UNIVERSIDAT POLITÈCNICA DE VALÈNCIA

INSTITUTO UNIVERSITARIO DE INGENIERÍA DE ALIMENTOS

PARA EL DESARROLLO

DIFFERENT STRATEGIES TO OBTAIN ANTIMICROBIAL BIODEGRADABLE FILMS FOR FOOD APPLICATIONS, USING STARCH AND/OR CHITOSAN WITH OR WITHOUT ESSENTIAL OILS

DOCTORAL THESIS

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UNIVERSIDAD POLITÈCNICA DE VALÈNCIA

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PARA EL DESARROLLO

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CONSIDERAN: que la memoria titulada **"DIFFERENT STRATEGIES TO OBTAIN ANTIMICROBIAL BIODEGRADABLE FILMS FOR FOOD APPLICATIONS, USING STARCH AND/OR CHITOSAN WITH OR WITHOUT ESSENTIAL OILS"**, que presenta **D^{a}. Cristina Encarnación Valencia Sullca**, para aspirar al grado de Doctor por la Universitat Politècnica de València, realizada en el Instituto de Ingeniería de Alimentos para el Desarrollo (IuIAD–UPV) bajo su dirección, reúne las condiciones adecuadas para constituir su tesis doctoral, por lo que **AUTORIZAN** a la interesada su presentación.

Valencia a 31 de mayo de 2017

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A Dios

A mis amados padres (Elena y Ubaldo)

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PREFACE

DISSERTATION OUTLINE

This Doctoral Thesis is structured in five sections: Introduction, Objectives, Chapters, General Discussion and Conclusions.

The INTRODUCTION section revises the state of the art concerning the properties of starch films and the antimicrobial action of chitosan and essential oils used to develop biodegradable films. Encapsulation techniques with interest for developing edible films are also reviewed.

The OBJECTIVES section presents the general and specific objectives of the Thesis, which is focused on the development of biodegradable films based on hydrocolloids (starch and/or chitosan) with antimicrobial properties that were produced by different techniques (compression molding and/or casting).

The obtained results are organized in five CHAPTERS, each one corresponding to a scientific publication that includes the usual sections: introduction, materials and methods, results and discussion and conclusion.

Chapter 1, entitled **"Physical characterization of cassava starch – chitosan films obtained by compression molding"**, evaluated the effect of the incorporation of chitosan, glycerol and polyethylene glycol (PEG) as plasticizers on the physical and antimicrobial properties of cassava starch films obtained by compression molding. Results showed that chitosan incorporation provided the films with only a slight antimicrobial capacity and thus, in the subsequent chapters, different strategies to overcome this drawback were used.

Chapter 2, entitled **"Thermoplastic cassava starch – chitosan films containing essential oils"**, was focused in chitosan films (containing or not different essential oils as active ingredients) that were obtained by casting. The casted films were used to develop bilayer films with cassava starch films produced by compression molding. The bilayer films were effective at controlling the bacterial growth in pork meat samples, but the thermal treatment used to obtain the bilayers reduced its effectiveness. Therefore, in chapters 3 to 5 other strategies were developed to incorporate essential oils in the chitosan matrix and to improve the final retention of active ingredients at the same time that a control release in different food systems is promoted.

Chapter 3, entitled **"Influence of liposome encapsulated essential oils on properties of chitosan films"**, is centered in the encapsulation of active ingredients (eugenol or cinnamon leaf essential oil) in lecithin liposomes that were added to chitosan films obtained by casting. Films were evaluated in terms of their physical properties and antimicrobial performance in different food systems, as compared to chitosan films that included non-encapsulated active ingredients.

Chapter 4, entitled **"Release kinetics and antimicrobial properties of chitosan-essential oils films as affected by encapsulation within lecithin nanoliposomes"**, evaluated the antimicrobial properties of chitosan films containing active ingredients (eugenol or cinnamon leaf essential oil) in free form or encapsulated in lecithin liposomes encapsulated. The release kinetics of eugenol and the specific migration of the films in different food simulants were also analyzed.

Chapter 5, entitled **"Chitosan films containing encapsulated eugenol in alginate microcapsules"**, showed another encapsulation strategy to improve the functionality and retention of active compound in chitosan–based matrices. The films were evaluated in their physical and antimicrobial properties as well as the kinetics of eugenol release in food simulants.

Finally, the most important CONCLUSIONS of the Thesis are shown.

DISSEMINATION OF RESULTS

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PREDOCTORAL STAYS AT FOREIGN INSTITUTIONS

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ABSTRACT

The development of biodegradable active packaging materials is one of the challenges of society in order to solve the environmental problems associated with plastic waste while improving food preservation. In the present Doctoral Thesis, different strategies have been approached for the preparation and characterization of biodegradable films based on hydrocolloids (cassava starch (CS) and chitosan (CH)) with antimicrobial properties. Bioactive films have been obtained by adding essential oils with proven antimicrobial activity, namely cinnamon leaf (CLEO), oregano (OEO) and eugenol (EU). The active agents were incorporated into the polymer matrix by homogenization or encapsulation in lecithin liposomes or alginate microspheres, and films were obtained either by casting. The physicochemical properties of the films were analyzed as a function of their composition, as well as their antimicrobial activity through *in vitro* and *in vivo* tests.

Blend CS-CH films were obtained by compression molding, at two different polymers: plasticizer ratios (70:30 and 60:40). The structural, thermal and physical properties of CS films were affected by the incorporation of CH and the polymer: plasticizer proportion. The films with the highest plasticizer ratio had higher moisture content, were more permeable to water vapor, less rigid and less resistant. The incorporation of CH had a positive effect on the mechanical properties of the films, which became stiffer and more resistant to break but less stretchable. However, CS and CH exhibited lack of miscibility by melt blending, and the films exhibited a heterogeneous structure. PEG was crystallized to great an extent in the films, thus limiting its plasticizing effect. CH incorporation provided the films with only a slight antimicrobial capacity.

Bilayer films were obtained by melt blending thermoplastic CS and adding a second layer of casted CH. The two exhibited good interfacial adhesion and the bilayer films had better mechanical resistance than starch monolayers, although they were less stretchable due to the interfacial control of the film fracture. Chitosan was effective at controlling the bacterial growth in meat pork, although the thermal treatment used to obtain the bilayers reduced its effectiveness, revealing the loss of amino groups during treatment. The addition of CLEO and OEO did not promote any antimicrobial action in CH mono and bilayer films applied to pork meat.

CLEO and EU were incorporated into CH films by forming lecithin nanoliposomes, which acted as carriers of bioactive components. The encapsulation of eugenol or CLEO in lecithin liposomes led to the films retaining a higher amount of incorporated eugenol, whereas only 1 % – 2 % was retained when the active compounds was incorporated by direct emulsification. The overall migration of the films in hydrophilic simulants exceeded the established legal limit for food packaging materials. However, encapsulation in nanoliposomes reduced migration in all simulants. In *in vitro* antimicrobial efficacy assays, all films were effective against *L. innocua* and *E. coli*, with no significant effect of the active compound or mode of incorporation. However, encapsulation led to controlled and sustained release over time in pork samples stored for 13 days at 10°C.

Alginate microspheres containing eugenol (MEU) were obtained and incorporated into CH films, whose structural and physical properties were affected. The microspheres were visible in the film structure by SEM analysis. MEU incorporation improved the oxygen barrier properties while increasing the water vapor permeability of the films. Microspheres incorporation also modified the mechanical behavior of the films, reducing their stiffness and strength at break while increasing their stretchability. The fastest EU release was observed 3 % acetic acid food simulant, which was attributed to the higher solubility of the chitosan matrix in this solvent. The addition of the microcapsules conferred antimicrobial capacity to the films against *L. innocua* and *E. coli*.

RESUMEN

El desarrollo de materiales de envase biodegradables activos es uno de los retos de la sociedad para resolver los problemas medioambientales asociados a los residuos plásticos y mejorar la conservación de los alimentos, alargando su vida útil. En la presente Tesis Doctoral, se han analizado diferentes estrategias para la obtención y caracterización de películas biodegradables a base de hidrocoloides (almidón de yuca (A) y quitosano (Q)) con características antimicrobianas. Se obtuvieron películas bioactivas gracias a la incorporación de aceites esenciales de capacidad antimicrobiana comprobada: hoja de canela (AC), orégano (AO) y eugenol (EU). Los agentes activos se incorporaron en la matriz polimérica de quitosano por homogenización o encapsulación en liposomas de lecitina o microesferas de alginato, y las películas se obtuvieron mediante casting. Las propiedades fisicoquímicas de las películas se analizaron en función de su composición, así como su actividad antimicrobiana mediante análisis *in vitro* e *in vivo*.

Se obtuvieron películas por termo-compresión, a base de mezclas A-Q, con dos proporciones polímeros:plastificante (70:30 y 60:40). Las propiedades estructurales, térmicas y físicas de las películas de almidón de yuca obtenidas se vieron afectadas por la incorporación de Q y la proporción polímero: plastificante. Las películas con la mayor proporción de plastificante tuvieron mayor contenido en humedad y fueron más permeables al vapor de agua, menos rígidas y menos resistentes a la rotura. La incorporación de Q tuvo un efecto positivo sobre las propiedades mecánicas de las películas, que aumentaron su rigidez y resistencia a la fractura, reduciéndose su extensibilidad. Sin embargo, el A y Q presentaron una miscibilidad limitada por mezclado en fundido, y las películas exhibieron una estructura heterogénea. A su vez, el polietilenglicol se cristalizó en gran medida en las películas, lo que limitó su efecto plastificante. La incorporación de quitosano proporcionó a las películas sólo una ligera actividad antimicrobiana.

Se obtuvieron películas bicapa por procesado en seco de A y vertido/secado de una capa de Q. Ambos polímeros mostraron buena adhesión interfacial, y las bicapas mostraron mejor resistencia mecánica que las monocapas de almidón, aunque fueron menos extensibles debido al efecto de la interfase sobre la fractura. El quitosano fue efectivo en el control del crecimiento bacteriano en carne picada de cerdo, aunque su eficiencia se vio reducida debido al tratamiento térmico en las bicapas, lo que parece indicar la pérdida de grupos amino

durante el tratamiento. La incorporación de los aceites esenciales (AC y AO) no mejoró la acción antimicrobiana en las monocapas y bicapas de CH al aplicarse sobre carne de cerdo picada.

Se incorporaron aceite esencial de hoja de canela (AC) y eugenol (EU) en películas de Q utilizando nanoliposomas de lecitina. La encapsulación permitió una elevada proporción de retención de compuestos volátiles. La migración total de las películas en simulantes hidrofílicos superó el límite legal establecido para materiales de envase en contacto con alimentos. Sin embargo, la encapsulación en nanoliposomas redujo la migración en todos los simulantes. En ensayos in vitro de eficacia antimicrobiana, todas las películas fueron efectivas frente a *L. innocua* y *E. coli*, sin efecto significativo del compuesto activo ni del modo de incorporación. Sin embargo, la encapsulación propició una liberación controlada y sostenida en el tiempo en muestras de carne de cerdo almacenadas durante 13 días a 10°C.

Se obtuvieron microesferas de alginato con eugenol y se incorporaron en películas de quitosano, cuyas propiedades físicas y estructurales se vieron afectadas. Las microesferas fueron visibles en la estructura de las películas por SEM. La incorporación de las microesfereas promovió una mejora significativa en las propiedades barrera al oxígeno, a la vez que un aumento en la permeabilidad al vapor de agua de las películas. La adición de las microesferas también modificó el comportamiento mecánico de las películas, disminuyendo su rigidez y elasticidad, y aumentando su extensibilidad. La liberación más rápida de eugenol se observó en el simulante ácido acético (3 %), lo que se atribuyó a la mayor disolución de la matriz de quitosano en este solvente. La adición de las microcápsulas confirió capacidad antimicrobiana a los films de quitosano contra *L. innocua* y *E. coli*.

RESUM

El desenvolupament de materials d'envàs biodegradables actius és un dels reptes de la societat per a resoldre els problemes mediambientals associats als residus plàstics i millorar la conservació dels aliments, allargant la seua vida útil. En la present Tesi Doctoral, s'han analitzat diferents estratègies per a l'obtenció i caracterització de pel·lícules biodegradables de hidrocoloids (midó de mandioca i quitosano amb característiques antimicrobianes. Es van obtenir pel·lícules bioactives gràcies a la incorporació d'olis essencials de capacitat antimicrobiana comprovada: fulla de canyella (AC), orenga (AO) i eugenol (EU). Els agents actius es van incorporar en la matriu polimèrica de quitosano per homogenització o encapsulació en liposomes de lecitina o microesferes d'alginato, i les pel·lícules es van obtenir per *"casting"*. Les propietats fisicoquímiques de les pel·lícules es van analitzar en funció de la seua composició, així com la seua activitat antimicrobiana mitjançant anàlisi *in vitro* i *in vivo*.

Es van obtenir pel·lícules per termo- compressió de mescles midó-quitosano, amb dues proporcions polímers:plastificant (70:30 i 60:40). Les propietats estructurals, tèrmiques i físiques de les pel·lícules de midó de yuca obtingudes es van veure afectades per la incorporació de quitosano i la proporció polímer:plastificant. Les pel·lícules amb la major proporció de plastificant van tenir major contingut en humitat i van ser més permeables al vapor d'aigua, menys rígides i menys resistents al trencament. La incorporació de quitosano va tenir un efecte positiu sobre les propietats mecàniques de les pel·lícules, que van augmentar la seua rigidesa i resistència a la fractura, reduint-se la seua extensibilitat. No obstant açò, el midó i quitosano van presentar una miscibilidad limitada per termoprocessat, i les pel·lícules van exhibir una estructura heterogènia. El polietilenglicol va cristal·litzar en gran manera en les pel·lícules, la qual cosa va limitar el seu efecte plastificant. La incorporació de quitosano va proporcionar a les pel·lícules només una lleugera activitat antimicrobiana.

Es van obtenir pel·lícules bicapa formades per una capa de midó obtinguda per processament en sec i un altra capa de quitosano obtinguda per *"casting"*. Tots dos polímers van mostrar bona adhesió interfacial, i les bicapes van mostrar millor resistència mecànica que les monocapes de midó, encara que van ser menys extensibles a causa de l'efecte de la interfase sobre la fractura. El quitosano va ser efectiu en el control del creixement bacterià en carn picada de porc, encara que la seua eficiència es va veure reduïda a causa del tractament tèrmic en les bicapes, la qual cosa sembla indicar la pèrdua de grups amino durant el

tractament. La incorporació dels olis essencials (AC i AO) no va millorar l'acció antimicrobiana en les monocapes i bicapes de CH en aplicar-se sobre carn de porc.

Es van incorporar oli essencial de fulla de canyella (AC) i eugenol (EU) en pel·lícules de quitosano utilitzant nanoliposomes de lecitina. L'encapsulació va permetre una elevada proporció de retenció de compostos volàtils. La migració total de les pel·lícules en simulants hidrofílics va superar el límit legal establert per a materials d'envàs en contacte amb aliments. No obstant açò, l'encapsulació en nanoliposomes va reduir la migració en tots els simulants. En assajos in vitro d'eficàcia antimicrobiana, totes les pel·lícules van ser efectives enfront de *L. innocua* i *E. coli*, sense efecte significatiu del compost actiu ni de la manera d'incorporació. No obstant açò, l'encapsulació va propiciar un alliberament controlat i sostinguda en el temps en mostres de carn de porc emmagatzemades durant 13 dies a 10 ^{o}C.

Es van obtenir microesferes d'alginato amb eugenol i es van incorporar en pel·lícules de quitosano, les propietats físiques i estructurals de les quals es van veure afectades. Les microesferes van ser visibles en l'estructura de les pel·lícules per SEM. La incorporació de les microesferes va promoure una millora significativa en les propietats barrera a l'oxigen, alhora que un augment en la permeabilitat al vapor d'aigua de les pel·lícules. L'addició de les microesferes també va modificar el comportament mecànic de les pel·lícules, disminuint la seua rigidesa i elasticitat, i augmentant la seua extensibilitat. L'alliberament més ràpid de eugenol es va observar en el simulant àcid acètic (3 %), la qual cosa es va atribuir a la major dissolució de la matriu de quitosano en aquest solvent. L'addició de les microcàpsules va conferir capacitat antimicrobiana als films de quitosano contra *L. innocua* i *E. coli*.

I. INTRODUCTION

Petroleum-based conventional plastics are used for a wide variety of applications, such as food packaging, due to their durability, good mechanical and barrier properties, ease of processing and low cost. However, in recent years an increasing concern has arisen due to the environmental implications, given that these materials take hundreds of years to decompose (Sun et al., 2013; Debiagi et al., 2014).

Aiming to solve this problem, biodegradable polymers such as chitin, lignin, cellulose, starch and proteins have been widely studied. All these natural polymers have proved to be ideal raw materials for preparing biodegradable composites (Garlotta, 2001), hence may be an alternative to polluting synthetic polymers. Apart from being biodegradable, they are non-toxic and edible, and they can serve as carriers of antimicrobial and antioxidant agents (Balaguer et al., 2014), which allows for the development of active food packaging materials.

I.1 Starch as base material for biodegradable packaging applications

Starch is one of the most promising materials for biodegradable packaging applications, given its low cost, easy renewability, wide availability, sustainable production and good processability by means of conventional techniques (Shen et al., 2009; Jiménez et al., 2012; Cai et al., 2014; Garcia et al., 2014; López et al., 2014; Soares et al., 2014; Cano et al., 2014; Alves et al., 2015; Lourdin et al., 1995). It is one of the most abundant natural polysaccharide raw materials. For instance, the starch content is around 30 to 80 % in cereals (maize, wheat and rice), 25 to 50 % in legumes (pea and bean) and 60 to 90 % in tubers (potato and cassava) (Espinosa, 2008).

Starch granules can vary in shape, size, structure and chemical composition, depending on the source (Smith, 2001). Native starch is chemically composed of two main macromolecular components: amylose and amylopectin (**Figure I.1**). Amylose is a nearly linear polymer of α-1,4 anhydroglucose units that has excellent film-forming ability, rendering strong, isotropic, odorless, tasteless and colorless films (Campos et al., 2011). Amylopectin is a highly branched polymer of short α-1, 4 chains linked by α-1, 6 glucosidic branching points occurring every 25-30 glucose units (Durrani & Donald, 1995; Liu, 2005).

Native starches take the form of granules where both amylose and amylopectin are structured by hydrogen-bonding, containing crystalline and non-crystalline regions in alternating layers (Jenkins et al., 1993). Amylose and the branching points of amylopectin form the amorphous regions while the short-branched chains in the amylopectin are the main crystalline components. So, the higher content of amylopectin in native starch means greater crystallinity

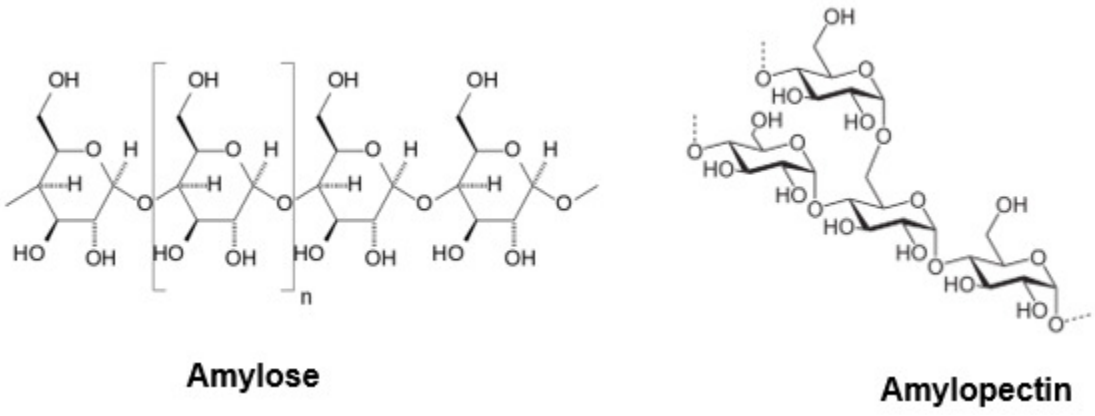

Figure I.1 Chemical structure of amylose and amylopectin.

(Cheetham & Tao, 1998). Starch generally contains 20 to 25 % amylose and 75 to 80 % amylopectin (Brown & Poon, 2005). For instance, wheat, corn and potato starches contain 20-30 % amylose, while its content in waxy starches is lower than 5 % and in high-amylose starches is as high as 50-80 % (Liu, 2005). The amylose:amylopectin ratio, and hence the starch origin, significantly impact its mechanical strength and flexibility, since these are affected by the strength of the crystalline region (Ruiz, 2006).

Starch granules are insoluble in cold water due to the fact that strong hydrogen bonds hold the starch chains together (Jiménez et al., 2012). However, when starch is heated in water, the crystalline structure is disrupted and water molecules interact with the hydroxyl groups of amylose and amylopectin, producing the partial solubilisation of starch (Hoover, 2001). Heating starch suspensions in an excess of water or another solvent with the ability to form hydrogen bonds and at high temperatures (between 65 and 100°C approximately, depending on the type of starch) provokes an irreversible gelatinization (de-structuration) process. This process is greatly affected by the kind of solvent and the starch/solvent ratio, and it introduces irreversible changes in the starch granules, such as lixiviation of amylose, loss of crystallinity, water absorption and swelling of the granules (Zhong et al., 2009; Carvalho, 2008). The gelatinization process initiates at low temperature and continues until the granules are completely disrupted, according to the following steps (Ratnayake & Jackson, 2007): (1) the absorption of water by starch granules promotes an increase in starch polymer mobility in the amorphous regions; (2) Starch polymers in the amorphous regions rearrange, often forming new intermolecular interactions; (3) with increasing hydrothermal effects, the polymers become more mobile and lose their intermolecular interactions and overall granular structure. At the end of the process, low molecular weight amylose chains are highly hydrated, including aggregates, which are also hydrated. After the gelatinization, there is a spontaneous

recrystallization process, when the linear chains of amylose and amylopectin re-associate by hydrogen bonds (Cano, 2015).

Thus, although native starch is not a thermoplastic material, thermoplastic starch (TPS) can be obtained after gelatinization (i.e. heat treatment with plasticizers). Subsequently, TPS can be processed like conventional polymers, and starch films can be obtained by two main techniques: solution casting followed by drying (wet method) and thermoplastic processing (dry method) (Romero-Bastidas et al., 2005; Paes et al., 2008).

When using the wet or casting process, the granules of native starch have to be disrupted through a gelatinization process in an excess of water in order to obtain TPS, hence enabling starch to form a film (Carvalho, 2008). The complete process could be divided into several steps: gelatinization and dispersion of the raw material, homogenization of the blends, casting on leveled petry or Teflon® dishes, and drying under controlled temperature and relative humidity. Usually, the presence of plasticizers (such as glycerol) is also necessary in order to reduce the brittleness of the pure starch films obtained by casting. Some authors mentioned that in the films preparation from starch it is crucial to add plasticizers that facilitate the flow and increase the flexibility of the film, increasing its resistance to breakage (Sothornvit & Krochta, 2005; Habitante et al., 2008). For this purpose polyalcohols such as glycerol, sorbitol, mannitol, sucrose, invert sugar, propylene glycol and polyethylene glycol are used (Gontard et al., 1993; Mali et al., 2006).

When dealing with dry processes, raw materials with thermoplastic properties that can be molded by thermal or mechanical processes with the aid of plasticizers are used so that the obtained materials do not present fragility at room temperature (Forsell et al., 1997). In this sense, Carvalho, (2008) described thermoplastic starch (TPS) as an amorphous or semi-crystalline material composed of gelatinized or destructurized starch containing one or a mixture of plasticizers. TPS can be repeatedly softened and hardened so that it can be moulded/shaped by the action of heat and shear forces, thus allowing its processing to be conducted with the techniques commonly used in the plastics industry.

TPS can be processed with the standard equipment's used for synthetic polymers such as compression molding, extrusion, co-extrusion, injection molding, blowing extrusion, flat film extrusion and blowing radiation (Van Soest et al., 1996b; Zhai et al., 2004).

The final properties of the starch materials are affected by the type of starch, chemical modifications and processing conditions (Chaudhary et al., 2008), but this polymer generally

yields an odourless, colourless and transparent polymer matrix with very low oxygen permeability, which can protect food products by forming an oxygen barrier (Vásconez et al., 2009). However, it also exhibits some disadvantages such as a strong hydrophilic character, a relatively high water vapour permeability (Lafargue et al., 2007; Chen et al., 2008; Phan The et al., 2009; Wu et al., 2010) as compared to synthetic polymers (Curvelo et al., 2001; Avérous & Boquillon, 2004; Ma et al., 2009; Castillo et al., 2013), and unstable mechanical properties due to the phenomenon of recrystallization throughout time (López et al., 2013; Taghizadeh & Favis, 2013; Salaberria et al., 2014).

Different strategies have been tested aiming to improve the properties of starch-based materials, such as thermo-mechanical processing and the incorporation of plasticizers (Ortega-Toro, 2015; Moscicki et al., 2012; López et al., 2013; Yu et al., 2013), starch modification in which the hydroxyls have been replaced by ester or ether groups (e.g. carboxymethyl starch and hydroxypropylated starch) (Olsson et al., 2014), reinforcement through fibers (Behall et al., 1998; Wollerdorfer & Bader, 1998), nanoparticles (Matsui et al., 2004; Souza et al., 2012), or clays (Huang et al., 2005; Wang et al., 2002), or blending with nanoclays to form starch nanocomposites (Liu et al., 2009), bilayer or multilayers formation (Ortega-Toro et al., 2015; Dole et al., 2005), using a compatibilizer to enhance interfacial adhesion of starch (Wang & Wang, 2003; Zuo et al., 2015). Depending on the qualities of the components, blending with other biodegradable polymers may lead to improved physical properties of the starch materials, such as reduced water vapor and oxygen permeabilities, mechanical improvement or better optical properties (Cuq et al., 1998; Wang et al., 2003; Avérous & Boquillon, 2004; Villada et al., 2007; Cano et al., 2014). Chitosan has proved its efficiency in this respect, mainly due to its more hydrophobic character as compared to starch (Ouattara et al., 2000a; Chillo et al., 2008; Lazaridou & Biliaderis, 2002; Fajardo et al., 2010; Pelissari et al., 2011; Pelissari et al., 2012; Lopez et., 2014; Shapi'I & Othman, 2016).

I.2 Cassava starch

Cassava starch has been used to produce environmentally safe food packaging films with excellent properties such as high transparency and low oxygen permeability (Poovarodom & Praditdoung, 2003). Coherently with the starch properties reported above, it is a cheap and abundant material, able to form a continuous polymer matrix (Bergo et al., 2012; Bergo et al., 2010a; Bergo et al., 2010b; Bergo et al., 2008), and flexible, tasteless, odourless, colorless, transparent, nontoxic and biologically degradable films (Chiumarelli & Hubinger, 2014; Belibi et al., 2014; Chang et al., 2000), even without plasticizer (Vicentini et al., 2005). Cassava starch

is appreciated for its paste clarity, low gelatinization temperature and good gel stability (Sedas & Kubiak, 1994). The suitability of cassava starch for films production was demonstrated by Hernández-Medina et al. (2008), since it presented great swelling power (Cheng et al., 1996; Moorthy, 2002), elasticity and water absorption capacity when compared to makal, sweet potato and sago starches. These results were coherent with Cheng et al. (1996), Moorthy (2002), Gujska et al. (1994), Bello-Pérez (1995) and Novelo & Betancur (2005). **Table I.1** gives an overview of the most relevant results recently reported for cassava starch films obtained by either casting or thermoplastic processing.

In spite of the advantages described, the application of cassava starch films is still limited by its high solubility in water, brittleness and difficult processing, hence requiring the improvement of its mechanical and barrier properties (Pelissari et al., 2009). A commonly used approach to overcome these drawbacks and provide further functional properties is to blend starch with other natural biopolymers in order to formulate composite materials (Jagannath et al., 2003; Yu et al., 2006) as well as testing the effectiveness of the plasticizer added at different ratios. Parra et al. (2004) observed improved mechanical and water barrier behavior when polyethylene glycol (PEG) and glutaraldehyde (GLU) were added to cassava starch films. Some authors found that glycerol and different sugars were compatible with cassava starch, improving their flexibility and avoiding the formation of fractures during the manipulation of the film, but they greatly diminished its barrier power, which improved with the addition of nanoparticles of clays (Su et al., 2010; Souza et al., 2012). Brandelero et al. (2011) reported that the structural properties of the films directly influence their barrier properties, since more compact films gave rise to lower water vapour permeability and manufacturing defects, such as pores and cracks, increased this property.

Several studies have been focused on blending cassava starch with other polymers, either synthetic or biodegradable, in order to improve their physical properties. Acosta et al. (2015) studied the incorporation of gelatin into cassava starch matrices containing glycerol as a plasticizer, and concluded that the blended films exhibited significantly higher hardness, resistance to break and extensibility than pure starch films. Other authors reported that by increasing the concentration of carboxymethylcellulose in cassava starch films, tensile strength was increased, whereas elongation at break and solubility in water were reduced. (Tongdeesoontorn et al., 2011). In another study, Chillo et al. (2008) reported that the mechanical and barrier properties of cassava starch-based films obtained by casting were influenced by chitosan and glycerol concentrations. The chitosan addition had a possitive

effect on the mechanical properties, whereas glycerol had a positive influence on the barrier properties. Similar results were obtained by Dang et al. (2015) for films obtained by blow extrusion. Likewise, Pelissari et al. (2012) reported that small amounts of chitosan (<5 %) increased the mechanical resistance of cassava starch films obtained by extrusion.

Soares et al., (2013) studied thermopressed TPS and poly (lactic acid) (TPS/PLA) blends coated with chitosan by spraying and immersion, and found that the chitosan coating reduced the water solubility. Soares et al. (2014) tested the effect of slow cooling on the same formulations. They found that the mechanical properties did not improve with the slow cooling, but water permeability was decreased. Shirai et al. (2013) mentioned that PLA reduced the water permeability and the opacity, while increasing the rigidity of films obtained by blow extrusion. Wootthikanokkhan et al. (2012) developed maleated thermoplastic cassava starch /PLA blends obtained by compression moulding. Besides the blending ratio, time and temperature affected the mechanical, rheological and morphological properties of blends.

Teixeira et al. (2012) found that the fiber present in the cassava bagasse acted as a reinforcement in the thermoplastic starch-PLA blends obtained by extrusion, thus increasing the tensile strength. Olivato et al. (2012) studied the influence of citric acid, malic acid and tartaric acid in starch/poly (butylene adipate co-terephthalate) films obtained by blow extrusion. Organic acids improved the properties of the blends by crosslinking the polymeric chains, yielding more resistant and less permeable films.

Prachayawarakorn & Pomdage (2014) observed a significant improvement in the mechanical properties due to carrageenan addition on TPS/low-density polyethylene composite blends reinforced by cotton fibres and processed by compounding and injection moulding. Müller et al. (2012) incorporated nanoclays on cassava starch films obtained by compression moulding, and reported the increased strength and decreased water permeability due to nanoclays. Similar results were reported by Lomelí-Ramírez et al. (2014) by incorporating green coconut fibre into cassava starch. Melo et al. (2011) obtained cassava starch films with xanthan gum and glycerol by extrusion and casting. Xanthan gum addition improved the permeability to water vapor, being better by casting. Also, they observed that there were no significant differences in mechanical properties in the tests performed at different relative humidities.

Table I.1 Summary of results obtained for cassava starch films obtained by different techniques (casting and thermoplastic processing).

Other film compounds (X)	CS: X ratio	Plasticizer	Applied method	Films conditioned	Relevant result	References
Gelatin	0-100:100-0	Glycerol: 30 %	Casting	54 % R.H. at 25°C- 48h.	Good mechanical properties	Acosta et al. (2015)
Carboxymethylcellulose	5:0-40	Glycerol: 30 %	Casting	53 % R.H. at 25°C- 24h.	Good mechanical properties	Tongdeesoontorn et al. (2011)
Chitosan	4:0-1	Glycerol: 0-1.25 %	Casting	57.5-57.7 % R.H. at 25°C-48h.	Good mechanical properties	Chillo et al. (2008)
Nanoclays	0-96:0-6	Glycerol: 25 %	Compression moulding	75-0 % R.H.	Effect on the WVP	Müller et al. (2012)
Coconut fiber	0-8:0-30	Glycerol: 30 %	Compression moulding	60 % R.H. at 25°C.	Good thermal stability	Lomelí-Ramírez et . al. (2014)
Poly (lactic acid) (PLA)	20-40:60-80	Glycerol: 25 %	Compression moulding	N.R.	Good mechanical properties	Wootthikanokkhan et al. (2012)

N.R: Not Reported

Table I.1 (Continued).

Other film compounds (X)	CS: X ratio	Plasticizer	Applied method	Films conditioned	Relevant result	References
Poly (lactic acid) (PLA), Chitosan	70:30+CH (spray)	Glycerol: 30 %	Compression moulding	N.R.	PLA stabilized the WVP	Soares et al. (2013)
Poly (lactic acid) (PLA), Chitosan	70:30+CH (0.1 %)	Glycerol: 30 %	Compression moulding	N.R.	Effect on the WVP	Soares et al. (2014)
Chitosan	70-82:0-5	Glycerol: 18-25 %	Extrusion	64 % R.H. at 25°C-48h.	More rigid films	Pelissari et al. (2012)
Clay nanoparticles	5:0-0.1	Glycerol: 0.75-1.25 %	Extrusion	75 % R.H. at 25°C- 7d.	Effect on the WVP	Souza et al. (2012)
Poly (lactic acid) (PLA), Cassava bagasse	80:20	Glycerol: 30 %	Extrusion	53 % R.H.	Good mechanical properties	Teixeira et al. (2012)
Butylene adipate coterephthalate (PBAT)	0-80:0-50	Glycerol: 30 %	Blow extrusion	90-64 % R.H. at 48h-30d.	Good mechanical properties	Brandelero et al. (2011)

N.R: Not Reported

Table I.1 (Continued).

Other film compounds (X)	CS: X ratio	Plasticizer	Applied method	Films conditioned	Relevant result	References
Butylene adipate co-terephthalate PBAT)/ Tartaric, malic and citric acid	55:45+0.75-1.5 % (Tartaric, malic and citric acid)	Glycerol: 18 %	Blow extrusion	75-33 % R.H.	WVP were improved when the acids were added	Olivato et al. (2012)
Chitosan	70:30+CH (0.1 %)	Glycerol: 30 %	Blow extrusion	62-42 % R.H.-2 d.	Good mechanical properties	Dang et al., (2015)
Butylene adipate co-terephthalate PBAT)/ Poly (lactic acid)(PLA)	60:40 PBAT 75:25 PLA	Glycerol: 32 %	Blow extrusion	62-42 % R.H.-48h.	WVP were WVP	Shirai et al. (2013)
Blends with non-biodegradable plastics/ Carrageenan (CG) and cotton fibers (CF)	70:0-10 %(CG) +5 %(CF)	Glycerol: 30 %	Injection molding	60 % R.H. at 23°C - 24h.	Good mechanical properties	Prachayawarakorn et al. (2014)
Xanthan gum co-terephthalate PBAT)/ Poly (lactic acid)(PLA)	20:0-10 %	Glycerol: 20 %	Extrusion and casting	60 % R.H. at 25°C - 7d.	Xanthan gum addition improved the WVP	Melo et al. (2011)

I.3 Chitosan and its applications in biodegradable packaging

CH is a linear co-polymer cationic polysaccharide consisting of β (1-4)-linked 2-amino-2-deoxy-D-glucose (D-glucosamine) and 2-acetamido-2-deoxy-D-glucose (N-acetyl-D-glucosamine) units (**Figure I.2**). The properties, biodegradability and biological role of chitosan is frequently dependent on the relative proportions of N-acetyl-D-glucosamine and D-glucosamine residues (George & Abraham, 2006).

This renewable, non-toxic, biodegradable, biocompatible polymer (Shahidi et al., 1999; Beverlya et al., 2008; Barikani et al., 2014) is obtained from chitin's partial deacetylation by alkaline hydrolysis or enzymatic method (Morley et al., 2006; Sagheer et al., 2009). Chitin is the primary structural component of the outer skeletons of crustaceans, and is also found in many other species such as molluscs, insects and fungi (George & Abraham, 2006).

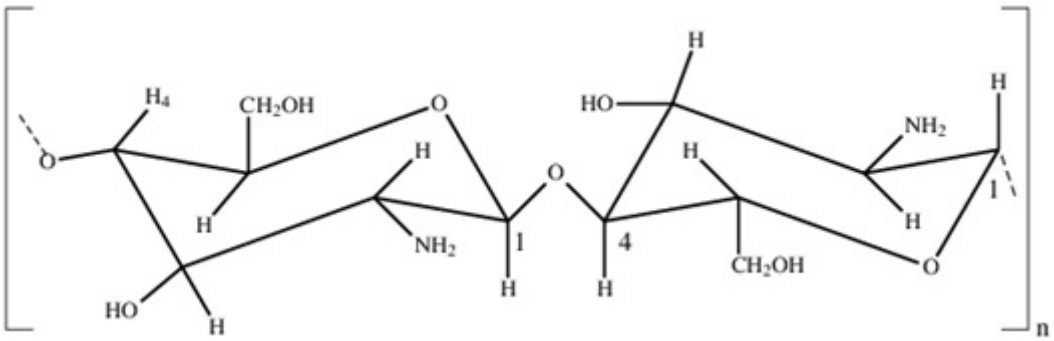

Figure I.2 Chemical structure of chitosan.

Chitosan can exhibit different degrees of deacetylation (DDA), defined as the percentage of primary amino groups in the polymer backbone, as well as different average molecular weights (Roberts, 1992). The protonation of amino groups increases the polyelectrolyte charge, leading to changes in structure, properties and applications. In general, an N-deacetylation degree between 55 % and 70 % is considered as low, 70 to 85 % is considered as medium, 85 to 95 % is referred to as high and more than 95 % is called ultrahigh (He et al., 2016). Likewise, different types of CH can be found according to its molecular weight: low (50,000 - 190,000 Da), medium (190,000 - 310,000 Da) and high (310,000 - 375,000 Da) (Shahidi & Abuzaytoun, 2005).

Most chitosans are insoluble in water and in most common organic solvents (van den Broek et al., 2015). However, they can be easily dissolved in acidic aqueous solutions below pH 6.3, although at concentrations above >2 wt % they become very viscous (Kaur & Dhillon, 2014; Yeul & Rayalu, 2013). The conformation of CH in solution greatly depends on both the

structural parameters of the molecule (DDA and chain length) and solution parameters, such as ionic strength, pH and dielectric constant of the solvent (Sorlier et al., 2002). Therefore, the properties of the solvent play a very important role in the behaviour of chitosan-based film-forming solutions or dispersions. Kim et al. (2006) reported that the water permeability was significantly affected by the different DDA of chitosan, solvent pH and type of acid (formic acid, lactic acid, acetic acid and propionic acid), which interacted strongly with each other. Higher water permeability was measured for highly de-acetylated chitosan.

Since chitosan has shown to have antimicrobial properties against bacteria, yeasts, moulds and fungi (Friedman & Juneja, 2010; Rabea et al., 2003; Saggiorato et al., 2012; Avila-Sosa et al., 2012, Perdones et al, 2012, 2014 & 2016; Alves-Silva et al., 2013; Roselló et al., 2015), it finds application not only as a component of packaging material but also as a food additive or preservative to both retard microorganisms growth in food and ultimately improve the quality and shelf life of the product (Kong et al., 2010).

The mode of action of chitosan is influenced by different factors (Kong et al., 2010) such as its molecular weight (Kong et al., 2010; Park et al., 2008, 2011; Zheng & Zhu, 2003) and degree of deacetylation (Verlee et al., 2017), as well has type of microorganism. Some works reported that chitosan is most active at the cell surface of fungi or bacteria leading to permeabilization (Verlee et al., 2017), thus leading to leakage of intracellular material resulting in cell death (Chung & Chen, 2008; Costa et al., 2012; Liu et al., 2004; Raafat et al., 2008).

The mechanism of antibacterial action of CH against Gram-positive and Gram-negative bacteria is different because of the structural differences of the bacteria (Kong et al., 2010). In the case of Gram negative bacteria, there are two mechanisms which are believed to be active at the outer membrane (Verlee et al., 2017). The first mechanism is linked to the chelation effect of chitosan with different cations when the pH is above the pK_a (Bassi et al., 1999; Goy et al., 2009; Wang et al., 2005). Chitosan (pK_a 6.3-6.5) has the best antimicrobial properties at low pH due to the protonated amino groups. This can result in the disruption of the cell wall integrity and disturbs the uptake of important nutrients (Ca^{2+}, Mg^{2+}, etc.) (Goy et al., 2009; Kong et al., 2010). The second effect is the electrostatic interaction of chitosan and the anionic parts of the lipopolysaccharide at the outer membrane (Helander et al., 2001; Liu et al., 2004).

For Gram positive bacteria, it has been described that chitosan binds non-covalently with teichoic acids incorporated in the peptidoglycan layer (Raafat et al., 2008). These teichoic acids on the cell surface are important for cell division and other fundamental aspects of the

Gram positive bacterial physiology (Brown et al., 2013). Most likely, the electrostatic interaction of chitosan with these teichoic acids disrupts the functioning of the teichoic acids, and this probably leads to disruption of the cell functioning. Other cationic bactericides have also shown activity towards Gram positive bacteria due to these anionic teichoic acids (Neuhaus and Baddiley, 2003). Electrostatic interactions between the anionic cell surface and chitosan are important factors determining the antimicrobial activity of chitosan against fungi and bacteria (Verlee et al., 2017).

Apart from these, other mechanisms have been suggested, such as chitosan affecting DNA expression by binding to nucleic acids (Galvan Marquez et al., 2013; Junguang et al., 2007; Park et al., 2011) or chitosan working as chelating agent of essential minerals (Benhabiles et al., 2012; Jing et al., 2007; Goy et al., 2009; Martinez-Camacho et al., 2013)

Due to chitosan's antimicrobial activity, non-toxicity and low permeability to oxygen, chitosan films have a great potential to be used as active packaging material (Kanatt et al., 2012; Leceta et al., 2013). In fact, this polymer presents an excellent film-forming ability with no need for plasticizing compounds (Domard & Domard, 2001; Kittur et al., 1998; Vargas & González-Martínez, 2010). Therefore, chitosan can be used as a carrying material in films with bioactive substances or antimicrobial agents, these gradually migrating from the packaging onto the surface of the food (Elsabee & Abdou, 2013) and hence controlling microbial contamination on fresh or processed foods. Moreover, the incorporation of chitosan into biodegradable films based in other materials can provide them with antimicrobial character, offering protection against contamination and microbial spoilage.

Chitosan presents excellent biocompatibility with other biopolymers and lipids and, as compared with starch, presents more hydrophobic character (Mendes et al., 2016; Dang & Yoksan, 2015). Therefore, blending TPS and chitosan represents an alternative route to obtain more humidity resistant materials (van den Broek et al., 2015). It has been reported that increasing concentrations of chitosan affect the mechanical properties of starch films, resulting in an increase in tensile strength. Also, water absorption and water vapor permeability of cassava starch films may be reduced due to the hydrophobic character of chitosan (Bangyekan et al., 2006). In this sense, Vásconez et al. (2009) reported that the addition of chitosan reduced water vapor permeability and water solubility of cassava starch films, whereas the mechanical properties were significantly improved as the chitosan ratio increased in the films, these being more resistant and extensible. Moreover, chitosan seemed to inhibit starch retrogradation. Similar results were observed by Pelissari et al. (2012), who reported

that a higher concentration of chitosan favored the formation of more rigid and opaque, and less permeable films. Likewise, Dang et al. (2015) observed that the incorporation of chitosan caused increased tensile strength, rigidity and thermal stability, as well as reduced water absorption and surface stickiness. The same effect on the mechanical properties was observed by Zhai et al. (2004) in corn starch/chitosan films. In addition, X-ray diffraction and scanning electron microscope analysis of the blend films indicated the interaction between the polymers. Other authors reported that the incorporation of chitosan resulted in an increase in film solubility, tensile strength and elongation at break, along with a decrease in Young's modulus and water vapor permeability (Ren et al., 2017).

In other study, Shen et al. (2010) developed antimicrobial films with sweet potato starch and chitosan, where chitosan incorporation improved the tensile strength and elongation at break while decreasing the oxygen permeability, water vapor permeability and water solubility. Similar results were observed for potato films containing chitosan (Mollah et al., 2016). In rice starch/chitosan films, Bourtoom & Chinnan (2008) observed an increase in tensile strength and water vapor permeability, along with decreased elongation at break and film solubility due to chitosan incorporation. However, too high a concentration of chitosan yielded phase separation between starch and chitosan. On the other hand, Bonilla et al. (2013a) found that chitosan addition into starch formulations yielded glossier and thicker films, while improving the mechanical properties and inhibiting starch retrogradation. Also, a notable antimicrobial effect was detected in the blend films when the proportion of chitosan was 50 %.

I.4 Essential oils and their application into biodegradable films

In recent years there has been a considerable pressure by consumers to reduce or eliminate chemically synthesized additives in foods. On the other hand, the interest in the possible use of natural alternatives to food additives to prevent bacterial and fungal growth has notably increased (Lanciotti et al., 2004). In this sense, the use of natural products such as essential oils (EOs), as food preserving agents is being promoted (Quesada et al., 2016). EOs have shown antioxidant properties as well as antimicrobial effects against mold, yeasts, bacteria and viruses, mainly due to their bioactive components such as flavonoids, terpenes, carotenes, etc. (Burt, 2004; Kuorwel et al., 2011). In addition, most of them are classified as Generally Recognized As Safe (GRAS) (Ruiz-Navajas et al., 2013).

EOs are aromatic liquids obtained from plant materials (flowers, buds, seeds, leaves, bark, etc.). Steam distillation is the most common method for extracting EOs without loss of their

properties. EOs can include more than sixty individual components but major components can constitute up to 85 % of the oil, whereas other components are present only as a trace (Burt, 2004). The main constituents of EOs are terpenes (such as limonene, β-pinene, p-cymene, β-linalool, citral, γ-terpinene and estragol) and phenols (such as carvacrol, thymol and eugenol). The small terpenoids and phenolic compounds found in their chemical composition appear to be responsible for the antimicrobial activity of different EOs (Helander et al., 1998), since the pure compounds are able to significantly decrease pathogenic bacteria counts in foods (Burt, 2004). The antimicrobial effect of EOs against bacteria has been extensively studied and reported (Smith-Palmer et al., 1998; Burt, 2004; Bakkali et al., 2008; Sivakumar & Bautista-Baños, 2014; Rivera Calo et al., 2015). Gram-positive bacteria have been reported to be slightly more sensitive to EOs than Gram-negative (Aguirre et al., 2013; Burt, 2004; Corrales et al., 2014; Martucci et al., 2015; Smith-Palmer et al., 2001). This difference is related to the presence of an additional, relatively impermeable, outer membrane surrounding the cell wall in Gram-negative bacteria, which restricts the diffusion of hydrophobic compounds through its lipopolysaccharide covering (Burt, 2004; Sánchez-González et al., 2011b).

Rather than a single specific mechanism, the combined effect of different substances explains the antimicrobial activity of EOs (Burt, 2004; Corrales et al., 2014). Some authors indicated that phenolic compounds of EOs, such as carvacrol, eugenol and thymol, can attack the phospholipid cell membrane leading to an increase in both the permeability and leakage of the cytoplasm, or an interaction with enzymes located on the cell wall (Burt, 2004; Campos et al., 2011; Zivanovic et al., 2005; Matan et al., 2006).

Clove, cinnamon, oregano and rosemary are considered as the most common spices and herbs with strong antimicrobial activity (Weerakkody et al., 2010). Their EOs contain carvacrol, cinnamaldhyde, eugenol and camphor, which have been identified as the major chemical components responsible for exerting antimicrobial activity (El-Massry et al., 2008; Kordali et al., 2008; Singh et al., 2007; Zawirska-Wojtasiak & Wasowicz, 2009). **Table I.2** shows the main components of oreganum and cinnamon leaf essential oils, and **Figure I.3** shows the chemical structure of their major compounds (eugenol and carvacrol).

Of the essential oils, oregano has been found to be one of the most effective against microbial growth, as demonstrated in numerous studies (Aguirre et al., 2013; Emiroğlu et al., 2010; Zivanovic et al., 2005). Carvacrol, thymol, γ-therpinene and p-cymene are its principal constituents (Benavides et al., 2012; Burt, 2004; Jouki et al., 2014; Kuorwel et al., 2011; Sadaka et al., 2014; Sánchez-González et al., 2011b) and the antimicrobial activity is

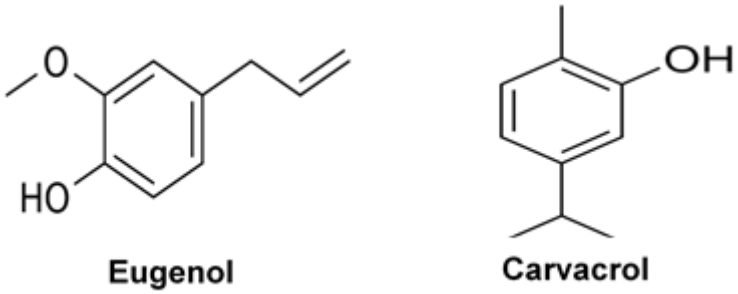

Figure I.3 Chemical structure of eugenol and carvacrol.

Table I.2 Main constituents of cinnamon leaf and oregano essential oils and their wt. percentage range, as reported by several authors.

Essential oil	CS: Compounds	%	References
Cinnamon leaf	Eugenol	73.27-83.04	Ribeiro-Santos et
(Cinnamomun	β-caryophyllene	5.38	al. (2017)
zeylanicum)	Benzylbenzoate	4.04-3.84	Ribes et al. (2017)
	Linalool	3.31	
	Cinnamyl acetate	2.53 -1.09	
Oregano	Carvacrol	43.24-71.8	Morshedloo et al.
(Origanum vulgare)	γ-terpinene	13.52-14.16	(2017)
	p-cymene	11.46-11.63	Pesavento et al.
	Thymol	1.6-4.51	(2015)
			Azevedo et al.
			(2015)

related to the synergic effect established among them. Carvacrol is a phenolic compound with demonstrated antimicrobial activity against bacteria (Periago et al., 2004), fungi (Daferera et al., 2003) and yeasts (Arora & Kaur, 1999), showing a high potential to improve the shelf life and safety of perishable foods (Holley & Patel, 2005). This compound disintegrates the outer membrane of Gram-negative bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane to ATP (Burt, 2004).

Cinnamon leaf essential oil (CLEO) is recognized for its flavor and aroma in addition to its antimicrobial properties (Singh et al., 2007; Ayala-Zavala et al., 2008). Antifungal and antioxidant properties of CLEO are due to volatile components such as cinnamaldehyde and eugenol (Combrinck et al., 2011). Its main component, eugenol, has been long known for its analgesic, local anesthetic, anti-inflammatory and antibacterial effects (Hemaiswarya &

Doble, 2009). It is a known antibacterial agent against pathogens including *Escherichia coli, Listeria monocytogenes* (Blaszyk & Holley, 1998), *Campylobacter jejuni, Salmonella enterica, Staphylococcus aureus, Lactobacillus sakei* and *Helicobacter pyroli* (Friedman et al., 2002; Walsh et al., 2003). Du et al. (2009) prepared apple films with either cinnamon, allspice or clove bud essential oils and placed them on media plates inoculated with either *E. coli, S. enterica or L. monocytogenes*. For all three bacteria, cinnamon oil had the largest zone of inhibition, which indicates its antimicrobial power. The FDA (1997) classified clove, oregano, thyme, nutmeg, basil, mustard and cinnamon essential oils as GRAS. The main component of cinnamon leaf essential oil, eugenol (4-allyl-2-methoxyphenol), and the main component of oregano EO (carvacrol) have also been recognized as GRAS (Hemaiswarya & Doble, 2009; Hyldgaard et al., 2012, FDA 2016).

Despite their effectiveness and safety at low concentrations making them suitable food preservatives, the main drawbacks of EOs are their high volatility and their notorious odor, which can alter organoleptic properties of fresh food, thus limiting their utilization (Burt & Reinders, 2003; Corrales et al., 2014). Also, EOs are very sensitive to thermal effects and oxidation (Piletti et al., 2017; Ribeiro- Santos et al., 2017). Inclusion in a biodegradable polymer film could be an effective strategy aiming to solve these disadvantages. This way, active packaging materials incorporating EOs as natural preservatives could be formulated via either direct incorporation into the packaging (by coating, adsorption or direct blend with the polymer, Sadaka et al., 2014) or microencapsulation using different techniques (Bakry et al., 2016). In this sense, packaging materials, edible films and coatings can be fortified with these antimicrobials in order to create a protective barrier to help to control the growth of pathogenic and spoilage microorganisms and to prevent post-contamination of foods (Kristo et al., 2008; Azeredo, 2009). The antimicrobial activity of EOs would then take place as their components, entrapped in the polymeric matrices, are released either by direct contact with the food or into the headspace surrounding it. The controlled release of these antimicrobials into the food product may then prolong its shelf life, quality and safety. However, it should be noted that the release may be affected by many factors, including electrostatic interactions between the antimicrobial agent and the polymer chains, structural changes induced by the presence of antimicrobial, and environmental conditions (Avila-Sosa et al., 2012).

Antimicrobial biodegradable films can be formulated by using inherently antimicrobial polymers as matrix material, such as chitosan. EOs could then contribute to preserve the film, at the same time that their compounds interact with the polymer molecules and can

act as plasticizers (Ruiz-Navajas et al., 2013). Chitosan-based films and coatings have been formulated by incorporation of different EOs such as lemon, basil, thyme, cinnamon leaf and oregano (Bonilla et al., 2013b; Bonilla et al., 2014; Sánchez-González et al., 2011a, 2011b, 2011c, 2011d and 2011e; Wang et al., 2011; Randazzo et al., 2016; Abdollahi et al., 2012; Hosseini et al., 2009; Fernández-Pan et al., 2015: Perdones et al., 2012; Ojagh et al., 2010; Pelissari, et al. 2009). The effect of the EOs incorporation on the film properties depends on the concentration, on the type of molecules, the structure and stability of the film-forming emulsion (lipid droplets in the chitosan aqueous phase) (Perdones, 2015) and the homogenization technique (Atarés & Chiralt, 2016). **Table I.3** shows a representative selection of recent studies dealing with chitosan based films incorporated with EOs by homogenization, in order to give an overview of the tests usually performed when dealing with the effect of EOs addition on the structure and physical properties of CH films.

When EOs are incorporated into the film forming dispersion by emulsification or homogenization techniques, fine emulsions are obtained containing polymer at the continuous aqueous phase. Droplet reduction favours the intimate EO incorporation into the polymer matrix, giving rise to increased interactions between the polymer and the oils, which may result in a less cohesive polymer matrix (Bonilla et al., 2012). Ojagh et al. (2010) studied the incorporation of cinnamon EO into chitosan films, and observed sheet-like structures stacked in compact layers when the oil was added. The films with this lipid showed improved water barrier properties, higher tensile strength and lower stretchability. Similar results were observed by Shen & Kamdem (2015) for CH films containing citronella and cedarwood essential oils. Bonilla et al. (2012) found that the induced close contact polymer-oil caused by high pressure homogenization gave rise to more fragile films of increased stretchability, due the weakening effect on the chitosan matrix where chain interaction forces are reduced. Other authors observed that adding non-encapsulated essential oils only caused a slight elastic modulus reduction of CH films, which can be explained by the low lipid ratio and the arrangement of the lipid droplets in the film matrix (Jiménez et al., 2014; Valencia-Sullca et al., 2016). The tensile strength decrease may be accompanied by either an increase or a decrease of percentage elongation (Valencia-Sullca et al., 2016). In this sense, the EOs proportion determines its effect on elongation at break (Pranoto et al., 2005).

Recent studies have analysed the effect of EO incorporation on barrier properties of CH films. Reyes-Chaparro et al. (2015) reported a positive effect of clove extract incorporation on the water vapour barrier properties of chitosan films. WVP reduction, coherently with the

concentration of EO, was also observed by Pelissari et al. (2009). Due to the hydrophobic character and complexity of EOs, as these are incorporated the increased film hydrophobicity will reduce the water absorption hence increasing oxygen permeability (Atarés & Chiralt, 2016). Perdones et al. (2014) reported a significant increase in oxygen permeability of chitosan films when oleic acid or CLEO were incorporated, especially at the highest lipid content.

The optical properties of chitosan-based films are also affected by the nature and concentration of the added lipids. Moradi et al. (2012) reported that the incorporation of *Zataria multiflora Boiss* EO did not affect the colour in the CH films, while the incorporated grape seed extract had gave them a brownish color. Ojagh et al. (2010) elaborated chitosan films with cinnamon EO and reported a yellowness increase. Likewise, Siripatrawan & Harte (2010) incorporated green tea extract into chitosan films, and found significant colour changes as the extract concentrations increased. A transparency reduction caused by EOs addition was also observed by López-Mata et al. (2013) when working with carvacrol incorporated CH films. The same effect was observed by Peng & Li (2014), when lemon, thyme and cinnamon EOs were added into CH films. The gloss of the films is linked to the morphology of their surface; the smoother the surface, the higher the gloss. EOs led to a decrease in the gloss of CH films, regardless of EO concentration (Perdones, 2015). This gloss decrease might be explained by the increase in the surface roughness of the composite films. Being highly volatile mixtures, EOs incorporation may lead to rough coating surfaces, presenting plenty of voids and contributing to gloss reduction (Sánchez-González et al., 2010b).

Table I.3 Recent papers studying the effect of essential oils on CH films properties (Scanning Electron Microscopy (SEM), elastic modulus (EM, MPa), tensile strength (TS, MPa), elongation at break (E, %), water vapour permeability (WVP), oxygen permeability (OP), gloss and transparency.

Essential oils	Matrix : oil proportion	T- %RH conditioning	SEM	EM	TS	E	WVP	OP	Gloss 60°	Ti	References
Cinnamon	2:0-2 %	25°C-51 %	+	-	+	+	+	-	-	-	Ojagh et al. (2010)
Zataria multiflora Boiss, Grape seed	20:0-20	25°C -52 %	-	-	+	+	+	-	-	-	Moradi et al. (2012)
Tea tree	1:0-2 %	20°C-54.4 %	+	+	+	+	+	-	+	+	Sánchez-González et al. (2010a)
Bergamot	1:0-3 %	20°C-54.4 %	-	+	+	+	+	-	-	-	Sánchez-González et al. (2010b)
Green tea extract	2:0-20 %	25°C-50 %	-	-	+	+	-	-	-	+	Siripatrawan & Harte (2010)
Basil,thyme	1:0-1 %	5°C-58 %	+	+	+	+	+	-	+	-	Bonilla et al. (2011) Bonilla et al. (2012)
Cinnamon Leaf	0:0-1 %	5-20°C-60 %	+	+	+	+	+	+	+	-	Perdones et al. (2014)

Table I.3 (Continued).

Essential oils	Matrix : oil proportion	T- %RH conditioning	SEM	EM	TS	E	WVP	OP	Gloss 60°	Ti	References
Basil,thyme	1:0-1 %	5°C-59 %	-	+	+	+	+	-	+	+	Perdones et al. (2016)
Thyme,clove, Cinnamon	1:0-1.5 %	25°C-51 %	+	-	+	+	+	-	-	-	Hosseini et al. (2009)
Rosemary	2:0-1.5 %	25°C-50 %	+	-	+	+	+	-	-	+	Abdollahi et al. (2012)
Carvacrol	2:0-1 %	25°C-50 %	-	-	+	+	+	-	-	-	Yuan et al. (2015)
Cinnamon/Clove bud	2:0-10 %	25°C-50 %	+	-	+	-	+	-	-	-	Wang et al.(2011)
Caraway	1:0-2 %	23°C-50 %	-	-	+	+	+	+	-	-	Hromis et al. (2015)
Lemon/Thyme/Cinnamon	2:0-1 %	25°C-53 %	+	-	+	+	+	-	-	-	Peng and Li (2014)

Table I.4 summarizes some recent studies dealing with the effect of EOs addition on the antimicrobial activity of CH films or coatings. The antimicrobial effect provided by EO incorporation is highly dependent on the EO and microorganism selected. Several studies report that the incorporation of the EO enhanced the antimicrobial efficiency of chitosan against different strains (*E. coli, L. monocytogenes, S. aureus, Salmonella typhimurium, Bacillus cereus*) whereas it had little effect on the physico-chemical properties of films (Pranoto et al., 2005; Zivanovic et al., 2005). Contrarily, Sánchez-González et al. (2011c) reported that when CH films were tested against *E.coli*, the incorporation of essential oils (tea tree, bergamot and lemon) into the films led to a significant decrease of their antimicrobial effectiveness. This was attributed to the dilution effect of CH when EO is present, thus being less available for microorganisms, and it reflects the milder antimicrobial effect of the EO compounds as compared to CH.

CH films containing cinnamon bark EO showed strong antimicrobial activity against food-borne pathogens, particularly Gram-positive *L. monocytogenes* which, shows the potential of this oil to be incorporated in biopolymer antimicrobial films to improve microbiological safety (Ma et al., 2016). Likewise, Hafsa et al. (2016) confirmed the antimicrobial efficiency of chitosan films containing *Eucalyptus globulus* EO, which could be used as active films due to its excellent antimicrobial and antioxidant activities. Perdones et al. (2014) reported that CLEO incorporated CH films showed antifungal activity against *A. niger, B. cinerea and R. stolonifer* and allowed for a significantly increased shelf-life of strawberries infected with *R. stolonifer*. The effect of mint and thyme essential oils against *L. monocytogenes* in cabbage was also reported, and it was found that the antimicrobial effect of chitosan films was even more pronounced with the addition of EOs (Jovanovic et al., 2016a). Similarly, Wang et al. (2011) prepared chitosan films incorporated with cinnamon, clove and anise EOs, and they found that cinnamon oil - chitosan films exhibited a synergistic effect, which was related to the constant release of cinnamon EO. Quesada et al. (2016) stored meat samples covered with CH films containing thyme EO. In this case, the incorporation of EO resulted in decreased yeast populations, whereas aerobic mesophilic bacteria, lactic acid bacteria, and enterobacteria were not affected. However, EO improved the protection against lipid oxidation and gave a better appearance to the packaged meat.

The antimicrobial efficacy of EOs incorporated biodegradable films is highly dependent on the controlled release of the active EO compounds. In this sense, it was reported that the antimicrobial efficacy of chitosan-films containing bergamot EO could be improved by

maintaining higher water content in the films, hence favouring the diffusion of limonene towards the product surface. The solubility of limonene is higher in non-polar foods media, such as fats, but its release from the film is slowed down by contact with non-polar food simulants, due to the lack of film hydration which would facilitate mass transfer (Sánchez-González et al., 2011d). Many studies reported that EOs efficiency could be affected by the partial evaporation of the volatile compounds during the film preparation (Sánchez-González, 2010b; Perdones et al., 2014; Zhaveh et al., 2015; Valencia-Sullca et al., 2016). In this sense, the use of encapsulation techniques represents a new approach to overcome this limitation, as will be described below.

Table I.4 Recent studies dealing with the effect of EOs addition on the antimicrobial activity of CH films or coating.

Essential oils	Matrix : oil proportion	Microorganisms tested	Food system	Main effect	References
Eucalyptus globulus	2:0-4 %	*Escherichia Coli* *Staphylococcus aureus* *Pseudomonas aeruginosas* *Candida albicans* *Candida parapsilosis*	Agar disk diffusion method	Inhibition of growth	Hafsa et al. (2016)
Cinnamon bark	1:0-3 %	*Escherichia Coli* *Listeria Monocytogenes* *Salmonella enterica*	Agar disk diffusion method	Inhibition of growth	Ma et al. (2016)
Anise, Basil, coriander, oregano	1.5:0-4 %	*Escherichia Coli* *Listeria Monocytogenes*	Agar disk diffusion method	Oregano exhibited the strongest antimicrobial activity	Zivanovic et al. (2005)
Lemon, thyme, cinnamon	2:0-1 %	*Escherichia Coli* *Staphylococcus aureus*	Agar disk diffusion method	Good antimicrobial activity	Peng & Li (2014)

Table I.4 (Continued)

Essential oils	Matrix : oil proportion	Microorganisms tested	Food system	Main effect	References
Garlic	1:0-4 %	*Escherichia coli* *Salmonella typhimurium* *Pseudomonas aeruginosas* *Bacillus cereus*	Agar dilution method	Inhibition of growth	Pranoto et al. (2005)
Bergamot, lemon, tea tree	1:0-3 %	*Escherichia coli* *Listeria Monocytogenes* *Staphylococcus aureus*	Agar dilution method	Tea tree exhibited the highest antimicrobial activity	Sánchez-González et al. (2011c)
Cinnamon tea tree	2:0-2 %	*Escherichia coli* *Listeria Monocytogenes* *Lactobacillus platarum* *Lactobacillus sakie* *Pseudomonas fluorescens*	Agar disk diffusion method	Good antimicrobial activity	Sánchez-González et al. (2011c)
Carvacrol	0.4: 0-1.5 %	*Escherichia coli* *Salmonella typhimurium*	Agar disk diffusion method	Good antimicrobial activity	López-Mata et al. (2013)

Table I.4 (Continued)

Essential oils	Matrix : oil proportion	Microorganisms tested	Food system	Main effect	References
Tea tree	1:0-2 %	*Listeria Monocytogenes* *Penicillum italicum*	Agar dilution method	Good antimicrobial activity	Sánchez-Gonzáles et al. (2010a)
Basil, thyme	1:0.5-1 %	Aspergillus niger *Botrytis cinerea* *Rhizopus stolonifer*	Contact method and head space method	Did not inhibit the growth	Perdones et al. (2016)
Cinnamon leaf	1:0-1 %	*Aspergillus niger* *Botrytis cinerea* *Rhizopus stolonifer*	Agar disk diffusion method Strawberries coating	Good antifungal activity	Perdones et al. (2014)
Oregano,coriander,basil, anis	1:0-4 %	*Listeria Monocytogenes* *Escherichia Coli*	Agar disk diffusion method Meat	Reduction the bacterial on meat	Zivanovic et al. (2005)
Thyme	1:0-2 %	Yeast, aerobic mesophilic, lactic acid, enterobacteria	Pork meat	Protects against lipid oxidation	Sánchez-Gonzáles Quesada et al. (2016)

Table I.4 (Continued)

Essential oils	Matrix : oil proportion	Microorganisms tested	Food system	Main effect	References
Mint,thyme	0.5-2:0-0.2 %	*Listeria Monocytogenes*	Fresh cabbage and black radish	Reduction of growth bacterial	Jovanovic et al. (2016a) Jovanovic et al. (2016b)
Basil,thyme	2:0-1.5 %	*Listeria Monocytogenes* *Escherichia coli* Coliforms, total aerobial count	Pork meat	Decrease in antibacterial infection levels	Bonilla et al. (2014)
Lemon	1: 0-3 %	*Botrytis cinerea*	Strawberries with or without coating	Inhibition of growth	Perdones et al. (2012)
Tea tree, thyme, bergamot	1:0-1 %	*Penicillum italicum*	Orange (Coating)	Tea tree shown antibacterial	Cháfer et al. (2012)
Oregano	5-10:1-5 %	*Aspergillus niger* *Rhizopus stolonifer*	Grapes with or without coating	Inhibition of growth	dos Santos et al. (2012)

I.5 Improving the functional properties of essential oils by encapsulation techniques

The main drawbacks affecting the applicability of EOs as food preservatives (high volatility, strong odor and chemical sensitivity) could be mitigated by including them in the formulation of biodegradable films and coatings. Moreover, controlled release could be attained by using the encapsulation technology, a process where tiny particles or droplets are surrounded by a coating wall, or are embedded in a homogeneous or heterogeneous matrix, to form small capsules (Gharsallaoui et al., 2007; Calvo et al., 2011).

Encapsulation has been defined as the packaging technology of a specific component (solid, liquid or gas material) within a matrix (proteins, polysaccharides, etc.), forming small particles called microcapsules (Desai & Park, 2005; Gharsallaoui et al., 2007). Hence, it consists of coating or entrapping a material or mixture (guest) into other materials or systems (host/carrier), aiming for protection against oxidation and/or heat degradation, controlled release rate under specific conditions, and convenience in the handling of active ingredients (Bhandari et al., 1998; Fang & Bhandari, 2010; Shrestha et al., 2017). Depending on the physico-chemical properties of the core, the wall composition, and the microencapsulation technique used, the types, size and shape of particles can be modulated. In addition, all these conditions could also affect the functional properties and the potential applications of the encapsulated components (Gibbs et al., 1999; Gharsallaoui et al., 2007; Bakry et al., 2016). Morphology of different types of microcapsules are shown in **Figure 1.4**. In its simplest form, a microcapsule is a small sphere with a uniform wall, where the core may be a crystalline material, a jagged adsorbent particle, an emulsion, a suspension of solids or a suspension of smaller microcapsules (Gharsallaoui et al 2007).

Generally, microcapsules are sized between 1 and 1000 μm comprising an active agent surrounded by a natural or synthetic polymeric membrane (Martins et al., 2014). The encapsulated substance can be called the core, fill, active, internal or payload phase. The substance that is encapsulating is often called the coating, membrane, shell, capsule, carrier material, external phase or matrix (Wandrey et al., 2009; Fang & Bhandari, 2010).

The carrier material of encapsulates used in food products or processes should be food grade and able to form a barrier for the active agent and its surroundings (Zuidam & Shimoni, 2010). In the food industry, flavours, antimicrobials, antioxidants, vitamins and probiotics account for some examples of substances that may be encapsulated in order to control its action (Nedovic et al., 2011).

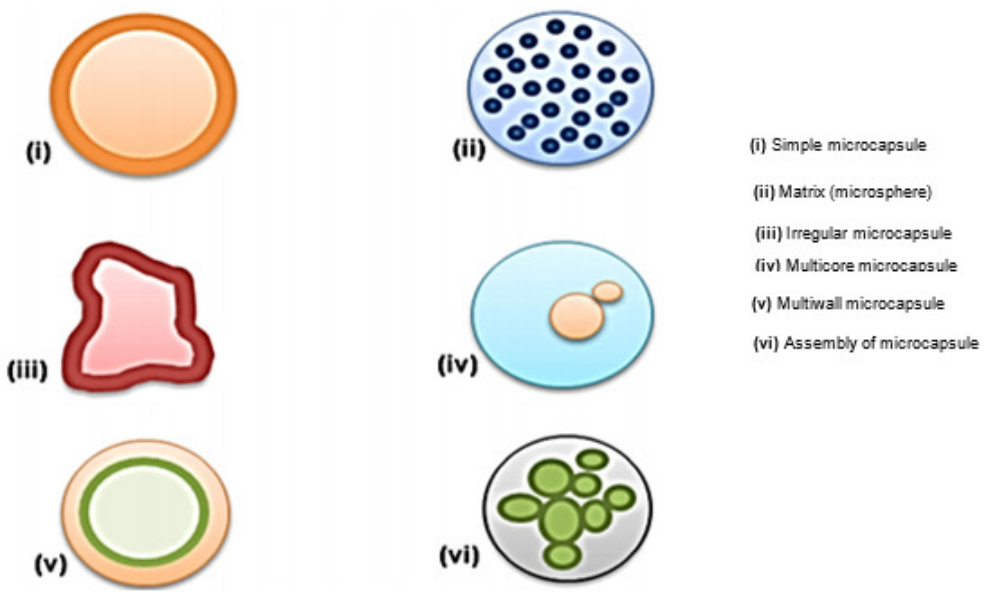

Figure I.4 Morphology of different types of microcapsules (Bakry et al., 2016).

By creating a physical barrier between the core and wall materials, encapsulation protects sensitive ingredients from the external environment, particularly moisture, pH and oxidation (Nesterenko et al., 2013), thus increasing the stability of the bioactive compounds during processing and storage, and preventing undesirable interactions with the food matrix (McClements & Lesmes, 2009). Microencapsulation can be carried out using a wide range of techniques, providing isolation, entrapment, protection or controlled release of sensitive or reactive materials (Martins et al., 2014). It is one of the most effective methods to date to achieve controlled release of the compounds (Moretti et al., 2004; Hussain & Maji, 2008). The core material is gradually diffused through the capsule walls, thereby offering controlled release properties under desired conditions (Fang & Bhandari, 2010).

The wall material determines the stability of microparticles, the process efficiency and the degree of protection for the core (Bakry et al., 2016). The encapsulated agent can be released by several mechanisms such as mechanical action, heat, diffusion, pH, biodegradation and dissolution. The selection of the technique and shell material depends on the final application of the product, considering physical and chemical stability, concentration, required particle size, release mechanism and manufacturing costs (Martins et al., 2014).

A wide variety of substances may be used to coat or encapsulate the payload phase. However, due to legal restrictions, different compounds widely accepted for drug encapsulation but not certified as GRAS, cannot be used in the food industry. Several criteria must be taken into account when selecting the wall material for a given application. Some of

these are linked to its physico-chemical properties, such as solubility, molecular weight, glass/melting transition, crystallinity, diffusibility, film forming and emulsifying properties, mechanical strength, compatibility with the food product, appropriate thermal or dissolution behaviour, release profile and particle size (Brazel, 1999). The wall material must be food-grade, biodegradable and able to form a barrier between the internal phase and its surroundings (Bakry et al., 2016). It should also allow for encapsulation providing functionality to the final product. Moreover, the costs should be also considered.

The materials most widely used for encapsulation in food applications are plant polysaccharides (starch and derivatives, cellulose and derivatives), plant exudates and extracts (gums, galactomannans, pectin, soluble soybean polysaccharides), microbial and animal polysaccharides (dextran, chitosan, xanthan and gellan), lipid materials (fatty acids, fatty alcohols, waxes, glycerides and phospholipids) and others (Poly (N-vinyl-2-pyrrolidone), paraffin, shellac) (Nevodic et al., 2011; Wandrey et al., 2009). Apart from these, recent studies have been found on the use of alginate, lecithin, inulin and whey protein. **Table I.5** summarizes the advantages and disadvantages of using different wall materials for encapsulation, on the basis of recent studies on this area.

Alginate has traditionally been used as a thickening agent, a gelling agent and a colloidal stabilizer in the food and beverage industry. Due to several unique properties, it can be used as a matrix for the entrapment and/or delivery of a variety of proteins and cells. These properties include: (i) a relatively inert aqueous environment within the matrix; (ii) a mild room temperature encapsulation process free of organic solvents; (iii) a high gel porosity which allows for high diffusion rates of macromolecules; (iv) the ability to control this porosity with simple coating procedures and (v) dissolution and biodegradation of the system under normal physiological conditions (Gombotz & Wee, 2012). Also, alginate hydrogels are extensively used in microcapsules because of their simplicity, non-toxicity, biocompatibility and low cost (Rowley et al., 1999; Krasaekoopt et al., 2003; Vishwakarma et al., 2016).

Alginate is a family of unbranched binary copolymers comprising a backbone of (1-4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues (Draget et al., 2006) (**Figure. I.5**). Depending on the algae source, the composition and the sequence in D-mannuronic and L-guluronic acids vary widely, thus influencing its functional properties. G-units have a bucked shape while M-units tend to extended band. Two G-units aligned side-by-side result in the formation of a hole with specific dimension, which is able to bind selectively divalent cations (Kasra-Kermanshahi et al., 2010).

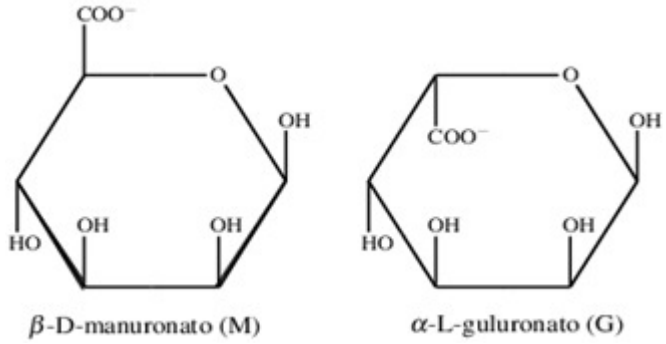

Figure I.5 Chemical structure of β-D-mannuronic acid and α-L-guluronic acid.

The most attractive application of alginate is the calcium-induced gelation resulting from specific and strong interactions between calcium ions and guluronate residues in alginate (Grant et al., 1973). To prepare alginate capsules, sodium alginate droplets fall into a solution containing a multivalent cations (usually Ca^{2+}). The droplets form gel spheres instantaneously, forming a three dimensional structure due to a polymer crosslinking by exchange of sodium ions from the guluronic acids with divalent cations (Ca^{2+}, Sr^{2+} or Ba^{2+}). This results in a chain-chain association to form the “egg box model” (**Figure I.6**) which has been accepted as a general model to describe the gel formation (Rousseau et al., 2004). The process yields very porous alginate capsules, which are also sensitive to acidic environment (Gouin, 2004; Mortazavian et al., 2008).

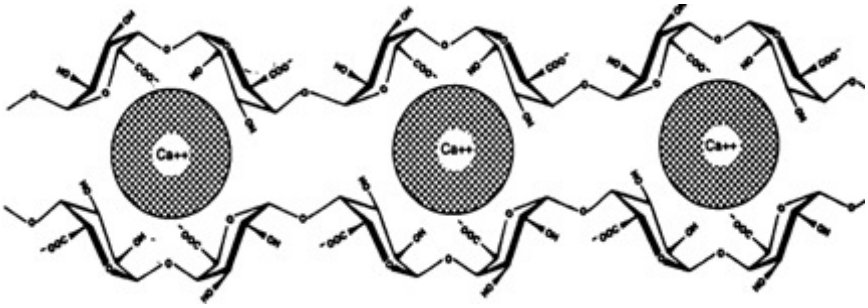

Figure I.6 The “Eggs-box"model for alginate gelation with calcium ions (Rousseau et al., 2004).

Lecithin is the name given to a mixture of glycolipids, triglycerides, and phospholipids (such as phosphatedylcholine, phosphatidylethanolamine, and phosphatidylinositol) (Machado et al., 2014). The major source of lecithin is soy beans, followed by eggs, milk, sunflower seeds and rapeseed (Szuhaj, 2005). As defined in the guidelines of European Community Regulation N°. 1272/2008, lecithin is recognized as a non-hazardous compound, commonly used as an emulsifying and surfactant agent, and does not have any specific limitations on its use in food (Jatoi et al., 2017). The effectiveness of lecithin as a nanoemulsion stabilizer is attributed to

the self-assembly of the molecule at the oil-in-water (O/W) interface and subsequent formation of a thick viscoelastic film strengthened by hydrogen bonding between phosphate groups on neighboring molecules (Shchipunov, 2001). Lecithin has been successfully used for essential oils encapsulation (Valencia-Sullca et al., 2016).

Table I.6 shows a representative list of recent studies on the encapsulation of essential oils utilizing different methods. Beirão-da-Costa et al. (2013) developed inulin microcapsules containing oregano EO, by spray drying at different solids concentration and drying temperatures. The EO was efficiently encapsulated showing different releasing profiles as a function of structural dissimilarities produced by drying conditions. Similar results were observed by Alvarenga et al. (2012) for oregano EO microcapsules obtained by spray drying. Hosseini et al. (2013b) studied the oregano EO encapsulation in chitosan nanoparticles by emulsification-ionic gelation of chitosan with sodium tripolyphosphate (TPP) and found that the content of OEO in the nanoparticles influenced its release rate. Mint based oil microcapsules were successfully produced by spray drying using guar gum modified with n-octenyl succinic anhydride and oleic acid (Sarkar et al., 2013). Dong et al. (2011) developed gelatin/gum arabic microcapsules containing peppermint oil by the coacervation technique, using transglutaminase as hardening agent, and observed that only 7 % of peppermint essential oil was released from microcapsules during 40 days of storage in cold water, thereby showing excellent storage stability.

Rosemary oil-arabic gum microencapsules were prepared by spray-drying (de Barros Fernandes et al., 2014), and it was found that microencapsulation improved the functional activity of EO through high volatiles retention. Previously, de barros Fernandes et al. (2013) had reported that the chemical composition of encapsulated rosemary oil was not affected by the encapsulation process. Chatterjee & Bhattacharjee, (2013) microencapsulated eugenol-rich clove extracts in maltodextrin and gum arabic matrices using spray drying, and attained controlled release of the clove extract antioxidants. The same method was used by Dima et al. (2016) to study the encapsulation of coriander essential oil in various materials (chitosan, alginate, chitosan/alginate, and chitosan/inulin). The microcapsules obtained were resistant to pH and temperature variations, ensuring a slow release of the essential oil. Chitosan/poly (ethylene oxide) nanofibers mats containing cinnamaldehyde were obtained by electrospining (Rieger & Schiffman, 2014). The intrinsic antibacterial activity of chitosan along with the quick release of cinnamaldehyde enabled high inactivation rates against *Escherichia coli* and *Pseudomonas aeruginosa*.

The encapsulation of thyme has been widely studied using different methods (Benavides et al., 2016; Ghaderi-Ghahfarokhi et al., 2016; Chung et al., 2013). In this sense, Benavides et al. (2016) reported that thyme-loaded alginate microspheres can be successfully prepared by an oil-in-water emulsion by ionic gelation. The microencapsulation process did not affect the thyme antimicrobial activity. Similar results were observed by Ghaderi-Ghahfarokhi et al. (2016), who stated that the encapsulation of thyme essential oil in chitosan nanoparticles may be a promising technology for the control of microbial growth. Microcapsules containing thyme oil were prepared by in situ polymerization, using melamine-form aldehyde prepolymer as a wall material (Chung et al., 2013). The release rate of thyme oil was sustained for a long time, and depended on both the storage temperature and the emulsifier type. Microencapsulation of pimento EO using chitosan and k-carrageenan resulted in antimicrobial activity against *Candida utilis, Bacillus cereus* and *Bacillus subtilis*. The release rate of oil increased with the chitosan content, wich suggested that encapsulated essential oil can be used in the meat industry to increase the functionality of meat products (Dima et al., 2014).

Table I.5 Advantages and disadvantages of wall material to encapsulation.

Wall material	Advantages	Disadvantages	References
Alginate	Simplicity Non toxicity Biocompatibility Low cost The highest mucoadhesive strength	Very porous Alginate beads are sensitive to the acidic environment	Soliman et al.(2013) George & Abraham (2006) Pasparakis & Bouropoulos (2006) Baimark & Srisuwan (2014) Paques et al. (2014)
Chitosan	Useful for encapsulation of hydrophilic macromolecules Biodegradable Possesses heat shrinking property Biocompatibility Low toxicity Used as coating materials as well	The poor water solubility The easy dissolution of chitosan in the low pH	George & Abraham (2006) Don et al. (2001) Sriupayo et al. (2005) Hosseini et al. (2013a) Martín et al. (2010)
Lecithin	Non-hazardous compound It has been used as encapsulant material for hydrophobic compounds. Their amphiphilic nature, availability and excellent functionality		Pires et al. (2017) Cheng et al. (2017)

Table I.5 (Continued).

Wall material	Advantages	Disadvantages	References
Gum arabic	Produces the most stable emulsions with oils Compatible with most gums, starches, carbohydrates and proteins.	High costs Limited supply Quality variations	Alves et al. (2014) Lv et al. (2014) Gharsallaoui et al. (2007)
Maltodextrin	Low cost High solubility in water Good flavor	Agglomeration Low glass transition temperature (Crystallization) Poor emulsifying capacity	Alves et al. (2014) Bae & Lee (2008) Gharsallaoui et al. (2007)
Guar gum	Low cost	Demands alteration of its chemical architecture or chain size It lacks emulsifying activity	Sarkar et al. (2013) Thimma & Tammishetti (2003)
Pectin	Can be used to form biopolymer particles in combination with other polymers Is used as gelling agent in food	High degradation rate	Joye & McClements (2014) de Vos et al. (2010) George & Abraham (2006)

Table I.5 (Continued).

Wall material	Advantages	Disadvantages	References
Gelatin	Clear, elastic, transparent, and thermo-reversible gels Biodegradable Water-solubility Emulsifying ability Low cost	High deformation of capsules Low the values of viscoelastic parameters	Sutaphanit & Chitprasert et al. (2014) Zuidam & Nedovic (2010)
β-Cyclodextrin	Protects active compounds against oxidation, heat degradation, and evaporation	It has low thermal stability	Ayala-Zavala et al. (2008) Martín et al. (2010)
Inulin	Non-toxic Biocompatible Water soluble Biodegradable Cheap polymer	High molecular weight More viscous when dissolved The higher molecular weight also correlates with a lower solubility	Garcia et al. (2013) Mensink et al. (2015)
Whey protein	Gelling and emulsification properties Excellent encapsulating agent of oils Mild gelation Biocompatibility Biodegradability Non-toxicity	Not stable at high protein concentrations	Gunasekaran et al. (2006) Bae & Lee (2008) Gbassi et al. (2009) Gunasekaran et al. (2007) Sağlam et al. (2014)

Table I.5 (Continued).

Wall material	Advantages	Disadvantages	References
Cellulose	**a.- Cellulose acetate phthalate (CAP) :**		
	Non-toxic	Soluble at higher pH ($\geq$ 6)	Joye & McClements (2014)
	Insoluble in acid media (pH $\leq$5)		
	b.- Carboxymethylcellulose (CMC):		
	Low cost	High hydrophilic character	Arion (2000)
	Biocompatible and biodegradable and water-soluble.		Ke et al. (2014)
	Anti-adhesive property		Sakai et al. (2009)
	c.- Ethylcellulose:		Gunduz et al. (2013)
	Has been used as a hydrophobic polymer to produce	Is not biodegradable	Montes et al. (2011)
	hollow-shell particles and particles with a solid core.	Hydrophobic polymer	Muschert et al. (2009)
	Non-toxic, non-allergenic and non -irritant		

Table I.6 A representative list of encapsulated essential oils and other compounds with different methods for encapsulation.

Core material (Essential oils)	Wall material	Core : wall ratio	Methods	Relevant result	References
Mint	Guar gum (GA) hydrolyzate modified with N-octenyl succinic anhydride (OSA)	20 % (GA) w/v: 15 %	Spray drying	GA-OSA microcapsules showed better microcapsules	Sarkar et al.(2013)
Rosemary	Modified starch, maltodextrin	29.4 : 20.9 % (w/w)	Spray drying	The rosemary oil maintained all of its main constituents	de Barros Fernandes et al. (2014)
Eugenol-rich clove extract	Maltodextrin(M), gum arabic (GA)	2,5 g : 12 g (M)-6 g (GA)	Spray drying	Controlled release of the antioxidant	Chatterjee & Bhattacharjee (2013)
Coriander	Sodium alginate, chitosan	5 % : 20 %	Spray drying	The CH microcapsules release rate at pH 2.5 and alginate at pH 6.5	Dima et al. (2016)
Thymol, cinnamaldehyde	β-Cyclodextrin	N.R.	Freeze drying	Release was detected at RH <84 %.	Ponce Cevallos et al. (2010)

N.R: Not Reported

Table I.6 (Continued).

Core material (Essential oils)	Wall material	Core : wall ratio	Methods	Relevant result	References
Cinnamaldehyde	Chitosan, poly (ethylene oxide)	0-5 % : 0.42 %	Electrospinning	Inhibition of growth	Rieger & Schiffman (2014)
Thyme	Sodium alginate	1 % : 1 %	Emulsification-Ionic gelation	Good antimicrobial activity	Benavides et al. (2016)
Thyme	Chitosan	0.8 - 1	Emulsification-Ionic gelation	Good thermal stability and antioxidant activity	Ghaderi-Ghahfarokhi et al. (2016)
Oregano	Chitosan	0.04 -0.32 g : 1 % (CH)	Emulsification-Ionic gelation	The content of oregano influenced the release rate	Hosseini et al. (2013a)
Clover, cinnamon bark,lemongrass	Sodium Tripolyphosphate Cellulose nanocrystals	0.04 -1.28g : 0.5 %	Emulsification-Ionic gelation	Good mechanical and antibacterial the release rate	Zhang et al. (2017)
Satureja hortensis	Sodium alginate	1-3 %:1 %	Multiple emulsification -Ionic gelation	Good antioxidant activity and inhibitory effect	Hosseini et al. (2013b)

Table I.6 (Continued).

Core material (Essential oils)	Wall material	Core : wall ratio	Methods	Relevant result	References
Eugenol	Chitosan, poly	1:0-1.25	Emulsification -Ionic gelation	Good antioxidant activity	Woranuch & Yoksan (2013)
Thyme	Poly(vinylalcohol) (PVA)	N.R.	In situ polymerization	The sustained release properties for a long time	Chung et al. (2013)
Cinnamon leaf, eugenol	Lecithin	5wt % : 5wt %	Liposome entrapment	Inhibition of growth	Valencia-Sullca et al. (2016)
Clove, thyme, cinnamon	Sodium alginate	1- 0.3	Extrusion	Inhibition of growth	Soliman et al. (2013)
Basil	Gelatin, glutaraldehyde	N.R.	Simple Coacervation	Good protection against physical and chemical	Sutaphanit et al. (2014)
Pimiento	Chitosan, k-carrageenan	30-67:1	Complex coacervation	Release rate increased with the increase of CH content	Dima et al. (2014)
Jasmine	Gelatin, gum arabic	1 %(w/v) : 1	Complex coacervation	It exhibited perfect m orphology	Lv et al. (2014)

N.R: Not Reported

I.6 Microencapsulation methods

A large number of microencapsulation methods have been developed, which can be adapted to different types of active agents and shell materials, generating particles with a variable range of sizes, shell thicknesses and permeability, providing a tool to modulate the release rate of the active principle (Martins et al., 2014). According to their nature, encapsulation techniques can be classified into three classes: chemical processes (ionic gelation, in situ polymerization); physicochemical techniques (coacervation, liposome encapsulation,) and physical processes (spray drying, spray chilling/cooling) (Gibbs et al., 1999; Zuidam & Heinrich, 2009; Martín et al., 2010; Carvalho et al.,2016). The microencapsulation method should generally be simple, reproducible, fast, effective and easy to implement on industrial scale. Their choice depends on aspects such as physicochemical properties of the encapsulated and encapsulating material, the release characteristics of the encapsulated compound, purpose and cost (Carvalho et al., 2016). Some of the most important and usual microencapsulation methods to produce humid and dry capsules are discussed below. Likewise, different microencapsulation technologies and their advantages and disadvantages are shown in the **Table 1.7**.

I.6.1 Methods to produce humid capsules

Ionic gelation / **extrusion** has attracted great interest due to its highly compatible, non-toxic, organic solvent free, convenient and controllable benefits (Zhang et al., 2017). It has been applied utilizing different polysaccharides as encapsulating materials such as alginate, k-carrageenan, gellan gum, xanthan, pectin, chitosan and gelatin (Abdel-Hafez et al., 2014; Benavides et al., 2016; Yoksan et al., 2010; Calvo et al., 1997; Hosseini et al., 2013a; Ghaderi-Ghahfarokhi et al., 2016; Nagavarma et al., 2012). This is the most popular method for humid microcapsules production (Green et al., 1996; Koyama and Seki 2004; Özer et al., 2008). This technique has been used successfully for microencapsulation of essential oils such as *Satureja hortensis* (Hosseini et al., 2013b), thyme (Benavides et al., 2016), *Lippia sidoides* (Ghaderi Ghahfarokhi et al., 2016), oregano (Paula et al., 2011) and eugenol (Woranuch & Yoksan 2013).

Extrusion has been recently used to encapsulate some vegetable and essential oils, including olive, clove, thyme and cinnamon, for food and pesticide industries (Sun-Waterhouse et al., 2011; Soliman et al., 2013). Extrusion consists of dropping droplets of an aqueous solution of polymer, which may contain an active ingredient, into a gelling bath (in the case of alginate,

gelling bath would be Ca^{2+} solution) (**Figure I.7**). The dripping tool can be simply a pipette, a syringe, a vibrating nozzle, a spraying nozzle, jet cutter or atomizing disk (Wandrey et al., 2009). The principal advantage of extrusion microencapsulation of oils is the stability of the oils against oxidation, low surface oil and a prolonged shelf-life compared to that of spray-dried essential oils (Gouin, 2004).

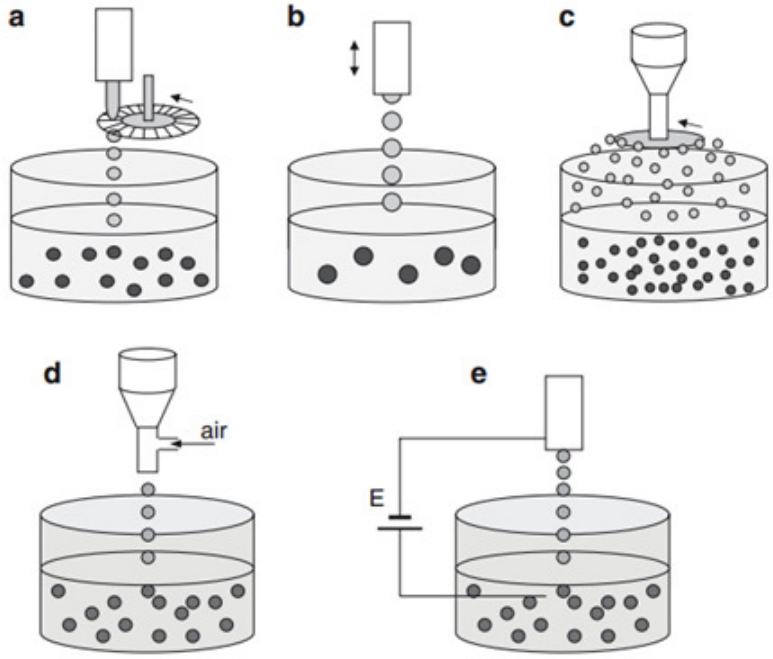

Figure I.7 Set-ups of different ways of making alginate microspheres by extrusion. Aqueous solution alginate and active are atomized by jet-cutter (a), pipette or vibrating nozzle (b), atomizing disk (c), coaxial air-flow (d), or electrostatic potential (e). The droplets fall into a batch of Ca^{2+}, resulting in instantaneous formation of calcium alginate microspheres (Zuidam & Nedovic, 2010).

Contrarily, this method provides capsules of large sizes and different shapes, which limits the use of extruded essential oils in various applications (Desai & Jin Park, 2005; Reis et al., 2006). Although ionic gelation is a simple and mild technique, the gels produced are porous, which can accelerate oxygen permeation through the matrix or allow for the release of active compounds with low molar mass that are inserted into the gels (Sezer & Akbuga, 1999). To circumvent this limitation and improve the functionality of the gels in film or microparticle form, several authors suggest mixing with other biopolymers (Devi & Kakati, 2013) or coating with a layer of polyelectrolytes with the opposite charge by electrostatic complexation (Gbassi et al., 2011; Hébrard et al., 2010), as well as coating with a second polymer to provide better protection (Kailasapathy, 2009).

Co-Extrusion might be utilized to prepare spherical microbeads with a hydrophobic core and a hydrophilic or hydrophobic shell (Zuidam & Shimoni, 2010). This technology is based on a laminar liquid jet that is broken into equally sized droplets by a vibrating nozzle (Prüsse et al., 2008; Del Gaudio et al., 2005). Co-extrusion consists of a concentric feed tube through which wall and core materials are pumped separately to the nozzles mounted on the outer surface of the device (Desai & Jin Park, 2005). While the core material flows through the center tube, wall material flows through the outer tube (Bakry et al., 2016). The droplets are then gelled in a cross-linking solution. The diameter of microcapsules is controlled by two main factors, namely the flow rate and the polymer solution viscosity (Del Gaudio et al., 2005). The coextrusion method was used in the encapsulation of olive oil using alginate microspheres (Sun-Waterhouse et al., 2011), and the encapsulated oil was found to be more stable during storage. Similar results were reported for canola oil (Wang et al., 2013).

Liposome entrapment or liposomal microencapsulation is based on the formation of liposomes, i.e. spherical vesicles formed of an aqueous core and amphiphilic lipid bilayer (Gulati et al., 1998; Walde & Ichikawa, 2001). Being biodegradable, biocompatible, nontoxic and nonimmunogenic (Voinea & Simionescu, 2002), liposomes have attracted attention as carriers for pharmaceutical drugs (Torchilin & Weissig, 2003), given that active agents can be entrapped within their aqueous compartment at a low yield, or within or attached to the membrane at a high yield (Sherry et al., 2013).

Liposomes are formed when phospholipids such as lecithin or cholesterol are dispersed in aqueous media and exposed to high shear rates by using microfluidization, sonication or colloid mill. The underlying mechanism for the formation of liposomes is basically the hydrophilic-hydrophobic interactions between phospholipids and water molecules. Bath sonication is preferably chosen for preparation of liposomes as a relatively mild process (Dua et al., 2012). Frequency, power input and sonication time are the main factors that control the size distribution of the final emulsion product.

Liposome applicability in the food industry is still limited due to their chemical and physical instability upon storage (Were et al. 2003; Gouin 2004; Taylor et al. 2005; Kosaraju et al. 2006; Mozafari et al. 2006; Takahashi et al. 2007; Zuidam et al., 2003). However, there are several studies using liposomal encapsulation of ciprosine to avoid loss during cheese processing (Picón et al., 1994) and liposomal nanoencapsulation of phenolic compounds (de Assis et. al., 2014). Sherry et al. (2013) reported that encapsulation in liposomes is a valuable tool in order to preserve the anti-microbial activity of essential oils.

Liposomes are usually classified according to their lamellarity and size. Small unilamellar vesicles range between 20 and 100 nm, large unilamellar vesicles are bigger than 100 nm, and multilamellar vesicles excede 0.5 μm (Mishra et al., 2011; Rongen et al., 1997). Nanoliposomes are liposomes with a diameter at nanometer scale (Zhang et al., 2012). The good biocompatibility of phospholipids makes nanoliposomes an ideal carrier system with applications in different fields including food, cosmetics, pharmaceutics and tissue engineering (Mozafari, 2010; Nirmala et al., 2011). The combinations of incorporated bioactive compounds can be protected against degradation and improve their stability and solubility (such as solubilization of hydrophilic components in hydrophobic matrices and vice versa) (Klaypradit & Huang, 2008; Jafari et al., 2008; de Assis et al., 2014). Also, nanoliposomes can provide controlled release of bioactive agents, including food and nutraceutical ingredients, in the right place at the right time, so they increase the efficiency and cellular uptake of the encapsulated material. Reactive, sensitive or volatile additives (vitamins, enzymes, antioxidants, weight loss etc.) can be transformed into stable ingredients using nanoliposomes (Mozafari, 2007; Machado et al., 2014). Nanoliposome formation has been used successfully to protect cinnamon leaf essential oil and eugenol (Valencia-Sullca et al., 2016), as well as rapeseed essential oil (Jiménez et al., 2014).

Coacervation consists of the separation of a polymeric solution into two liquid phases, a polymer-rich phase called coacervate and a diluted phase called equilibrium solution, induced by media modifications (specific pH, temperature, ionic strength and polyion concentrations), followed by the coating of the coacervate phase around suspended core particles or suspended droplets (Martín et al., 2010). Therefore, coacervation is a three-step process: (i) formation of an oil-in-water (o/w) emulsion (active compound is dispersed in the aqueous phase and polymer is dissolved in the organic phase); (ii) deposition of the liquid polymer coating upon the core material; and (iii) stabilization and hardening the coating material to form a self-sustaining microcapsules (Lazko et al., 2004; Carvalho et al., 2016).

The most important factors for the coacervation technique are the volume of dispersed phase, the ratio of incompatible polymer to coating polymer, the stirring rate of the dispersion and the core material to be encapsulated (Nihant et al., 1995). In the coacervation technique, the composition and viscosity for polymers solution in supernatant phases act on size distribution, surface morphology and internal porosity of the microcapsules (Nihant et al., 1994; Nihant et al., 1995).

Coacervation avoids the use of high-temperature, which is an advantage for heat-labile active ingredients (Dima et al., 2014). It can provide protection against degradative reactions, prevent the loss of volatile aromatic ingredients, control release and improve the stability of the flavour and essential oils core materials (Xiao et al., 2014). However, due to the high processing cost and complexity of procedure, coacervation is not used much at industrial scale. Also, it cannot be used for producing very small microspheres (John et al., 2011) and the most common problem is agglomeration of microcapsules (Lam et al., 2014). Complex coacervation has been used to microencapsulate pimiento, clove, jasmine and sweet orange essential oils (Dima et al., 2014; Thimma & Tammishetti, 2003; Lv et al., 2014; Jun-Xia, et al., 2011). The microencapsulation efficiency of jasmine oil by complex coacervation with soybean protein gelatin/gum arabic varied from 76 % to 81 % (Lv et al., 2014).

Other more recent methods for humid capsules obtentnion such as interfacial polymerization and in situ polymerization have been included in **Table I.7**.

I.6.2 Methods to produce dry capsules

Microencapsulation by **spray-drying** is one of the oldest encapsulation methods, as well as one of the most common and cheapest techniques for producing microcapsules (Martín et al., 2010). Spray drying microencapsulation is a simple, flexible, rapid, low cost process and easy scaling-up technique (Aniesrani Delfiya et al., 2014). In this method, the material for encapsulation is homogenized with the carrier material. Then the mixture is fed into a spray dryer and atomized with a nozzle or spinning wheel. The contact between drop and hot air takes place during atomization. Rapid evaporation of solvent (usually water) maintains droplet temperature at low level and allows quasi-instantaneously entrapping the active compound. The microcapsules are then collected after they fall to the bottom of the drier (Gibss et al., 1999; Martín et al., 2010). There are different parameters to optimize spray-drying such as air flow, feed rate, feed temperature, inlet air temperature and outlet air temperature (Vega & Roos, 2006; O'Riordan et al., 2001). Spray drying is widely used for the drying of heat-sensitive food products, pharmaceuticals and other substances, including essential oils (Sarkar et al., 2013; de Barros Fernandes et al., 2014; (Chatterjee et al., 2013; Dima et al., 2016; Bringas-Lantigua et al., 2012; Beirão-da-Costa et al., 2013; Beirão-da- Costa et al., 2012).

Spray-drying disadvantages are linked to the high temperature used to dry, which can cause the loss of some low-boiling point compounds or the oxidation of some food components such as poly unsaturated fatty acids (Heinzelmann et al., 2000). Plus, the wall material should

have low viscosity at relatively high concentrations (Carvalho et al., 2016). It produces a very fine powder which needs further processing, such as agglomeration by fluidized bed coating process (Turchiuli et al., 2005).

Freeze-drying is used for the dehydration of almost all heat-sensitive materials and aromas such as oils. Besides protecting heat-sensitive core materials, freeze-drying is simple and easy to operate (Bakry et al., 2016). In the freeze-drying process solvent is removed from a frozen solution by vacuum sublimation, maintaining the drying chamber pressure and temperature bellow the triple point of solvent (Santivarangkna et al., 2007; Solanki et al., 2013). Freeze-dried materials seem to have the maximum retention of volatile compounds in comparison to that of spray-drying (Krokida & Philippopoulos, 2006). This technique has been used successfully for microencapsulating some oils such as fish (Tamjidi et al., 2013), flaxseed (Karaca et al., 2013), walnut (Calvo et al., 2011), and olive (Silva et al., 2013), as well as thymol and cinnamaldehyde (Ponce Cevallos et al., 2010).

The major disadvantages of freeze-drying are the high energy input and long processing time. In addition, during processing a barrier with an open porous structure between the active agent and its surroundings is formed, offering poor protection when prolonged release of an active is required (Zuidam & Shimoni 2010).

Spray-freeze-drying method combines processing steps that are common to freeze-drying and spray-drying (Anandharamakrishnan et al., 2010). Spray-freeze drying was used for the first time for producing docosahexaenoic acid (DHA) in microencapsulated form. Stability of the spray-freeze-dried microencapsulated DHA oil was higher under the storage conditions. The main advantages of spray-freeze-drying techniques are controlled size and high capsules surface area. This technique has nevertheless some disadvantages including the high use of energy, the long processing time and the cost, which is 30-50 times higher than spray drying (Zuidam & Nedovic, 2010).

Spray-chilling, spray-cooling are similar processes as spray-drying, but no water is evaporated and the air used is cold, which enables particle solidification (Alvim et al., 2016). As spray drying, this technique is rapid and takes place in one step (Lamprecht & Bodmeier, 2012). It is based on the atomization of a mixture of the active substance and a melted lipid material in a cold chamber (temperature below the lipid's melting point) in which the droplets in contact with the cool air solidify to form solid lipid microparticles that retain and protect the active substance (Zuidam & Shimoni, 2010). These processes are suitable to encapsulate

water soluble materials which can be volatilized during thermal processing. They are also very used for encapsulation of aroma compounds to improve heat stability and control release. This encapsulation technique has potential for industrial scale manufacture (Gouin, 2004). It can generate small beads, which are desirable in food processing. The major limitations are the high process costs, the special handling and storage conditions required (Madene et al., 2006), the low capacity of encapsulation and the release of core material during storage (Sato & Ueno, 2005). The production of lipid microcapsules containing tocopherol was possible in lipid matrices using spray chilling, showing high efficiency of microencapsulation and high levels of retention of the active product (Diaz Gamboa et al., 2011). Likewise, Alvim et al. (2016) reported similar results in microparticles containing ascorbic acid.

Fluid bed coating is an encapsulation method where a coating is applied onto powder particles in a batch processor or a continuous set-up. The powder particles are suspended by an air stream at a specific temperature and sprayed with an atomized, coating material (Joye & McClements 2014). The coating material might be an aqueous solution of cellulose or starch derivatives, proteins and gums (Dewettinck & Huyghebaert, 1999). The fluid bed processing is widely used in cosmetic industry, particularly for encapsulating spray-dried flavours (Hede et al., 2008). This method can also be used to produce multilayer coatings (Champagne et al., 2010). This method allows for control over temperature, and the particles obtained by this technique are uniform in size. On the other hand, it is difficult to master for long duration and may take long time (Carvalho et al., 2016). Fluid bed coating has been used to improve the stability against oxidation of fish oil (Anwar et al., 2010).

Electrospinning is a combination between two techniques, namely electrospray and spinning. In this technique, a high electric field is applied to a fluid which comes out from the tip of a die that acts as one of the electrodes. This leads to droplet deformation and finally to the ejection of a charged jet from the tip towards the counter electrode, hence to the formation of continuous capsules (cylinder). Cinnamaldehyde was successfully electrospun and delivered from chitosan/poly(ethylene oxide) nanofiber mats, without the use of a surfactant, which enabled high inactivation rates against Escherichia coli and Pseudomonas aeruginosa (Rieger & Schiffman, 2014). The main advantage of electrospinning technique is that capsules are very thin with large surface areas (Agarwal et al., 2008).

Table I.7 Advantages and disadvantages of different microencapsulation technologies .

Encapsulation methods	Advantages	Disadvantages	References
Ionic gelation/Extrusion	Simple and high encapsulation efficiency. Increased stability of oils against oxidation. Reduce the evaporation rate of essential oils.	Polymerization reaction is difficult to control Expensive (double the cost) than spray drying Inefficiency in producing large microspheres Limited choice of wall material	Bansode et al. (2010) Lam & Gambari (2014) Gouin (2004) Soliman et al.(2013) Desai & Jin Park (2005) Reis et al. (2007)
Liposome entrapment	Biodegradable, biocompatible, nontoxic and nonimmunogenic.	Chemical and physical instability upon storage in especially emulsified food products	Voinea & Simionescu (2002) Mozafari et al. (2006) Takahashi et al. (2007)
Co-Extrusion	Size-controlled microspheres Stability during storage.	Require special handling conditions	Del Gaudio et al. (2005) Sun-Waterhouse et al. (2011) Wang et al. (2013)
Coacervation : **Simple coacervation** **Complex coacervation**	High encapsulation efficiency. Efficient control of particle size.	Is not used much in industrial scale Expensive method Aggregation of particles (agglomeration) Hard scaling-up Evaporation of volatiles Possibility of dissolution of active compound into the processing solvent Oxidation of product Not be used for producing small microspheres	Madene et al. (2006) Xiao et al. (2014) Estevinho et al. (2013) Sutaphanit & Chitprasert (2014) Dima et al. (2014) Lam et al. (2014) John et al. (2011)

Table I.7 (Continued) .

Encapsulation methods	Advantages	Disadvantages	References
Spray Drying	Simple and versatile Can be operated on a continuous basis Applied on a large scale Suitable for industrial application Rapid and relatively low cost	Non-uniform particles Low oil loading level Require further processing Possibility of lost low-boiling point aromatics The oxidation of poly unsaturated fatty acids due to high temperatures operations	Madene et al. (2006) Lam & Gambari (2014) Estevinho et al. (2013) Gharsallaoui et al. (2007) Fuchs et al. (2006) Heinzelmann et al. (2000)
Freeze Drying	Simple and easy to operate Protects heat-sensitive core materials More resistant to oxidation	The high energy input and long processing time. An open porous structure between the active agent and its surroundings is formed	Bakry et al. (2016) Velasco et al. (2003) Zuidam & Shimoni (2010)
Spray Freeze Drying	Controlled size Larger specific surface area than spray-dried capsules	The use of high energy The long processing time The cost which is 30- 50 times expensive than spray-drying	Zuidam & Nedovic (2010) Semyonov et al. (2010)

Table I.7 (Continued) .

Encapsulation methods	Advantages	Disadvantages	References
Spray chilling/ Spray cooling	Suitable for water-soluble materials Cheapest encapsulation technology Potential of industrial scale manufacture Generates smaller beads	High process costs Require special handling and storage conditions	Madene et al. (2006) Gouin et al. (2004) Diaz Gamboa et al. (2012) Sato & Ueno (2006)
Fluid bed coating	Low operational costs High thermal efficiency process Total temperature control Easy scale-up	Long time process Technology difficult to control for longer duration	Madene et al. (2006) Lam & Gambari (2014) Carvalho et al. (2016) Champagne et al. (2010)
Electrospinning	Production of very thin capsules Large surface areas	The ability to spin an oil or another water-immiscible phase is limited	Agarwal et al. (2008) Rieger & Schiffman (2014)

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II. OBJECTIVES

The general objective of this work was to develop active biodegradable films based on hydrocolloids (starch and chitosan), using different strategies. Films were obtained either by melt blending or casting. Antimicrobial compounds (eugenol, oregano essential oil or cinnamon leaf essential oil) were incorporated into the polymer matrix by homogenization or encapsulation in lecithin liposomes or alginate microspheres.

For this purpose, several specific objectives were defined:

- To characterize the structural, physical and antimicrobial properties of films based on thermoplastic cassava starch and chitosan, obtained by compression moulding, as affected by plasticizer/starch/chitosan ratios (Chapter 1).
- To characterize the physical, structural and antimicrobial properties of bilayer consisting of one layer of thermoplastic cassava starch films obtained by melt blending and a second layer of casted chitosan films. The effect of the addition of essential oils (oregano or cinnamon leaf) in the chitosan layer was evaluated (Chapter 2).
- To assess the effect of the incorporation technique on the retention of essential oils (eugenol and cinnamon leaf essential oil) in casted chitosan films. The active ingredients were incorporated either in free form or encapsulated within lecithin liposomes and the impact of the addition technique on film microstructure and, physical properties and antimicrobial activity and functionality when applied to pork meat were also evaluated (Chapter 3 and 4).
- To develop microcapsules containing eugenol by ionic gelation of alginate and incorporate them into chitosan casted films to evaluate their effect on film physical properties, eugenol release kinetics and antimicrobial activity (Chapter 5).

III. RESULTS

III.1. CHAPTER 1: PHYSICAL CHARACTERIZATION OF CASSAVA STARCH - CHITOSAN FILMS OBTAINED BY COMPRESSION MOLDING.

III.2. CHAPTER 2: THERMOPLASTIC CASSAVA STARCH-CHITOSAN FILMS CONTAINING ESSENTIAL OILS.

III.3. CHAPTER 3: INFLUENCE OF LIPOSOME ENCAPSULATED ESSENTIAL OILS ON PROPERTIES OF CHITOSAN FILMS.

III.4. CHAPTER 4: RELEASE KINETICS AND ANTIMICROBIAL PROPERTIES OF CHITOSAN-ESSENTIAL OILS FILMS AS AFFECTED BY ENCAPSULATION WITHIN LECITHIN NANOLIPOSOMES.

III.5. CHAPTER 5: CHITOSAN FILMS CONTAINING ENCAPSULATED EUGENOL IN ALGINATE MICROCAPSULES

III.1 CHAPTER 1

PHYSICAL CHARACTERIZATION OF CASSAVA STARCH – CHITOSAN FILMS OBTAINED BY COMPRESSION MOLDING

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ABSTRACT

Biodegradable cassava starch-chitosan blend films were obtained by melt bending and compression molding at 160°C, using glycerol and poliethylene-glicol as plasticizers. Both the starch:chitosan and the polymer:plasticizer ratios were varied in order to analyze the effect of both on the properties of the films. Additionally, the antimicrobial activity of 70:30 polymer:plasticizer films was tested. Both polymers had limited miscibility by melt blending, which resulted in heterogeneous film structure, as observed by SEM. All film components were thermally stable up to 200°C, which garranted their thermostability during processing, although fims with chitosan exhibited browning promoted by thermal process. Poliethylene-glicol partially crystallized in the thermoprocessed films, to a greater extent as the chitosan ratio increased, which limited its plasticizing effect. This indicates its phase separation in the film, mostly provoked by the starch substitution with chitosan. As expected, the films with the highest plasticizer ratio had higher moisture content and were more permeable to water vapour, less rigid and less resistant to break. As the chitosan proportion increased, the films became more rigid, more resistant, less stretchable and had a more saturated yellowish color, related with some component reaction. Chitosan incorporation provided the films with only a slight antimicrobial capacity, contrarily to previous results in casting films.

Keywords: biodegradable films, blend films, chitosan, cassava starch, compression molding.

1. INTRODUCTION

In recent years, increasing interest on biodegradable materials has developed mainly due to concerns over the disposal of conventional synthetic plastic materials derived from petroleum. Being resistant to microbial attack and biodegradation, synthetic plastic materials take a long time to be degraded and most of them end up over-burdening the environment. In this sense, recent investigations have focussed on the development and characterization of biodegradable films from natural polymers (Arvanitoyannis, 1999; Fang et al., 2005). Starch has been widely used for the preparation of biodegradable films because of its abundance, low cost, renewable nature, biocompatibility, non-toxicity (Carvalho, 2008) and low oxygen permeability (Jiménez et al., 2012a; Zhang et al., 2014). However, starch-based materials show several disadvantages, which reduce their applicability as packaging material, such as their highly hydrophilic character, limited mechanical properties and the retrogradation phenomena that occur during aging (Ortega-Toro et al., 2015). The utilization of a plasticizer, which normally is the second major component of starch based films, helps to overcome film brittleness caused by intermolecular forces.

Aiming to improve starch film characteristics, it has been combined with both synthetic and natural polymers (López et al., 2014). Amongst them, chitosan has led to positive results in films obtained by casting (Chillo et al., 2008; Fajardo et al., 2010; Lazaridou & Biliaderis, 2002; Quattara et al., 2000). Incorporating chitosan into starch-based films has been reported to reduce their water affinity and improve their mechanical properties, due to the formation of intermolecular hydrogen bonds between the amino and hydroxyl groups of chitosan and the hydroxyl groups of starch (Xu et al., 2005). Moreover, the antibacterial and antifungal properties of chitosan, which have been studied by several authors (Friedman & Juneja, 2010; Rabea et al., 2003; Avila-Sosa et al., 2012), could provide the blend films with antimicrobial properties.

Native starch can be converted into thermoplastic starch (TPS) by the disruption of the polymeric chains interactions, under specific conditions of temperature and /or mechanical energy, in the presence of a plasticizer (Tomé et al., 2012; Chivrac et al., 2009). In order to obtain TPS, scaling-up processing methods using equipments designed for synthetic polymers is indispensable (Sothornvit et al., 2007; Thunwall et al., 2008). For example, extrusion, blowing, injection and thermocompression are viable alternatives due to their energy-efficiency and high productivity (Flores et al., 2010; Pellissari et al., 2012; Thunwall et al., 2006). As opposed to casting, these techniques are suitable for film production at an

industrial scale (Tomé et al., 2012).

The aim of this work was to characterize the structural, physical and thermal behaviour of starch-chitosan blend films with glycerol and polyethylene glycol (PEG) as plasticizers. The polymer:plasticizer and the starch:chitosan ratios were varied in order to assess their effect on the properties of the films obtained by compression molding. The effect of storage time and the antimicrobial properties of the films were also studied.

2. MATERIALS AND METHODS

2.1. Materials

Cassava starch (CS) was supplied by Quimidroga S.A. (Barcelona, Spain). Chitosan (CH) of high molecular weight (practical grade, >75 % deacetylation degree, Batch MKBP1333V) and polyethylene glycol 4000 (PEG) were purchased from Sigma-Aldrich (Madrid, Spain). Glycerol (Gly) and Mg $(NO_3)_2$ were provided by 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. Buffered peptone water, Violet Red Bile Agar and Plate Count Agar were provided by Scharlau (Barcelona, Spain).

2.2. Film preparation

Eight formulations based on CS, CH, with Gly and PEG as plasticizer (P), were prepared. In all cases, plasticizers were mixed in a constant Gly: PEG mass ratio 75:25. Two series of formulations were prepared with polymer:plasticizer mass ratios 70:30 and 60:40. In each series, CS:CH mass ratios were 100:0, 90:10, 80:20 and 70:30.

After weighing, CS, CH and plasticizers were dispersed in distilled water. The formulations were hot-mixed on a two-roll mill (Model LRM-M-100, Labtech Engineering, Thailand) at 160°C and 10 rpm for 30 minutes until a homogeneous paste was obtained. Before compression molding, the paste was conditioned at 25°C and 53 % RH using Mg $(NO_3)_2$ oversaturated solutions for 72 h. The films were obtained by compression molding (Model LP20, Labtech Engineering, Thailand). Four grams of the paste were put onto steel sheets and preheated on the heating unit for 5 min. The films were performed at 160°C for 2 min at 50 bar, followed by 6 min at 120 bar; thereafter, a cooling cycle (40°C /min) was applied for 3 min. The films obtained were conditioned at 25°C and 53 % RH for 1 or 5 weeks before characterization.

2.3. Characterization of the films

2.3.1 Microstructural analysis

The microstructural analysis of the cross-sections of the films was carried out by means of a scanning electron microscope (JEOL JSM-5410, Japan, model JSM-5410). The film samples were maintained in desiccators with P_2O_5 in order to eliminate film moisture. Film pieces (5x5 mm approximately) were cryofractured and mounted on cupper stubs. After gold coating, the samples were observed using an accelerating voltage of 10 kV.

2.3.2 Thermogravimetric analysis (TGA)

The thermal stability of the films and their components was analyzed using a thermogravimetric analyzer (TGA/SDTA 851e, Mettler Toledo, Schwerzenbach, Switzerland), equipped with an ultra-micro weighing scale ($\pm$0.1 μg), under nitrogen flow (50 mL/min). The analysis was carried out during heating from 25 to 600°C at 10°C/min. Approximately 3 mg of sample were used in each test, considering at least two replicates per formulation. Initial degradation temperature (T_0), i. e. the temperature at which 10 % mass loss is registered, and maximum degradation rate temperature (T_{max}) were recorded.

2.3.3 Differential scanning calorimetry (DSC)

The thermal properties were analyzed using a differential scanning calorimeter (DSC 1 Star System, Mettler- Toledo, Inc., Switzerland) with a 20 mL/min nitrogen flow. Film samples were desiccated with P_2O_5 and crushed with a mortar. Two samples (approximately 10 mg) per formulation were placed into aluminium pans and sealed. An empty sample pan was taken as a reference. A first heating step was done from 0°C to 160°C at 50°C/min to remove any residual water, followed by a cooling step to 0°C at the same speed, and a second heating to 200°C at 10°C /min.

2.3.4 Moisture content and water vapour permeability (WVP)

The moisture content of film samples previously conditioned at 53 % RH was determined with a gravimetric method. Five samples per formulation were considered. Water was eliminated from them using a two-step method: desiccation in a vacuum oven (60°C - 24 h), and storage in desiccators with P_2O_5 until constant weight was reached. The results were expressed as g of water per 100 g of dry film.

The water vapour permeability (WVP) of the film samples was determined by means of the ASTM E96-95 (ASTM, 1995) gravimetric method, taking the modification proposed by McHugh et al. (1993) into account. Distilled water was placed in Payne permeability cups (Elcometer SPRL, Hermelle/s Argenteau, Belgium) of 3.5 cm in diameter. Six round samples per formulation were cut, and the thickness was measured to the nearest 0.0025 mm in six points per sample (Electronic Digital Micrometer, Comecta S.A., Barcelona, Spain). WVP was determined at 25°C and 53-100 % RH gradient which was generated by using an oversaturated Mg $(NO_3)_2$ solution and pure water, respectively. The cups were weighed every 1.5 h, for 24 h with an analytical scale (ME36S Sartorius, Alemania). Once the steady state was reached, the slope obtained from the weight loss vs. time was used to calculate WVP ($g.mm.KPa^{-1}.h^{-1}.m^{-2}$).

2.3.5 Mechanical properties

A universal test machine (TA-XT plus, Stable Micro Systems, Surrey, United Kingdom) was used to determine the elastic modulus (EM), tensile strength at break (TS) and percentage of elongation at break (%E) of the film samples. These parameters were obtained from stress-Hencky strain curves, following ASTM standard method D882 (ASTM, 2001). Ten film stripes (25 mm wide and 100 mm long) per formulation were tested. Film thickness was measured in four positions along the stripe to the nearest 0.0025 mm with a hand-held digital micrometer (Electronic Digital Micrometer, Comecta S.A., Barcelona, Spain). Equilibrated samples were mounted in the film-extension grips of the testing machine and stretched at 50 mm min^{-1} until breaking. The relative humidity of the environment was held constant, at approximately 53 % during the tests, which were performed at 25°C.

2.3.6 Optical properties

The surface reflectance spectra of the films were obtained using a MINOLTA spectrocolorimeter, model CM-3600d (Minolta CO., Tokyo, Japan), illuminant D65 and observer 10°. The Kubelka-Munk theory for multiple scattering was applied to the reflection spectra to determine

the film's transparency (Hutchings, 1999). The surface reflectance spectra were determined from 400 to 700 nm using both a white and a black background. The measurements were taken at four random points. The internal transmittance (T_i) of the films was determined using Eq. (1), where R_0 is the reflectance of the sample measured on a black background. Parameters a and b were calculated by means of Eqs. (2) and (3), where R is the reflectance of the sample backed by a standard white standard background and R_g is the reflectance of the white background.

$$T_i = \sqrt{(a - R_0)^2 - b^2} \qquad \text{Eq.1}$$

$$a = \frac{1}{2}(R + \frac{R_0 - R + R_g}{R_0 R_g}) \qquad \text{Eq.2}$$

$$b = \sqrt{a^2 - 1} \qquad \text{Eq.3}$$

Coordinates lightness (L*), chrome (C^*_{ab}) and hue (h^*_{ab}) of the films were obtained from the surface reflectance spectra. Moreover, the whiteness index (WI) was calculated according to Atarés et al. (2010). The gloss was determined at a 60° incidence angle by means of a flat surface gloss meter (Multi Gloss 268, Minolta, Germany), following the ASTM standard D523 method (ASTM, 1999). The measurements of each sample were taken in triplicate and four films were measured per formulation. The results were expressed as gloss units (GU), relative to a highly polished surface of black glass standard with a value near to 100 GU.

2.3.7 Antimicrobial properties

The four film formulations with a polymer: platicizer mass ratio 70:30 were tested in their antibacterial properties using pork meat and following a methodology adapted from Bonilla et al. (2014). Each test sample was obtained by molding 10 g of meat in a petri dish (5 cm diameter), and coating with the films. Non-coated samples (control) and samples coated were stored in duplicate at 10°C for 7 days. To perform the microbiological analyses, each sample was homogenized in a Stomacher (Bag Mixer 400, Interscience) with 90 mL of sterile buffered peptone water for 2 min. Then, serial dilutions were made and plated out. Total viable and coliform microorganism counts were determined at 0, 1, 4 and 7 days. Total aerobic counts were determined in Plate Count Agar incubated at 37°C for 48 h. While coliforms were determined in Violet Red Bile Agar incubated at 37°C for 48 h. All tests were made in triplicate.

2.4 Statistical analysis

The statistical analysis of the data was performed through analysis of variance (ANOVA) using Statgraphics Centurion XVI. II. Fisher's least significant difference (LSD) procedure was used.

3. RESULTS AND DISCUSSION

3.1 Microstructural analysis

Figure 1.1 shows the micrographs of the cross section of the films after a 1 week storage period at 25°C and 53 % RH. Starch samples showed a quite homogenous structure where no starch granules were observed, which indicates that they were gelatinized during the roller mill step. Nevertheless, all samples exhibited micro-cracks, which reflects the sample fragility after dessication with P_2O_5.

The increase in the plasticizer content did not change this behavior. Incorporation of chitosan into the film matrix was not homogenous due to the lack of miscibility of polymers by melt blending. Although a homogeneous matrix was obtained when starch-chitosan blend films were obtained by casting the aqueous solutions of both macromolecules at different ratios (Bonilla et al., 2013), no total compatibility of polymers was obtained in dry conditions. This may be due to difficulties in the melt blending, where the much higher viscosity of the medium and the difficulties for chain extension inhibit the establishment of adequate interactions between the polymer segments. Therefore, some flakes with different sizes of chitosan in the starch matrix can be observed in samples regardless the ratio of chitosan. These were observed in different zones of the films with stochastic distribution. Even at the lowest chitosan ratio, immiscibility of melt polymers was evident.

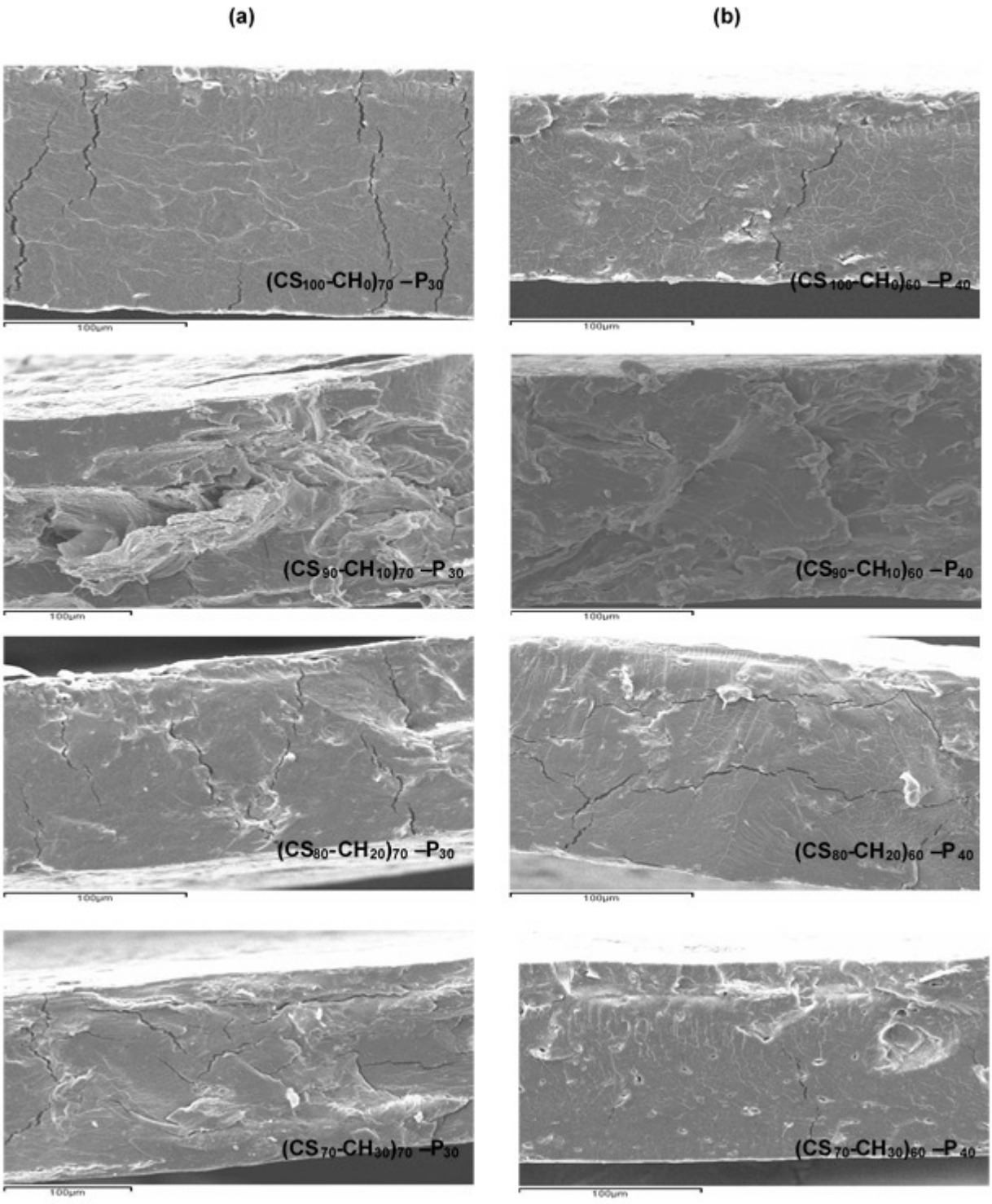

Figure 1.1. Scanning electron microscopy micrographs of the cross-sections of the films. **(a)** Polymer: plasticizer proportion 70:30. **(b)** Polymer: plasticizer proportion 60:40.

3.2 Thermogravimetric analysis (TGA)

TGA is considered as the best method for studying the thermal stability of polymers (Abdelrazek et al., 2010). **Figure 1.2** shows the mass loss curves of all samples (films and film components) over heating and its first derivative. **Table 1.1** shows the values of the initial degradation temperature (T_0) and maximum degradation rate temperature (T_{max}) of the films components and films, as well as the percentage mass loss of all samples at the end of the TGA test (600°C). The Tmax values of the pure compounds (native CS, CH, Gly and PEG) were 321°C, 302°C, 251°C and 407°C, respectively. Similar results have been reported previously (Pelissari et al., 2009; Dou et al., 2009; Zampori et al., 2012). Therefore, the T_0 values of all film components were higher than 200°C, which guarantees their stability in the elaboration of the films by compression molding.

Table 1.1 Thermal properties of the films analyzed by TGA (T_0, T_{max}, % Mass loss over degradation). Mean values and standard deviation.

Samples	T_0 (°C)	T_{max} (°C)	% Mass loss
Native CS	294.5 ± 0.4	321.3 ± 0.4	90.5 ± 0.3
CH	278.93 ± 0.08	302.5 ± 0.5	76.12 ± 0.14
Gly	220.7 ± 0.3	250.8 ± 0.4	96.95 ± 0.07
PEG	372.8 ± 0.3	406.91 ± 0.11	98.32 ± 0.11
$(CS_{100}\text{-}CH_0)_{70}\text{-}P_{30}$	$299.5 \pm 0.3^{(e)}$	$319.3 \pm 0.5^{(ab)}$	$92.43 \pm 0.07^{(f)}$
$(CS_{90}\text{-}CH_{10})_{70}\text{-}P_{30}$	$291.1 \pm 1.4^{(cd)}$	$318.82 \pm 0.13^{(ab)}$	$84.20 \pm 0.10^{(cd)}$
$(CS_{80}\text{-}CH_{20})_{70}\text{-}P_{30}$	$281.2 \pm 3.5^{(b)}$	$319.8 \pm 0.6^{(bc)}$	$82.23 \pm 0.19^{(a)}$
$(CS_{70}\text{-}CH_{30})_{70}\text{-}P_{30}$	$268.7 \pm 1.4^{(a)}$	$319.91 \pm 0.11^{(bc)}$	$81.7 \pm 0.6^{(a)}$
$(CS_{100}\text{-}CH_0)_{60}\text{-}P_{40}$	$296.2 \pm 1.4^{(de)}$	$318.1 \pm 1.5^{(a)}$	$86.61 \pm 0.30^{(e)}$
$(CS_{90}\text{-}CH_{10})_{60}\text{-}P_{40}$	$272.5 \pm 6.4^{(a)}$	$319.1 \pm 0.7^{(ab)}$	$83.3 \pm 0.5^{(b)}$
$(CS_{80}\text{-}CH_{20})_{60}\text{-}P_{40}$	$286.3 \pm 0.5^{(bc)}$	$322.92 \pm 0.12^{(d)}$	$84.76 \pm 0.05^{(d)}$
$(CS_{70}\text{-}CH_{30})_{60}\text{-}P_{40}$	$293.1 \pm 1.2^{(de)}$	$321.1 \pm 0.7^{(c)}$	$83.60 \pm 0.15^{(bc)}$

Different superscript letters (a,b,c,..) within the same column indicate significant differences among film formulations ($p < 0.05$).

The mass loss curves of the film samples (**Figure 1.2**) showed three separate steps. The first corresponds to the initial weight loss attributed to the loss of water. The percentage mass reduction was coherent with the water content of these (about 10-15 %). The second stage at around 200°C can be attributed to the degradation of glycerol (Dick et al., 2015; Dou et al., 2009). The third mass loss step at about 320°C corresponds to the degradation of the polymers, and it supossed the most important mass loss.

The addition of chitosan up to the mass ratio 70:30 (CS:CH) did not affect the thermal stability of the films. Both pure polymers showed similar degradation pattern and their blend degradation were mainly affected by the presence of plastizicers. Degradation process in the blend films was more extended, starting at lower temperature due to the early glycerol degradation. Pelissari et al. (2009) also reported similar Tmax values for cassava starch-chitosan films produced by extrusion. The reported values of T_0 and $T_{\max}$ of the starch films, regardless the plasticizer proportion, were similar to those reported for corn starch and cassava starch films obtained by compression molding and extrusion (Ortega-Toro et al., 2014; Dang & Yoksan, 2015). Additionally, it was observed that, as chitosan proportion was increased in the film formulation, the percentage mass loss at 600°C was reduced, in agreement with the greater mass residue of CH. This is coherent with previous studies (Dang & Yoksan, 2015) on cassava starch-chitosan films.

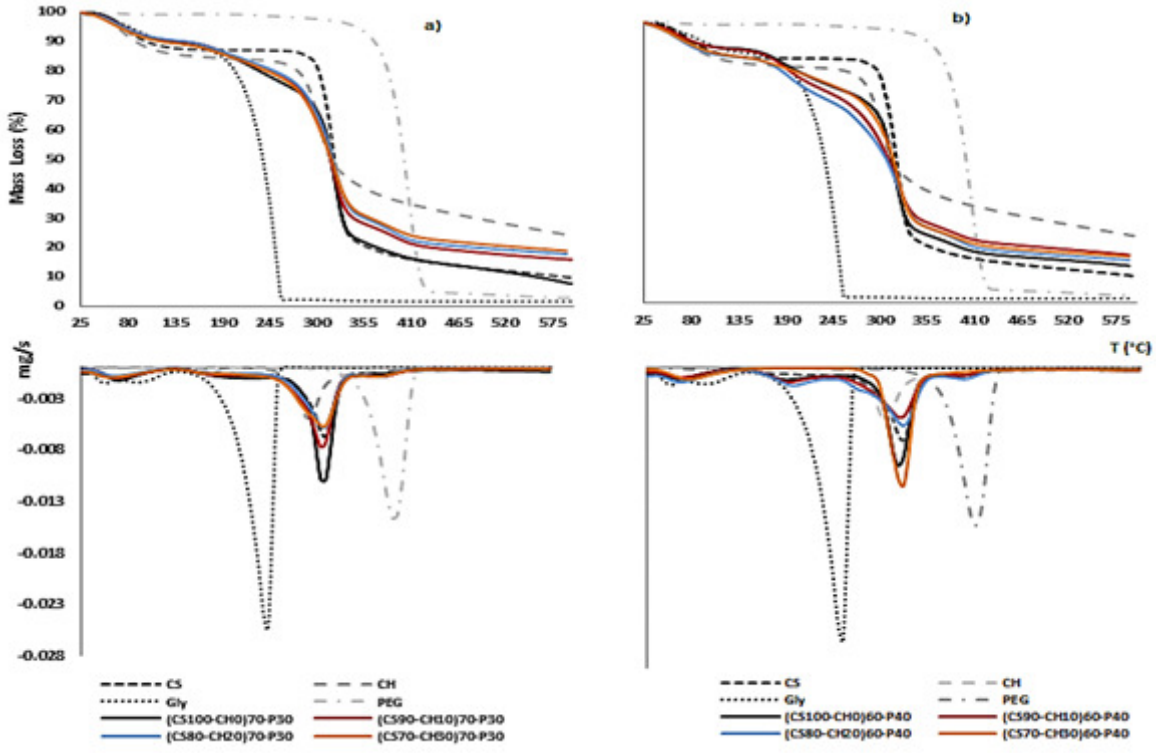

Figure 1.2. Typical thermogravimetric curves (mass loss vs. temperature) and first derivative (mg/s vs temperature) for **a)** Polymer: plasticizer proportion 70:30 and **b)** Polymer: plasticizer proportion 60:40.

3.3. Differential scanning calorimetry (DSC)

Table 1.2 shows the thermal properties (T_m, ΔH_m and % crystalinity of PEG) of the studied films conditioned for 1 week at 25°C and 53 % RH, along with those of pure PEG. No glass transition or melting of polymers were observed in the temperature range analysed, but both in the first and the second heating steps, a melting endotherm attributable to PEG was observed at the temperatures (T_m) reported in Table 1.2, with the corresponding ΔH_m values. Pure PEG sample shows an endothermic peak at 62.5°C, with a melting enthalpy of 194 J/g. Similar results were obtained by Song et al. (2008) and Boscá et al. (2002).

Table 1.2 Thermal properties of films analyzed by DSC. Mean values and standard deviation.

Samples	T_m (°C)		ΔH_m (J/g PEG)		% Crystallinity (PEG)	
	1^{st} heating	2^{nd} heating	1^{st} heating	2^{nd} heating	1^{st} heating	2^{nd} heating
$(CS_{100}\text{-}CH_0)_{70}\text{-}P_{30}$	$56.4 \pm 0.6^{(a)(x)}$	$50.4 \pm 1.1^{(bc)(y)}$	$68.6 \pm 0.4^{(f)(x)}$	$28.9 \pm 0.9^{(e)(y)}$	$35.4 \pm 0.7^{(b)(x)}$	$8.8 \pm 0.2^{(b)(y)}$
$(CS_{90}\text{-}CH_{10})_{70}\text{-}P_{30}$	$58.1 \pm 0.6^{(bc)(x)}$	$52.4 \pm 0.3^{(d)(y)}$	$73.4 \pm 0.9^{(e)(x)}$	$47.3 \pm 0.5^{(c)(y)}$	$37.9 \pm 0.6^{(c)(x)}$	$24.97 \pm 0.01^{(g)(y)}$
$(CS_{80}\text{-}CH_{20})_{70}\text{-}P_{30}$	$56.84 \pm 0.03^{(ab)(x)}$	$50.8 \pm 0.2^{(bc)(y)}$	$81.2 \pm 1.4^{(d)(x)}$	$38.9 \pm 1.4^{(d)(y)}$	$42.0 \pm 0.7^{(d)(x)}$	$20.1 \pm 0.4^{(e)(y)}$
$(CS_{70}\text{-}CH_{30})_{70}\text{-}P_{30}$	$58.6 \pm 1.2^{(c)(x)}$	$51.0 \pm 0.7^{(cd)(y)}$	$104 \pm 4^{(b)(x)}$	$57 \pm 2^{(b)(y)}$	$53.4 \pm 0.5^{(f)(x)}$	$29.70 \pm 0.5^{(h)(y)}$
$(CS_{100}\text{-}CH_0)_{60}\text{-}P_{40}$	$58.51 \pm 0.02^{(c)(x)}$	$48.8 \pm 0.4^{(a)(y)}$	$73.8 \pm 1.4^{(e)(x)}$	$14.59 \pm 0.12^{(g)(y)}$	$38.2 \pm 0.7^{(c)(x)}$	$6.40 \pm 0.06^{(a)(y)}$
$(CS_{90}\text{-}CH_{10})_{60}\text{-}P_{40}$	$60.6 \pm 1.7^{(d)(x)}$	$50.5 \pm 0.4^{(bc)(y)}$	$91.4 \pm 2.1^{(c)(x)}$	$41 \pm 0.6^{(d)(y)}$	$47.2 \pm 0.9^{(e)(x)}$	$21.8 \pm 0.2^{(f)(y)}$
$(CS_{80}\text{-}CH_{20})_{60}\text{-}P_{40}$	$60.2 \pm 1.2^{(d)(x)}$	$50.79 \pm 0.11^{(bc)(y)}$	$95 \pm 2^{(c)(x)}$	$30.1 \pm 0.9^{(e)(y)}$	$48.9 \pm 1.1^{(e)(x)}$	$17.57 \pm 0.13^{(d)(y)}$
$(CS_{70}\text{-}CH_{30})_{60}\text{-}P_{40}$	$57.3 \pm 0.6^{(abc)(x)}$	$49.4 \pm 0.5^{(ab)(y)}$	$62 \pm 4^{(g)(x)}$	$21.3 \pm 1.4^{(f)(y)}$	$32.2 \pm 0.5^{(a)(x)}$	$11.31 \pm 0.15^{(c)(y)}$
PEG	$62.5 \pm 0.3^{(e)(x)}$	$56.4 \pm 0.5^{(e)(y)}$	$194 \pm 3^{(a)(x)}$	$174 \pm 3^{(a)(y)}$		

Different superscript letters (a,b,c,..) within the same column indicate significant differences among formulations ($p < 0.05$). Different superscript letters (x,y) within the same formulation indicate significant differences between different heating ($p < 0.05$).

Over the first heating step of the film samples, this endotherm was observed at slightly lower temperatures, the T_m values ranging between 56 and 61°C. T_m reduction was also observed over the second heating step when the thermal history of the sample had been deleted. This reduction in the melting temperature, with respect to that of pure PEG, suggests that some film component are partially miscible with PEG, depressing its melting point, coherently with previous reports (Song et al., 2008). In a compatible blend, the melting temperature of one component is often reduced owing to the increasing of lattice defects resulted from the partial miscibility of the noncrystalline phase. In fact, previous studies demonstrated attractive interactions between chitosan and PEG by viscometry analysis (Jiang & Han, 1998).

The degree of crystallinity of PEG in the films was estimated from the enthalpy values of pure compound assuming complete crystallization in this case. In general, crystallization degree increased when the CH ratio rose in the film, which could be related with the previously commented interactions between these components. Nevertheless, sample with the higesth CH ratio and 40 % plasticizers exhibit the lowest crystallization degree. Higher plasticization promotes molecular mobility and so the polymer crystallization. The PEG crystallization in the films will limit its plasticizing effect since the crystallized fraction is in a separated phase without interactions with the polymer chains. Then, the reduction of PEG ratio in the films could be considered.

3.4. Thickness, moisture content and water vapour permeability (WVP)

Table 1.3 shows the values of thickness along with water vapour permeability (WVP) and moisture content for 1 and 5 week storage at 53 % RH-25°C. As can be observed, the film thickness increases when CH was incorporated into the film (from 170 μm to 260 μm). Similar results were reported by López et al. (2014) and Pelissari et al. (2009) studying starch and chitosan films obtained by extrusion. As observed in **Figure 1.1**, the parcial incompatibility of both polymers leads to more heterogeneous structure and this in turn results in a film thickening effect.

Table 1.3 Thickness, moisture content and water vapour permeability (WVP) of films equilibrated at 53 % RH. Mean values and standard deviation.

Film	Thickness (μm)	WVP (g mm kPa^{-1} h^{-1} m^{-2})		Moisture content (g water/ 100g dry film)	
		Week 1	Week 5	Week 1	Week 5
$(CS_{100}\text{-}CH_0)_{70}\text{-}P_{30}$	$199 \pm 12^{(b)}$	$16 \pm 3^{(abc)(1)}$	$15.1 \pm 0.9^{(bc)(1)}$	$10.4 \pm 0.4^{(a)(1)}$	$11.8 \pm 0.4^{(b)(2)}$
$(CS_{90}\text{-}CH_{10})_{70}\text{-}P_{30}$	$216 \pm 5^{(c)}$	$15.3 \pm 0.8^{(ab)(1)}$	$15.5 \pm 0.4^{(bc)(1)}$	$9.8 \pm 0.5^{(a)(1)}$	$11.8 \pm 0.8^{(b)(2)}$
$(CS_{80}\text{-}CH_{20})_{70}\text{-}P_{30}$	$237 \pm 6^{(d)}$	$14.10 \pm 0.11^{(a)(1)}$	$13.2 \pm 0.6^{(a)(1)}$	$9.7 \pm 1.1^{(a)(1)}$	$10.2 \pm 0.5^{(a)(1)}$
$(CS_{70}\text{-}CH_{30})_{70}\text{-}P_{30}$	$260 \pm 12^{(f)}$	$14.7 \pm 1.5^{(a)(1)}$	$14.5 \pm 0.9^{(ab)(1)}$	$9.9 \pm 0.8^{(a)(1)}$	$10.7 \pm 0.2^{(a)(2)}$
$(CS_{100}\text{-}CH_0)_{60}\text{-}P_{40}$	$170 \pm 6^{(a)}$	$21 \pm 3^{(e)(1)}$	$16.9 \pm 1.2^{(de)(2)}$	$12.4 \pm 0.5^{(b)(1)}$	$13.7 \pm 1.2^{(c)(2)}$
$(CS_{90}\text{-}CH_{10})_{60}\text{-}P_{40}$	$193 \pm 8^{(b)}$	$19 \pm 2^{(cd)(1)}$	$17.1 \pm 1.1^{(de)(1)}$	$12.8 \pm 0.4^{(b)(1)}$	$15.0 \pm 1.1^{(d)(2)}$
$(CS_{80}\text{-}CH_{20})_{60}\text{-}P_{40}$	$229 \pm 4^{(d)}$	$20 \pm 2^{(de)(1)}$	$16.3 \pm 1.1^{(cd)(1)}$	$12.2 \pm 0.4^{(b)(1)}$	$14.6 \pm 0.2^{(cd)(2)}$
$(CS_{70}\text{-}CH_{30})_{60}\text{-}P_{40}$	$247 \pm 10^{(e)}$	$18 \pm 2^{(bcd)(1)}$	$18 \pm 2^{(e)(1)}$	$12.4 \pm 0.5^{(b)(1)}$	$14.4 \pm 0.7^{(cd)(2)}$

Different superscript letters (a,b,c..) within the same column indicate significant differences among formulations ($p < 0.05$). Different superscript numbers (1,2) within the same row indicate significant differences for the same formulation with different storage time. ($p < 0.05$).

The moisture content values ranged between 10 and 15 g water / 100 g dry film. It was significantly increased after 5 storage weeks ($p < 0.05$), indicating that films equilibrate slowly with the ambient relative humidity, even though storage time did not affect WVP significantly. Similar results have been reported previously by Ortega-Toro et al. (2014) for CS-HPMC films obtained by compression molding, and by Jiménez et al. (2012b) in CS- fatty acids films obtained by casting. In these studies, storing the films for 5 weeks resulted in a significant increase ($p < 0.05$) in the film moisture content with no effect on the WVP values, regardless of the film composition. Very small differences in the moisture content of the films were observed for the different CS:CH ratios, although the increase in the plasticizer content promoted moisture gain.

WVP is an important property that is directly related to film capacity to hinder the water vapor transport applied to foods. In this sense, Ma et al. (2008) pointed out that WVP must be as low as possible to avoid the water transference. The polymer: plasticizer ratio had a significant effect on both the moisture content and WVP ($p < 0.05$), and regardless the storage time, as the plasticizer proportion was increased, both properties rose. Previous works have reported this effect of plasticizer on the moisture content on the films (Mali et al., 2006) and WVP (Alves et al., 2007; Chillo et al., 2008). The addition of plasticizer modifies the molecular organization making the structure less compact and therefore more permeable. An increase in inter-chain spacing due to inclusion of plasticizer molecules may promote water vapor diffusivity through the film, hence enabling water vapor transmission (Yang & Paulson, 2000). Contrarily, the CS:CH mass ratio did not have a significant effect on WVP.

3.5. Mechanical properties

The elastic modulus (EM), tensile strength at break (TS) and percentage elongation at break (%E) are useful parameters to describe the mechanical properties of films, and closely related to the film microstructure (Ninnemann, 1968; Mc Hugh & Krochta, 1994). EM indicates the rigidity of the material, TS represents the film resistance to break, whereas elongation at break (%E) is determined at the point where the film breaks under tensile testing and gives information about the film flexibility and stretchability (Fabra et al., 2008). **Table 1.4** shows these parameters for each film formulation after 1 and 5 week storage at 25°C and 53 % RH, and **Figure 1.3** represents the corresponding stress-strain curves of the films. The polymer: plasticizer ratio had a significant effect on both EM and TS ($p < 0.05$), whereas no effect on %E was found.

Table 1.4 Tensile properties (elastic modulus: EM, tensile strength: TS and deformation: E %, at break) of all films equilibrated at 53 % RH after 1 and 5 week storage. Mean values and standard deviation.

Films	EM (MPa)		TS (MPa)		% (E (%))	
	Week 1	Week 5	Week 1	Week 5	Week 1	Week 5
$(CS_{100}\text{-}CH_0)_{70}\text{-}P_{30}$	$321 \pm 19^{(b)(1)}$	$542 \pm 28^{(e)(2)}$	$7.4 \pm 0.5^{(a)(1)}$	$13.3 \pm 0.8^{(b)(2)}$	$7.9 \pm 0.6^{(h)(1)}$	$5.2 \pm 0.3^{(e)(2)}$
$(CS_{90}\text{-}CH_{10})_{70}\text{-}P_{30}$	$422 \pm 12^{(d)(1)}$	$761 \pm 49^{(f)(2)}$	$13.7 \pm 0.7^{(e)(1)}$	$14.5 \pm 0.4^{(c)(2)}$	$4.7 \pm 0.3^{(f)(1)}$	$2.0 \pm 0.2^{(a)(2)}$
$(CS_{80}\text{-}CH_{20})_{70}\text{-}P_{30}$	$596 \pm 22^{(g)(1)}$	$930 \pm 20^{(g)(2)}$	$16.8 \pm 0.3^{(f)(1)}$	$18.1 \pm 0.5^{(d)(2)}$	$3.3 \pm 0.2^{(e)(1)}$	$1.88 \pm 0.12^{(a)(2)}$
$(CS_{70}\text{-}CH_{30})_{70}\text{-}P_{30}$	$732 \pm 16^{(h)(1)}$	$1118 \pm 36^{(h)(2)}$	$20.0 \pm 0.9^{(g)(1)}$	$22.2 \pm 0.8^{(e)(2)}$	$2.8 \pm 0.2^{(d)(1)}$	$1.91 \pm 0.13^{(a)(2)}$
$(CS_{100}\text{-}CH_0)_{60}\text{-}P_{40}$	$245 \pm 6^{(a)(1)}$	$145 \pm 11^{(a)(2)}$	$9.3 \pm 0.3^{(b)(1)}$	$8.6 \pm 0.2^{(a)(2)}$	$6.4 \pm 0.5^{(g)(1)}$	$8.5 \pm 0.3^{(g)(2)}$
$(CS_{90}\text{-}CH_{10})_{60}\text{-}P_{40}$	$344 \pm 15^{(c)(1)}$	$281 \pm 16^{(b)(2)}$	$9.8 \pm 0.4^{(bc)(1)}$	$8.3 \pm 0.5^{(a)(2)}$	$2.31 \pm 0.14^{(bc)(1)}$	$5.6 \pm 0.4^{(f)(2)}$
$(CS_{80}\text{-}CH_{20})_{60}\text{-}P_{40}$	$463 \pm 14^{(e)(1)}$	$350 \pm 20^{(c)(2)}$	$10.1 \pm 0.6^{(c)(1)}$	$8.1 \pm 0.7^{(a)(2)}$	$2.1 \pm 0.2^{(ab)(1)}$	$4.4 \pm 0.3^{(d)(2)}$
$(CS_{70}\text{-}CH_{30})_{60}\text{-}P_{40}$	$546 \pm 32^{(f)(1)}$	$459 \pm 35^{(d)(2)}$	$10.8 \pm 0.8^{(d)(1)}$	$8.5 \pm 0.3^{(a)(2)}$	$1.93 \pm 0.13^{(a)(1)}$	$2.6 \pm 0.2^{(bc)(2)}$

Different superscript letters (a,b,c..) within the same column indicate significant differences among formulations ($p < 0.05$). Different superscript numbers (1,2) within the same row indicate significant differences for the same formulation with different storage time. ($p < 0.05$).

The films with higher plasticizer ratio (60:40) were significantly less rigid and less resistant (lower EM and TS) than those with ratio the 70:30, both after 1 and 5 week storage. This is in agreement with previous reports. Da Róz et al. (2006) observed that the quantity and type of plasticizer influenced the mechanical properties of thermoplastic starch (TPS) obtained by melt processing, and a softening effect caused by the plasticizing of the amorphous phase was observed. Sothornvit et al. (2007) observed a significant reduction of EM and TS as the glycerol ratio increased in compression molded whey protein films. The same trend was reported in other studies (Alves et al., 2007; Mali et al., 2004; Sobral et al., 2001). The molecular size, configuration and total number of functional hydroxide groups of the plasticizer as well as its compatibility with the polymer could affect the interactions between the plasticizer and the polymer (Yang & Paulson, 2000). Being a small molecule, glycerol can get into the polymer chains and weaken the interactions between them (Su et al., 2010).

The CS:CH ratio also affected the mechanical properties significantly, and as the CH ratio was increased, the films became stiffer, more resistant and less stretchable ($p < 0.05$), regardless the storage time (**Table 1.4**, **Figure 1.3**). In fact, no plastic deformation was observed for CH containing films (**Figure 1.3**).

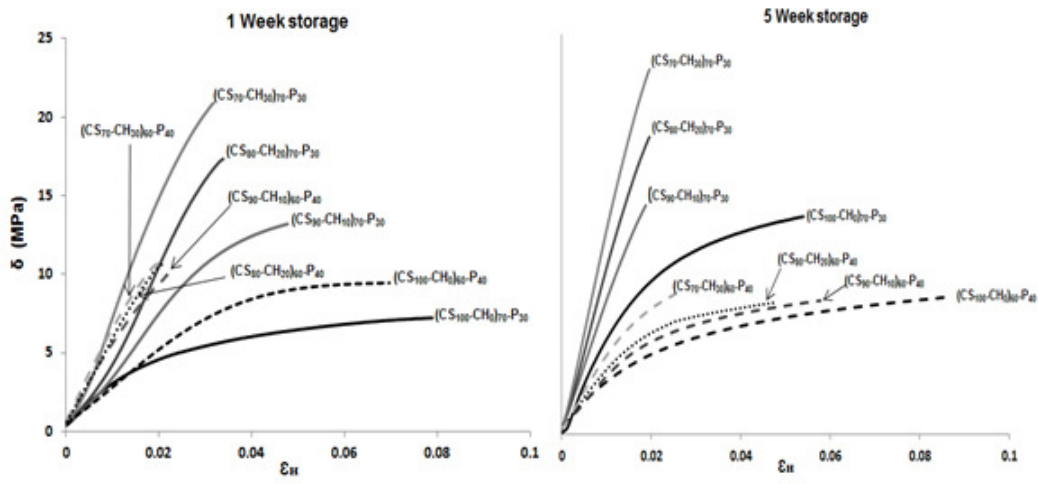

Figure 1.3. Typical stress-strain curves of the films after 1 week (left) and 5 weeks (right) of storage at 53 % RH.

The improvement of the tensile parameters in starch films caused by chitosan addition had previously been observed both in films obtained by casting (Bonilla et al., 2013; Chillo et al., 2008) and by extrusion (López et al., 2014; Bourtoom & Chinan, 2008; Pelissari et al., 2012). This effect could be attributed to the intermolecular hydrogen bonding between starch -OH groups with chitosan and chitin - NH_2 groups (López et al., 2014). As the chitosan proportion increased in the film formulation, more -NH2 groups are available to form hydrogen bonds with starch, hence improving the film resistance (Pelissari et al. 2012). The stretchability reduction in starch blend films caused by chitosan addition has been previously reported by several authors (Bourtoom & Chinan, 2008; Pelissari et al., 2012; Xu et al., 2005; Lóopez et al., 2014). Pelissari et al. (2012) justified this reduction by pointing out the potential increase of the starch crystallinity caused by chitosan, although no starch melting was observed in the studied formulations at the ususal temperature (150-200°C, López et al. 2014). The mixture effect increasing the structural heterogeneity could also result in some film shortening and an additional effect of the PEG's reduced plasticizing efficiency, due to its greater crystallization in CH containing films, could also be pointed out.

Storage time did not modify the tensile parameters following a clear pattern. In fact, the effect of plasticizer content and CH was more coherent at 5 storage weeks probably by the complete film equilibration, which avoid variability associated to differences in the moisture content. The small changes observed in tensile behavior must be attributed to the moisture content equilibration more than to relevant changes in the polymer matrix during storage.

3.6 Optical properties: transparency, colour and gloss

Figure 1.4 shows typical internal transmittance spectra of films, from 400 to 700 nm, as a transparency indicator. Higher values of internal transmittance (T_i) are related with higher structural homogeneity and more transparent films (Villalobos et al., 2005). Of the films tested, those with no CH were the most transparent, regardless the plasticizer ratio and the storage time (**Figure 1.4**). Cassava starch has previously shown high transparency in a comparative study reported by Cano et al. (2014).

On the other hand, the incorporation of CH brought about a decrease in T_i, closely related to the chitosan proportion incorporated. This is in accordance to the increased heterogeneity of the films caused by chitosan incorporation, as previously described. The heterogeneous structure implies the occurrence of changes in the refractive index and higher light dispersion resulting in transparency loss, which is especially remarkable at low wavelength. This transparency reduction of starch films caused by chitosan addition was previously observed by López et al. (2014), Pelissari et al. (2012) and Dang & Yoksan (2015). The selective reduction of Ti at low wavelengths agrees with the yellowness development in the films during thermoprocessing, as commented on below.

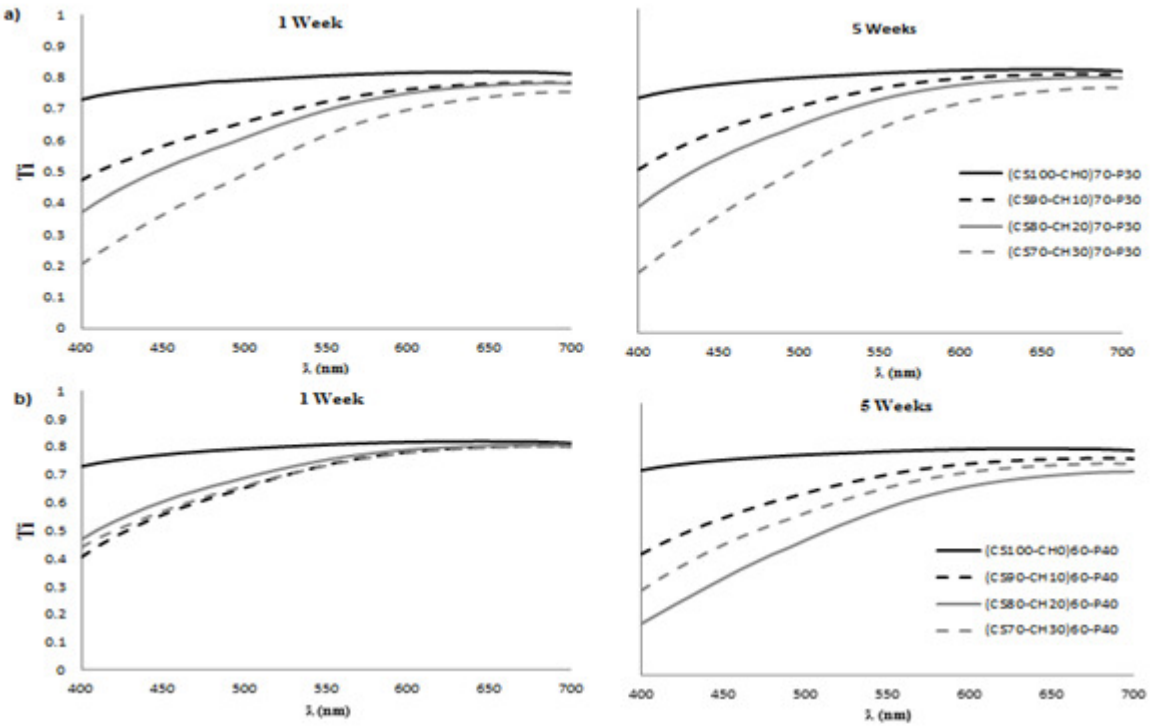

Figure 1.4. Spectral distribution of the internal transmittance (Ti) of the films. **(a)** Ratio polymer: plasticizer 70:30. **(b)** Ratio polymer: plasticizer 60:40. After 1 week and 5 weeks of storage.

The results of the optical parameters (lightness, chrome, hue, whiteness index and gloss) of the films after 1 and 5 weeks storage at 25°C and 53 % RH are reported in **Table 1.5**. Regardless of the plasticizer proportion and the storage time, increasing the chitosan proportion resulted in a lightness and hue reduction, as well as a chroma increase ($p < 0.05$). In fact, the films with increased CH proportion became more saturated yellowish color. WI was reduced consequently. Similar results were reported when chitosan was incorporated in cassava starch films obtained by extrusion (Dang & Yoksan, 2015), cassava starch films were processed by blown film extrusion (Khanh & Rangrong, 2015) and corn starch films were obtained by thermo-compression (López et al., 2014).

This color change has been attributed to the occurrence of Maillard reactions between amino groups of CH and the present carbonyl groups in the starch fraction (López et al., 2014). This reaction is promoted by the processing temperature and, in the early stage, involves the formation of conjugates between the carbonyl and amine groups, producing Schiff bases, which subsequently cyclizes in the Amadori compounds and insoluble polymeric compounds, referred as melanoidins. It is remarkable that films with 40 % plastizicer exhibited less browning after processing, but the reaction progressed during the film storage.

The film gloss is related to the surface morphology (Sánchez-González et al., 2010). In this study, all film formulations had low gloss values at both 1 and 5 storage weeks. This is in agreement with previous results on starch-HPMC thermo-compressed films (Ortega-Toro et al., 2014). The limited compatibility of both polymers, as observed in section 3.1., may be responsible for the films surface roughness and the consequent reduced gloss.

Table 1.5 Lightness (L*), chroma (C^*_{ab}), hue (h^*_{ab}), whiteness index (WI) and gloss at 60° after 1 and 5 week storage. Mean values and standard deviation.

	Films	L*	C^*_{ab}	h^*_{ab}	WI	Gloss (60°)
Week 1	$(CS_{100}\text{-}CH_0)_{70}\text{-}P_{30}$	$74.4 \pm 0.3^{(e)(1)}$	$8.1 \pm 0.3^{(a)(1)}$	$84.41 \pm 0.12^{(f)(1)}$	$73.1 \pm 0.4^{(g)(1)}$	$6.4 \pm 1.1^{(a)(1)}$
	$(CS_{90}\text{-}CH_{10})_{70}\text{-}P_{30}$	$62.7 \pm 0.5^{(d)(1)}$	$24.5 \pm 0.6^{(b)(1)}$	$73.9 \pm 0.4^{(d)(1)}$	$55.3 \pm 0.7^{(e)(1)}$	$7.1 \pm 0.6^{(b)(1)}$
	$(CS_{80}\text{-}CH_{20})_{70}\text{-}P_{30}$	$58.8 \pm 0.7^{(b)(1)}$	$29.8 \pm 0.6^{(e)(1)}$	$69.9 \pm 0.4^{(b)(1)}$	$49.2 \pm 0.9^{(b)(1)}$	$9.0 \pm 0.9^{(d)(1)}$
	$(CS_{70}\text{-}CH_{30})_{70}\text{-}P_{30}$	$52.8 \pm 0.2^{(a)(1)}$	$32.2 \pm 0.7^{(f)(1)}$	$66.7 \pm 0.4^{(a)(1)}$	$43.20 \pm 0.03^{(a)(1)}$	$9.3 \pm 1.2^{(d)(1)}$
	$(CS_{100}\text{-}CH_0)_{60}\text{-}P_{40}$	$73.3 \pm 0.3^{(f)(1)}$	$8.3 \pm 0.2^{(a)(1)}$	$85.4 \pm 0.4^{(g)(1)}$	$72.0 \pm 0.3^{(f)(1)}$	$5.9 \pm 0.7^{(a)(1)}$
	$(CS_{90}\text{-}CH_{10})_{60}\text{-}P_{40}$	$59.1 \pm 0.4^{(b)(1)}$	$28.8 \pm 0.6^{(d)(1)}$	$71.7 \pm 0.5^{(c)(1)}$	$50.0 \pm 0.7^{(b)(1)}$	$7.3 \pm 0.8^{(b)(1)}$
	$(CS_{80}\text{-}CH_{20})_{60}\text{-}P_{40}$	$63.0 \pm 0.3^{(d)(1)}$	$27.5 \pm 0.5^{(c)(1)}$	$74.4 \pm 0.3^{(e)(1)}$	$54.0 \pm 0.5^{(d)(1)}$	$7.4 \pm 0.3^{(b)(1)}$
	$(CS_{70}\text{-}CH_{20})_{60}\text{-}P_{40}$	$62.1 \pm 1.1^{(c)(1)}$	$29.50 \pm 0.12^{(e)(1)}$	$71.5 \pm 0.4^{(c)(1)}$	$51.7 \pm 0.9^{(c)(1)}$	$8.0 \pm 0.8^{(c)(1)}$
Week 5	$(CS_{100}\text{-}CH_0)_{70}\text{-}P_{30}$	$73.6 \pm 0.4^{(f)(1)}$	$8.6 \pm 0.6^{(a)(1)}$	$83.8 \pm 1.2^{(f)(1)}$	$72.3 \pm 0.3^{(g)(2)}$	$6.6 \pm 0.6^{(bc)(1)}$
	$(CS_{90}\text{-}CH_{10})_{70}\text{-}P_{30}$	$65.5 \pm 1.1^{(e)(2)}$	$26.4 \pm 0.4^{(b)(2)}$	$76.6 \pm 1.1^{(e)(2)}$	$56.8 \pm 0.4^{(e)(2)}$	$6.9 \pm 0.4^{(d)(1)}$
	$(CS_{80}\text{-}CH_{20})_{70}\text{-}P_{30}$	$61.6 \pm 0.3^{(d)(2)}$	$30.7 \pm 0.5^{(d)(2)}$	$73.2 \pm 0.3^{(c)(2)}$	$50.8 \pm 0.5^{(c)(2)}$	$8.6 \pm 0.5^{(e)(1)}$
	$(CS_{70}\text{-}CH_{30})_{70}\text{-}P_{30}$	$54.5 \pm 0.7^{(b)(2)}$	$35.3 \pm 0.6^{(g)(2)}$	$68.5 \pm 0.6^{(a)(2)}$	$42.6 \pm 0.9^{(a)(1)}$	$8.7 \pm 0.5^{(e)(2)}$
	$(CS_{100}\text{-}CH_0)_{60}\text{-}P_{40}$	$72.8 \pm 0.2^{(g)(1)}$	$8.7 \pm 0.7^{(a)(1)}$	$83.7 \pm 0.9^{(f)(2)}$	$71.4 \pm 0.2^{(f)(1)}$	$6.5 \pm 0.6^{(b)(2)}$
	$(CS_{90}\text{-}CH_{10})_{60}\text{-}P_{40}$	$61.8 \pm 0.4^{(d)(2)}$	$27.8 \pm 0.7^{(c)(2)}$	$74.3 \pm 0.5^{(d)(2)}$	$52.7 \pm 0.7^{(d)(2)}$	$6.0 \pm 0.4^{(a)(2)}$
	$(CS_{80}\text{-}CH_{20})_{60}\text{-}P_{40}$	$53.4 \pm 0.4^{(a)(2)}$	$33.4 \pm 0.2^{(f)(2)}$	$68.3 \pm 0.2^{(a)(2)}$	$42.8 \pm 0.2^{(a)(2)}$	$6.2 \pm 0.4^{(a)(2)}$
	$(CS_{70}\text{-}CH_{30})_{60}\text{-}P_{40}$	$58.6 \pm 1.3^{(c)(2)}$	$32.7 \pm 0.5^{(e)(2)}$	$70.6 \pm 0.7^{(b)(2)}$	$47.2 \pm 1.2^{(b)(2)}$	$6.9 \pm 0.5^{(cd)(2)}$

Different superscript letters (a,b,..) within the same column indicate significant differences among formulations ($p < 0.05$). Different superscript numbers (1,2) within the same row indicate significant differences for the same formulation with different storage time. ($p < 0.05$).

3.7. Antimicrobial properties

Table 1.6 shows the progress over storage time (up to 7 days) of the total aerobic and coliform counts corresponding to the control (meat without film) and pork meat samples coated with the four films with the proportion of polymer: plasticizer (70:30). In all cases, the total aerobic and coliform counts increased over time, and the chitosan proportion in the films seemed to have a slight effect on the progression of the counts and the final values. Cassava starch films with no chitosan $(CS_{100}\text{-}CH_0)_{70}\text{-}P_{30}$ had no antibacterial effect, and the bacterial growth in this case was even slightly higher than that found for control samples ($p < 0.05$). After 7 days of storage, the lowest populations corresponded to the films with the highest chitosan proportion ($p < 0.05$).

Table 1.6 The antimicrobial activity of the films on pork meat and stored 7 days at 10°C. Mean values and standard deviation.

		Time (days)			
	Films	0	1	4	7
coliforms (log CFU/g)	Control	$0.27 \pm 0.02^{(a)(1)}$	$1.07 \pm 0.04^{(c)(2)}$	$3.50 \pm 0.02^{(d)(3)}$	$5.36 \pm 0.03^{(c)(4)}$
	$(CS_{100}\text{-}CH_0)_{70}\text{-}P_{30}$	$0.64 \pm 0.03^{(c)(1)}$	$1.30 \pm 0.04^{(e)(2)}$	$3.67 \pm 0.02^{(e)(3)}$	$5.59 \pm 0.03^{(d)(4)}$
	$((CS_{90}\text{-}CH_{10})_{70}\text{-}P_{30}$	$0.67 \pm 0.03^{(c)(1)}$	$1.17 \pm 0.05^{(d)(2)}$	$3.42 \pm 0.04^{(c)(3)}$	$5.37 \pm 0.04^{(c)(4)}$
	$(CS_{80}\text{-}CH_{20})_{70}\text{-}P_{30}$	$0.57 \pm 0.03^{(b)(1)}$	$0.99 \pm 0.04^{(b)(2)}$	$3.10 \pm 0.03^{(b)(3)}$	$5.14 \pm 0.03^{(b)(4)}$
	$(CS_{70}\text{-}CH_{30})_{70}\text{-}P_{30}$	$0.28 \pm 0.02^{(a)(1)}$	$0.69 \pm 0.05^{(a)(2)}$	$2.96 \pm 0.09^{(a)(3)}$	$4.53 \pm 0.11^{(a)(4)}$
Total aerobic counts (log CFU/g)	Control	$1.28 \pm 0.07^{(b)(1)}$	$2.68 \pm 0.08^{(c)(2)}$	$4.74 \pm 0.07^{(c)(3)}$	$6.43 \pm 0.04^{(d)(4)}$
	$(CS_{100}\text{-}CH_0)_{70}\text{-}P_{30}$	$1.45 \pm 0.04^{(c)(1)}$	$2.86 \pm 0.13^{(d)(2)}$	$5.12 \pm 0.03^{(e)(3)}$	$6.55 \pm 0.04^{(e)(4)}$
	$(CS_{90}\text{-}CH_{10})_{70}\text{-}P_{30}$	$1.88 \pm 0.03^{(e)(1)}$	$3.21 \pm 0.14^{(e)(2)}$	$4.53 \pm 0.04^{(d)(3)}$	$5.47 \pm 0.02^{(c)(4)}$
	$(CS_{80}\text{-}CH_{20})_{70}\text{-}P_{30}$	$1.75 \pm 0.03^{(d)(1)}$	$2.11 \pm 0.04^{(b)(2)}$	$4.15 \pm 0.04^{(b)(3)}$	$5.26 \pm 0.09^{(b)(4)}$
	$(CS_{70}\text{-}CH_{30})_{70}\text{-}P_{30}$	$1.13 \pm 0.06^{(a)(1)}$	$1.71 \pm 0.08^{(a)(2)}$	$3.79 \pm 0.09^{(a)(3)}$	$5.01 \pm 0.05^{(a)(4)}$

Different superscript letters (a,b,c..) within the same column indicate significant differences among formulations for the same microbiological analysis ($p < 0.05$). Different superscript numbers (1,2,3,4) within the same row indicate significant differences for the same formulation with different storage time. ($p < 0.05$).

However, the previously reported antimicrobial effect of chitosan was not remarkable in this study, since only a 1 log reduction was achieved. Probably the method for film preparation led to strong interactions between the hydroxyl groups in starch and amino groups in chitosan, thus affecting the diffusion phenomenon of chitosan from the film matrix into the model food system studied (López et al., 2014). Likewise, Pelissari et al. (2009) reported that chitosan incorporation into cassava starch films produced by extrusion did not result in antimicrobial action. Probably, the antimicrobial activity of chitosan in films produced by casting is due to the previous solution in acetic acid, which protonates the NH_2 groups of chitosan and enhances solubility. Whenever chitosan dissolution is unnecessary (extrusion, compression molding) the NH2 groups are not protonated, which could explain the reduced antimicrobial activity.

4. CONCLUSIONS

The structural, thermal and physical properties of cassava starch films were affected by the incorporation of chitosan and the polymer:plasticizer proportion. Starch and chitosan exhibited lack of miscibility by melt blending, and the films exhibited a heterogeneous structure. All film components degraded over 200°C, guaranteeing their thermal stability over thermal processing. Chitosan incorporation did not notably modify the thermal stability of the films, although heat induced Maillard reaction occurred during the films' thermoprocessing, causing films yellowing. PEG was crystallized to great an extent in the films which will limit its plastizicing effect. The films with the highest plasticizer ratio had higher moisture content, were more permeable to water vapour, less rigid and less resistant. As the CH ratio increased, the films became stiffer, more resistant to break but less stretchable, which could be attributed to the intermolecular hydrogen bonding between starch and chitosan. Moreover, increasing the chitosan proportion resulted in lightness and hue reduction, as well as chroma increase. Contrarily to previous results in casting films, chitosan incorporation provided the films with only a slight antimicrobial capacity, probably linked to the film thermal processing, which could limit the CH diffusion into the food.

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III.2 CHAPTER 2

THERMOPLASTIC CASSAVA STARCH – CHITOSAN FILMS CONTAINING ESSENTIAL OILS

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ABSTRACT

Starch-chitosan bilayer films, containing or not essential oils in the casted chitosan layer were obtained by thermo-compression. Bilayer films exhibited a good interfacial adhesion and better mechanical resistance than starch monolayers, although they were less stretchable and less transparent. Starch-chitosan films were effective at controlling the bacterial growth in pork meat, but the thermal treatment applied to obtain the bilayers reduced their antimicrobial properties. The addition of essential oils did not promote any antimicrobial action in CH mono and bilayer films applied to pork meat. The final amount of essential oils in the films was very limited by the potential losses occurred during film processing method. Other strategies to incorporate the essential oils into chitosan-based films must be used in order to improve their final retention in the film matrix and their effective release into the coated food.

Keywords: thermoplastic starch; chitosan, thermal degradation, tensile properties, antimicrobial.

1. INTRODUCTION

In recent years, the interest in biodegradable films has grown mainly due to general concern about the disposal of conventional synthetic plastic materials since their full degradation generally requires a long period of time (Xu et al., 2005). Starch has been considered for many years as a biodegradable polymer with a high potential for packaging applications (Doane et al., 1992; Shogren, 1998). Biodegradable films based on hydrocolloids such as starch can act as barriers to control the transfer of moisture, oxygen, carbon dioxide, lipids, and flavor components, thus preventing quality deterioration and increase the shelf-life of food products (Ghanbarzadeh & Oromiehi, 2009; Naushad Emmambux &Stading, 2007). The use of starch from different sources (corn, cassava, wheat, rice, potato, pea, etc.) to obtain biodegradable plastics is being extensively studied since starch is abundant and accessible at a relatively low cost (Mali et al., 2006). Several authors have shown the possibility to transform native starch into thermoplastic-like products under restructuring and plasticization conditions (Tomka, 1991; Swanson, et al., 1993). Furthermore, the feasibility of processing starch by using plastic-processing equipment has long been demonstrated (Tomka, 1991).

Thermoplastic starch (TPS) can be processed in the same way as synthetic plastics through extrusion and injection units (Averous et al., 2000) and thermocompression (Flores et al., 2010; Pellissari et al., 2012; Thunwall et al., 2006). The thermocompression method is useful as a processing method because of its simplicity and capability of producing films (Lopez, et al., 2014). During the extrusion of starch, the combination of shear, temperature and plasticizers allows for producing a molten thermoplastic material by disruption of the native crystalline granular structure and plasticization. This plasticized starch could be suitable for injection moulding or thermoforming (Avérous et al., 2001). TPS is a very hydrophilic product (Averous et al., 2000) and yields films with high water sensitivity (Zobel, 1988) and poor mechanical properties (Van Soest, 1996), which change with time (crystallization due to ageing and plasticization by water adsorption). The combination of starch with chitosan (CH) led to the improvement of the functional properties of the films while conferring them antimicrobial properties (Bonilla et al., 2014).

Chitosan is a biodegradable biopolymer mainly extracted from chitin from shrimp waste (Wong et al., 1992). Chitosan has shown excellent properties such as bio-compatibility, non-toxicity and adsorption to negatively charged interfaces since it is positively charged in acid media (Lertsutthiwong et al., 2002; Weska et al., 2007 ; Youn, et al., 2007; Dutta et al., 2004).

The use of films as carriers of antimicrobial agents, such as essential oils, represents an interesting approach for the external incorporation of such active ingredients onto food system surfaces. Essential oils, which exhibit both antimicrobial and antioxidant capacity (Bakkali et al., 2008), were incorporated into the formulation of antimicrobial films based on chitosan to extend the shelf-life of pork meat (Bonilla et al., 2014). Oregano essential oil (OEO) has been shown to possess higher antimicrobial activity than other essential oils such as thyme or basil essential oil (Burt, 2004), and it has been effective at inhibiting the microbial growth of some foodborne microorganisms, such as Staphylococcus aureus, Escherichia coli and Bacillus subtilis (Lv et al., 2011). Carvacrol, the main compound of OEO, was effective in inhibiting the growth and survival of Listeria monocytogenes, Aeromonas hydrophila, and Pseudomonas fluorescens (de Sousa et al., 2012). Cinnamon leaf essential oil (CLEO) is recognized for its aroma and medicinal properties (Ayala-Zavala et al., 2008; Singh et al., 2007). The main component of CLEO is eugenol (70-95 %), followed by cinnamaldehyde, which can be present in a proportion of 1-5 % (Vangalapati, et al., 2012). In addition, CLEO antimicrobial and antifungal properties have also drawn great attention in many studies (Chang et al., 2001, Kim et al., 2004, Park et al., 2000; Singh et al., 1995). Essential oils and their active ingredients can diffuse from the film into the coated food to control target microorganisms. In this sense, the combination of biodegradable polymers (Starch- CH) as bilayers, where CH encapsulates the antimicrobial agent and is not submitted to the heat blending step, can be an interesting approach to obtain new biodegradable films with antimicrobial activity. However, there are not previous studies on the development of these types of bilayer films, to the best of our knowledge.

The aim of this work was to characterize the thermal behavior, optical, barrier, mechanical and antimicrobial properties of bilayer films prepared with thermoplastic starch and chitosan, containing or not oregano or cinnamon leaf essential oil in the chitosan layer.

2. MATERIALS AND METHODS

2.1. Materials

Cassava starch was supplied by Quimidroga S.A. (Barcelona, Spain), high molecular weight chitosan (practical grade, >75 % deacetylation degree, Batch MKBP1333V), polyethylene glycol (PEG), Tween 85 and Glycerol (Gly) were supplied by Sigma-Aldrich (Madrid, Spain). Oregano (OEO) and cinnamon leaf (CLEO) essential oils were provided by Herbes del Molí (Alicante, Spain) and Mg $(NO_3)_2$ was obtained from Panreac Química, S.A. (Castellar del

Vallés, Barcelona, Spain). Pork meat, which was used for the microbiological study, was purchased in a local supermarket. 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. Film preparation

Chitosan-based films were obtained by casting as previously described by Bonilla et al., (2013). Chitosan (1.0 % w/w) was dispersed in an aqueous solution of glacial acetic acid (1.0 % v/w) under magnetic stirring at 40°C for 24 hours. The film-forming dispersions (FFDs) were obtained by adding OEO or CLEO at 0.25 % (w/w) and Tween 85 at 0.1 % (w/w). FFDs were homogenized with a rotor-stator (13500 rpm, 4 minutes, Yellow Line DL 25 Basic, IKA, Janke y Kunkel, Germany) and degassed at room temperature with a vacuum pump. Subsequently, FFDs were cast in a framed and leveled polytetrafluorethylene (PTFE) plate (diameter = 15 cm, 5.6 mg solids/cm2) and dried at room temperature at 45 % RH. Sample codes for chitosan films, with or without OEO and CLEO were: CH, CH:OEO, and CH:CLEO.

Cassava starch films (CS) were obtained by melt blending and compression molding. Cassava starch, glycerol (Gly) and polyethyleneglycol (PEG) were dispersed in water in a polymer: plasticizer mass ratio of 1:0.3. Both plasticizers were mixed in a Gly: PEG mass ratio of 3:0.05 The formulations were melt blended on a two-roll mill (Model LRM-M-100, Labtech Engineering, Thailand) at 160°C for 30 minutes and afterwards were submitted to compression molding with a hot plates hydraulic press (Model LP20, Labtech Engineering, Thailand) at 160°C and 1.2 x 10^7 Pa. This film was named as CS. Chitosan-starch bilayer films were obtained by compressing CS film and chitosan based films, at 100°C for 2 minutes by means of the hot plate hydraulic press. The obtained bilayer films were named as follows: CS-CH; CS-CH:OEO and CS:CH:CLEO. Taking into account the relative weight of each layer with the same area, the ratio S:CH sheets was 3:1.

All films were stored at 25°C and 53 % relative humidity (RH) for one week prior performing the analysis of thermal, barrier, optical and mechanical properties. 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.

2.3. Field emission scanning electron microscopy (FESEM)

The microstructural analysis of the cross-sections of the bilayer films was carried out by means of a field emission scanning electron microscope (Ultra 55, Zeiss, Oxford Instruments, UK). The film samples were maintained in desiccators with P_2O_5 to guarantee that water was not present in the sample and two samples per film formulation were analyzed. Film pieces, 0.5 cm^2 approximately in size, were cryofractured from films and fixed on copper stubs, gold coated, and observed using an accelerating voltage of 2 kV.

2.4. Thermogravimetric analysis

A thermogravimetric analyzer (TGA/SDTA 851e, Mettler Toledo, Schwerzenbach, Switzerland), equipped with an ultra-micro weighing scale ($\pm$ 0.1 μg), was used to determine the thermal stability of the film samples under nitrogen flow (50 mL/min). The analysis was carried out using the following temperature program: heating from 25 to 600°C at a 10°C/min heating rate. Approximately 3 mg of each sample were used in each test, considering at least two replicates for each one. Initial degradation temperature (T_0) and the temperature of the maximum degradation rate (T_{max}), were registered from the first derivative of the resulting weight loss curves.

2.5. Mechanical properties

Mechanical properties were measured with a Universal Test Machine (TA.XT plus, Stable Micro Systems, Haslemere, England), following the ASTM standard method D882 (ASTM, 2001). Equilibrated film samples (25 mm wide and 100 mm long) were mounted in the film-extension grips (A/TG model), which were set 50 mm apart. Tension test was performed at 50 mm/min. Stress-strain curves were obtained and the tensile strength at break (TS), percentage of elongation at break (ϵ (%)) and elastic modulus (EM) were calculated. Ten replicates carried out per formulation.

2.6. Water vapor and oxygen permeability

The water vapor permeability (WVP) of the films was determined by using the ASTM E96-95 (ASTM, 1995) gravimetric method, taking into account the modification proposed by Mc Hugh et al. (1993). Films were selected for WVP tests based on the lack of physical defects such as cracks, bubbles, or pinholes. Distilled water was placed in Payne permeability cups (3.5 cm diameter, Elcometer SPRL, Hermelle /s Argenteau, Belgium) to expose the film to 100 % RH on one side. Once the films were secured, each cup was placed in a relative

humidity equilibrated cabinet at 25 °C, with a fan placed on the top of the cup to reduce resistance to water vapor transport. RH of the cabinets (53 %) was held constant using oversaturated solutions of magnesium nitrate-6-hydrate (Panreac Química, SA, Castellar del Vallés, Barcelona). The cups were weighed every 1.5 h for 24 h with an analytical scale (ME36S Sartorius, Germany, 0.0001 g) and water vapor transmission rate was determined from the slope obtained from the regression analysis of weight loss data versus time, once the steady state had been reached, divided by the film area. From this data, water vapor permeability values were obtained, taking into account the average valus of film thickness in each case. The equation proposed by McHugh et al. (1993) was used to correct the effect of concentration gradients established in the stagnant air gap inside the cup.

The oxygen barrier capacity of the films was evaluated by measuring oxygen permeability (OP) by means of an Ox-Tran 1/50 system (Mocon, Minneapolis, USA) at 25°C (ASTM Standard Method D3985-95, 2002). Measurements were taken at 53 % HR in films previously equilibrated at the same RH. Films were exposed to pure nitrogen flow on one side and pure oxygen flow on the other side. The OP was calculated by dividing the oxygen transmission rate by the difference in the oxygen partial pressure on the two sides of the film, and multiplying by the average film thickness. At least three replicates per formulation were taken into account.

2.7. Optical properties

The transparency of the films was determined by applying the Kubelka-Munk theory (Hutchings, 1999) for multiple scattering to the reflection spectra. When the light passes through the film, it is partially absorbed and scattered, which is quantified by the absorption (K) and the scattering (S) coefficients. Internal transmittance (T_i) of the films was quantified using (Eq.1). In this Equation R_0 is the reflectance of the film on an ideal black background. Equation (2) and (3) are used to calculate a and b parameters, respectively. R in equation 2 is the reflectance of the sample layer backed by a known reflectance (R_g). The surface reflectance spectra of the films were determined from 400 to 700 nm with a spectrocolorimeter CM-5 (Konica Minolta Co., Tokyo, Japan) on both a white and a black background. All measurements were performed at least in triplicate for each sample on the free film surface during its drying. In bilayer films, measurements were carried out on the CH layer.

$$T_i = \sqrt{(a - R_0)^2 - b^2} \qquad \text{Eq.1}$$

$$a = \frac{1}{2}(R + \frac{R_0 - R + R_g}{R_0 R_g}) \qquad \text{Eq.2}$$

$$b = \sqrt{a^2 - 1} \qquad \text{Eq.3}$$

Color coordinates of the films, L*, C^*_{ab} (Eq.(4)) and h^*_{ab} (Eq.(5)) from the CIELAB color space were determined, using D65 illuminant and 10° observer and taking into account R_∞ (Eq.(6)), which is the reflectance of an infinitely thick layer of the material.

$$C^*_{ab} = \sqrt{a^{*2} + b^{*2}} \qquad \text{Eq.4}$$

$$h^*_{ab} = \arctan \frac{b^*}{a^*} \qquad \text{Eq.5}$$

$$R_\infty = a - b \qquad \text{Eq.6}$$

Whiteness index (WI) was determined according to equation 7.

$$WI = 100 - ((100 - L^*)^2 + a^{*2} + b^{*2})^{0,5} \qquad \text{Eq.7}$$

Gloss was measured using a flat surface gloss meter (MultiGloss 268, Minolta, Langenhagen, Germany) at an angle of 600 with respect to the normal to the film surface, according to the ASTM standard D523 (ASTM, 1999). Prior to gloss measurements, films were conditioned at 25°C and 53 % RH for one week. Gloss measurements were carried out over a black matte standard plate and were taken in triplicate. Results were expressed as gloss units, relative to a highly polished surface of standard black glass with a value close to 100. For bilayer films, gloss was measured on the CH layer. For CH casted monolayer films, gloss measurements were performed on the free film surface for water evaporation.

2.8. Antimicrobial properties

The antimicrobial capacity of the films (monolayers and bilayers) was tested in pork meat, as described in previous studies (Bonilla et al., 2013). Films were put in contact with the meat surface by the CH side. Pork (about 10 g) was molded by using petri dishes (5 cm in diameter) to obtain the test samples. The surface of the meat was coated with the films. Non-coated samples (control) and samples coated with the different types of films were stored in duplicate at 10°C for 7 days in a thermostat cabinet (Aqualytic GmbH & Co, Dortmund, Germany). To perform microbiological analyses, 10 g of each sample were aseptically obtained and homogenized in a Stomacher (Bag Mixer 400, Interscience) 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 aerobial and coliform microorganism counts were determined at 0, 1, 4 and 7 days. Total aerobial counts were determined in Plate Count agar incubated at 37°C for 48 h. Coliform microorganisms were determined in Violet Red Bile Agar incubated at 37°C for 48 h. All tests were performed in triplicate.

2.9. Statistical analysis

Statistical analyses were performed through analysis of variance (ANOVA) using Statgraphics Centurion XVI- II. Fisher's least significant difference (LSD) procedure at 95 % was used.

3. RESULTS AND DISCUSSION

3.1 Microestructure

Figure 2.1 shows the FESEM micrographs of the cross-section bilayer films, where the two polymer layers can be clearly distinguished.

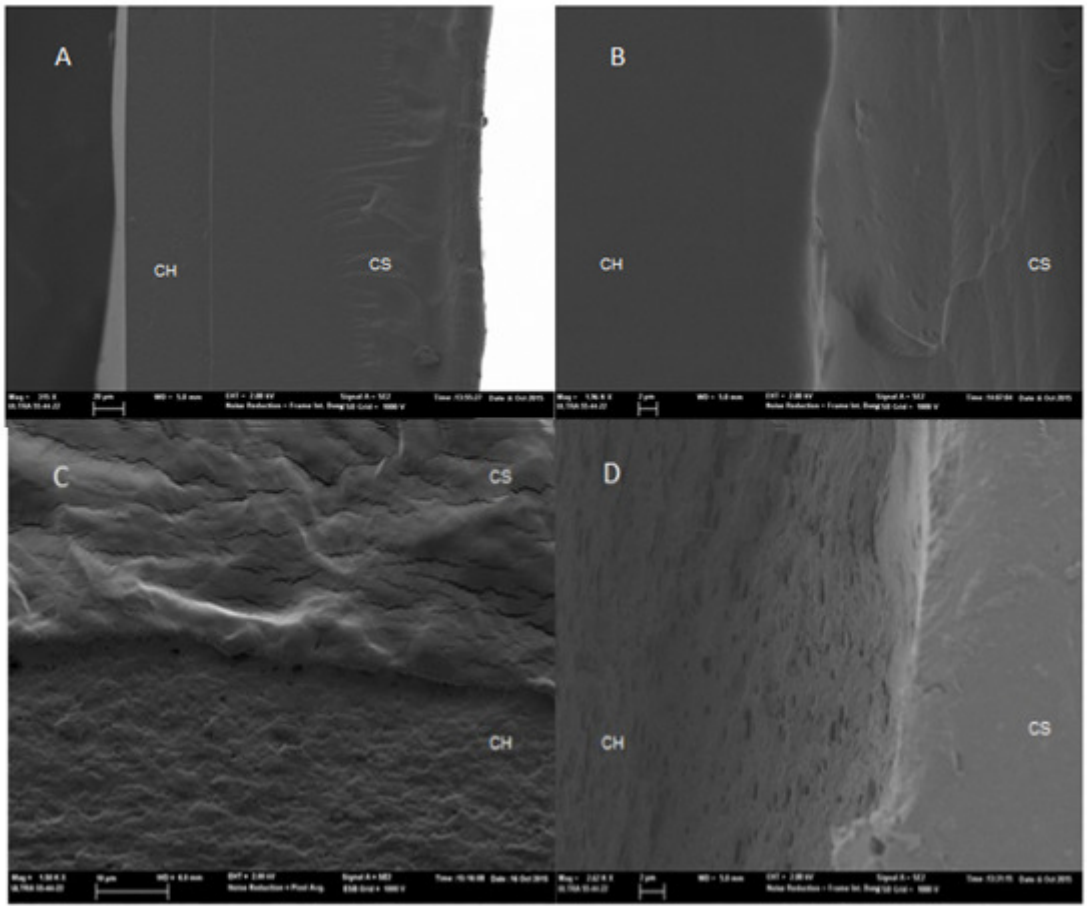

Figure 2.1. FESEM micrographs of cross-sections of bilayer films A and B) CS-CH at different magnifications C) CS-CH-OEO showing the layer interface, D) CS-CH-CLEO showing the layer interface.

The estimated thickness of each monolayer agreed with the initial values of the respective monolayers, thus maintaining the 3:1 ratio for starch and chitosan layers. The micrographs showed very good adhesion of both polymer layers and no detachment was observed, which confirmed the good compatibility of polymers at the interface. CS:CH bilayers exhibited a continuous homogeneous aspect at both phases, while starch layer showed some micro-cracks, which are related with the more brittle nature of this film under the observation conditions, (theoretical 0 water content), as reported by Jiménez et al. (2012) for starch films. Moreover, the absence of starch granules, points to the effectiveness of the shear-thermoprocessing at gelatinizing the starch granules. In the CS:CH bilayers with oregano or clove essential oils, the oil droplets or their voids created after cryofracture can be clearly distinguished in the CH layer.

3.2. Thermal characterization

The thermal degradation of the films was analyzed by TGA, which provides information about the thermal stability of polymers and the potential effect of the essential oils added, due to their potential diffusion into both layers and interactions with each macromolecule (Ortega-Toro et al., 2014). **Figure 2.2** shows the typical curves of the initial mass losses of the monolayers and bilayers, and the derivative curves with the peaks associated with the different weight losses caused by thermal degradation.

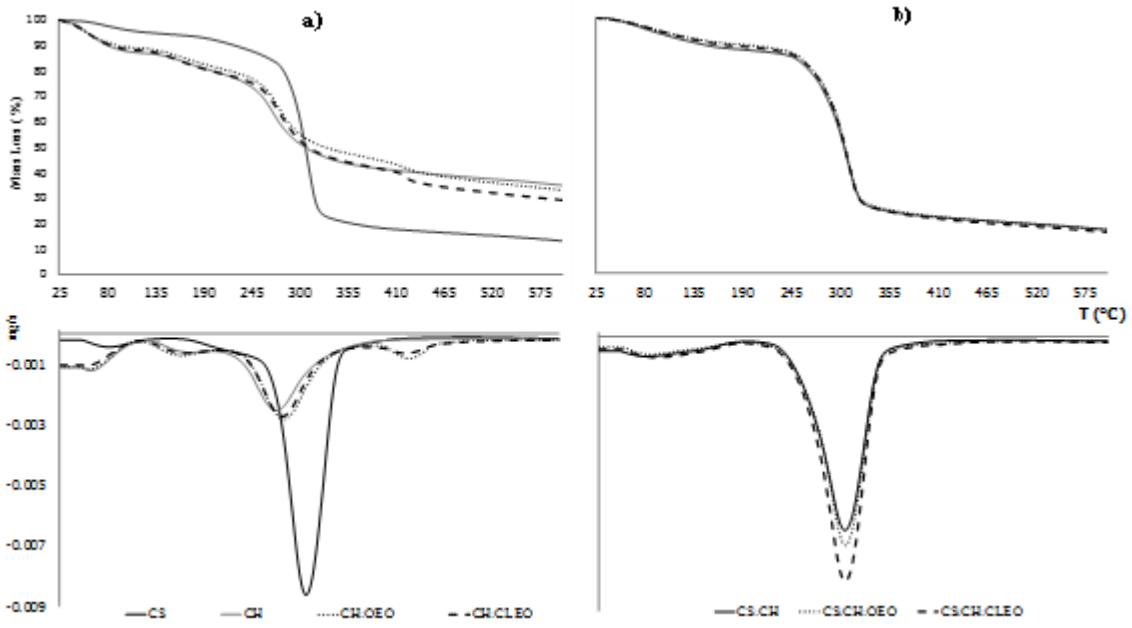

Figure 2.2. Typical thermogravimetric curves (weight loss vs. temperature) and first derivative (mg/s vs temperature) for a) monolayer films and b) bilayer films.

All films exhibited weight loss below 130°C, which must be attributed to the loss of adsorbed and bound water. By comparing monolayer samples, CH films exhibited weight loss between 130-240°C (about 10 %) regardless the presence of EO, which could be due to the partial deamination of the chains at this temperature range (de Brito & Campana-Filho, 2007). Afterwards, polymer chains degrade in a faster step between 270°C and 320°C showing a less sharp pattern than starch monolayers. T_{max} of pure starch films was 308°C, which is in agreement with previous studies (Pelissari et al., 2009; Dang & Yoksan, 2015). In contrast, T_{max} of CH monolayers was 269°C, near to that reported by other authors (Tripathi et al., 2009; Chen et al., 2008; Lewandowska, 2009; Bonilla et al., 2013). As seen in **Table 2.1**, no great effect of the EO was observed in thermodegradation pattern of CH, except the small loss (about 5 %) occurred at very high temperature (400-450°C) and a slight increase in temperature of the maximum degradation rate (T_{max}).

Table 2.1 Thermal properties of the films and essential oils (T_0, T_{max}, Mass loss during degradation). Mean values and standard deviation.

		Time (days)			
		Films	T_0 (°C)	T_{max} (°C)	%Mass loss
Monolayer	films	CS	$279.44 \pm 0.18^{(g)}$	$308.40 \pm 0.14^{(h)}$	$86.94 \pm 0.01^{(g)}$
		CH	$241.21 \pm 0.05^{(c)}$	$269.13 \pm 0.06^{(c)}$	$65.15 \pm 0.01^{(a)}$
		CH:OEO	$248.34 \pm 0.42^{(d)}$	$276.49 \pm 0.01^{(e)}$	$70.79 \pm 0.02^{(c)}$
		CH:CLEO	$247.4 \pm 0.2^{(d)}$	$275.82 \pm 0.01^{(d)}$	$67.07 \pm 0.01^{(b)}$
Bilayer	films	CS:CH	$271.50 \pm 0.17^{(e)}$	$308.05 \pm 0.07^{(g)}$	$82.92 \pm 0.06^{(e)}$
		CS: CH:OEO	$274.4 \pm 0.4^{(f)}$	$307.82 \pm 0.02^{(f)}$	$82.81 \pm 0.01^{(d)}$
		CS:CH:CLEO	$274.6 \pm 0.4^{(f)}$	$307.81 \pm 0.03^{(f)}$	$83.84 \pm 0.02^{(f)}$
		Oregano oil	$131.2 \pm 0.9^{(a)}$	$151.09 \pm 0.12^{(a)}$	$82.83 \pm 0.01^{(d)}$
		Cinnamon leaf oil	$133.3 \pm 0.6^{(b)}$	$171.75 \pm 0.11^{(b)}$	$82.84 \pm 0.01^{(d)}$

Different superscripts letters (a,b,c,..) within the same column indicate significant differences among formulations ($p < 0.05$).

The small weight loss in films containing EO could be attributed to the losses of strongly bonded molecules of the essential oils, which are delivered after the polymer degradation. Phenolic compounds, such as the main of components of oregano and clove essential oils, have the ability to crosslink with amino groups of the CH chains, becoming bonded to the matrix (Pelissari et al., 2009; Reyes-Chaparro et al., 2015). This behavior has been also observed by Ramos et al. (2012) and Reyes-Chaparro et al. (2015) for essential oils included

in CH matrix. No losses of free essential oils compounds were observed at their volatilization temperature (150°C-170°C, **Table 3.1**), which could indicate that the main part of non-bonded compounds evaporated during the film drying step by the steam drag effect associated to the water evaporation, as observed in previous studies for similar film composition (Perdones et al., 2016). **Table 3.1** shows the temperatures for initial and maximum degradation rates of polymers, where the lower values of CH than starch can be observed, as well as the small increase in T_{max} of CH films promoted by essential oils. This effect could be due to the polymer bonding of the EO phenolic compounds, which can slightly modify thermal resistance.

As concerns bilayer films, thermodegradation pattern was very similar and closer to that of starch monolayer, in agreement with the greatest ratio of this polymer in the films. The lack of appreciable differences due to the essential oils, as observed for CH monolayers, can be related with the small weight fraction of the essential oil in the double sheet. Nevertheless, an additional loss of essential oil compounds could occur during the bilayer thermocompression, mainly due to the steam drag effect associated to the residual water evaporation. Therefore, the processing parameters, in particular temperature and time should be optimized to avoid excessive evaporation and loss of these compounds incorporated (Dobkowski, 2006; Ramos et al., 2012).

3.3 Mechanical properties

As shown in **Table 2.2**, CS film exhibited the lowest elastic modulus and tensile strength values and the highest elongation at break. In monolayer films, the addition of EOs to CH films improved the stretchability and reduced the film stiffness. This effect can be attributed to the developed interactions CH-phenolic compounds that weaken the CH chain interaction forces, causing interruptions of the polymer chain aggregation in the matrix, which favors the sliding of the chains during film stretching and reduce the strength of the matrix (Bonilla et al., 2012).

The obtained values for pure CH monolayers were similar to those previously reported by Bonilla et al., (2012) and Vargas et.al., (2011), while the incorporation of OEO and CLEO provoked similar effects to that previously observed by Bonilla et al., (2012) for CH films containing basil or thyme essential oils. The discontinuities introduced in the chitosan matrix by oil droplets (**Figure 1C** and **1D**), will also contribute to the loss of the film cohesion and mechanical resistance.

Table 2.2 Tensile properties (elastic modulus: EM, tensile strength: TS and elongation: E %, at break) of all films equilibrated at 53 % RH after 1 week storage. Mean values and standard deviation.

	Films	EM (MPa)	TS (MPa)	E (%)
Monolayer films	CS	$565 \pm 12^{(a)}$	$17.3 \pm 0.4^{(a)}$	$7.5 \pm 0.3^{(f)}$
	CH	$1521 \pm 52^{(f)}$	$51 \pm 5^{(f)}$	$4.7 \pm 0.3^{(c)}$
	CH:OEO	$1078 \pm 21^{(d)}$	$40 \pm 3^{(d)}$	$6.2 \pm 0.5^{(e)}$
	CH:CLEO	$1417 \pm 27^{(e)}$	$43 \pm 3^{(e)}$	$5.9 \pm 0.4^{(d)}$
Bilayer films	CS:CH	$921 \pm 80^{(c)}$	$20\pm 2^{(c)}$	$2.28 \pm 0.14^{(b)}$
	CS: CH:OEO	$910 \pm 38^{(b)}$	$17 \pm 1^{(a)}$	$1.9 \pm 0.2^{(a)}$
	CS:CH:CLEO	$918 \pm 4^{(d)}$	$18\pm 2^{(b)}$	$2.4 \pm 2.1^{(b)}$

Different superscript (a,b,c,..) within the same column indicate significant differences among formulations ($p < 0.05$).

All bilayer films showed higher values of EM and TS than CS monolayers, but lower than the CH monolayer. Likewise, they exhibited lower extensibility than pure CS films and CH-based monolayers, especially when the CH layer contained essential oils. This behavior demonstrated the reinforcement effect produced by CH monolayer in the prevalently starch films, despite its lower ratio in the double sheet. However, bilayers lost flexibility probably due to the controlling effect of the S-CH interface at film fracture. The strong CH-starch bonding at the interface could make the chain sliding at this zone difficult, provoking film fracture instead of plastic deformation. In this sense, the enhanced film extensibility by EO was not evidenced in thermo-compressed bilayers since the interfacial adhesion of the monolayers controlled the film fracture. Nevertheless, with the CH layer adhesion, starch films gained stiffness and resistance to fracture.

3.4 Film thickness and barrier properties

Table 2.3 shows the thickness values of the monolayer and bilayer films. Film thickness of the CH monolayers slightly decreased when OEO or CLEO was incorporated into the film formulation (from 66 μm to 52 μm), which is in agreement with the volatile losses during film formation since a similar total solid amount per surface unit was casted in all cases.

Table 2.3 Thickness, water vapor permeability (WVP) and oxygen permeability (OP) of the films. Mean values and standard deviation.

		Film	Thickness (μm)	WVP (g mm kPa^{-1} h^{-1} m^{-2})	OP (cm^{3}m^{-1}s^{-1} Pa^{-1}) x 10^{-13}
Monolayer	films	CS	$171 \pm 5^{(c)}$	$9.38 \pm 0.11^{(f)}$	$0.601 \pm 0.009^{(b)}$
		CH	$66 \pm 3^{(b)}$	$5.35 \pm 0.14^{(a)}$	$0.781 \pm 0.011^{(c)}$
		CH:OEO	$54 \pm 3^{(a)}$	$6.9 \pm 0.2^{(d)}$	-
		CH:CLEO	$55 \pm 2^{(a)}$	$5.9 \pm 0.3^{(b)}$	-
Bilayer	films	CS:CH	$221 \pm 5^{(f)}$	$7.3 \pm 0.6^{(c)}$	$0.097 \pm 0.003^{(a)}$
		CS: CH:OEO	$209 \pm 10^{(e)}$	$8.03 \pm 0.18^{(e)}$	-
		CS:CH:CLEO	$207 \pm 9^{(e)}$	$7.87 \pm 0.14^{(e)}$	-

Different superscript (a,b,c,..) within the same column indicate significant differences among formulations ($p < 0.05$).

A reduction in film thickness due to essential oil addition was previously reported in similar studies, and was explained by important losses of essential oils during the drying step of the films, mainly due to the steam drag effect associated to water evaporation (Perdones et al., 2016). As a consequence, the thickness of the bilayers that incorporated EO were also slightly lower than that of CS:CH bilayer. Nevertheless, no significant flow of the polymer layers occurred during the thermocompression step, as revealed the final bilayer thickness, which was close to the sum of the respective monolayers. FESEM observations (**Figure 1**) corroborate that in bilayer films the thickness of each film layer practically maintained their initial value, being the S:CH proportion about 3:1. In fact, the low temperature applied in thermocompression (100°C) would not justify the polymer flow. However, at this temperature the residual water content of the film sheets could evaporate also leading to losses of the EOs by steam drag effect.

Table 2.3 also shows the barrier properties of the films. CH-based monolayer films showed the lowest WVP values, and the addition of essential oils (OEO or CLEO) slightly increased the WVP of CH films. This effect was also observed by Bonilla et al. (2012) and Vargas et al. (2009) and was attributed to the loss of CH matrix cohesion associated to the chain interactions with the essential oil compounds. As expected, bilayer films exhibited values of WVP between those found for CS and CH monolayers, and essential oils also slightly promoted an increase in the WVP values of bilayer films. As previously reported by Bonilla et al. (2013), the oxygen permeability of pure CS and CH films was very low, but the oxygen

barrier properties of CS:CH bilayers were ever more improved with respect to CS or CH monolayers. This effect could be due to the polymer interactions at the interface, which generate a perpendicular layer to mass transfer in the bilayer, with great resistance to the gas transport.

3.5 Optical properties

Figure 2.3 shows the spectral distribution curves of the internal transmittance (T_i) of the monolayer and bilayer films.

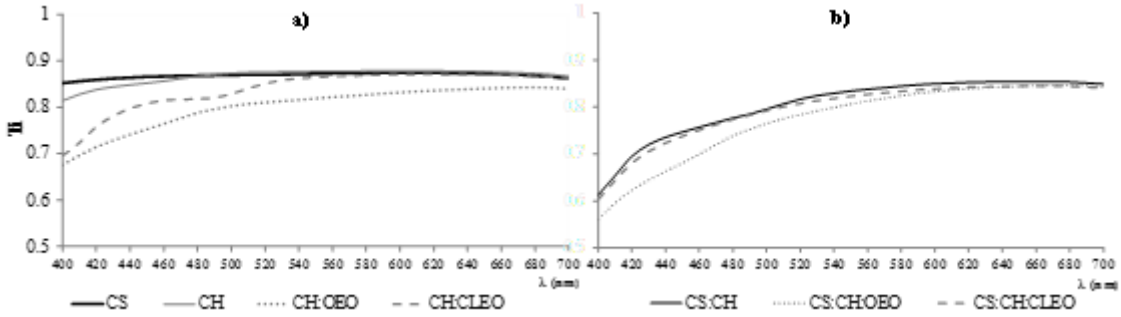

Figure 2.3. Spectral distribution curves of internal transmittance (T_i) of the (a) monolayer and (b) bilayer films.

The results obtained revealed significant differences between films. CS monolayer was the most transparent, whereas CH monolayers showed a higher opacity, especially when they contained essential oils, whose dispersion in the matrix promotes light scattering, thus reducing film transparency to a different extent depending on the essential oil. Oregano EO provoked greater reduction in transparency than cinnamon leaf EO, according to their different refractive index and coloration of their constituents, which provokes light selective absorption. As expected, bilayer films were less transparent, since an additional change in the film refractive index occurs at the polymer interface, enhancing light scattering.

From the reflectance spectra of an infinite film thickness, lightness (L*), hue (h^*_{ab}), chroma (C^*_{ab}) and whiteness index (WI) of each film were calculated (**Table 2.4**). Cassava starch films showed higher lightness and WI than CH monolayers. The incorporation of essential oils led to a significant reduction in both WI and lightness values of CH films and yielded films with a yellower, more saturated color (lower hue values and higher chroma). This affected both mono and bilayer films. Therefore, bilayer films were darker than CS monolayers with a more saturated yellowish, especially when incorporated essential oils.

Table 2.4 Lightness (L*), chroma (C^*_{ab}), hue (h^*_{ab}), whiteness index (WI) and gloss at 60°. Mean values and standard deviation.

	Films	L*	C_{ab}	h_{ab}	WI	Gloss (60°)
Monolayers	CS	$86.4 \pm 0.7^{(d)}$	$5.5 \pm 0.2^{(a)}$	$81 \pm 2^{(c)}$	$84.9 \pm 0.2^{(e)}$	$41 \pm 2^{(e)}$
	CH	$68 \pm 1^{(c)}$	$10.6 \pm 0.3^{(b)}$	$106.9 \pm 0.6^{(f)}$	$66.4 \pm 0.9^{(d)}$	$49 \pm 2^{(g)}$
	CH:OEO	$63.3 \pm 0.4^{(a)}$	$17.64 \pm 0.12^{(c)}$	$88.2 \pm 0.2^{(e)}$	$59.1 \pm 0.4^{(b)}$	$54 \pm 1^{(h)}$
	CH:CLEO	$63.5 \pm 0.5^{(a)}$	$18.6 \pm 0.3^{(d)}$	$88.7 \pm 0.2^{(e)}$	$59.1 \pm 0.3^{(b)}$	$47 \pm 2^{(f)}$
Bilayers	CS:CH	$70.9 \pm 0.5^{(d)}$	$25.4 \pm 0.6^{(f)}$	$79.9 \pm 0.4^{(b)}$	$61.4 \pm 0.5^{(c)}$	$23 \pm 1^{(a)}$
	CS:CH:OEO	$66.1 \pm 0.8^{(b)}$	$26.83 \pm 0.13^{(g)}$	$77.4 \pm 0.5^{(a)}$	$57 \pm 1^{(a)}$	$26 \pm 1^{(c)}$
	CS:CH:CLEO	$69 \pm 2^{(c)}$	$22.7 \pm 0.5^{(e)}$	$83.1 \pm 0.7^{(d)}$	$62 \pm 1^{(c)}$	$23 \pm 2^{(a)}$

Different superscript (a,b,c,..) within the same column indicate significant differences among formulations ($p < 0.05$).

The gloss of the films is linked to the morphology of their surface and generally, the smoother the surface, the glossier the film. Table 4 shows the gloss values at 60° of all monolayer and bilayer films. Incorporation of essential oils did not notably affect the gloss of the CH monolayers despite their potential effect on the film surface morphology due to their dispersed nature in the films, which could increase the surface roughness (Bonilla et al., 2012).

Thermo-compression in bilayers, greatly reduced the gloss of the CH face, which can be attributed to the rearrangement of the chains near the surface, where temperature was reach the highest value, provoking a less oriented more disordered chain arrangement and affecting the surface roughness.

3.6 Antimicrobial properties

Figure 2.4 shows the progress over storage time (up to 7 days) of coliform counts in non-coated and coated pork meat. CH monolayers were the most effective at reducing the growth of total aerobial and coliform microorganisms during the whole storage period, and the growth inhibition coincides with that reported by Bonilla et al. (2014) pork meat.

The incorporation of essential oils into the monolayer films caused a slight increase in the microbial counts. This indicates that CH was more effective than the essential oils, at their final concentration in the films, to inhibit the bacterial growth. The reduction of the effective ratio of chitosan in films containing essential oils explains the decrease in the antimicrobial effectiveness caused by a dilution effect (Sánchez-González et al. 2011).

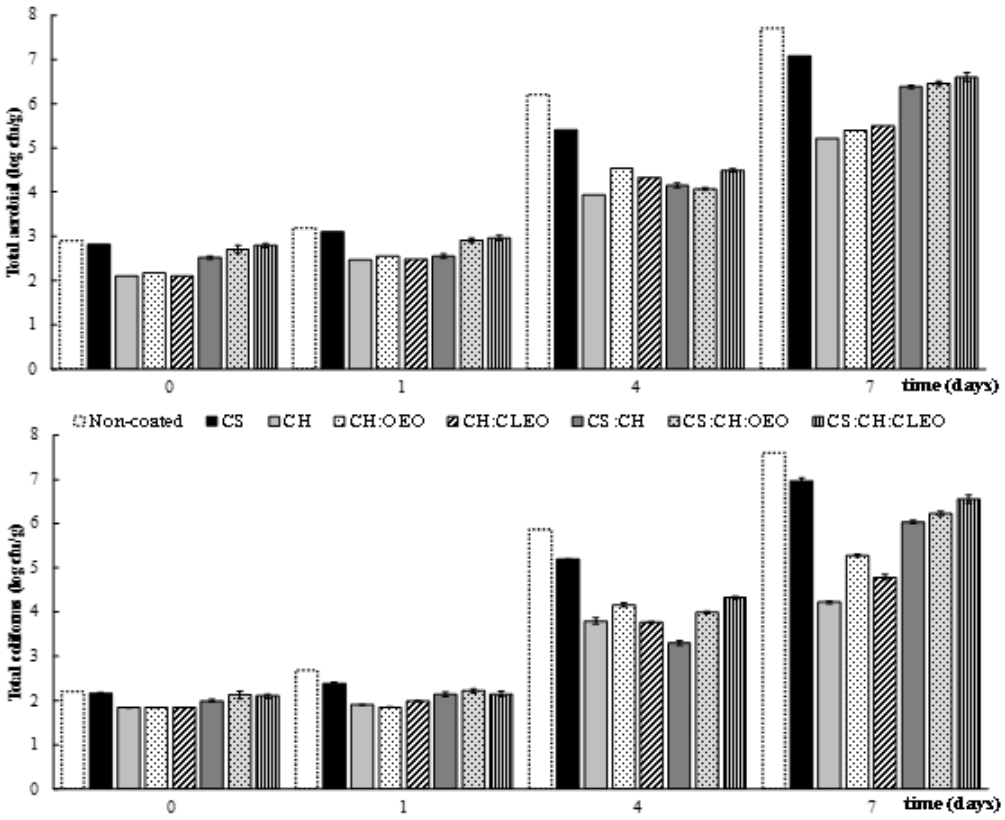

Figure 2.4. Total aerobial and coliform counts of non-coated pork samples and samples coated with the films. Mean values and standard deviation.

Bilayer films were slightly less effective than CH monolayers at controlling the bacterial growth, especially at the end of incubation time, where more marked differences between films were observed. The loss of antimicrobial capacity of CH in bilayers could be due to the partial deamination of the CH chains during the thermo-compression step, as deduced from the thermal analysis. TGA of the bilayers do not exhibit the CH weight loss occurred in CH monolayers between 130-200°C, which could indicate that this event occurred during the film processing. Several authors indicated the role of amino groups in the antimicrobial activity of CH (Kong et al., 2010; Verlee et al., 2017). At the end of storage, all films led to a reduction in microbial load as compared to non-coated samples and samples coated with pure CS films, revealing the antimicrobial action of CH, affected to a different extent by the dilution effect in the matrix and thermal treatment.

5. CONCLUSION

It was possible to obtain starch-chitosan bilayer films, containing or not essential oils in the chitosan layer, by thermo-compression, exhibiting a good interfacial adhesion between the polymer layers. Starch-chitosan bilayer films showed better mechanical resistance than starch monolayers, although they were less stretchable due to the interfacial control of the film fracture. Bilayer films were slightly less transparent but showed acceptable optical properties. Chitosan was effective at controlling the bacterial growth in meat pork. However, the thermal treatment used to obtain the bilayers reduced its effectiveness, revealing the loss of amino groups during treatment, as it was also confirmed by thermal analyses. Essential oils did not exhibit antimicrobial action in CH mono and in bilayers when applied to pork meat. The final amount of essential oils in the films was very limited by the potential losses occurred during the casting and thermoprocessing methods that were used for film production. Other strategies to incorporate the antimicrobial essential oils in the films must be utilized in order to improve the final retention of essential oils in polymer matrices and their effective release into the food media to exceed the minimally inhibitory concentration.

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III.3 CHAPTER 3

INFLUENCE OF LIPOSOME ENCAPSULATED ESSENTIAL OILS ON PROPERTIES OF CHITOSAN FILMS

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ABSTRACT

The effect of the encapsulation of eugenol and cinnamon leaf essential oil (CLEO) in lecithin liposomes on the losses of these compounds during the chitosan film formation process by casting was evaluated. Film-forming dispersions and films with eugenol or CLEO (either free or encapsulated) were obtained and characterized. The content of eugenol in active films was quantified by means of solvent extraction and gas chromatograph (GC) analysis. The encapsulation of eugenol or CLEO in lecithin liposomes led to the films retaining 40-50 % of the incorporated eugenol, whereas only 1-2 % was retained when eugenol was incorporated by direct emulsification. Films with liposomes exhibited a lamellar microstructure which improved film extensibility and increased water vapour barrier capacity with respect to those with free emulsified compounds. Liposomes also modified the optical properties of the films, reducing their gloss, increasing colour saturation and making them redder in colour. The encapsulation of volatile active compounds in liposomes appears to be a good strategy for obtaining active films with essential oils.

Keywords: chitosan, eugenol, liposome, lecithin, cinnamon leaf essential oil, encapsulation.

1. INTRODUCTION

Biopolymer films containing antioxidant/antimicrobial compounds, useful for the development of active packaging materials, are of great interest for the purposes of food quality and safety preservation and as a means of extending shelf-life. In this sense, the use of biodegradable polymers is advisable in order to reduce the environmentally harmful effects caused by the use of synthetic polymer-based packages and in order to limit the exploitation of constantly shrinking oil reserves (Jiménez et al., 2013).

Chitosan (CH) is a cationic, non-toxic, biodegradable polysaccharide, compatible with other biopolymers, which film-forming properties have been extensively studied (Zhai et al., 2004). This natural biopolymer can be obtained from the deacetylation of the chitin present in crustacean exoskeletons, and it has potential applications in the food industry on the basis of its described characteristics and its antimicrobial properties. CH films exhibit good mechanical and structural properties and constitute a good barrier to gases and aromas.

The incorporation of essential oils (EO) into the chitosan matrix could improve its functionality for food preservation purposes, since antimicrobial/antioxidant properties would be enhanced at the same time as the water barrier capacity of the films was improved, in line with the increase in the films' hydrophobic fraction (Perdones et al., 2014). Particularly, cinnamon leaf essential oil (CLEO) and its main compound, eugenol, have been described as antibacterial and antifungal agents at relatively low concentrations in previous studies (Singh et al., 2007; De Martino et al., 2009; Bajpai et al., 2012). The minimal inhibitory concentration (MIC) of eugenol against *Listeria monocytogenes* and *Escherichia coli* is 1.5 g/L and 1.0 g/L, respectively (Shah et al., 2013). Eugenol has been recognized as safe by the FDA (Food and Drug Administration, 2009) and approved by the European Union as a safe savoring agent for foods (Sebaaly et al., 2015).

Different essential oils have been widely used in the formulation of active biodegradable films (Atarés & Chiralt,2016) but, on top of their potential sensory impact on the coated or packaged product, the losses of these volatile compounds during film preparation represent an added problem (Sánchez-González et al., 2011; Bakkali et al., 2008). Incorporation of EOs into hydrophilic biopolymer films has been carried out by emulsification in the aqueous film-forming dispersion of the polymer before film formation by casting and drying (Wu et al., 2015; Imran et al., 2012). Nevertheless, during the film drying step, oil droplets flocculate, coalesce and cream to the top of the drying film, where oil components volatilize together with water at a lower temperature than their boiling point (steam distillation) (Perdones et al., 2014).

The encapsulation of essential oil compounds before film preparation can mitigate both the losses and the sensory impact of EOs, also contributing to modulate the release kinetics of actives into the product. In this sense, the use of liposomes or nanoliposomas (Zhang et al., 2012), that can act as carrier systems of a wide range of compounds, represent an interesting alternative. Nevertheless, the presence of these lipid structures in the film matrix may affect the functional (mechanical, barrier or optical) film properties as packaging material.

The aim of this study was to assess the effect of the encapsulation of eugenol (Eu) and cinnamon leaf essential oil (CLEO) within lecithin nanoliposomes on their retention in chitosan films during film formation, as compared to free compounds. Likewise, the effects of the incorporation of nanoencapsulated compounds on the film structure and physical (barrier, tensile and optical) properties were analysed, in comparison with the free-form incorporation.

2. MATERIALS AND METHODS

2.1. Materials

High molecular weight chitosan (practical grade, >75 % deacetylation degree, Batch MKBP1333V, supplied by Sigma-Aldrich Chemie, Steinheim, Germany) was used as film-forming polymer. Glacial acetic acid, magnesium nitrate-6-hydrate and diethyl ether (ethanol stabilized) were supplied by Panreac Química SLU (Castellar del Vallés, Barcelona, Spain). For nanoliposome formulation, non-GMO sunflower seed lecithin with 20 % phosphatidylcholine, supplied by Lipoid H20 (Lipoid gmbh, Ludwigshafen, Germany) was used. Cinnamon leaf oil (Herbes de Molí, Coop. V., Benimarfull, Alicante, Spain) and its main component, eugenol (Sigma Aldrich Química S.L., Madrid, Spain) were used as antimicrobial agents. SephadexR G50 and anhydrous sodium sulphate were purchased from Sigma Aldrich Química S.L., (Madrid, Spain) and Triton X100 from Carl Roth GmbH (Karlsruhe, Germany).

2.2. Preparation of nanoliposome dispersions

Preparation of nanoliposome dispersions was carried out according to a previously described method (Zhang et al., 2012; Jiménez et al., 2014). Lecithin was dispersed in distilled water (5 wt %) and stirred for 4 hours at 700 rpm. Eugenol or CLEO at 5 wt % was incorporated to the lecithin dispersion and afterwards three different liposome samples were obtained (Lec, Lec-Eu and Lec-CLEO) by sonication at 20 kHz for 10 minutes with one-second pulses. The ultrasound probe was placed in the center of the sample.

The encapsulation efficiency in the nanoliposome dispersions was determined. To this end, Sephadex® gel filtration was conducted. Sephadex G50 (0.5g) was added to swell in deionized water (10 mL) for 6h. A layer of about 5 cm of gel was formed. In order to eliminate the excess water, the column was centrifuged at 1,500 rpm for 7 min (Medifriger-BL, P-Selecta, Barcelona, Spain). Finally, 1 ml Lec-Eu or Lec-ClEO liposome sample was added on the top of the column and the centrifugation was repeated. The gel-filtered liposomes were destabilized by the addition of 3 mL of 0.15 w/v % Triton X100 followed by vortexing.

The active compounds were recovered by extraction with 2 ml diethylether and centrifugation at 2000 rpm for 10 min, which were repeated three times. The extract was dehydrated with anhydrous sodium sulphate, afterwards filtered and dry nitrogen flow was used to eliminate the remaining solvent. Finally, the extract was stored in a desiccator with silicagel, and the mass of encapsulated active compound was determined. The results of encapsulation efficiency were expressed as the ratio between the encapsulated and the incorporated active compound.

2.3. Preparation of chitosan films with active compounds

A solution of 1 % chitosan (w/w) was prepared in a 1 % (v / w) acetic acid solution. This was stirred at room temperature for 24 hours and then filtered with a sieve (120 μm pore size). The active compounds (Eu and CLEO) were incorporated in two different forms: either encapsulated in lecithin liposomes or by direct emulsification. For this purpose, 0.5 g of active compound (Eu or CLEO), either as free form or as lecithin liposomes (in this case 1.0 g of the active liposome dispersion) were added to 90 g of the chitosan solution. In this way, a 26 or 36 wt % of Eu or CLEO in the film dry solids was obtained for films with and without lecithin, respectively. This percentage was selected to overcome the values of the minimal inhibitory concentration (MIC) of actives against some typical patogens or food spoilage microorganisms (Shah et al., 2013; Olasupo et al., 2003) according to previuos studies with chitosan films containg CLEO (Perdones et al., 2014). A control film with lecithin liposomes without active compound was also obtained by incorporating to 0.5 g of lecithin as liposome dispersion (26 wt % of the film solids).

Film-forming dispersions with liposomes were kept under stirring for 2 hours before casting, while those containing free active compounds were homogenized in an Ultraturrax homogenizer (Yellow Line Model DI 25 basic, IKA, Germany) at 13,500 rpm for 4 minutes.

Thus, six film-forming dispersions (FFD) were obtained: pure chitosan (CH), control with lecithin (CH/Lec), films with eugenol or cinnamon leaf essential oil, non-encapsulated (CH/Eu

and CH/CLEO) and encapsulated in lecithin (CH/Lec-Eu and CH/Lec-CLEO). The FFDs were poured into 150 mm diameter Teflon plates (1 g of solids per plate). The films were obtained by drying at 25°C and 45 % relative humidity.

Prior to characterization, the films were conditioned for one week at 53 % relative humidity, using saturated solutions of Mg $(NO_3)_2$, at 25°C.

2.4. Characterization of nanoliposome and film-forming dispersions

Both nanoliposome and film-forming dispersions (ND and FFD) were characterized in triplicate as to their density, ζ-potential and particle size. The density of the different samples was measured using the pycnometer method. In order to determine the diameter (z-average) and ζ-potential of nanoliposomes, the samples were diluted in distilled water (1:100) and measured at 25°C in a Zetasizer Nano-Z device (Nano series model Zen 2600, Malvern Instruments, Worcestershire, United Kingdom). This equipment measures the electrophoretic mobility through light scattering caused by the dispersed particles, and the ζ-potential is determined using the Smoluchowsky model. All of the samples were measured in triplicate.

2.5. Film characterization

2.5.1. Eugenol retention in the films

Retention of the EO compounds in the films during the film formation was determined through the total eugenol content in the films analysed by GC. To this end, 0.3 g film samples were extracted with 10 mL of diethyl ether for 24 hours. This time was set after preliminary tests as the total extraction time. 2 μl of extracts were injected (injection temperature, 50°C) in a gas chromatograph (Hewlett Packard 6890 FID GC System) with flame ionization detector (GC-FID) using a DB-5 column (30 mm x 0.32 mm of internal diameter); 0.25 μm film thickness (V J & Scientifics, Agilent, Palo Alto, USA). Helium (1 mL / min) was used as carrier gas. The heating schedule was: heating at 5°C/min from 50°C to 130°C, and holding at 130°C for 5 min, followed by heating at 10°C/min up to 200°C (Alma et al., 2007). For the purposes of eugenol quantification, a calibration curve using eugenol solutions in ethyl ether in the range of 50-2000 ppm was obtained. The wt % of Eu in CLEO was also determined through GC analysis under the same conditions. The amount of retained Eu in the films was obtained. For films contining CLEO, the quantified Eu peak in each extract and the corresponding Eu percentage in the CLEO were taken into account. Results were expressed as the percentage of retained eugenol in the film (mass of extracted compound/mass of incorporated compound).

2.5.2. Thickness

Film thickness was measured using a digital electronic micrometer (Palmer model, Comecta S.A., Barcelona, Spain) to the nearest 0.0005 mm. Six measurements per sample were taken performed in random positions, and the results were used to analyze the tensile and barrier properties of the films.

2.5.3. Tensile properties

The tensile behaviour of films was tested by using a universal test Machine (TA.XTplus model, Stable Micro Systems, Haslemere, England). The elastic modulus (EM), tensile strength (TS) and elongation at fracture (%E) were determined following ASTM standard method D882 (ASTM, 2001). These parameters were obtained from the stress-Henky strain curves calculated from the force-distance data.

Equilibrated film strips (25 mm wide, 100 mm long) were mounted in the film-extension grips of the testing machine and stretched at 50 mm min^{-1} until breaking. At least eight replicates were obtained per formulation.

2.5.4. Moisture content, water vapor permeability and solubility

The moisture content of the films was determined using a gravimetric method. Firstly, the film samples were dried in a convection oven at 60 °C for 24 h, and then in a vacuum oven (Vacioterm-T, JP-selecta S.A., Barcelona, Spain) at 60°C and 0.8 bar for 48 h. Finally, in order to remove the residual moisture, the samples were conditioned in a dessicator with P_2O_5 till constant weight. The results were expressed as g of water per 100 g of dry film.

The water vapour permeability (WVP) of the films was determined by using the ASTM E96-95 (ASTM, 1995) gravimetric method, taking into account the modification proposed by other authors McHugh et al. (1993).Films were selected based on the lack of physical defects, such as cracks, bubbles, or pinholes. Six samples per formulation were cut, and distilled water was placed in Payne permeability cups (3.5 cm diameter, Elcometer SPRL, Hermelle /s Argenteau, Belgium) to expose the film to 100 % RH on one side. Once the films were secured, each cup was placed in a relative humidity equilibrated cabinet at 25°C, with a fan placed on the top of the cup in order to reduce resistance to water vapor transport. The RH of the cabinets (53 %) was held constant using oversaturated solutions of magnesium nitrate-6-hydrate. The cups were weighed every 1.5 h for 24 h with an analytical scales (ME36S Sartorius, Alemania - 0.0001 g). Once the steady state had been reached, water vapor

transmission rate was determined from the slope obtained from the regression analysis of weight loss data versus time, divided by the film area. WVP was obtained taking into account the average film thickness in each case. The equation proposed by other studies McHugh et al. (1993) was used to correct the effect of concentration gradients established in the stagnant air gap inside the cup.

In order to determine the film solubility, film samples were immersed in double distilled water (film: water mass ratio 1:200), and kept at 25 °C for 24 hours. After this, the films were dried in a convection oven (JP Selecta, SA Barcelona, Spain) at 60°C for 24 hours. Finally, the film samples were dried in a vacuum oven at 60°C to constant mass. Solubility was expressed as the percentual loss of the dry solids of the film by water solution, as rrefered to the initial mass of the dry film.

2.5.5. Optical properties

The opacity of the films was determined by applying the Kubelka-Munk theory for multiple scattering (Judd & Wyszecki, 1975; Hutchings, 1999). A spectrocolorimeter (CM-3600d Minolta CO., Tokyo) was used to obtain the reflection spectra of the films on a white (R) and a black (R_0) background between 400 and 700 nm, as well as the spectrum of the white background used (R_g). From these spectra, the internal transmittance (T_i, a transparency indicator) and R_∞ (the reflectance of an infinitely thick film), were calculated using equations (1) to (4).

$$T_i = \sqrt{(a + R_0)^2 - b^2} \qquad \text{Eq.1}$$

$$a = \frac{1}{2}(R + \frac{R_0 - R + R_g}{R_0 R_g}) \qquad \text{Eq.2}$$

$$b = \sqrt{a^2 - 1} \qquad \text{Eq.3}$$

$$R_\infty = a - b \qquad \text{Eq.4}$$

Three measurements were taken on each film and three films were considered per formulation. From R_∞ spectra, the CIEL*a*b* color coordinates were determined using the 10° observer and the D65 illuminant as reference (Hutchings, 1999). Moreover, hue (hab*) and chroma (C^*_{ab}) were calculated by using equations (5) and (6).

$$h^*_{ab} = \arctan \frac{b^*}{a^*} \qquad \text{Eq.5}$$

$$C^*_{ab} = \sqrt{a^{*2} + b^{*2}} \qquad \text{Eq.6}$$

Gloss was measured using a flat surface gloss meter Multi Gloss 268 (Minolta, Langenhagen, Germany) at a 60° angle of incidence according to ASTM D523 standard (ASTM, 1999). The film samples were placed on a matte black surface, and nine measurements per formulation were taken on the side of the film that was exposed to the atmosphere during drying.

2.5.6. Scanning electron microscopy (SEM)

The microstructural analysis of the cross-sections and surface images of the films was carried out using a scanning electron microscope (model JEOL JSM-5410, Japan). The film samples were maintained in desiccators with P_2O_5 in order to eliminate film moisture. Film pieces (0.5 cm^2 approximately) were cryofractured by immersion in liquid nitrogen and mounted on copper stubs. After gold coating, the samples were observed using an accelerating voltage of 10 kV.

2.5.6. Thermogravimetric analysis (TGA)

The thermal stability of the films was studied by TGA using a Mettler-Toledo thermobalance (model TGA / SDTA 851e, Schwarzenbach, Switzerland). Crushed film samples (3mg) were gradually heated at 7°C / min from room temperature to 600°C under nitrogen flow (50 mL / min). The onset temperature (T_0) and maximum degradation rate temperature (T_{max}) of the films were registered in two replicates per formulation.

2.6. Antimicrobial activity

Listeria innocua (CECT 910) and *Escherichia coli* (CECT 101) were supplied by the Spanish Type Culture Collection (CECT, Burjassot, Spain). These bacterial cultures were regenerated (from a culture stored at -25°C) by transferring a loopful into 10 mL of Tryptone Soy Broth (TSB, Scharlab, Barcelona, Spain) and incubating at 37°C for 24 hours. From this culture, a 10 μL aliquot was again transferred into 10 mL of TSB and grown at 37°C for 24 hours more in order to obtain a culture in exponential phase of growth. Afterwards, this bacterial culture was appropriately diluted in TSB tubes to get a target inoculum of 10^5 CFU/mL. Circular samples of 55 mm in diameter, obtained from the different types of film formulations were placed on inoculated Tryptose Soya Agar plates (solid medium test) and in Tryptose Soya Agar tubes (liquid medium test). Inoculated tubes and plates without film were used as control samples. Immediately after the inoculation and after 6 days at 10°C the microbial counts on Tryptone Soy Agar (TSA, Scharlab, Barcelona, Spain) plates were determined. In the liquid medium tests (broth tubes), serial dilutions were made and poured onto TSA dishes which were incubated for 24 hours at 37°C. For the solid medium tests (agar plates) the dishes content

was removed aseptically and placed in a sterile plastic bag with 90 mL of Buffered Peptone Water. The bags were homogenized with a Stomacher (Interscience BagMixer Stomacher 400 W Homogenizer, France). Serial dilutions were made and then poured into TSA Petri dishes. Samples were incubated at 37°C for 24 hours before colonies were counted. All the tests were run in triplicate.

2.7. Statistical analysis

The statistical analysis of the data was performed through an analysis of variance (ANOVA) using Statgraphics Centurion XVI.II. Fisher's least significant difference (LSD) procedure was used.

3. Results and discussion

3.1. Properties of nanoliposome and film-forming dispersions

The encapsulation degree in the nanoliposome dispersions was 98.2 $\pm$ 0.7 %, without significant differences for the type of active (Eu or CLEO). So, the non-encapsulated oil ratio was negligible in both cases, which indicates that the direct incorporation of ND into the FFD is an adequate method for the formulation of films with nanoliposomes.

The density and ζ-potential values of ND and FFD are shown in **Table 3.1**. No significant differences were found for the density values of nanoliposome dispersions with essential oil or eugenol. The lecithin nanoliposomes exhibited negative ζ-potential values, with a greater negative charge when the active compounds were incorporated. The FFD had high positive values of ζ-potential due to the charges of the chitosan chain with protonated amine groups at low pH (Falguera et al., 2011;Leceta et al., 2013).The FFD with liposomes exhibited the highest values of positive ζ-potential, without no significant differences between them, which indicates that the positively charged chitosan molecules adsorbed on the negatively charged nanoliposomes, thus giving rise to a reversed surface charge and greater values of ζ-potential than pure CH dispersions and those containing emulsified compounds.

Table 3.1 Density and ζ-potential of nanoliposome dispersions (ND) and film-forming dispersions (FFD). Mean values and standard deviation. Lec: lecithin, Eu: eugenol, CLEO: cinnamon leaf essential oil, CH: chitosan.

ND	ρ (kg/m3)	ζ- Potencial (mV)
Lec	1007 $\pm$ 2$^{(a)}$	-35.1 $\pm$ 1.6$^{(c)}$
Lec-Eu	1009 $\pm$ 1$^{(a)}$	-43.0 $\pm$ 0.7$^{(a)}$
Lec-CLEO	1009 $\pm$ 2$^{(a)}$	-41.4 $\pm$ 0.5$^{(b)}$
FFD	ρ (kg/m3)	ζ (mV)
CH	1004 $\pm$ 3$^{(a)}$	30.3 $\pm$ 1.4$^{(a)}$
CH/Eu	1006 $\pm$ 1$^{(a)}$	43 $\pm$ 3$^{(b)}$
CH/CLEO	1006 $\pm$ 1$^{(a)}$	57 $\pm$ 2$^{(c)}$
CH/Lec	1018 $\pm$ 2$^{(b)}$	65 $\pm$ 3$^{(d)}$
CH/Lec-Eu	1005 $\pm$ 1$^{(a)}$	66.4 $\pm$ 0.3$^{(d)}$
CH/Lec-CLEO	1005 $\pm$ 1$^{(a)}$	67.3 $\pm$ 0.5$^{(d)}$

Different superscript letters (a, b, c, d) within the same column indicate significant differences among formulations ($p < 0.05$).

On the other hand, the addition of emulsified active compounds also promoted the increase in ζ-potential values, which indicates that CH molecules also adsorb on the droplet surface, but to a lesser extent than on negatively charged liposomes, due to the electrostatic interactions with the positively charged chains. CH adsorption will contribute to the stability of the liposomes during the film drying, in which water loss can lead to phase transitions in the lipid structures (Krog, 1990; Larsson & Dejmek,1990) releasing the encapsulated material. The size distribution of lipid particles dispersed in the film-forming emulsions affects the properties of the final film, such as water vapor permeability and mechanical properties (Pérez-Gago & Krochta, 2001).

Figure 3.1 shows the particle size distribution in the obtained liposome dispersions, and the zeta average size values in the three nanoliposome dispersions, where significant differences can be seen ($p < 0.05$). Incorporating eugenol or essential oil to liposomes led to an increase in the size of the smaller particles, hence an increase in the average zeta size. Nevertheless, no notable differences were observed for size distributions of liposomes loaded with both components. Pure lecithin nanoliposomes had the smallest average size (90.6 nm) and the incorporation of the active compounds resulted in larger particles due to their incorporation in the liposome core. The size of lipid associations in an aqueous medium tends to increase as

the hydrophobic nature of their molecules increases (Fabra et al., 2008; Sánchez-González et al., 2009).

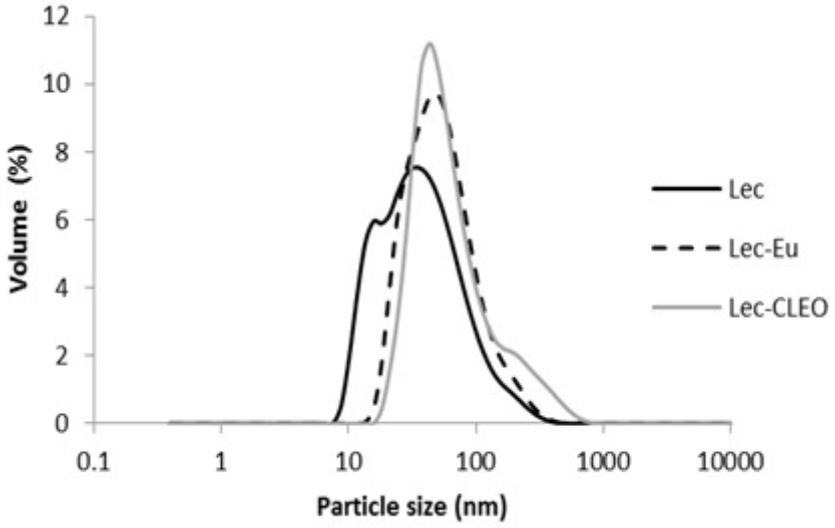

Figure 3.1. Typical particle size distribution curves of the nanoliposome dispersions Lec: lecithin, Eu: eugenol, CLEO: cinnamon leaf essential oil.

3.2. Film properties

3.2.1. Eugenol and essential oil retention

Table 3.2 shows the amount of active compound extracted from the films, the nominal mass of active compound added to the film sample and the respective percentage retention (with respect to the initial amount) in each sample. The encapsulation of the active compounds in lecithin liposomes greatly reduced the loss of the active compounds during the film drying step, and 40-50 % of the incorporated eugenol was retained in the film. This indicates the effectiveness of this strategy as a means of preventing the losses of volatile compounds during the film drying.

The highest eugenol retention (51 %) was obtained from the films containing encapsulated cinnamon leaf oil, which may be due to the predominant loss of other, more volatile essential oil compounds during the drying step. On the other hand, in the films with non-encapsulated active compounds, eugenol losses reached 99 % of the added amount, which implies a final content in the dried film of 0.6 wt % of the total solids. This amount is very low and suggests that a part of the active could be bonded to the polymer matrix and it was not extracted by the solvent. Nevertheless, the obtained results demonstrate the efficacy of liposome encapsulation to limit volatile losses during the film preparation process.

Table 3.2 Mass fraction of eugenol in the dried films (mg/g film solids), extracted in the dried film and initially incorporated, and percentage retention (extracted with respect to the initially added). Lec: lecithin, Eu: eugenol, CLEO: cinnamon leaf essential oil, CH: chitosan.

Film	Extracted	Incorporated	% Retention
CH/Eu	$4.9 \pm 1.0^{(b)}$	357	1
CH/Lec-Eu	$108.9 \pm 1.3^{(c)}$	263	41
CH/CLEO	$5.4 \pm 1.3^{(d)}$	318	2
CH/Lec-CLEO	$118 \pm 3^{(e)}$	234	51

Different superscript letters (a, b, c) within the same column indicate significant differences among formulations ($p < 0.05$).

3.2.2. Thickness and tensile properties

Table 3.3 shows the thickness and tensile parameters of the films. The chitosan film thickness ranged between 33 and 49 μm, and it decreased when free essential oils were incorporated. This is coherent with the partial volatilization of the oils, involving an effective reduction in the amount of solids per area unit of the film. The addition of encapsulated compounds provoked an increase in the films' thickness with respect to pure chitosan in line with the lower losses of volatiles and a different microstructural arrangement of the film components as previously observed by other authors (Jiménez et al., 2014).

Table 3.3 Thickness and tensile parameters (elastic modulus, EM; tensile strength, TS; percentage elongation, %E) of the films. Mean values and standard deviation. Lec: lecithin, Eu: eugenol, CLEO: cinnamon leaf essential oil, CH: chitosan.

Film	Thickness (μm)	EM (MPa)	TS (MPa)	%E
CH	$43 \pm 2^{(b)}$	$1660 \pm 170^{(d)}$	$53 \pm 4^{(b)}$	$6 \pm 1^{(a)}$
CH/Eu	$33 \pm 2^{(a)}$	$1623 \pm 70^{(d)}$	$46 \pm 4^{(ab)}$	$8 \pm 3^{(a)}$
CH/CLEO	$35 \pm 2^{(a)}$	$1460 \pm 30^{(c)}$	$42 \pm 3^{(a)}$	$7 \pm 4^{(a)}$
CH/Lec	$45 \pm 1^{(b)}$	$1145 \pm 4^{(b)}$	$41 \pm 1^{(a)}$	$11 \pm 3^{(a)}$
CH/Lec-Eu	$48 \pm 2^{(c)}$	$840 \pm 80^{(a)}$	$651 \pm 10^{(b)}$	$31 \pm 8^{(b)}$
CH/Lec-CLEO	$49 \pm 2^{(c)}$	$860 \pm 70^{(a)}$	$43 \pm 3^{(a)}$	$27 \pm 4^{(b)}$

Different superscript letters (a, b, c) within the same column indicate significant differences among formulations ($p < 0.05$).

The elastic modulus (EM), tensile strength (TS) and deformation at break (%E) describe the tensile properties of the films, which are closely related to their structure (Mc Hugh & Krochta, 1994). The EM significantly decreased subsequent to the addition of lecithin liposomes, both single and loaded with actives, which can be attributed to the interruptions in the polymer matrix introduced by lipid particles. This leads to a loss of network cohesion, as observed by other authors when studying different matrices (Fabra et al., 2008; Sánchez-González et al., 2009). Adding non-encapsulated oils only caused a slight EM reduction, which can be explained by the lower lipid ratio and the arrangement of the lipid droplets in the film matrix. Whereas TS and %E were not affected by the addition of non-encapsulated oils, the films with liposomes exhibited increased extensibility, with similar TS values to the CH control film. Pure chitosan films were mechanically stronger and less extensible than films with liposomes. This different behavior may be explained by the different structural arrangement of components, as commented on below.

3.2.3. Moisture content, water vapor permeability and solubility

Water vapor permeability (WVP) is a relevant property directly related to the usefulness of the film in food applications, and should be as low as possible to prevent the transfer of water (Ma et al., 2008). **Table 3.4** shows the moisture content, water vapor permeability and solubility of the films.

Table 3.4 Water content (Xw), water vapor permeability (WVP) and solubility (g of solubilized solids/100 g of initial solids) of the films. Mean values and standard deviation. Lec: lecithin, Eu: eugenol, CLEO: cinnamon leaf essential oil, CH: chitosan.

Films	Xw (g water/ 100 g dry film)	WVP (g.mm. $kPa^{-1}.h^{-1}.m^2$)	Solubility (%)
CH	$39 \pm 2^{(f)}$	$4.3 \pm 0.2^{(d)}$	$67 \pm 2^{(d)}$
CH/Eu	$25.4 \pm 0.8^{(ab)}$	$3.7 \pm 0.2^{(c)}$	$43 \pm 1^{(c)}$
CH/CLEO	$25.6 \pm 1.2^{(ab)}$	$4.6 \pm 0.4^{(e)}$	$40 \pm 1^{(bc)}$
CH/Lec	$30.1 \pm 1.7^{(d)}$	$2.4 \pm 0.2^{(b)}$	$40 \pm 2^{(b)}$
CH/Lec-Eu	$24.2 \pm 0.6^{(a)}$	$2.2 \pm 0.2^{(a)}$	$37 \pm 2^{(a)}$
CH/Lec-CLEO	$26.9 \pm 0.8^{(cd)}$	$1.90 \pm 0.2^{(a)}$	$35 \pm 1^{(a)}$

Different superscript letters (a, b, c, d) within the same column indicate significant differences among formulations ($p < 0.05$).

The equilibrium moisture content of the films decreased significantly when both free and encapsulated lipid compounds were added, since the proportion of active sites for water adsorption per unit dry mass decreases (Fabra et al., 2010). Likewise, possible interactions between the chitosan chains and the active compounds could reduce the availability of the chitosan polar groups to form hydrophilic bonds with water molecules (Shen & Kamdem, 2015). As compared to the films with non-encapsulated active compounds, lecithin incorporation resulted in a significant WVP decrease. A different final amount of lipid in the films, as well as the differences in their structural arrangement, may explain their different effectiveness at reducing water vapor permeability. Film solubility in water was also significantly reduced by the addition of lipids (lecithin or EO compounds); the greater the total lipid content, the lower the solubility values, coherently with the increased hydrophobic character of the film (Ojagh et al., 2010).

3.2.4. Optical properties

According to other studies (Hutchings, 1999), the transparency and brightness of the material are the most important optical properties with which to assess the direct impact on the appreciation of the color and appearance of a coated/packaged product. **Table 3.5** shows the values of lightness (L*), chroma (C^*_{ab}), hue (h^*_{ab}) and gloss at 60° of the different samples. Due to the typical color of lecithin, films with liposomes were darker, with a more saturated reddish color than the pure chitosan films.

Table 3.5 Lightness (L*), chroma (C^*_{ab}), hue (h^*_{ab}) and gloss (60°) of the films. Mean values and standard deviation. Lec: lecithin, Eu: eugenol, CLEO: cinnamon leaf essential oil, CH: chitosan.

Films	L*	C^*_{ab}	h^*_{ab}	Gloss (60°)
CH	$91 \pm 2^{(e)}$	$4 \pm 2^{(a)}$	$109 \pm 5^{(c)}$	$51 \pm 6^{(d)}$
CH/Eu	$75 \pm 4^{(cd)}$	$14 \pm 6^{(b)}$	$66 \pm 6^{(a)}$	$19 \pm 2^{(b)}$
CH/CLEO	$77.3 \pm 1.3^{(d)}$	$15.0 \pm 1.3^{(b)}$	$66 \pm 2^{(a)}$	$15 \pm 3^{(a)}$
CH/Lec	$67.4 \pm 1.1^{(a)}$	$23.0 \pm 0.2^{(c)}$	$80.4 \pm 0.7^{(c)}$	$33 \pm 2^{(c)}$
CH/Lec-Eu	$69.6 \pm 1.1^{(ab)}$	$23.0 \pm 0.3^{(c)}$	$78.4 \pm 0.3^{(c)}$	$15 \pm 2^{(a)}$
CH/Lec-CLEO	$72.0 \pm 0.3^{(bc)}$	$26.3 \pm 0.4^{(c)}$	$80.0 \pm 0.2^{(c)}$	$20 \pm 3^{(b)}$

Different superscript letters (a, b, c, d) within the same column indicate significant differences among formulations ($p < 0.05$).

This effect was also previously observed in starch-sodium caseinate films with lecithin liposomes (Jiménez et al., 2014).The gloss was decreased by incorporating active compounds, especially in free form, which can be attributed to the increase in surface roughness associated with the creaming of lipids during drying, as previously observed for lipid containing films (Sánchez-González et al., 2009; Fabra et al., 2009).

Figure 3.2 shows the spectral distribution curves of T_i. The incorporation of free lipids, and particularly the incorporation of liposomes, reduced the Ti of the films; in line with the presence of a dispersed phase in the matrix which enhances light scattering. In the case of liposomes, the absorbance of lecithin also contributes to lower the transmittance of the films.

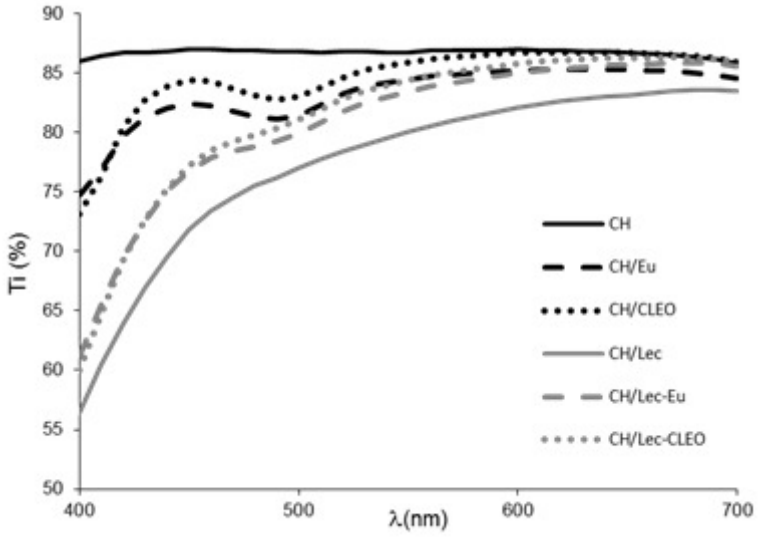

Figure 3.2. Spectral distribution curves of internal transmittance (T_i) of the films. Lec: lecithin, Eu: eugenol, CLEO: cinnamon leaf essential oil, CH: chitosan.

3.2.5. Microstructural properties

A microstructural study of the films allows the arrangement of the components in the dry films to be observed, and correlates these observations with other functional-characteristics, such as the barrier, mechanical and optical properties (Cano et al., 2014). **Figures 3.3** and **3.4** show the micrographs of the cross section and surface of the obtained films, respectively. The holes corresponding to eugenol or oil droplets in the cross section of the films can be seen when these are incorporated in free form (CH/Eu, CH/CLEO). On the surface of the films with these free compounds, some droplet footprint can also be observed, whereas the films with liposomes show a smoother surface. These holes were probably caused by the evaporation of the volatile compounds during the film drying or during SEM observation under high vacuum conditions.

On the other hand, when active compounds are incorporated in liposome form, no visible drops can be seen in the structure whereas laminar formations can be observed, where void layers intercalate with polymer fibrous arrangements. This appearance suggests that lamellar liposomal lipid associations were formed during the film drying step, in line with the liotropic mesomorphism of lipid associations (Larsson & Dejmek, 1990), which would be inserted between the polymer layers producing a laminated structure. In this arrangement, eugenol or cinnamon essential oil would occupy the hydrophobic core of the laminar structures. CH/Lec films also exhibited a laminar structure, but the separation between layers expands when there is eugenol or essential oil in the formulation. So, as the water content in the FFD is reduced during film drying, the micellar associations undergo phase transitions and lamellar structures tend to form, containing lipids at the hydrophobic core (Krog, 1990).

This laminar structure explains the greater elongation capability of the films containing liposomes during the tensile test, especially those containing eugenol or essential oil, since the layers can easily slide during the test, hence allowing for a greater deformation before fracture. However, the weaker cohesive force between layers, as compared to that acting between polymer chains, would lead to softer films (less resistant to deformation) with lower elastic modulus, as shown in **Table 3.3**. Likewise, the formation of lipid layers perpendicular to the mass transport in the film, also explains the great reduction in the water vapour permeability and water solubility of the films when liposomes were added to the film formulation.

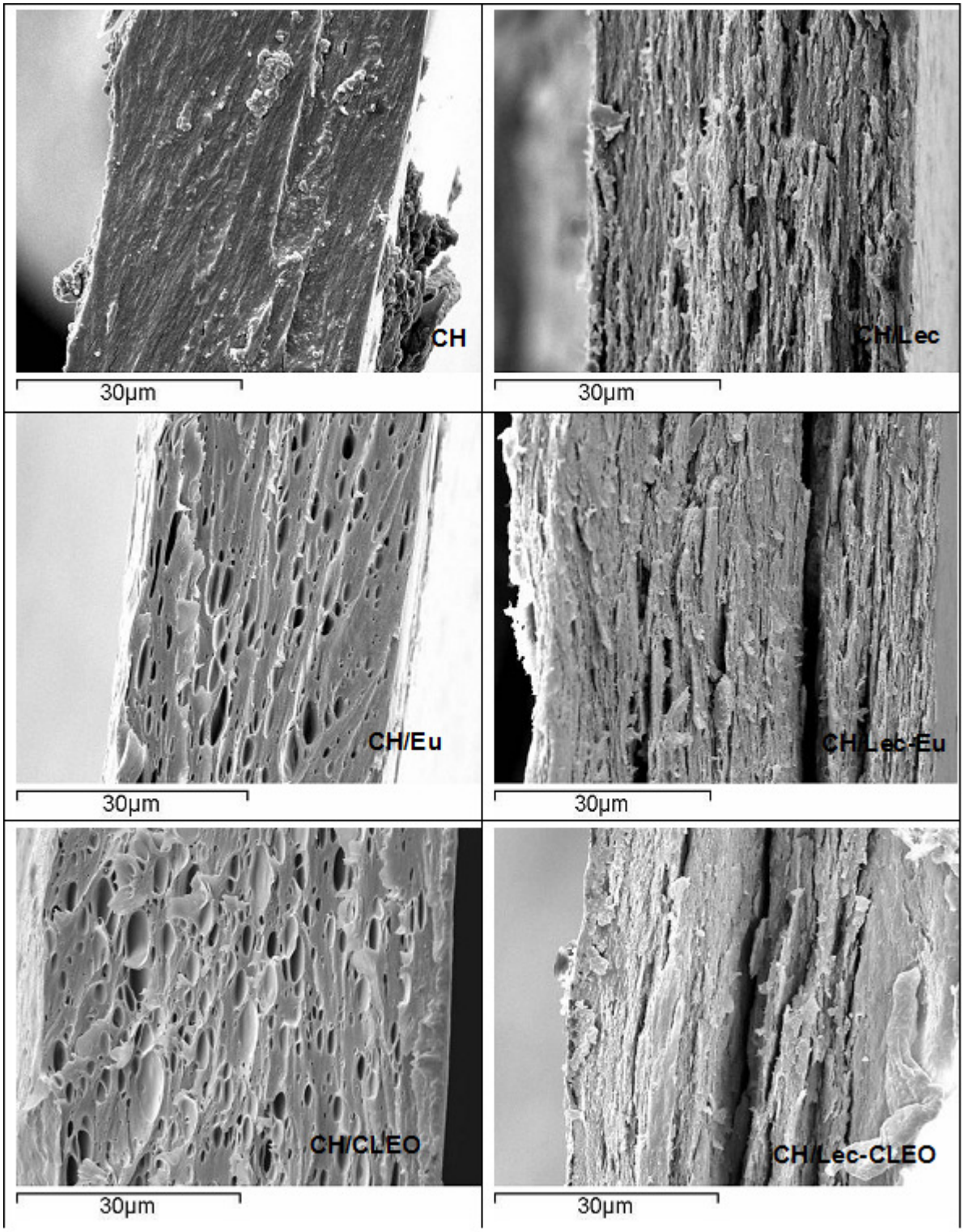

Figure 3.3. SEM micrographs of the cross section of the chitosan films with and without eugenol and cinnamon leaf essential oil in free form (left) or lecithin liposomes (right). Lec: lecithin, Eu: eugenol, CLEO: cinnamon leaf essential oil, CH: chitosan.

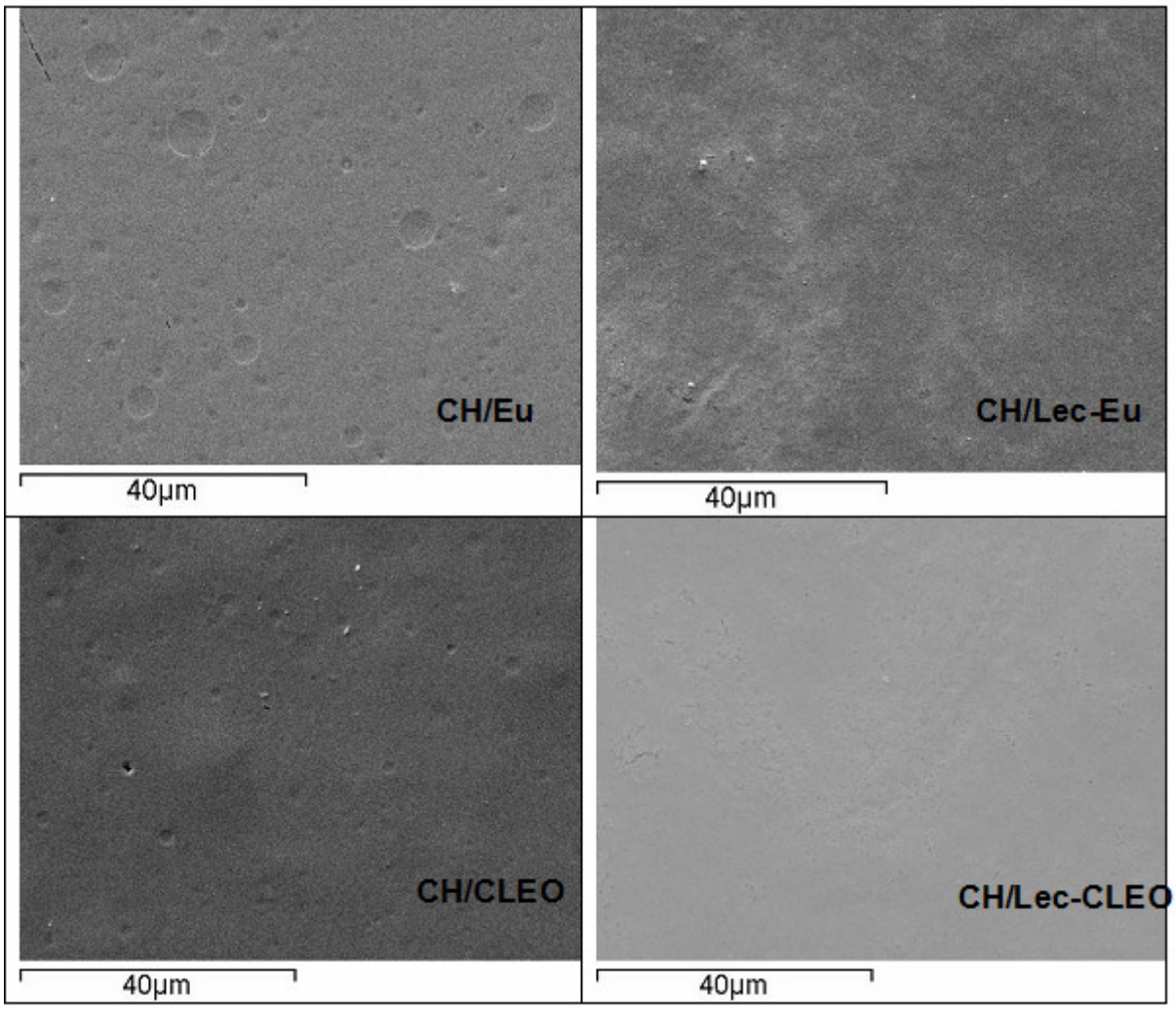

Figure 3.4. SEM micrographs of the surface of the chitosan films with eugenol (top) and cinnamon leaf essential oil (below) in free form (left) or lecithin liposomes (right). Lec: lecithin, Eu: eugenol, CLEO: cinnamon leaf essential oil, CH: chitosan.

3.2.6. Thermogravimetric analysis (TGA)

Figure 3.5 shows the thermal degradation curves (derivative curve: DTGA) of chitosan films, where three stages can be observed. The first mass loss step below about 100°C is due to the evaporation of residual water from the films. The second step can either be attributed to the degradation/volatilization of low molecular weight fractions, such as volatile compounds and lecithin, or to the losses of some groups (such as NH_2) from chitosan chains, and the third step is associated with the decomposition of polymer units, as observed by other authors (Shen & Kamdem, 2015). Films containing lecithin exhibited greater weight loss in the second stage, which can be attributed to the gradual decomposition of lecithin (about 20 % weight loss at 270°C) as deduced from the TGA of pure lecithin. The boiling point of eugenol is 253°C (Van Roon et al., 2002), which implies that when Eu or CLEO are present in the film, their thermal release would overlap with the polymer's degradation temperature range.

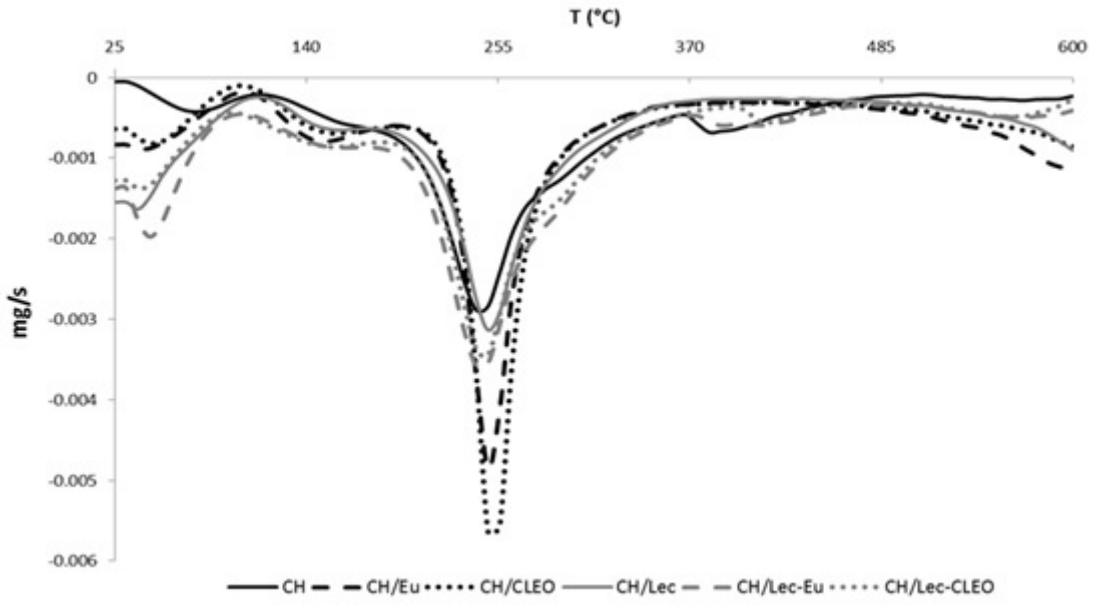

Figure 3.5. First derivative of weight loss vs. temperature curves obtained from TGA. Lec: lecithin, Eu: eugenol, CLEO: cinnamon leaf essential oil, CH: chitosan.

Table 3.6 shows the onset temperature (T_0) and maximum degradation rate temperature (T_{max}) of the polymer for the different films. The onset and Tmax temperature values of polymer decomposition were hardly affected by the lipid presence, which indicates that no strong interactions occurred among lipid molecules and polymer chains. Nevertheless, the thermal degradation of lipid components affected the thermogram shape. The films with free eugenol or CLEO had the highest onset temperature and degradation rate of the polymer, which could indicate that the eugenol remaining in the film may be strongly bonded to the

polymer chains, thus affecting their thermal behavior. In this sense, its extraction could be limited, this contributing to the very low retention determined, as previously comented.

Table 3.6 Onset temperature (T_0) and maximum degradation rate temperure of the films. Mean values and standard deviation. Lec: lecithin, Eu: eugenol, CLEO: cinnamon leaf essential oil, CH: chitosan.

Films	T_0 (°C)	T_{max} (°C)
CH	$193 \pm 4^{(a)}$	$248 \pm 3^{(a)}$
CH/Eu	$214 \pm 3^{(b)}$	$249 \pm 2^{(a)}$
CH/CLEO	$212 \pm 3^{(b)}$	$248 \pm 6^{(a)}$
CH/Lec	$193 \pm 2^{(a)}$	$243 \pm 2^{(a)}$
CH/Lec-Eu	$193 \pm 4^{(a)}$	$245 \pm 2^{(a)}$
CH/Lec-CLEO	$195 \pm 5^{(a)}$	$247 \pm 2^{(a)}$

Different superscript letters (a, b, c) within the same column indicate significant differences among formulations ($p < 0.05$).

3.3. Antimicrobial activity

Table 3.7 and **3.8** show *E. coli* and *L. innocua* counts, respectively immediately after the inoculation of the bacterial culture (0 days) and after 6 days of cold storage. Microbial counts were significantly reduced by film application right after the inoculation. The higher antibacterial effect (lower bacterial counts) was obtained for *E. coli*, which is in agreement with the greatest antimicrobial effect of chitosan against Gram - negative bacteria (Devlieghere et al., 2004), as compared to Gram -positive bacteria such as *L. innocua*.

Table 3.7 *Escherichia coli* counts in liquid (TSA Broth) and solid media (TSA Agar) at 10Â°C. Mean values ± standard deviation Lec: lecithin, Eu: eugenol, CLEO: cinnamon leaf essential oil, CH: chitosan. ng: no growth.

Film	*Escherichia coli* (log cfu/g)			
	Broth		Agar	
	0 days	6 days	0 days	6 days
Control	$5.7 \pm 0.3^{(ax)}$	$8.15 \pm 0.03^{(ay)}$	$5.58 \pm 0.07^{(ax)}$	$6.69 \pm 0.07^{(ay)}$
CH	$2.7 \pm 0.3^{(bx)}$	$2.16 \pm 0.02^{(by)}$	$3.63 \pm 0.03^{(b)}$	$4.02 \pm 0.04^{(b)}$
CH/Eu	ng	ng	$2.56 \pm 0.08^{(c)}$	ng
CH/CLEO	ng	ng	$2.55 \pm 0.06^{(c)}$	ng
CH/Lec	$2.95 \pm 0.04^{(bx)}$	$2.07 \pm 0.02^{(by)}$	$3.92 \pm 0.02^{(b)}$	$3.92 \pm 0.04^{(b)}$
CH/Lec-Eu	ng	ng	$2.70 \pm 0.08^{(c)}$	ng
CH/Lec-CLEO	ng	ng	$2.74 \pm 0.03^{(c)}$	ng

Different superscript letters (a, b, c) within the same column indicate significant differences among formulations ($p < 0.05$). Different superscript letters (x, y) within the same column indicate significant differences due to storage time.

For both bacteria and in liquid medium, the application of films containing the active ingredient led to no bacterial growth during the whole period of cold storage. This can be explained by the solution of the film matrix and the rapid release of the active ingredient. In the agar plates, immediately after inoculation, samples coated with films containing eugenol or cinnamon leaf essential oil led to a signficant reduction in the growth of both bacteria as compared to non-coated (control) and samples coated with CH or CH/Lec In samples coated with films containg the active ingredient no growth was detected at the end of the storage period.

Table 3.8 *Listeria innocua* counts in liquid (TSA Broth) and solid media (TSA Agar). Mean values ± standard deviation Lec: lecithin, Eu: eugenol, CLEO: cinnamon leaf essential oil, CH: chitosan. ng: no growth.

Film	*Listeria innocua* (log cfu/g)			
	Broth		Agar	
	0 days	6 days	0 days	6 days
Control	$5.40 \pm 0.02^{(ax)}$	$7.81 \pm 0.03^{(ay)}$	$5.38 \pm 0.07^{(ax)}$	$7.04 \pm 0.01^{(ay)}$
CH	$4.44 \pm 0.03^{(bx)}$	ng	$4.16 \pm 0.01^{(b)}$	$4.20 \pm 0.02^{(b)}$
CH/Eu	ng	ng	$2.83 \pm 0.05^{(c)}$	ng
CH/CLEO	ng	ng	$2.89 \pm 0.04^{(c)}$	ng
CH/Lec	$4.46 \pm 0.02^{(by)}$	ng	$4.28 \pm 0.02^{(b)}$	$4.32 \pm 0.01^{(b)}$
CH/Lec-Eu	ng	ng	$2.97 \pm 0.05^{(c)}$	ng
CH/Lec-CLEO	ng	ng	$2.97 \pm 0.03^{(c)}$	ng

Different superscript letters (a, b, c) within the same column indicate significant differences among formulations ($p < 0.05$). Different superscript letters (x, y) within the same column indicate significant differences due to storage time.

4. CONCLUSIONS

The incorporation of lecithin liposomes containing eugenol or cinnamon leaf essential oil into chitosan films obtained by casting allowed for a high retention ratio (40-50 %) of volatile compounds, as compared to the 1-2 % which is retained when they are free incorporated by emulsification. Films with liposomes exhibited a lamellar microstructure where lipid lecithin layers seem to alternate with polymer layers, due to the liposome phase transitions during the progressive film drying. This microstructure improved the extensibility of the films, while reducing their firmness and water vapor permeability. Liposome incorporation modified the optical properties of the films; the gloss was reduced, chrome was increased and the films became reddish, coherently with the chromatic properties of lecithin. The addition of the active ingredients enhanced the antimicrobial activity of pure chitosan films. The incorporation of lecithin as encapsulating agent did not affect the antimicrobial effectiveness of the films. Therefore, the encapsulation of volatile active compounds in liposomes emerges as a good strategy for the obtention of active films with essential oils.

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III.4 CHAPTER 4

RELEASE KINETICS AND ANTIMICROBIAL PROPERTIES OF CHITOSAN-ESSENTIAL OILS FILMS AS AFFECTED BY ENCAPSULATION WITHIN LECITHIN NANOLIPOSOMES

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ABSTRACT

The antimicrobial capacity of CH-based films containing eugenol (EU) or cinnamon leaf essential oil (CLEO), as affected by their encapsulation within lecithin nanoliposomes, was tested against *Escherichia coli* and *Listeria innocua*. Films were able to reduce microbial load all types of samples (liquid and solid media and pork meat), and more significantly for *E. coli*. The evaluation of the overall migration in the films showed that in hydrophilic simulants (ethanol 10 % and acetic acid 3 %), the limit that the EU Directive stablishes was exceeded and thus the potential application of these films would be limited to lipophilic food products.

The retention capacity of the active ingredients in the polymer matrix was revealed by the kinetics of eugenol release in four food simulants (ethanol 10 %, ethanol 50 %, acetic acid 3 % and isooctane). For all of them, the rate constant (K_1) obtained by applying Peleg's model, was higher for films containing lecithin, whereas diffusivity values were reduced. Films were able to slow down the changes in color parameters and increase the shelf-life of cold stored pork meat.

Keywords: biopolymer, essential oil, cinnamon, eugenol, antimicrobial, migration.

1. INTRODUCTION

Chitosan (CH) is a cationic, non-toxic, biodegradable film-forming polysaccharide, compatible with other biopolymers, which can be obtained from the deacetylation of the chitin present in crustacean exoskeletons, and shows antimicrobial properties (Zhai et al., 2004). CH-based films exhibit good mechanical and structural properties and constitute a good barrier to gases and aromas. The incorporation of active ingredients such as essential oils into CH-based films could improve their functionality, since the antimicrobial properties of the film would be enhanced (Vargas et al., 2012). Cinnamon leaf essential oil (CLEO) and its main compound, eugenol (EU), have been described as antibacterial and antifungal agents at relatively low concentrations in previous studies (De Martino et al., 2009; Bajpai et al., 2012). Eugenol has been recognized as safe by the US Food and Drug Administration (US FDA, 2009) and approved by the European Union as a safe flavoring agent for foods (Sebaaly et al., 2015).

Different essential oils have been widely used in the formulation of active biodegradable films based on chitosan. Essential oils have been incorporated into hydrophilic biopolymer films by emulsification in the aqueous film-forming dispersion (FFD) of the polymer before film formation by means of different homogenization methods and the films were obtained by casting (Sánchez-González et al., 2009; Bonilla et al., 2011). However, the losses of volatile compounds during casting technique shows an important drawback since during the film drying step, oil droplets flocculate, coalesce and cream to the top of the drying film, where oil components volatilize together with water at a lower temperature than their boiling point (Perdones et al., 2016). The encapsulation of essential oil compounds before film preparation can reduce the losses of volatile compounds at the same time that the sensory impact of essential oils is minimized. Moreover, encapsulation can modulate the release kinetics of active compounds into the food product surface. In this sense, the use of liposomes or nanoliposomes, which can act as carrier systems of a wide range of compounds, represent an interesting alternative (Zhang et al., 2012).

The antimicrobial effect of films containing liposomes depends on both the nature of the active ingredients (essential oils) and on the capacity of the film to release enough active compound to the coated food at a determined contact time and at equilibrium (partition coefficient). Moreover, the release kinetics of liposomes depends on interactions between the encapsulated active ingredients and the polymer matrix together with the solubility of the active compounds in the food system. In this sense, the release kinetics should be evaluated in systems with different polarities, which simulate different kinds of foods, such as standard food simulants (European Commission Regulation, 2011).

In order to guarantee antimicrobial effectiveness and food safety it is essential to evaluate the release kinetics of the active compound into the food throughout the storage time (Tehrany and Desobry, 2007). The time needed to reach a concentration level of active in the food that surpasses the minimum inhibitory concentration of the target microorganisms must be estimated to ensure food safety. For prediction purposes, and to evaluate the compound release rate from the films, and the concentration reached in the food simulant Fick equation (Del Nobile et al., 2008; Sánchez-González, et al., 2012) or first order kinetics models have been used (Barba et al., 2015; Biddeci et al., 2016).

The aim of this work was to evaluate the antimicrobial effect of chitosan films containing eugenol or cinnamon essential oil as affected by their encapsulation within lecithin nanoliposomes.

2. MATERIALS AND METHODS

2.1. Reagents

Chitosan (CH) and eugenol (EU) were supplied by Sigma-Aldrich Chemie (Steinheim, Germany). Cinnamon leaf essential oil (CLEO) was provided by Herbes del molí (Alicante, Spain). Sunflower Lecithin (Lec), Lipoid H20- 20 % - phosphaatidilcoline no-GMO, was supplied by LipoidGmbH (Frigenstraβe, Germany). Methanol, ethanol, glacial acetic acid, isooctane, glycerol and magnesium nitrate was purchased from Panreac Química SLU (Castellar del Vallés, Barcelona; Spain).

L. innocua (CECT 910) and *E. coli* (CECT 10) lyophilized strains were obtained from Colección Española de Cultivos Tipo (CECT, Burjassot, Spain). Buffered peptone water and culture media were supplied by Scharlab S.L. (Mas d'EnCisa, Barcelona, Spain).

2.2. Preparation of the nanoliposome dispersions

The preparation of nanoliposome dispersions was carried out according to a previously described method (Jiménez et al., 2014). Lecithin was dispersed in distilled water (5 wt %) and stirred for 4 h at 700 rpm Eugenol or CLEO at 5 wt % was incorporated into the lecithin dispersion and afterwards three different liposome samples were obtained (Lec, Lec-Eu and Lec-CLEO) by sonication at 20 kHz for 10 min with 1 s pulses. The ultrasound probe was placed in the centre of the sample. An aqueous solution of lecithin was prepared (5 wt %) by stirring at room temperature during 3-4 hours.

2.3. Preparation of the film-forming dispersions and films

A solution of 1 % CH (w/w) was prepared in a 1 % (v/w) acetic acid solution by stirring at room temperature for 24 h. The active compounds (eugenol or CLEO) were incorporated in two different forms: encapsulated in liposomes or by direct emulsification. These FFDs were named as CH-EU, CH-CLEO, CH-Lec-Eu, and CH-Lec-CLEO and were obtained by adding 0.5 g of active compound (eugenol or CLEO) as free form or 10 g of the liposome dispersion to 90 g of the CH solution. The amount of active was selected to overcome the values of the minimal inhibitory concentration of actives against some typical pathogens or food spoilage microorganisms according to previous studies with CH films containing CLEO (Perdones et al., 2014).

CH FFDs with and without lecithin nanoliposomes free of active ingredients were also prepared. These FFDs were name as CH and CH-Lec, respectively.

FFDs were homogenized with a rotor-stator (Yellow Line DI 25 basic, IKA, Germany) at 13500 rpm for 4 minutes. Films were obtained by casting the FFDs in Teflon® leveled plates (150 mm in diameter, 1 g solids/plate), which were dried at room temperature and 46 % RH for 24-48h.

Films were peeled of from the casting plates and conditioned in hermetic jars with an oversaturated Mg $(NO_3)_2$ solution (53 % RH) during a week prior performing the analysis.

2.4. Overall migration in food simulants

The overall migration test was carried out, according to Regulation 10/2011/EC (European Directive, 14/1/2011) for food contact materials. The overall migration test, which determines the migration of a specific packaging material in food simulants, was carried out with simulant A (ethanol 10 % v/v, aqueous food), simulant B (acetic acid 3 % w/v, aqueous food with pH lower than 4.5) and simulant D2 (isooctane, food with a fatty continuous phase). The ratio film surface/ volume simulant was 6 dm^2/kg, and the test was carried out at 20°C in duplicate for 10 days. Afterwards, the solvent was transferred to cups and evaporated at 60°C until a constant weight was reached. (24-48h). The overall migration of each film formulation was determined as the weight of residue after drying and expressed as mg/dm^2 of film. All analyses were carried out in duplicate.

2.5. Kinetics of eugenol release in food simulants

The kinetics of release of EU from the films was tested in four food simulants: A (ethanol 10 % v/v, aqueous food), simulant B (acetic acid 3 % w/v, aqueous food with pH lower than 4.5), simulant D1 (ethanol 50 % v/v, mimics alcoholic food and oil-in water emulsions and simulant D2 (isooctane, food with a fatty continuous phase). Film samples (4 cm^2) were placed in flasks with 100 mL of the corresponding simulant at 20°C and under continuous stirring. After different contact times up to equilibrium (24 h), the samples were taken from the flasks, and the absorbance at 282 nm was measured (Spectrophotometer Helios Zeta UVeVis, Thermo Fisher Scientific, UK). Thus, the EU profile concentration in each simulant over time could be determined by the absorbance measurements using the standard calibration curve. All analyses were performed in triplicate for three different flasks containing the different film samples. The liquid phase in contact with the active-free films was used as blank for the absorbance measurements, for each simulant and time.

Peleg's model (Eq. 1), was applied to experimental data in order to predict the release kinetics (Peleg,1988).

$$M_t = M_0 + \frac{t}{k_1 + k_2 t} \qquad \text{Eq.1}$$

where M_t is total eugenol released at time t, M_0 is the initial amount of eugenol released, k_1 is the kinetic constant of the model that is inversely related to the mass transfer rate at the beginning of the process, and k_2 is a constant of the model that is related to the asymptotic value, which can be related to the equilibrium value ($1/M_\infty$ where M_∞ is the amount of active released at equilibrium).

Fick's second law was considered to model the diffusion process of EU in the films bilayer films towards the food simulants. Film samples can be considered as infinite plane sheets with the half thickness as a characteristic dimension, where the active compound diffuses only in an axial direction. The diffusional long-time equation for an infinite plane sheet (Crank, 1975), considering with eight terms, was used to determine the values of diffusion coefficient (D) of EU into the different solvents (Eq. 2):

$$\frac{M_t}{M_\infty} = 1 - \frac{8}{\pi^2} \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} exp\left(\frac{-D(2n+1)^2 \pi^2 t}{L^2}\right) \qquad \text{Eq.2}$$

Where:

D: diffusion coefficient (m^2/s)

M_t : mass of eugenol released at time t (g)

M_∞: the mass of compound released at equilibrium (g)

L: half thickness of film (m)

2.6 Antimicrobial properties of the films

Listeria innocua (CECT 910) and Escherichia coli (CECT 101) were supplied by the Spanish Type Culture Collection (CECT, Burjassot, Spain). These bacterial cultures were regenerated (from a culture stored at -25°C) by transferring a loopful into 10mL of Tryptone Soy Broth (TSB) (Scharlab, Barcelona, Spain) and incubating at 37°C for 24h. From this culture, a 10 μL aliquot was again transferred into 10 mL of TSB and grown at 37°C for 24h more in order to obtain a culture in the exponential phase of growth. Afterwards, this bacterial culture was appropriately diluted in TSB tubes to get a target inoculum of 10^5 CFU mL^{-1}. Circular samples (55 mm in diameter) of the films were sterilized under shortwave UV light and aseptically placed in TSB tubes (liquid medium test), on inoculated Tryptose Soya Agar (TSA) (Scharlab) plates (solid medium test) and on pork meat sample slices. Inoculated broth tubes, agar plates and meat slices without film were used as control samples. Immediately after the inoculation and after 6 days at 10°C the microbial counts on TSA plates were determined. In the liquid medium tests (broth tubes), serial dilutions were made and poured onto TSA dishes which were incubated for 24 h at 37°C. For the solid medium tests (agar plates) and the raw pork meat slices, the dish content was removed aseptically and placed in a sterile plastic bag with 90 mL of Buffered Peptone Water.

The bags were homogenized with a Stomacher (Interscience BagMixer Stomacher 400 W Homogenizer, St Nom la Bretèche, France). Serial dilutions were made and then poured into TSA Petri dishes. Samples were incubated at 37°C for 24 h before colonies were counted. All the tests were run in triplicate.

2.7. Effect of the films on the shelf-life of pork meat

The effect of film application on the shelf-life of pork meat was tested, as described in previous studies (Bonilla et al., 2013). Pork meat was purchased in a local supermarket and slices of 5 cm in diameter (about 10 g) were obtained. The surface of the meat slices was coated with the films. Non-coated samples (control) and samples coated with the different types of films were stored in duplicate at 10°C for 7 days in a thermostat cabinet (Aqualytic GmbH & Co, Dortmund, Germany).

In order to evaluate the specific migration of eugenol into the coated meat samples, at different times of cold storage, the films containing EU were peeled off the coated pork meat slices and eugenol was extracted with methanol and quantify by means of the measurement of absorbance. To this end, film samples (4 cm^2) were kept in 100 mL methanol under stirring (300 rpm) for 24 h at 20°C. The extract was filtered and quantitatively diluted to measure the absorbance at 282 nm using a UV-visible spectrophotometer (Helios Zeta UVeVis, Thermo Fisher Scientific, UK), using the methanol extract of eugenol-free CH films as blank solution. All analyses were carried out in duplicate.

To perform microbiological analyses, 10 g of each sample were aseptically obtained and homogenized in a Stomacher (Bag Mixer 400, Interscience) 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 aerobial counts were determined in Plate Count agar incubated at 37°C for 48 h. Coliform microorganisms were determined in Violet Red Bile Agar incubated at 37°C for 48 h. All tests were performed in triplicate. Color changes in meat slices during cold storage were obtained by using a spectrocolorimeter CM-3600d (Minolta Co, Tokyo, Japan) with a 30 mm diameter window. In coated samples, films were peeled off the coated pork meat slices prior performing the measurements. Reflection spectra were obtained over a black (R_0) and a white (R) standard background, of known reflectance (R_g) in 6 different areas of the meat sample. From these spectra, the infinite reflection spectra of the samples (R_∞) was obtained (Eq. 3-5)

$$R_\infty = a - b \qquad \text{Eq.3}$$

$$a = \frac{1}{2}(R + \frac{R_0 - R + R_g}{R_0 R_g}) \qquad \text{Eq.4}$$

$$b = \sqrt{a^2 - 1} \qquad \text{Eq.5}$$

From R_∞= spectra, and using D65 illuminant /10° observer, CIE-L* a* b* coordinates (CIE, 1986), and chromatic parameters hue (h^*_{ab}, Eq.6) and chroma (C^*_{ab}, Eq.7), and the total color difference as regard a non-coated sample were obtained (ΔE, Eq.8) The color of each sample was measured in quintuplicate.

$$C^*_{ab} = \sqrt{a^{*2} + b^{*2}} \qquad \text{Eq.6}$$

$$h^*_{ab} = \arctan \frac{b^*}{a^*} \qquad \text{Eq.7}$$

$$\Delta E = \sqrt{\Delta L^* + \Delta a^* + \Delta b^*} \qquad \text{Eq.8}$$

2.8. Statistical analysis

Statistical analyses were performed through analysis of variance (ANOVA) using Statgraphics Centurion XVI-II. Fisher's least significant difference (LSD) procedure at 95 % was used.

3. RESULTS AND DISCUSSION

3.1. Total migration of eugenol

Table 4.1 shows total migration of material in three food simulants of different polarity and pH (3 % aqueous acetic acid solution, 10 % aqueous ethanol solution and isooctane).

Table 4.1 Total migration (10 days, 20°C) of material from the films. Mean values and standard deviation.

Film	Total migration (mg/ dm^2 film)		
	Ethanol 10 %	Acetic acid 3 %	Isooctane
CH	$485 \pm 2^{(a1)}$	$655 \pm 8^{(a2)}$	$9.2 \pm 0.3^{(a3)}$
CH-Lec	$144 \pm 2^{(b1)}$	$309 \pm 5^{(b2)}$	$3.30 \pm 0.14^{(b3)}$
CH-EU	$397 \pm 3^{(c1)}$	$567 \pm 10^{(c2)}$	$5.30 \pm 0.14^{(c3)}$
CH-CLEO	$383 \pm 4^{(d1)}$	$561 \pm 4^{(c2)}$	$4.5 \pm 0.12^{(c3)}$
CH-Lec-EU	$93.5 \pm 1.5^{(e1)}$	$139 \pm 2^{(d2)}$	$0.9 \pm 0.4^{(d3)}$
CH-Lec-CLEO	$91.0 \pm 1.3^{(e1)}$	$134 \pm 5^{(d2)}$	$0.9 \pm 0.2^{(d3)}$

Different letters in the same column (a-e) indicate significant differences ($p<0.05$) among films. Different numbers in the same row (1-3) indicate significant differences ($p<0.05$) among simulant for the same film.

Total migration was affected by both the pH and polarity of the simulant together with the solubility of film components in the release media. In hydrophilic simulants, specific migration values exceeded the limit of 10 mg /dm^2 regulated by the European Directive for food contact materials (European Commission, 2011). Moreover, as expected, for all films, the highest migration levels were obtained in 3 % acetic acid (simulant A), since acid media promote chitosan solution and hence the diffusion of film ingredients into the release solvent. On the contrary, in isooctane migration levels were lower than the regulated limit of 10 mg/dm^2, especially in films containing lecithin nanoliposomes. The encapsulation of essential oils in lecithin nanoliposomes reduced significantly migration levels, especially as compared to pure CH films. This can be explained by the reduction in swelling promoted by the addition of lipid compounds in the polymer matrix (Hromiš et al., 2015). In all solvents, no significant differences in the migration levels were detected due to the type of added active ingredient.

The reported results agree with the effect of film microstructure in migration levels (Valencia-Sullca et al., 2016). In this previous study, microstructure observations revealed that the addition of active ingredients encapsulated in lecithin nanoliposomes led to the formation of laminar structures, where the essential oils were located at the core of the nanocapsules. These laminar structures containing lecithin limited the interactions between the film ingredients and the solvent, thus reducing total migration.

3.2. Kinetics of eugenol release in food simulants

Figure 4.1 shows the mass of eugenol released, as regards of the mass released at equilibrium, as a function of time, together with the Peleg's model fit, obtained for films containing pure eugenol (encapsulated or not in lecithin nanoliposomes).

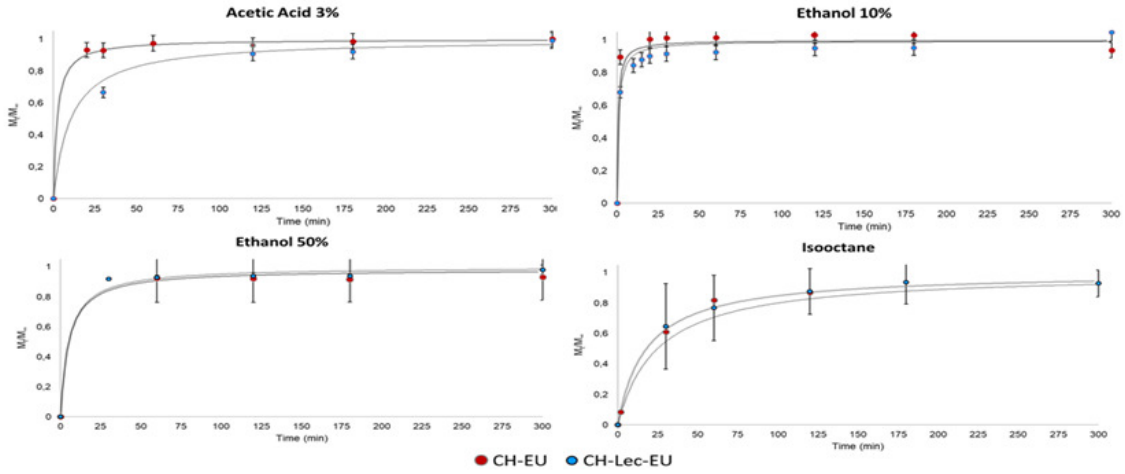

Figure 4.1. Kinetics of eugenol release from CH-EU and CH-Lec-EU films in different food simulants.

The kinetics of eugenol release from CH-EU films in hydrophilic simulants was fast. Release occurs in two steps: (1) fast release and diffusion of eugenol from film surface and (2) slower release diffusion of eugenol in the polymer matrix. In isooctane, the maximum release was achieved after 20 minutes, and a lower amount of eugenol was released. Films containing nanoliposomes (CH-Lec-EU) showed smoother curves, which is in agreement with a slower release kinetics. The above mentioned laminated microstructure makes eugenol diffusion difficult, leading to lower release rates.

Table 4.2 shows Peleg's model parameters together with the diffusivity values predicted by Fick Model. Diffusivity values of eugenol were significantly affected by the type of simulant and by its encapsulation with lecithin ($p<0.05$), which reduced diffusion levels in all solvents except for 50 % ethanol. In films formulated with EU in free form, diffusivity values significantly decrease with the decrease in polarity of the solvent, which reveals eugenol affinity for hydrophilic media. In films containing lecithin-eugenol nanoliposomes, the opposite trend was observed: the reduction in polarity promoted an increase in diffusivity values except for isooctane, where the lowest diffusivity value was obtained, which is consistent with the reported kinetics of release (**Figure 1**).

Table 4.2 Apparent diffusivity (D) and parameters of Peleg's model: amount of active compound released at equilibrium in the simulant (M_∞) and its release rate ($1/k_1$), and maximum release ratio (M_∞/M_0, mass of active released at equilibrium in the simulant related to the initial mass of the active in the film expressed with respect to the amount determined by methanol extraction (1) or by acetic acid extraction (2).

Film	Simulant	D x 10^{13} (m^2/s)	$1/k_1$ (mg/min)	$M_\infty = 1/k_2$ (mg/100 g film)	M_∞/M_0 [1]	R^2
CH-EU	Ethanol 10 %	$5.33 \pm 0.03^{(c)(x)}$	$0.00410 \pm 0.00011^{(d)(x)}$	$2.686 \pm 0.122^{(b)(x)}$	$0.636 \pm 0.026^{(b)}$	>0.999
	Acetic acid 3 %	$8.50 \pm 0.11^{(d)(x)}$	$0.00152 \pm 0.00005^{(c)(x)}$	$2.969 \pm 0.054^{(c)(x)}$	$0.713 \pm 0.028^{(c)}$	>0.998
	Ethanol 50 %	$4.84 \pm 0.08^{(b)(x)}$	$0.00076 \pm 0.00003^{(b)(x)}$	$3.503 \pm 0.067^{(d)(x)}$	$0.828 \pm 0.032^{(d)}$	>0.999
	Isooctane	$3.02 \pm 0.07^{(a)(x)}$	$0.000068 \pm 0.000003^{(a)(x)}$	$1.256 \pm 0.043^{(a)(x)}$	$0.035 \pm 0.002^{(a)}$	>0.996
CH-Lec-EU	Ethanol 10 %	$2.17 \pm 0.07^{(c)(y)}$	$0.00209 \pm 0.00007^{(d)(y)}$	$1.994 \pm 0.071^{(b)(y)}$	$0.754 \pm 0.032^{(b)}$	>0.999
	Acetic acid 3 %	$0.60 \pm 0.02^{(b)(y)}$	$0.00026 \pm 0.00001^{(b)(y)}$	$2.669 \pm 0.045^{(c)(y)}$	$1.000 \pm 0.039^{(c)}$	>0.998
	Ethanol 50 %	$6.32 \pm 0.09^{(d)(y)}$	$0.00059 \pm 0.00001^{(c)(y)}$	$2.095 \pm 0.047^{(b)(y)}$	$0.790 \pm 0.020^{(b)}$	>0.999
	Isooctane	$0.21 \pm 0.01^{(a)(y)}$	$0.000070 \pm 0.000003^{(a)(x)}$	$1.788 \pm 0.063^{(a)(y)}$	$0.671 \pm 0.016^{(a)}$	>0.995

Different letters in the same column (a-h) indicate significant differences ($p<0.05$) among films. Different numbers in the same column (1-2) indicate significant differences ($p<0.05$) among films for the same simulant.

Parameters of Peleg's model showed a more rapid release of eugenol from films with non-encapsulated eugenol (higher values of $1/k_1$) as compared to films containing lecithin nanoliposomes. This coincides with the results found by Wu et al. (2015) in gelatin films containing cinnamon leaf essential oil.

For all hydrophilic solvents, the plateau value of Peleg's model (M_∞) was lower in films containing nanoliposomes. This parameter is affected by the initial content of eugenol in the film (M_0), being 0.347 g EU/g dry film and 0.257 g EU/g dry film for CH-EU and CH-Lec-EU films, respectively. T_0 minimize the effect of the difference in initial eugenol content in the films, the ratio M_∞/M_0 after extraction in methanol was calculated.

3.3. Antimicrobial activity against Escherichia coli and Listeria innocua

Figure 4.2 shows the evolution of *E. coli* and *L. innocua* during cold storage in Triptose Soya broth (TSB), Tryptose Soya Agar (TSA) plates and pork meat slices, as affected by the application of the films.

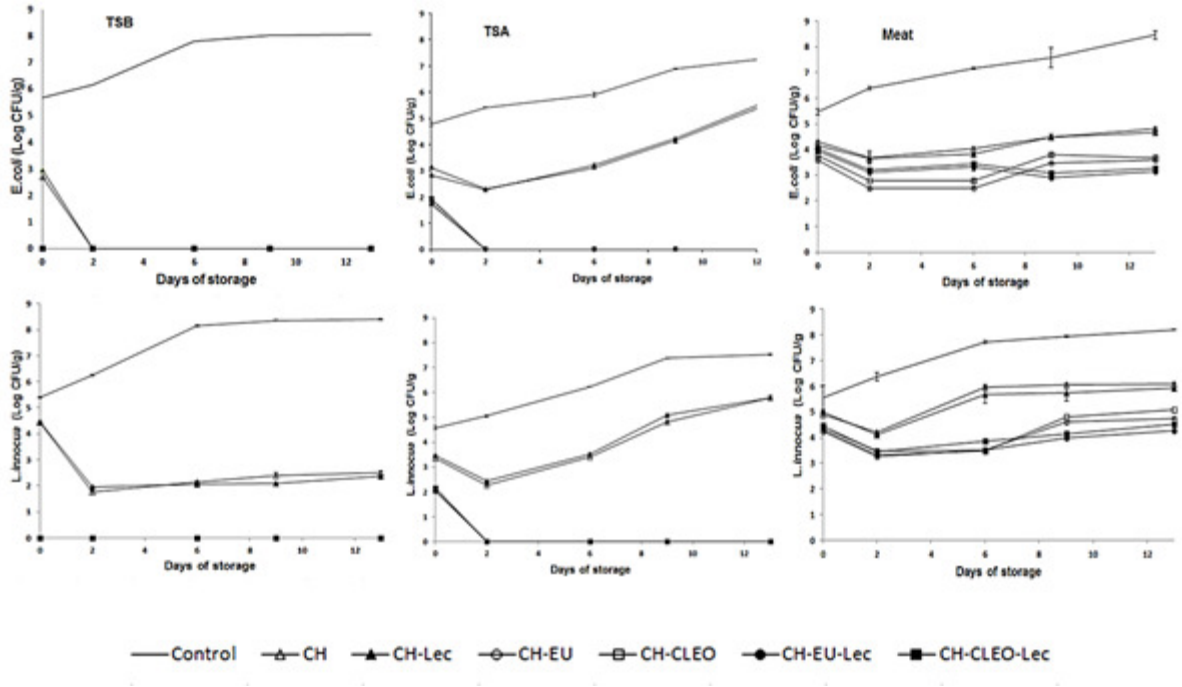

Figure 4.2. Microbial count of samples inoculated with *L. innocua* or *E. coli* during storage at 10°C. Mean values and standard deviations.

Chitosan films significantly reduced microbial growth in all types of samples, especially as regards *E. coli*. This coincides with previous studies that reported a more significant antibacterial effect of CH against Gram-negative bacteria, such as *E. coli*, due to the higher negative charge of cell membrane thus promoting the interactions of the cationic groups of chitosan (O'Callaghan & Kerry, 2016).

The antibacterial effect of CH was more marked in TSB, which is coherent with the highest solubility of CH in liquid media. On the contrary, in solid media polymer diffusivity is limited, which implied a lower antimicrobial effect, especially when films were applied to meat slices. In the three types of samples, there were not detected differences due to the addition of lecithin, which coincides with previous studies (Jiménez et al., 2015).

Films containing active ingredients (EU or CLEO) had a bactericidal effect against both microorganisms, and significantly in inoculated TSB and TSA media. Previous studies have shown a synergic effect of CH films and cinnamon leaf essential oil against *E. coli* (Wang et al., 2011). No significant effects were observed due to the encapsulation of the active ingredients in lecithin nanoliposomes.

3.4. Effect of the films on the shelf-life of pork meat

The amount of EU that was release from CH-EU and CH-Lec-EU films in contact with meat slices after different times of cold storage was obtained by extraction of the remaining EU in the film. The results are shown in **Table 4.3**.

Table 4.3 Total specific migration of eugenol (mg eugenol/g meat) from the films in meat samples stored at 10°C. Mean values and standard deviation, in brackets.

Film	0 days	2 days	6 days	13 days
CH-EU	$1.2 \pm {}^{0,4(a1)}$	$2.12 \pm 0.03^{(a2)}$	$2.90 \pm 0.03^{(a3)}$	$2.53 \pm 0.04^{(a23)}$
CH-Lec-EU	$0.710 \pm {}^{0,006(b1)}$	$2.133 \pm 0.004^{(a2)}$	$2.34 \pm 0.05^{(b3)}$	$2.38 \pm 0.03^{(b3)}$

Different letters (a, b) in the same column indicate significant differences between films at the same storage time ($p<0.05$). Different numbers (1, 2, 3) in the same row indicate differences due to storage time for the same film ($p<0.05$).

The amount of eugenol released to meat slices from the two types of films increased during storage. Encapsulation of eugenol in lecithin nanoliposomes delayed eugenol release, especially at the beginning of the storage, which suggested better results at long term as regards antimicrobial activity.

Figure 4.3 shows total aerobial and coliform microorganism counts in non-inoculated pork meat slices as affected by film application. All films showed antimicrobial activity and reduced total aerobial and coliform counts as compared to non-coated samples (control). Results agree with Bonilla et al. (2014). All films showed a bactericidal effect in meat slices at the beginning of storage. After two days, in samples coated with CH and CH-Lec, an increase in the microbial load was detected, with no significant differences between samples. Films formulated with active ingredients kept the bactericidal effect against coliforms during 9 days of storage, and no significant differences were detected due to the encapsulation of active compounds. During the whole storage period, films containing essential oils led to microbial loads lower than 5 CFU/g, which is maximum value allowed according the 88/657/EEC European Directive (Council Directive, 1988).

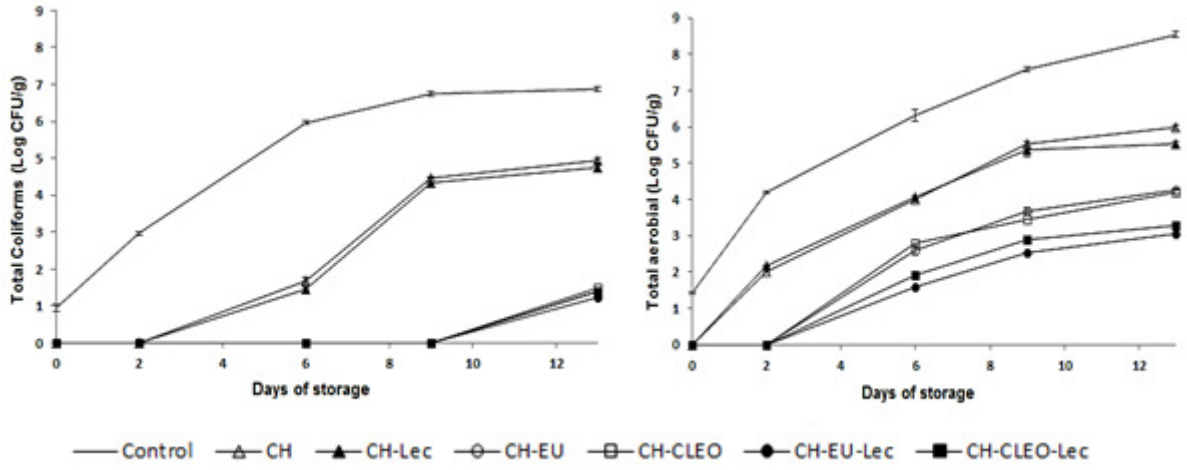

Figure 4.3. Total aerobial and coliform counts in pork meat slices during storage at 10°C. Mean values and standard deviation.

The antibacterial effect of the films was less significant in terms of total aerobial counts as compared to coliforms, which are the microorganism that are more affected by the antibacterial action of chitosan, as commented on above.

3.5. Color

Figure 4.4 shows the values of chromatic parameters and color change as regards non-coated samples after 13 days of cold storage.

Lightness of samples was reduced after 13 days of storage, due to surface dehydration, while hue values was slightly increased, which is in agreement with the loss of red color. Films containing cinnamon leaf essential significantly reduced the changes in color parameters during storage, especially in terms of total color difference. The latter could be explained by the antioxidant effect of cinnamon leaf essential oil films (Perdones et al., 2014).

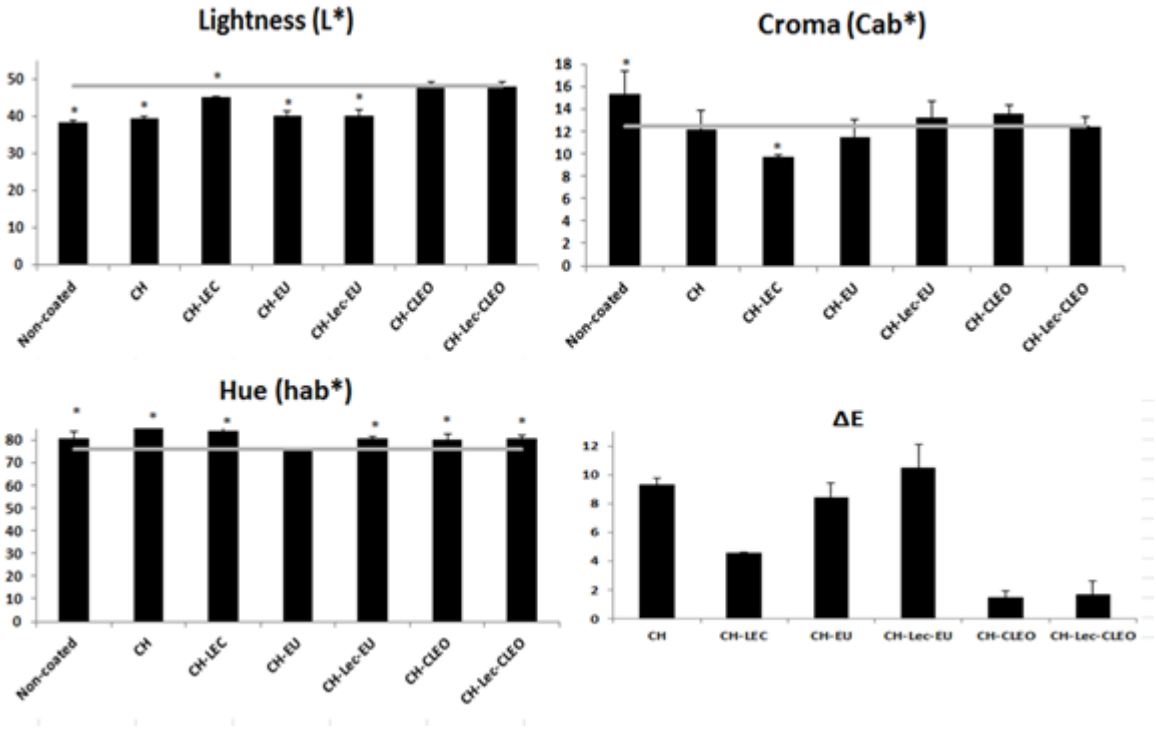

Figure 4.4. Chromatic parameters and total color difference as regards non-coated samples (ΔE) after 13 days of cold storage. The initial counts are indicated by a continuous line. *Indicates significant differences ($p<0.05$). The initial counts are indicated by a continuous line. *Indicates significant differences ($p<0.05$).

5. CONCLUSIONS

Encapsulation of eugenol and cinnamon leaf essential oil in lecithin nanoliposomes reduced total migration of chitosan-based films and led to a delayed release in food simulants. All films were effective at controlling and reducing the growth of *L. innocua* and *E. coli* both in liquid and solid media as well as in pork meat slices during cold storage. No significant differences in terms of antibacterial efficacy were obtained between films containing active ingredients.

The encapsulation of eugenol promoted its control release when chitosan films were applied to pork meat samples and thus the antimicrobial effect and protective effect in terms of color changes of such films was enhanced. Therefore, the encapsulation of eugenol and cinnamon leaf essential oils in lecithin nanoliposomes yielded active films that can extend the shelf-life of cold stored pork meat slices.

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III.5 CHAPTER 5

CHITOSAN FILMS CONTAINING ENCAPSULATED EUGENOL IN ALGINATE MICROCAPSULES

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ABSTRACT

Alginate microspheres containing eugenol as active ingredient were obtained by extrusion and ionic gelation. The effect of their incorporation on the mechanical and barrier properties of chitosan was evaluated as well as the kinetics of eugenol release kinetics and antimicrobial effectivity of the films. Microspheres increased the stretchability of chitosan the films at the same time that the oxygen barrier properties were improved. Microspheres were visible in the structure of the film and their release rate in food simulants was enhanced in 3 % acetic acid. However, the release rate of eugenol was reduced markedly in non-polar systems (isooctane). The incorporation of encapsulated eugenol into chitosan films enhanced their antimicrobial efficacy.

Keywords: microcapsules, eugenol, alginate, controlled release, encapsulation.

1. INTRODUCTION

In order to solve the problems generated by plastic waste, many efforts have been done to obtain an environmental friendly material (Almasi et al., 2010). Most of the research focuses on substitution of the petroleum-based plastics by biodegradable materials with similar properties and low cost (Mali et al., 2005; Mariniello et al., 2007; Larotonda et al., 2005).

Chitosan (CH), a cationic polysaccharide consisting of (1, 4)-linked-2- amino-deoxy-b-D-glucan, is the deacetylated form of chitin (Yuan et al., 2016). CH, which is non-toxic, biodegradable and compatible with other biopolymers, exhibits antibacterial and gel-forming properties, and has been employed for film preparation (Zhai et al., 2004; Dutta, et al., 2009; Kong et al., 2010; Cruz-Romero et al., 2013). Chitosan films are promising systems to be used as active ingredient carriers for their application in food preservation, since they can carry and release compounds with antimicrobial or antioxidant abilities (Elsabee and Abdou, 2013; Ruiz-Navajas et al., 2013; Perdones et al., 2016).

Essential oils (EOs) have also been used in the formulation of biodegradable films, since they exhibit antimicrobial and antioxidant capacities (Bakkali et al., 2008). They have also been utilized due to their antifungal, antiviral and insecticidal properties (Kordali et al., 2005; Pezo et al., 2006). Essential oils have been categorized as GRAS (Generally Recognized As Safe) by US Food and Drug Administration, which has triggered the interest of the food industry in their use as potential alternatives to synthetic additives (Soni et al., 2016). Essential oils have been widely used for food preservation (Viuda-Martos et al., 2010; Hyldgaard et al., 2012; Tongnuanchan and Benjakul, 2014) since their strong components can act against pathogenic bacteria (Nazzaro et al., 2013). EOs incorporation into a film matrix may improve its water-vapour barriers because of the hydrophobicity of these (Tongnuanchan and Benjakul, 2014; Vergis et al., 2013).

Eugenol (4-allyl-2-methoxyphenol), is the main component of cinnamon essential oil (Ribeiro-Santos et al., 2017; Ribes et al., 2017). This naturally occurring phenol is present in essential oils of many plants and has proved to be active against many pathogenic bacteria, fungi and viruses (Devi et al., 2010; Ali et al., 2005). Several authors have reported its activity against pathogens including *Escherichia coli* O157:H7, *Listeria monocytogenes, Campylobacter jejuni, Salmonella enterica, Staphylococcus aureus, Lactobacillus sakei and Helicobacter pyroli*, acting primarily by disrupting the cytoplasmic membrane (Gil & Holley, 2006; Devi et al., 2010). Moreover, the hydrophobic nature of eugenol (EU) enables it to penetrate the

lipopolysaccharide of the Gram-negative bacterial cell membrane and alter the cell structure, which subsequently results in the leakage of intracellular constituents (Hemaiswarya & Doble, 2009).

However, the antimicrobial activity of EOs in general is limited, since these are unstable and fragile volatile compounds. EOs compounds can be easily degraded by oxidation, volatilization, heating or interaction with light, and should be protected if their efficiency is to be preserved (Asbahani et al., 2015). Moreover, and specifically in film formulation, the incorporation of essential oils as active compounds may imply large volatile losses during the incorporation/casting/drying process. Over the drying step, the oil droplets can be unstable and grow by coalescence until a fraction of the lipids separate off and rise to the upper surface of the film rendering it less effective for shelf life extension (Jiménez et al., 2010).

An interesting approach aiming to reduce this limitation would be to incorporate the essential oil or active compound within polymer microcapsules, which would then be included in the formulation of the active film. This way, EOs stability could be increased by encapsulation, hence prolonging the benefits of the active compounds through controlled release (Hong and Park, 1999; Asbahani et al., 2015). The microencapsulation method should generally be simple, reproducible, fast, effective and easy (Carvalho et al., 2016). Also, their choice depends on aspects such as physico-chemical properties of the encapsulated and encapsulating material, the release characteristics of the encapsulated compound, purpose and cost (Krishnan et al., 2005; Kanakdande et al., 2007).

Alginate has been widely used as microencapsulation material as it is non-toxic, biocompatible, cheap, biodegradable and compatible with many food systems (Léonard et al., 2013; Gombotz & Wee, 2012; Lee & Mooney, 2012). The encapsulation of many different oils for nutrition, therapeutics, and flavoring or aromas in alginate matrices has been reported (Abang et al., 2012; Sun-Waterhouse et al.,2011; Wang et al.,2013). The aim of the present work was to prepare alginate microcapsules containing eugenol as active ingredient, and evaluate the effect of their incorporation into chitosan films on the properties of the films. Additionally, the analysis of eugenol release kinetics and antimicrobial effectivity of the films were carried out.

2. MATERIALS AND METHODS

2.1 Materials

Sodium alginate (Batch MKBH8463Vz) with an M/G ratio ≈ 1.56, chitosan (Batch SLBC2867V, Batch MKBP1333V), acetic acid (Batch SZBF1810V) and eugenol (Batch MKBH9640V) were supplied by Sigma-Aldrich Chemie (Steinheim, Germany), as well as the UV grade solvents (methanol, ethanol, acetic acid and isooctane. Calcium chloride dehydrate (batch V1F064083H) was purchased from International MgbH (Darmstadt, Germany). Phosphorus pentoxide and magnesium nitrate-6-hydrate were supplied by Panreac Química SLU (Castellar del Vallés, Barcelona, Spain). Tryptone Soy Broth (TSB) and Tryptose Soya Agar (TSA) were supplied by Scharlab (Barcelona, Spain).

2.2 Preparation and characterization of the microspheres

A sodium alginate solution (2 % wt) was prepared in distilled water at room temperature by magnetic stirring for 24 h at 250 rpm. Separately, an eugenol emulsion (2 % wt. in water) was prepared by homogenization at 13500 rpm-4 minutes, using a rotor-stator homogenizer (model DL 25 Basic, IKA, Janke and Kunkel, Germany). This emulsion was sonicated at 40 kHz (Sonicator Vibra cell 75115, 500 W, Bioblock Scientific Co.) at 40 % of full power for 600 s (30s on /30s off). The ultrasound probe was placed in the center of the sample.

Alginate solution (2 % wt.) and eugenol sonicated emulsion (2 % wt.) were mixed in a 1:1 weight ratio to obtain 1 % wt blend of each ($Alg_{1\,\%}$-$Eu_{1\,\%}$), which was used for the preparation of the microcapsules containing eugenol (MEU). Additionally, sodium alginate solutions (1 % and 2 % wt.) and eugenol sonicated emulsions (1 % and 2 % wt.) were prepared as control samples. These were referred to as $Alg_{1\,\%}$, $Alg_{2\,\%}$, $Eu_{1\,\%}$ and $Eu_{2\,\%}$.

Alginate solutions, eugenol emulsions and the blend ($Alg_{1\,\%}Eu_{1\,\%}$) were characterized in triplicate as to their density, pH, ζ-potential and particle size. The density was measured using the pycnometer method, and the pH by means of a pH meter model GLP +21 (Crison Instruments SA, Hospital del Llobregat, Barcelona, Spain). The diameter (z-average) and ζ-potential of the emulsions were measured using a Zetasizer Nano-Z device (Nano series model Zen 2600, Malvern Instruments, Worcestershire, United Kingdom), which measures the electrophoretic mobility through light scattering caused by the dispersed particles, and the ζ-potential is therefore determined using the Smoluchowski approximation. Before measurements, the samples were diluted with distilled water (1:100). Three readings were made per sample and each measurement was repeated three times at 25°C.

Microspheres were made using the Encapsulator B-395 Pro (BÜCHI Labortechnik, Flawil, Switzerland). The vibration frequency was adjusted at 1800 Hz to generate 1800 droplets per second. The flow rate was 12 mL.min^{-1}. The nozzle was 150 μm diameter. Droplets fell in a 5 % wt. $CaCl_2$ solution to allow for microspheres (ME_U) formation. These were maintained in the gelling bath for 10 min under magnetic stirring (200 rpm) at room temperature, after which they were filtered and washed with distilled water. In order to obtain microcapsules loaded with chitosan, a solution of chitosan (0.2 % wt.) was prepared in a 0.1 % (v / w) acetic acid solution, and stirred at room temperature for 24 hours. Alginate-Eugenol microcapsules (M_{EU}) were incubated in the chitosan solution (1:1 mass ratio) under magnetic stirring, for either 1 or 10 minutes to obtain MEU/CH1 and M_{EU}/CH_{10}. Alginate microcapsules (M) without eugenol were used as control. All the samples were stored at 4°C prior to microcapsules characterization or film preparation.

2.3 Characterization of microspheres

The particle size analyses of the emulsions were carried out using a laser diffractometer (Mastersizer, 3000; Malvern Instruments, Worcestershire, UK) with ultrasound application, in ten replicates per formulation. Results were reported as the volume weighted mean globule size (Eq.1):

$$D\,[4,3] = \sum n_i d_i^4 / n_i d_i^3 \qquad \text{Eq.1}$$

Where n_i is the number of particles; d_i is the diameter of the particle (μm).

The mechanical properties of the alginate microcapsules were investigated according to Bekhit et al. (2016). A rotational rheometer Malvern Kinexus pro (Malvern Instruments, Orsay, France) with a plate-and-plate (20 mm) geometry was used. Briefly, the microspheres were filtered and gently whipped with a filter paper to remove the excess of water and finally re-dispersed in water for a final weight ratio of 1:1. Next, the mechanical properties of the microspheres dispersions were investigated by linear rheology. Firstly, an amplitude sweep was conducted at a frequency of 1 Hz by changing the shear strain from 0.01 % to 10 % in order to determine the linear viscoelastic region. On the basis of this test, a value of strain within the linear regime (0.1 %) was then used in the subsequent frequency sweep with the change of frequency between 0.1 and 10 Hz. All measurements were performed in triplicate at 25°C.

2.4 Preparation and characterization of the films

Chitosan films were obtained by casting as it has been previously described by Bonilla et al. (2014). Chitosan (1.0 % wt.) was dispersed in an aqueous solution of glacial acetic acid (1.0 % v/w) under magnetic stirring at 40°C. The alginate-eugenol microspheres (M_{EU}) were incorporated into the chitosan film-forming solution in a chitosan: microspheres mass ratio of 1:1.5. Films were obtained by casting the film-forming solutions at a constant surface density (5.856 mg of dry solids/ cm^2) to obtain CH/ M_{EU} and control films (CH). Films were stored at 25°C and 53 % relative humidity (RH) for one week. Film thickness was determined in six points of the films, using a Palmer digital micrometer (Comecta, Barcelona, Spain) to the nearest 0.005 mm.

2.4.1 Mechanical behavior, moisture content and barrier properties

The mechanical properties were analyzed by means of tensile tests (ASTM standard method D882, ASTM, 2001). A universal testing Machine (TA.XTplus model, Stable Micro Systems, Haslemere, England) was used. Equilibrated film strips (25 mm wide, 100 mm long) were mounted in the film-extension grips of the testing machine and stretched at 50 mm min^{-1} until breaking. The true stress (σ) *vs.* Hencky strain (ϵH) curves were registered, from which the mechanical parameters elastic modulus (EM), tensile strength at break (TS) and elongation percentage at break (%E) were obtained. At least eight replicates were carried out per formulation.

The moisture content of the films was determined using a gravimetric method. Firstly, the film samples were dried in a convection oven at 60°C for 24 h, and then in a vacuum oven (Vacioterm-T, JP-selecta S.A., Barcelona, Spain) at 60°C and 0.8 bar for 48 h. Finally, in order to remove the residual moisture, the samples were conditioned in a dessicator with P_2O_5 to constant weight. The results were expressed as g of water per 100 g of dry film.

Water vapor permeability (WVP) of the films was determined gravimetrically using the ASTM E96-95 (ASTM, 1995) gravimetric method, taking into account the modification proposed by Mc Hugh et al. (1993). Films were selected for WVP tests based on the lack of physical defects such as cracks, bubbles or pinholes. Distilled water was placed in Payne permeability cups (3.5 cm diameter, Elcometer SPRL, Hermelle /s Argenteau, Belgium) to expose the film to 100 % RH on one side. Once the films were secured, each cup was placed in a relative humidity equilibrated cabinet at 25°C, with a fan placed on the top of the cup to reduce resistance to water vapor transport. RH of the cabinets (53 %) was held constant using

oversaturated solutions of magnesium nitrate-6-hydrate. The cups were weighed every 1.5 h for 24 h with an analytical scale (ME36S Sartorius, Alemania - 0.0001 g) and water vapor transmission rate was determined from the slope obtained from the regression analysis of weight loss data versus time, once the steady state had been reached, divided by the film area. From this data, water vapor permeability values were obtained, taking into account the average film thickness in each case. The equation proposed by Mc Hugh et al. (1993) was used to correct the effect of concentration gradients established in the stagnant air gap inside the cup.

The oxygen permeability (OP) of the films was determined at 53 % RH and 25°C using an OX-TRAN (Model 2/21 ML Mocon Lippke, Neuwied, Germany). The samples were conditioned at the relative humidity level of the test in a desiccator using magnesium nitrate-6-hydrate saturated solutions. Two samples were placed in the equipment for analysis, and the transmission values were determined every 20 min until equilibrium was reached. The 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 in every case.

2.4.2 Optical properties

The internal transmittance of the films was determined by applying the Kubelka-Munk theory (Hutchings, 1999) for multiple scattering to the reflection spectra. The surface reflectance spectra of the films were determined from 400 to 700 nm with a spectrocolorimeter CM-5 (Konica Minolta Co., Tokyo, Japan) on both a white and a black background. All measurements were performed at least in triplicate for each sample on the free film surface during its drying. When the light passes through the film, it is partially absorbed and scattered, which is quantified by the absorption (K) and the scattering (S) coefficients. Internal transmittance (T_i) of the films was quantified using (Eq.2). In this Equation R_0 is the reflectance of the film on an ideal black background. Eq.(3) and (4) are used to calculate a and b parameters, respectively. R in equation 2 is the reflectance of the sample layer backed by a known reflectance (R_g).

$$T_i = \sqrt{(a - R_0)^2 - b^2} \qquad \text{Eq.2}$$

$$a = \frac{1}{2}(R + \frac{R_0 - R + R_g}{R_0 R_g}) \qquad \text{Eq.3}$$

$$b = \sqrt{a^2 - 1} \qquad \text{Eq.4}$$

Color coordinates of the films, L*, C^*_{ab} (Eq.(5)) and h^*_{ab} (Eq.(6)) from the CIELAB color space were determined, using D65 illuminant and 100 observer, and taking into account R_∞ (Eq.(7)) which corresponds with the reflectance of an infinitely thick layer of the material.

$$C^*_{ab} = \sqrt{a^{*2} + b^{*2}} \qquad \text{Eq.5}$$

$$h^*_{ab} = \arctan \frac{b^*}{a^*} \qquad \text{Eq.6}$$

$$R_\infty = a - b \qquad \text{Eq.7}$$

Moreover, the Whiteness index (WI) was calculated by using Eq.8.

$$WI = 100 - \left((100 - L)^* + a^{*2} + b^{*2}\right)^{0,5} \qquad \text{Eq.8}$$

2.4.3 Scanning Electron Microscopy (SEM)

Microstructural analysis of the films was carried out by SEM using a scanning electron microscope JEOL JSM-5410 (Japan). Film samples were maintained in a desiccator with P_2O_5 for one week to ensure desiccation. Sample films were frozen in liquid nitrogen and cryofractured to observe the cross-section microstructure. Surface images were also obtained. Films were fixed on copper stubs, gold coated, and observed using an accelerating voltage of 10 kV.

2.4.4 Thermogravimetric analysis (TGA)

The thermal stability of the films was analyzed using a thermogravimetric analyzer (TGA/SDTA 851e, Mettler Toledo, Schwerzenbach, Switzerland). Approximately 3 mg of preconditioned freeze dried sample were used in each test. The sample was heated from room temperature to 600°C, under nitrogen flow (50 mL/min), at 10°C/min. Two replicates per formulation were run.

2.4.5 Eugenol retention in the films and eugenol release kinetics in different simulants

The amount of eugenol in the CH/M_{EU} films after one week conditioning (53 % RH and 25°C) was determined by triplicate with a UV-visible spectrophotometer (Helios Zeta UVeVis, Thermo Fisher Scientific, United Kingdom). Film samples (100 mg) were cut, introduced into 100 ml of acetic acid 3 % (w/v) and stirred overnight. After 24h, the absorbance at 282 nm was measured and transformed into eugenol content extracted from the film (mg Eu/g dry film), using the corresponding calibration curve. The retention percentage of eugenol in the films

was calculated by referring the mass of eugenol extracted to the mass of eugenol initially incorporated (Eq.9).

Retention of eugenol = (Extracted Eu (g))/(Incorporated Eu (g)).100 Eq.9

Release kinetics of eugenol was evaluated in four simulants, namely ethanol 10 % (v/v), acetic acid 3 % (w/v), ethanol 50 % (v/v) and isooctane (Regulation 10/2011/EC). Film samples (100 mg) were weighed, placed in flasks with 100 mL of the corresponding simulant and kept under stirring at room temperature up to 10h. At different contact times, the absorbance of the solvent was measured at 282 nm, aiming to quantify the released eugenol. All analyses were performed in triplicate.

The results of eugenol release over time (M_t) were fitted to Peleg model, Korsmeyer-Peppas equation and Fick diffusional model.

Peleg's model (Peleg, 1988) was used to describe the release of eugenol in the simulants. The experimental values (M_t *vs.* t) were fitted to the model (Eq.10) in order to calculate the parameters k_1 (min/g) and k_2 (g^{-1})

$$\frac{t}{M_t - M_0} = k_1 + k_2.t \qquad \text{Eq.10}$$

Where:

M_t: mass of eugenol released into the simulant in a time t

M_0: initial mass of eugenol released into the simulant

k_1: model parameter, inversely related to the eugenol release rate

k_2: model parameter, inversely related to the asymptotic value of M_t (M_∞)

Therefore, this model allows for the prediction of the asymptotic value of M_t (M_∞) released in each simulant, with Eq.11:

$$M_\infty = M_0 + 1/k_2 \qquad \text{Eq.11}$$

Additionally, M_∞ was divided by the mass of eugenol initially incorporated in the film, hence obtaining the partition coefficient. Korsmeyer-Peppas model (Siepmann and Peppas, 2011; Hosseini et al 2013; Tavares et al., 2016) (Eq.12), is generally used to analyze the release of active compounds through a polymer matrix.

$$\frac{M_t}{M_\infty} = k.t^n \qquad \text{Eq.11}$$

Where:

$\frac{M_t}{M_\infty}$: mass of eugenol released at time t referred to the asymptotic release

k : kinetic constant of the model

n: release exponent, which indicates the mechanism associated with the release of the active principle (Tavares et al., 2016). The values of n indicate the release mechanism involved. If n is close to 0.5, the release takes place through Fickian diffusion, while lower values indicate quasi-Fickian diffusion (Siepmann and Peppas, 2011; Maderuelo et al., 2011). Higher values are related to non-Fickian release or anomalous transport (Paula et al., 2011).

The release process can also be described by the diffusional model. Fick's second law of diffusion in a plane sheet (Crank, 1975) was applied to model the diffusion process of eugenol in the CH films towards the food simulants. The diffusional long-time equation for an infinite plane sheet (Eq. 12) was used to determine the diffusion coefficient (D) of eugenol into the solvents, considering 8 terms of the equation.

$$\frac{M_t}{M_\infty} = 1 - \frac{8}{\pi^2}\sum_{n=0}^{\infty}\frac{1}{(2n+1)^2}exp\left(\frac{-D(2n+1)^2\pi^2 t}{L^2}\right) \qquad \text{Eq.12}$$

Where:

D: diffusion coefficient (m^2/s)

M_t : mass of eugenol released at time t (g)

M_∞: the mass of compound released at equilibrium (g)

L: half thickness of film (m)

2.5 Antimicrobial activity

The antimicrobial activity of the films (CH and CH/M_{EU}) was tested againts *Listeria innocua* (CECT 910, Gram positive) and *Escherichia coli* (CECT 101, Gram negative) bacteria, both supplied by the Spanish Type Culture Collection (CECT, Burjassot, Spain). The bacterial cultures, initially stored at -25°C, were regenerated by transferring a loopful into 10 mL of TSB and incubating at 37°C for 24 hours. From this culture, a 10 μL aliquot was again transferred into 10 mL of TSB, which was incubated for 24 h at the same temperature to obtain a culture in exponential phase of growth. This bacterial culture was appropriately diluted in TSB tubes to get a target inoculum of 10^5 CFU/mL, which was used both for liquid medium and solid medium tests.

Antimicrobial tests in liquid medium were performed using tubes of TSB (9.9 mL) where 100 μL of the inoculum were added. UV treated film samples (55 mm diameter) were introduced in the test tubes. Over storage at 10°C up to 13 days, microbial counts were performed. To this aim, serial dilutions were made and poured onto TSA dishes which were incubated for 24 h at 37°C. Inoculated tubes without film sample were used as control.

Inoculated TSA plates (100 μL inoculum per plate) were used for the solid medium tests. UV treated film samples (55 mm diameter) were placed on the plates and stored at 10°C up to 13 days. Microbial counts were performed making serial dilutions onto TSA dishes, which were incubated for 24 h at 37°C. Inoculated plates without film sample were used as control.

2.6 Statistical analysis

The statistical analysis of the data was performed through analysis of variance (ANOVA) using Statgraphics Centurion XVI.II. Fisher's least significant difference (LSD) procedure was used.

3. RESULTS AND DISCUSSION

3.1 Physicochemical properties of alginate solution, eugenol emulsions and microspheres

The density, pH, ζ-potential and z-average size values of alginate solutions and eugenol emulsions are shown in **Table 5.1**. All samples exhibited negative ζ-potential values, especially $Alg_{1\%}$-$Eu_{1\%}$, and thus the electrostatic interaction with the positively charged chitosan solution to develop the coated microsphere will be promoted.

Table 5.1 Density, pH and ζ-potential of the dispersions an emulsion used to prepare the microcapsules. Mean values and standard deviation.

Samples	ρ (kg/m3)	pH	ζ-Potential (mV)	Z-average (nm)
$Alg_{1\%}$	$1004 \pm 2^{(b)}$	$7.01 \pm 0.03^{(d)}$	$-65.3 \pm 1.2^{(c)}$	-
$Alg_{2\%}$	$1009 \pm 3^{(d)}$	$6.98 \pm 0.04^{(d)}$	$-72.6 \pm 1.1^{(a)}$	-
$Eu_{1\%}$	$97 \pm 4^{(a)}$	$6.03 \pm 0.04^{(a)}$	$-25.7 \pm 0.6^{(e)}$	$333.1 \pm 0.6^{(a)}$
$Eu_{2\%}$	$998 \pm 3^{(a)}$	$6.32 \pm 0.03^{(b)}$	$-53.8 \pm 0.9^{(d)}$	$744.4 \pm 1.1^{(b)}$
$Alg_{1\%}$-$Eu_{1\%}$	$1006 \pm 2^{(c)}$	$6.74 \pm 0.03^{(c)}$	$-69.8 \pm 1.3^{(b)}$	$842.1 \pm 0.9^{(c)}$

Different superscript letters (a,b,c,..) within the same column indicate significant differences among formulations ($p < 0.05$).

All microspheres showed unimodal size distributions. The presence of eugenol in the encapsulating material, and the addition of the chitosan coating promoted an increase in

microcapsules size. The increase in particle size of microspheres by the addition of essential oil, has been reported in several studies (Banerjee et al., 2013; Hosseini et al., 2013; Lertsutthiwong et al., 2008; Dima et al. 2016). The broadest particle size distribution was reported for chitosan coated microspheres that were submitted to 10 minutes of incubation (M_{EU}/CH_{10}).

Figure 5.1 shows the particle size distribution of microspheres (M), microcapsules carrying eugenol (M_{EU}) and microcapsules coated with chitosan incubated for 1 or 10 minutes (M_{EU}/CH_1 and M_{EU}/CH_{10}, respectively).

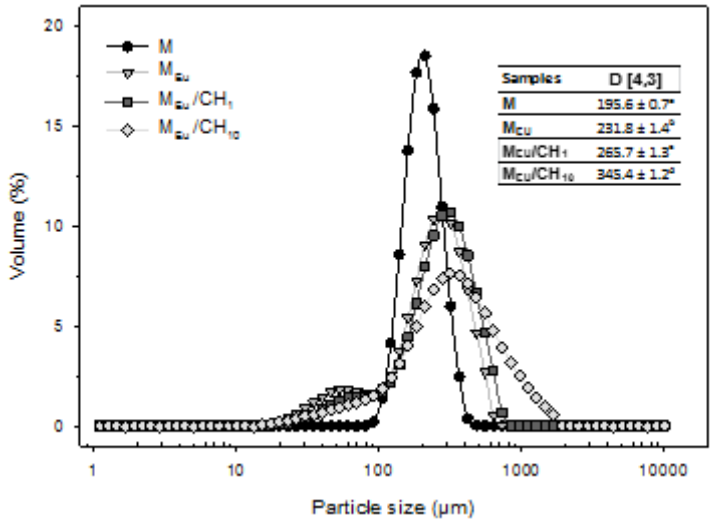

Figure 5.1. Typical particle size distribution curves of the microspheres.

3.2 Mechanical behavior of the microcapsules

The elastic (G') and viscous (G") moduli of M, M_{10} and M_{10} coated with chitosan during 1 and 10 minutes were investigated by dynamic mechanical analyses. Rheological frequency sweep tests were performed on the microcapsule dispersions at a constant s of 0.1 %, which was confirmed to be in the linear viscoelastic range for all microsphere dispersions as shown in the strain sweep results (**Figure 5.2a**).

As shown in **Figure 5.2b**, the G' vales were higher than G", confirming that the microsphere dispersions have essentially an elastic rather than a viscous character. This characteristic indicates that the deformation energy is recovered in the elastic stretching of chemical bonds (Stendahl, et al., 2006).

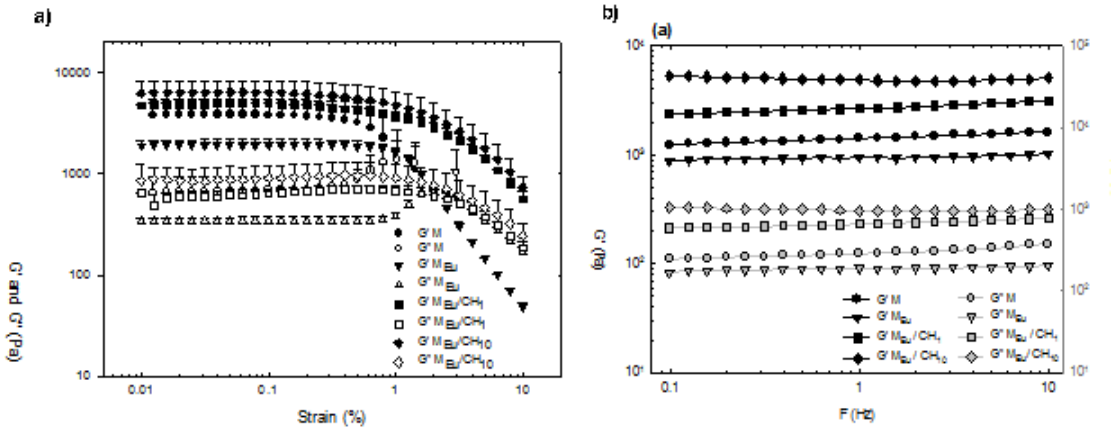

Figure 5.2. a) Strain sweep dependence of the storage moduli (G') and loss moduli (G") of the microspheres dispersions at 1 Hz and b) Frequency dependence of the storage moduli (G') and loss moduli (G").

All mechanical spectra show similar mechanical profile and a no frequency dependence behavior of G' and G", which indicates that the microsphere dispersions at the studied volume fraction exhibited a good mechanical stability. The coated microspheres showed the highest G' and G" values followed by simple the alginate microsphere dispersion (M).

Coating alginate microspheres with a chitosan layer via electrostatic interactions between the free alginate carboxylic groups and the amino groups of chitosan enhanced the mechanical stability of the microsphere dispersions. Indeed, the M/CH_{10} systems showed the highest G' value, followed by the system coated for 1 min (M/CH1), with no significant differences between them. From the mechanical data, it can be assumed that 1 min was enough to trigger the electrostatic interaction between the oppositely charged polysaccharides.

3.3 Mechanical and barrier properties of films

The tensile properties, elastic modulus (EM), tensile strength at fracture (TS) and percentage of elongation at break (%E), are shown in **Table 5.2**, together with thickness, water content and barrier properties.

Table 5.2 Elastic modulus (EM), tensile strength (TS), elongation at break (%E), water vapor permeability (WVP), oxygen permeability (OP) and moisture content. Mean values and standard deviation.

Film	Thickness	EM (MPa)	TS (MPa)	% E	WVP ($g.mm.kPa^{-1}.h^{-1}m^{-2}$)	OP ($cm^3.mm.m^{-2}.atm^{-1}day^{-1}$)	Xw (g-water/100 g dry film)
CH	$65.0 \pm 0.4^{(a)}$	$1668 \pm 29^{(a)}$	$52 \pm 3^{(a)}$	$6.0 \pm 1.6^{(a)}$	$4.42 \pm 0.18^{(a)}$	$0.54 \pm 0.08^{(a)}$	$14.1 \pm 1.1^{(a)}$
CH/M_{EU}	$125.0 \pm 0.6^{(b)}$	$927 \pm 17^{(b)}$	$27 \pm 2^{(b)}$	$11.0 \pm 2^{(b)}$	$8.79 \pm 1.09^{(b)}$	$0.35 \pm 0.07^{(b)}$	$16.3 \pm 1.2^{(b)}$

Different superscript letters (a,b) within the same column indicate significant differences among formulations ($p < 0.05$).

The obtained values for mechanical properties for pure CH films were similar to those reported by Bonilla et al. (2012). The incorporation of microcapsules caused a significant decrease in EM and TS ($p<0.05$), along with a significant increase ($p<0.05$) in %E, hence yielding more extensible films with lower rigidity and resistance to fracture. Similar results have been reported by Woranuch & Yoksan (2013) in films loaded with eugenol-chitosan nanoparticles with application for active packaging.

The addition of microcapsules significantly increased the WVP of the films ($p<0.05$), which can be explained by the change in the molecular organization of the film promoted by microcapsules addition that in turn promotes a less compact and more permeable structure with a higher moisture content. The incorporation of microcapsules into films implied a slight decrease in the oxygen permeability values. This can be explained by the decrease in oxygen permeability due to the change in molecular orientation of the polymeric chains (Salame & Steingiser, 1977), and the solubilization and diffusion of eugenol through the film surface (Woranuch & Yoksan, 2013).

3.4 Optical properties

Table 5.3 lightness, chroma, hue, whiteness index and internal transmittance at 450 nm of the films. The incorporation of the microcapsules slightly affected the lightness and chroma. However, hue, whiteness index and internal transmittance were not significant affected by the addition of microspheres.

Table 5.3 Lightness (L*), chroma (C^*_{ab}), hue (h^*_{ab}), whiteness index (WI) and internal transmittance (T_i) at 450 nm. Mean values and standard deviation.

Film	L*	C^*_{ab}	h^*_{ab}	WI	T_i (450 nm)
CH	$82.3 \pm 0.9^{(a)}$	$21.3 \pm 0.7^{(a)}$	$89.1 \pm 0.8^{(a)}$	$72.5 \pm 0.4^{(a)}$	$0.84 \pm 0.03^{(a)}$
CH/M_{EU}	$77.0 \pm 0.7^{(b)}$	$18.1 \pm 0.3^{(b)}$	$92.2 \pm 0.2^{(a)}$	$68.4 \pm 0.6^{(a)}$	$0.81 \pm 0.05^{(a)}$

Different superscript letters (a,b) within the same column indicate significant differences among formulations ($p < 0.05$).

3.5 Microstructure

The final microstructure of the film depends on the interactions of its components, and the drying conditions of the film-forming dispersions and has a great impact on the properties of the films (Mali et al., 2002). Also, the microstructural analysis provides relevant information about the organization of the components and helps to understand the values obtained for the barrier, mechanical and optical properties of the films. **Figure 5.3** show the surface and cross sections of the films, obtained by SEM. In general, pure CH film showed a homogeneous microstructure with a compact and ordered continuous matrix. When microcapsules are incorporated to the CH matrix, film surface microstructure exhibits globular forms as can be appreciated in their surface whereas the sections of CH/M_{EU} films exhibited holes corresponding to the incorporated microcapsules, which can be seen in globular forms.

3.6 Thermogravimetric analysis (TGA)

Figure 5.4 shows the obtained thermograms for the different films (CH and CH/M_{EU}). In general, two significant weight loss steps were observed in TGA curve of both films. The weight loss between 40-120°C is probably due to the vaporization of water and the residual acetic acid. The second weight loss occurred between 170-300°C may be due to the thermal degradation of CH, as previously reported by several authors (Tripathi et al., 2009; Bonilla et al., 2013) and the degradation of carboxylic groups present in the sodium alginate microcapsule structure in the case of CH/M_{EU}. Kulig et al. (2016) reported that the degradation of carboxylic groups of sodium alginate appeared at a temperature range between 178-190°C. In this temperature range, the degradation of the active compound (eugenol) can also occur, since the thermal degradation of pure eugenol has been reported in the range of 80-270°C (Choi et al., 2009).

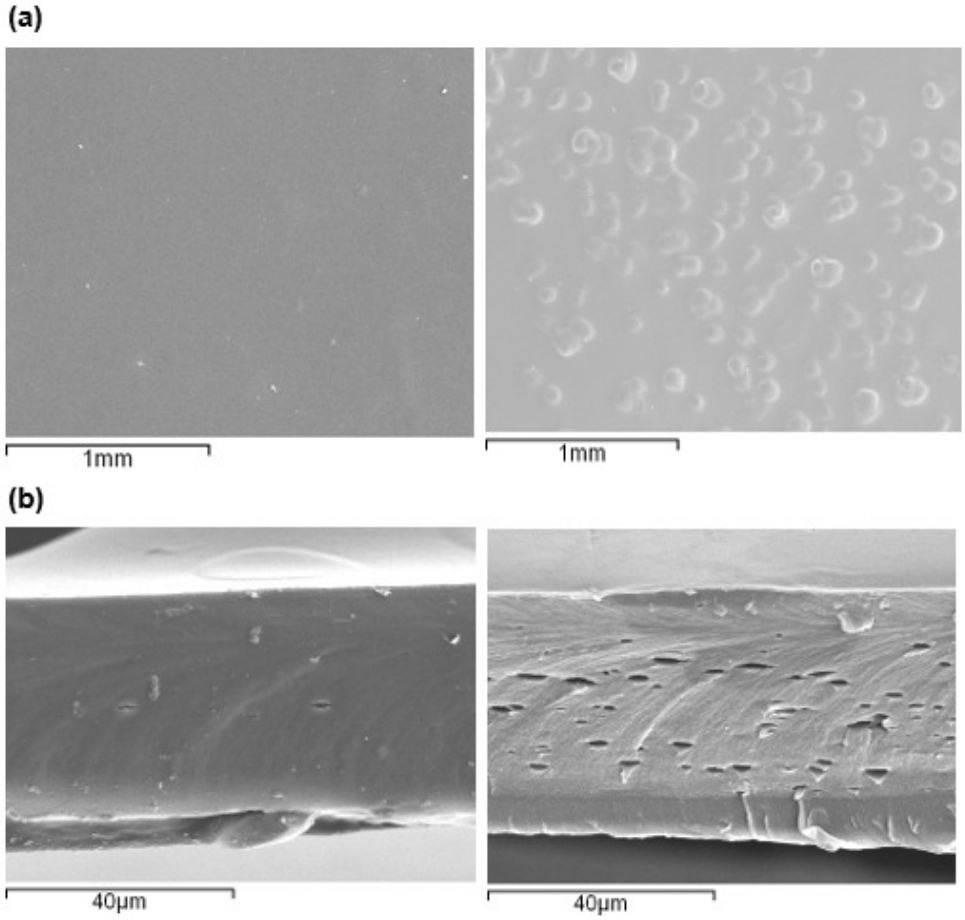

Figure 5.3. SEM micrographs of the (a) surface and (b) cross-section of chitosan film (left) and chitosan film with alginate-eugenol microcapsules (CH/M_{EU}) (right).

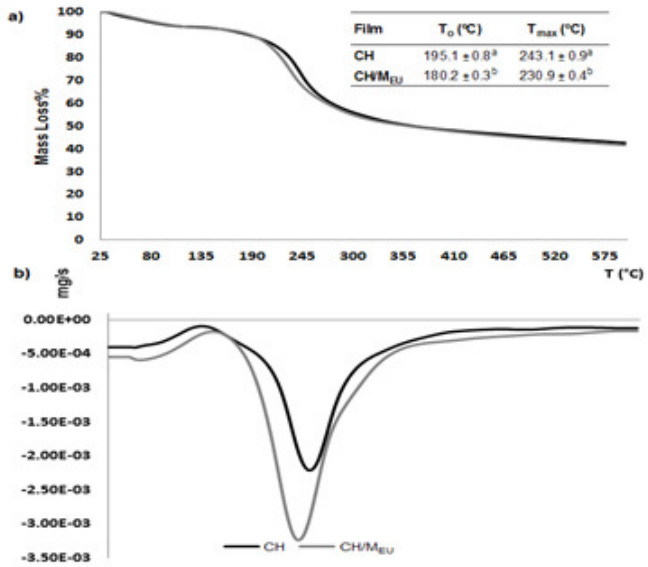

Figure 5.4. a) Typical thermogravimetric curves (mass loss *vs.* temperature) and b) first derivative (mg/s *vs.* temperature) for CH and CH/M_{EU} films.

3.7 Kinetics of release of eugenol

The mass of eugenol released at a time t referred to the mass of eugenol released in the equilibrium (M_t / M_∞) was plotted *vs.* time for the four simulants (**Figure 5.5**).

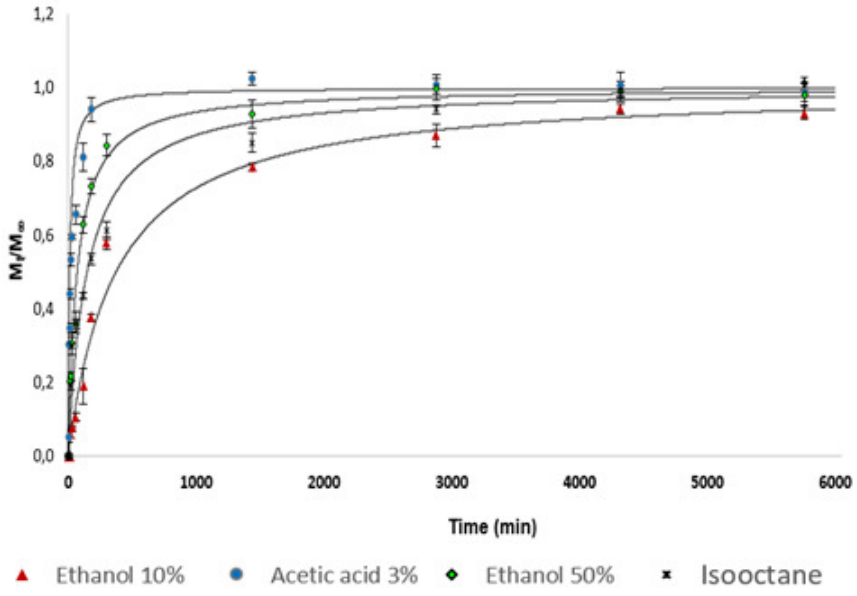

Figure 5.5. Eugenol release curves from chitosan films containing encapsulated eugenol-alginate microcapsules in the food simulants: ethanol 10 % (v / v), ethanol 50 % (v / v), acetic acid 3 % (w / v) and Isooctane. Experimental data (symbols) and Fitted Peleg's (continuous lines).

The fastest release of eugenol was observed in 3 % acetic acid, where a sudden increase and maintenance in the amount of eugenol released, close to the asymptotic value was observed. Acetic acid 3 % promoted the hydration and swelling of both the CH polymer matrix and the alginate microcapsules, thus resulting in the promotion of the active compound release. The slowest delivery rate occurred in ethanol 10 %.

Table 5.4 shows the values of Peleg's model parameters. A good model fitting was achieved in all cases (R^2 >0.99). As commented on above, the parameters of the Peleg's model showed a faster release of eugenol from the film in acid acetic 3 % (lower values of k_1) due to the greater accessibility of eugenol to the solvent, coherently with the obtained ratios (M_∞/M) for each simulant.

Release data were also fitted to Korsmeyer-Peppas model for (driving force, 1-M_t/M_∞, lower than 5) and the results are shown in **Table 5.5**, together with diffusivity predicted by Fick's model.

Table 5.4 Parameters of Peleg's model: amount of eugenol released from CH_{EU} at equilibrium in the simulant (M_∞) and its release rate ($1/k_1$), and maximum release ratio (M_∞/M_0): mass of active released at equilibrium in the simulant related to the initial mass of the active in the film (expressed with respect to the theoretical incorporated amount (^{1}) and with respect to the amount determined by acetic acid extraction (^{2}).

Simulant	$1/k_1$ (mg/min)	$M_\infty = 1/k_2$ (mg/100 g film)	M_∞/M_0^1	M_∞/M_0^2	R^2
Ethanol 10 %	$0.0036 \pm 0.0002^{(a)}$	$1.35 \pm 0.03^{(b)}$	$0.74 \pm 0.03^{(b)}$	$0.61 \pm 0.03^{(b)}$	>0.997
Á. Acético 3 %	$0.1743 \pm 0.0003^{(d)}$	$1.82 \pm 0.05^{(d)}$	$1.00 \pm 0.03^{(d)}$	$0.75 \pm 0.02^{(d)}$	>0.999
Ethanol 50 %	$0.0222 \pm 0.0004^{(c)}$	$1.57 \pm 0.05^{(c)}$	$0.86 \pm 0.03^{(c)}$	$0.65 \pm 0.03^{(c)}$	>0.999
Isooctane	$0.0044 \pm 0.0002^{(b)}$	$0.64 \pm 0.02^{(a)}$	$0.35 \pm 0.02^{(a)}$	$0.27 \pm 0.01^{(a)}$	>0.998

*M_0 = 12.9 ± 0.4 mg EU/g dry film (retention is 60.2 ± 0.7 %).

Different superscript letters (a, b,c,d) within the same column indicate significant differences among formulations ($p < 0.05$).

According to Korsmeyer-Peppas model, a value of n equal to 0.5 means that the release is related to a Fickian diffusion mechanism of first order; on the contrary, when n is equal to 1, polymer relaxation or degradation occurs, leading to zero-order release. An anomalous Fickian diffusion occurs when n values are between 0.5 and 1 (Mandal, Mann and Kundu, 2009). As shown in **Table 5.5**, n values indicated Fickian behavior when the release occurred in ethanol 50 %. The low value of n reported in acetic acid can be explained by the partial film solution in these solvents, which contributes to a non-Fickian eugenol release.

Table 5.5 Diffusion coefficient (D) and parameters of the Korsmeyer-Peppas model (rate constant (k) and diffusional exponent (n)).

Film	Simulant	D x 10^{13} (m^2/s)	R^2	n	k	R^2
CH/M_{EU}	Ethanol 10 %	$0.170 \pm 0.002^{(a)}$	0.992	$0.867 \pm 0.004^{(d)}$	$0.134 \pm 0.006^{(a)}$	>0.981
	Acetic acid 3 %	$1.147 \pm 0.038^{(d)}$	0.996	$0.274 \pm 0.004^{(a)}$	$0.682 \pm 0.006^{(d)}$	>0.974
	Ethanol 50 %	$0.370 \pm 0.010^{(c)}$	0.996	$0.503 \pm 0.010^{(c)}$	$0.402 \pm 0.011^{(c)}$	>0.975
	Isooctane	$0.253 \pm 0.012^{(b)}$	0.986	$0.391 \pm 0.008^{(b)}$	$0.341 \pm 0.005^{(b)}$	>0.942

Different superscript letters (a, b) within the same column indicate significant differences among formulations ($p < 0.05$).

3.8 Antimicrobial activity

The effect of different (CH and CH/M_{EU}) films on the growth and survival of *E. coli* and *L. innocua* microorganisms at 10°C is shown in **Figure 5.6**.

In the liquid medium, the application of CH and CH/M films showed a higher antibacterial effect for *E. coli* through practically the whole storage period, as microbial counts were significantly reduced. This can be explained by the reported more significant effect of CH against Gram-negative bacteria like *E. coli* (Cárdenas et al., 2008., Goy et al., 2016). The incorporation of microspheres (containing the active compound) into the CH films had a significant effect in the growth of both *L. innocua* and *E. coli*, as compared to non-coated samples (control) and sample coated with pure CH films. This can be explained by the combined effect of low temperature and eugenol release. Some authors reported that eugenol disrupts the bacteria cytoplasmic membrane nd increases its non-specific permeability (Bennis et al., 2004; Gill & Holley, 2006; Hemaiswarya & Doble, M, 2009; Nazzaro et al., 2013).

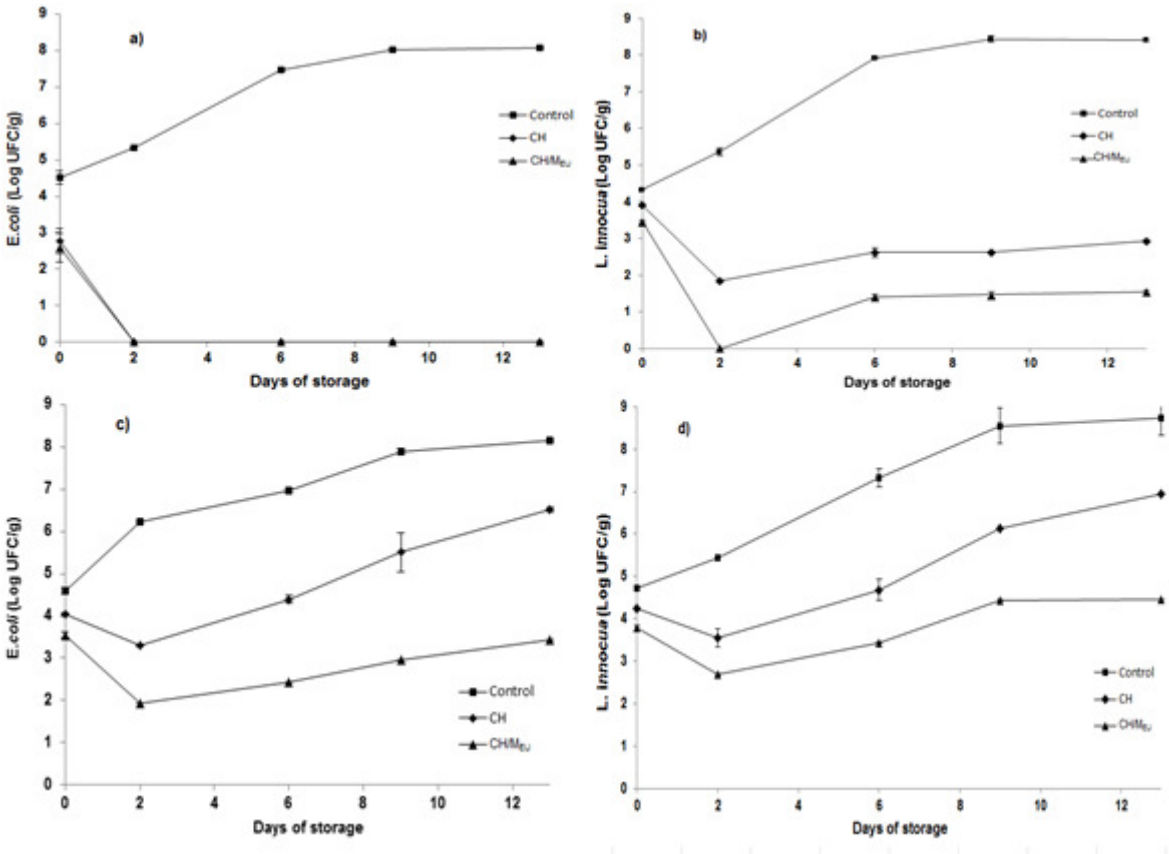

Figure 5.6. Antimicrobial activity of the different films against *L. Innocua* and *E.Coli* at 10°C. Figures a) and b): assays in liquid medium. Figures c) and d): solid media assays.

4. CONCLUSION

The addition of alginate microspheres containing eugenol into CH films increased the stretchability of the films at the same time that the oxygen barrier properties were improved. Microcapsules were visible in the structure of the film and their release rate in food simulants was enhanced when 3 % acetic acid was used, but it fell markedly in ethanol 10 %. Incorporating antimicrobial agents such as eugenol encapsulated into chitosan film enhanced the antimicrobial efficacy of chitosan, as the active ingredients diffused to hydrophilic food systems.

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IV. GENERAL DISCUSSION

There is a considerably growing interest on the development of biodegradable packaging materials obtained from renewable and natural sources, such as starch and chitosan. These materials could eventually replace conventional plastic polymers partially or entirely, since they are low cost, renewable and environmentally friendly. However, the use of starch biodegradable materials is often limited by its high hydrophilicity and its poor mechanical properties when compared with conventional materials. Different strategies (use of plasticizers or blending with other polymers such as chitosan) aim to improve these inconveniences, thus enabling a wide range of materials with diverse properties and a broad range of applications.

Research in food packaging have also focused on the incorporation of essential oils aiming to extend food products shelf-life. However, their use is limited by their strong flavor, low water solubility, high volatility and proneness to oxidation. Encapsulation techniques could help improving the stability of essential oils, allowing for higher efficacy and controlled release into the food matrix, which is why these new techniques are gaining global interest across the food industry and research community.

However, more studies are required to develop practical applications of starch or chitosan based films for food preservation. Research should be also focused on the selection of specific active compounds to target pathogenic microorganisms and products, subsequently studying the effect of these compounds on the film functional properties, release kinetics (into food simulants and specific foods), and antimicrobial studies (both in vitro and in vivo).

The following figures summarize the main physical properties of all films obtained and studied in this doctoral thesis, allowing for a straightforward comparison of the results and the analysis of the composition's main effects on the out coming parameters. For the purpose of comparison, the films obtained in the different chapters will be named as different chapter, as described in **Table IV.1**.

Table IV.1: compilation of all the films obtained in this doctoral thesis, organized by chapter.

Chapter	Description	Relative mass proportion (dry basis)								Notation
		CS	CH	P	OEO	CLEO	Eu	Lec	Alg	
1	Cassava starch (CS)-chitosan (CH) blend films obtained by compression molding, with varying proportion of plasticizer (P)	0.7	0	0.3	-	-	-	-	-	$(CS_{100}\text{-}CH_0)_{70}\text{-}P_{30}$
		0.63	0.07	0.3	-	-	-	-	-	$(CS_{90}\text{-}CH_{10})_{70}\text{-}P_{30}$
		0.56	0.14	0.3	-	-	-	-	-	$(CS_{80}\text{-}CH_{20})_{70}\text{-}P_{30}$
		0.49	0.21	0.3	-	-	-	-	-	$(CS_{70}\text{-}CH_{30})_{70}\text{-}P_{30}$
		0.6	0	0.4	-	-	-	-	-	$(CS_{100}\text{-}CH_0)_{60}\text{-}P_{40}$
		0.54	0.06	0.4	-	-	-	-	-	$(CS_{90}\text{-}CH_{10})_{60}\text{-}P_{40}$
		0.48	0.12	0.4	-	-	-	-	-	$(CS_{80}\text{-}CH_{20})_{60}\text{-}P_{40}$
		0.42	0.18	0.4	-	-	-	-	-	$(CS_{70}\text{-}CH_{30})_{60}\text{-}P_{40}$
2	Monolayer and bilayer films where the chitosan (CH) layer was obtained by casting, and the cassava starch (CS) layer was obtained by compression molding. Oregano essential oil (OEO) or cinnamon leaf essential oil (CLEO) were incorporated in the CH layer	1	-	0.3	-	-	-	-	-	CS (monolayer)
		-	1	0	-	-	-	-	-	CH (monolayer)
		-	1	-	0.25	-	-	-	-	CH:OEO (monolayer)
		-	1	-	-	0.25	-	-	-	CH:CLEO (monolayer)
		1	0.33	0.3	-	-	-	-	-	CS:CH (bilayer)
		1	0.33	0.3	0.08	-	-	-	-	CS:CH:OEO (bilayer)
		1	0.33	0.3	-	0.08	-	-	-	CS:CH:CLEO (bilayer)
3/4	Chitosan (CH) films with cinnamon leaf essential oil (CLEO) or eugenol (Eu), either incorporated in free form or encapsulated in lecithin (Lec) liposomes, obtained by casting	-	0.9	-	-	-	0.5	-	-	CH/Eu
		-	0.9	-	-	0.5	-	-	-	CH/CLEO
		-	0.9	-	-	-	-	0.5	-	CH/Lec
		-	0.9	-	-	-	0.5	0.5	-	CH/Lec-Eu
		-	0.9	-	-	0.5	-	0.5	-	CH/Lec-CLEO
5	Chitosan (CH) films with eugenol (Eu) encapsulated in alginate microcapsules (M), obtained by casting	-	1	-	-	-	0.0225	-	0.0225	CH/M_{EU}

Mechanical properties

Figure IV.1 shows the correlation between EM (Elastic Modulus, MPa) and TS (Tensile Strength, MPa) of all the films.

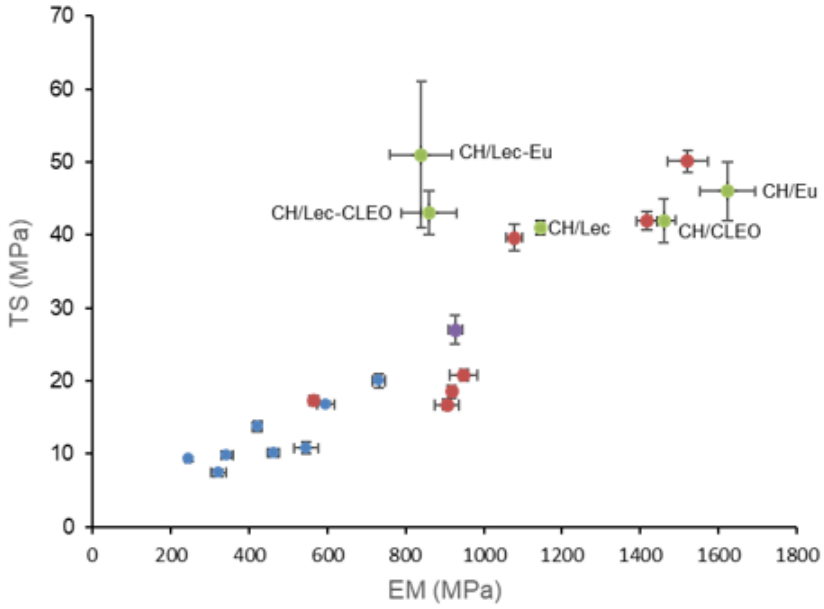

Figure IV.1 Mechanical properties (Tensile Strength *vs.* Elastic Modulus) of the films (● chapter 1, ● chapter 2, ● chapter 3/4, ● chapter 5).

As these two parameters are plotted, a strong correlation between the two is noticeable. The least rigid and resistant films were those obtained in the first study (chapter 1), based on CS-CH blends, with high plasticizer content and obtained by compression molding. The addition of chitosan and plasticizers into cassava starch films reduced the intermolecular interaction between polymeric chains of starch and chitosan, when were obtained by melting and compression molding, thus resulting in materials with low rigidity and poor tensile strength. Films with improved mechanical properties were obtained in the following studies, when films with lower plasticizer proportion, or formulated without plasticizer, were obtained by casting. That was the case of chapter 3, where the incorporation of Lec and Eu or CLEO resulted in a reduced plasticizing effect, and the films showed increased rigidity and resistance. The formulations with Eu and CLEO encapsulated into lecithin liposomes escaped from the general trend observed, showing higher TS. Probably, the specific interactions between the CH, the lecithin and the essential oils in the complex structure of these films contributed to their high tensile strength.

In chapter 2, a two-fold behavior can be observed: bilayer and CS films showed lower EM and TS than monolayer CH films, which is most likely attributable to the plasticizer incorporated in the CS layer (1:0.3 polymer:plasticizer mass ratio). Monolayer CH films with or without essential oils (study 2) showed improved mechanical properties, with high rigidity and resistance. The absence of plasticizer in these formulations is most likely responsible for these results. In addition, the incorporation of essential oils into CH films (chapter 2) provoked a light decrease of EM and TS, an effect attributable to the breakup of film network caused by the addition of essential oils. **Figure IV.2** shows the dispersion graph for percentage elongation (%E) *versus* EM.

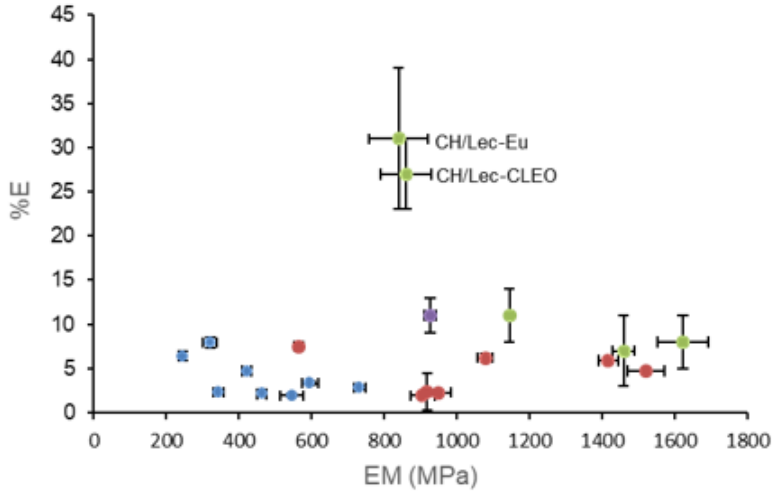

Figure IV.2 Mechanical properties (stretchability *vs.* Elastic Modulus) of the films (● chapter 1, ● chapter 2, ● chapter 3/4, ● chapter 5).

All the films obtained had reduced stretchability (less than about 10 %), except for two formulations of chapter 3, namely CH/Lec-Eu and CH/Lec-CLEO, both containing active compounds encapsulated into lecithin liposomes. Apparently, the incorporation of essential oils in encapsulated form contributed to the film structure with the formation of matrix discontinuities. The interactions between them, lecithin and chitosan affected the mechanical behavior of the films, significantly increasing the stretchability of the structure.

Water barrier properties

Figure IV.3 shows the water vapor permeability (WVP) values of all the films, aiming for a comparison between the chapter.

As **Figure IV.3** shows, the water vapor permeability of films in chapter 1 was significantly higher than that of the rest. This is probably attributable to the high plasticizer proportion of the formulations in this study, where polymer:plasticizer mass ratios were as high as 1:0.43 and 1:0.67, aiming for the obtention of films by compression molding. Surely, the incorporation

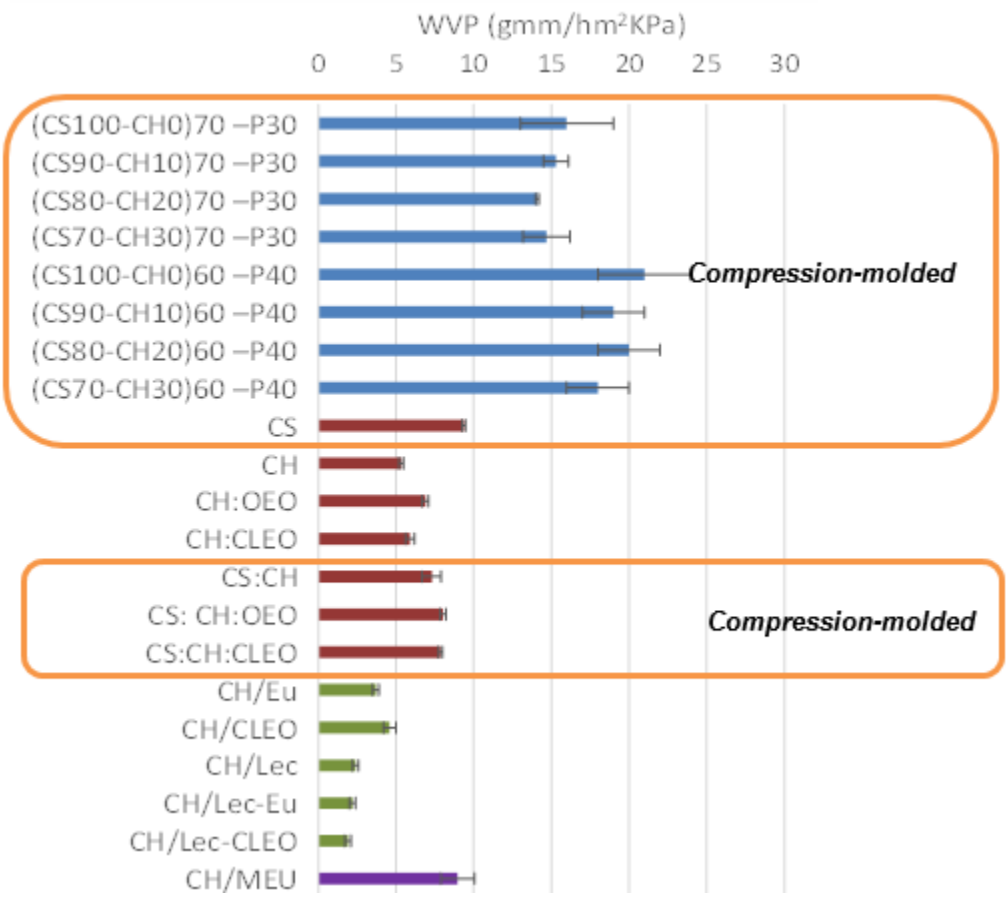

Figure IV.3 Values of the water vapour permeability (WVP) of the films.

of a high proportion of plasticizers in to the CS- CH blends, contributed to the high water permeability of the films, which also became higher as plasticizer concentration increased. The hygroscopic character of the plasticizers leading to an increase the hydrophilic group availability and in free volume of the system, increasing the water vapour transmission rate through the films.

On the other hand, in chapter 2, only CS layer had plasticizer (1:0.3 polymer:plasticizer mass ratio), and no plasticizer was needed for fllm obtention in the rest of the studies. This resulted in an improvement of the water barrier properties of the films in the remaining chapter, all of which were obtained by casting.

As compared with monolayer CH formulation in chapter 2, the incorporation of Eu or CLEO and more so the addition of nanoliposomes containing these essential oils into the CH matrix (chapter 3 and 4) provoked a significant decrease of WVP. The hydrophobic nature of essential oils could affect the hydrophilic or hydrophobic property of the film, giving as result a decrease of WVP. Moreover, the hydrogen and covalent interactions between the chitosan network and essential oils limit the availability of hydrogen groups to form hydrophilic bonding with water, subsequently leading to a decrease in the affinity of chitosan film to water and decreasing

WVP. The additional WVP reduction by nanoliposome incorporation is probably due to the interaction of the polar-head groups located in the outer membrane of nanoliposomes with the chitosan matrix, resulting in an even dispersion of nanoliposomes in the film, where the water diffusion rate was reduced.

Figure IV.4 shows the strong inverse correlation found between the stiffness/strength of the films and their water permeability. As commented, the most to water vapor permeable films were those obtained by blending of CS and CH (chapter 1), especially at the highest plasticizer content. These films also showed the lowest rigidity and resistance. As the chitosan proportion in the films was increased, and the plasticizer proportion was reduced or eliminated, the films became more stiff and resistant, as well as a better barrier to water vapor, which was additionally improved by the presence of fatty materials (lecithin, eugenol and CLEO).

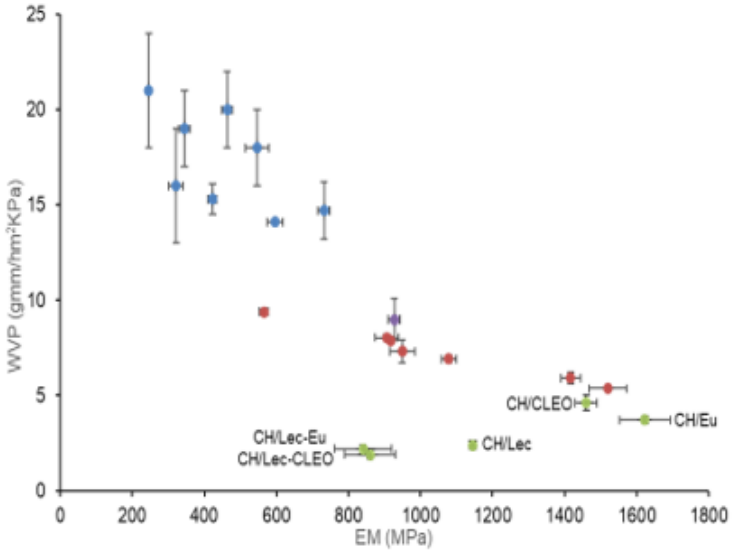

Figure IV. 4 Water Vapor Permeability *vs.* Elastic Modulus of the films (● chapter 1, ● chapter 2, ● chapter 3/4, ● chapter 4).

Optical properties

Figure IV.5 shows the chromatic parameters (hue vs. chroma) for the different film formulations.

The hue of all films ranged between 60 and 110, corresponding to yellowish color. The most reddish hue was found for CH/Eu and CH/CLEO (chapter 3), whose color modification can be attributed to the active agents incorporated. The films with the lowest chrome were those of CS with no CH. As CH was incorporated in the formulations, and as the active compounds were added, the color saturation was increased.

Essential oils and particularly the incorporation of liposomes reduced provoked small changes in the color parameters and whiteness index. On the one hand, a displacement of the hue to

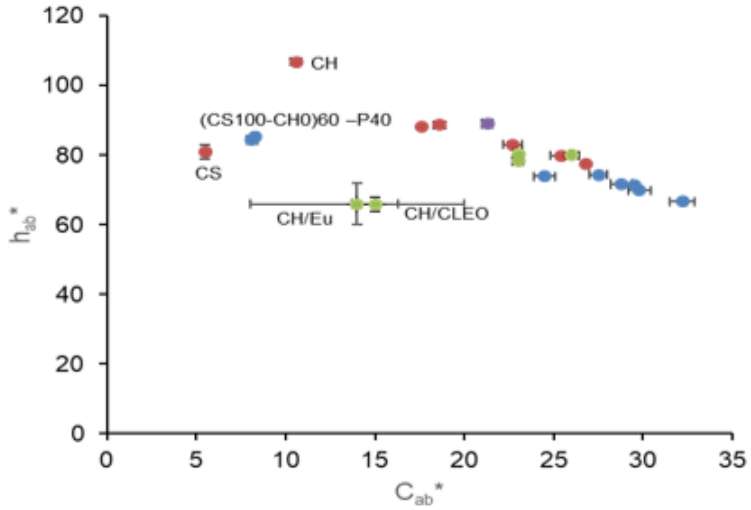

Figure IV.5: Hue *versus* chroma of the films (● chapter 1, ● chapter 2, ● chapter 3/4, ● chapter 5).

more yellow color and an increase in color saturation was observed when essential oils in free form were present in the chitosan films. On the other hand, films with liposomes were darker, with a more saturated reddish color than the pure chitosan films. This effect can be due to the presence of a dispersed phase in the CH film matrix, which promoted light dispersion through the film and at the film surface, thus affecting transparency and gloss, respectively.

On the other hand, not only did the essential oils incorporation affect the film optical properties, but also the film processing method. In this sense, thermo-compression in bilayers, greatly reduced the gloss of the CH face and films were less transparent than monolayers, especially when incorporated essential oils. As previously commented on, this effect can be due the thermal treatment and the formation of conjugated Schiff's bases, which are intermediate products of the Maillard reaction between amino groups of CH and the present carbonyl groups in the starch fraction, leading to a decrease in transparency.

Thermal properties

Figure IV.6 shows the correlation between T_{max} and T_0, obtained from thermogravimetric analyses of all the films.

There was a very sharp correlation between the T_0 and the T_{max} obtained from the thermogravimetric analyses. The highest thermal resistance was found for the films in chapter 1, obtained by compression molding. These were closely followed by bilayers and CS monolayer in chapter 2, where the same process was applied. The CH casted monolayers in chapter 2 were less thermally resistant. Finally, the films obtained in chapter 3 and 4 showed the weakest resistance to heat.

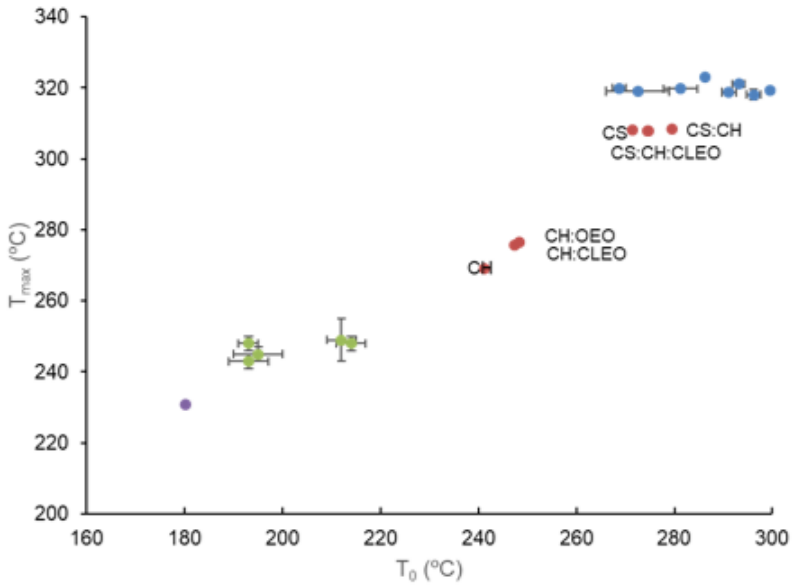

Figure IV.6 T_{max} versus T_0, obtained from thermogravimetric analyses of all the films (● chapter 1, ● chapter 2, ● chapter 3/4, ● chapter 4).

Antimicrobial capacity

The antimicrobial capacity of the films was in each case determined by the composition and the film obtention process. **Table IV.2** shows the reduction in microbial counts in coated meat samples (inoculated or not), as regards to non-coated samples.

Table IV.2: Reduction in microbial counts (Microbial count t = 7 days - Microbial counts t = 0 days) in cold stored pork meat samples.

	Microbial reduction at 7 days (Log cfu/g)	Chapter 1	Chapter 2	Chapter 3/4
Non-inoculated pork meat samples	*Total aerobial*	0.96-1.42	0.65-1.33	2.24-4.72
	Coliform	0.22-0.83	0.63-1.67	4.29-5.97
Inoculated pork meat samples	*Listeria innocua*	-	-	1.76-4.22
	Escherichia coli	-	-	3.13-4.66

CS-CH blend films in chapter 1 showed very weak antimicrobial activity (lower than 2 logs), which was attributed to the inactivation of chitosan due to the compression molding process. A reduced antimicrobial activity was also observed from films in chapter 2, especially from CS:CH bilayer films obtained by the same process. Better antimicrobial activity was obtained from casted CH films with or without OEO or CLEO (chapters 3 and 4), where no deamination of the chitosan would be expected. In these chapters, CH films with no essential oils added, showed antimicrobial activity against *E. coli* and *L. innocua* and the incorporation of essential

oils (both free and encapsulated) was effective at inhibiting or reducing the microbial growth. The same trend was observed against the natural aerobic microbiota in pork meat. The antibacterial activity of chitosan casted films was furtherly improved by the incorporation of alginate microcapsules loaded with eugenol, as seen in Chapter 5.

The antimicrobial activity of active compounds is dependent on their own nature, the type of bacteria targeted and the matrix where they are incorporated. In this sense, in chapter 4 it was shown a more significant antibacterial activity in liquid medium as compared to solid medium and pork meat samples. In this respect, release studies into food simulants and real foods are necessary before practical applications of active films.

V. CONCLUSIONS

1. The structural homogeneity and physical properties of blend films obtained by compression molding were affected by the cassava starch:chitosan ratio. The increase in the plasticizer content did not improve film homogeneity. As the plasticizer content was increased, both the moisture content and water vapor permeability rose, coherently with a less compact and therefore more permeable structure. Moreover, the films with higher plasticizer ratio were significantly less rigid and less resistant. Chitosan addition improved film stiffness and strength. Chitosan incorporation provided the films with only a slight antimicrobial capacity, probably due to thermal processing, which could limit chitosan diffusion into the food system. Moreover, deamination of chitosan taking place during thermal processing led to a reduced antimicrobial activity.

2. The thermo-compression of one layer of melt blended cassava starch and a second layer of casted chitosan, containing or not essential oils (oregano or cinnamon leaf) yielded bilayer films with good interfacial adhesion. Bilayers showed a better mechanical resistance as compared to starch monolayers, although bilayers were less stretchable as compared to chitosan and starch monolayers, due to the interfacial control of the film fracture. Essential oil addition promoted an increase in the water vapor permeability values of bilayer films, while the oxygen permeability was reduced. The thermal treatment used to obtain the bilayers reduced chitosan film effectiveness, revealing the loss of amino groups of chitosan during treatment, which was confirmed by thermal analyses.

3. The encapsulation of eugenol or cinnamon leaf essential oil in lecithin liposomes allowed for a high retention ratio of volatile compounds in chitosan films, as compared to films with non-encapsulated essential oils. Films with liposomes exhibited a lamellar microstructure, which improved the extensibility of the films, while reducing their firmness and water vapor permeability. Liposome incorporation modified film optical properties, coherently with the chromatic properties of lecithin. Films showed antimicrobial activity proven in liquid and solid media. However, when films were applied on meat samples, the interactions with the complex food matrix led to a little decrease in its antimicrobial capacity. Owing to the controlled-release, films containing encapsulated active compounds showed a sustained antimicrobial activity. Color changes were better preserved by the application of films containing cinnamon leaf essential oil. The encapsulation of volatile active compounds in lecithin liposomes was proved to be a good strategy for developing chitosan-based active films with essential oils.

4. The incorporation of alginate-eugenol microspheres into chitosan films obtained by casting caused a significant improvement in the oxygen barrier properties, while increasing water

vapor permeability. The mechanical behavior of the films was affected, since microsphere led to reduced stiffness and elasticity. The release of the eugenol encapsulated through the polymer layer and into liquid and solid media enhanced the antimicrobial capacity of chitosan films.