4% w/w) and variable concentrations of chitosan and glycerol, and also found shear thinning behaviour in all cases. Moreover, these authors observed a positive effect of chitosan concentration on the apparent viscosity of the blend dispersions, which is coherent with the trend found in the studied WS-CH FFD. The increase in CH proportion also led to a significant increase in the consistency index (K), which is coherent with the observed increase in the shear stress-shear rate relationship.

Table V.1. ζ-potential and rheological parameters (K, n and apparent viscosity at 100s^{-1}) of all FFD.

FFD	**pH**	**ζ-potential***	**K (mPa s^{n})**	**N**	**η_{ap} (Pa s) at 100 s^{-1}**
WS$_{100}$	5.33 (0.01)^{e}	-1.8 (1.4)^{a}	0.00117 (0.00016)^{a}	1.12 (0.02)^{a}	0.00195 (0.00007)^{a}
WS$_{90}$:CH$_{10}$	4.02 (0.03)^{c}	58.3 (1.5)^{b}	0.0179 (0.0015)^{b}	0.940 (0.008)^{b}	0.0131 (0.007)^{b}
WS$_{80}$:CH$_{20}$	3.95 (0.03)^{c}	73 (3)^{c}	0.0511 (0.0006)^{c}	0.8906 (0.0013)^{c}	0.0299 (0.0002)^{c}
WS$_{70}$:CH$_{30}$	3.87 (0.02)^{b}	74.2 (1.9)^{cd}	0.0901 (0.0006)^{d}	0.8619 (0.0004)^{d}	0.0461 (0.003)^{d}
WS$_{60}$:CH$_{40}$	3.95 (0.02)^{c}	75 (3)^{cd}	0.1221 (0.0013)^{e}	0.8508 (0.0008)^{d}	0.0594 (0.004)^{e}
WS$_{50}$:CH$_{50}$	3.79 (0.01)^{a}	77 (3)^{de}	0.1277 (0.0017)^{e}	0.8593 (0.0016)^{d}	0.0647 (0.004)^{f}
CH$_{100}$	3.84 (0.02)^{b}	79 (3)^{e}	0.626 (0.011)^{f}	0.7594 (0.0017)^{e}	0.1994 (0.0021)^{g}

The same superscript $^{(abc)}$ means homogeneous group in LSD test. *In the case of ζ-potential mean values, standard deviation values were obtained from three different distributions.

3.2. Characterization of the films

3.2.1. Thickness

The results of the thickness of the films conditioned at 25ºC and 53% RH are shown in Table V.2. Even though all films were casted at a constant mass/surface ratio (5.6 mg solids/cm^2), the final thickness of these differed significantly ($p<0.05$) as a function of the composition (chitosan ratio and moisture content). The films of WS_{100} were the thinnest, with a thickness of about 50µm, whereas CH_{100} films are the thickest (about 80µm) and film thickness tends to increase when the CH ratio increases in the film. In the same sense, moisture content of the films increased as the CH proportion increases in the film (0.084 g/g film for WS_{100} and 0.1417 g/g film for CH_{100}). The films formulated with both polysaccharides showed intermediate thickness and moisture content values.

These results are probably linked to the differences in the polymer packing as the drying of the films takes place with different water holding capacity. Being positively charged, pure chitosan chains have wide hydration layers with highly retained water molecules which take part in the film structure thus inhibiting the chain approximation and giving rise to thicker films. The hydration layers in WS are thinner due to the neutral character of the chains, which implies lower water content and thickness of the films. The greater the CH ratio, the more open the matrix due to the greater hydration level of the films.

3.2.2. Microstructure, surface roughness and gloss

Figure V.2 shows the SEM microstructural images of cross sections of the. Figure V.3 shows AFM images of the surface topography of some film formulations (WS_{100}, WS_{80}:CH_{20}, WS_{50}:CH_{50}, CH_{100}). The corresponding roughness parameters can be found in Table V.2. From the cross-section images, it can be observed that the inner structure of the films was uniform, which suggests that wheat starch and chitosan form a homogeneous blend and do not tend to separate.

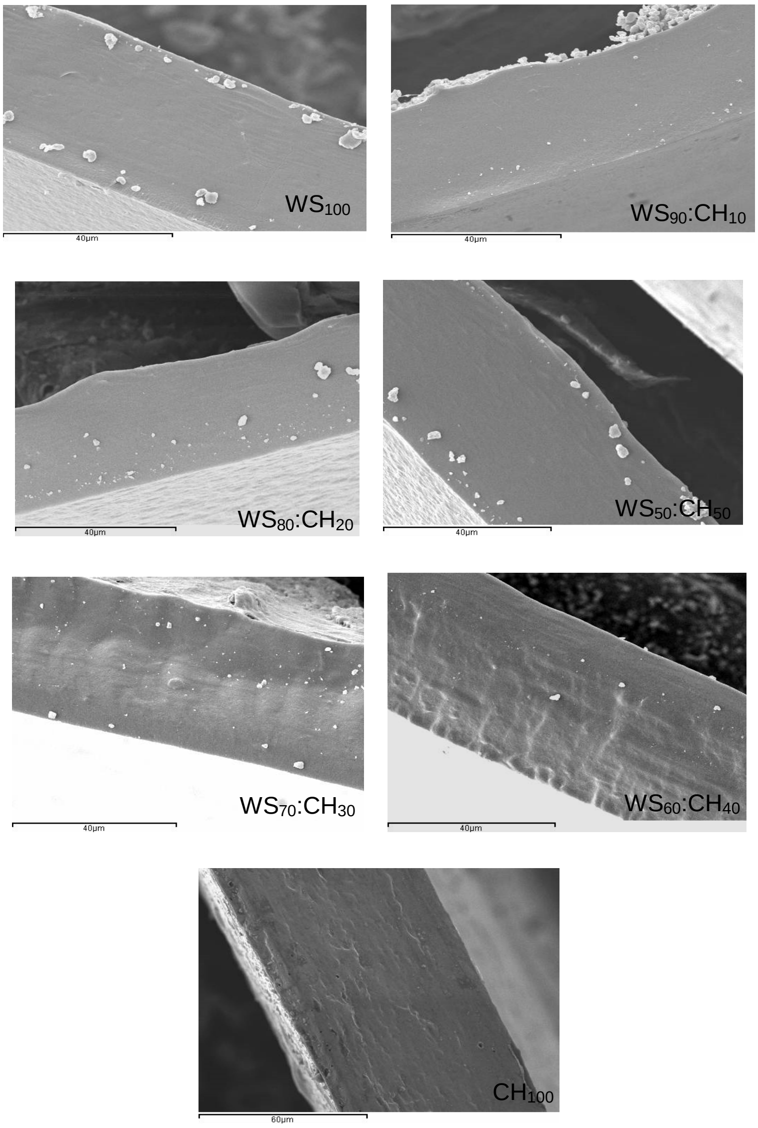

Figure V.2. Scanning electron microscopy (SEM) images of cross-sections of the films. CH: chitosan. WS: wheat starch. The percentage of each polymer in the film-forming dispersion is indicated as a subscript.

This good compatibility of chitosan, starch and the plasticizer was also observed by Mathew & Abraham (2008), when working with mixtures of chitosan and potato starch with glycerol. Xu et al. (2005) also found a good molecular miscibility between these two polymers. On the other hand, the presence of small fragments of material was probably caused by the film cryofracture prior to the SEM analysis.

Tapping mode AFM images yielded information about the surface features of the films (Figure V.3). It was found that the inherent roughness of the WS films was diminished as the chitosan proportion was increased as can be seen in Table V.2. Pure chitosan films were the smoothest (Figure V.3) and showed the lowest values of all roughness parameters reported in Table V.2. All the films showed good structural integrity and no pores or cracks were observed.

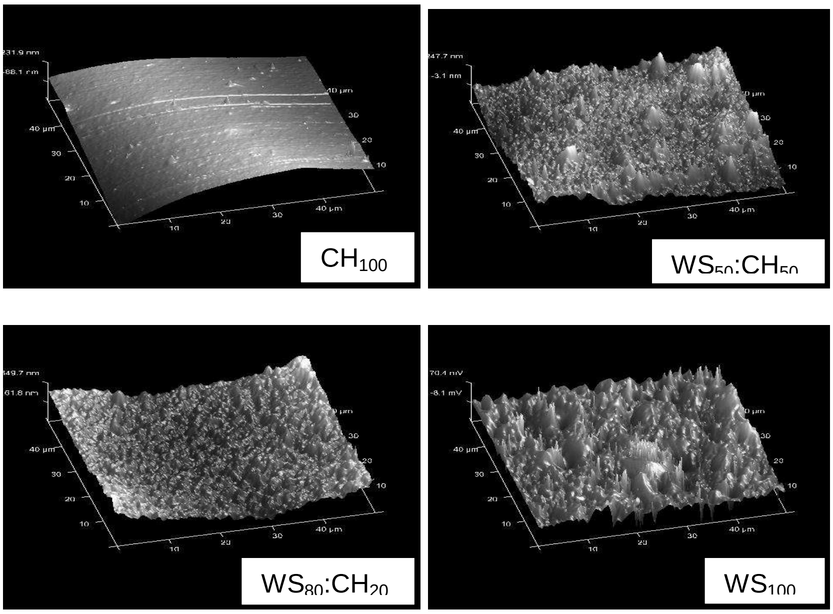

Figure V.3. AFM images of surface topography of films from pure polymers and selected CH-WS blends. CH: chitosan. WS: wheat starch. The percentage of each polymer in the film-forming dispersion is indicated as a subscript.

The results of gloss at 60° are reported in Table V.2. This angle was chosen, as indicated in the norm, because the results obtained were neither too high nor too low. Pure WS films, being the roughest, were also the least glossy. As a higher proportion of chitosan was incorporated into the formulations, the roughness was decreased and the films became significantly glossier. This also points to the compatibility between the two polymers, since phase separation could cause greater surface irregularities and roughness.

Table V.2. Thickness, gloss at 60º and roughness parameters (R_a, R_q and ISAD) of the films.

	Thickness (µm)	**Gloss**	**R_a (nm)**	**R_q (nm)**	**ISAD (%)**
WS_{100}	53 (3)^{a}	11 (4)^{a}	321 (105)^{a}	446 (163)^{a}	1.6 (0.6)^{a}
WS_{90}:CH_{10}	63 (3)^{cd}	10.9 (1.4)^{a}	-	-	-
WS_{80}:CH_{20}	61 (4)^{bc}	13.0 (1.6)^{a}	113 (38)^{b}	147 (41)^{b}	0.8 (0.3)^{b}
WS_{70}:CH_{30}	59 (4)^{b}	26 (7)^{b}	-	-	-
WS_{60}:CH_{40}	64 (2)^{cd}	39 (11)^{c}	-	-	-
WS_{50}:CH_{50}	65 (4)^{d}	62 (15)^{c}	54 (9)^{b}	69 (10)^{b}	0.3 (0.1)^{bc}
CH_{100}	79 (3)^{e}	38 (12)^{d}	57 (23)^{b}	72 (30)^{b}	0.1 (0.1)^{c}

The same superscript (^{abc}) means homogeneous groups in LSD test.

3.2.3. Tensile properties

Biodegradable or edible films must withstand the normal stress encountered during their application and subsequent shipping and handling of the food in order to maintain its integrity and barrier properties. High tensile strength -the maximum tensile stress sustained by the sample during the tension test- is generally required, (Bourtoom & Chinnan, 2008).

Typical true stress-Henky strain curves obtained from the tensile tests are shown in Figure V.4. Blend films exhibit plastic deformation which contributes to their stretchability, whereas this does not occur for pure wheat starch films.

The tensile properties (Elastic Modulus EM, tensile strength at fracture, TS and percentage of elongation at fracture, %E) of the films after 7 days of storage at 58% RH are shown in Table V.3. It was found that, as the chitosan ratio increased in the film, with the subsequent reduction of the overall glycerol content, the TS showed a significant increase ($p<0.05$), i.e. films became more resistant. Chillo et al. (2008) studied the mechanical properties of tapioca starch-chitosan films with different concentrations of glycerol, and they found that the plasticizer proportion had a negligible effect on the tensile strength, whereas this variable increased coherently with the chitosan proportion. The same trend was observed by other authors working with blend films of chitosan and starches of different origin, such as rice (Bourtoom & Chinnan, 2008), corn (Xu et al., 2005) and sweet potato (Shen et al., 2010), where the plasticizer proportion was constant.

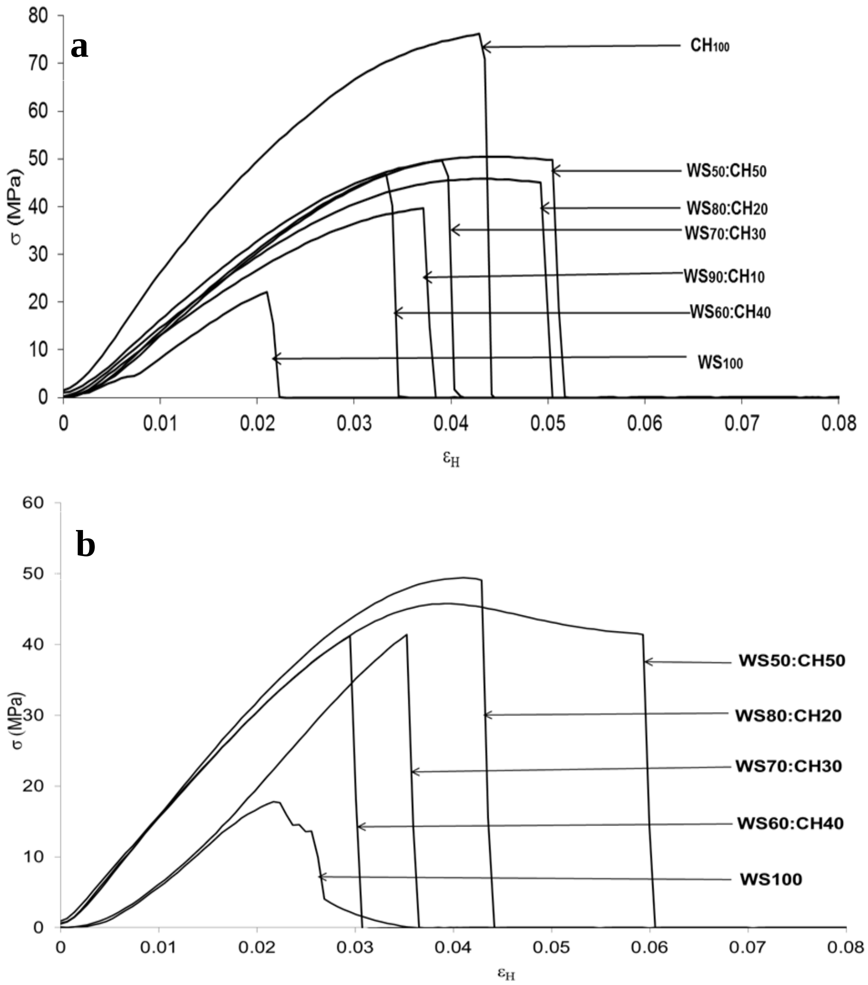

Figure V.4. Typical true stress (σ) vs. Hencky strain (ε_H) curves obtained in tensile tests carried out on pure biopolymer and WS-CH blend films a) after days of storage, b) after 90 days of storage. CH: chitosan. WS: wheat starch. The percentage of each polymer in the film-forming dispersion is indicated as a subscript.

From the values of mechanical parameters of CH and WS it is evident that cohesion forces in the CH matrix are more intense than in the WS matrix as a result of the higher intermolecular forces. Being CH a charged chain, van der Waals forces, including hydrogen bonds, are enhanced involving water molecules in a cooperative network. These effects are of lower magnitude in neutral polymer chains where bonds trough water molecules are weaker giving rise to a less cohesive network. The incorporation of CH to the blend films seems to contribute to increase the cooperative van der Waals forces in the matrix as a consequence of its greater capacity to form hydrogen bonds. In this sense, the charged amino groups plays an important role since their net charge contribute to polarize other chain bonds, thus promoting the attractive forces between chains and constitutional water molecules.

Elongation at break (%E) is an indication of the films’ flexibility and stretchability, which is determined at the point where the film breaks under tensile testing. It is expressed as the percentage of change of the original length of the specimen between the grips of a film to stretch. Chillo et al. (2008) observed that, as the glycerol proportion was increased, the elongation at break of their starch-chitosan blend films increased. We observed that the starch chitosan blend films, even their reduced glycerol content, tended to elongate slightly more than starch films, which are typically very brittle (Xu et al., 2005). The addition of chitosan plays a plasticizing role in the blend films, favoring the film extension and plastic deformation. This is the result of an increase of the feasibility for the chain slippage during the film stretching, which could be associated with a greater amount of water molecules between chains, acting as a "lubricant", with great capacity to break and form successive hydrogen bonds (flow of water molecules) as the chains move as a result of tensile forces. The thinnest hydration layers in the neutral starch chains and their greater capacity to form intra-molecular bonds, make the chain slippage more difficult and film breaks at lower deformation, despite the presence of greater amount of glycerol, which is less effective than water in the promotion of molecular flow.

Mechanical parameters of the films after 90 days storage under the same RH conditions are also shown in Table V.3. In most cases, the mechanical parameters of blend films did not exhibit significant differences between 7 and 90 days of storage ($p>0.05$) which indicates that starch retrogradation did not occur in these cases. Nevertheless, pure starch film showed a notable decrease in the film stretchability (50% reduction of deformation at break) with the subsequent decrease of tensile at break. This change can be associated with the increase in the intramolecular bonds of amylose chains forming crystalline forms (Carvalho, 2008) which are mechanically more brittle, thus decreasing the film extension capacity. To better understand the kinetics of starch retrogradation in this kind of wheat starch films, its mechanical properties were also measured immediately after drying and equilibration at 58% RH (2 days after casting). It was ensured that the film was completely equilibrated by controlling its moisture content.

Table V.3. Tensile properties (EM, TS and E%) of all films (58% RH). Mean values and 95% LSD intervals in brackets.

	EM (MPa)		**TS (MPa)**		**E (%)**	
	7 days	**90 days**	**7 days**	**90 days**	**7 days**	**90 days**
WS$_{100}$	1356 (189)^{a,x}	1299 (174)^{a,x}	33 (9)^{a,x}	12 (5)^{a,y}	3.5 (0.9)^{a,x}	1.8 (0.7)^{a,y}
WS$_{90}$:CH$_{10}$	1414 (45)^{ab}	-	38.4 (1.6)^{ab}	-	3.7 (0.6)^{a}	-
WS$_{80}$:CH$_{20}$	1451 (159)^{ab,x}	1715 (224)^{b,y}	45 (3)^{bc,x}	47 (11)^{c,x}	4.3 (0.8)^{ab,x}	3.6 (0.7)^{b,x}
WS$_{70}$:CH$_{30}$	1465 (261)^{ab,x}	1552 (172)^{ab,x}	47 (6)^{c,x}	34 (18)^{bc,x}	4.2 (0.6)^{ab,x}	3.8 (0.5)^{b,x}
WS$_{60}$:CH$_{40}$	1586 (104)^{b,x}	1530 (255)^{ab,x}	48 (2)^{c,x}	32 (13)^{b,y}	4.6 (1.3)^{ab,x}	3.6 (0.8)^{b,x}
WS$_{50}$:CH$_{50}$	1593 (117)^{b,x}	1559 (259)^{ab,x}	46 (2)^{c,x}	45 (6)^{c,x}	5 (2)^{b,x}	5.6 (1.3)^{c,x}
CH$_{100}$	2451 (250)^{c}	-	74 (11)^{d}	-	4.6 (0.8)^{ab}	-

The same superscript (^{abcd} to be compared vertically and xy to be compared horizontally) means homogeneous group in LSD test.

The changes in the three mechanical parameters of WS100 films that take place over time are shown in Figure V.5. Immediately after drying, the films were less rigid and more extensible than after storage. After only 7 days stored at 58% RH, the films became significantly more rigid and less extensible, which seems to indicate that some starch retrogradation had already occurred. The recrystallization or retrogradation of starch is known to occur after gelatinization (Bertuzzi et al., 2007) and, in starch films, it was inhibited when blends of starch with other polymers are used in the films (Wu et al., 2009; Jiménez et al., 2012a,b). In WS films, the incorporation of CH contribute to improve mechanical resistance and stretchability at the same time that inhibit the retrogradation process, probably due to the fact that the presence of the neighbor chains of CH represent an steric hindrance for aggregation of helical conformation of amylose.

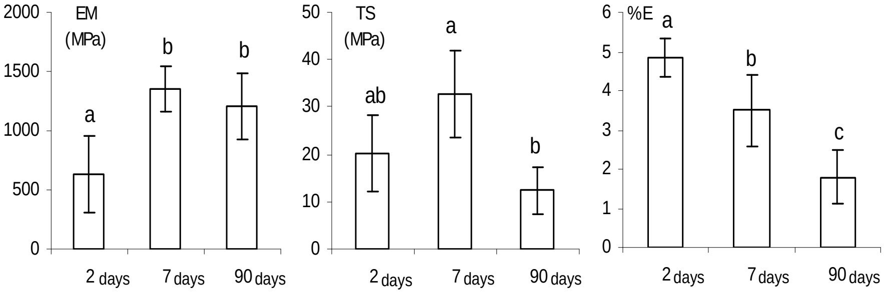

Figure V.5. Effect of storage time on mechanical properties of pure wheat starch films. Mean values and standard deviation. Different letters indicate 95% significant differences.

3.2.4. Equilibrium moisture content and water vapour permeability

Table V.4 shows the moisture content of the films at the two temperatures (5 and 25ºC) where water vapor permeability was determined and the values of water vapor permeability. Permeability values of hydrophilic materials are highly affected by both moisture content and temperature. Water acts as a plasticizer and, as stated by Bertuzzi et al. (2007), the main functional properties of hydrophilic materials usually depend on their water content, and hence on the surrounding humidity. Barrier properties depend heavily on water content. An increase of water content and/or temperature, implies an increase in the molecular mobility and therefore, in all diffusion dependent properties, such as permeability values (Gontard, et al., 1993; Gennadios & Weller, 1994; Mehyar & Han, 2004). For all film formulations WVP tends to increase with the temperature, despite the slight decrease in the film water content, whereas differences among films are hardly appreciated from a practical point of view. Nevertheless, the addition of CH tends to increase the WVP values, as the same time that the moisture content of the films increases. This was also observed by other authors for starch:chitosan blend films. The increase of permeability can be associated with the greater water binding capacity of CH molecules, as previously commented. The latter favours the transfer of water molecules through the more open matrix of the film. The overall reduction of glycerol content in the films, when the CH ratio increases, probably mitigates the potential permeability increase due to CH addition. Being a plasticiser agent, glycerol dilutes and softens the structure, increasing chain mobility and consequently promoting mass transfer across the films. Bourtoom & Chinnan (2008) formulated blend films of starch:chitosan with proportions ranging between 2:1 and 0.5:1, where the proportion of added plasticizer (sorbitol) was 40% of solids, and found that WVP increased with the chitosan ratio. Xu et al. (2005) found the same trend when working with corn starch and chitosan blend films. The increase of WVP at low proportions of CH may be due to the differences in the hydration layers of both polymers, as commented on above, which make the CH containing matrices more open to water transfer. The scarce influence of CH in the studied WS-CH films can be attributed to the progressive reduction of glycerol as CH increase in the film. Glycerol promotes water transfer in starch films and its reduction, in line with the CH increase in the blend, mitigates the effect of the incorporation of a more hydrophilic polymer with greater water capacity such as CH. Nevertheless, this represents an advantage since whereas other properties, such as mechanical resistance, were improved, water vapour mass transfer was not enhanced significantly.

Table V.4. Moisture content (MC, g water/g dry film) of films stored at 5ºC-58%RH and 25ºC-58%RH. Water vapour permeability (WVP) of the films at 58-100% RH and 5ºC and 25ºC.

	MC 5ºC-59%RH	**WVP (5ºC) x 10^{10} (g s^{-1} m^{-1} Pa^{-1})**	**MC (db) 25ºC-53%RH**	**WVP (25ºC) x 10^{10} (g s^{-1} m^{-1} Pa^{-1})**
WS_{100}	0.089 (0.007) ^{x,a}	4.0 (0.3)^{x,a}	0.075 (0.003)^{y,a}	13 (2)^{y,a}
WS_{90}:CH_{10}	0.094 (0.008)^{x,ab}	4.7 (0.3)^{x,ab}	0.079 (0.003)^{x,ab}	14(4)^{y,a}
WS_{80}:CH_{20}	0.104 (0.009)^{x,bc}	5.2 (0.5)^{x,bc}	0.083 (0.003)^{x,b}	18 (2)^{y,a}
WS_{70}:CH_{30}	0.108 (0.013)^{x,cd}	7.4 (0.7)^{x,d}	0.092 (0.007)^{x,c}	16 (3)^{y,a}
WS_{60}:CH_{40}	0.111 (0.007)^{x,cd}	6.0 (1.3)^{x,c}	0.092 (0.005)^{x,c}	16 (5)^{y,a}
WS_{50}:CH_{50}	0.116 (0.012)^{x,d}	5.81 (0.17)^{x,c}	0.102 (0.005)^{x,d}	16 (4)^{y,a}
CH_{100}	0.162 (0.009)^{x,e}	4.9 (0.2)^{x,abc}	0.133 (0.006)^{x,e}	19 (3)^{y,a}

Different letters indicate 95 % significant differences due to temperature and RH conditions ($^{x, y}$) or to film formulation ($^{a, b, c, d}$).

3.2.5 Oxygen permeability (OP)

Starch films are able to act as oxygen barriers, thus lengthening the shelf life of the food (Shen et al., 2010). The results of the oxygen permeability of the films at 10ºC and 25ºC are shown in Table V.5, where the greatest OP of pure CH films can be seen. Nevertheless, the incorporation of CH to WS films did not provoke a significant increase in their OP values at 5ºC. At 25ºC, OP values tend to increase as the CH content is increased in the film, although this was only significant when more than 30% CH is present in the blend and the differences were very small. The temperature increase did not promote a significant ($p>0.05$) increase of the OP values in the studied films, although a slight tendency to increase was appreciated, as expected from the promotion of molecular mobility. This behavior also represents an advantage of the blend films, since CH incorporation led to an enhancement of starch films, inhibiting retrogradation and increasing mechanical resistance, but did not improve their barrier properties notably. The lower requirement of glycerol in blend films plays an important role because glycerol contributes to promote mass transfer processes due to the promotion of molecular mobility. With lower ratio of glycerol, chitosan containing films showed similar barrier properties to wheat starch-glycerol films.

Table V.5. Oxygen permeability (OP) of the films at 58-100% RH and 10ºC and 25ºC.

	OP 10ºC (cm^3*mm/(m^2*atm*day)	**OP 25ºC (cm^3*mm/(m^2*atm*day)**
WS_{100}	$0.045\ (0.015)^{x,a}$	$0.061\ (0.011)^{x,a}$
$WS_{90}:CH_{10}$	$0.0468\ (0.0009)^{x,a}$	$0.055\ (0.008)^{x,a}$
$WS_{80}:CH_{20}$	$0.038\ (0.013)^{x,a}$	$0.067\ (0.007)^{x,a}$
$WS_{70}:CH_{30}$	$0.04\ (0.02)^{x,a}$	$0.069\ (0.014)^{x,ab}$
$WS_{60}:CH_{40}$	$0.056\ (0.003)^{x,a}$	$0.0764\ (0.0008)^{y,ab}$
$WS_{50}:CH_{50}$	$0.055\ (0.032)^{x,a}$	$0.08\ (0.02)^{x,ab}$
CH_{100}	$0.15\ (0.04)^{x,b}$	$0.10\ (0.02)^{x,b}$

The same superscript (^{abc}) to be compared vertically the different formulations and (^{xy}) horizontally the same formulation in LSD test.

3.2.6. Antimicrobial properties

Figure V.6 shows the progress over storage time (up to 14 days) of the total aerobial and coliform counts corresponding to the control (C: minced meat without film) and minced pork meat samples coated with the four films being tested (WS_{100}, WS_{80}:CH_{20}, WS_{50}:CH_{50}, CH_{100}). The two films with the highest chitosan contents exhibited the most important reduction in the microbial load as compared to the control treatment, both for coliforms and total aerobial counts, showing some bactericidal effect after two days of cold storage. The films with only a little proportion of chitosan, or none at all, did not show a significant antibacterial effect. These results suggest that the antimicrobial effect of the films is directly related with the concentration of CH, which has proven its antibacterial properties in previous studies performed both in vitro (Sanchez-González et al. 2010) and on pork meat hamburgers (Vargas et al., 2011).

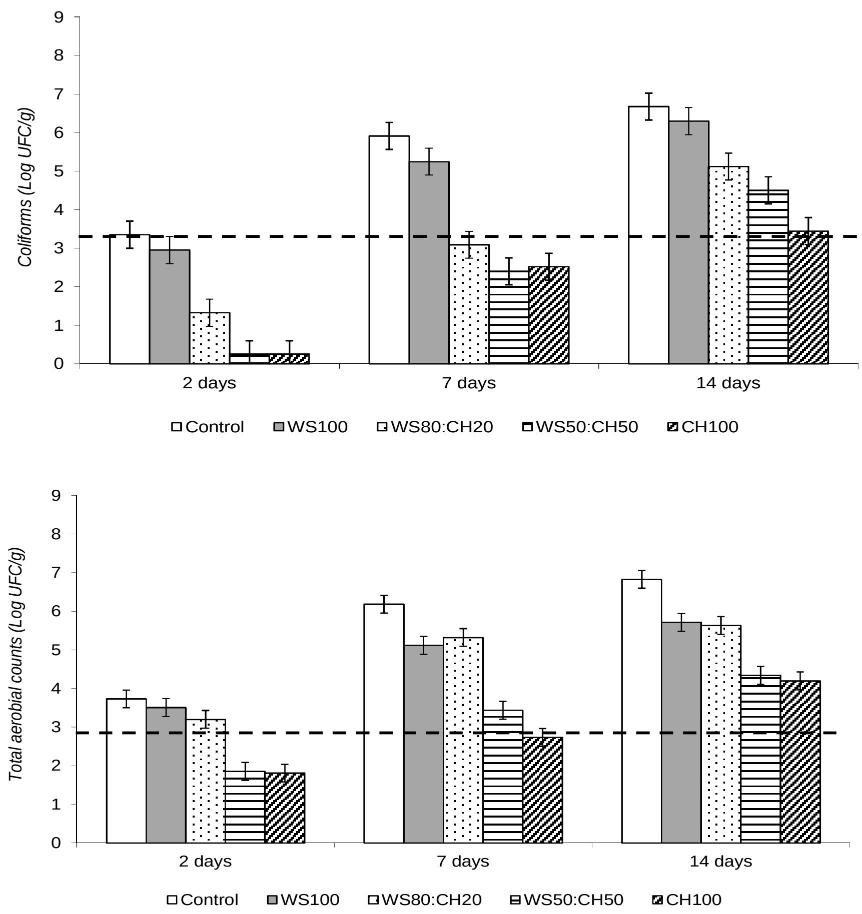

Figure V.6: Total aerobial and coliform counts of non-coated minced pork samples (control) and samples coated with WS, CH and WS-CH blend films. Mean values and 95% LSD intervals. Dashed line indicates initial counts in minced meat samples. CH: chitosan. WS: wheat starch. The percentage of each polymer in the film-forming dispersion is indicated.

4. Conclusions

The replacement of wheat starch-glycerol with chitosan affected the properties of film forming dispersions and films. Chitosan addition led to a significant increase of the particle surface charge and apparent viscosity of the FFD and yielded glossier and thicker films. The mechanical properties were significantly improved as the chitosan ratio increased in the films, these being more resistant and extensible, at the same time that chitosan seems to inhibit starch retrogradation. Both, WVP and OP values, tend to slightly increase when chitosan was incorporated to the film, although induced differences are not relevant. A notable antimicrobial effect was detected in the blend films when the proportion of chitosan was 50%. The obtained results suggest that it would be possible to formulate edible antibacterial films with thermoplastic properties by combining wheat starch and chitosan.

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EFFECT OF THE INCORPORATION OF ANTIOXIDANTS ON THE PROPERTIES OF WHEAT STARCH-CHITOSAN FILMS

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Abstract

Blend edible films were prepared from wheat starch (WS) and chitosan (CH) with glycerol as plasticizer. Four active ingredients (antioxidants) were added, namely basil essential oil, thyme essential oil, citric acid and α-tocopherol. The starch:antioxidant mass ratio was 1:0.1. Prior the characterization, the films were conditioned at 25ºC-53%RH as to their structural, mechanical, optical and barrier properties. The antioxidant capacity of the active ingredients was determined by means a spectrophotometric method. The incorporation of antioxidants led to a heterogeneous film microstructure, mainly in those containing α-tocopherol, which affected the surface roughness. Yellowness was induced in films when α-tocopherol was added and no notable colour changes were observed in the other cases, although all the antioxidants increased the transparency of the films. Despit the fact that the mechanical properties were barely affected by the incorporation of antioxidants, citric acid promoted an increase in the elastic modulus but a decrease in film stretchability. The water vapour barrier properties of the films were only slightly improved when citric acid and α-tocopherol were added, whereas the oxygen barrier properties were significantly improved in all cases. The greatest antioxidant capacity of the films was expected for films containing α-tocopherol, which exhibited the highest antioxidant power.

Keywords: edible films, wheat starch, chitosan, basil essential oil, thyme essential oil, α-tocopherol, citric acid, ABTS.

1. Introduction

Edible films or coatings are defined as thin layers of material suitable for consumption, which act as a barrier against different agents (water vapour, oxygen and moisture) thus increasing the shelf-life of the product (Guilbert et al., 1996). Their use is a promising alternative in the field of food preservation, allowing for the reduction of waste plastics. Additionally, the incorporation of additives to these coatings, such as antimicrobials or antioxidant agents, is potentially of interest (Baldwin et al., 1996).

Biodegradable polymers based on natural polysaccharides, particularly starch, can be produced at low cost and on a large scale. The starch-based materials can reduce the use of non-renewable resources. Furthermore, starch is one of the most abundant, natural biopolymers. The starch granule is essentially composed of two polysaccharides, amylose and amylopectin, and some minor components such as lipids and proteins. This polymer has attracted considerable attention as a biodegradable thermoplastic polymer (Angellier et al., 2006). The films prepared from starch represent promising applications in food packaging due to their biodegradability, low cost, flexibility and transparency. However, several authors have observed that, despite their ease of preparation, starch films have some drawbacks, such as their poor mechanical properties. For this reason, starch films require the addition of plasticizing compounds (Parra et al., 2004).

Chitosan is a cationic polysaccharide derived from the deacetylation of chitin, a component of the shells of crustaceans.

It has been shown to provide antimicrobial activity (Dutta et al., 2009; Kumar, 2000), but its application is currently limited by its relatively high price and the fact that it is highly permeable to water vapour (Butler et al., 1996). Bonilla et al. (2013) characterized films with varying proportions of wheat starch and chitosan, and observed that the films a chitosan proportion of 20% (w/w) exhibited good antimicrobial properties.

The addition of essential oils (basil and thyme) and other components with antioxidant activity, such as α-tocopherol (liposoluble antioxidant) or citric acid, can improve the functional properties of edible films and increase their potential uses in the preservation of foods with a high fat content. Although the incorporation of bioactive compounds can modify the barrier properties (water vapour and oxygen) of the films, doing so provides additional advantages, such as protection against microbial growth and lipid oxidation (Gutierrez et al., 2008; Ahn et al., 2008). In this sense, there is the challenge of finding appropriate formulations to delay the oxidation of lipids that are easily oxidized in high-fat products (such as meat, fish or derivatives).

The aim of this work was to study the effect of adding different ingredients - essential oils (basil and thyme), citric acid and α-tocopherol - on the microstructure, mechanical behaviour, water vapour and oxygen permeabilities and optical properties of wheat starch-chitosan films. Additionally, the antioxidant properties of the active compounds were quantified by means of a spectrophotometric method.

2. Materials and methods

2.1. Raw materials

The raw materials used were: native wheat starch (WS) with 21% amylose (Roquette Laisa SA Benifaió, Valencia, Spain), high molecular weight chitosan (CH) (Batch MKBB0585, Sigma - Aldrich, Madrid, Spain), basil (B) and thyme (Th) essential oils (Herbes of Moli, Alcoy, Alicante, Spain), α-tocopherol (Tp) and citric acid (CA), (Sigma-Aldrich, Madrid, Spain), glycerol, acetic acid and $Mg(NO_3)_2$ (Panreac Química SA, Castellar del Vallès, Barcelona, Spain).

The reactants for the antioxidant capacity assay -Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), $K_2S_2O_8$ and ABTS (2'2-azino-bis-[3 etilbenzotiazol-6-sulfonic acid])- were supplied by Sigma-Aldrich (Madrid, Spain).

2.2. Experimental design and preparation of the films

WS was diluted at 1% (w/w) in distilled water, and glycerol was added in a starch:glycerol ratio of 1:0.15. This dispersion was heated in a water bath at 95°C for 40 minutes to promote starch gelatinization. CH (1% w/w) was dispersed in an aqueous solution of acetic acid (1% v/w) under magnetic stirring at 40ºC and 450 rpm for 24h. The two solutions were mixed in a mass ratio WS:CH of 4:1. The active ingredients were added at 0.1 g/g of starch. Hence, five film-forming dispersions (FFD) were obtained, namely WS:CH (control formulation), WS:CH:B, WS:CH:Th, WS:CH:CA and WS:CH:Tp. All of these were homogenized using rotor-stator equipment (Yellow Line DL 25 Basic, IKA, Janke y Kunkel, Germany) at 13,500 rpm for 4 minutes and degassed at room temperature by means of a vacuum pump. In order to obtain the films, the FFD were cast in framed and leveled polytetrafluorethylene (PTFE) plates (150 mm of diameter), at constant total solid mass (1g per plate). After drying for 48 hours at 25ºC and 50%RH, the films were removed from the plates and conditioned at 5°C-58%RH or 25°C-53%RH in cabinets containing saturated solutions of $Mg(NO_3)_2$.

2.3. Characterization of the films

2.3.1. Film thickness

The film thickness was measured using a Palmer digital micrometer (Comecta, Barcelona, Spain) to the nearest 0.001 mm. Four to six random positions were taken from each film sample and considered for the mechanical and barrier property assays.

2.3.2. Microstructure of the films

The microstructural arrangement of the film components was studied by combining two techniques: scanning electron microscopy (SEM) and atomic force microscopy (AFM). SEM micrographs of the surfaces and cross-sections of the films were obtained using a JEOL JSM-5410 electron microscope (Japan). To observe the sections of the films, the specimens were cryofractured by immersion in liquid nitrogen. The samples were mounted on copper stubs, gold coated and observed using an accelerating voltage of 10 kV.

The surface of the films was analyzed by AFM using a Nanoscope III.a, Scanning Probe Microscope (Digital Instruments, Inc. Santa Barbara, California). For each formulation, three samples were cut (1x1 cm^2). A three-dimensional image of a film surface area (50 x 50 μm) was obtained in each test, and three images were captured per formulation. Following the ASME B46.1 (1995) method, the following statistical parameters describing the surface roughness were calculated:

- Average roughness (R_a): the arithmetic average of the absolute values of the height deviations with respect to a mean surface (1).

$$R_a = \frac{1}{N}\sum_{J=1}^{N}\left|Z_j\right| \qquad (1)$$

- Root–mean–square roughness (R_q): root–mean–square average of height deviations taken from the mean data plane (2).

$$R_q = \sqrt{\frac{\sum_{J=1}^{N} Z_J^2}{N}} \qquad (2)$$

- Image Surface Area Difference (ISAD, %): relative difference between the real and the geometric surface area.

2.3.3. Optical properties: translucency, colour and gloss

The surface reflectance spectra of the films were obtained using a MINOLTA spectrocolorimeter, model CM-3600d (Minolta CO., Tokyo, Japan), illuminant D_{65} and observer 10º. The measurements were taken at three random points on the surface exposed to the atmosfhere during the drying step. The level of transparency/opacity of the samples was determined through internal transmittance (T_i) calculated according to equations 3 to 5 (Hutchings, 1999), where R is the reflectance of the sample layer backed by a known reflectance R_g and R_0 is the reflectance of the film on an ideal black background.

$$a = \frac{1}{2}\left(R + \frac{R_0 - R + R_g}{R_0 \cdot R_g}\right) \quad (3)$$

$$b = \sqrt{a^2 - 1} \quad (4)$$

$$T_i = \sqrt{(a - R_0)^2 - b^2} \quad (5)$$

The reflectance of an infinitely thick layer of the material was determined by means of equation 6, prior to obtaining the colour coordinates: Lightness (L*), Chroma (C^*_{ab}) (eq. 7) and hue (h^*_{ab}) (eq. 8). Moreover, the Whiteness index (WI) was calculated using eq. 9.

$$R_\infty = a - b \quad (6)$$

$$C^*_{ab} = \sqrt{\left(a^{*2} + b^{*2}\right)} \quad (7)$$

$$h^{*}_{ab} = \text{arctg}\left(\frac{b^{*}}{a^{*}}\right) \tag{8}$$

$$WI = 100 - ((100 - L^{*})^{2} + a^{*2} + b^{*2})^{1/2} \tag{9}$$

Following ASTM D523 (1999), a glossmeter (Multi-Gloss 268, Konica Minolta, Langenhagen, Germany) was employed to measure the gloss at 20°, 60° and 85° on two films of each formulation, previously conditioned at 25°C- 53% RH. Six measurements were taken on the face exposed to the atmosphere during the drying process.

2.3.4. Tensile properties

The mechanical properties of the films were determined using a Universal Testing Machine (Stable Micro Systems, TA.XT plus, Haslemere, England) following the ASTM D882 (2001) standard method. After conditioning at 25ºC-53%RH, 8 rectangular samples (25mm x 100mm) were cut per formulation, and the thickness of each specimen was determined at 4 different positions. The films were mounted on film-extension grips and stretched at 50 mm min^{-1} until breakage. The curves of force versus distance obtained in the test curves were transformed into stress (σ) versus Hencky strain (ε_H) curves. The parameters, EM (elastic modulus, MPa), TS (tensile strength at break, MPa), and %E (deformation at the fracture point, expressed in percentage with respect to the initial gage length), were obtained following ASTM standard method D882.

2.3.5. Moisture content, water vapour permeability and oxygen permeability

Once equilibrated, three film samples were taken per formulation to determine the moisture content by means of a gravimetric method. Firstly, the specimens were desiccated in a vacuum oven at 60 ºC for 24h, and secondly, they were stored in cabinets containing P_2O_5 until they reached constant weight. The moisture content (MC) was expressed as the g of water per 100 g of the dried film.

The water vapour permeability of the films was determined at 25ºC and 53-100 RH gradient. The WVP was determined through a modification of the ASTM E96-95 gravimetric method for flexible films (McHugh et al., 1993). Three circular samples were cut (3.5 cm in diameter) per formulation, selecting films without visible defects such as cracks or pores. The thickness of each sample was determined at 6 random positions. Payne permeability cups of 3.5 cm in diameter (Elcometer SPRL, Hermelle/s Argenteau, Belgium) were filled with 5 ml distilled water (RH = 100%). Saturated solutions of magnesium nitrate were used to generate 53% RH inside the cabinets. The shiny side of the films was exposed to the atmosphere at the lowest RH (53% RH at 25ºC). The cups were weighed using an analytical balance (ME36S, Sartorius, Germany) (±0.00001 g) at intervals of 1.5 hours for 24hours. The slope of the weight loss vs. time plot was divided by the exposed film area to calculate the water vapour transmission rate (WVTR). The vapour pressure on the film's inner surface was obtained from equation 10, which corrects the effect of the concentration gradients established in the head space of the cup.

$$\mathrm{WVTR} = \frac{\mathrm{P} \cdot \mathrm{D} \cdot \mathrm{Ln}\left[{(\mathrm{P} - \mathrm{p}_2)}/{(\mathrm{P} - \mathrm{p}_1)} \right]}{\mathrm{R} \cdot \mathrm{T} \cdot \Delta z} \qquad (10)$$

Where: P= total pressure (atm); D= diffusivity of water through air at 25ºC (m^2/s); R= gas law constant ($82.057 \cdot 10^{-3}$ m^3 atm $kmol^{-1}$ K^{-1}); T= absolute temperature (K); Δz= mean height of stagnant air gap (m), considering the initial and final value; p1= water vapour pressure on the solution surface (atm); p2= corrected water vapour pressure on the film's inner surface in the cup (atm).

Finally, WVP was obtained using equation 11, where p_3 is the water vapour pressure on the outer side of the film.

$$\mathrm{WVP} = \frac{\mathrm{WVTR}}{(\mathrm{p}_2 - \mathrm{p}_3)} \cdot \mathrm{thickness} \qquad (11)$$

The oxygen permeability (OP) test was conducted following the ASTM Standard Method D3985–05, (2010). The films were exposed to oxygen and nitrogen flows using the Ox-Tran equipment (Model 1/50, Mocon, Minneapolis, USA) at 25°C-

53% RH. The tests were performed in continuous mode. The OP was calculated by dividing the oxygen transmission rate ($cm^3 \cdot mm\ m^{-2} \cdot day^{-1} \cdot atm^{-1}$) by the difference in oxygen partial pressure between the two sides of the film, and multiplying this by the average film thickness. Two replicates were made per formulation.

2.4. Antioxidant activity

The antioxidant capacity of the active ingredients was determined by means of a spectrophotometric method described by Re et al. (1998). This method is based on comparing the antioxidant activity of the sample with that of a standard antioxidant, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a vitamin E analogue.

ABTS (2,2’-azinobis(3- ethylbenzothiazoline- 6-sulfonic acid) diammonium salt) was dissolved in water at 7 mM and allowed to react with a 2.45 mM potassium persulfate solution (final concentrations) in the dark, for 16 h. During this period, ABTS radical cation ($ABTS^{\bullet+}$), a blue chromophore, was produced. The tests were performed with ethanolic dilutions of the $ABTS^{\bullet+}$

solution, whose absorbance at 734 nm was 0.70 (± 0.02). The antioxidants were diluted in ethanol prior to the determination. Dilutions were made so that the addition of a 10 μl-aliquot to 990 μl of $\text{ABTS}^{\bullet +}$ dilution would produce a decrease in absorbance of 20– 80% within 6 min. Absorbance at 734 nm (A_{734}) was registered every minute during the test. For the calibration, Trolox standard solutions of up to 2mM were prepared and the same procedure was followed. The TEAC of the compounds was determined by comparing the corresponding percentage of absorbance reduction to the Trolox concentration–response curve and expressed as the mass of the compound which produces the same percentage of absorbance reduction as 1mM Trolox solution. All the determinations were carried out three times using a Beckman Coulter DU 730 (England) spectrophotometer, with ethanol as the blank.

2.5. Statistical analysis

The statistical analysis of the data was performed through an analysis of variance (ANOVA) using Statgraphics Plus

(Manugistics Corp., Rockville, MD). Fisher's least significant difference (LSD) procedure was used.

3. Results and discussion

3.1. Microstructure

The microstructure of the films was qualitatively studied, with the aim of observing the effect active ingredients have on the arrangement of the film components. Figure VI.1 shows SEM micrographs of the film surfaces in contact with air during drying and Figure VI.2 shows the cross section images of the cryofractured films. The film without antioxidants (WS:CH) exhibited a compact and ordered structure, without pores, which suggests that wheat starch and chitosan are compatible polymers, able to form a homogeneous structure. The fact that the two polymers are highly compatible was previously observed by Bonilla et al., 2013. The addition of active ingredients, which represent almost 7% of the total film solids, brought about some structural changes, as a function of the nature of the added compound(s). Essential oils were integrated into the polymer matrix to differing degrees. The film with thyme essential oil (WS:CH:Th) did not show a marked phase separation, and it was only on the film surface that coated lipid droplets were exhibited, resulting from the coalescence and creaming of some droplets during the film drying step; in the cross of the film, it was barely possible to observe very small lipid droplets. However, the film with basil essential oil (WS:CH:B) exhibited phase separation and the creaming of the oil was evident on the film surface, while oil droplets could be observed in the cross section of the polymer matrix. The incorporation of citric acid did not lead to a highly heterogeneous starch-chitosan films (WS:CH:CA) structure, although the film surface and cross section appear coarser than for antioxidant-free films. The greatest irregularities were observed in the films with α- tocopherol (WS:CH:Tp), due to its low miscibility with the polymers and its different interactions with each one. In fact, cross section micrographs of these films showed a two-layer separation: a smooth bottom layer, seeming to contain a smaller amount of lipids, and a lipid-rich top layer.

Likewise, big droplets of one polymer phase containing a greater or smaller lipid fraction can be observed in the other. This is especially clear at surface level and could indicate that polymers become more incompatible in the presence of α-tocopherol due to the different interactions of this lipid with the polymer chains. A similar structural effect was observed for corn starch-sodium caseinate blend films when α-tocopherol was added (Jiménez et al. 2013).

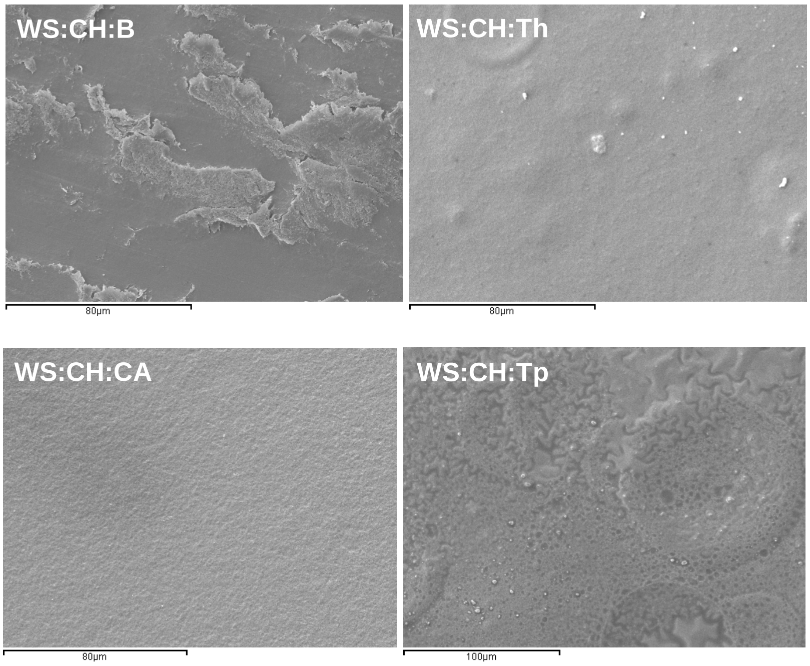

Figure VI.1. Scanning electron microscopy micrographs of the films surface.

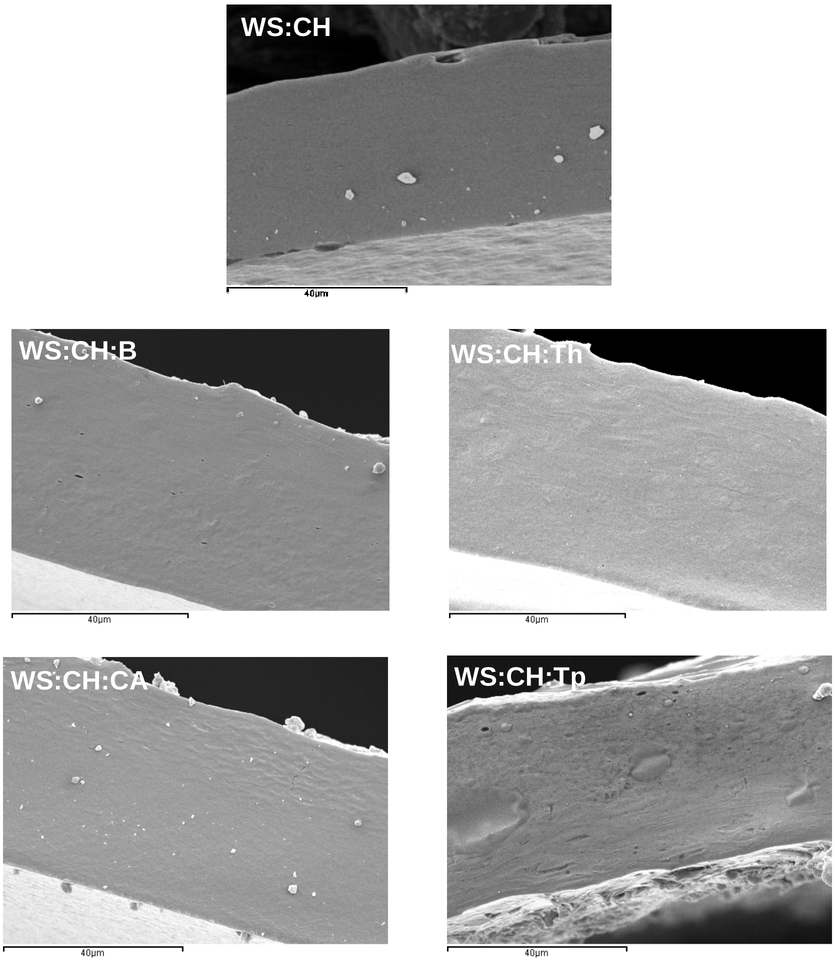

Figure VI.2. Scanning electron microscopy micrographs of the cross-sections of the films.

AFM images were obtained with the aim of quantifying the impact of active ingredients on the surface topography of the films (figure VI.3). The corresponding roughness parameters are shown in table VI.1. The incorporation of the active compounds did not have a relevant effect on the topography of the film surface-and, coherently, the roughness parameters were not affected with the only exception of α-tocopherol. WS:CH:Tp films exhibited a rough surface, with an important increase in all roughness parameters ($p<0.05$), which coincides with the phase previously described phase separation phenomena.

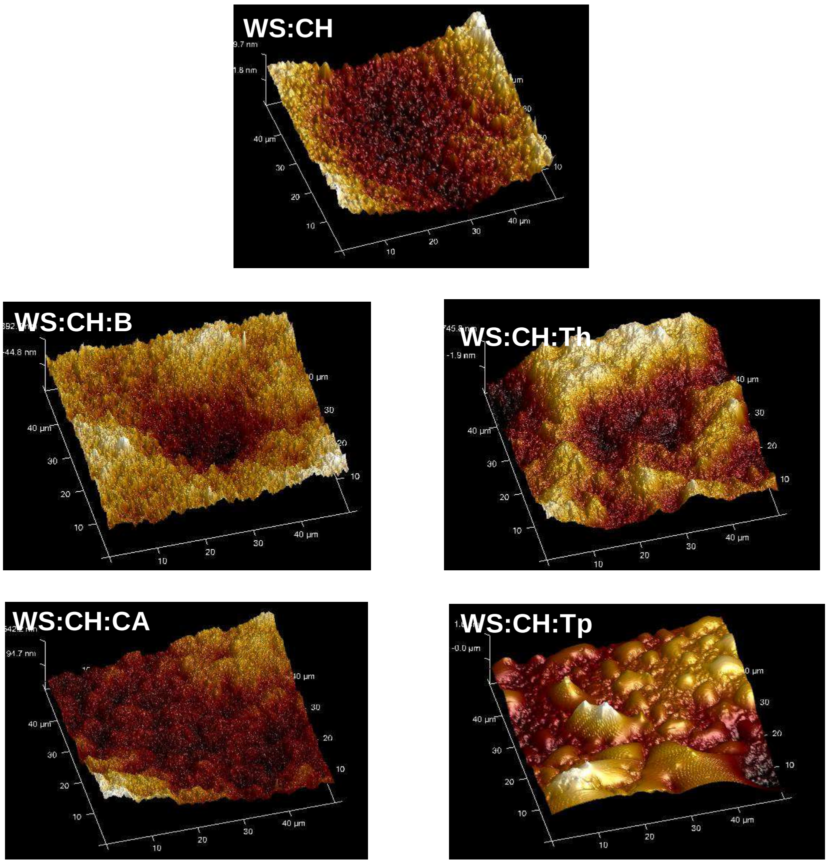

Figure VI.3. Typical AFM images of the surface topography of the films.

Table VI.1. Roughness parameters (R_a, R_q and ISAD) and gloss at 60º of the films. Mean values and standard deviation in brackets.

Samples	R_q (nm)	R_a (nm)	ISAD (%)	Gloss (60°)
WS:CH	147 (41)^{ab}	113 (38)^{ab}	0.8 (0.3)^{a}	51 (16)^{c}
WS:CH:B	109 (35)^{a}	85 (27)^{a}	1.4 (0.2)^{ab}	19 (14)^{a}
WS:CH:Th	212 (42)^{b}	172 (37)^{b}	1.22 (0.07)^{ab}	13 (2)^{a}
WS:CH:CA	109 (23)^{a}	86 (18)^{a}	0.89 (0.03)^{a}	31 (18)^{b}
WS:CH:Tp	486 (125)^{c}	371 (124)^{c}	4 (3)^{b}	13 (3)^{a}

The same superscript (^{abc}) means homogeneous groups in LSD test.

3.2. Optical properties

The film gloss is related to the surface morphology (Sánchez-González et al. 2010) and generally, the smoother their surface, the glossier the films are. The gloss values at 60º are shown in table VI.1. This angle was selected following norm ASTM D523 (1999). A significant reduction in gloss ($p<0.05$) was observed to take place after the addition of every active ingredient, but mainly after those which were lipid in nature. Essential oils and α-tocopherol provoked a reduction in the gloss of the WS:CH films of over 50%, which is coherent with the less miscible nature of these compounds with the polymers and the subsequent effect of the dispersed particles on the film surface. Although AFM did not detect a significantly higher degree of roughness in films with essential oils, this could be due to the low mechanical resistance of the lipid droplets at the surface, which made it impossible to detect during the surface scanning of the probe.

Table VI.2 shows the lightness, chroma and hue of the films. The incorporation of the antioxidants did not affect these variables very markedly. However, the most intense yellow colour and the highest chroma were observed in films containing α-tocopherol. As a consequence, the whiteness index was the lowest in these films.

Table VI.2. Lightness (L*), hue (h^*_{ab}), chroma (C^*_{ab}) and whiteness index (WI) values of the film. Average values and standard deviations in brackets.

Samples	L*	h^*_{ab}	C^*_{ab}	WI
WS:CH	82 (2)^{a}	96.6 (0.6)^{a}	10.3(0.9)^{a}	79 (2)^{ab}
WS:CH:B	88 (3)^{b}	98.2 (0.9)^{a}	11 (5)^{a}	84 (6)^{b}
WS:CH:Th	84.4 (0.4)^{ab}	95.2 (0.2)^{a}	12.8 (0.6)^{ab}	79.8 (0.6)^{ab}
WS:CH:CA	86.9 (0.6)^{b}	97.8 (0.6)^{a}	10.0 (0.4)^{a}	83.5 (0.5)^{b}
WS:CH:Tp	83.0 (0.4)^{a}	89.5 (0.6)^{a}	15.7 (0.9)^{b}	76.8 (0.9)^{a}

The same superscript (^{abc}) means homogeneous groups in LSD test.

Typical spectra of the internal transmittance of the films between 400 and 700 nm are shown in figure VI.4. There were similarities not only between the shape of films with antioxidants, but also between these and that of the WS:CH film, which is coherent with the slight colour differences observed for every film. However, it was found that the antioxidants brought about some increase in the internal transmittance values. This could be explained by the formation of a less compact structure, with a lower refraction index, which, in turn, became less opaque. This is coherent with the measured film thickness (table VI.3) which reveals that thicker films were obtained when antioxidants were present in their formulation, despite the constant amount of solid per surface unit. This effect that the compact nature of the matrix had on the film transparency was partially mitigated in the case of the film containing α-tocopherol, due to the greater light dispersion provoked by the more heterogeneous film structure.

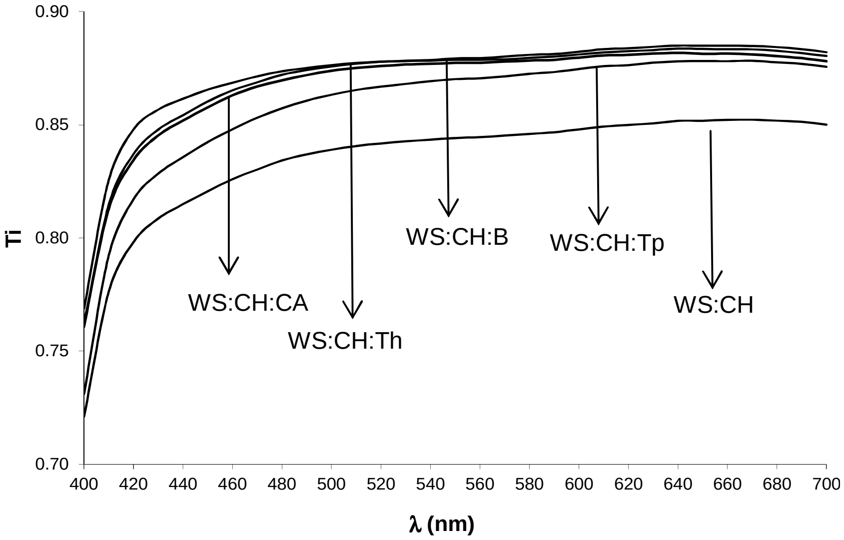

Figure VI.4. Typical spectra of internal transmittance (T_i) of the films.

3.3. Mechanical properties

Mechanical parameters, elastic modulus (EM), and tensile (TS) and deformation (E,%) at break are shown in table VI.3 for every film obtained. The mechanical behaviour of WS:CH films is similar to that reported by Bonilla et al. (2013) for films of similar composition. The incorporation of active ingredients had variable effects on the mechanical parameters, depending on their specific nature and effects on the film microstructure. Thyme essential oil did not significantly affect the mechanical response of the films, apart from a slight decrease in stretchability. The films containing basil essential oil or α-tocopherol showed some increase in EM and TS, as compared to WS:CH films, with no effect on the elongation at break. On the other hand, the addition of citric acid led to an increase in the elastic modulus and some reduction in film resistance and break and extensibility. This may be due to a cross-linking effect with the starch molecules, as previously described by Reddy & Yang, (2010).

Table VI.3. Tensile properties (Elastic modulus: EM, tensile strength: TS and deformation: E %, at break) and thickness of all films equilibrated at 58% RH. Mean values and standard deviations in brackets.

Samples	EM (MPa)	TS (MPa)	E (%)	Thickness (μm)
WS:CH	1458 (151)^{a}	44 (3)^{b}	4.3 (0.8)^{b}	52.7 (1.6)^{a}
WS:CH:B	1712 (46)^{b}	54.6 (1.2)^{c}	4.3 (0.6)^{b}	57 (5)^{b}
WS:CH:Th	1450 (201)^{a}	43 (7)^{b}	3.6 (0.6)^{a}	55 (11)^{b}
WS:CH:CA	1418 (605)^{a}	34 (12)^{a}	3.0 (1.0)^{a}	52 (8)^{a}
WS:CH:Tp	1598 (106)^{b}	47 (10)^{b}	4.4 (1.4)^{b}	58 (5)^{b}

The same superscript (^{abc}) means homogeneous groups in LSD test.

3.4. Moisture content and barrier properties.

The equilibrium moisture content of the films stored at 53%RH-25ºC is shown in table VI.4, together with the water vapour permeability (WVP) and oxygen permeability (OP) values. Film composition was observed to have no notable effect on the equilibrium moisture content, probably due to the relatively low level (6.7 % of total solids) of hydrophobic compound incorporated.

WVP was barely affected by the incorporation of active compounds. Although the incorporation of α-tocopherol or citric acid led to some WVP reduction, essential oils had no significant impact. In the case of citric acid, the above-mentioned cross-linking effect may explain what can be observed, since a more closed matrix is formed, thus inhibiting the transfer of water molecules. The polymer phase separation induced in the film containing α-tocopherol, which enhances the tortuosity index for mass transport in the matrix (Perez-Gagó & Krochta, 2001) may also explain the observed decrease in the WVP values.

Oxygen permeability values at 25ºC-53%RH are also shown in table VI.4. The incorporation of antioxidants caused a significant reduction (of nearly 50%) of OP in every case, as compared to WS:CH films. A chemical oxygen blocking effect produced by these compounds could be responsible for this improvement in the oxygen barrier properties, as has been previously observed for corn-starch-sodium caseinate films containing α-tocopherol (Jimenez et al. 2013).

Table VI.4. Moisture content (%MC, g water/100g dry film) of films equilibrated at 25ºC-53% RH. Water vapor permeability (WVP, g mm $kPa^{-1}h^{-1}m^{-2}$) of the films at 58–100% RH and 25ºC and Oxygen permeability (OP, $cm^3mm^{-2}atm^{-1}day^{-1}$) at 25ºC-53% RH. Mean values and standard deviation in brackets.

Samples	WVPx10^{10}	OPx10^2	%MC
WS:CH	18 (2)^a	6.7 (0.7)^a	8.4 (0.5)^a
WS:CH:B	19.1 (1.4)^a	3.7 (1.0)^b	8.67(0.13)^a
WS:CH:Th	18.2 (0.5)^a	3.2 (0.2)^b	8.62(0.16)^a
WS:CH:CA	15.6 (1.0)^b	3.8 (0.7)^b	8.8 (1.3)^a
WS:CH:Tp	15.6 (1.3)^b	3.8 (0.7)^b	8.2 (0.3)^a

The same superscript (^{abc}) means homogeneous groups in LSD test.

3.5. Antioxidant activity

Table VI.5 shows the TEAC of the essential oils, citric acid and α-tocopherol, defined as the amount (mg) of the ingredient that produces the same absorbance reduction as 1mM Trolox solution. Of all the antioxidants, the lowest antioxidant capacity was found for basil essential oil and citric acid, since the greatest amounts of these are necessary to produce the same percentage of absorbance reduction as 10 μl of 1mM Trolox solution. Thyme essential oil exhibited a slightly higher antioxidant power, and α-tocopherol was the most effective antioxidant, as revealed by the test. The expected antioxidant capacity of the films will be related with that obtained for the incorporated compounds, although their possible oxidation during the film formation and the interactions with the film matrix may also contribute to this capacity.

Table VI.5. Trolox equivalent antioxidant capacity (TEAC) of the incorporated antioxidant compounds, expressed as the amount (mg) of the compound which gives the same absorbance reduction as 1mM Trolox solution.

Ingredient	TEAC (mg)
B	1.933
Th	1.575
CA	1.933
Tp	0.538

CONCLUSIONS

The incorporation of the antioxidants (basil and thyme essential oils, citric acid and α-tocopherol) at 6.7 % in wheat starch:chitosan (4:1) films gave rise to microstructural changes that were depend on the different affinity of components. The greatest structural change was induced by α-tocopherol which promoted polymer phase separation in the film. All the antioxidants provoked a loss in film gloss, but a gain in transparency, while α-tocopherol induced yellowness and a reduction of the whiteness index. Although the mechanical properties of the films were barely affected by the antioxidants, citric acid promoted an increase in the elastic modulus but a decrease in the tensile and deformation at break, probably due to cross-linking with starch chains. Although the water vapour barrier properties of the films were only slightly improved when citric acid and α-tocopherol were added, the oxygen barrier properties of the films were significantly improved in every case, probably due to a chemical oxygen blocking effect. The greatest antioxidant capacity of the films was expected for films containing α-tocopherol, which showed the highest antioxidant power of all the tested compounds.

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PHYSICAL, STRUCTURAL AND ANTIMICROBIAL PROPERTIES OF POLY VINYL ALCOHOL– CHITOSAN BIODEGRADABLE FILMS

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Food Hydrocolloids

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Abstract

Chitosan (CH) was blended with poly vinyl alcohol (PVA) in different compositions to obtain biodegradable films (PVA90:CH10, PVA80:CH20, PVA70:CH30). Blends and neat polymer films (PVA100, CH100) were characterized for their thermal behavior, structural, mechanical and barrier properties as well as antimicrobial activity. Both polymers showed good compatibility, as demonstrated by FESEM images and thermal behavior. A reduction in crystallinity of the blend was observed as the chitosan content was increased. Moreover, chitosan addition strongly reduced the film stretchability while increased the film rigidity and resistance to fracture, mainly at 70:30 PVA:CH ratio. Additional advantages of chitosan incorporation to PVA films are the reduction of the UV-transmittance while providing antimicrobial properties.

Keywords: Bio-nanocomposites; Chitosan; Poly vinyl alcohol; barrier properties.

1. Introduction

Several biopolymers have been used to develop eco-friendly food packaging materials. Usually, films based on biopolymers are highly sensitive to environmental conditions and generally present low mechanical resistance. As a result, several studies have been carried out to develop films based on mixtures of biopolymers and synthetic polymers (Kanatt et al., 2012). Environmentally degradable plastics have attracted growing attention because of their potential use in the replacement of traditional non-degradable plastic items deriving from fossil fuel feed stocks. Poly vinyl alcohol (PVA) has been widely utilized for the preparation of blends and composites with several natural, renewable polymers (Chiellini et al., 2003). PVA is a semicrystalline polymer, extensively studied because of its interesting physical properties, which arise from the presence of OH groups and the hydrogen bond formation (Abdelrazek et al., 2010). It is a water-soluble synthetic polymer and it has good biodegradability, excellent chemical resistance and good mechanical properties (Chen et al., 2008).

Antimicrobial packaging is one of the most promising active packaging systems. The use of such packaging is not meant to be a substitute for good sanitation practices, but it should enhance the safety of food as an additional hurdle for the growth of pathogenic and/or spoilage microorganisms (Trypathi et al., 2009). Chitosan (CH) is a cationic polysaccharide with excellent film forming properties. It is obtained from chitin by deacetylation in the presence of alkali (Bonilla et al., 2012). Chitosan films have a great potential to be used as active packaging material due to its antimicrobial activity, non-toxicity and low permeability to oxygen (Kanatt et al., 2012). The positive charge of chitosan affords the polymer numerous and unique physiological and biological properties (Honary et al., 2011). Several studies have demonstrated the effect of molecular weight and concentration of chitosan on antibacterial and antifungal activities (Honary et al., 2011; Sánchez-González et al. 2011). Since chitosan contains hydroxyl and amine groups, it is potentially miscible with PVA due to the formation of hydrogen bonds (Arvanitoyannis et al., 1997; Chen et al., 2008; Chiellini et al., 2003). Polymer blending is one of the most effective methods to have new material with desired properties. Films formed by blending of polymers usually show modified physical and mechanical properties as compared to films made of individual components. Since synthetic polymers are easily obtained and have low production cost, blending of natural (such as CH) and synthetic (such as PVA) polymers may improve the cost performance ratio of the resulting films (Bahrami et al., 2003).

Several studies have been carried out on the properties of PVA-CH blends, describing their diverse biomedical applications (Zhuang et al., 2012; Ionita and Iovu, 2012; Sundaramurthi et al., 2012; Gholipour-Kanani et al., 2012) and their bacteriostatic effects (Li et al., 2012). Nevertheless, PVA-CH blend films have not been characterized as to their mechanical and barrier properties under conditions normally used in food packaging (such as low and intermediate relative humidity), nor in their antimicrobial properties against foodborn microorganisms.

The aim of this work was to study the physical, antimicrobial and barrier properties of PVA films containing chitosan in different ratios. The films were characterized in their thermal behavior, structural and mechanical properties and in their water vapor barrier properties. Their protective ability against microbial growth was evaluated aiming to a potential use as antimicrobial packaging material.

2. Materials and methods

2.1. Reagents and raw materials

Poly vinyl alcohol (PVA) (Mw: 89,000-98,000, degree of hydrolyzing > 99%, viscosity: 11.6-15.4 cPs) and high molecular weight chitosan (CH) (practical grade, Batch MKBB0585, degree of deacetylation > 77%, viscosity: 1220 cPs), were supplied by Sigma-Aldrich Quimica (Madrid, Spain). Glacial acetic acid and $Mg(NO_3)_2$ were provided from Panreac Química, S.A (Castellar del Vallés, Barcelona, Spain). Millipore water was used to prepare the film-forming dispersions (FFD).

For the microbiology study, pork meat was purchased in a local supermarket and processed at the laboratory. Tryptone Phosphate Water (peptone buffered water), Violet Red Bile Agar (VRBA) and Plate Count Agar (PCA) were provided by Scharlau Microbiology (Barcelona, Spain).

2.2. Preparation of the film-forming dispersions and casting of the films

PVA was diluted in millipore water at 1.5% (w/w) under magnetic stirring at 90°C for 3h. Chitosan (0.15% w/w) was dissolved in water with glacial acetic acid (1% v/v) under magnetic stirring at 40°C for 12h. The film-forming dispersions (FFD) were obtained by mixing PVA and CH solutions at different mass ratios, where part of the PVA was gradually replaced by CH, up to 30%. Formulations were named as follows, the numbers indicating the PVA:CH mass ratio: PVA100, PVA90:CH10, PVA80:CH20, PVA70:CH30 and CH100. All film-forming dispersions were prepared by using a ultrasound homogenizer at 40% of amplitude for 5 min, at room temperature. The FFD, were cast in framed and leveled polytetrafluorethylene (PTFE) petri dishes (9cm diameter, 15g of FFD per plate) and dried in an oven at 40°C for 24-48h. The films were peeled off from the casting plates and conditioned at 58% RH and room temperature in chambers containing saturated solutions of $Mg(NO_3)_2$ prior to all analyses.

2.3. Characterization of the films

2.3.1. Film thickness

The film thickness was measured using a digital micrometer to the nearest 0.001 mm. Six random positions in each film sample were considered.

2.3.2. Microstructure

Microstructure of the cross-sections of the films was obtained by Field emission scanning electron microscope, FESEM, using a Supra 25-Zeiss electron microscope. Film specimens were cryofractured by immersion in liquid nitrogen and mounted on copper stubs perpendicularly to their surface. Samples were gold coated and observed, using an accelerating voltage of 5 kV.

2.3.3. Fourier transform infrared spectroscopy (FTIR)

FTIR spectroscopy, based on the identification of absorption bands concerned with the vibrations of functional groups present in PVA and CH macromolecules, was used to analyze the chain interactions in blend films. Jasco FT-IR 615 spectrometer was used to record the FTIR spectra between wave numbers 400 and 4000cm^{-1}. The transmission mode was used, and the resolution was 1 cm^{-1}. All tests were performed at room temperature.

2.3.4. Ultraviolet-visible spectrophotometry (UV-VIS)

UV-Vis absorption spectra of the samples (1x1 cm^2) were recorded in the wavelength range of 250 to 900 nm using a Perkin Elmer Instruments (Lambda 35) UV-Vis spectrophotometer.

2.3.5. Tensile properties

According to UNI ISO 527-1 (ISO, 2012), a Universal Testing Machine (digital Lloyd instrument) was used to obtain the true stress-Hencky strain curves for the tensile tests of the films. From these curves, Young modulus (Eyoung), tensile strength (TS) and elongation at break (%E) of the films were evaluated. Rectangular samples (1 x 5 cm^2) were cut and stored at room temperature in cabinets containing $Mg(NO_3)_2$ saturated solution (58% RH). Equilibrated film samples were mounted in the film-extension grips and stretched at 50 mm min^{-1} until breakage, with a crosshead speed of 5mm min^{-1} and a load cell of 500 N. The measurements were done at room temperature and six samples for each formulation were tested.

2.3.6. Water vapour permeability (WVP)

Water vapour permeability (WVP) was determined at 10°C and 53–100% RH gradient using a modification of the ASTM E96-95 gravimetric method (1995) for flexible films (Mc Hugh et al., 1993). Round samples were cut (three per formulation) and four random thickness measurements were taken per sample. Payne permeability cups of 3.5cm in diameter (Elcometer SPRL, Hermelle/s Argenteau, Belgium) were filled with 5 ml distilled water (RH = 100%). Once the films were secured, each cup was placed in a pre-equilibrated cabinet fitted with a fan to provide a strong driving force across the film for water vapour diffusion. The RH of the cabinet was held constant at 53% using $Mg(NO_3)_2$ over-saturated solution. The shiny side of the films was exposed to the atmosphere at the lowest RH, and the cabinets were stored at the mentioned temperature. The cups were weighed periodically (±0.00001g) after steady state was reached. The slope of the weight loss vs. time plot was divided by the exposed film area to calculate the water vapour transmission rate (WVTR). The vapour pressure on the film inner surface was obtained by means of the method proposed by Mc Hugh et al. (1993) in order to correct the effect of concentration gradients established in the stagnant air gap inside the cup.

2.3.7. Thermogravimetric analysis (TGA)

A thermogravimetric analyzer (TGA, Seiko Exstar 6300) was utilized to measure the thermal weight loss (TG) and derivate (DTG) of the PVA100, CH100 and PVA:CH blend films in the temperature range between 30°C and 900°C, with a heating rate of 10°C/min under nitrogen stream. Maximum thermal degradation temperature (Tmax) was evaluated.

2.3.8. Differential scanning calorimetry (DSC)

Film samples of 2-5 mg were tested by using a differential scanning calorimeter (DSC, Mettler Toledo 822/e8). Measurements were carried out under nitrogen flow in the temperature range from -25°C to 220°C. After a first heating step, cooling was performed at 10°C/min, and finally a second heating step at the same rate. Data were recorded both during the cooling and second heating steps. The glass transition temperature (T_g) was taken as the inflection point of the specific heat increment at the glass–rubber transition while the melting temperature (T_m) and the crystallization temperature (T_c) was taken as the peak temperature of the endotherm and exothermic, during the cooling and the heating, respectively. Three samples were used to characterize each material. The cristallinity degree was calculated as:

$$\chi = \frac{1}{(1-m_f)}\left[\frac{\Delta H}{\Delta H_0}\right]100 \qquad \text{(Eq. 1)}$$

Where ΔH, is the enthalpy for melting or crystallization, ΔH_0 is enthalpy of melting for a 100% crystalline PVA sample, taken as 156 J/g (Tubbs, 1965), and (1-) is the weight fraction of PVA in the sample.

2.3.9. Antimicrobial properties

Three of the film formulations (PVA100, PVA80:C20 and CH100) were tested in their antibacterial properties using minced pork meat. The meat was ground by using a mincer (Severin Elektrogeräte GmbH, Sundern, Germany) and 10g of minced meat were molded into hamburgers (5cm diameter). The surface of the minced meat samples was coated with the stand-alone films that were previously prepared by casting, as described in section 2.2. Duplicates of the non-coated (control) and coated samples were placed in uncovered petri dishes and stored at 10ºC. To perform microbiological analyses, 10g of each sample were aseptically obtained and homogenized in a Stomacher with 90mL of sterile buffered peptone water for 2min. Aliquots were serially diluted in buffered peptone water and plated out following standard methodologies. Total viable and coliform microorganism counts were determined at 0 and 7 days. For the viable counts, Plate Count Agar plates were incubated at 37ºC for 24h. Coliform counts were determined using Violet Red Bile Agar plates incubated at 37ºC for 48h.

2.4. Statistical analysis

The statistical analysis of the data was performed through an analysis of variance (ANOVA) using Statgraphics Plus (Manugistics Corp., Rockville, MD) Fisher's least significant difference (LSD) procedure was used.

3. Results and discussion

3.1 Film microstructure

Table VII.1 shows the thickness of the films after equilibration at 58% RH. CH100 films were the thinnest (39µm in average), which is directly linked to their lowest solid surface density (8 g/m^2) and PVA100 films were much thicker, due to the much higher solid surface density (80 g/m^2).

In the blends, the films thickness is controlled by the PVA fraction. However, the thickness of blend films increase with the chitosan content due to the wider hydration layers of the charged chitosan chains. Bonilla et al., (2013) found an increase in the film thickness when the CH ratio increased in starch-CH blend films, which was attributed to the enhancement of the water bonding capacity of the films when they contain CH. Moreover, previous studies reported the water sorption properties of PVA-CH blend films and concluded that, as the chitosan proportion increased in the films, their water bonding capacity increased (Ludwiczak and Mucha, 2010; Srinivasa et al., 2003).

The final microstructure of the films results from the interactions between the polymers in the blend. Their microstructure was qualitatively analyzed through the FESEM images. Characteristic micrographs of the film cross sections of all formulations are shown in Fig. VII.1, where a continuous, homogeneous phase in both the pure polymer films and the blends can be observed, without any evidence of phase separation. Moreover, no irregularities, such as air bubbles, pores, cracks or droplets were detected. This indicates the high compatibility of the two polymers, such as has been previously observed by other authors (Trypathi et al., 2009).

Table VII.1. Thickness, tensile properties (E_{Young}, TS and E%) and WVP of the films. Average values and standard deviations, in brackets. The same superscript means homogeneous group in LSD test.

Samples	Thickness (μm)	E_{Young} (MPa)	TS (MPa)	E (%)	WVP (g/s*m*Pa) x 10^{10}
PVA100	0.25 (0.05)^{a}	263 (35)^{a}	23 (3)^{ab}	265 (17)^{a}	12 (2)^{a}
PVA90:CH10	0.19 (0.04)^{b}	172 (15)^{a}	28 (4)^{b}	173 (20)^{b}	19 (3)^{b}
PVA80:CH20	0.24 (0.03)^{a}	1390 (68)^{b}	43 (5)^{c}	81 (6)^{c}	12 (2)^{a}
PVA70:CH30	0.31 (0.04)^{c}	1090 (30)^{c}	24 (2)^{ab}	32 (7)^{d}	21.5 (1.3)^{b}
CH100	0.039 (0.002)^{d}	533 (15)^{d}	20 (7)^{a}	6 (2)^{e}	6.4 (0.9)^{c}

Different letters indicate significant differences ($p<0.05$) due to film formulation (a, b, c, d).

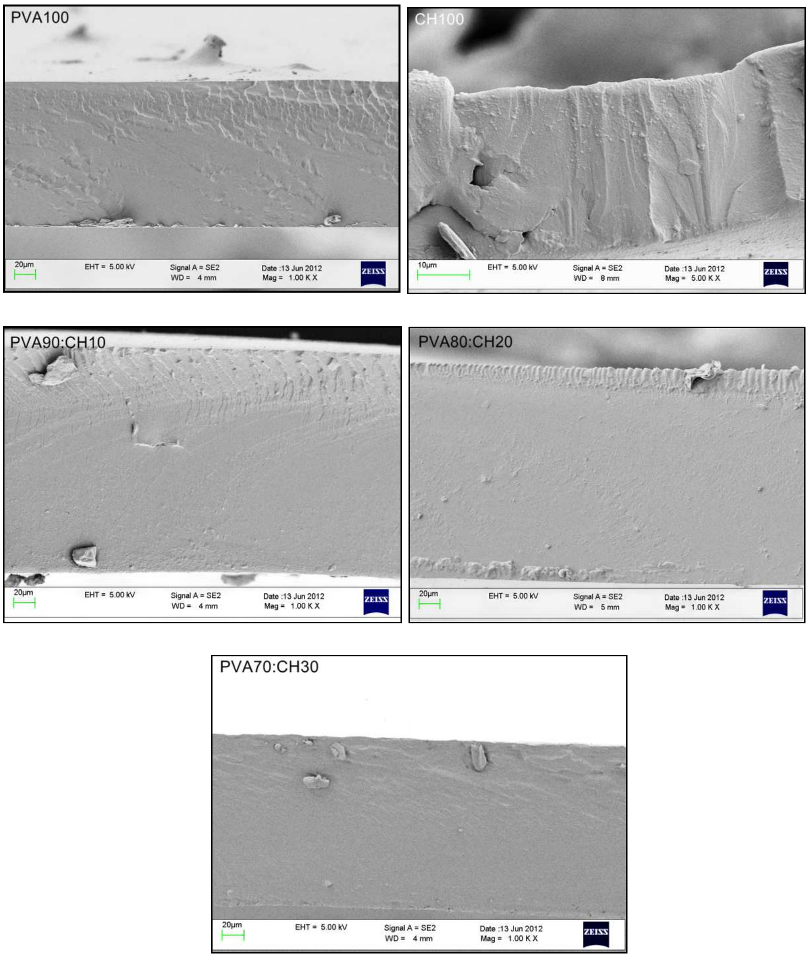

Figure VII.1. Field emission scanning electron microscopy (FESEM) images of cross-sections of the films. PVA: Polyvinyl alcohol, CH: chitosan.

3.2 Film chemical and transparency properties

Since the molecules of both CH and PVA are capable of forming hydrogen bonds, it is expected that some specific interactions could be formed between the different molecular groups, thus affecting the sample spectra. FTIR spectra of pure PVA, pure CH and PVA:CH films are shown in Fig VII.2a. For pure PVA, the band at about 3455cm^{-1} is assigned to –OH stretching (Abdelrazek et al., 2010; Costa-Junior et al., 2009a), and that at 1430cm^{-1} corresponds to OH bending vibration of hydroxyl group (Abdelrazek et al., 2010; Kumar et al. 2010). The band corresponding to methylene group (CH_2) asymmetric stretching vibration occurs at about 2933cm^{-1} (Costa-Junior et al., 2009a; Abdelrazek et al., 2010; Kumar et al. 2010). The vibrational band at about 1650-1700cm^{-1} corresponds to C=C stretching of PVA (Abdelrazek et al., 2010; Costa-Junior et al. 2009 a). The band at about 1100cm^{-1} corresponds to C-O stretching (Abdelrazek et al., 2010; Kumar et al. 2010; Buraidah and Arof, 2011), and that at about 1760cm^{-1} to C=O stretching of acetyl groups present on the PVA backbone (Abdelrazek et al., 2010; Costa-Junior et al. 2009; Buraidah and Arof, 2011).

For pure chitosan, the main characteristic bands are assigned to saccharide structures at 890, 1020 and 1150cm^{-1} and strong amino characteristic bands at around 3430, 1660 and 1560cm^{-1} assigned to –OH stretching, amide I and amide II bands, respectively (Costa-Junior et al., 2009a, Abdelrazek et al., 2010, Kumar et al., 2010, Buraidah and Arof, 2011). According to Kumar et al. (2010), the peak at about 1250cm-1 corresponds to amino group.

For the spectra of the PVA:CH blends, as the chitosan content was increased a clear decrease in the intensity of the band around 3380cm^{-1} was observed. This may be attributed to a hydroxyl group (-OH) stretching vibration of PVA with a secondary amine (-NH) of chitosan (Abdelrazek et al., 2010, Costa-Junior et al., 2009a). According to Kumar et al. (2010), this absorption peak, concerned with –OH and –NH stretching vibrations, shifted to a lower wave number with the increase of PVA content in the blends. Compared with FT-IR spectra of pure CH, the absorption peak of PVA:CH at 1250cm^{-1} disappeared. The band at around 1077cm^{-1} indicates the presence of a hydroxyl group (-OH) with polymeric association and a secondary amine (-NH) (Abdelrazek et al., 2010).

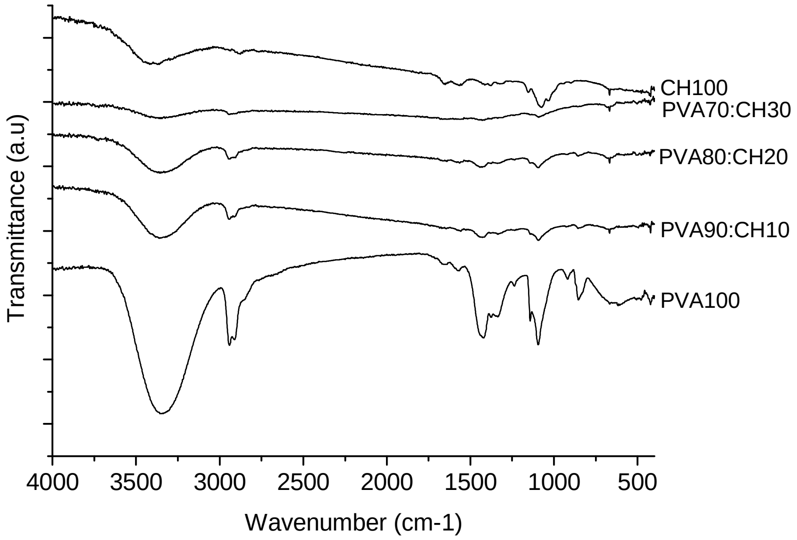

Figure VII.2a. FTIR spectra for pure PVA, pure CH and PVA:CH blend films.

UV-VIS spectra of PVA, and PVA:CH films are shown in Fig VII.2b. The films showed an absorption band at about 273-280nm which comes from unsaturated bonds mainly C-O (Abdelrazek et al., 2010), which increased in absorption intensity when CH ratio increased. This reflects the promotion of the interactions between CH and PVA chains in the film.

From the practical point of view, it is remarkable that blend films have better barrier to ultraviolet light. One of the desired characteristics of packaging material is that it should protect food from the effects of light, especially UV radiation (Kanatt et al., 2012). Amongst the blend films, PVA70:CH30 exhibited the lowest UV light transmission still maintaining the well transparent/white color of the original polymers. The low levels of transmission in the UV range can make PVA-CH based films an excellent barrier to prevent UV light-induced lipid oxidation when applied in food systems.

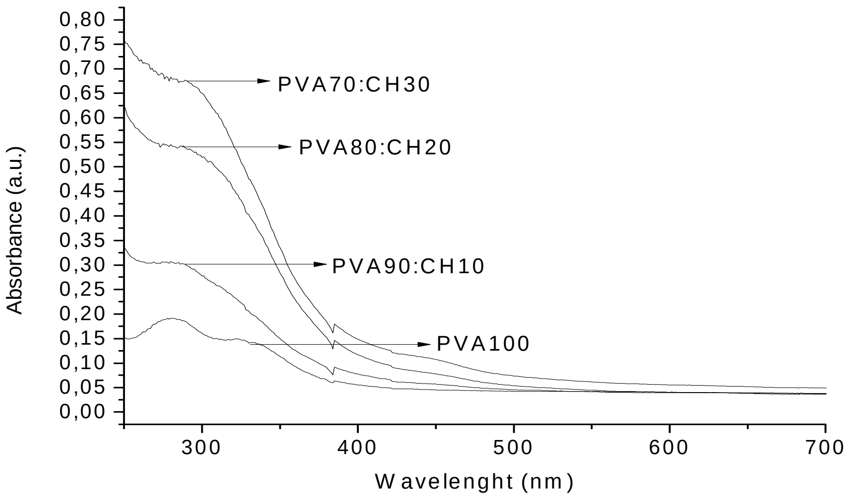

Figure VII.2b. UV-VIS spectra for pure PVA and PVA:CH blend films.

3.4 Tensile properties

Films for food packaging are required to maintain their integrity in order to withstand the stress that occurs during shipping, handling and storage (Kanatt et al., 2012). Typical true stress-Hencky strain curves obtained from the tensile tests are shown in Figure VII.3. Chitosan films proved to be the least stretchable, as opposed to those of PVA. Blend films showed a marked plastic deformation which greatly decreased as the chitosan ratio increased.

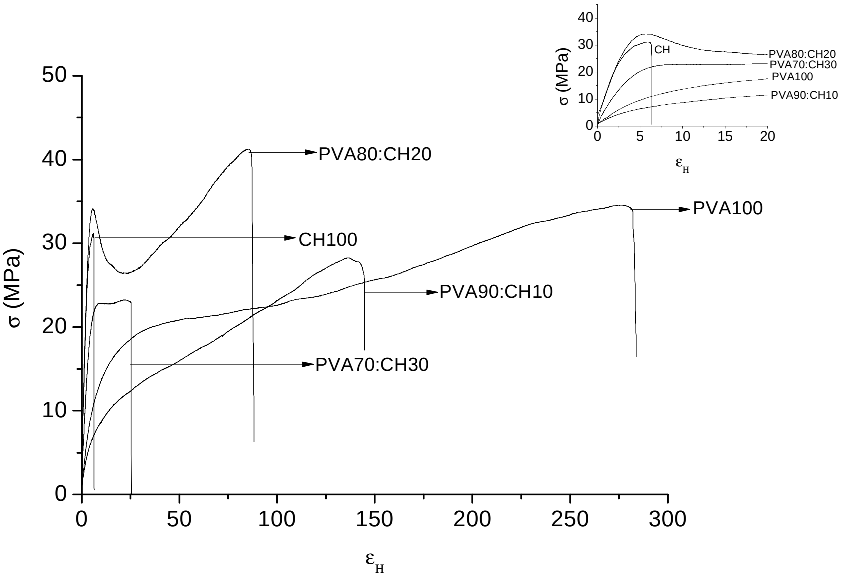

Figure VII.3. Typical true stress (σ) vs. Hencky strain (ε_H) curves obtained in tensile tests carried out on pure PVA, pure CH and PVA:CH blend films.

The tensile properties, elastic modulus (E), tensile strength at fracture (TS) and percentage of elongation at fracture (%E) of the films are shown in Table VII.1. PVA100 and CH100 showed similar TS values, comparable to those reported by other authors for pure chitosan films (Chen and Hwa, 1996) and pure PVA films (Costa-Junior et al., 2009b). As the chitosan ratio increased in the film up to 20%, the TS showed a significant increase ($p<0.05$). The same trend was observed by other authors working with blend films of PVA:CH, who reported the good mechanical properties of the PVA:CH blend (Kanatt et al., 2012; Bahrami et al., 2003). This behavior suggests the establishment of bonds between the chains of both polymers in the blends that reinforce the network structure, thus improving the mechanical strength of the films. Bahrami et al., 2003, suggested that the interaction between -OH groups of PVA with the -NH2 groups of CH, is able to improve the mechanical properties of the blend films.

Elongation at break (%E), which is determined at the point where the film breaks under tensile testing, gives information about the film flexibility and stretchability. The elongation at break of CH100 films was similar to that found by Bonilla et al. (2012), whereas that found for PVA100 films was comparable to that reported by Costa-Junior (2009)b. Coherently with the different behavior of the two pure polymers, the elongation at break of the PVA:CH blend films greatly decreased when the CH content increased (Table VII.1), which agrees with that reported by Bahrami et al. (2003) for PVA:CH blend films. So, the PVA:CH blend films, specially with ratio 80:20, are stronger and more resistant to fracture than the pure polymer films, although their extensibility are reduced considerably as compared with PVA films. This behavior agrees with the formation of inter-chain bonds that reinforce cohesion of the polymer network but limit the slippage of polymer chains during the tensile test, thus reducing the film stretchability.

3.5 Water vapor permeability (WVP)

WVP results are shown in Table VII.1. Water vapor is one of the main permeants studied in food packaging applications, because it may transfer from the internal or external environment through the polymer package wall, resulting in product quality and shelf-life changes (Kanatt et al., 2012). The WVP of pure chitosan films was similar to the reported by Bonilla et al. (2012) ($6 \cdot 10^{10}$ g $s^{-1}m^{-1}Pa^{-1}$), and that of pure PVA films was comparable to that obtained by Jipa et al. (2012) ($17 \cdot 10^{10}$ g $s^{-1}m^{-1}Pa^{-1}$). WVP of PVA was significantly ($p<0.05$) higher than that of CH, coherently with the highly hydrophilic nature of PVA (Hassimi et al., 2008). Nevertheless, the incorporation of CH to the PVA films did not provoke any reduction of the WVP values, but a significant increase, except for the ratio 80:20 where no change was induced. This can be to the formation of a more open matrix where polymer chains are less densely packed due to the wider hydration layers of CH, as can be also deduced from the values of the film thickness. In this situation, transfer of water molecules through the network is facilitated. The formation of a reasonably high number of OH-NH2 bonds between the two polymers in the films with 80:20 PVA:CH ratio, as can be deduced from their mechanical behavior, can contribute to limit the transfer rate of water molecules and so the WVP. Nevertheless, the water barrier properties of PVA films were not improved in any case by the CH addition.

3.6 Thermal behavior

TGA is considered as the most important method for studying the thermal stability of polymers (Abdelrazek et al., 2010). The weight loss (TG) and derivate (DTG) curves for the TGA tests of PVA100, CH100 and PVA:CH blend films are reported in Figure VII.4. Two significant weight loss stages are observed in the TG curve of CH100 film. The small weight loss at 50–120°C (about 10%) is probably due to the loss of adsorbed and bound water and the residual acetic acid. The second weight loss step (50-55%) at 170–350°C is attributed to the thermal degradation of CH, as previously reported by several authors (Trypathi et al., 2009, Chen et al., 2008, Lewandowska et al., 2009).

On the other hand, the thermal behavior of PVA and PVA:CH films proceeds in three stages. The first weight loss (about 10%) at 100–170°C is due to the moisture vaporization (Lewandowska et al., 2009). The second weight loss step at 200–300°C is due to the thermal degradation of PVA and CH

(Trypathi et al., 2009, Chen et al., 2008). One third weight loss step at 380–500°C occurs due to the degradation of the by-products generated by PVA during its thermal degradation process (Chen et al., 2008; Lewandowska et al., 2009). According to Holland and Hay (2001), thermal degradation resulted in the formation of aldehyde and alkene end-groups in the molten state. In the range between 200 and 300ºC, chitosan showed more thermal stability (lower weight loss) than PVA. Consequently, as the chitosan ratio increased in the blend, the residual mass increased from 15% to 38%. The temperature at the maximum decomposition rate (T_{max}) was determined (Table VII.2), and it was found that chitosan addition resulted in a T_{max} increase of the blends, which indicates that the PVA films showed higher thermal stability due to the chitosan addition. Similar results were observed by Lewandowska (2009) and Peesan et al. (2003).

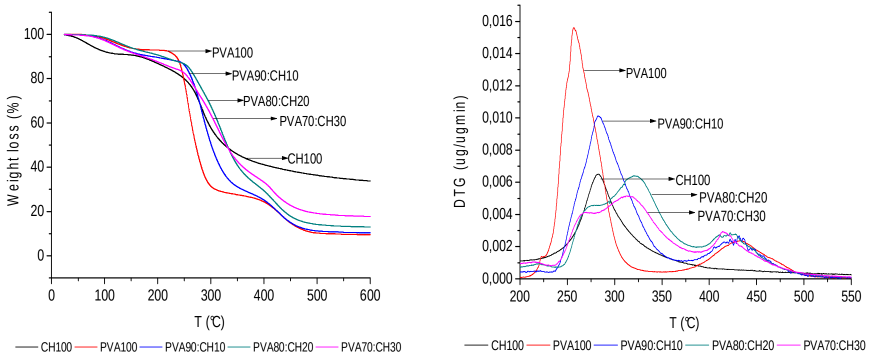

Figure VII.4. a) TG and b) DTG typical curves of pure PVA, pure CH and PVA:CH blend films.

The thermal behavior of chitosan, and especially its glass transition temperature (T_g), has been subject of controversy. Apparently, variables such as the source or the extraction method strongly influence T_g (Neto et al., 2005). Sakurai et al. (2000) observed the T_g of chitosan at 203ºC, while Kittur et al (2002) found no evidence for T_g, suggesting that T_g for chitosan could lie at a higher temperature, where degradation prevents its determination. According to Trypathi (2009), DSC thermogram of chitosan exhibited a sharp exothermic peak at 290°C, associated with the decomposition of chitosan. In the present work, chitosan did not show any significant transition in the temperature range of the DSC scans. Figure VII.5 shows the DSC curves obtained for PVA100 and PVA:CH blend films.

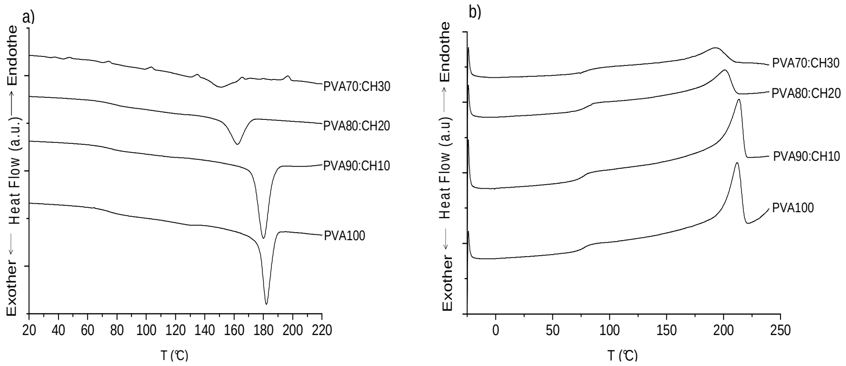

Figure VII.5. DSC thermograms a) Cooling scan and b) Second heating scan for pure PVA and PVA:CH blend films.

The thermal parameters obtained from the second heating scan (T_g, T_m, and ΔH_m), for both blend films and PVA, are shown in Table VII.2. T_g values of the blends showed some increase as the chitosan content increased. This agrees with the polymer interactions through the hydrogen bond formation, which implies an effective increase in the mean molecular weight and so in the T_g values. The mixture effect of compatible polymers on T_g implies that its values are intermediate between the respective values of the pure polymers. In this sense, and in agreement with those reported values for CH, the obtained increase in T_g values in the blends also points to the good miscibility of the both macromolecules. On the other hand, a notable depression of T_m value as the CH content increased ($p<0.05$) was found, which points to the good miscibility of the polymers that affect crystallization process of PVA. This reduction of the crystallization or melting temperature is considered as a measure of the blend compatibility (Lewandowska et al., 2009; Kumar et al., 2010).

The calculated percentage of crystallinity is also shown in Table VII.2, where the decrease in crystallinity of PVA when the CH ratio increased in the blend can be observed. This result agrees with the WVP studies for PVA:CH blens and underlines the miscibility of polymers and the increase in the steric hindrance of PVA chains to reorient in crystalline zones.

Table VII.2. Thermal characteristics of PVA, CH and PVA:CH blend films.

Thermal characteristics of PVA, CH and PVA-CH blend films					
	Second Heating Scan				
Samples	**T_g (°C)**	**T_m (°C)**	**ΔH_m (J/g)**	**X (%)**	**T_{max}(°C)**
PVA100	69 (4)^a	221 (2)^a	55.4 (1.2)^a	36 (1)^a	255.75
PVA90:CH10	77.0 (1.0)^b	213.2 (1.1)^b	41.3 (0.8)^b	29 (1)^b	280.53
PVA80:CH20	79 (3)^b	199.5 (1.1)^c	24 (2)^c	19 (2)^c	320.22
PVA70:CH30	80 (2)^b	199.5 (1.1)^c	24 (11)^c	15 (1)^d	313.64
CH100	-	-	-	-	283.07

T_g: glass transition temperature; T_m: melting temperature; ΔH_m: enthalpy of fusion; X: crystallinity; T_{max}: temperature at maximum decomposition rate. Different letters indicate significant differences ($p<0.05$) due to film formulation ($^{a, b, c, d}$).

3.9 Antibacterial properties

Figure VII.6 shows how the application of the films affected the growth of total aerobic mesophilic and coliform microorganisms over storage at 10ºC. The addition of chitosan in the formulation entailed a growth inhibition effect through the reduction of microbial counts after a 7 day storage period. According to numerous authors, one of the reasons for the antimicrobial character of CH is its positively charged amino group which interacts with negatively charged microbial cell membranes, leading to the leakage of proteinaceous and other intracellular constituents of the microorganisms (Kanatt et al., 2012, Möller et al., 2004, Honary et al., 2011). Coherently with this, PVA:CH films showed significant ($p<0.05$) antimicrobial activity against total aerobic mesophilic and coliform microorganisms as compared to non-coated samples. Significant differences in microbial growth inhibition were observed between PVA80:CH20 and CH100 films, as compared to the control sample ($p<0.05$). At the end of storage, PVA80:CH20 and CH100 films led to a significant reduction in microbial load for coliforms with respect to the non-coated samples (about 3 and 3.5 log CFU/g, respectively). Previous studies also showed the antimicrobial activity of PVA:CH films against Gram negative (E. Coli) (Zhao et al., 2003; Trypathi et al., 2009) and Gram positive (S. aureus and B. subtilis) microorganisms (Trypathi et al., 2009).

On the other hand, at the end of storage, PVA100, PVA80:CH20 and CH100 films led to a significant reduction in total aerobial counts as compared to non-coated samples (about 2.3, 2.4 and 3.8 log CFU/g, respectively). Although no antimicrobial activity has been previously reported for PVA films, the obtained reduction in aerobial counts with respect to the control sample could be attribute to the strong oxygen barrier that PVA film suppose (Gaume et al., 2012), which will contribute to growth inhibition of aerobial microorganisms.

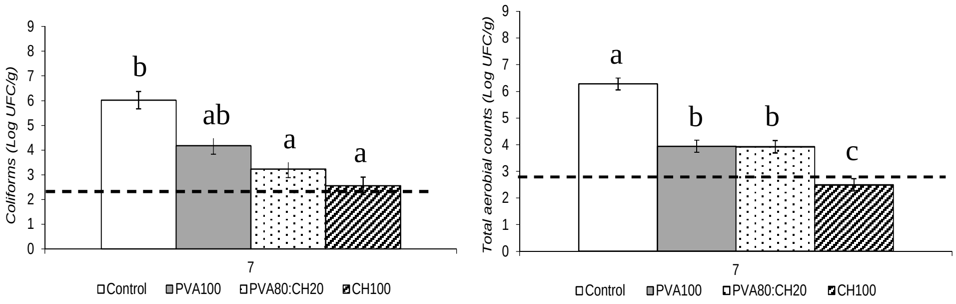

Figure VII.6. Microbial counts of minced pork meat samples coated with PVA:CH films after 7 days storage at 10ºC. Mean values and 95% LSD intervals. Control = non-coated samples. Different letters indicate significant differences ($p<0.05$) due to film formulation ($^{a, b, c}$).

4. Conclusions

The blending of chitosan and PVA rendered films of homogeneous structure, due to the high compatibility of both materials, as revealed by thermal behavior of the blends. Chitosan addition greatly reduced the extensibility of the PVA films, while increasing their rigidity and strength. A reduction of the crystallinity degree was also observed together with an increase in the thermal stability. Incorporation of CH to PVA provoked a reduction of the UV light transmission of the films, associated to the polymer interactions, which could suppose and advantage to their use in food packaging at the same time that provide to the films antimicrobial activity. The blending of chitosan into PVA films seems to be a promising strategy to obtain antimicrobial, biodegradable packaging for food products.

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PHYSICOCHEMICAL AND ANTIMICROBIAL PROPERTIES OF EXTRUDED POLYLACTIC ACID FILMS AS AFFECTED BY CHITOSAN ADDITION

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Abstract

Films based on polylactic acid (PLA) and different amounts of high molecular weight chitosan (CH) were prepared by extrusion. The effects of CH particle size and the amount of chitosan incorporated in the PLA matrix were investigated in terms of physicochemical characteristics and antimicrobial activity of the films. The incorporation of CH particles led to less rigid and less stretchable films. Thermal properties of PLA were not affected by chitosan addition. Water vapour permeability of the composite films was higher than pure PLA films. PLA:CH composite showed significant antimicrobial activity against total aerobial and coliform microorganisms, especially when the particle size of CH was reduced.

Keywords: polylactic acid (PLA), chitosan, polymer blends, antimicrobial, mechanical properties, water vapour permeability.

1. Introduction

The growth of environmental concerns over non-renewable and non-biodegradable petrochemical-based plastic packaging materials has raised interest in the use of biodegradable alternatives originated from renewable sources (Rhim, 2008). Blending two or more polymers has gradually become an important approach to develop new materials that exhibit properties that could not be achieved by using each individual polymer (Chen et al., 2008). Thermoplastic polymers exhibit properties that make them ideal for developing packaging materials such as low process temperature (compared to metal and glass), tunable barrier properties, printability, heat seal ability, and ease of processing into different forms (Lim, 2008). One of the most promising biodegradable thermoplastic materials is polylactic acid (PLA), a polyester produced from L-lactic acid (LA) which is obtained from the fermentation of corn starch and other polysaccharide sources (Fortunati, 2010). PLA has a significant potential for the packaging industry because it yields stiff films of high transparency and can be processed using readily available production technologies. However, its thermal properties, toughness, water vapour and gas barrier properties are inferior to those of conventional petroleum-based polymers (Petersen, 2001).

Extrusion is the most important technique for continuous melt processing of PLA. The plasticizing extruder can be part of the forming machine systems for injection molding, blow molding, film blowing and melt spinning (Lim, 2008).

Chitosan (CH) is a cationic polysaccharide obtained from chitin by deacetylation in the presence of alkali. Besides its biodegradability and biocompatibility, chitosan is reported to be an active polymer with antimicrobial activity (Cuero et al., 1999). Chitosan cannot be processed by melting (Grande and Carvalho, 2011) and thus chitosan-based films can only be obtained by solving in acid media (commonly acetic acid) and casting techniques (Vargas et al., 2009; Vargas et al, 2011). Chitosan films exhibit a high sensitivity to moisture and low water barrier properties. Therefore, for most food applications, it is required to associate this polysaccharide with a more moisture-resistant polymer, while maintaining the overall biodegradability of the product. Many efforts have been made to produce new polymeric systems incorporating chitosan as a bioactive material. Some studies deal with the development of CH-PLA biodegradable composites by solution mixing and film casting procedure (Chen et al., 2005; Grande and Carvalho, 2011;Li et al., 2003; Sébastien et al. 2006). Other recent approach to obtain CH incorporated PLA films was to prepare PLA sheets by extrusion, coat them with a CH solution and immerse in crosslinking agents such as gluteraldehyde (Soares et al., 2013).

Some attempts to produce composite systems based on PLA with CH by melt processing have been made (Correlo et al., 2005). However, to the best of our knowledge, the studies on the properties of melt processed PLA-CH films are still scarce and there is no published information on the antimicrobial effect of such films.

The aim of this study was to prepare PLA:CH composite films by extrusion and to evaluate the effects of the chitosan particle size and chitosan content on the physicochemical and antimicrobial properties of PLA:CH composite films.

2. Materials and methods

2.1. Reagents and raw materials

Polylactic acid (PLA 3051D, in pellets, degree of hydrolyzing > 99%, density of 1.25 g/cm^3, molecular weight of 1.42x10^4 g/mol, melt flow index of 7.75 g/10 min at 210°C, 2.16 kg) was supplied by Nature Works®, USA. High molecular weight chitosan (CH) (practical grade, Batch MKBB0585, degree of deacetylation > 77%, viscosity: 1220 cPs), was supplied by Sigma-Aldrich Quimica (Madrid, Spain). Acetic acid and $Mg(NO_3)_2$ were provided from Panreac Química, S.A (Barcelona, Spain). Millipore water was used to prepare the film-forming dispersions (FFD). For the microbiology study, pork meat was purchased in a local supermarket and processed at the laboratory. Tryptone Phosphate Water (peptone buffered water), Violet Red Bile Agar (VRB agar) and Plate Count Agar (PCA) were provided by Scharlau Microbiology (Barcelona, Spain).

2.2. Preparation of film formulations and film processing

PLA pellets were dried in a vacuum oven at 98°C for 3 h. CH powder (particle size of about 715 μm) was grinded to reduce its particle size to 180 μm, by using a cutter grinder. PLA and CH (5 and 10 % w/w in the composite) were used for prepare the different films by extrusion. Film formulations were named as follows: PLA, G1:PLA_{95}:CH_5, G1:PLA_{90}:CH_{10}, G2: PLA_{95}:CH_5, G2: PLA_{90}:CH_{10}. Where G1 and G2 stand for the particle size of the CH powder (original or reduced particle size, respectively) and the subscripts indicate the PLA:CH ratio in the extruded films. Pure PLA and PLA:CH composites were manufactured by using a twin-screw microextruder (DSM Explorer 5&15 CC Micro Compounder, Geleen, The Netherlands). After several preliminary tests, a screw speed of 150 rpm, head force of 2500

N and mixing time of 3 min were used in order to improve the properties of the obtained films. The temperature profile of the mixing process (170°C-180°C-185°C) was chosen in order to prevent thermal degradation of PLA. After mixing, the film-forming process was carried out by using a speed roll of 600 mm/min, a head force of 3000 N and a maximum temperature of 200°C. Films were conditioned at room temperature and 37% RH in chambers containing saturated solutions of $Mg(NO_3)_2$ prior to all analyses. Film thickness was measured using a Palmer digital micrometer (Comecta, Barcelona, Spain) to the nearest 0.001 mm. Six random positions in each film sample were considered.

2.3. Characterization of the films

2.3.1. Ultraviolet-visible spectrophotometry (UV-VIS)

UV-Vis absorption spectra of the samples (1x1 cm^2) were recorded in the wavelength range from 250 to 900 nm with Perkin Elmer Instruments (Lambda 35) UV-Vis spectrophotometer.

2.3.2. Microstructure

The microstructure of the chitosan flakes and the surface of the films was obtained by field emission scanning electron microscope (FESEM) using a Supra 25-Zeiss electron microscope (Germany). A JEOL JSM-5410 (Japan) electron microscope was used to obtained scanning electron microscope (SEM) images of the cross-section of the films. The films were equilibrated in P_2O_5 to eliminate water, and then mounted on copper stubs. Samples were gold coated and observed, using an accelerating voltage of 5 kV (FESEM) or 15 kV (SEM).

2.3.3. Thermogravimetric analysis (TGA)

A thermogravimetric analyzer (Seiko Exstar 6300, Japan) was used to measure the thermal weight loss of the pure PLA, and PLA:CH composite films in a temperature range between 30°C and 900°C with a heating rate of 10°C/min under nitrogen stream.

2.3.4. Differential scanning calorimetry (DSC)

Films samples of 5 mg were tested by differential scanning calorimeter (DSC, Mettler Toledo 822/e, Switzerland).Measurements were carried out under nitrogen flow in the temperature range from -25°C to 220°C. After a first heating step, cooling at 10°C/min, and finally a second heating step at the same rate were performed. Data were recorded both during the cooling and second heating steps. Glass transition temperature (midpoint, T_g), crystallization temperature (T_c), crystallization enthalpy (ΔH_c) melting temperature (T_m) and melting enthalpy (ΔH_m) were determined. The crystallinity (X) degree was calculated by considering the melting enthalpy of a 100% crystalline PLA sample (93 J/g, Rhiga et al., 2004) and the weight fraction of PLA in the sample (m_f), by applying eq. 1.

$$\chi = \frac{1}{(1-m_f)}\left[\frac{\Delta H}{\Delta H_0}\right]*100 \qquad \text{(Eq. 1)}$$

2.3.5. Tensile properties

True stress-Hencky strain curves of film samples were obtained by using a Universal Testing Machine (Digital Lloyd instrument, Lloyd Instruments Ltd, Bognor Regis, West Sussex, UK), according to UNI ISO 527-1 (ISO, 2012). From these curves, the elastic modulus (EM), tensile strength at the yield point (σ_y) and break (σ_b) and elongation at yield point (E_y%) and break (E_y%) of the films were evaluated. Rectangular samples (1x5 cm^2) were cut and stored at room temperature in cabinets containing $Mg(NO_3)_2$ saturated solution. Equilibrated film samples were mounted in the film-extension grips and stretched at 50 mm/min until breakage, with a crosshead speed of 5 mm/min with a load cell of 500 N. The measurements were done at room temperature and six samples for each formulation were tested.

2.3.6. Water vapour permeability (WVP)

Water vapour permeability (WVP) was determined at 10°C and 58–100% RH gradient using a modification of the ASTM E96-

95 gravimetric method (1995) for flexible films (Mc Hugh et al., 1993). Round samples were cut (three per formulation) and six random thickness measurements were taken per sample. Payne permeability cups of 2.24 cm in diameter (Elcometer SPRL, Hermalle/s Argenteau, Belgium) were filled with 5 ml distilled water (RH = 100%). Once the films were secured, each cup was placed in a pre-equilibrated cabinet fitted with a fan to provide a strong driving force across the film for water vapour diffusion. The RH of the cabinet was held constant at 53%, using $Mg(NO_3)_2$ over-saturated solution. The shiny side of the films was exposed to the atmosphere at the lowest RH, and the cabinets were stored at the mentioned temperatures. The cups were weighed periodically (±0.00001g) after steady state was reached. The slope of the weight loss vs. time plot was divided by the exposed film area to calculate the water vapour transmission rate (WVTR). The vapour pressure on the film's inner surface was obtained by means of the method proposed by Mc Hugh et al. (1993) to correct the effect of concentration gradients established in the stagnant air gap inside the cup.

2.3.7. Antimicrobial properties

PLA, G1:PLA_{90}:CH_{10} and G2:PLA_{90}:CH_{10} composites were applied to minced pork meat to evaluate their antimicrobial properties. Pork meat was ground by using a mincer (Severin Elektrogeräte GmbH, Sundern, Germany) and samples of 10 g of minced meat were molded (5 cm diameter). The surface of both sides of the minced pork meat samples was coated with the films that were previously prepared by extrusion, as described in section 2.2. Non-coated (control) and coated samples were placed in uncovered petri dishes and were stored in duplicate at 10ºC. To perform microbiological analyses, 10 g of each sample were aseptically obtained and homogenized in a Stomacher with 90 mL of sterile buffered peptone water for 2 min. Aliquots were serially diluted in buffered peptone water and plated out following standard methodologies. Total viable and coliform microorganism counts were determined at 0 and 7 days. For the viable counts, Plate Count Agar plates were incubated at 37ºC for 24h. Coliform counts were determined using Violet Red Bile Agar plates incubated at 37ºC for 48h.

2.4. Statistical analysis

The statistical analysis of the data was performed through an analysis of variance (ANOVA) using Statgraphics Plus (Manugistics Corp., Rockville, MD) Fisher's least significant difference (LSD) procedure was used.

3. Results

3.1. Visual appearance and UV-VIS spectra

The visual appearance of pure PLA films and PLA:CH composites films is shown in Fig. 1. The neat PLA extruded film was more transparent than the composites in agreements with observations reported previously by Fortunati et al., (2010). CH particles dispersed in PLA matrix films are visually observed in Figure 1, implying a reduction in optical transparency of the composite films, as compared to pure PLA samples. The blend films were slightly yellow which could be due to a partial miscibility of the CH which affects the color of the continuous matrix.

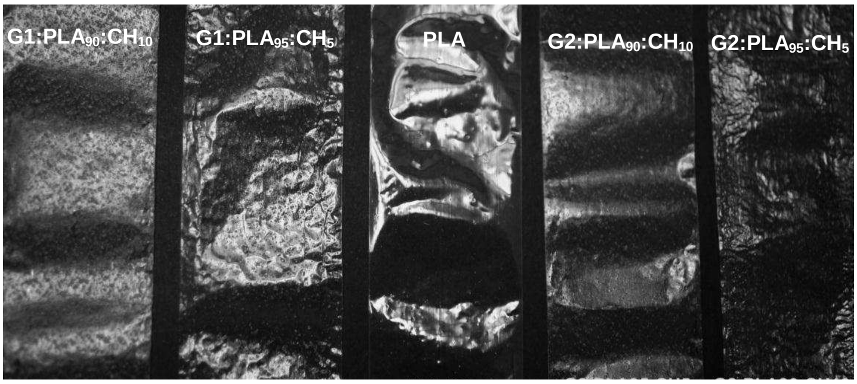

Figure VIII.1. Images of pure PLA films and PLA:CH composite films. PLA: Polylactic acid, CH: chitosan. The percentage of each polymer is indicated as a subscript.

This was reflected in the UV-VIS spectra of the films (Figure 2) where a higher absorbance in the wavelength range of 200-400 nm was detected for PLA:CH blend films. Pure PLA films showed lower absorbance than PLA:CH films, which suggests that blend films are a better barrier to the ultraviolet light, which in turn can contribute to inhibit lipid oxidation in food systems (Kanatt et al., 2012).

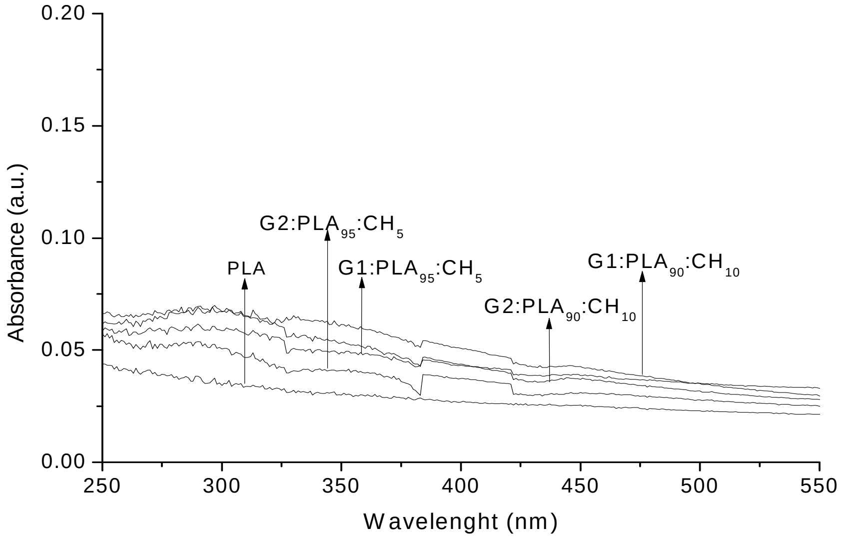

Figure VIII.2. UV-VIS spectra of pure PLA and PLA:CH composite films. PLA: Polylactic acid, CH: chitosan. The percentage of each polymer is indicated as a subscript.

3.2. Microstructure

FESEM micrographs of CH powder samples of different particle size are shown in Fig. 3. The shape of CH flakes was irregular, having initial sizes between 600 and 800 μm before grinding. No chitosan aggregates were detected. After mechanical grinding, the size of CH particles was reduced to sizes around 180 μm, maintaining their original shape and absence of evident agglomeration.

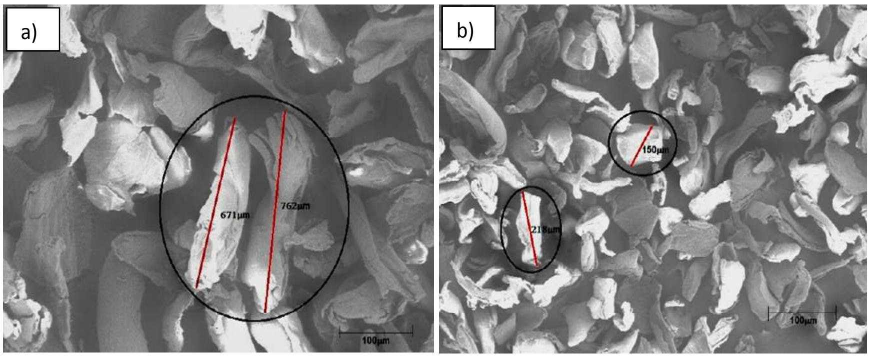

Figure VIII.3. Field emission scanning electron microscopy (FESEM) images of different granulometries of chitosan: a) G1 and b) G2.

Characteristic FESEM images of the pure PLA film and PLA:CH composite surfaces are shown in Fig. 4a. Pure PLA films had a homogeneous structure and no irregularities were detected. On the contrary, PLA:CH films showed increased roughness in their surface appearance, associated to the presence of CH particles in the PLA matrix. FESEM micrographs of the cross-section of pure PLA and PLA:CH films were obtained by fracturing the film by stretching them on the opposite direction of the flow in the screw, since the cryofracture was not possible due to their plastic properties. FESEM images are shown in Figure 4b. Pure PLA films showed a more homogeneous oriented structure with some aggregates whereas the addition of CH particles led to the introduction of irregularities and discontinuities in the oriented PLA matrix, that were more marked as the size of the particles and the proportion of CH increased.

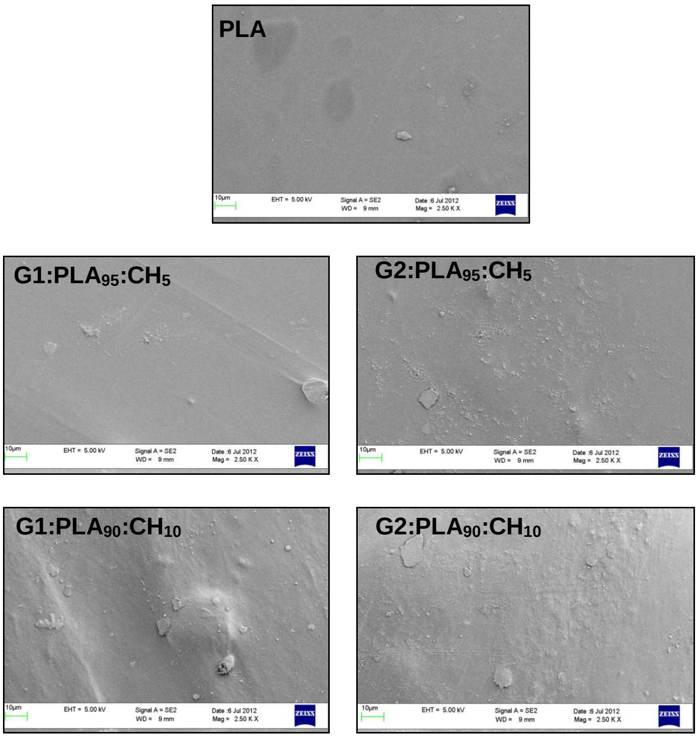

Figure VIII.4a. Field emission scanning electron microscopy images of the surface of PLA and PLA:CH composite films. Polylactic acid, CH: chitosan. The percentage of each polymer in the film is indicated as a subscript.

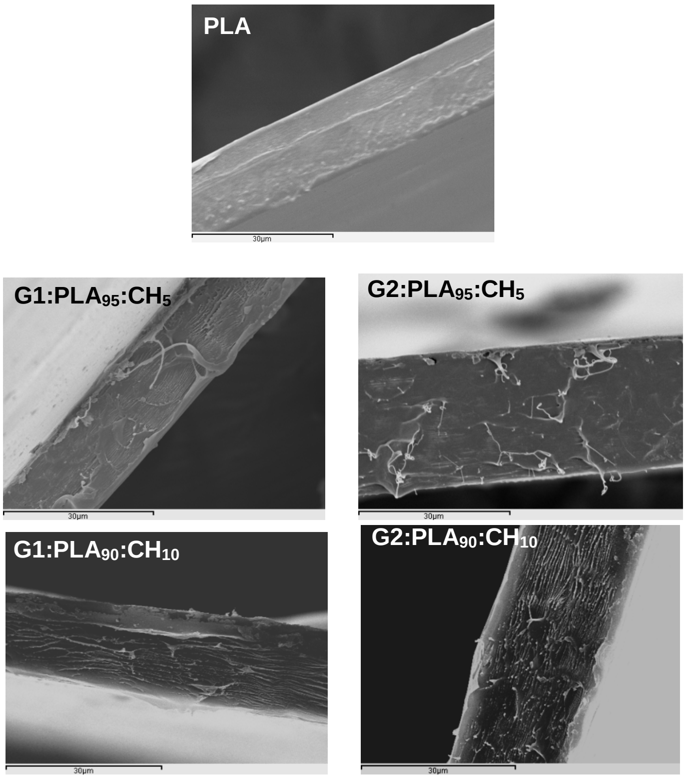

Figure VIII.4b. Scanning electron microscopy images of the cross-sections of PLA and PLA:CH composite films. Polylactic acid, CH: chitosan. The percentage of each polymer in the film is indicated as a subscript.

3.3. Thermal behavior

The weight loss (TG) and derivative (DTG) curves of pure PLA films and PLA:CH composites are reported in Fig 5. A complete weight loss in a single step with a maximum at 358°C for pure PLA was detected. This coincides with the results reported by

Fortunati et al. (2010). The incorporation of CH particles did not promote significant changes in the thermal behavior of pure PLA.

Previous studies showed that the thermal degradation of pure CH is characterized by two significant weight losses. One corresponds to moisture vaporization, the loss of adsorbed and bound water and the residues of acetic acid (Trypathi et al., 2009; Chen et al., 2008; Lewandowska, et al., 2009). The second weight loss, which was observed at 300-400°C could be attributed to the degradation of the saccharide structure of CH molecule, including the dehydration of saccharide rings and the polymerization and decomposition of the acetylated and deacetylated units of chitin (Paulino et al., 2006). However, in the present study, a single degradation step at around 350ºC was detected for both composite and pure PLA films (Figure 5b). The relatively low ratio of CH in the blends could explain the observed behavior.

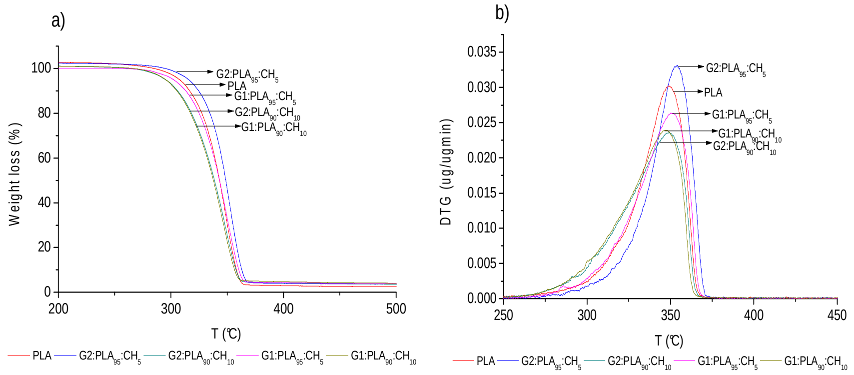

Figure VIII.5. a) TG and b) DTG curves of PLA and different PLA:CH composite films. PLA: Polylactic acid, CH: chitosan. The percentage of each polymer is indicated as a subscript.

DSC analysis was used to analyse the glass transition and melting properties of PLA and PLA:CH composite systems. The DSC curves are shown in Fig. 6. The values of thermal parameters obtained in the first heating scan for all films are reported in Table 1. PLA extruded films have an average glass transition (T_g) value of 61ºC. This result is similar to that reported in other studies (Correlo et al., 2005; Fortunati et al., 2010). No significant effects of the CH addition on the T_g values was observed, which can be due to the lack of miscibility of both polymers which determines that both polymers are distributed in separated phases, although only one T_g was detected. This can be explained by the relative low concentration of CH. Moreover, it is known that chitosan is a semicrystalline polymer due to its strong intramolecular hydrogen bonds on the backbone, and it also has a rigid amorphous phase because of its heterocyclic units. As a result, when CH is heated within a certain range of temperature below its decomposition temperature, the variation in heat capacity corresponding to the change in specific volume near the T_g is probably too small to be detected by the DSC technique (Wan et al. 2006).

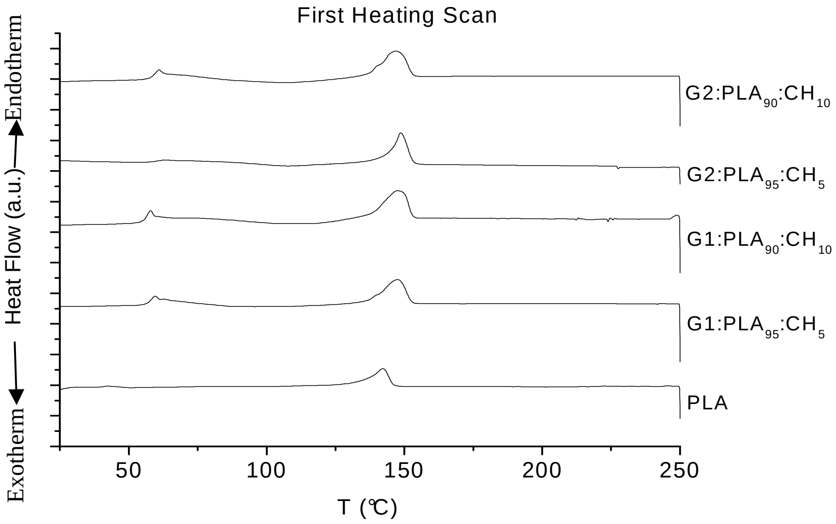

Figure VIII.6. First heating DSC scan for pure PLA and PLA:CH composite films. PLA: Polylactic acid, CH: chitosan. The percentage of each polymer is indicated as a subscript.

Crystallization temperature (T_{cc}) of the films significantly increased ($p<0.05$) with the addition of CH particles ($p<0.05$). According to Correlo et al., (2005), this may indicate that chitosan acts as a nucleating agent, promoting a faster crystallization of PLA. However, the addition of CH did not significantly affect the melting temperature and enthalpy which also agrees to the polymer incompatibility. Coherently with that, the degree of crystallinity of PLA was not affected by the CH addition.

Table VIII.1. Thermal properties of pure PLA and PLA:CH composite films (first heating scan): Glass transition temperature (T_g); crystallization temperature (T_c); enthalpy of crystallization (ΔHc); Melting temperature (T_m); Enthalpy of melting (ΔH_m); crystallinity degree (X).

Film	**T_g (°C)**	**T_c (°C)**	**ΔH_c (J/g)**	**T_m (°C)**	**ΔH_m (J/g)**	**X (%)**
PLA	61.0 (0.1)^a	86 (1)^a	16.15 (0.04)^a	146 (1)^a	27 (4)^a	29 (4)^a
G1:PLA_{95}:CH_5	58 (2)^a	95 (4)^b	14 (4)^a	147 (1)^a	21 (2)^a	24 (2)^a
G1:PLA_{90}:CH_{10}	58 (2)^a	114 (3)^c	16(2)^a	148 (1)^a	22 (3)^a	27 (4)^a
G2:PLA_{95}:CH_5	58 (1)^a	97 (3)^b	16 (8)^a	147 (1)^a	26 (4)^a	29 (4)^a
G2:PLA_{90}:CH_{10}	59 (1)^a	105 (1)^d	17 (5)^a	147 (1)^a	24 (1)^a	29 (2)^a

PLA: Polylactic acid, CH: chitosan. The percentage of each polymer is indicated as a subscript. Different letters ($^{a,\ b,\ c,\ d}$) indicate significant differences ($p<0.05$) due to film formulation.

3.4. Tensile properties

The typical tensile curves of pure PLA and PLA:CH films are plotted in Fig. 7. It is remarkable that composite films showed a less plastic deformation than pure PLA films, which made them less stretchable. Average tensile strength (σ_b) and percentage elongation at break (E_b%), yield stress and strain (σ_Y, E_y) and elastic modulus (EM) are shown in Table 2.

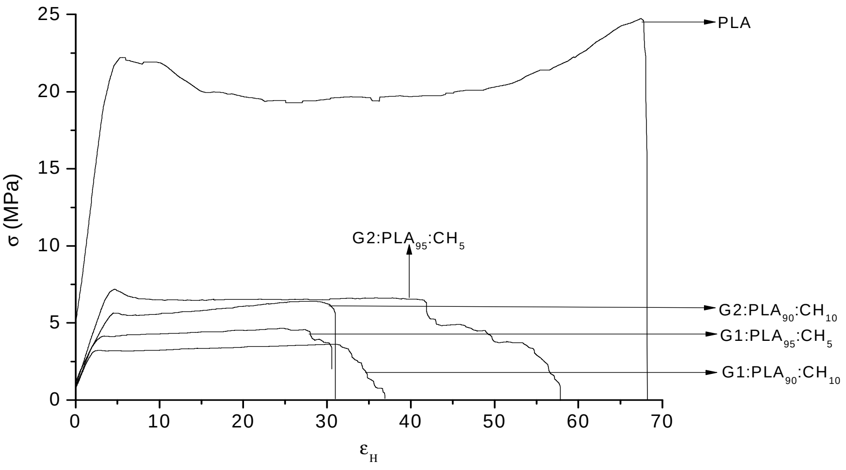

Figure VIII.7. Typical true stress (σ) vs. Hencky strain (ε_H) curves obtained in tensile tests carried out on PLA and PLA:CH composite films. PLA: Polylactic acid, CH: chitosan. The percentage of each polymer is indicated as a subscript.

The values of pure PLA films were in the range of those reported by Fortunati et al. (2012). A decrease in the elongation at break with respect to pure PLA films was detected for all PLA:CH systems, which coincides with the results observed in PLA extruded films containing 50% CH (Correlo et al., 2005). The elongation at break was affected by the volume fraction of the added CH when particle size of CH was reduced (G2), but no effect was observed for the greatest particle size. Colom et al., (2003) observed that particle volume fraction and the interactions between the particles and the matrix affect film extensibility. The addition of CH led to a significant decrease in tensile strength, yield stress and strain and elastic modulus. These results highlight that chitosan did not have a reinforcement effect when added to PLA matrix since PLA:CH blend films were more fragile than PLA films. At higher chitosan content, the filler inclusions can form aggregates. The decrease in tensile strength with the increase in CH content could be explained by the lack of compatibility between PLA and CH particles and the generation of discontinuities in the PLA matrix which decreased the overall cohesion forces in the matrix and make it more sensible to flow and break.

3.5. Water vapour permeability (WVP)

WVP values are showed in Table 2. PLA films showed lower WVP than composite films, which can be due to the greater water affinity of CH which could favor the transport of water molecules through the film. According with Suyatma et al., (2004), water barrier properties of PLA:CH films increased as

PLA content was higher as a result of the well-known hydrophobicity of PLA. Nevertheless, it is remarkable that the film sample which contains the greatest amount of CH with low particle size showed a WPV in the order of that of pure PLA films, which have a no clear explanation.

Table VIII.2. Tensile properties (25ºC-58%RH) and water vapour permeability (WVP) at 10ºC and 58-100%RH gradient of pure PLA and PLA:CH composite films. Average values and standard deviations, in brackets.

Film	**σ_y (MPa)**	**E_y (%)**	**σ_b (MPa)**	**E_b (%)**	**EM (MPa)**	**WVP ($g \cdot s^{-1} \cdot m^{-1} \cdot Pa^{-1}$) x 10^{11}**
PLA	20 (3)^{c}	5 (1)^{b}	23 (6)^{c}	68 (13)^{c}	445 (44)^{d}	4 (3)^{a}
G1:PLA_{95}:CH_5	4.0 (0.2)^{ab}	7 (1)^{c}	4 (1)^{a}	34 (5)^{ab}	66 (4)^{a}	21.1 (0.3)^{b}
G1:PLA_{90}:CH_{10}	3.1 (0.4)^{a}	3 (1)^{a}	3 (1)^{ab}	35 (4)^{ab}	143 (2)^{b}	20 (2)^{ab}
G2:PLA_{95}:CH_5	7.6 (0.4)^{b}	4 (1)^{b}	8 (1)^{b}	44 (4)^{b}	294 (10)^{c}	74 (1)^{c}
G2:PLA_{90}:CH_{10}	6 (1)^{ab}	4 (1)^{b}	7 (1)^{ab}	31 (6)^{a}	155 (21)^{b}	7 (0.3)^{ab}

PLA: Polylactic acid, CH: chitosan. The percentage of each polymer is indicated as a subscript. Different letters ($^{a,\ b,\ c,\ d}$) indicate significant differences ($p<0.05$) due to film formulation.

3.6. Antimicrobial properties

Microbial counts of non-coated (control) and coated minced pork meat samples at the beginning and after 7 days of cold storage are shown in Figure 8. All films showed an antimicrobial activity against total aerobic mesophilic and coliform microorganisms as compared to non-coated samples ($p<0.05$). The slight reduction in bacterial counts detected in pure PLA films can be related to their low oxygen permeability (Fortunati et al., 2010). PLA:CH films led to greater a reduction of microbial counts, which was significant for coliform microorganisms (about 2 log CFU/g), associated to the antibacterial activity of CH. These antimicrobial properties of CH have been explained by the interaction of the positive charges present in the polymeric chain of chitosan with the negative charges from the residues of macromolecules (lipopolysaccharides and proteins) in the membranes of microbial cells, which, in turn, can interfere with the nutrient exchange between the exterior and interior of the cell (Martínez-Camacho, 2010; Möller et al., 2004). Previous researches have also shown the antimicrobial activity of CH films applied to meat products (Rao et al., 2005; Vargas et al., 2011b).

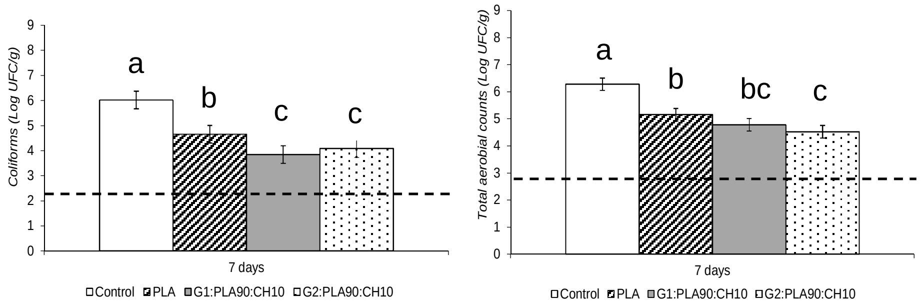

Figure VIII.8. Microbial counts of minced pork meat samples coated with PLA and PLA:CH after 10 days of cold-storage. Mean values and 95% LSD intervals. Control = non-coated samples. Initial count is indicated by the dashed line.

4. Conclusions

Chitosan was efficiently incorporated into PLA matrices by extrusion in 5 and 10 % of the matrix. Both polymers are no compatible and due to the lack of the fusion of CH during processing, particles of this polymer remain in the final films. Therefore CH did not affect either thermal behavior of the blend films or total crystallinity degree of PLA. Nevertheless, the presence of CH particles increased the crystallization temperature and reduced the mechanical resistance and extensibility of the films as well as the water vapor barrier. The antimicrobial effect of chitosan was proven in the composite films, which showed a greater reduction of the microbial counts when applied on minced meat samples, especially when their particle size was lower, since this favors the diffusion of polymer to the sample surface.

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CONCLUSIONS

CONCLUSIONS

Basil and thyme essential oils could be incorporated to chitosan films with different homogenization techniques of the film forming emulsions. Microfluidization at 165 MPa greatly affected the properties of the emulsions and films. This technique produced a reduction in oil droplet size and viscosity, while promoted the adsorption of chitosan on the oil-water interface. The induced close contact polymer-oil compounds gives rise to more fragile films of increased stretchability due the weakening effect on the CH matrix where chain interaction forces are reduced. This effect was also observed for the films obtained from mild homogenized dispersions, but with less intensity. Water barrier properties of the films were dependent on the ratio of essential oil and the homogenization technique. High-pressure homogenization increased WVP of pure CH films while oil incorporation was only effective to reduce WVP when they were incorporated at the lowest ratio and when high pressure was used in the homogenization of the film-forming emulsions. Gloss was reduced by the essential oil addition, whereas high pressure homogenization tended to yield glossier films.

The addition of basil and thyme essential oils to chitosan films increased their potential antioxidant effects, although it did not improve their antibacterial efficiency in pork meat samples. The improvement in the antioxidant properties of chitosan-based films by adding essential oils occurs in spite of the worsening of their oxygen barrier properties, which points to the specific chemical action of the oil's antioxidant compounds. The reduction in the oxygen availability in minced meat provoked by sample coating leads to the expected changes in colour associated with the conversion of myoglobin into metmyoglobin, but other quality aspects related to food safety are improved. Therefore, chitosan-essential oil films may be formulated in order to be applied to meat products, increasing the product's shelf life and safety.

Blend films with wheat starch and chitosan could be obtained without phase separation since polymers showed high compatibility. The replacement of wheat starch-glycerol with chitosan affected the properties of film forming dispersions and films. Chitosan addition led to a significant increase of the particle surface charge and apparent viscosity of the film forming dispersions and yielded glossier and thicker films. The mechanical properties were significantly improved as the chitosan ratio increased in the films, these being more resistant and extensible, at the same time that chitosan seems to inhibit starch retrogradation. Both, water vapor and oxygen permeability values, tend to slightly increase when chitosan was incorporated to the film, although induced differences are not relevant. A notable antimicrobial effect in minced pork meat was detected in the blend films when the proportion of chitosan was 50%. The obtained results suggest that it would be possible to formulate edible antibacterial films with thermoplastic properties by combining wheat starch and chitosan.

The incorporation of antioxidants (basil and thyme essential oils, citric acid and α-tocopherol) at 6.7 % in wheat starch:chitosan (4:1) films gave rise to microstructural changes depending on the different affinity of components. The greatest structural change was induced by α-tocopherol which promoted polymer phase separation in the film. All antioxidants provoked a loss of gloss, but a transparency gain in the films, while α-tocopherol induced yellowness and a reduction of the film whiteness index. The mechanical properties of the films were hardly affected by the antioxidants, but citric acid promoted an increase of elastic modulus but a decrease in the tensile and deformation at break, probably by cross-linking with starch chains. Water vapour barrier properties of the films were only slightly improved when citric acid and α-tocopherol were added, whereas the oxygen barrier properties of the films were significantly improved in all cases, probably due to a chemical oxygen blocking effect. The greatest antioxidant capacity of the films was expected for films containing α-tocopherol which showed the highest antioxidant power among the proved compounds.

The blending of chitosan and polyvinyl alcohol rendered films of homogeneous structure, due to the high compatibility of both materials, as revealed by thermal behavior of the blends. Chitosan addition greatly reduced the extensibility of the polyvinyl alcohol films, while increasing their rigidity and strength. A reduction of the crystallinity degree of polyvinyl alcohol was also observed together with an increase in the thermal stability. Incorporation of chitosan to polyvinyl alcohol provoked a reduction of the UV light transmission of the films, associated to the polymer interactions, which could suppose and advantage to their use in food packaging at the same time that provide to the films antimicrobial activity. The blending of chitosan into polyvinyl alcohol films seems to be a promising strategy to obtain antimicrobial, biodegradable packaging for food products.

Chitosan was efficiently incorporated into polylactic acid matrices by extrusion in 5 and 10 % of the matrix. Both polymers are no compatible and, due to fact that no total fusion of chitosan occurs during the extrusion process, particles of the polymer remain in the final films. Therefore chitosan did not affect either thermal behavior of the blend films or crystallinity degree of polylactic acid. The presence of CH particles reduced the mechanical resistance and extensibility of the films as well as the water vapor barrier. However, the reduction of chitosan particle size seemed to limit this negative impact. The antimicrobial effect of chitosan was proven in the blend films, which showed a greater reduction of the microbial counts when applied on minced meat samples, especially when their particle size was lower, since this favors the diffusion of polymer to the sample surface.

In general, chitosan blend films with other polymers can be obtained due to the polymer compatibility with other matrices or it can act as filler with particles of different size. This allows us to modulate the film properties in order to adequate the film functionality and to impart antimicrobial properties to the obtained film. Other components, such as antimicrobial or antioxidant compounds could also be incorporated to pure or blend chitosan films to obtain active packaging materials with good functional properties.