UNIVERSIDAD POLITÉCNICA DE VALENCIA

DEPARTAMENTO DE TECNOLOGÍA DE ALIMENTOS

INSTITUTO UNIVERSITARIO DE INGENIERÍA DE ALIMENTOS PARA EL DESAROLLO

Caracterización y aplicación de recubrimientos antimicrobianos a base de polisacáridos y aceites esenciales

TESIS DOCTORAL

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CONSIDERAN: que la memoria titulada **CARACTERIZACIÓN Y APLICACIÓN DE RECUBRIMIENTOS ANTIMICROBIANOS A BASE DE POLISACÁRIDOS Y ACEITES ESENCIALES** que presenta **Dª. Laura Sánchez González** para aspirar al grado de Doctor por la Universidad Politécnica de Valencia, reúne las condiciones adecuadas para constituir su tesis doctoral, por lo que **AUTORIZAN** a la interesada para su presentación.

Valencia, 15 de noviembre de 2010

Fdo: Chelo González Martínez

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Resumen

En los últimos años, la industria agroalimentaria ha priorizado la sustitución de los aditivos químicos convencionales por compuestos naturales como respuesta a la demanda creciente de los consumidores de una alimentación más sana, segura y respetuosa con el medio ambiente. El diseño de formulaciones formadoras de recubrimiento a base de biopolímeros biodegradables como el quitosano (CH) o la hidroxipropilmetilcelulosa (HPMC) combinados con antimicrobianos naturales representa una técnica innovadora que puede garantizar la seguridad y prolongar la vida útil de los alimentos. En este sentido, numerosas investigaciones destacan el amplio espectro de actividad antimicrobiana de los aceites esenciales (AE) frente a diferentes cepas de hongos, levaduras, virus y bacterias. En el presente estudio, se diseñaron formulaciones formadoras de recubrimiento (FFR) a base de CH y HPMC con diferentes concentraciones de aceites esenciales de árbol de té (TTO), limón (LO) y bergamota (BO). Se evaluó el efecto de la incorporación de estos AE sobre las propiedades fisicoquímicas de las FFR y de los films secos aislados. En estos films, se analizó además la efectividad antimicrobiana frente a diferentes cepas de hongos y bacterias. A partir del análisis de todas las propiedades caracterizadas y variables analizadas (tipo de matriz, tipo y cantidad de aceite), se seleccionaron films para los posteriores estudios. Se profundizó en el conocimiento de los mecanismos de difusión de los compuestos activos de estos antimicrobianos mediante técnicas cromatograficas. También se evaluó el efecto de la aplicación de recubrimientos a base de CH, HPMC y BO, en la calidad y seguridad de uvas almacenadas en refrigeración. Los resultados de la caracterización de las FFR mostraron que la incorporación de AE en CH y HPMC supuso cambios significativos en la viscosidad, tamaño de partícula y potencial-ζ. Todas las FFR presentaron un comportamiento pseudoplástico independiente del tiempo. En cuanto a los films secos aislados, la incorporación de AE mejoró las propiedades

barreras al vapor de agua de los films puros de CH y HPMC. Se observaron también cambios significativos en las propiedades ópticas, mecánicas y microestructura de los films. El análisis estadístico multivariable, realizado para todas las variables y propiedades, reveló que la matriz es la que más influye en las propiedades caracterizadas, tanto de los FFR como de los film aislados. Además para un polímero dado, el tipo y el nivel de AE incorporado son, respectivamente, los factores que más contribuyen en todas las propiedades (FFR y films). En cuanto a las propiedades antimicrobianas, en general los films diseñados a base de CH o HPMC y AE presentaron una capacidad antimicrobiana significativa frente a *Escherichia coli*, *Staphylococcus aureus* y *Listeria monocytogenes*. En los films a base de CH o HPMC y TTO, se observó una inhibición completa del crecimiento de la cepa Gram negativo, *E.coli*. En cuanto a las dos bacterias Gram positivo, *L.monocytogenes* y *S.aureus*, se obtuvieron los mejores resultados con los films de HPMC-TTO y HPMC-BO, respectivamente. El análisis de los fenómenos de difusión de los AE, realizado en films de CH y BO, a través del seguimiento del limoneno, mostró la mayor difusión de este compuesto en etanol al 95%. Se observó una relación lineal entre los valores de coeficientes de difusión en etanol al 95% y el contenido en aceite esencial del film de CH. Además para un nivel de BO determinado, el coeficiente de difusión disminuye al aumentar el espesor del film. Las pérdidas de volátiles durante el proceso de secado de los films fueron mayores al aumentar la cantidad de AE incorporada a los films. Finalmente, algunas FFR seleccionadas, fueron aplicadas a uvas (var. Moscatel) y almacenadas en refrigeración. Los resultados mostraron que, la aplicación de los recubrimientos supone mejoras en algunos parámetros de calidad del fruto como las pérdidas de peso y firmeza. Los mejores resultados se obtuvieron en los recubrimientos con CH y BO ya que, además de presentar una mayor capacidad antimicrobiana, consiguieron reducir la actividad respiratoria y un interesante efecto barrera frente a las pérdidas de agua.

Abstract

Substitution of chemical additives by natural compounds is one of the current trends in the food industry. The design of film forming dispersions based on biodegradable biopolymers such as chitosan (CH) or hydroxypropylmethylcellulose (HPMC) in combination with natural antimicrobials appears as an innovative technology that can ensure the safety and prolong the shelf life of food. Essential oils present significant antimicrobial properties. Recent articles reported the large spectrum of action of these compounds, since they present an antimicrobial activity against different strains of fungi, yeasts, viruses and bacteria. Thus in the present study film forming dispersions (FFD) based on CH and HPMC with different concentrations of tea tree, lemon and bergamot essential oils were designed. The effect of the incorporation of these essential oils on the physicochemical properties of FFD and dry films and its diffusion in food simulants by gas chromatography were evaluated. The effect of the application of coatings based on CH, HPMC and essential oil of bergamot in the quality of grapes refrigerated was also studied.

Results showed that the addition of essential oils in CH and HPMC resulted in significant changes in viscosity, particle size and ζ-potential. All FFD showed pseudoplastic non-time dependant rheological behaviour. The addition of essential oils improved barrier properties to water vapour of CH and HPMC pure films. Significant changes were also observed in terms of optical, mechanical and microstructure of films. Multivariate statistical analysis revealed a greater influence of matrix nature on the physicochemical properties of FFD and films analyzed. In addition for a given polymer, the nature and level of essential oil are the determinants built respectively for the properties of FFD and films. Generally films designed based on CH or HPMC and essential oils (tea tree, lemon, bergamot) showed significant antimicrobial properties against *Escherichia coli*,

Staphylococcus aureus and *Listeria monocytogenes*. A complete inhibition of the growth of Gram-negative bacteria, *E. coli*, was observed for films based on CH or HPMC and tea tree oil. As for the two Gram positive bacteria, *L. monocytogenes* and *S. aureus*, the best results were obtained with films of HPMC – tea tree oil and HPMC - bergamot oil respectively.

Volatiles losses during the drying process of films were determined by headspace gas chromatography. Losses increased with the amount of essential oil. The phenomenon of diffusion of essential oils incorporated into films to food is a complex mechanism dependent on several factors such as film structure and polarity, solubility of food simulant and migrant. Results showed that diffusion of limonene, the major component of bergamot essential oil, is higher in ethanol 95%. A linear relationship was observed between the values of diffusion coefficients in ethanol 95% and amount of essential oil in chitosan film. For a given essential oil level, diffusion coefficient decreases with increasing film thickness.

Finally, some of the FFD studied were applied in grapes (cv. Moscatel). The coatings accounted for significant changes in some physicochemical properties of fruit such as weight losses and firmness. The best results were obtained with CH coating enriched with bergamot oil; they showed significant antimicrobial properties, barrier effect against water losses and interesting reduction in respiratory rate.

Resum

En els últims anys, la indústria agroalimentària ha prioritzat la substitució dels additius químics convencionals per compostos naturals com a resposta a la demanda creixent dels consumidors d'una alimentació més sana, segura i respectuosa amb el medi ambient. El disseny de formulacions formadores de recobriment a base de biopolímers biodegradables com el quitosà (CH) o l'hidroxipropilmetilcel.lulosa (HPMC) combinats amb antimicrobians naturals representa una tècnica innovadora que pot garantir la seguretat i prolongar la vida útil dels aliments. En este sentit, nombroses investigacions destaquen l'ampli espectre d'activitat antimicrobiana dels olis essencials (AE) enfront de diferents ceps de fongs, rents, virus i bacteris. En el present estudi, es van dissenyar formulacions formadores de recobriment (FFR) a base de CH i HPMC amb diferents concentracions d'olis essencials d'arbre de te (TTO), llima (LO) i bergamota (BO). Es va avaluar l'efecte de la incorporació d'estos AE sobre les propietats fisicoquímiques de les FFR i dels films secs aïllats. En estos films, es va analitzar a més l'efectivitat antimicrobiana enfront de diferents ceps de fongs i bacteris. A partir de l'anàlisi de totes les propietats caracteritzades i variables analitzades (tipus de matriu, tipus i quantitat d'oli), es van seleccionar films per als posteriors estudis. Es va aprofundir en el coneixement dels mecanismes de difusió dels compostos actius d'estos antimicrobians per mitjà de tècniques cromatogràfiques. També es va avaluar l'efecte de l'aplicació de recobriments a base de CH, HPMC i BO, en la qualitat i seguretat de raïm emmagatzemat en refrigeració. Els resultats de la caracterització de les FFR van mostrar que la incorporació d'AE en CH i HPMC va suposar canvis significatius en la viscositat, grandària de partícula i potencial- ζ. Totes les FFR van presentar un comportament pseudoplàstic independent del temps. Quant als films secs aïllats, la incorporació d'AE va millorar les propietats barreres al vapor d'aigua dels films purs de CH i

HPMC. Es van observar també canvis significatius en les propietats òptiques, mecàniques i microestructura dels films. L'anàlisi estadístic multivariable, realitzat per a totes les variables i propietats, va revelar que la matriu és la que més influïx en les propietats caracteritzades, tant dels FFR com dels film aïllats. A més per a un polímer determinat, el tipus i el nivell d'AE incorporat són, respectivament, els factors que més contribuïxen en totes les propietats (FFR i films). Quant a les propietats antimicrobianes, en general els films dissenyats a base de CH o HPMC i AE van presentar una capacitat antimicrobiana significativa enfront d'*Escherichia coli*, *Staphylococcus aureus* i *Listeria monocytogenes*. En els films a base de CH o HPMC i TTO, es va observar una inhibició completa del creixement del cep Gram negatiu, *E.coli*. Quants als dos bacteris Gram positiu, *L.monocytogenes* i *S.aureus*, es van obtindre els millors resultats amb els films de HPMC-TTO i HPMC-BO, respectivament. L'anàlisi dels fenòmens de difusió dels AE, realitzat en films de CH i BO, a través del seguiment del limoneno, va mostrar la major difusió d'este compost en etanol al 95%. Es va observar una relació lineal entre els valors de coeficients de difusió en etanol al 95% i el contingut en oli essencial del film de CH. A més per a un nivell de BO determinat, el coeficient de difusió disminuïx a l'augmentar la grossària del film. Les pèrdues de volàtils durant el procés d'assecat dels films van ser majors a l'augmentar la quantitat d'AE incorporada als films. Finalment, algunes FFR seleccionades, van ser aplicades a raïm (var. Moscatell) i emmagatzemades en refrigeració. Els resultats van mostrar que, l'aplicació dels recobriments suposa millores en alguns paràmetres de qualitat del fruit com les pèrdues de pes i fermesa. Els millors resultats es van obtindre en els recobriments amb CH i BO ja que, a més de presentar una major capacitat antimicrobiana, van aconseguir reduir l'activitat respiratòria i un interessant efecte barrera front a les pèrdues d'aigua.

ÍNDICE PÁGINA

Justificación e interés del trabajo

La tecnología de los recubrimientos "comestibles" es una alternativa muy prometedora para la industria alimentaria, ya que ofrece soluciones viables y sostenibles para la conservación de alimentos. Esta tecnologia tiene un potencial infinito ya que la selección de las materias primas para el diseño de las formulaciones formadoras de los recubrimientos (FFR), compuestos naturales y biodegradables, se hace a medida del sistema en el que van ser aplicados. En este sentido, se trata de recubrimientos "inteligentes" puesto que son activos y selectivos con una aplicabilidad muy amplia. En el sector de las frutas y hortalizas, por ejemplo, se aplican para alargar la vida útil, reducir las pérdidas de humedad y ralentizar los procesos de maduración de los frutos ya que actúan como barrera al intercambio gaseoso. También se utilizan para aportar brillo al producto, mejorar su integridad mecánica así como protegerle frente a la manipulación posterior, confiriéndole un aspecto más apetecible en el punto de venta. Por otra parte, los recubrimientos también pueden servir como vehículo para la incorporación de compuestos que actúen como antimicrobianos o antioxidantes, entre otros. En cuanto al coste, aunque en principio es más elevado que el de los films de polietileno o polipropileno, son del mismo orden que los recubrimientos multicapa o los envases activos con los que son más comparables a efecto de funcionalidad.

En la actualidad, las nuevas tendencias en el sector del envase hacia el uso de materiales biodegradables, ha impulsado el desarrollo de films y recubrimientos a base de polisacáridos solubles en agua. En este sentido, las soluciones de hidroxipropilmetilcelulosa (HPMC) y quitosano (CH) forman films resistentes a aceites y grasas, flexibles, transparentes, sin olor y sabor. Su permeabilidad selectiva a gases permite el retraso de la madurez en las frutas, al reducir la concentración interna de O_2, sin causar una acumulación excesiva de CO_2. Sin

embargo, su alta permeabilidad al vapor de agua hace necesaria la incorporación de compuestos lipídicos para mejorar esta propiedad. Si el compuesto lipídico tiene además actividad antimicrobiana, como es el caso de los aceites esenciales, la funcionalidad añadida será doble. La actividad antimicrobiana de los aceites esenciales ha sido ampliamente estudiada *in vitro* y su aplicación a film o recubrimientos de uso alimentario ha sido, hasta el momento, bastante limitada. De ahí que, en este trabajo se hayan desarrollado recubrimientos a base de HPMC/CH con diferentes tipos de aceites esenciales, compuestos de naturaleza lipídica con los que se podría conseguir una adecuada optimización de las propiedades funcionales del film.

La efectividad de los films composite de hidrocoloides y antimicrobianos de carácter lipídico (aceites esenciales) depende de numerosos factores. Por su carácter lipídico la concentración relativa de ambas fracciones, el estado físico del lípido, la longitud, el grado de insaturación y de ramificación de la cadena hidrocarbonada, así como de la distribución que alcancen los componentes lipídicos en la estructura final: tamaño de los glóbulos grasos y nivel de agregación. Además, la forma de preparación y la composición de la emulsión de partida influye en gran medida en el tamaño y distribución de tamaños de gotas y por tanto, también en las propiedades finales del film. En el caso de los aceites esenciales esta complejidad aumenta todavía más, ya que se componen de varios centenares de compuestos simples. No obstante, es crucial considerar el aceite esencial en su conjunto, ya que desde el punto de vista de control microbiano, se ha demostrado que crean menos problemas de resistencia que cuando se aplican sus componentes simples aislados. La gran problemática de los aceites es su gran volatilidad, olor, baja persistencia y alto coste. Por ello su incorporación a diferentes matrices biodegradables que sirvan de soporte ayudaría a minimizar esta problemática a la vez que abaratar los costes de aplicación. El papel de estas matrices (quitosano o hidroxipropilmetilcelulosa) será clave en las propiedades

finales del film, tanto fisicoquímicas como antimicrobianas, así como en su posterior aplicación, y deberá ser analizado.

En definitiva, los films composite ofrecen grandes ventajas en cuanto a la posibilidad de modificación y adecuación de las propiedades funcionales a determinadas aplicaciones. Sin embargo, existen pocos estudios donde se analicen las propiedades fisicoquímicas y antimicrobianas de formulaciones formadoras de recubrimiento y films secos (aislados) a base de aceites esenciales y el efecto que la matriz polimérica tiene sobre éstas. En este trabajo, se pretende analizar dichas propiedades para establecer criterios de formulación de recubrimientos que permitan su adecuación a determinados usos en productos alimentarios (como frutas).

I. INTRODUCCIÓN

La introducción se divide en dos partes, un capitulo de libro y un artículo de revisión que tratan, por una parte, la aplicación de recubrimientos comestibles en la conservación de frutas y hortalizas, y por otra, la funcionalidad y usos de los aceites esenciales y en especial el efecto de su incorporación a la formulación de films.

En el capitulo de libro, se presentan las mayores ventajas de la aplicación de recubrimientos comestibles al área de la conservación de frutas y hortalizas ya que además de prevenir los daños físicos, se reducen las pérdidas de humedad del producto. Los recubrimientos comestibles constituyen además una vía interesante para la reutilización de subproductos de la industria alimentaria (proteínas, polisacáridos) a la vez que pueden ser utilizados como vehículo para introducir compuestos activos como antimicrobianos, antioxidantes, aromas, nutrientes, etc. Por otra parte, la utilización de estos recubrimientos permitiría reducir la cantidad de envases sintéticos no biodegradables con el consiguiente beneficio medioambiental. En este trabajo también se exponen los principales inconvenientes y limitaciones de la utilización de los recubrimientos en la conservación de frutas y hortalizas.

El artículo de revisión presenta las principales propiedades de los aceites esenciales como su actividad antioxidante y poder antimicrobiano, centrándose en el efecto que la incorporación de los mismos tiene en las propiedades de los films y recubrimientos biodegradables. Estas propiedades antioxidantes y antimicrobianas han sido ampliamente estudiadas, pero los mecanismos de acción, probablemente relacionados con su carácter hidrófobo, aun quedan por describir de forma clara y precisa. Los trabajos de aplicación de los aceites esenciales como antimicrobianos naturales, ponen de manifiesto sus principales limitaciones en la conservación de alimentos. A nivel económico, el coste sigue siendo relativamente elevado y otro

problema importante está relacionado con la modificación de las propiedades organolépticas de los alimentos debido al intenso aroma de estos compuestos. La incorporación de los aceites esenciales en recubrimientos permitirá minimizar estos problemas, ya que esta tecnología reduce la cantidad de aceite necesaria para garantizar calidad y seguridad del producto. En este sentido, el aceite incorporado a la matriz polimérica difunde hacia el alimento de forma controlada, prolongándose así la actividad del compuesto antimicrobiano. El artículo expone además las ventajas y limitaciones de estos recubrimientos.

EDIBLE COATINGS FOR FRESH AND MINIMALLY PROCESSED FRUITS AND VEGETABLES

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ABSTRACT

The main interest of applying edible coatings to fruits and vegetables is to reduce moisture losses, prevent physical damage, enhance product appearance and to act as a carrier of different food ingredients such as colorants, flavours or nutrients. Their functionality can be broadened by incorporating antimicrobials to protect food products from microbial spoilage, thus extending their shelf-life and enhancing their safety. The benefits gained by using this technology are connected to the fact that some by-products may be re-used by the food industry (like proteins, polysaccharides…), and to the fact that the environmental impact is reduced due to the smaller quantity of synthetic packaging wasted, as it can be eaten together with the product. The major achievement is to obtain a coated product with the same sensory attributes as the uncoated one or to develop a coating with interesting properties from a nutritional or sensory point of view.

1. INTRODUCTION

Coatings may be defined as a thin layer of material that covers the surface of the food, while *films* are formed previously and later applied to the product. Those that can be eaten as part of the whole product are called edible. The composition of edible coatings must therefore conform to the regulations that apply to the food product concerned (Guilbert et al., 1995). According to both the European Directive (1995; 1998) and the USA Food and Drug Administration (FDA 21CFR172 2006), edible coatings are those coatings that are formulated with food-grade additives. Among the ingredients that can be incorporated into the formulation of edible coatings, the European Directive (1995; 1998) includes the following: arabic and karaya gum, pectins, shellac, beeswax, candelillawax, carnaubawax, lecithin, polysorbates, fatty acids and fatty acid salts. The Food and

Drug Administration, on the other hand, mentions other additives used as components of protective coatings applied to fresh fruits and vegetables like morpholine, polydextrose, sorbitan monostearate, sucrose fatty acid esters, cocoa butter, and castor oil (Vargas et al., 2008).

The main function of these coatings when applied to fruit and vegetables is to extend their shelf life by acting as a barrier not only against water vapour transport but also against oxygen, carbon dioxide or other gasses involved in the response of fruits to ripening. This contributes to limit the weight losses (also usually related to firmness decay) of products throughout storage and distribution and to slow down their metabolic activity. These effects have been proven to be similar to those occurring under controlled or modified atmospheres (Park et al., 1999), because of the modification of the internal atmosphere of the coated product. Other common functions of these coatings are to prevent physical damage and to enhance product appearance.

The main advantages of this technology are the possibility of incorporating preservatives (i.e. antimicrobials) in order to control the growth of microorganisms or other compounds, such as functional or bioactive substances, and the possible reduction of the amount of synthetic packaging wasted as, in some cases, they can completely substitute the conventional plastic packaging by using natural biodegradable raw materials in their formulation. This is in line with the changes taking place both in legislation and consumer trends in the consumption of natural products, free of chemical-based preservatives.

A combination of hydrocolloids (polysaccharides or proteins) and lipids usually constitutes the basic composition of the formulation of edible coatings for fresh fruit and vegetables, known as composite coatings or films. These composite coatings take advantage of the special functional characteristics of each group, reducing their drawbacks (Greener and Fennema 1994), as commented on below. In this sense, several studies have remarked on the effectiveness of polysaccharide

films as water vapour barriers and their enhancement by the incorporation of lipids (Vargas et al., 2006; Rojas-Argudo et al., 2009; Valencia-Chamorro et al., 2009; Pastor et al., 2010).

Nevertheless, their composition may also include a very wide range of minor compounds, such as plasticizers, acids, bases or salts to regulate the pH and a number of additives to improve the coating functionality, such as antimicrobials and antioxidants, and certain nutrients like vitamins, volatile precursors, flavours, firming agents and colorants.

Hydrocolloids tend to form hydrophilic networks, usually being good barriers to oxygen and carbon dioxide but poor barriers to water vapour. Of the hydrocolloids, the polysaccharides are the most commonly used components of edible coatings for fruits (Kester and Fennema 1986; Krochta and De Mulder Johnston 1997), as they are present in the majority of the commercially available formulations. The main reason could be related to the better microbial and physical stability throughout time of polysaccharide in comparison with protein films, especially in high relative humidity environments. In general, lipids impart hydrophobicity to the hydrocolloid matrix, thus improving the films' capacity as a water vapour barrier but decreasing their mechanical resistance. To find the proper lipid/hydrocolloid ratio that complements these abilities is key in the successful development of edible coatings for fruit and vegetable application.

Plasticizers are minor components which are added to improve the mechanical properties of brittle films. The most commonly used plasticizers are glycerol, polyethyleneglycol and sorbitol, among others, and water is considered to be a universal plasticizer, especially in hydrophilic films. Its addition allows the intermolecular forces between the polymer chains to be reduced while increasing the molecular mobility of the continuous polymer matrix, leading to more flexible and stretchable films that are easier to handle. For example, casein based films are

very brittle and the addition of plasticizer, usually in a protein: plasticizer ratio of around 1:0.2 to 1:1, is required to reduce rigidity (Fabra, 2010).

These composite films are usually applied by spraying or by immersing the product in the film forming dispersion, where the lipid is dispersed in the hydrophilic phase of an emulsion/dispersion. This technique allows a monolayer composite edible coating to be formed but, the application of two layers, one formed by the hydrocolloid and a second one formed by the lipid may also be found. Examples of the application of a one-layer composite film are found in some works by Vargas et al. (2006, 2009a) where chitosan-oleic acid films were applied to strawberries and fresh cut carrots. On the other hand, Wong et al. (1994) applied two-layer film coatings to fresh cut apple cylinders, one being a mix of different polysaccharides (pectin, carrageenan, alginate and cellulose) and the other, an acetylated monoglyceride layer. Emulsified coatings are less efficient than bilayers due to the non-homogeneous distribution of the lipid dispersed phase but they have received more attention because they need only one drying step instead of the two for bilayer films. Moreover, both their hydrophilic and lipophilic nature permits their adhesion onto any support, whatever its polarity, and exhibit good mechanical resistance (Quezada et al., 2000).

Coatings began to be applied to preserve fruits and vegetables in the last century. Most of these traditional coatings were not edible (i.e. some waxes applied to citrus fruit and apples), and offered limited benefits, mainly associated to the glossy appearance and the reduction in weight loss while the coated fruit was in storage. This fact, together with the possibility of reducing the amount of waste generated by the plastic packaging industry, have been the major driving forces involved in the development of edible coatings. Furthermore, some research has demonstrated that some active substances improve their efficiency when applied as a part of the whole coating, if compared with their direct surface application by dipping or spraying the foodstuff. This is because the active substance can react with food

components and can evaporate or diffuse into the food showing reduced activity, resulting in the need for large concentrations to be applied (Vojdani and Torres, 1990; Baldwin et al., 1996; Kristo et al., 2008). This has been explained by the fact that the coatings can maintain the effective concentration of the active compounds (an antimicrobial, for example) on the product surface for longer, where the microbial growth is prevalent. Another advantage is the possibility of controlling the release of the active compound as a function of time (by means of encapsulation techniques).

The main interest of using an edible coating for preserving fruits and vegetables centres around the possibility of developing tailor-made coatings, depending on the needs and requirements of each product. The possibilities are infinite, i.e., from a typical coating to control water loss to a complex one offering antimicrobial activity, new flavours/tastes or nutritional benefits due to the incorporation of functional ingredients. So, the importance of knowing how different factors influence their properties in order to design the most suitable film for a determined use and functionality is highlighted.

2. IMPROVEMENT OF PHYSICOCHEMICAL AND FUNCTIONAL PROPERTIES OF EDIBLE COATINGS

There are different requirements that an ideal coating should fulfil in order to obtain the maximum benefits when applied to a food product. The most relevant properties of a coating to be applied to fruit and vegetables are summarised below (Table1).

The key factor to be able to improve the film properties in order to set the basis for obtaining new and highly functional edible coatings is to understand how the nature of the compounds of the film forming dispersion (FFD), its molecular

arrangement and the interactions between the components affect the physicochemical properties of the films.

When developing edible coatings, the classical approach has been to characterize their properties when they are cast and then peeled off from a plate. This approach can be very useful to compare coatings, but a major weakness is that it does not take into account the interactions between a coating and a fruit surface and its subsequent influence on coating properties (Vargas et al., 2008).

The most important properties to be measured when characterizing the films to be applied to coat fruit and vegetables are the following.

Table 1. Desirable properties of edible coatings.

Property	Response	Desirable value
Water barrier	Limit weight losses	High
O_2/CO_2 barrier	Delay in metabolic activity	Moderate
Mechanical resistance	Prevent physical damage	Hard, flexible and stretchable
Glossy and transparent	Enhance appearance	High
Antimicrobial activity	Extension of shelf life	High
Sensory properties	Odourless, tasteless	High
Water sorption capacity	Film higroscopicity and solubility	Low
Production Cost	Viability	Low

2.1. Water vapour permeability (WVP)

For the selection and optimization of coatings, one of the most commonly used parameters has been the water vapour permeability (WVP) of the dry film, measured under controlled environmental conditions (temperature, relative humidity), as this parameter allows the moisture exchange between the coated commodity/product and the surrounding environment to be controlled. So, as commented on in Table 1, low WVP values are desirable to minimize weight losses

in the coated product which, in turn, also usually directly affects product firmness and appearance.

The usual WVP values of films are in the order of 400 10^{-11} to 0.017 10^{-11} (g m^{-1} s^{-1} Pa^{-1}) for some pure polysaccharide/protein and pure candelilla wax films, respectively, as shown in Table 2. The high values are in accordance with the greater hydrophilic nature of polysaccharides. Furthermore, these values go up as the level of plastification of the film rises due to the great mobility of the polymer chains inside the matrix.

Composite films present WVP values halfway between both types of films, due to the increase of hydrophobic compounds, which leads to an improvement in the WVP values. The efficiency which the lipid compound demonstrates in reducing the WVP depends on several factors, such as the chemical nature (length of the chain and degree of unsaturation…) related to the degree of hydrophocibity, concentration, physical state, size and structure of the lipids in the polymer network (Morillon et al., 2002; Fabra, 2010).

Figure 1 shows the WVP of chitosan (CH) films as affected by the incorporation of a complex lipid compound, such as an essential oil (bergamot oil).

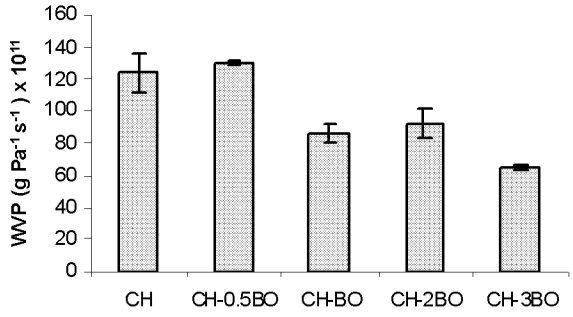

Figure 1. Water vapour permeability of chitosan (CH) and CH with different levels of bergamot essential oil (BO) composite films, determined at 100/54.4 RH gradient and 20°C. Mean values and 95% LSD intervals.

As expected, pure CH films without hydrophobic compounds showed poor moisture barrier properties, but the incorporation of bergamot oil (BO) led to an improvement in the water vapour permeability. Thus, the incorporation of 3% BO caused a reduction of about 48% in the WVP of pure CH films at 20º C.

Table 2. Water vapour, oxygen and carbon dioxide permeability of films based on different polysaccharides, lipids and proteins. (adapted from Vargas et al. 2008)

Film	WVP x 10^{11} (1)	RH gradient (%/%)	T (ºC)	PO_2 (2)	PCO_2 (2)	RH (%)	T (ºC)
Methylcellulose	7.55	0/75	25	1.12	64.19	0	30
Hydroxypropylcellulose	11	-	-	1.01	62.03	0	30
Hydroxypropyl methylcellulose	10.5	0/85	27	0.01-0.1	-	50	25
Starch	217	74/50	23	137.5	2523.7	63.8	20
Chitosan	360	100/50	25	0.90	15.33	93	25
Pectin	-	-	-	2.55	40.78	96	25
Carrageenan	190	100/50	25	0.362	-	-	-
Zein	11	0/85	21	0.25	1.13	60	20
Gluten	4.3	0/50	23	1.88	46.88	91	25
Soy	354	100/50	25	0.067	-	50	25
Whey proteins	417	100/55	25	0.001- 0.01	-	50	25
Sodium caseinate	42.5	0/81	25	0.76	4.56	77	25
Candelilla wax	0.017	0/100	25	0.537	2.04	60	30
Shellac	0.58	0/100	30	0.083	0.29	60	20
Low Density Polyethylene	0.07-0.097	0/90	38	1.92	10.36	90	25
High Density Polyethylene	0.024	0/90	38	0.024			

(1) $g \cdot m^{-1} \cdot s^{-1} \cdot Pa^{-1}$

(2) $mL.\mu m/m^2.d.Pa$

2.2. Oxygen and carbon dioxide permeability

Oxygen and carbon dioxide permeability of coatings is also an important characteristic to take into account when the respiration or oxidation reactions could affect product quality, which is the case of fresh or minimally processed fruits and vegetables. In this sense, under controlled or modified atmospheres, edible coatings can have similar effects to storage.

Scientific literature provides a great amount of information on gas barrier properties of edible films with different compositions; however, comparing the coatings is sometimes difficult or impossible due to differences both in the types of equipment used and the test conditions during measurements (temperature/relative humidity). Table 2 shows the oxygen and carbon dioxide permeability values of edible, hydrocolloid-based films, where reported permeability values together with measurement conditions have been indicated. In addition, the values of common synthetic polymers used for food packaging are shown for comparison purposes.

As mentioned above, it is difficult to compare the values, as the permeability properties are greatly affected by the temperature and relative humidity conditions (which determines the water content in hygroscopic films, such as hydrocolloid-based ones), moisture content, which induces changes in the solubility and diffusivity of gases in the biopolymer matrix. In dry conditions, edible films present low permeabilities. However, although the oxygen permeability of most synthetic films remains constant irrespective of the relative humidity, that of edible films and coatings usually increases significantly in the presence of moisture (Debeaufort and Voilley, 2009).

2.3. Mechanical properties

The mechanical properties of edible coatings depend on several factors, as the interactions between their components and the polymer matrix are strongly affected by the physical, chemical and temperature conditions, which in turn influence film stability and flexibility. Moreover, it is well known that the environmental conditions during the production, storage and usage of these materials affect their mechanical properties. Ageing phenomena that occur within the useful lifetime of the films also cause great losses to mechanical properties, particularly as regards film elongation (Garcia et al., 2009).

The mechanical properties of edible films can be improved by combining hydrocolloids with other ingredients. For instance, Peña et al. (2010) incorporated tannins to gelatine-based films, which promoted an increase of the elastic modulus and tensile strength of the films when using a chitosan-tanning ratio of up to 1:2. The increase in the strength of the composite films could be linked to the formation of a more stable polymer network due to attractive interactions between gelatin and tannins.

In the case of whey protein-based films, the mechanical properties can be improved by denaturation (Perez-Gago and Krochta, 2001). Another more recent approach to improve the mechanical properties of films is to incorporate nanoparticles into the film-forming solutions. In this sense, de Moura et al. (2010) incorporated chitosan/tripolyphosphate nanoparticles into hydroxypropylmethylcellulose (HPMC)-based films, which led to a significant improvement in their mechanical properties. The nanoparticles tend to occupy the empty spaces in the pores of the HPMC matrix, leading to the collapse of a greater amount of pores and thereby improving the film tensile properties. The smaller the nanoparticles, the more the mechanical properties improve.

Hydrocolloids are often combined with lipid compounds, to obtain composite films with improved mechanical properties. In such films, the way in which the lipid is added (dispersed in the hydrocolloid aqueous solution to obtain an emulsion in a single packaging layer or cast on an existing packaging to form a bilayer film) strongly influences the film's mechanical properties.

The microstructure of emulsified films is greatly affected by the nature of the lipid compound (saturated or unsaturated fatty acids or oils), as well as the size and shape of the lipid aggregates in the initial film-forming emulsion and their development during film drying, which in turn has a strong influence on the mechanical properties of the dry film. For example, the addition of oleic acid to the HPMC or chitosan matrix caused a significant decrease in the elastic modulus (EM) and tensile strength (TS) due to the introduction of discontinuities in the polymer matrix (Vargas et al., 2009b; Jiménez et al., 2010). In films prepared with HPMC and saturated fatty acids (lauric, miristic and palmitic), EM and TS decreased when the lipid layers increased in size, which can be attributed to the greater mechanical resistance of the small particles, taking into account the solid state of the lipids under the conditions of the mechanical test (Jiménez et al., 2010).

2.4. Optical properties

The appearance of the coatings is of relevance since their commercial acceptance depends mainly on this attribute. An ideal coating has to be transparent, glossy and colourless. These attributes are usually measured through the internal transmittance (by means of a spectrophotometer) and gloss (by means of a glossimeter) of the dry films and are greatly affected by the internal structure and surface roughness of the film, respectively. Thus, a colourless, homogeneous internal structure and a surface free of irregularities are usually well correlated with transparent and glossy films.

Hydroxypropylmethylcellulose (HPMC) films are considered to be highly transparent and glossy due to their high internal transmittance and gloss values (Sánchez-González et al., 2009), which in turn are responsible for the brightness of the fruit and vegetables coated with these films. The presence of a dispersed phase in the continuous HPMC matrix of the film (with a different refractive index) usually leads to a decrease in the transparency and gloss of these composite films, which could also affect the external appearance of the coated product.

2.5. Antimicrobial properties

As has been commented on above, films could serve as carriers of antimicrobial compounds, enhancing their functionality by preventing the growth of spoilage microorganisms on fruit and vegetables.

A great number of works have been published dealing with the addition of different antimicrobial agents to a film matrix. The list of antimicrobials used is very long, differing according to nature and source.

In the last few years, one of the research priorities of the food industry has been to look for healthier, safer and more sustainable alternative treatments and compounds to substitute the traditional chemical products used for food preservation, partially due to increasing consumer demand. Coatings based on edible/biodegradable hydrocolloids, incorporating natural compounds with antimicrobial activity to control fungal growth in fresh fruits and/or bacterial growth in minimally processed products is, nowadays, a promising technology used in food preservation. Natural compounds with antimicrobial activity include a wide number of products from plants (i.e. essential oils), sea organisms (chitosan) or microorganisms (nisin, lysozyme…) and their incorporation into the film forming dispersions (FFDs) to obtain antimicrobial coatings allows the functionality of the films to be extended, while improving the efficiency and persistency of the antimicrobial compound on the product surface.

Chitosan is a biodegradable cationic polysaccharide with antimicrobial activity and excellent film forming ability (Vargas and González-Martinez, 2010). This makes it particularly suitable for the formulation of edible coatings, which have proved to be effective at extending the shelf-life of fruits and vegetables (Han, Zhao, Leonard, & Traber, 2004; Vargas, Albors, Chiralt, & Gonzalez-Martinez, 2006). The antimicrobial activity of chitosan films depends on the type of microorganism and is caused by the electrostatic interaction between $-NH_3^+$groups of chitosan acetate and carbonyl and phosphoryl groups of the phospholipid components of cell membranes, which increases the permeability of the outer and inner membranes, with the release of the cellular content. Figure 2 shows the effect of different films on the in vitro growth and survival of (a) *Escherichia coli* and (b) *Penicilium italicum.* The major antimicrobial response against *E.Coli* of pure chitosan films can be observed, population did not exceed 2 logs UFC/cm^2 during the 12 days of storage. Nevertheless, this response was not observed when using P *italicum* (Figure 2b).

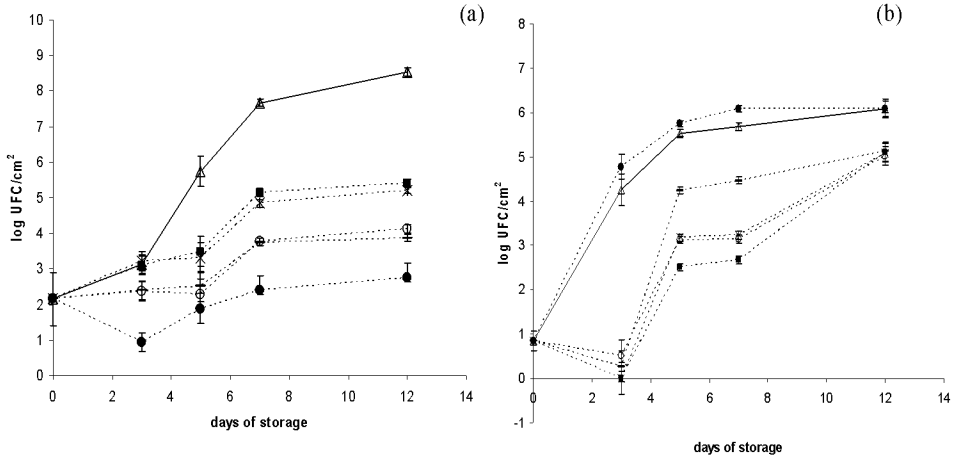

Figure 2. Effect of different films of chitosan (CH) and CH with different levels of lemon essential oil (LO) based films on the growth and survival of (a) *Escherichia coli* and (b) *Penicilium italicum.* Mean values and 95% LSD intervals for each sample time (Δ control, • CH, - CH-0.5LO, ○ CH-1LO, * CH-2LO, ■ CH-3LO).

Natural plant extracts (mainly essential oils or pure substances derivated from them) have also been extensively used to formulate natural antimicrobial films using different polymer matrices (Zivanovic et al., 2005; Ponce et al., 2008; Sánchez-González et al., 2009, 2010a, 2010b), in line with the increasing consumer demand for a more restricted use of chemicals on minimally processed fruits and vegetables. Figure 2 (b) shows that the incorporation of lemon essential oil (LO) enhanced the antimicrobial activity of pure CH films, as in this case the pure CH films did not exhibit any antifungal activity against the fungus *P.italicum*. On the contrary, in Figure 2(a), where the CH films presented a high antimicrobial effectiveness against *E.Coli*, this effect was not observed in the composite films, possibly because of the dilution effect of CH when EO is present, thus being less available for microorganisms.

Other very common antimicrobial compounds used in edible films have been nisin, incorporated into HPMC (Coma et al., 2001; Sebti et al., 2002), methylcellulose (MC) (Cha et al., 2001), corn zein (Hoffman et al., 2001; Padgett et al., 1998), wheat gluten (Ko et al., 2001), whey protein (Talarico et al., 1989; El-Ziney et al., 1999) and soy protein (Cleveland et al., 2001), lysozyme into soy and corn protein films (Karaman et al., 2001; Wan et al., 1998; Cleveland et al., 2001), potassium sorbate into HPMC (Grande et al., 2006), and some other acids such as benzoic, sorbic and lactic acid.

The task of controlling the antimicrobial release from the edible films is very important, but sometimes a very complex one. The diffusion of these antimicrobials is influenced by the the film's nature (type, physical characteristics, manufacturing procedure), food (pH, water activity) and storage conditions (temperature, relative humidity) (Cagri et al., 2004). The degree of interactions between the active agent and the film matrix could also affect the release of the active compound and thus, it has to be taken into account. Some studies have provided some insight into the diffusion of antimicrobials (Cagri et al., 2004).

Sánchez-González et al. (2010b) studied the effect of the incorporation of three essential oils (EOs) on the microbial properties of two polymer based films (chitosan and HPMC) against three foodborne pathogens, *Escherichia coli, Listeria monocytogenes* and *Staphylococcus aureus*. The result showed that both HPMC-EO and CH-EO composite films presented a significant antimicrobial activity against the three pathogens studied. The authors pointed out that the nature of the EOs, the structure of the film and the possible interactions existing between the polymer and active constituents of EOs must be considered in order to design biodegradable films with antimicrobial activity and also that the choice of the polymer was a determining factor, more important than the selection of EO.

Furthermore, the antimicrobial agent might influence some properties of the film, i.e. mechanical response, water sorption and water barrier properties, among others. Thus, natural essential oils have been shown to exert antimicrobial activity while decreasing the WVP of the composite films and reducing the mechanical resistance (Sánchez-González et al., 2009, 2010a).

3. APPLICATION OF EDIBLE FILMS TO FRESH AND MINIMALLY PROCESSED FRUITS AND VEGETABLES: BENEFITS AND LIMITATIONS

The response of the coated fruits and vegetables depends on both the properties of the fruit/vegetable to be preserved or enhanced and on the film properties. An optimum formulation can be achieved by a precise control of gas permeability, texture and colour changes by means of quantitative or qualitative changes in coating formulation.

By consulting the literature, the use of coatings can be seen to yield relevant results in the field of fruit preservation. Composite films are usually used, as can be observed in Table 3. These coatings usually permit fruits or vegetables to reduce

water loss and slow down respiration rates, while preserving colour and firmness, leading to a longer product shelf life, as is deduced from Table 3.

It is important to remark on the fact that, once applied onto the commodity surface, the degree of coating uniformity is of special importance because it determines the efficiency of the coating as a barrier. For example, when applying HPMC-propolis based films to table grapes, Pastor et al. (2010b) observed differences in the film's extensibility onto the fruit skin and the subsequent degree of coating uniformity which caused inefficiency of the film in a stochastic way.

Table 3. Application of edible coatings to fresh fruits and vegetables

Type of Coating	Composition	Application	Main Response	References
Polysaccharide	Chitosan and Tween 80	Strawberry Raspberry Sweet cherry Tomato Table grape Litchi Carrot Citrus Peach Japanese Pear Kiwi Fruit Longan Lettuce Mango Papaya	Reduction of fungal infection, retention of fruit firmness, extension of shelf-life	Romanazzi *et al.* 2002; Romanazzi *et al.* 2003; Devlieguere *et al.* 2004; Han *et al.* 2004; Park and Zhao 2004; Park *et al.* 2005; Caro and Joas 2005; Joas *et al.* 2005; Ruoyi *et al.* 2005; Chien *et al.* 2007; Jiang et al., 2005; Chien et al., 2007; Meng et al., 2008; Ali et al., 2010; Liu et al., 2007; Meng et al., 2010; Badawy and Rabea, 2009; Campaniello et al.,2008; Vargas et al., 2009; Xu et al., 2007
Polysaccharide	Alginate Alginate and glycerol	Mushroom Apple Onion	Extension of shelf-life, reduction of moisture loss and better appearance	Falcao-Rodrigues et al. 2007 Olivas et al., 2007
Polysaccharide	Cactus mucilage extract and glycerol	Strawberry	Extension of shelf-life maintaining sensory properties	Del-Valle *et al.* 2005
Polysaccharide	*Aloe Vera* L. gel	Sweet cherry Table grape	Extension of storability	Valverde *et al.* 2005; Martínez-Romero *et al.* 2006 Castillo et al., 2010
Polysaccharide	Alginate, pectin and gellan	Melon	Decrease in moisture loss and preservation of mechanical properties	Oms-Oliu et al., 2008b
Polysaccharide	MC and glycerol	Strawberry Avocados	Delay in senescence	Park *et al.* 2005; Maftoonazad and Ramaswamy 2005

(cont.)

Polysaccharide	Medium or high amylase content starch and glycerol	Strawberry	Delay in senescence and improvement of overall organoleptic conditions	García *et al.* 1998a; García *et al.* 1998b
Polysaccharide	MC, lauric acid, stearic acid, palmitic acid and PEG[2]	Bean Strawberry	Reduction of weight loss	Ayranci and Tunç 1997
Polysaccharide	Locust bean gum	Mandarin	Reduction of weight loss and ethanol content	Rojas-Argudo et al., 2009
Polysaccharide	Chitosan, starch, glycerol	Carrot	Extension of shelf-life	Durango et al., 2006
Polysaccharide	Chitosan, MC, Tween 80	Strawberry	Delay in senescence	Vargas *et al.*, 2006b
Protein	Soy protein, glycerol, malic acid, lactic acid	Apple	No significant effect on sensory quality	Eswaranandam *et al.* 2006
Protein-Polysaccharide	CMC, WPI[1], caseinates and glycerol	Strawberry	Reduction of mold infection	Vachon *et al.* 2003
Protein-Polysaccharide	Galactomannans and collagen	Mango Apple	Reduction of gas transfer rate	Lima et al., 2010
Polysaccharide-lipid	Pullulan, non-ionic sucrose fatty acid ster and sorbitol	Strawberry Kiwi	Reduction in internal O_2, firmness and colour retention; increase in internal ethylenene in kiwi fruits	Diab *et al.* 2001
Polysaccharide-lipid	Maltodextrin, CMC, propylenglicol, fatty acid sters Sodium benzoate	Mango	Extension of postharvest storage	Díaz-Sobac *et al.* 1996
Polysaccharide-lipid	HPMC, shellac, stearic acid and glycerol	Plum	Effects were only noticeable at long term storage	Pérez-Gago *et al.* 2003
Polysaccharide-lipid	MC, [2]PEG, stearic acid, citric acid, ascorbic acid	Apricot Green pepper	Decrease in moisture loss	Ayranci and Tunc 2004

(cont.)

Polysaccharide-lipid	Chitosan, oleic acid, Tween 80	Strawberry	Delay in senescence and fungal infection	Vargas et al. 2006a
Polysaccharide-lipid	CMC, paraffin wax, beeswax, soybean oil, oleic acid and sodium oleate	Mandarin Peach Pear	Delay in ascorbic acid loss of mandarins and extension of shelf-life	Togrul and Arslan 2004a, 2004b
Polysaccharide-lipid	Alginate, gellan with sunflower oil	Papaya	Decrease in moisture loss and preservation of mechanical properties	Tapia et al., 2008
Protein-Lipid	WPI[1], sodium caseinate, beeswax and glycerol	Green Bell peppers	No significant effect on moisture loss, respiration rate or colour	Lerdthanangkul and Krochta 1996
Protein-lipid	Wheat gluten, glycerol, estearic acid, palmitic acid and beeswax	Strawberry	Lipids reduced weight loss and preserved mechanical properties	Tanada-Palmu and Grosso 2005
Hydrocoloids-antimicrobial/antioxidant agent	Oleoresins with sodium caseinate, Chitosan, CMC	Romaine lettuce, butter lettuce and butternut squash	Antimicrobial and antioxidant protection of minimally processed squash	Ponce et al, 2008
Polysaccharide-antibrowning agent	chitosan with 1-methylcyclopropene	Indian jujube	Antioxidant protection	Qiuping and Wenshui, 2007
Polysaccharide-antibrowning agent	Alginate, pectin and gellan with N-acetylcysteine, glutathione.	Pear	Microbial growth reduction, prevention from browning and preservation of sensory attributes	Oms-Oliu et al., 2008a
Polysaccharide-antibrowning agent	Alginate, gellan with N-acetylcysteine	Apple	Extension of shelf-life	Rojas-Graü et al., 2008
Polysaccharide-antimicrobial agent	chitosan with rosemary extract	Pear	Extension of shelf-life	Xiao et al., 2010

(cont.)

Polysaccharide-antimicrobial agent	Chitosan, methyl cellulose and vanillin	Cantaloupe, pineapple	Reduction of microorganisms level, quality attributes remain acceptable	Sangsuwan et al., 2008
Polysaccharide-antimicrobial agent	Essential oils with apple puree-alginate	Apple	Extension of shelf-life	Rojas-Graü et al., 2007
Polysaccharide-antimicrobial agent	Malic acid and essential oils with alginate	Melon	Extension of shelf-life	Raybaudi-Massilia et al., 2008
Polysaccharide-antimicrobial agent	Tapioca starch-decolorized hsian-tsao leaf gum with green tea extract	Fruit-based salad, romaine heart	Extension of shelf-life	Chiu and Lai, 2010

[1]Whey Protein Isolate; [2]Polyethilene glycol

3.1. Weight loss reduction

The weight loss reduction throughout storage is due to the greater water vapour resistance (parameter that allows us to determine whether the coating has the expected water barrier properties when applied to the product's surface) of coated products, related to the hydrophobic nature of the film when forming a continuous matrix around the product. The thickness of the film, related to the total amount of solids in the formulation, also plays an important role in the water transference as can be deduced from Figure 3, for hydroxypropylmethylcellulose (HPMC) coated strawberries. In this Figure, the thickest films promoted the greatest water transference resistance.

It is also relevant to take the tissue-film component interactions into account, as the coating could alter the cell tissue, leading to cellular damage and making the coatings useless. For example, coating fresh peeled mandarin segments with HPMC based films led to them having a greater water vapour resistance than uncoated ones, surely due to the modification in the permeability of mandarin segment tissue (Pastor, 2010).

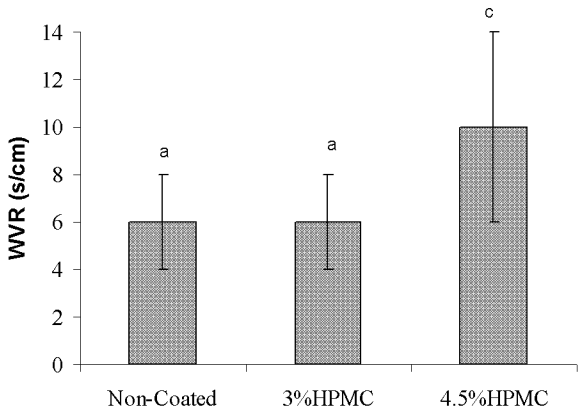

Figure 3. Water vapour resistance of strawberries (*cv. Camarosa*) coated with HPMC based edible coatings. Mean values and standard deviation. Different letters imply 95% significant differences.

3.2. Ripening delay

The changes observed in the ripening process of some coated commodities are usually due to the modification of the respiration rates of the coated product.

If the coating is working properly, the metabolic pathway of the product is slowed down and, as a result, the ripening process is also delayed.

Edible coatings can delay the ripening of fruits and vegetables by modifying their internal atmospheres by means of selective permeability to metabolic gases (decreasing O_2 and/or increasing CO_2, as well as inhibiting ethylene biosynthesis and action). This effect is mainly achieved by blocking a greater or lesser proportion of the pores on the fruit surface (Banks et al., 1997; Hagenmaier and Baker, 1995), and depends on coating composition. The reduction of respiration rates caused by coatings has been described for grapes (Valverde et al., 2005; Pastor et al., 2010b) and other fruits, such as avocado (Maftoonazad and Ramaswamy, 2005) and sweet cherry (Alonso and Alique, 2004).

Choosing a proper coating is complex, because it depends on the specific respiration and transpiration rates of the commodity and outside environmental conditions. Commodities with different skin characteristics might be expected to have very distinct types of interactions with a surface coating. In fact, the level of modification of the internal atmosphere mainly stems from how fruit skin, the coating and the respiration rate of the commodity interact. It is also essential to know the total solid content in the coating formulation (related to the thickness of the film) so that coated products may not have an excessive restriction of gas exchange through the skin, resulting in anaerobiosis and the further development of off-flavors. For instance, Bai et al. (2003) studied coatings for apples and found that although shellac is the best coating for Delicious apples, it caused anaerobiosis in Braeburn and Granny Smith apples. In this way, the coatings developed for one variety of fruit may not be appropriate for another. For each fruit or vegetable, it is

necessary to know the optimum oxygen concentration at which the rate of consumption is minimized without promoting the development of anaerobic respiration.

As shown in Figure 4, coating application led to a significant decrease in the respiration rate of coated tomatoes, especially in terms of oxygen consumption. The respiration quotient was near to one in all cases, thus indicating that the fermentation process has not been promoted by coating application.

Furthermore, slowing down the respiration rate by means of a polymer coating could also explain the delay in the use of some components such as organic acids in the enzymatic reactions of respiration, for example in coated strawberries (Hernandez-Muñoz et al., 2006). In this sense, the titratable acidity of chitosan or starch-based coated strawberries kept in cold storage has been reported to decrease with time, but to a lesser extent than that of uncoated fruit (Garcia et al., 1998a; Zhang and Quantick, 1998)

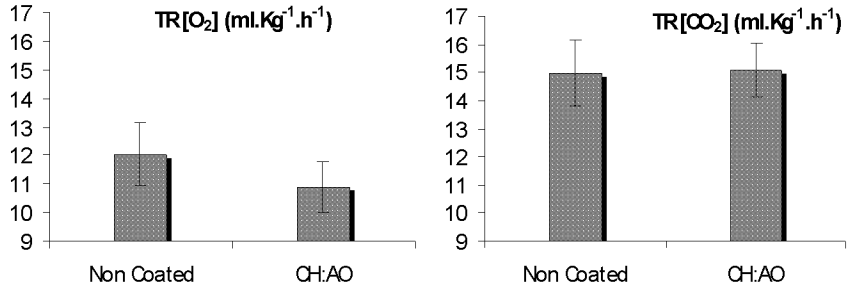

Figure 4. Respiration rates of tomatoes (*cv Cherry*) non coated and coated with chitosan-oleic coatings after two days of storage at 20°C. Mean values and 95% LSD intervals.

Although, Vargas et al. (2008) also observed that the anthocyanin content significantly decreased throughout storage in all chitosan coated strawberries ($p < 0.05$), no significant changes were observed in control samples at the end of the storage period, probably due to the dense structure of the chitosan film which proved to be a very effective gas barrier (Wong et al., 1994).

3.3. Firmness and colour preservation.

Edible coatings can delay fruit senescence, by decreasing both the respiration rate and water loss (as commented on above), which can be evaluated from the changes in the mechanical response and colour development of coated and non-coated samples during storage. Figure 5 shows the resistance to fracture of fresh strawberry, before and after coating, throughout cold storage. Chitosan coated strawberries presented a greater resistance to fracture, surely due to the smaller amount of water lost by the coated samples. In general, coating formulations that minimize weight loss are also better at maintaining firmness, since the firmness attribute is highly influenced by water content. When water is lost, the turgor of the produce decreases, as does firmness.

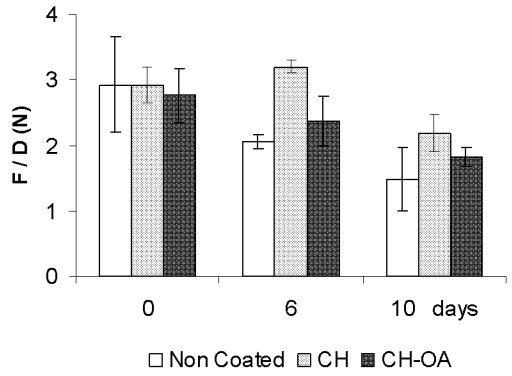

Figure 5. Resistance to fracture of strawberries (*cv. Camarosa*) stored at 5°C as affected by chitosan based coatings. Mean values and 95% LSD intervals (CH: Chitosan; CH-OA: Chitosan-Oleic acid).

The optical properties of a product are usually evaluated in terms of colour coordinates, luminosity, hue and crome (L*, hab*, Cab*), and brightness. When applying coatings, the appearance of the coated commodity depends on the film's optical properties. For instance, Vargas et al. (2006) observed a decrease in the luminosity of chitosan coated strawberries, which became significant ($p < 0.05$) with the addition of oleic acid. This was explained by the changes in the surface reflection properties when the fruit was coated.

Some coatings are not visually perceived and can enhance the product appearance. Figure 6 shows the photograph of uncoated and HPMC coated table grapes, where the great brightness of the coated samples can be observed, which is a consequence of the optical properties of the films, as was commented on above.

Nevertheless, some coatings can modify the optical or colour attributes of the product, leading to a poor appearance which limits its commercial application. This is because the optical properties of the dry film are poor (opaque, with colour, pale…), affecting the commodity's appearance to a greater or lesser extent, depending on the product's surface features.

a) Non-coated b) Coated

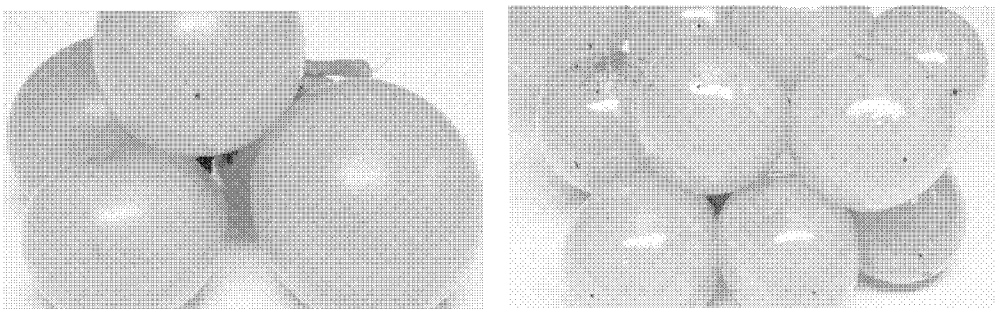

Figure 6. Photographs of a) non-coated and b) hydroxypropylmethyl cellulose table grapes.

When changes in the colour of the commodities are attributed to surface drying, coatings can prevent them by reducing water loss. For instance, the development of the white blush in cut carrot, which is caused, among other factors, by surface dehydration, may be delayed by maintaining an excess of moisture on the carrot's surface. In this sense, the use of highly hydrophilic coatings, which lead to the existence of a wet film that covers the cut surface of fresh-cut carrot, can reduce the incidence of white blush, as can be observed in Figure 7 (Vargas et al., 2009a). This Figure shows the lower white index (WI) of coated cut-carrot in comparison with non-coated ones.

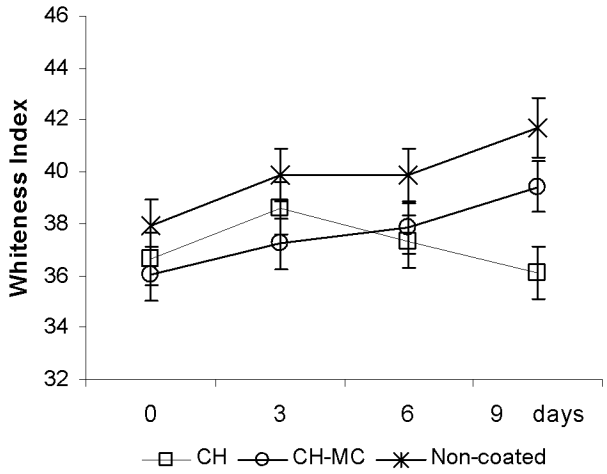

Figure 7. Whiteness index of carrot slices uncoated and coated with chitosan based films during cold storage. Mean values and 95% LSD intervals (CH: Chitosan; CH-MC: Chitosan- hydroxypropylmethylcellulose).

3. 4. Microbial stability

The incorporation of some antimicrobial agents into the coatings could lead to effective antimicrobial activity, promoting the fruit's microbial stability throughout storage. The literature provides some promising examples that use different

antimicrobial agents. Thus, Pastor et al. (2010b) found a lower microbial growth in table grapes coated with HPMC films incorporating propolis than in uncoated samples, for both mesophilic, aerobic counts and yeast and mould counts. Working with table grapes coated with HPMC/CH and CH-bergamot oil based films, Sánchez-González et al. (2010c) found that as far as moulds and yeasts were concerned, coatings with CH and bergamot oil (BO) reduced the initial counts of the samples and coatings with HPMC and BO inhibited growth throughout the whole storage period. Lee et al. (2003) extended the shelf-life of minimally processed apples by means of edible coatings based on carrageenan, whey protein concentrate and carboxymetylcellulose and antibrowning agents such as citric, oxalic and ascorbic acid. These authors showed that edible coating treatment was effective at reducing the levels of microbial count on PCA medium, as neither mesophiles nor psychrotrophs exceeded 10^4 cfu/g for any coated apples after 2 weeks' storage, while the psychrotroph count of non-coated apples was over 106 cfu/g. A carboxymethyl cellulose coating with preservatives and an acidulant seemed to have a synergistic effect on the control of microbial growth on apple slices (Baldwin et al., 1996). Additionally, these coating treatments also showed positive sensory analysis results.

One of the main advantages of using antimicrobial coatings is that the antimicrobial effect is mainly localized on the food surface as opposed to mixing the active compound directly with the food, which is of limited effectiveness owing to the low availability of the antimicrobial substance on the surface where the contamination is prevalent. In this sense, Kristo et al. (2008) demonstrated that sodium caseinate films containing nisin enhanced the antimicrobial activity of nisin and contributed to the long term efficacy of this antimicrobial in *in vitro* studies.

Finally, interactions between coatings and food matrixes have to be studied as the availability of the antimicrobial could also be influenced by them. As usual, correlations between *in vitro* (films) and *in vivo* (applications) antimicrobial studies are not always positive and fundamental research studies are needed to elucidate the established interactions and how they affect the mechanisms of action of the compounds.

3.5. Antioxidant protection

Some coatings can impart an antioxidant protection to the coated commodities, as can be observed in Table 3. In general, the observed antioxidant effect is due to the limitation of oxygen, as a consequence of the low oxygen permeability of the film. Thus, the use of proper coatings can be an alternative to reduce the rate of oxygen dependent chemical reactions.

4. MAIN LIMITATIONS

The main limitations arise from the impact of the coatings on the sensory quality of the product and this is highlighted as one of the factors which is key to the success of this preservation strategy. The most relevant sensory attributes of the coated product are the appearance and flavour. Coated fruits and vegetables usually become less aromatic, as the coatings are a more or less efficient barrier for gasses and volatile compounds. This becomes an important limitation when coating highly aromatic products such as in the case of strawberries (Vargas et al., 2006), but it seems not to be as relevant in other kinds of products, such as citrus fruits.

In studies carried out on table grapes coated with HPMC and HPMC-propolis coatings, Pastor (2010) found that coated grapes were significantly glossier than the uncoated ones. Significant differences in odour and flavour appreciation were found between uncoated samples and those coated with HPMC containing

propolis, due to the impact of the taste of propolis on the overall flavour and odour. Nevertheless, the differences were much less appreciable than those detected when pure propolis was used to cover the grapes directly (unpublished results), which indicates that HPMC encapsulates propolis compounds, thus diminishing their sensory appreciation (Pastor et al., 2010b).

5. CONCLUSION

Edible coatings are considered as one of the most promising technologies, not only in the post-harvest of fruit and vegetables, but also in pre-harvest applications. Recent research has focused on the analysis of the different interactions between film components and the food matrix and on the control of the release of the active agents. There is great possible potential for the application and success of this technology but further effort is needed to find the optimum formulation of natural and biodegradable ingredients which leads to a longer shelf life of fresh and minimally processed fruits or vegetables with an excellent sensory quality.

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USE OF ESSENTIAL OILS IN BIOACTIVE EDIBLE COATINGS – A REVIEW

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ABSTRACT

Antimicrobial and antioxidant properties of essential oils have previously been reviewed extensively. The mechanisms of action of essential oils have not been clearly identified but they seem to be related with their hydrophobic nature. Application of these natural compounds in food industry could be a potential alternative but its application costs and other problems, such as their intense aroma and potential toxicity limit their use in the area of food preservation.

An interesting strategy to reduce doses of essential oils while maintaining their effectiveness could be the incorporation of these natural compounds into bioactive films. This review discusses the use of essential oils as natural antimicrobial and antioxidant compounds as active components in coatings. Advantages and limitations were also reviewed.

Keywords: film, antimicrobial, biodegradable, food preservation.

1. INTRODUCTION

Consumer requirements guide to more natural products, minimally processed. To satisfy these exigencies, one of the major challenges in the food industry consists on the reduction of conventional chemical additives. For this reason the exploitation of natural products from plant origin has been receiving more and more attention.

Among natural antimicrobials, essential oils have been widely studied. Essential oils (EO) present a large spectre of action. Thus, spoilage microorganisms, foodborne and postharvest pathogens were sensible to these antimicrobials (Burt, 2004; Bakkali et al., 2008).

Despite the great potential of essential oils, their use in food preservation remains limited mainly due to their intense aroma and toxicity problems. Changes in the organoleptic properties of the food when these oils are used have been reported by several authors. To minimise the required doses, one interesting option would be the use of edible coatings as vector of these natural compounds.

Edible coatings have recently gained more interest in the food preservation area due to the promising results obtained. Biodegradable films can improve the quality of food products. For instance, in meat preservation Gennadios et al. (1997) reported a reduction of moisture loss and lipid oxidation, leading to an improvement of the product appearance.

This paper details the reasons of the growing interest existing today for essential oils and the effect of its incorporation into bioactive coatings. Examples of the use of bioactive edible films enriched with essential oils in fruit, meat and fish preservation are detailed. Finally, advantages and limitations of this promising technology are discussed.

2. INTEREST OF ESSENTIAL OILS FOR FOOD APPLICATIONS

2.1. Extraction-Composition

Steam distillation is the most commonly used method for producing essential oils. Other technologies exist but remain little used; it is the case of hydrodistillation, microwave or solvent extraction. Supercritical carbon dioxide appears as an interesting method since thermal and hydrolytic degradation of labile compounds is avoided. Moreover with this technology, time process is significantly reduced and the presence of toxic solvent residues avoided. Extraction with liquid carbon dioxide under low temperature and high pressure produces a more natural organoleptic profile but remains expensive (Moyler, 1998).

The extraction method influences both composition and antimicrobial activity of EO. Packiyasothy and Kyle (2002) showed that EO extracted by hexane presented a greater antimicrobial activity than the corresponding steam stilled distilled EO. Bendahou et al. (2008) reported that composition of oregano oil changes with extractions method. Authors compared composition of oregano essential oil obtained with 3 different extraction methods, hydrodistillation, microwave-assisted extraction and solvent-free microwave extraction. An important difference was observed in terms of thymol amount, the level of this component was significantly higher with the last method cited (81.1% instead of 41.6 and 65.4% with hydrodistillation and microwave-assisted extraction respectively). Moreover oregano oil extracted by solvent-free microwave extraction presented an antimicrobial activity more accused than effect of EO obtained by hydrodistillation.

Other factors such as harvesting season, geographical source, ripeness can affect the composition of the EO for same plant specie. Shanjani et al. (2010) showed both the harvesting season and the choice of fresh or dried materials are critical factors for the composition of Juniperus excelsa essential oil. Autumn is the most

desirable season for harvesting both foliage and berry essential oils because yields reached their higher concentrations in this period. Sari et al. (2006) reported that chemical composition, antimicrobial and antioxidant properties of oregano essential oil change with the EO origin. The degree of maturity is equally important; Msaada et al. (2007) studied the EO composition of coriander fruits at three stages of maturity. An accumulation of monoterpene alcohols and ketones was observed during fruit maturation process. UV-A radiation (360 nm) affects equally EO chemical composition (Maffei et al., 1999).

Composition of essential oils

EO contain 85-99% volatile and 1-15% non-volatile components. The volatile constituents are a mixture of terpens, terpenoids and other aromatic and aliphatic constituents, all characterized by low molecular weights (Smith-Palmer et al., 2001; Bakkali et al., 2008). Terpens are made from combinations of several 5 carbon-base (C5) units called isoprene. The main terpens are the monoterpenes (C10) and sesquiterpenes (C15). Terpenoids are terpens containing oxygen. Monoterpenes, formed from the coupling of two isoprene units, are the most representative molecules constituting 90% of the EO. Aromatic compounds are derived from phenylpropane. Aldehydes (cinnamaldehyde), alcohols (cinnamic alcohol), phenols (eugenol), methoxy derivatives (anethole, estragole) and methylene dioxy compounds (myristicine, apiole) are examples of aromatic components.

The major components of a number of EO are presented in Table 1.In this table were equally reported methods used to determine antioxidant properties and microorganisms tested. This information will be expanded later in the document.

Table 1. Latin name, major components and properties of different essential oils.

Common name of EO	Latin name of plant source	Major components	Antioxidant properties	Antifungal properties	Antibacterial properties	References
Bergamot	*Citrus bergamia*	Limonene Linalool	ABTS		Pathogenic bacteria	Moufida and Marzouk (2003) Fisher and Phillips (2006) Mantle et al. (1998)
Cinnamon	*Cinnamomum zeylandicum*	Trans-cinnamaldehyde	ABTS	*Aspergillus* *Fusarium* *Penicillium*	Pathogenic bacteria	Goñi et al. (2009) Mantle et al. (1998) Singh et al. (2007)
Coriander	*Coriandrum sativum* (seeds)	Linalool	DPPH	*Saccahromyces*	Pathogenic bacteria	Msaada et al. (2007) Wangensteen et al. (2004) Delaquis et al. (2002)
Clove	*Syzygium aromaticum*	Eugenol Eugenyl acetate	DPPH	*Aspergillus*	Pathogenic bacteria	Wenqiang et al. (2007) Gülçin et al. (2004) Goñi et al. (2009) Omidbeygi et al. (2007)
Eucalyptus	*Eucalyptus globulus*	Eucalyptol	Thiobarbituric acid DPPH	*Candida* *Saccharomyces* *Rhodotorula*	Pathogenic bacteria	Amakura et al. (2002) Oyedeji et al. (1999) Sacchetti et al. (2005) Delaquis et al. (2002)
Lemon	*Citrus limon*	Limonene Valencene Ocimene	ABTS	*Aspergillus* *Penicillium*	Pathogenic bacteria	Moufida and Marzouk (2003) Mantle et al. (1998) Viuda-Martso et al. (2008) Fisher and Phillips (2006)

(cont.)

Oregano	*Origanum vulgare*	Carvacrol Thymol γ-Terpinene p-Cymene	Thiobarbituric acid DPPH	*Botrytis* *Fusarium* *Clavibacter* *Candida* *Saccharomyces* *Rhodotorula*	Pathogenic bacteria	Kulisic et al. (2004) Souza et al. (2007) Zivanovic et al. (2005)
Rosemary	*Rosemarinus officinalis*	α-pinene Bomyl acetate Camphor 1,8-cineole	ABTS DPPH	*Candida* *Saccharomyces* *Rhodotorula*	Pathogenic bacteria	Mantle et al. (1998) Gachkar et al. (2007) Sacchetti et al. (2005)
Sage	*Salvia officinalis L.*	Camphor α-pinene β-pinene 1,8-cineole α-thujone Borneol Viridiflorol	ABTS	*Candida* *Aspergillus* *Penicillium* *Fusarium*	Pathogenic bacteria	Ben Taarit et al. (2009) Mantle et al. (1998) Pinto et al. (2007) Longaray-Delamare at al. (2007)
Thyme	*Thymus vulgaris*	Thymol Carvacrol γ-Terpinene p-Cymene	Aldehyde Carboxylic acid ABTS DPPH	*Aspergillus* *Candida* *Saccharomyces* *Rhodotorula*	Pathogenic bacteria	Mantle et al. (1998) Bagamboula et al. (2004) Oussalah et al. (2007) Sacchetti et al. (2005)
Tea Tree	*Melaleuca alternifolia*	Terpinen-4-ol γ-Terpinene α-Terpinene 1,8-Cineole	DPPH	*Fusarium* *Pyrenophora* *Candida*	Pathogenic bacteria	Brophy et al. (1998) Kim et al. (2004) Terzi et al. (2007) Juliano et al. (2008) Moreira et al. (2005) Messager et al. (2005)

2.2. Essential oils properties

Essential oils and their components commonly used as flavouring in the food industry, also present interesting antibacterial, antifungal and antioxidant properties. Numerous studies reported antimicrobial and antioxidant effects of essential oils (Table 1).

All components of EO present activity but some studies try to determine what components are responsible for major antioxidant or antimicrobial effect. Carvacrol, thymol, eugenol are for instance the main components responsible for antioxidant activity of basil and thyme oils (Lee et al., 2005).

2.2.1. Cytotoxicity

Even if the EO mechanism of action is not clearly described, it seems that the antimicrobial activity is essentially due to their hydrophobicity. Terpens, the major compounds of EO, have the ability to disrupt and penetrate the lipid structure of the cell wall bacteria, leading to denaturing of proteins and destruction of cell membrane (Turina et al., 2006). Lambert et al. (2001) reported that essential oils present stronger antibacterial properties against food-borne pathogens containing a high percentage of phenolic compounds such as carvacrol, eugenol and thymol. These compounds are able to disintegrate the outer membrane of Gram-negative bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane to ATP. Ultee et al. (2000a) confirmed that cellular membranes became more fluid in presence of carvacrol. This compound forms channels through the membrane by pushing apart the fatty acid chains of the phospholipids, allowing ions to leave the cytoplasm. Lambert et al. (2001) observed a leakage of phosphate ions from *Staphylococcus aureus* and *Pseudomonas aeruginosa* in presence of oregano oil. Eugenol, the main component of clove oil, was equally studied. Thoroski et al. (1989) reported cell wall deterioration and high degree of cell lysis in presence of eugenol.

But not all the volatile components of essential oils present the same mechanism of action. For instance, cinnamaldehyde is not able to induce disruption of cellular membrane but inhibited the activity of *Enterobacter aerogenes* amino acid decarboxylase enzymes involved in cell metabolic pathways (Wendakoon and Sakaguchi, 1995).

As commented above, the destruction of cell membranes leads to cytoplasmic leakage and cell lysis. De Souza et al. (2010) showed that the Origanum vulgare L. essential oil induces an alteration of cell surfaces morphology of *Staphylococcus aureus* with a loss of cytoplasmic material. Action of EO not only affects cytoplasmic membrane but equally mitochondrial membrane. (Rasooli et al., 2006). Morphological changes of bacterial membrane in presence of EO were observed. For instance, the outer membrane of both *Escherichia coli* and *Salmonella typhimurium* disintegrates following exposure to carvacrol and thymol (Helander et al., 1998). In prokaryotic cells, the permeabilization of membranes is associated with loss of ions and reduction of membrane potential, collapse of the proton pump and depletion of the ATP pool (Turina et al., 2006; Di Pasqua et al., 2006). In eukaryotic cells, EO can provoke depolarisation of the mitochondrial membranes by decreasing the membrane potential. Ionic channels and proton pump were affected. Membranes became permeable and cellular lysis was induced.

Moreover it seems that EO present an action on bacterial toxins. De Souza et al. (2010) showed that Origanum vulgare L. essential oil suppresses the synthesis of staphylococcal enterotoxins.

Cytotoxic effects were observed in vitro in most of the microorganisms. Gram-positive bacteria resulted to be slightly more sensitive to EO than Gram-negative bacteria. This difference is certainly due to the relatively impermeable outer membrane that surrounds Gram-negative bacteria (Smith-Palmer et al., 2001). However, not all studies on essential oils conclude than Gram-positive bacteria are

more susceptible. For instance, a study testing 50 commercially available EO against 25 genera didn't found evidence for a difference in sensitivity between Gram-negative and Gram-positive bacteria (Deans and Ritchie, 1987). Dorman and Deans (2000) postulate that individual components of essential oils exhibit different degree of activity against Gram-negative and Gram-positive bacteria, and as the chemical composition of EO vary with different factors as geographical origin and harvesting period, variation in term of composition would be sufficient to explain the variability in the degree of susceptibility of Gram-negative and Gram-positive bacteria.

The total antimicrobial activity of EOs can not be attributed to a mix of the main components which present antimicrobial activity. EOs are complex mixtures of numerous molecules, their biological effects are the result of a synergism of all components. Several studies concluded that essential oils were more effective in terms of antimicrobial activity than the major components mixed (Gill et al., 2002; Mourey and Canillac, 2002). So it seems that minor components play an important role, and certainly phenomena of synergism exist. For instance p-Cymene is not an effective antibacterial when used alone (Dorman and Deans, 2000) but in association with carvacrol synergism was observed against *Bacillus cereus* both *in vitro* and in rice (Ultee et al., 2000b). Another example of synergism was described by Lambert et al. (2001), carvacrol and thymol present an additive effect *in vitro* when tested against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Until now, essentially due to these phenomena of synergism, resistance or adaptation has not been described.

2.2.2. Antioxidant activity

Essential oils include terpenolic and phenolic compounds which present antioxidant activity. Antioxidant activity of EO has been largely studied *in vitro* by

physico-chemical methods. In Table 1 were reported some EO and corresponding tests used to quantify their antioxidant activity. Mantle et al. (1998) determined for instance free radical scavenging properties by three complementary assay procedures, attenuation of the generation of $ABTS^{\bullet+}$ radical, inhibition of iodophenol enhanced chemiluminescence by a horseradish peroxidise/perborate/luminol system and protection of a target enzyme against oxidative damage by $^{\bullet}OH$ or $O_2^{\bullet-}$ generated by $Co^{60}\gamma$ radiolysis. Cinnamon, pimento and bay essential oils showed substantial antioxidant activity. None of the plant extracts tested present significant antioxidant protective activity against $^{\bullet}OH$ or $O_2^{\bullet-}$ species. Antioxidant activity of Citrus oils as bergamot or lemon is rather slight. Other authors use DPPH assay to determine antioxidant capacity, this method is based on the scavenging of the stable DPPH radical by the antioxidant. Mantle et al. (1998) insist on the influence of the method used on the determination of the antioxidant activity. In fact the apparent antioxidant capacity of free radical scavenging agents depends entirely on the assay method used and particular free radical species generated. Therefore it seems important to determine antioxidant activity by several methods. Previous studies mentioned some essential oils possess good antioxidant properties comparable with that of well-known antioxidant butylated hydroxytoluene (BHT) (Politeo et al., 2007; Estévez et al., 2007).

2.3. Use of essential oils for food preservation / purposes in food technology

Essential oils have been largely employed for their properties already observed in nature, i.e., for their antibacterial, antifungal and insecticidal activities. The potential use of EO as natural antimicrobials and antioxidants has been reported in meat, fish, fruits, vegetables and dairy products (Tables 2 and 3). It also is possible to find in the literature a number of potential synergists to increase the EO effectiveness.

Table 2. Examples of essential oils use as natural antimicrobials in foodstuffs

Food group	Food	Essential oil	Microorganisms	References
Meat	Minced beef	Oregano	Natural flora	Skandamis and Nychas (2001)
	Beef filet		*Listeria monocytogenes*	Tsigarida et al. (2000)
	Cooked chicken sausage	Mustard	*Escherichia coli*	Lemay et al. (2002)
	Minced beef	Thyme	*Escherichia coli*	Solomakos et al. (2008)
	Hot dog	Thyme Clove	*Listeria monocytogenes*	Singh et al. (2003)
	Minced mutton	Clove	*Listeria monocytogenes*	Vrinda Menon and Garg (2001)
	Mortadella (bologna-type sausage)	Thyme Rosemary	Natural flora	Viuda-Martos et al. (2010)
Fish	Salmon fillet / Cod fillet	Oregano	*Photobacterium phosphoreum*	Mejlholm and Dalgaard (2002)
	Mediterranean swordfish fillet	Thyme	Natural flora	Kykkidou et al. (2009)
Dairy	Mozzarella cheese	Clove	*Listeria monocytogenes*	Vrinda Menon and Garg (2001)

(cont.)

	Eggplant salad	Oregano	*Escherichia coli* O157:H7	Skandamis and Nychas (2000)
Vegetables	Lettuce Carrot	Oregano Thyme	Natural flora	Gutierrez et al. (2009)
	Tomato paste	Thyme Summer savory Clove	*Aspergillus*	Omidbeygi et al. (2007)
Fruit	Peach	Lemongrass Thyme	*Botrytis* *Penicillium* *Rhizopus*	Arrebola et al. (2010)
	Strawberry	Thyme	*Botrytis* *Rhizopus*	Bhaskara Reddy et al. (1998)
Cereals	Maize grain	Anise Thyme Clove Boldus Poleo	*Aspergillus*	Bluma and Etcheverry (2008)

For instance Tassou et al. (1995) observed a synergism between NaCl and mint oil against *Salmonella enteritidis* and *Listeria monocytogenes*. Several authors reported a synergistic action of nisin and EO or pure components of essential oils (Pol and Smid, 1999; Yamazaki et al., 2004; Solomakos et al., 2008). The combination of thyme essential oil at 0.6% and nisin at 500 or 1000 IU/g showed a synergistic activity against *Listeria monocytogenes* in minced beef during refrigerated storage (Solomakos et al., 2008).

The oxygen availability and temperature also modifies the EO antimicrobial activity. The antibacterial activity of oregano and thyme oils against *Salmonella typhimurium* and *Staphylococcus aureus* was enhanced at low oxygen levels (Paster et al., 1990). Therefore the use of vacuum packing in combination with EO appears as a good foodstuff preservation strategy. Atrea et al. (2009) evaluated the use of vacuum packaging with oregano essential oil as an antimicrobial treatment for shelf-life extension of fresh Mediterranean octopus stored under refrigeration for 23 days. Authors observed that the combination of vacuum packaging with EO (0.4%v/w) permit a shelf-life extension of approximately 17 days in comparison with the untreated fresh product. Frangos et al. (2010) studied the same combination (MAP-oregano essential oil) with salt on the shelf-life of refrigerated trout fillets. This combination permits a significant shelf-life extension, 11-12 days approximately. On the other hand, EOs are showed to be more effective at low temperatures because the higher permeability of the cell membrane at these temperatures, which allows the EOs to dissolve easier in the lipidic bilayer (Lopez-Gomez et al., 2009).

The combination of EO and Modified Atmosphere Packaging (MAP) has been largely documented last years. Kostaki et al. (2009) evaluated the combined effect of MAP and thyme oil on the quality and shelf-life extension of fresh filleted sea bass. The combined used of thyme oil (0.2% v/w) and MAP ($60\%CO_2/30\%N_2/10\%O_2$) permitted a shelf-life extension of 11-12 in comparison

with the fresh product. Chouliara et al. (2007) investigated the combined effect of oregano oil and MAP technology (30%CO_2/70%N_2 and 70%CO_2/30%N_2) on shelf-life extension of fresh chicken meat stored at 4ºC. Oregano oil and MAP exhibited an additive preservation effect; a shelf-life extension of 5-6 days was achieved. Valero et al. (2006b) proved the combination of MAP and eugenol or thymol was an interesting tool to preserve the quality, safety and functional properties of table grapes.

The combination of different EO can equally lead to synergism. Gutierrez et al. (2005) observed oregano in combination with thyme oil shows a greater activity than when assessed individually. More precisely, the combination of oregano with sage or thyme increased the lag phase of *Escherichia coli*, by comparison with the individual EO, but the combined used of oregano and rosemary not induces synergistic effects.

Some considerations must be considered before use of EO in food preservation. One of them is the possible existence of interactions between EO and food components. This problem must be considered in a possible application. Gutierrez et al. (2005) studied interactions of food ingredients with EO. The antimicrobial activity of thyme was increased in high protein concentrations, leading to a significantly longer lag phase from 3 to 12 % of protein with respect to the control ($p<0.05$). Presence of starch modifies equally EO activity, low concentrations of this carbohydrate has a positive influence on the EO antimicrobial activity. The fat content of food products affects equally EO antimicrobial effectiveness. Mejlholm and Dalgaard (2002) suggested that if the EO are dissolved in the lipid phase, they are less available to act on microorganisms present in the aqueous phase. High concentrations of sunflower oil have a negative influence on the antimicrobial activity of oregano and thyme oils. Cava et al. (2007) observed that the antimicrobial activity of cinnamon and clove oils against *Listeria monocytogenes* was reduced in milk samples with higher fat content. Smith-Palmer et al. (2001)

also observed that EO are less effective in products with high fat content, in this case the study was realised in soft cheese with different fat content. Previously mint oil was found to exhibit little antibacterial effect against *Listeria monocytogenes* and *Salmonella enteritidis* in high fat products (Tassou et al., 1995). The pH of food products appears as a non-negligible parameter since EO antimicrobial activity is affected by this parameter. The susceptibility of bacteria to EO increases with lower pH values since the hydrophobicity of EO increases at these pH values, consequently enabling easier dissolution in the lipids of the cell membrane of target bacteria (Juven et al., 1994).

Finally the stability of EO during food processing should be equally considered. Tomaino et al. (2005) studied the influence of heating on antioxidant activity of several EO as clove, thyme or cinnamon; this activity was better maintained if food and more precisely, oil, was heated at 180ºC for 10 minutes.

Table 3. Examples of essential oils use as natural antioxidants in foodstuffs

Food group	Food	Essential oil	References
Meat	Porcine and bovine meat	Oregano Sage	Fasseas et al. (2007)
	Mortadella (bologna-type sausage)	Thyme Rosemary	Viuda-Martos et al. (2010)
Fish	Sea bream fillets	Oregano	Goulas and Kontominas (2007)
Dairy	Butter	*Satureja cilicica*	Ozkan et al. (2007)
Vegetables	Leafy vegetables	Eucalyptus Tea tree Melisa Roomer Clove Lemon	Ponce et al. (2004)
Fruit	Raspberries	Tea tree oil	Chanjirakul et al. (2006)

3. BIOACTIVE EDIBLE COATINGS

3.1. Interest of bioactive edible coatings

Edible films and coatings have been widely studied; various reviews have been written about properties and potential applications of films (Kester and Fenema, 1986; Gennadios et al., 1997; Debeaufort et al., 1998; Morillon et al., 2002).

Edible coatings can act as moisture and gas barriers; they could preserve the colour, texture and moisture of the product. Edible films have received considerable attention in recent years in part because, unlike most of traditional packaging, they are biodegradable and they contribute to reduce environmental pollution. Coatings materials that are currently used include polysaccharides (cellulose derivatives, starch, chitin, gums), proteins (soy, milk, gelatin, corn zein, wheat gluten) and lipids (oils, waxes, resins). The use of plasticizers (sorbitol, glycerol) at minimum amount can be interesting to improve film mechanical properties.

The possibility of incorporating active compounds (antimicrobials, antioxidants, nutraceuticals, flavours, colorants) in polymeric matrices remains one of the main advantages of coatings. The number of recently patents and research articles dealing on active packaging has notably increased. Antimicrobials among the most studied include organic acids (acetic, lactic, propionic, malic), metals (silver), bacteriocins (nisin, lacticin), enzymes (lysozyme, lactoperoxidase), peptides and natural antimicrobials (spices, essential oils, propolis). Combination of several antimicrobials has also been investigated. Antimicrobial coatings inhibit the development of spoilage and pathogenic bacteria by controlled the release of the active compound.

Promising results have been obtained with the use of bioactive coatings alone or in combination with other non-thermal methods as modified atmosphere packaging (Kostaki et al., 2009; Serrano et al., 2008).

3.2. Effect of the incorporation of essential oils into films properties

As commented above, the incorporation of EO into polymeric matrices gives them interesting antimicrobial/antioxidant properties. Examples of bioactive films enriched with essential oils with antimicrobial effectiveness are reported on table 4. The major advantage of this technology is that the diffusion rate of the antimicrobial agent can be slowed down, keeping high concentrations of the active compounds on the product surface (where the contamination is prevalent) for extended periods of time and thus, making the process more effective in reducing the levels of microorganism than when applied directly on the surface of the product via a spray solution. (Qintavall et al., 2002; Kristo et al., 2008). However, further efforts must be conducted to control the diffusion rate of these active compounds to the product surface during storage.

In addition to confer antimicrobial properties to edible films, the incorporation of EO supposes modifications in terms of physical films properties. These modifications usually are similar to those presented when adding more simple lipids to the films matrix (i.e. oleic acid). Nevertheless, interactions established between EO components and the polymeric matrix become more complex and to optimize the composition of bioactive coatings, it's important to take them into account.

Film water vapour permeability (WVP) is a determinant factor to understand moisture exchanges between the coated product and the surrounding environment. Low WVP values are desirable to minimize weight losses in the coated product

which, in turn, also directly affects product firmness and appearance. The incorporation of EO into polymeric matrices leads to an improvement of the film WVP because of the increment in the hydrophobic compound fraction in the film. Usually WVP values linearly with the increase of EO concentration (Sánchez-González et al., 2010a; Sánchez-González et al., 2010b). For instance pure CH films without hydrophobic compounds showed poor moisture barrier properties at 20ºC, but the incorporation of bergamot oil (3%) induces a significant reduction of WVP of nearly 50% (Sánchez-González et al., 2010b).

Oxygen and carbon dioxide permeabilities of coatings are also important film properties. Composite films with EO seem to result to be a better barrier to gases but little information has been found in the literature. Rojas-Graü et al. (2007) reported a slight decreased in oxygen permeability of the films based on alginate - apple puree with lemongrass oil.

The mechanical properties of edible coatings depend on several factors, as the interactions between their components and the polymer matrix are strongly affected by the physical, chemical and temperature conditions, which in turn influence film stability and flexibility. The incorporation of EO into a continuous polymeric matrix decreases its mechanical resistance to fracture because of the structural discontinuities caused by the oil dispersed phase. Elongation at break of pure chitosan films was for instance reduced when cinnamon, tea tree or bergamot oil was incorporated (Ojagh et al., 2010; Sánchez-González et al., 2010a; Sánchez-González et al., 2010b).

Table 4. Examples of films enriched with essential oils with antimicrobial properties

Polymer	Essential oil	Microorganisms	References
Alginate	Garlic oil	*Escherichia coli* *Salmonella typhimurium* *Staphylococcus aureus* *Bacillus cereus*	Pranoto et al. (2005a)
	Oregano, lemongrass, cinnamon oil	*Escherichia coli*	Rojas-Graü et al. (2007)
Whey protein	Oregano oil	Spoilage bacteria	Zinoviadou et al. (2009)
	Oregano, rosemary and garlic oils	*Salmonella enteriditis* *Listeria monocytogenes* *Staphylococcus aureus* *Escherichia coli* *Lactobacillus plantarum*	Seydim and Sarikus (2006)
Chitosan	Garlic oil	*Escherichia coli* *Listeria monocytogenes* *Staphylococcus aureus* *Bacillus cereus*	Pranoto et al. (2005b)
	Oregano oil	*Salmonella typhimurium* *Escherichia coli* *Listeria monocytogenes*	Zivanovic et al. (2005)
	Cinnamon oil	*Escherichia coli* *Listeria monocytogenes* *Lactobacillus plantarum* *Lactobacillus sakei* *Pseudomonas fluorescens*	Ojagh et al. (2010)
	Clove oil	*Escherichia coli* *Pseudomonas fluorescens* *Listeria innocua* *Lactobacillus acidophilus*	Gómez-Estaca et al. (2010)
	Tea tree oil	*Penicillium italicum* *Listeria monocytogenes*	Sánchez-González et al. (2010a)
	Bergamot oil	*Penicillium italicum*	Sánchez-González et al. (2010b)

Moreover, the use of EO induces modifications in terms of film transparency, gloss and colour. The appearance of the coatings is of relevance since their commercial acceptance depends mainly on this attribute. Usually, the incorporation of EO into films decreases their gloss and transparency (Sánchez-González et al., 2010a; Sánchez-González et al., 2010b) due to the increase of the surface roughness of the composite films as a consequence of the migration of droplets or aggregates to the top of the film during film drying, which leads to surface irregularities. Nevertheless, observed differences in terms of colour are not significant when low concentrations of EOs are used in bioactive films (Pranoto et al., 2005; Zinoviadou et al., 2009).

4. APPLCATION OF BIOACTIVE EDIBLE FILMS ENRICHED WITH ESSENTIAL OILS

4.1. Fruits

The use of coatings can be seen to yield relevant results in the field of fruit preservation. Some examples have been reported on Table 5. These coatings usually permit fruits to reduce water loss and slow down respiration rates, while preserving colour and firmness.

The weight loss reduction throughout storage is due to the greater water vapour resistance (parameter that allows us to determine whether the coating has the expected water barrier properties when applied to the product's surface) of coated products, related to the hydrophobic nature of the film when forming a continuous matrix around the product. As commented on above, the hydrophobic nature of EO explains the improvement in terms of weight loss reduction with composite coatings. Thus, du Plooy et al. (2009) reported a significant reduction in terms of weight loss for oranges coated with commercial coating amended with *Lippia*

scaberrima oil. Similar results were observed in table grapes coated with hydroxypropylmethylcellulose / chitosan enriched with bergamot oil (Sánchez-González et al., 2010c).

The changes observed in the ripening process of some coated commodities are usually due to the modification of the respiration rates of the coated product. Edible coatings can delay the ripening of fruits and vegetables by modifying their internal atmospheres by means of selective permeability to metabolic gases (decreasing O_2 and/or increasing CO_2, as well as inhibiting ethylene biosynthesis and action). The reduction of respiration rates caused by coatings has been described for grapes (Sánchez-González et al., 2010c), apples (Rojas-Graü et al., 2007) and fresh-cut melon (Raybaudi-Massilia et al., 2008). Those authors reported a lower oxygen consumption and carbon dioxide production when EO were added to coatings probably due to a major resistance by the coating to gases diffusion due to the lipophilic nature of essential oils.

In general, coating formulations that minimize weight loss are also better at maintaining firmness, since the firmness attribute is highly influenced by water content. Nevertheless, the incorporation of some essential oils as palmarosa and lemongrass into coatings can sometimes affect negatively the fruit firmness. This phenomenon was observed by Raybaudi-Massilia et al. (2008). When high concentrations of EO were used, a decrease of the fresh-cut melon firmness was observed. Authors explain this result by a possible action of cinnamon, lemongrass, palmarosa oils over the cell tissue of the fruit, which produce structural changes.

The coating application on the fruit appearance can affect optical and colour attributes of the product. For instance, Raybaudi-Massilia et al. (2008) observed that high concentrations of EO tested reduced whiteness of fresh-cut melon during the first hours, nevertheless no significant differences were observed during the storage period.

Sanchez-Gonzalez at al. (2010c) observed that the incorporation of bergamot oil into pure chitosan coatings increased colour saturation of the grapes due to the contribution of coating to the selective light absorption. Authors mentioned that these coatings slightly enhanced the brownish aspect of grapes.

Furthermore the incorporation of EO into coatings leads to effective antimicrobial activity, promoting the fruit's microbial stability throughout storage. The literature provides some promising examples that use bioactive films with different EO to coat fruits and vegetables. Thus Bosquez-Molina et al. (2010) evaluated the antimicrobial effect of thyme and Mexican lime oils incorporated in mesquite gum against *Colletotrichum gloeosporioides* and *Rhizopus stolonifer* in stored papaya fruit. These coatings allow a reduction of decay induce by tested microorganisms by up to 50 and 40% compared with the 100% infection observed with non-treated papayas. Working with table grapes coated with HPMC/CH and bergamot oil (BO) based films, Sánchez-González et al. (2010c) found that as far as moulds and yeasts were concerned, coatings with CH and BO reduced the initial counts of the samples, and coatings with HPMC and BO inhibited growth throughout the whole storage period. Concerning fresh-cut products, Raybaudi-Massilia et al. (2008) reported a significant extension of the shelf-life of melon, more than 21 days, with the use of alginate based coatings enriched with cinnamon leaf, palmarosa or lemongrass oils.

However, when applying bioactive coatings containing EO to fruits and vegetables one of the limiting factors is the impact of such components in the sensory characteristics of the coated products, mainly due to the great amount of volatile compounds of the EO which mask the natural flavour of fruits and vegetables. In this sense, Rojas-Graü et al. (2007) evaluated the sensory quality of fresh-cut apples coated with edible coatings based on apple puree and alginate and containing lemongrass oil and oregano essential oil. Sensory analyses indicated that oregano essential oil led to a decrease in the overall preference of samples and

residual aromatic herbal taste was detected after 2 weeks of storage, despite the low concentration of oregano oil used (0.1% w/w). Raybaudi-Massilia et al. (2008) mentioned the incorporation of cinnamon oil induces lower acceptation of fresh-cut melon in comparison with palmarosa or lemongrass oil.

The use of compatible EO-foodstuff could be also a good alternative, i.e., composite films based on citrus EOs (lemon, bergamot..) applied to lemon, orange or grapefruit to minimize the sensory impact of essential oils on fruits.

Table 5. Application of bioactive edible films containing EO in food preservation

Essential oil	Coating composition	Application	References
Bergamot	Chitosan / Hydroxypropyl methylcellulose	Table grapes	Sánchez-González et al. (2010c)
Cinnamon leaf (*Cinnamomum zeylanicum*), palmarosa (*Cymbopogon martini*), lemongrass (*Cymbopogon citrates*)	Alginate	Melon	Raybaudi-Massilia et al. (2008)
Oregano, lemongrass	Alginate with apple puree	Apple	Rojas-Graü et al. (2007)
Thyme, Mexican lime	Mesquite gum	Papaya	Bosquez-Molina et al. (2010)
Lippia scaberrima	Commercial coating (Carnauba Tropical®)	Oranges	du Plooy et al. (2009)
Rosemary, oregano, olive, capsicum, garlic, onion, cranberry	Chitosan	Squash slices	Ponce et al. (2008)
Oregano	Chitosan, Tween 20	Bologna	Chi et al. (2006)
Anise, basil, coriander and oregano	Chitosan, Tween 20	Bologna	Zivanovic et al. (2005)
Pimento and oregano	Calcium caseinate, whey protein isolates, Carboxymethylcellulose, modified starch	Beef	Oussalah et al. (2004)
Oregano	Whey protein isolate	Beef	Zinoviadou et al. (2009)
Cilantro	Gelatine gel	Vacuum packed ham	Gill et al. (2002)
Thyme, oregano	Soy protein isolate	Ground Beef	Emiroğlu et al. (2010)
Cinnamon	Alginate	Snakehead fish fillets	Lu et al. (2010)
Cinnamon	Chitosan	Trout	Ojagh et al. (2009)
Rosemary, oregano	Gelatine	Sardine	Gómez-Estaca et al. (2007)
Clove	Gelatine - chitosan	Cod fillets	Gómez-Estaca et al. (2010)

4.2. Meat and fish

Although the use of bioactive coatings enriched with EO appears as a promising technology in fish and meat preservation, few studies have been published to date. Some examples have been reported on Table 5. The application of such coatings usually led to a reduction or inhibition of microbial growth and an extension of the shelf-life of the coated product. Moreover, in meat and seafood, edible coatings enriched with EO lead to a decrease in lipid oxidation, without compromising significantly the sensory quality of the coated product (Ojagh et al., 2009). For instance, Oussalah et al. (2004) evaluated the ability of a milk protein-based film containing 1% essential oils of oregano, pimiento or a mixture of both to control *Pseudomonas* spp. And *E.Coli* 0157:H7 growth on surface-inoculated beef muscle. Results showed that films containing essentials oils reduced the growth of microorganism during seven storage days, when compared to the controls (coated beef with free-essential oil films and non-coated samples). Moreover, the most effective films against both bacteria were those incorporating oregano EO, while pimiento-based films presented the highest antioxidant activity.

5. BENEFITS AND LIMITATIONS OF ESSENTIAL OIL USE

5.1. Benefits

The use of EO can be beneficial for human health. According to Clark (2002), antioxidants and more precisely EO are antimutagenic and anticarcinogenic due to their radical scavenging properties (Surh, 2002; Ferguson et al., 2004; Collins, 2005). The dose seems to be an important parameter since at high concentrations problems of cellular DNA damage can appear. Cardile et al. (2009) showed the EO from *Salvia bracteata* and *Salvia rubifolia* used at non-toxic concentrations in normal cells exhibited an inhibitory effect on human cancer cells (M14 human

melanoma cells). Menichini et al. (2009) reported equally an antitumor activity for EO from *Teucrium* in addition of its anti-inflammatory effect.

Moreover EO by intermediate of their volatile compounds as terpens, terpenoids and phenolic compounds can act as prooxidants (Fujisawa et al., 2002). Atsumi et al. (2005) equally observed that some components of EO, eugenol and isoeugenol, present prooxidant and antioxidant activities.

5.2. Limitations

5.2.1. Organoleptic aspects

One of the major limitations to the use of EO in food preservation is the persistence of strong aromas which could affect the organoleptic properties of foodstuff. Sensory tests must be carried out using instrumental analysis or trained individuals to evaluate the acceptability of the product. Chouliara et al. (2007) observed that oregano oil at a concentration of 1% in combination with MAP technology imparted a very strong taste to fresh chicken breast meat stored at 4ºC. Gutierrez et al. (2009) evaluated the efficacy of oregano and thyme essential oils for control the natural spoilage microflora on ready-to-eat lettuce and carrots. Even if microbiological results were positive, sensory quality remained a problem. So, panellists rejected lettuce washed with the EO treatments at the end of the storage period for overall appreciation.

Combining EO with other natural preservatives might minimize doses and reduce the impact on organoleptic properties of food products. Dose of EO is important in term of toxicity and sensory quality product. Kostaki et al. (2009) reported the presence of thyme oil improved the sensory quality of sea bass fillets when used in combination with MAP technology.

Valero and Giner (2006a) suggested that at low concentrations (inferior to 6 µl.100 ml^{-1}) cinnamaldehyde enhanced the taste of carrot broth without inducing adverse effect on the taste or smell of the product.

5.2.2. Toxicity

EO are generally recognised as safe (GRAS) at flavouring concentrations. Several studies reported problems of toxicity with EO (Peter, 2004). Carson and Riley (1993) reported the acute oral toxicity for tea tree essential oil (1.9-2.6 $mL.Kg^{-1}$). This toxicity is similar for other EO such as eucalyptus and authors indicate these EO should not be administrated orally (Altman, 1990). It seems that cinnamaldehyde, carvacrol, carvone and thymol didn't present significant effects in vivo in spite in vitro behaviour is completely different; a non-negligible toxic effect at cellular level was observed (Stammati et al., 1999).

In 2005 a scientific based guide was published to evaluate the safety of naturally occurring mixtures, particularly EO, for their use as flavour ingredients (Smith et al., 2005). The approach relies on the complete chemical characterization of the EO. Different components are classified in chemical groups and the safety of the intake of each group from consumption of the EO is evaluated according to data on absorption, metabolism and toxicology of members of the chemical groups. But ingestion of higher doses of these natural compounds can induce serious problems of oral toxicity. It is necessary to find a balance between the effective EO dose and the risk of toxicity. Dusan et al. (2006) showed that EO doses with the ability of completely inhibited bacterial growth (0.05%) present a relatively high cytotoxicity to intestinal-like cells cultured *in vitro*. The incidence of both necrotic and apoptotic cells in the Caco-2 population significantly increase. Lower doses (0.01%) present a limited antimicrobial activity but their damaging effect on Caco-2 cells is modest.

Moreover it seems that some EO can induce problems of allergy and particularly allergic contact dermatitis (Carson and Riley, 2001). This problem is related with the lipophilic nature of EO and their capacity to penetrate skin. Altman (1990) observed irritation problems when oil was applied to intact and abraded skin.

Problems of carcinogenicity can occurred with some EO. Estragole, one of the constituent of *Ocimum basilicum* and *Artemisia dracunculus* essential oils, presents carcinogenic properties in rat and mouse (Miller et al., 1983; Anthony et al., 1987). Another example described in the literature is limonene, a monoterpene largely represented in Citrus oils (NTP, 1990).

5.2.3. Economical aspects

The use of EO in foodstuffs preservation remains expensive. Incorporation of these natural compounds on formulation of coating appears as a good strategy to reduce cost of application since EO amounts can be reduced.

5.2.4. Legal aspects

The International Standard ISO 9235:1997 deals with aromatic natural raw materials. An official definition of essential oils is presented. ISO standards more specific are equally available. For instance the standards ISO 3520:1998 and 4730:2004 specify some characteristics of bergamot and tea tree oils essentially in terms of chemical composition.

The use of some components of EO only as flavourings in foodstuffs is authorised by European Union. The Council Directive 88/388/EEC sets out the definition of flavourings, the general rules for their use, the requirements for labelling and the maximum levels authorised. A positive list of authorised flavouring substances is available (Commission Decision 1999/217/EC). The use of limonene, carvacrol and linalool is for instance permitted.

6. FUTURE TRENDS

Among active packaging, edible films enriched with essential oils offer many possibilities in food preservation area. New applications of these bioactive coatings

are currently studied to scale laboratory. Among these applications, nuts (almond, walnuts) can be cited for instance. To improve these coatings properties several research lines are currently under study.

Many studies are focused on interactions between polymer and active compounds. It proves important to understand these mechanisms to optimize active coatings formulation.

The incorporation of EO into edible coatings reduces the required quantities to guarantee food safety. However during the drying stage of the film significant losses of volatile compounds occur. Micro and nanoencapsulation of EO could be a solution to minimize this problem and improve the effectiveness of active coatings enriched with essential oils.

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II. OBJETIVOS

1. OBJETIVOS

1.1. OBJETIVO GENERAL

El objetivo general del trabajo consiste en desarrollar, caracterizar y aplicar recubrimientos a base de hidroxipropilmetilcelulosa/quitosano y aceites esenciales para su uso en diferentes sistemas alimentarios. El criterio para el diseño y selección de estos recubrimientos será la optimización de las propiedades fisicoquímicas y antimicrobianas del film teniendo en cuenta las variables: tipo de matriz, y tipo y concentración de aceite.

1.2. OBJETIVOS ESPECÍFICOS

- Diseño de formulaciones formadoras de recubrimiento (FFR) a base de hidroxipropilmetilcelulosa (HPMC) y quitosano (CH) puros y mezclas con diferentes aceites esenciales (bergamota, limón, y árbol de té).

- Caracterización de las FFR, mediante el estudio del comportamiento reológico, de las propiedades superficiales y del tamaño y carga de las partículas dispersas.

- Evaluación de las características de los denominados films (recubrimientos secos y aislados), mediante el estudio de las propiedades de sorción y permeabilidad al vapor de agua, propiedades mecánicas, ópticas, microestructurales y antimicrobianas frente a bacterias Gram positivas, Gram negativas y hongos.

- Análisis conjunto de todas las propiedades caracterizadas en las FFR y films en aras a definir el efecto de las variables (tipo de matriz y tipo y concentración de aceite) sobre las mismas y así poder seleccionar y adecuar a determinadas aplicaciones.

- Análisis de los mecanismos difusionales de los componentes activos de los aceites esenciales desde la matriz polimérica hacia el alimento y de la pérdida de componentes volátiles. Este estudio se llevó a cabo en films previamente seleccionados por su mejor funcionalidad sobre alimentos modelos recomendados por la EFSA.

- Aplicación de recubrimiento a base de aceites esenciales, seleccionados por su funcionalidad y adecuación al fruto, en la calidad y seguridad de uvas almacenadas en refrigeración. Para ello se determinan los principales parámetros de calidad de las uvas recubiertas (pérdida de peso, composición, propiedades ópticas y mecánicas), actividad metabólica y alteración microbiológica.

III. RESULTADOS Y DISCUSIÓN

Los resultados se han organizado en cuatro capítulos. En el **primer capitulo**, se han caracterizados films a base de polisacáridos (hidroxipropilmetilcelulosa y quitosano) y aceites esenciales (árbol de té, bergamota) en cuanto a propiedades de las formulaciones formadoras de recubrimientos (FFR) y de los films secos aislados. Se analizaron la densidad, tamaño de partícula, pH, potencial-ζ y propiedades reológicas de las FFR. En cuanto a los films aislados, se caracterizaron sus propiedades mecánicas, ópticas, barrera al vapor de agua (isotermas de sorción, permeabilidad al vapor de agua) y microestructura. Se evaluó la efectividad antimicrobiana de algunos de estos films frente a las cepas *Penicillium italicum* y *Listeria monocytogenes*.

El **capitulo II** tiene como objetivo general la determinación de la influencia del tipo de matriz, naturaleza y concentración de aceite esencial sobre las propiedades de las FFR y films aislados. Para la consecución de este objetivo, se diseñaron recubrimientos a base de hidroxipropilmetilcelulosa y quitosano al 1% enriquecidos con diferentes niveles de aceites esenciales (árbol de té, bergamota, limón). Se utilizo como técnica estadística multivariable, un análisis discriminante para analizar la existencia de diferencias significativas entre los diferentes tipos de FFR y films. Para complementar este estudio sobre el efecto del tipo de matriz y aceite esencial, se analizó la efectividad antimicrobiana de dichos films frente a tres patógenos (*Escherichia coli, Listeria monocytogenes, Staphylococcus aureus*). Los resultados obtenidos sentaron las bases del diseño y selección de los films a utilizar y/o aplicar en los siguientes capítulos.

El gran carácter volátil de los compuestos activos de los aceites esenciales hace necesario profundizar en el análisis de los fenómenos de difusión desde la matriz polimérica hacia el alimento. Este análisis se realizó en films a base de quitosano y aceite esencial de bergamota que demostraron una mejor funcionalidad y

aplicabilidad. El estudio del comportamiento de estos films se realizó en cinco alimentos modelos recomendados por la EFSA (agua destilada, iso-octano y etanol diluido al 10, 50 y 95%) y se presenta en el **capitulo III**. El análisis de la difusión hacia los alimentos modelos se realizó a través del seguimiento, por cromatografía de gases, del limoneno, compuesto mayoritario del aceite esencial de bergamota. Los resultados se modelizaron mediante la segunda ley de Fick, determinándose los coeficientes de difusión de este compuesto para cada tipo de film y alimento modelo. Además también se evaluaron, mediante cromatografía, las perdidas de volátiles durante el secado de estos films.

Por ultimo, el **capitulo IV** consta de un artículo de aplicación de recubrimientos a base de hidroxipropimetilcelulosa / quitosano y aceite esencial de bergamota en la conservación refrigerada de uva de mesa, var. *Moscatel*, y tras un período de mercado de 2 días. Para ello, se caracterizaron los principales parámetros de calidad del fruto a diferentes tiempos de almacenamiento como la pérdida de peso, composición, propiedades ópticas y mecánicas, actividad respiratoria y análisis microbiológico. En este trabajo, se evalúo el efecto del tipo de matriz y tiempo de almacenamiento en las propiedades caracterizadas así como su posible correlación con las propiedades obtenidas en los films secos aislados.

Capítulo I

Propiedades fisicoquimicas y poder antimicrobiano de films a base de polisacáridos y aceites esenciales

CHARACTERIZATION OF EDIBLE FILMS BASED ON HYDROXYPROPYLMETHYLCELLULOSE AND TEA TREE ESSENTIAL OIL

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ABSTRACT

Edible films based on hydroxypropylmethylcellulose (HPMC) and different concentrations of tea tree essential oil (TTO) were prepared. Film-forming dispersions (FFD) were characterized in terms of rheological properties, particle size distribution and ζ-potential. In order to study the impact of the incorporation of TTO into the HPMC matrix, the water sorption isotherms, water vapour permeability (WVP), mechanical and optical properties of the dry films were evaluated. Results showed that the increase in TTO content promoted significant changes in the size and surface charge of the FFD particles. With regards to the film properties, the higher the TTO content, the lower the WVP and the moisture sorption capacity. In general, the addition of TTO into the HPMC matrix leads to a significant decrease in gloss and transparency and a decrease in the tensile strength and elastic modulus of the composite films. The properties of the films were related with their microstructure, which was observed by SEM.

Keywords: water vapour permeability, transparency, mechanical properties, microstructure, particle size distribution, ζ-potential.

1. INTRODUCTION

One of the current trends in the food industry consists of the substitution of chemical additives for natural compounds, especially in the area of food preservation. In response to this consumer requirement, the innovative concept of active packaging appears to be an interesting strategy. So, the development of bioactive edible coatings containing biodegradable polymers, such as cellulosic derivatives, combined with natural antimicrobial and/or antioxidant compounds is one of the most promising technologies for maintaining the quality and the safety of food products during the storage period. Recently, the exploitation of natural products of plant origin has been receiving more and more attention. For instance, essential oils compounds, which have a well documented antimicrobial activity against spoilage microorganisms, foodborne and postharvest pathogens, are of great potential use in bioactive coatings (Burt, 2004; Bakkali, Averbeck, Averbeck & Idaomar, 2008).

The essential oil of Melaleuca alternifolia, also known as tea tree oil (TTO), is widely available and has been investigated as a possible antimicrobial and antioxidant agent (Kim, Chen, Wu, Wang, Chung & Jin, 2004). Recent studies emphasise the wide spectrum of action of TTO, which is effective against fungi, yeasts, viruses and bacteria (Carson & Riley, 1993). TTO is a complex mixture of terpen hydrocarbons and tertiary alcohols and its composition is regulated by an international standard which sets levels of 14 components (Hammer, Carson, Riley & Nielsen, 2006). The main compounds responsible for the antimicrobial activity are terpinen-4-ol and 1.8-cineole. The mechanisms of action have not been clearly identified but they seem to be related with the hydrophobic nature of the terpens (Burt, 2004; Bakkali, Averbeck, Averbeck & Idaomar, 2008).

Cellulose derivatives are interesting film forming compounds, as they are odourless, tasteless and biodegradable (Krochta & Mulder-Johnston, 1997). In

addition, their cost of application is low. Hydroxypropylmethylcellulose (HPMC) presents excellent film-forming properties (Nisperos-Carriedo, 1994; Villalobos, Hernández-Muñoz & Chiralt, 2006), with very efficient oxygen, carbon dioxide and lipid barriers. However, HPMC films are highly permeable to water vapour, which is an important drawback that limits its application (Krochta & Mulder-Johnston, 1997), since an effective control of moisture transfer is a desirable property.

In order to improve water barrier properties, lipid compounds such as fatty acids, natural waxes, surfactants and resins are frequently incorporated into hydrocolloid-based films (Kester & Fennema, 1986; Baldwin, Nisperos, Hagenmaier, & Baker, 1997; Fabra, Talens, & Chiralt, 2008; Vargas, Albors, Chiralt & González-Martínez, 2009). For instance, the properties of HPMC-based films have been improved by incorporating surfactants (Villalobos, Hernández-Muñoz & Chiralt, 2006). This decreased the equilibrium moisture contents of the films and improved their water barrier properties.

In films containing lipids, the stability of the film forming dispersion (FFD) greatly affects the final microstructure of the film matrix, which defines its properties to a greater extent. In this sense, the characterization of some properties of the FFD contributes to the understanding of the differences in the final film properties. In particular, the surface charge of the particles and their size distribution will greatly affect the further development of the system during the film drying process since emulsion destabilization phenomena depend heavily on these factors (Morillon, Debeaufort, Blond, Capelle, & Voilley, 2002). The analysis of rheological behaviour of FFDs is useful not only to establish stability criteria, but also to define the technique used when FFDs are applied to a particular product (Fellows, 1990).

The properties of a film which are relevant when defining its suitability for a specific target application are barrier (water, oxygen, CO_2, etc.), mechanical and optical properties. The latter affect the appearance of the coated product, which is

an important quality factor. In this sense, it is important to ensure that gloss and transparency of the films are in an adequate range.

The aim of this work is to analyse the effect of TTO incorporation on the characteristics of hydroxypropylmethylcellulose-based films such as water sorption, mechanical, optical and barrier properties. Some relevant properties of the film-forming dispersions were also characterized and related with the film properties.

2. MATERIALS AND METHODS

2. 1. Materials

Hydroxypropylmethylcellulose (HPMC, E464, Methocel Food grade, Dow Chemical Company, Midland, USA), tea trea essential oil, supplied by Herbes del Molí (Alicante, Spain) and Tween 85 (Fluka, Sigma Aldrich, Madrid, España), were used to prepare the film-forming dispersions.

2.2. Preparation of the film-forming dispersions

Hydroxypropylmethylcellulose 5% wt was dispersed in deionised water at 80ºC. After the dissolution of the polysaccharide, Tween 85 and TTO were added in the ratios indicated in Table 1. HPMC-TTO mixtures were emulsified at room temperature using a rotor-stator homogenizer (Ultraturrax DI 25 basic-Yellowline, Janke & Kunkel, Staufen, Germany) at 13,500 rpm for 4 minutes. These emulsions were vacuum degasified at room temperature with a vacuum pump (Diaphragm vacuum pump, Wertheim, Germany).

Table 1. Composition of the film-forming dispersions (FFD)

FFD	HPMC (%p/p)	Tween 85 (%p/p)	TTO (% p/p)
HPMC	5	-	-
HPMC-0.5TTO	5	0.1	0.5
HPMC-1TTO	5	0.2	1
HPMC-2TTO	5	0.4	2

2.3. Characterization of the film-forming dispersions

Density of the FFD was measured by means of a digital densimeter DA-110M, (Mettler Toledo, Barcelona, Spain). A pH-meter C831 (Consort, Tumhout, Belgium) was used to determine the pH of the FFD at 20ºC.

2.3.1. ζ- potential measurements

In order to perform ζ- potential measurements, FFD were diluted to a droplet concentration of 0.02% TTO using deionised water. ζ-potential was determined by using a Zetasizer nano-Z (Malvern Instruments, Worcestershire, UK). The Smoluchowsky mathematical model was used to convert the electrophoretic mobility measurements into ζ-potential values.

2.3.2. Particle size measurements

Particle size analysis of the FFD was carried out by using a laser diffractometer (Mastersizer 2000, Malvern Instruments, Worcestershire, UK). The samples were diluted in deionised water at 2,000 rpm until an obscuration rate of 10% was obtained. The Mie theory was applied by considering a refractive index of 1.52 and

absorption of 0.1 for TTO. Three samples of each FFD were measured in quintuplicate.

2.3.3. Rheological behaviour

The rheological behaviour of FFD was analysed in triplicate at 25ºC by means of a rotational rheometer (HAAKE Rheostress 1, Thermo Electric Corporation, Karlsruhe, Germany) with a sensor system of coaxial cylinders type Z34DIN Ti. Rheological curves were obtained after a stabilization time of 5 minutes at 25ºC. The shear stress (σ) was measured as a function of shear rate ($\dot{\gamma}$) from 0 to 512 s^{-1}, using 5 minutes to reach the maximum shear rate and another 5 minutes to attain zero shear rate. The power law model (Eq. 1) was applied to determine the consistency index (K) and the flow behaviour index (n). Apparent viscosities were calculated at 100 s^{-1}.

$$\sigma = K \cdot \dot{\gamma}^{n} \qquad \text{(Eq. 1)}$$

2.4. Preparation of films

FFD were poured onto a framed and levelled polytetrafluorethylene (PTFE) plate (ϕ = 15 cm) and were dried in atmospheric conditions for 48 hours. Film thickness was controlled by pouring the amount of FFD that will provide a surface density of solids in the dry films of 56 g/m^2 in all formulations. Dry films were peeled off from the casting surface and preconditioned in desiccators at 20ºC and 54.4% relative humidity (RH) prior to testing. A hand-held digital micrometer (Palmer - Comecta, Spain, ± 0.001 mm) was used to measure film thickness in at least three different points of the same sample.

2.5. Water sorption isotherms

For the water adsorption experiments, film pieces of about 3 cm in diameter were transferred to vacuum chambers containing P_2O_5 to complete drying. Afterwards, film specimens, in triplicate, were placed in hermetic chambers containing oversaturated salt solutions (LiCL, $MgCl_2$, K_2CO_3, $Mg(NO_3)_2$, NaBr, KI, NaCl, KCl) at 20ºC to maintain the relative humidity at a constant level (Greenspan, 1977). Samples were weighed periodically till a constant value ($\Delta m \approx 0.0005$ g) was reached, where the equilibrium was assumed to be achieved (Spiess & Wolf, 1983). Finally, the equilibrium moisture content was determined by drying them to a constant weight in a vacuum oven at 70ºC.

2.6. Water vapour permeability

Water vapour permeability (WVP) was measured in dry film discs (ϕ = 7 cm), which were equilibrated at 54.4% RH and 20ºC, according to the "water method" of the ASTM E-96-95 (ASTM, 1995), using Payne permeability cups (Elcometer SPRL, Hermelle /s Argenteau, Belgium). Deionised water was used inside the testing cup to achieve 100% RH on one side of the film, while an oversaturated magnesium nitrate solution was used to control the RH on the other side of the film. During WVP testing, the side of the film in contact with the PTFE plate was placed in contact with that part of the test cup having the highest RH. This situation tries to simulate the case of a film applied on the wet surface of a fresh cut vegetable or fruit. A fan placed on the top of the cup was used to reduce resistance to water vapour transport. Water vapour transmission rate measurements (WVTR) were performed at 20ºC. To calculate WVTR, the slopes of the steady state period of the curves of weight loss as a function of time were determined by linear regression. For each type of film, WVP measurements were replicated three times and WVP was calculated according to Villalobos, Hernández-Muñoz & Chiralt (2006).

2.7. Mechanical properties

Mechanical properties were measured by using a Texture Analyser TA-XT-plus (Stable Micro Systems, Surrey, UK), with a 50 N load cell equipped with tensile grips (A/TG model). Sample films were cut into 25.4 mm wide and 100 mm long strips, according to the ASTM D-882 standard (ASTM, 2001). Grip separation was set at 50 mm and cross-head speed was 50 mm/min. Tensile strength (TS) and percentage of elongation (% E) at break, and elastic modulus (EM) were evaluated in eight samples from each type of film.

2.8. Optical properties

Gloss was measured using a flat surface gloss meter (Multi-Gloss 268, Minolta, Langenhagen, Germany) at an angle of 60º with respect to the normal to the film surface, according to the ASTM standard D523 (ASTM, 1999). Prior to gloss measurements, films were conditioned in desiccators at 20ºC and 54.4% RH. Gloss measurements were performed over a black matte standard plate and were taken in quintuplicate. Results were expressed as gloss units, relative to a highly polished surface of standard black glass with a value close to 100.

Colour measurements were carried out on film specimens equilibrated at 20ºC and 54.4% RH. The transparency of the films was determined through the surface reflectance spectra in a spectrocolorimeter CM-3600d (Minolta Co, Tokyo, Japan) with a 10 mm illuminated sample area. Measurements were taken from three samples in each formulation by using both a white and a black background. The transparency was determined by applying the Kubelka-Munk theory for multiple scattering to the reflection spectra. As each light flux passes through the layer, it is affected by the absorption coefficient (K) and the scattering coefficient (S). Transparency (K/S) was calculated, as indicated by Hutchings (1999), from the reflectance of the sample layer on a known reflectance background and on an ideal black background. Moreover, CIE-L* a* b* coordinates, (CIE, 1986) were

obtained by the infinite reflection spectra of the samples, using D65 illuminant/10º observer. The whiteness index (WI) of the samples was also calculated by using Eq. 2.

$$WI = 100 - \left((100 - L^*)^2 + a^{*2} + b^{*2} \right)^{0.5} \quad \text{(Eq. 2)}$$

2.9. Scanning electron microscopy

Microstructural analysis of cross-sections of the dry films (previously conditioned in desiccators with P_2O_5 for at least 15 days) was carried out using SEM technique in a JEOL JSM-5410 (Japan) electron microscope. Pieces of 6 x 1mmwere cut from films and mounted in copper stubs. Samples were gold coated and observed using an accelerating voltage of 10 kV.

2.10. Statistical analysis

Results were analysed by multifactor analysis of variance with 95% significance level using Statgraphics®Plus 5.1. Multiple comparisons were performed through 95% Least Significant Difference intervals.

3. RESULTS

3.1. Characterization of the film-forming dispersions

3.1.1. Density, pH, ζ-potential and particle size distribution

Density, pH, and ζ-potential values are reported in Table 2. The incorporation of tea tree oil (TTO) supposed a slight decrease in the density and the pH of the film-forming dispersions (FFD). The pH decrease may be related with the dissociation of some TTO compounds of an acid nature in the aqueous solution.

Table 2. Density (ρ), pH and ζ-potential values at 25 °C. Mean values and standard deviation

FFD	ρ (Kg/m^3)	pH	ζ (mV)
HPMC	1012.9 (0.1)	6.6 (0.2) [a]	-
HPMC-0.5TTO	1011.9 (0.1)	6.67 (0.17) [a,b]	-26.9 (0.8) [a]
HPMC-1TTO	1011.6 (0.1)	6.57 (0.12) [b]	-39 (3) [b]
HPMC-2TTO	1009.9 (0.1)	6.19 (0.19) [c]	-37 (2) [b]

[a, b, c, d] Different superscripts within a column indicate significant differences among formulations ($p < 0.05$).

The typical average particle size distributions of these FFD are plotted in Figure 1.

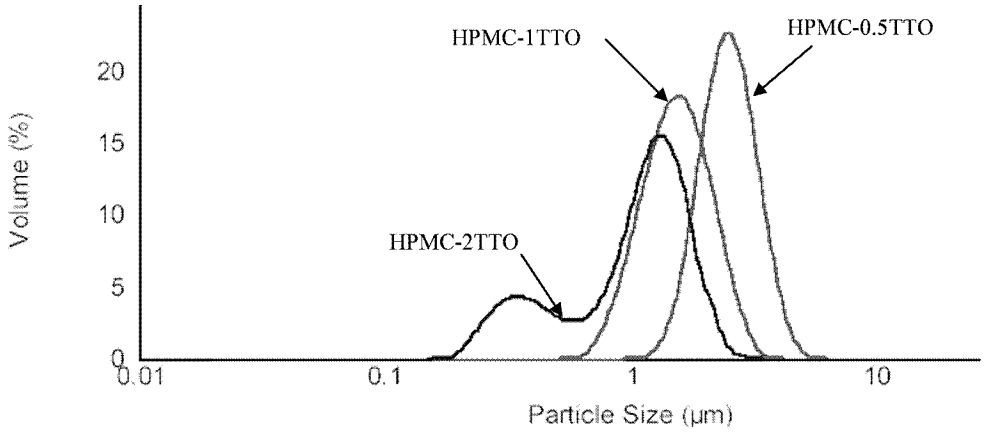

Figure 1. Particle size distribution of HPMC–TTO film-forming dispersions.

Particle size distributions were monomodal except for the formulation containing the highest TTO content, which showed a bimodal trend towards smaller particle sizes. This is also reflected in Table 3, where, for formulation HPMC-2TTO, the

two mean diameters (d_{43}, expressed as volume-length diameter and d_{32}, the area-volume mean diameter) are quite different. As can be observed, mean particle size values significantly decrease with the increase in TTO content ($p < 0.05$). This decrease can be related with the amount of emulsifier present in each case, which was incorporated in quantities proportional to the TTO percentage, which probably exceed the surface excess concentration ($\Gamma\infty$). This was done to assure the total recovery of the TTO droplets with multilayer formation to promote the emulsion stability. Nevertheless, when TTO increases in the mixture, the excess of Tween 85 in the system may lead to the formation of micelles of this compound smaller in size than the TTO droplets (Tcholakova, Denkov, & Danner, 2004; McClements, 2005; Nikiforidis & Kiosseoglou, 2007).

Table 3. Particle size (d_{43} and d_{32}), average surface area (A_N) and number of particles per m^3 (N) of the FFD at 25°C. Mean values and standard deviation.

FFD	d_{43} (μm)	d_{32} (μm)	$A_N \times 10^{-4}$ (m^2)	$N \times 10^{-15}$ (particles/m^3)
HPMC-0.5TTO	2.66 (0.16) [a]	2.46 (0.14) [a]	1.38 (0.08)[a]	0.584 (0.104)[a]
HPMC-1TTO	1.74 (0.15) [b]	1.59 (0.16) [b]	4.3 (0.4)[b]	4.39 (1.19)[b]
HPMC-2TTO	1.12 (0.13) [c]	0.75 (0.15) [c]	18 (3)[c]	32 (9)[c]

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

All dispersions showed a negative charge, as can be observed from the ζ-potential values in Table 2. The same dispersions but surfactant-free, also showed a similar negative charge (of around -2.14 mV). Several authors (Marinova et al., 1996; Hsu and Nacu, 2003; Pashley, 2003) have explained this effect by taking into account

the preferential adsorption of OH^- species from water onto the bare oil droplet surfaces, at pH>5. It is very common to have an appreciable electrical charge in emulsion droplets stabilized by non-ionic surfactants, such as Tween 85. These surfactants follow similar trends to bare oil droplets, but they tend to have more negative charges at the same pH, as has been observed in our experiments. This phenomenon has been attributed to the preferential adsorption of OH^- ions to the hydrophilic head groups of the surfactant (Hsu and Nacu, 2003, McClements, 2005). The electrical properties of oil droplets stabilized with the non-ionic surfactant are dominated by the electrical characteristics of the bare droplets, but are modified somewhat by the presence of the interfacial layer of adsorbed surfactant molecules. The increase in TTO content in the FFD caused a significant increase in the net electrical charge of the particles which can be attributed to a greater amount of the surfactant molecules at the interface (which retain more ions in the polar heads) in agreement with the hypothesis commented on above when analysing the particle size behaviour.

3.1.2. Rheological behaviour

Rheological data were fitted to the Ostwald de Waale model and the model parameters are shown in Table 4, together with apparent viscosity (η_{ap}) values at a shear rate of 100 s^{-1} and 400 s^{-1}. All FFD showed a pseudoplastic behaviour, with n values around 0.92 and no thyxotropic effects were observed from the comparison of the up and down curves. The increase in the TTO content in the FFD did not promote notable changes in the rheological pattern of the FFD in the range of the shear rate analysed. As expected, the apparent viscosity values slightly increased with the increase in TTO content, this being significant ($p<0.05$) for the FFD containing 2% TTO. Apart from the greater volume fraction of the dispersed phase, the smaller droplet size and the higher charge of the dispersed phase can also contribute to the viscosity increase.

Table 4. Ostwald de Waale model parameters and apparent viscosity of the FFD. Mean values and standard deviation.

FFD	$0 \leq \dot{\gamma} \leq 512\ s^{-1}$			
	n	k $(Pa \cdot s)^n$	η_{ap} (100 s^{-1}) (Pa·s)	η_{ap} (400 s^{-1}) (Pa·s)
HPMC	0.929 (0.003)[a]	0.1414 (0.0028)[a]	0.1023 (0.0017)[a]	0.0928 (0.0017)[a]
HPMC-0.5TTO	0.930 (0.004)[a]	0.1497 (0.004)[a]	0.1083 (0.0014)[a]	0.0983 (0.0013)[a]
HPMC-1TTO	0.9264 (0.0015)[a]	0.15 (0.04)[a]	0.1096 (0.0014)[a]	0.0989 (0.0012)[a]
HPMC-2TTO	0.9206 (0.0015)[b]	0.17 (0.04)[b]	0.1169 (0.0014)[b]	0.1047 (0.0016)[b]

[a, b, c, d] Different superscripts within a column indicate 99.99% significant difference among formulations.

3.2. Characteristics of the films

3.2.1. Water sorption isotherms

The water sorption isotherms (equilibrium moisture content in dry basis vs. water activity) of the HPMC and HPMC-TTO composite films at 20ºC are shown in Figure 2. The water sorption curves of HPMC based films are sigmoid in shape, showing a slower increase in equilibrium moisture content till aw 0.6, after which a steep rise in moisture content may be observed, associated to the promotion of solubilisation phenomenon. Similar behaviour has also been observed by other authors in HPMC based films (Sebti, Ham-Pichavant & Coma, 2002; Villalobos, Hernández-Muñoz & Chiralt, 2006; Sebti, Chollet, Degraeve, Noel & Peyrol, 2007). The incorporation of TTO supposed a decrease of the sorption capacity of the films.

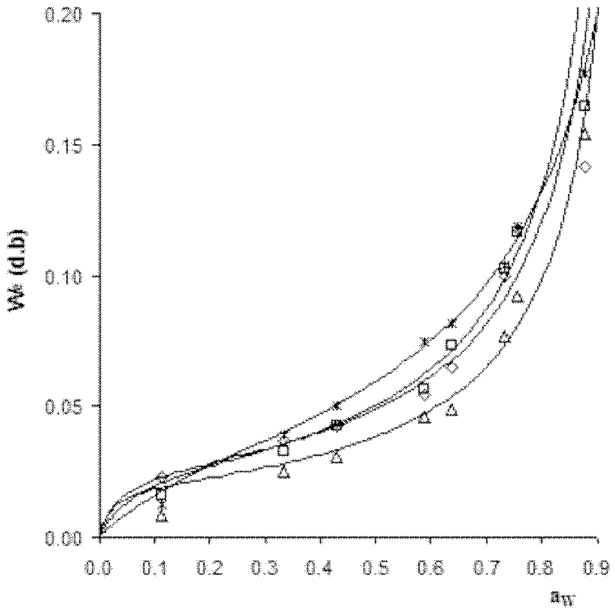

Figure 2. Water sorption isotherms (experimental points and GAB fitted model) of HPMC and HPMC-TTO composite films at 20ºC (∗ HPMC, □ HPMC-0,5TTO, ◇ HPMC-1TTO, □ HPMC-2TTO).

The Guggenheim-Anderson-de Böer (GAB) model (fitted in the entire aw range) was used to fit the water adsorption data of the films at 20ºC. As shown in Table 5 the monolayer moisture content (W_o) diminished with the increase of TTO content in the film. The constant C, related to the water-substrate interaction energy, increased with the incorporation of TTO. This seems to indicate that, as the film becomes more hydrophilic, the water molecules are adsorbed with less energy in the active sites. This behaviour has also been observed by Villalobos, Hernández-Muñoz and Chiralt (2006) in HPMC-surfactant based films.

Table 5. GAB parameters obtained from water sorption isotherms.

Film	T=20°C			
	W_0	C	K	r^2
HPMC	0.039	6.2	0.902	0.830
HPMC-0.5TTO	0.026	21.3	1.007	0.852
HPMC-1TTO	0.025	33.9	0.990	0.806
HPMC-2TTO	0.024	44.9	0.981	0.755

W_0 (g H_20/g d.m.)

In order to analyse the role of TTO in the adsorption behaviour of HPMC in the composite films, the equilibrium moisture content (We) of these films at each aw was compared with the value obtained by applying Eq. 3. In this equation, We was obtained from the equilibrium value of pure HPMC and its ratio in the dried film, by assuming that the lipophilic character of TTO meant that it adsorbed no water molecules.

$$W_{pred}\Big|_{a_w} = X_{HPMC} \cdot W_{eHPMC} \qquad \text{(Eq. 3)}$$

Where,

$W_{pred\,|\,aw}$, equilibrium moisture content of the composite film (d.b.) at a constant a_w.

X_{HPMC}, mass fraction of HPMC in the composite film.

W_{eHPMC}, equilibrium moisture content of pure HPMC film (d.b.) at a constant a_w.

Figure 3 shows experimental versus predicted equilibrium moisture content of HPMC-TTO composite films, where the good distribution of points on the diagonal suggests that TTO did not adsorb water molecules significantly and no appreciable

interactions between TTO and HPMC occur in the film; both components behave as separate phases.

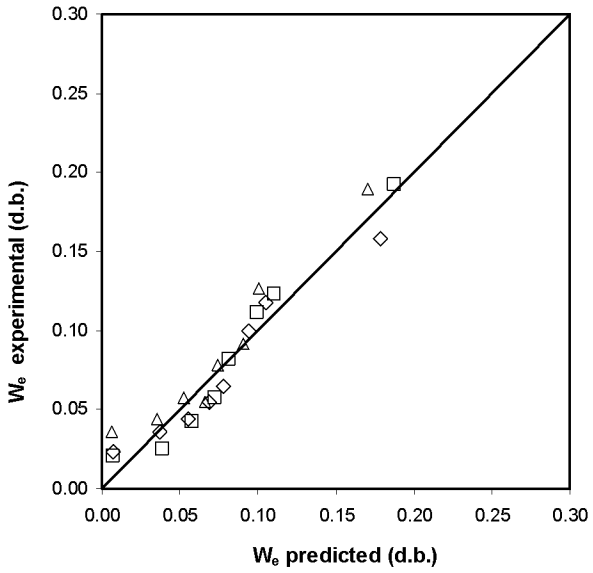

Figure 3. Comparison between experimental values of the equilibrium moisture content (W_e) and those predicted by the model (Eq. 3) for HPMC-TTO composite films (□ HPMC-0.5TTO, ◇ HPMC-1TTO, Δ HPMC-2TTO) at 20°C.

3.2.2. Water vapour permeability

The average thickness of the HPMC and HPMC-TTO composite films, which was used to determine water vapour permeability (WVP), was 44 μm (σ =8 μm). Figure 4 shows the WVP values, at 20°C, as a function of the TTO:HPMC ratio in the film, where a linear reduction was observed as this ratio increased . These values were in the range of those reported by other authors in films based on HPMC (Sebti, Ham-Pichavant, & Coma, 2002; Villalobos, Hernández-Muñoz & Chiralt, 2006). As expected, HPMC films without hydrophobic compounds showed poor moisture barrier properties, but the incorporation of TTO led to an improvement in

the water vapour permeability. The incorporation of 2% TTO caused a reduction of about 30% in WVP at 20ºC.

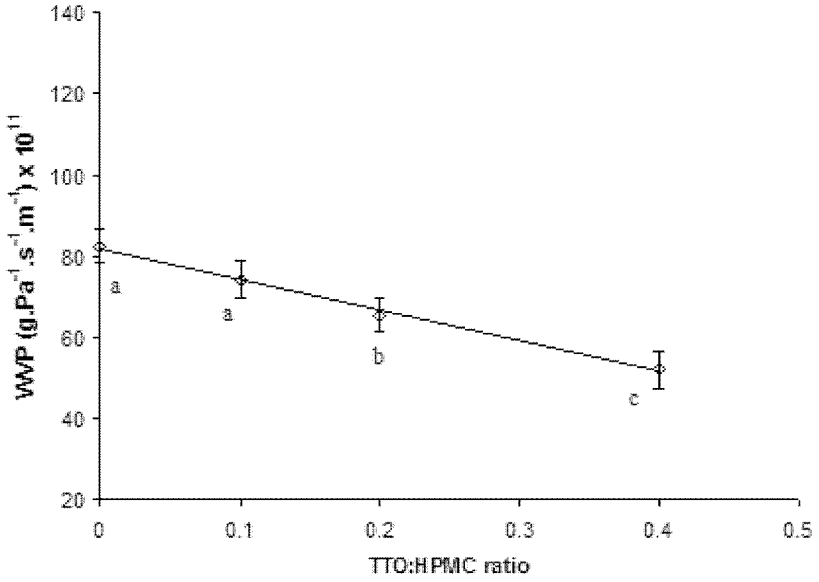

Figure 4. Water vapour permeability of films at 20ºC (100/54.4 RH gradient). Mean values and 95% LSD intervals. Different letters indicate significant differences ($p<0.05$).

3.2.3. Microstructure

The final microstructure that was developed by the different FFD after drying is influenced by the structural arrangement of the different components (HPMC, Tween 85 and TTO) in the initial dispersion, and their development during the drying process, where droplet flocculation, coalescence and creaming can occur. Figure 5 hows SEM micrographs of the cross-sections of the films, which show remarkable differences. While a continuous structure was observed for the HPMC (Figure 5a) film, the presence of TTO (Figure 5b-d) caused discontinuities

associated with the formation of two phases in the matrix: lipid droplets embedded in a continuous polymer network.

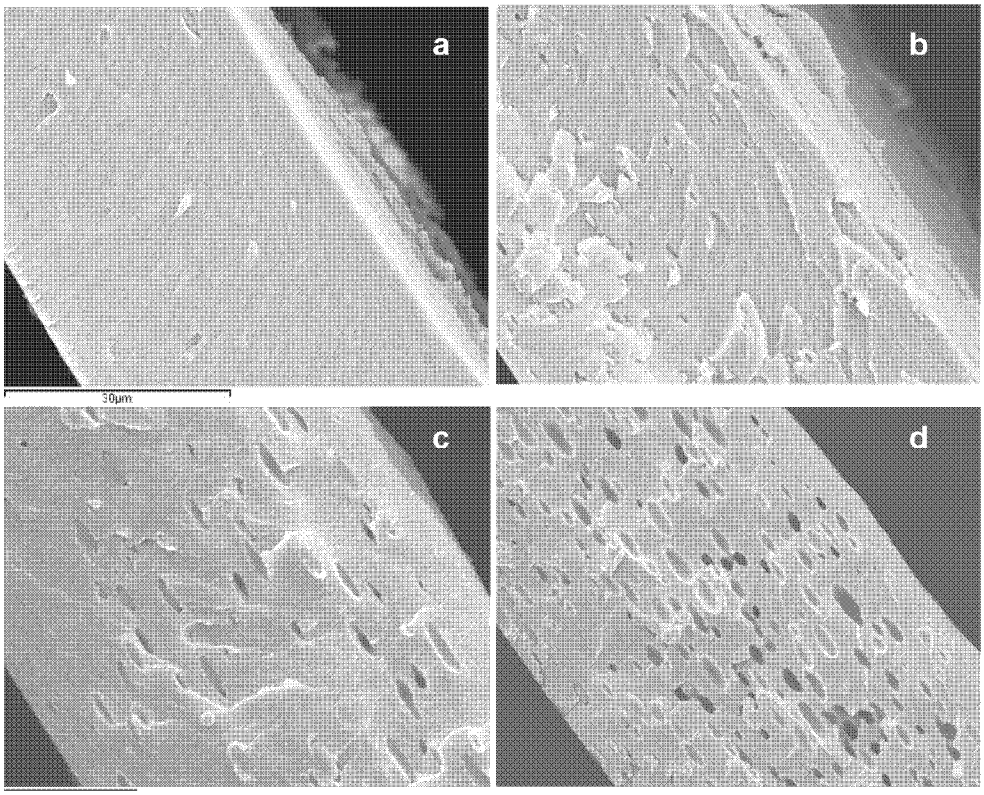

Figure 5. SEM micrographs of the cross-sections of the films. (a) HPMC, (b) HPMC-0.5TTO, (c) HPMC-1TTO, (d) HPMC-2TTO.

Lipid droplets, whose number increased with the TTO concentration, were homogenously distributed across the film. This reveals that very little creaming occurred during the film drying, probably due to the highly viscous effect of HPMC which, moreover, increased when drying progressed. Lipid droplets are

slightly enlarged probably due to the deformation forces that act during the polymer chain aggregation during the solvent evaporation. Despite the greater size of the droplets in the FFD with 0.5% TTO, the film showed the lowest droplet size. This is probably due to the lesser extent in which flocculation and coalescence occur during film drying, in agreement with the lower lipid concentration that limits the flocculation rate.

3.2.4. Optical properties

The gloss and transparency of the films are relevant properties since they have a direct impact on the appearance of the coated product. Film transparency was evaluated through the Kubelka-Munk K/S coefficient, defined as the ratio between light absorption and scattering. An increase in K/S can be assumed as an increase in transparency, although the effect of the selective absorption of components may also be considered (Hutchings, 1999). Figure 6a shows the values of K/S at $\lambda = 450$ nm and 600 nm. In contrast to K/S values measured at $\lambda = 600$ nm where no differences were observed among the samples, K/S values at $\lambda = 450$ nm were significantly affected by the amount of TTO ($p < 0.05$), the composite films with more than 1% of TTO being more opaque than HPMC films. This phenomenon is related with the light scattering provoked by lipid droplets (with a different refractive index) distributed throughout the film network; the higher the droplet concentration, the greater the light scattering intensity. This is also affected by the droplet size.

Figure 6b shows the whiteness index (WI) values, obtained from the infinite reflection spectra, of HPMC and HPMC-TTO composite films. The addition of TTO promoted a significant increase in the WI of the composite films in comparison with the HPMC film.

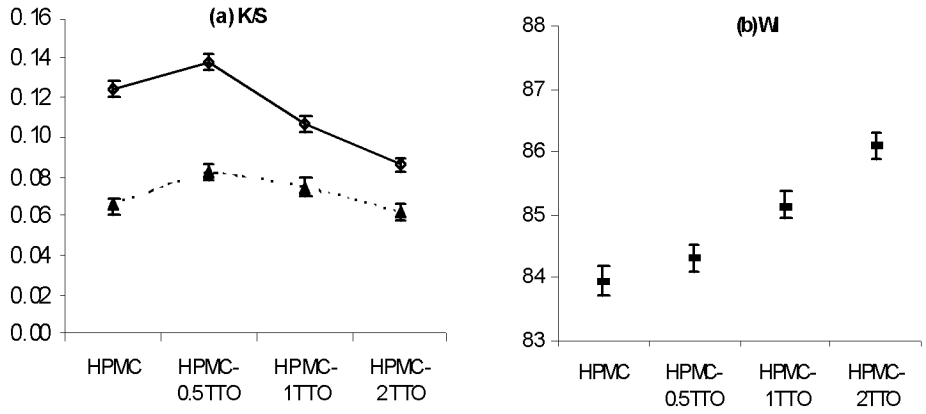

Figure 6. (a) K/S values at 450 nm (continuous line) and 600 nm (dashed line). (b) Whiteness index (WI) of HPMC and HPMC-TTO composite films at 54.4% relative humidity and 20°C. Mean values and 95% LSD intervals. Different letters (a, b, c) indicate significant differences ($p<0.05$).

Other authors have also described the same effect when lipid compounds are incorporated in the films (Yang & Paulson, 2000; Sebti, Chollet, Degraeve, Noel & Peyrol, 2007) and it is due to the increase in diffuse reflectance provoked by light scattering in the lipid droplets; the greater the light scattering intensity, the higher the whiteness index of the film.

The gloss values of the films measured at incidence angle values of 60º are shown in Figure 7.

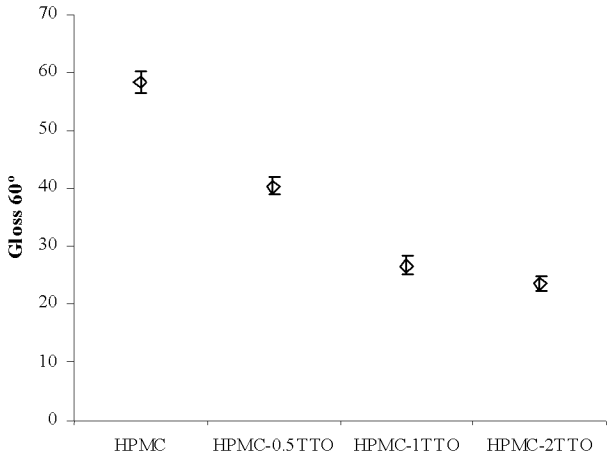

Figure 7. Gloss at 60° of HPMC and HPMC-TTO composite films. Mean values and 95% LSD intervals. Different letters indicate significant differences ($p<0.05$).

The addition of TTO to HPMC matrix supposed a decrease of the gloss as a function of the TTO concentration. Similar results have been obtained by different authors (Trezza & Krochta, 2000; Villalobos, Chanona, Hernández, Gutiérrez & Chiralt, 2005) in composite films containing lipids. The gloss of the films is related with the surface morphology reached during film drying. In general, the smoother the surface, the higher the gloss (Ward & Nussinovich, 1996). In this sense, the decrease in gloss in line with the increase in TTO content could be explained by an increase of the surface roughness of the composite films. This roughness appears as a consequence of the migration of droplets or aggregates to the top of the film which introduces irregularities in the surface. The increase in droplet concentration leads to a great number of irregularities which contribute to reduce the gloss.

3.2.5 Mechanical properties

The influence of TTO incorporation on film mechanical properties can be seen in table 6, which shows the percentage of elongation (E%) and tensile strength (TS) at break and elastic modulus (EM) of films equilibrated at 20ºC, 54.4% RH. These values were in the range of those reported by other authors in films based on HPMC (Sebti, Chollet, Degraeve, Noel & Peyrol, 2007; Li, Martelluci, Bruce, Kinyon, Hay & Higgins, 2002). The addition of TTO in the considered concentration range caused a significant decrease in the elastic modulus and in tensile strength at break, although no significant effect on deformation at break was observed when the TTO ratio increased. This coincides with the results reported by other authors when adding essential oil to a chitosan matrix (Pranoto, Rakshit & Salokhe, 2005; Zivanovic, Chi & Draughon, 2005) and is in agreement with the effect of the structural discontinuities provoked by the incorporation of the oil on the mechanical behaviour. These discontinuities reduced the film's resistance to fracture.

Table 6. Percentage of elongation at break (E), tensile strength (TS) and elastic modulus (EM) of HPMC and HPMC-TTO composite films. Mean values and standard deviation.

Film	E (%)	TS (MPa)	EM (MPa)
HPMC	0.10 (0.06)[a]	59 (6)[a]	1697 (80)[a]
HPMC-0.5TTO	0.09 (0.04)[a]	55 (10)[ab]	1289 (289)[b]
HPMC-1TTO	0.11 (0.05)[a]	52 (9)[ab]	1104 (252)[b]
HPMC-2TTO	0.11 (0.05)[a]	42 (2)[b]	956 (154)[b]

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

4. CONCLUSION

Tea tree essential oil appears as an interesting ingredient for the design of new film-forming dispersions based on hydroxypropyl methylcellulose. The increase of the TTO concentration supposed a change in the particle size of the emulsions without an overly marked increase of viscosity. The incorporation of TTO, at a ratio of 2:5 in the HPMC matrix, reduced the water vapour permeability by 30%, but decreased the film gloss and transparency. Likewise, the HPMC films' resistance to break were slightly reduced by TTO incorporation due to the presence of discontinuities in the film matrix that affect its mechanical response. Future work will be focused on understanding the structural characteristics and phase morphology of this composite film throughout theoretical models that could explain the structural transformation of these compounds in the glass transition region.

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PHYSICAL AND ANTIMICROBIAL PROPERTIES OF CHITOSAN-TEA TREE ESSENTIAL OIL COMPOSITE FILMS

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ABSTRACT

Antimicrobial films were prepared by incorporating different concentrations of tea tree essential oil (TTO) into chitosan (CH) films. Film-forming dispersions (FFD) were characterized in terms of rheological properties, particle size distribution and ζ-potential. In order to study the impact of the incorporation of TTO into the CH matrix, the water vapour permeability (WVP), mechanical and optical properties of the dry films were evaluated. The properties of the films were related with their microstructure, which was observed by SEM. Furthermore, the antimicrobial effectiveness of CH-TTO composite films against *Listeria monocytogenes* and *Penicillium italicum* was studied.

Keyword: *Penicillium italicum, Listeria monocytogenes*, water vapour permeability, microstructure, mechanical properties, particle size distribution, ζ-potential.

1. INTRODUCTION

Current research is trying to develop alternative strategies for reducing the use of chemical additives in the food industry. In this context, the use of natural compounds such as essential oils, with antimicrobial properties appears to be an interesting option. The antibacterial and antifungal activities of essential oils (EOs) have long been acknowledged, but the food industry has recently been paying more and more attention to their application as natural antimicrobials (Du Plooy et al., 2009). The main advantage of EOs application is their greater activity as compared with the effects of the individual active compounds, probably due to the synergistic effects (Bakkali et al., 2008).

The essential oil of *Melaleuca alternifolia*, also named as tea tree oil (TTO), is a complex mixture of terpen hydrocarbons and tertiary alcohols for which an international standard sets levels of 14 components (Hammer et al., 2006). The main compounds responsible for the antimicrobial activity are terpinen-4-ol and 1,8-cineole. Currently, the international standard regulation for TTO sets a minimum content of 30% terpinen-4-ol and a maximum content of 15% 1,8-cineole to meet the European Pharmacopoeia requirements (ISO 4730). The mechanisms of action of TTO have not been clearly identified but they seem to be related with the hydrophobic nature of the terpens (Burt, 2004; Bakkali et al., 2008).

The potential benefits of the uses of TTO are widely known. What is remarkable is how it can be used pharmaceutically, as it represents a good alternative to the most commonly used antifungal drugs, because yeasts often show resistance to them (Bagg et al., 2003; Juliano et al., 2008). In this sense, TTO has been used successfully in the management of oral candidosis in AIDS patients (Vazquez and Zawawi, 2002) and other oral fungal infections in patients suffering from advanced cancer (Bagg et al., 2006). Although TTO is considered as relatively safe, more studies should be undertaken in order to obtain the best type and means of

application, taking advantage of its potential benefits as an antimicrobial and antifungal agent (Juliano et al., 2008).

An interesting strategy to reduce the required doses of TTO while maintaining antimicrobial effectiveness could be by incorporating this into bioactive film coatings which would allow us to fix and retain the compound on the product surface, thus increasing its effectiveness. In these coatings, the major compounds are biodegradable polymers and a relatively reduced amount of EOs can be used. Consequently, the application costs of essential oils and/or other problems, such as the intense aroma and potential toxicity, could be minimised.

Chitosan is an interesting biodegradable polymer. This is a cationic polysaccharide, with an excellent film forming ability (Li et al., 1992). Moreover, this biopolymer possesses a great potential as antimicrobial packaging material owing to its antimicrobial activity and non-toxicity (Jung and Kim, 1999; No et al., 2001; Tharanathan and Kittur, 2003). Sao Pedro et al. (2009) have summarized a reduced number of works that reveal that chitosan acts by entrapping essential oils with diverse applications for food preservation, medical and cosmetic uses. Juliano et al. (2008) demonstrated that chitosan might have a potential medical application in the development of new formulations containing TTO essential oil, showing a synergistic, antimicrobial effect under in vitro conditions against Candida albicans.

Several authors observed in in vitro studies that the incorporation of different essential oil into chitosan matrix improved its antimicrobial properties. Zivanovic et al. (2005) and Pelissary et al. (2009) showed this effect in oregano-chitosan based film against several strains as *Escherichia coli, Listeria monocytogenes, Salmonella enteriditis, Bacillus cereus* and *Staphylococcus aureus*. In the results found by Zivanovic et al. (2005), pure chitosan films reduced *L. monocytogenes* by 2 logs, whereas the films enriched with 1% and 2% oregano oil decreased the numbers of *L. monocytogenes* by 3.6 to 4 logs and *E. coli* by 3 logs. The combination essential oil - chitosan results also effective to maintain microbial

quality of food products (*in vivo*). In this sense, Ojagh et al. (2009) and Giatrakou et al. (2010) observed a good microbial stability of refrigerated rainbow trout and chicken products coated with chitosan - cinnamon oil and chitosan - thyme oil, respectively. Oregano oil associated with chitosan films has also been proved to be suitable for meat preservation (Chami et al., 2005; Chi et al., 2006).

The incorporation of antimicrobial compounds can affect the film properties which are relevant for a specific target application such as acting as a barrier to water vapour, oxygen, CO_2 or aroma compounds and mechanical and optical properties. The latter is an important quality factor as they define the appearance of the coated product. The properties of the film are also affected by the stability of the film-forming dispersion (FFD) which affects the final matrix microstructure of the film and some of its functional properties. In this sense, the surface charge and size distribution of the oil particles and the rheological behaviour of FFDs had to be analysed in order to establish stability criteria and define the best technique for FFD application to a particular product (Fellows, 1990).

The aim of this work is to analyse the effect of TTO incorporation on the properties of chitosan-based films such as antimicrobial, mechanical, optical and barrier properties. Some relevant properties of the film-forming dispersions were also characterized and related with the film properties.

2. METHODS AND MATERIALS

2. 1. Materials

High molecular weight chitosan (CH) with a deacetylation degree of 82.7% (Batch 10305DD, Sigma-Aldrich Química, Madrid, Spain), 98% glacial acetic acid (Panreac, Barcelona, Spain) and tea tree essential oil, supplied by Herbes del Molí (Alicante, Spain), were used to prepare the film-forming dispersions.

2.2. Preparation of the film-forming dispersions

Chitosan (1% w/w) was dispersed in an aqueous solution of glacial acetic acid (0.5%w/w) at 25ºC. After an overnight agitation, tea tree essential oil (TTO) was added to the chitosan (CH) solution to reach a final concentration of 0%, 0.5%, 1% and 2% (w/w). CH-TTO mixtures were emulsified at room temperature using a rotor-stator homogenizer (Ultraturrax DI 25 basic-Yellowline, Janke & Kunkel, Staufen, Germany) at 13,500 rpm for 4 minutes. These emulsions were vacuum degasified at room temperature with a vacuum pump (Diaphragm vacuum pump, Wertheim, Germany).

The experimental design was made taking into account the maximum levels of TTO which could be incorporated into the matrix without oil phase separation during the film drying.

2.3. Characterization of the film-forming dispersions

Density of the FFD was measured by means of a digital densimeter DA-110M, (Mettler Toledo, Barcelona, Spain). A pH-meter C831 (Consort, Tumhout, Belgium) was used to determine the pH of the FFD at 20ºC.

In order to perform ζ- potential measurements, FFDs were diluted to a droplet concentration of 0.02% TTO using an aqueous solution of glacial acetic acid (0.5%w/w). ζ-potential was determined by using a Zetasizer nano-Z (Malvern Instruments, Worcestershire, UK). The Smoluchowsky mathematical model was used to convert the electrophoretic mobility measurements into ζ-potential values.

A particle size analysis of the FFD was carried out by using a laser diffractometer (Mastersizer 2000, Malvern Instruments, Worcestershire, UK). The samples were diluted in deionised water at 2,000 rpm until an obscuration rate of 10% was obtained. The Mie theory was applied by considering a refractive index of 1.52 and absorption of 0.1 for TTO. Three samples of each FFD were measured in quintuplicate. The rheological behaviour of FFD was analysed in triplicate at 25ºC

by means of a rotational rheometer (HAAKE Rheostress 1, Thermo Electric Corporation, Karlsruhe, Germany) with a sensor system of coaxial cylinders type Z34DIN Ti. Rheological curves were obtained after a stabilization time of 5 minutes at 25ºC. The shear stress (σ) was measured as a function of the shear rate ($\dot{\gamma}$) from 0 to 512 s^{-1}, taking 5 minutes to reach the maximum shear rate and another 5 minutes to attain zero shear rate. The power law model (Eq. 1) was applied to determine the consistency index (K) and the flow behaviour index (n). Experimental and predicted flow curves at shear rate of 0-320 s^{-1} were plotted. Apparent viscosities were calculated at 100 s^{-1}.

$$\sigma = K \cdot \dot{\gamma}^{n} \qquad \text{(Eq. 1)}$$

2.4. Preparation and characterization of films

Films were obtained by casting procedure as follows: FFDs were poured onto a framed and levelled polytetrafluorethylene (PTFE) plate (ϕ = 15 cm) and dried under atmospheric conditions for 48 hours. Film thickness was controlled by pouring the amount of FFD that will provide a surface density of solids in the dry films of 56 g/m^2 in all formulations. Dry films were peeled off from the casting surface and preconditioned in desiccators at 20ºC and at 54.4% relative humidity (RH) prior to testing. A hand-held digital micrometer (Palmer - Comecta, Spain, ± 0.001 mm) was used to measure film thickness at least three different points of the same sample.

2.4.1. Water vapour permeability

Water vapour permeability (WVP) was measured in dry film discs (ϕ = 7 cm), which were equilibrated at 54.4% RH and 20ºC, according to the "water method" of the ASTM E-96-95 (ASTM, 1995), using Payne permeability cups (Elcometer

SPRL, Hermelle /s Argenteau, Belgium). Deionised water was used inside the testing cup to reach 100% RH on one side of the film, while an oversaturated magnesium nitrate solution was used to control the RH on the other side of the film. During WVP testing, the side of the film in contact with the PTFE plate was placed in contact with that part of the testing cup with the highest RH. This situation tries to simulate the case of a film applied on the wet surface of a fresh cut vegetable or fruit. A fan placed on the top of the cup was used to reduce resistance to water vapour transport. Water vapour transmission rate measurements (WVTR) were performed at 20ºC. To calculate WVTR, the slopes of the steady state period of the curves of weight loss as a function of time were determined by linear regression. For each type of film, WVP measurements were replicated three times and WVP was calculated following Villalobos et al. (2006).

2.4.2. Mechanical properties

Mechanical properties were measured by using a Texture Analyser TA-XT-plus (Stable Micro Systems, Surrey, UK), with a 50 N load cell equipped with tensile grips (A/TG model). Sample films were cut into 25.4 mm wide and 100 mm long strips, according to the ASTM D-882 standard (ASTM, 2001). Grip separation was set at 50 mm and cross-head speed was 50 mm/min. Tensile strength (TS) and percentage of elongation (% E) at break, and elastic modulus (EM) were evaluated in eight samples from each type of film.

2.4.3. Optical properties

Gloss was measured using a flat surface gloss meter (Multi-Gloss 268, Minolta, Langenhagen, Germany) at an angle of 60º, according to the ASTM standard D523 (ASTM, 1999). Prior to gloss measurements, films were conditioned in desiccators at 20ºC and 54.4% RH. Gloss measurements were performed over a black matte standard plate and were taken in quintuplicate. Results were expressed as gloss

units, relative to a highly polished surface of standard black glass with a value close to 100.

The transparency of the films was determined through the surface reflectance spectra in a spectrocolorimeter CM-3600d (Minolta Co, Tokyo, Japan) with a 10 mm illuminated sample area. Measurements were taken from three samples in each formulation by using both a white and a black background. The transparency was determined by applying the Kubelka-Munk theory for multiple scattering to the reflection spectra. As each light flux passes through the layer, it is affected by the absorption coefficient (K) and the scattering coefficient (S). Transparency (K/S) was calculated, as indicated by Hutchings (1999), from the reflectance of the sample layer on a known reflectance background and on an ideal black background.

2.4.4. Microstructure

Microstructural analysis of cross-sections of the dry films (previously conditioned in desiccators with P_2O_5 for at least 15 days) was carried out using SEM technique in a JEOL JSM-5410 (Japan) electron microscope. Pieces of 6 x 1 mm were cut from films and mounted in copper stubs. Samples were gold coated and observed using an accelerating voltage of 10 kV.

2.4.5. Microbiological analysis

The methodology followed for the determination of antimicrobial effectiveness of films was adapted from Kristo et al. (2008).

Stock culture of *Penicillium italicum* (CECT 2294) and *Listeria monocytogenes* (CECT 932), supplied by Colección Española de Cultivos Tipos (CECT, Burjassot, Spain), were kept frozen (-25ºC) respectively in Potato Dextrose Browth (PDB, Scharlab, Barcelona, Spain) and Tryptone Soy Broth (TSB, Scharlab, Barcelona, Spain) supplemented with 30% glycerol (Panreac, Barcelona, Spain).

The fungus was inoculated on Potato Dextrose Agar (PDA) and incubated at 25ºC until sporulation. The cells were counted in a haemocytometer and diluted to a concentration of 10^5 spores per ml, that is the recommended concentration for the evaluation of the growth of green and blue moulds in postharvest treatments. Aliquots of PDA (20 g) were poured into Petri dishes. After the culture medium solidified, diluted spore solution was inoculated on the surface.

Listeria monocytogenes was regenerated by transferring a loopful of bacteria into 10 mL of TSB and incubating at 37ºC overnight. A 10 µl aliquot from the overnight culture was again transferred to 10 mL of TSB and grown at 37ºC to the end of the exponential phase of growth. This culture, appropriately diluted, was then used for inoculation of the agar plates in order to obtain a target inoculum of 10^2 UFC/cm^2. Tryptone Soy Agar (International Diagnostics, UK) with 3% NaCl (Sigma-Aldrich GmbH, Germany) was used as a model solid food system (TSANaCl). Aliquots of TSANaCl (20 g) were poured into Petri dishes. After the culture medium solidified, properly diluted overnight culture was inoculated on the surface.

The different test films of the same diameter as the Petri dishes (containing or not antimicrobial substance) were placed on the inoculated surface. Inoculated and uncoated TSANaCl and PDA Petri dishes were used as control. Plates were then covered with parafilm to avoid dehydration and stored for 12 days at 20ºC and 10ºC, for the *Penicillium* and *Listeria* strains respectively.

Microbial counts on TSANaCl and PDA plates were examined immediately after the inoculation and periodically during the storage period.

To this end, the agar was removed aseptically from Petri dishes and placed in a sterile plastic bag with 100 ml of tryptone phosphate water (Sharlab, Barcelona, Spain). The bag was homogenized for 2 minutes in a Stomacher blender (Bag Mixer 400, Interscience). Serial dilutions were made and then poured onto PDA or

TSA. PDA and TSA plates were incubated respectively for 5 days at 25ºC and 24 hours at 37ºC before colonies were counted. All tests were run in duplicate.

2.5. Statistical analysis

Results were analysed by means of a multifactor analysis of variance with 95% significance level using Statgraphics®Plus 5.1. Multiple comparisons were performed through 95% Least Significant Difference intervals (LSD).

3. RESULTS AND DISCUSSION

3.1. Characterization of the film-forming dispersions

Density (ρ), rheological characteristics (apparent viscosity at 100 s^{-1}, and power law parameters), particle size and ζ-potential values at 25ºC are reported in Table 1. The pH of every FFD (with or without TTO) had an average value of 4.30. The incorporation of tea tree oil (TTO) led to a logical decrease in the density of the film-forming dispersions (FFD), which was significant from 1% TTO dispersions.

Table 1. FFS characterization: density (ρ), Ostwald de Waale model parameters, apparent viscosity (η_{ap}) at 100 s^{-1}, particle size (d_{43}) and ζ-potential values at 25 °C. Mean values and standard deviation.

FFS	ρ (Kg/m^3)	$0 \leq \dot{\gamma} \leq 512\ s^{-1}$				d_{43} (μm)	ζ (mV)
		n	k (Pa·s)^n	η_{ap} (Pa·s)	r^2 *		
CH	1004.69 (0.14)^a	0.785 (0.007)^a	0.58 (0.02)^a	0.217 (0.002)^a	0.975	-	101 (3)^a
CH-0.5TTO	1004.62 (0.14)^a	0.817 (0.015)^b	0.47 (0.04)^b	0.203 (0.002)^b	0.970	5.73 (0.16)^a	88 (3)^b
CH-1TTO	1003.6 (0.2)^b	0.816 (0.008)^b	0.48 (0.02)^b	0.203 (0.002)^b	0.974	9.88 (0.08)^b	86.9 (1.3)^b
CH-2TTO	896.9 (0.3)^c	0.807 (0.006)^b	0.501 (0.005)^b	0.2013 (0.0015)^b	0.975	14.7 (0.4)^c	79 (3)^c

$^{a, b, c}$ Different superscripts within a column indicate significant differences among formulations ($p < 0.05$).
*correlation coefficient of the fitting.

Figure 2 shows the experimental flow curves (symbols) of the different FFD. Rheological data were fitted to the Ostwald de Waale model and model parameters and the corresponding correlation coefficients (r^2) of the fitting are included in Table 1 together with apparent viscosity (η_{ap}) values at a shear rate of 100 s^{-1}. The values of the correlation coefficient were in all cases around 0.97 and in Figure 2, the close fit of the model can be observed. The addition of TTO promoted a significant change in the rheological pattern of the FFD. The rheological behaviour of dispersions depends on several factors related to the dispersed phase characteristics such as volume concentration, particle size, distribution and shape of the particles and surface electrical charge (Rao, 1977).

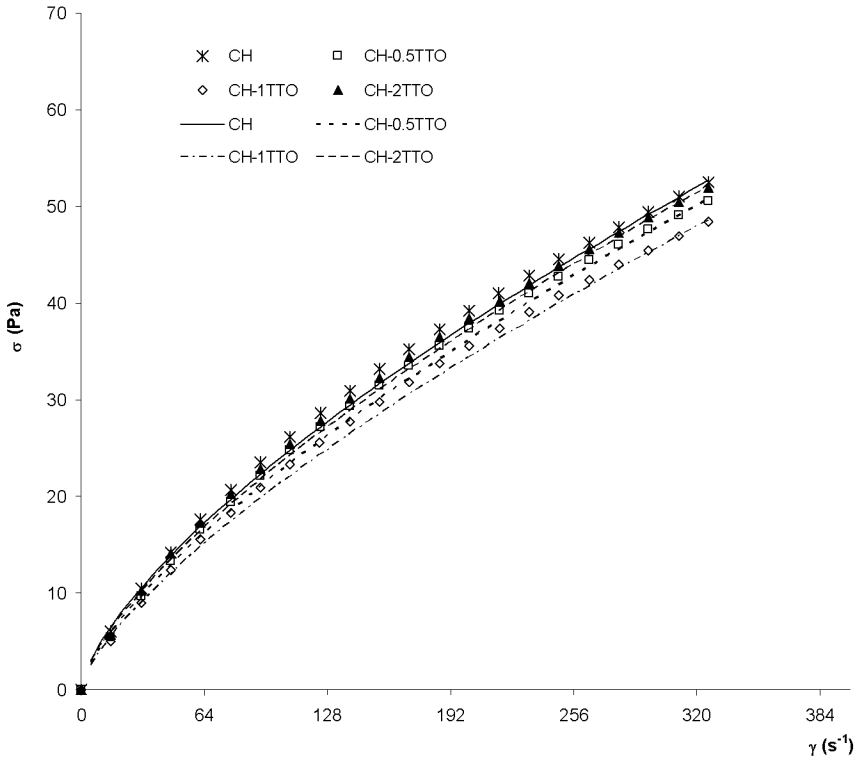

Figure 2. Experimental (symbols) and predicted (lines) flow curves obtained by Ostwald de Waale model for the different FFDs.

As shown in Table 1, CH solution showed a shear thinning fluid behaviour which was significantly affected by the incorporation of TTO. This caused a significant decrease in the consistency index (k) and gave rise to a less marked shear thinning fluid behaviour (higher n), as well as an unexpected decrease in the apparent viscosity η_{ap} ($p< 0.05$). This can be due to different causes: a) changes in the CH concentration in the continuous phase because of its adsorption at the oil-water interface, thus losing thickening capacity, b) changes in the electrical net charge of the particles (MClements, 2005). In this sense, the incorporation of TTO led to a decrease in measured ζ-potential (Table 1). This change in ζ-potential affects the

electroviscous effects and could also imply changes in the hydrodynamic volume of the particles, which directly affect the flow behaviour (Vargas et al., 2009). The non-significant influence of TTO concentration on flow behaviour could be related with the fact that as the concentration of the dispersed phase increases, the available level of CH in the continuous phase (greatly thickened) is reduced by the interfacial adsorption of the polymer in the increased surface area of the droplets. The average particle size distributions of the FFD are plotted in Figure 1.

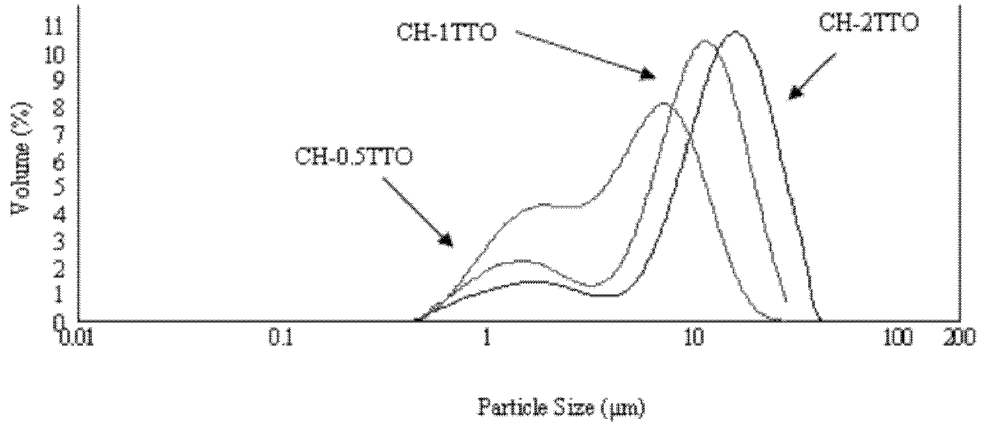

Figure 1. Particle size distribution of CH-TTO film-forming solutions.

Particle size distributions were bimodal for all the formulations containing TTO. There was an observed trend towards greater particle sizes as TTO content levels increased which coincided with that found by previous studies using CH and oleic acid (Vargas et al., 2009). This is also reflected in Table 1 through the d_{43} mean diameter values (expressed as volume-length diameter), where the mean particle size values significantly increased with the increase in TTO content ($p < 0.05$). This could be related with the lesser availability of CH for the interfacial adsorption as the TTO level increased. Thus, the increase in TTO content in the CH FFD led to

bigger emulsified droplets and a lower electrical net charge (ζ-potential of TTO measured in water was -30.60 mV). The reduction of the electrical net charge (decrease in ζ-potential) as the TTO content increases could be explained by the electrostatic interactions between CH and TTO compounds at the pH of the FFD (4.30), as was described in previous CH studies (Vargas et al., 2009). At this pH, the amino groups of chitosan are positively charged and could be partially neutralized through the interaction with some negatively charged groups of the TTO components when adsorbed on the oil droplet surface.

The particle size distribution, together with ζ-potential values, seems to point out that CH chains are extensively adsorbed on the TTO droplets leading to positively charged particles. The steric stabilization promoted by the CH interfacial absorption and the high value of ζ-potential (significantly higher than +30 mV) ensures the stability of the emulsified system (Roland et al., 2003) and so, minor changes in the oil droplet sizes would be expected during film drying.

3.2. Characteristics of the films

3.2.1. Water vapour permeability

The RH conditions used for measuring the WVP (100/54.4) of the films were established to simulate the environmental conditions when the films are applied as a coating for vegetables. The average thickness of the CH and CH-TTO composite films, which was used to determine water vapour permeability (WVP), was indicated in Table 2. This table also shows the WVP and the average value of the film equilibrium moisture content (We) at 20ºC, by considering an intermediate aw value between the two levels which define the RH gradient used in WVP determination. Water vapour permeability values were in the range of those reported by other authors working with films based on CH (Vargas et al., 2009).

Figure 3 shows the WVP values as a function of the TTO:CH ratio.

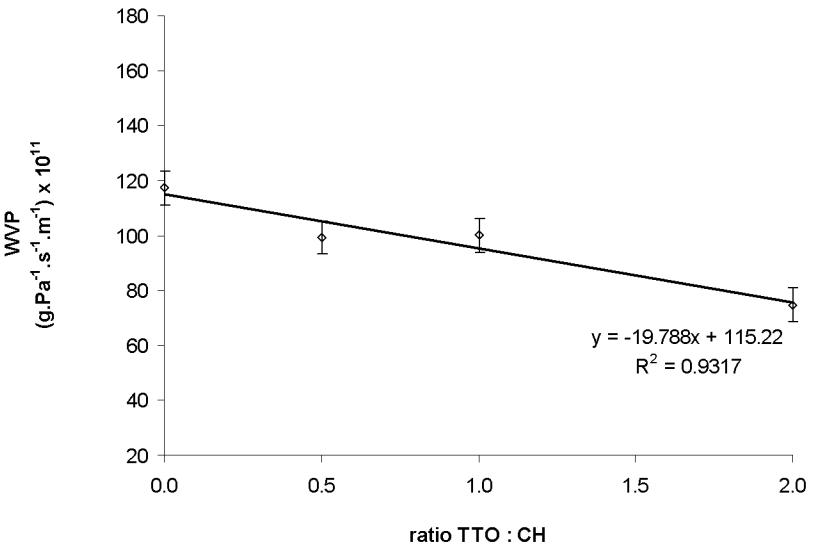

Figure 3. Water vapour permeability of films at 20ºC (100/54.4 RH gradient). Mean values and 95% LSD intervals.

The WVP values showed a significant decrease in line with the increase in TTO concentration, following a linear trend described by Eq.2 (r^2=0.931) reaching a maximum WVP reduction of about 40% with an incorporation of 2% TTO in the FFD. This behaviour is expected as an increase in the hydrophobic compound fraction usually leads to an improvement in the water barrier properties of films, as was previously reported for EO addition in CH films (Zivanovic et al., 2005)

WVP= -19.788 R +115.22 (Eq.2)

where WVP is the water vapour permeability $gm^{-1}s^{-1}Pa^{-1}$ and R is the TTO:CH ratio.

3.2.2. Mechanical properties

The influence of TTO incorporation on the film mechanical properties is shown in table 2, which shows the percentage of elongation (E%) and tensile strength (TS) at

break and elastic modulus (EM) of films equilibrated at 20ºC and 54.4% RH. Some of these values were in the range of those reported by Zivanovic et al., 2005: TS 105 MPa) but differed from other works (Vargas et al., 2009; Srinivasa et al., 2007). Several factors, such as the source of chitosan, the acid medium used to dissolve the polymer and the experimental conditions can explain the observed differences.

Table 2. Elongation (E), tensile strength (TS), elastic modulus (EM), WVP values, We estimated with GAB parameters (a_w=0.77) and thickness of CH and CH-TTO composite films at 20ºC. Mean values and standard deviation.

Film	E (%)	TS (MPa)	EM (MPa)	WVP ($g.Pa^{-1}.s^{-1}.m^{-1}$) x 10^{11}	We (g H_20/g d.m.)	Thickness (μm)
CH	22 (5)[a]	113 (20)[a]	2182 (277)[a]	124 (12)[a]	0.235	52(2)[a]
CH-0.5TTO	20 (8)[a]	75 (15)[b]	1447 (308)[b]	97 (4)[b]	0.210	40(3)[b]
CH-1TTO	17 (6)[a]	72 (12)[b]	1419 (322)[b]	100.9 (1.3)[b]	0.159	38(3)[b]
CH-2TTO	8 (2)[b]	54 (6)[c]	653 (157)[c]	74.8 (1.8)[c]	0.135	24(2)[c]

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

The addition of TTO in the considered concentration range caused a significant decrease not only in the elastic modulus, but also in the tensile strength and deformation at break, which underwent the greatest reduction of all mechanical parameters (higher than 50%) when 2% of TTO was incorporated. Nevertheless, additions of up to 1% of essential oil preserve the elongation capacity of pure CH films. The poor mechanical properties obtained by the addition of TTO may be related with the structural arrangement of the lipid phase into the CH matrix. Thus, the structural discontinuities provoked by the incorporation of the oil could explain

the lowest resistance to fracture of the composite films. Some of these results are in line with those reported by other authors when adding oils to a chitosan matrix (Zivanovic et al., 2005; Srinivasa et al, 2007; Vargas et al., 2009) but differ in some aspects due to the great influence of several, widely studied factors related to CH preparation (Burtler et al., 1996; Caner et al., 1998).

3.2.3. Optical properties

The gloss and transparency of the films are relevant properties since they have a direct impact on the appearance of the coated product. Film transparency was evaluated through the Kubelka-Munk K/S coefficient, defined as the ratio between light absorption and scattering. Figure 4 shows the values of K/S at λ = 450 nm (a) and gloss values (b) of the films measured at incidence angle values of 60º at 54.4% HR and 20ºC.

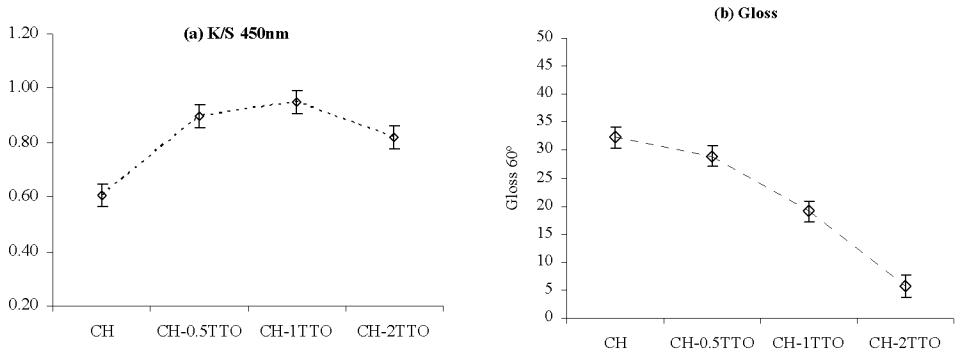

Figure 4. (a) K/S values at 450 nm (b) Gloss at 60º of CH and CH-TTO composite films at 54.4% relative humidity and 20ºC. Mean values and 95% LSD intervals.

K/S values at 450 nm were significantly affected by the amount of TTO. CH-TTO films were, in general, less opaque than CH films. The different transparency level is linked to the internal structure developed throughout the drying process, which is greatly affected by the initial structure of the FFD, i.e. volume fraction of the

dispersed phase and size of the oil droplets (Villalobos et al., 2005). The observed results can be explained by considering that the CH film is dense, closed and opaque. In the composite films, however, the TTO droplets are inserted between chitosan chains, interrupting the matrix and developing a more open structure. The interaction between TTO and water molecules modifies the refractive index of CH, thus affecting the film transparency as has been described in different studies (Vargas et al., 2009; Sánchez-González et al., 2009).

The addition of TTO to the CH matrix led to a decrease of the gloss as a function of the TTO concentration. Similar results have been obtained by different authors in composite films containing lipids (Trezza and Krochta, 2000; Villalobos et al., 2005) and, particularly, TTO essential oil (Sánchez-González et al., 2009). The gloss of the films is related with the surface morphology reached during film drying. In general, the smoother the surface, the higher the gloss (Ward and Nussinovich, 1996). In this sense, the decrease in gloss in line with the increase in TTO content could be explained by an increase of the surface roughness of the composite films. This roughness appears as a consequence of the migration of droplets or aggregates to the top of the film during film drying, which leads to surface irregularities. The increase in droplet concentration leads to a great number of irregularities which contribute to the gloss reduction.

3.2.4. Microstructure

The final microstructure that was developed by the different FFDs after drying is influenced by the structural arrangement of the different components (CH and TTO) in the initial dispersion, and their development during the drying process, where droplet flocculation, coalescence and creaming can occur. Figure 5 shows SEM micrographs of the cross-sections of the films, which show remarkable differences. While a continuous structure was observed for the CH (Figure 5a) film, the presence of TTO (Figure 5b-d) caused discontinuities associated with the

presence of two phases in the matrix: lipid droplets embedded in a continuous polymer network. Lipid droplets, whose number increased with the TTO concentration, were quite homogenously distributed across the film when the TTO:CH ratio was 2, whereas they are accumulated in the upper part of the film, near the film drying surface. The droplet size in the film increased as the TTO:CH ratio increased coinciding with the biggest particle size of the FFD and the greater progress of flocculation and coalescence during drying. Nevertheless, the great oil phase concentration contributes to inhibit creaming, thus giving rise to a more homogeneous droplet distribution across the film. This did not occur at low lipid concentrations where creaming was visible, as can be observed in the micrograph despite the lower droplet sizes. In every case, lipid droplets are slightly enlarged probably due to the deformation forces that act during the polymer chain aggregation during the solvent evaporation.

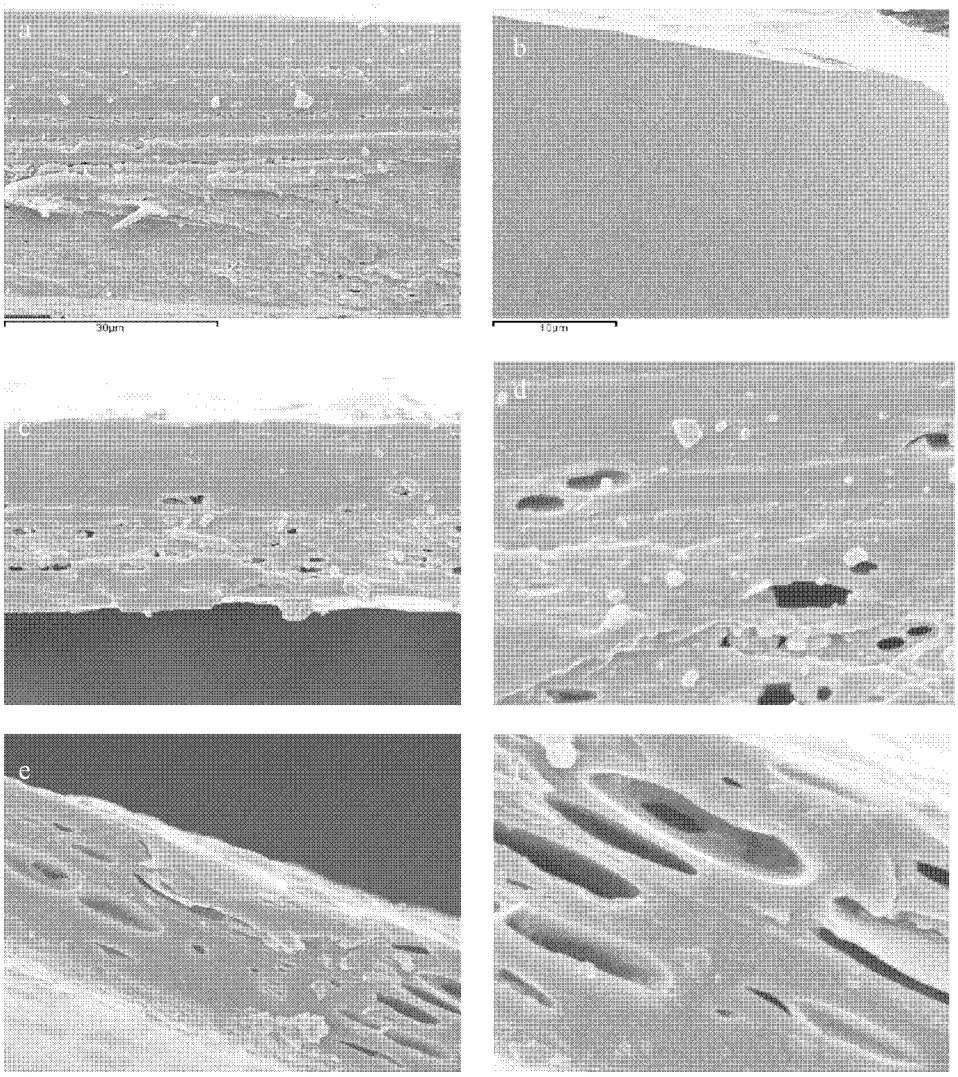

Figure 5. SEM micrographs of the cross-sections of the films. (a,b) CH, (c,d) CH-0.5TTO, (e,f) CH-2TTO.

3.2.5. Microbial effectiveness

3.2.5.1. *Penicillium italicum*

The possible antifungal effect against *Penicillium italicum* at 20ºC of CH and composite films was determined on PDA medium and shown in Figure 6.

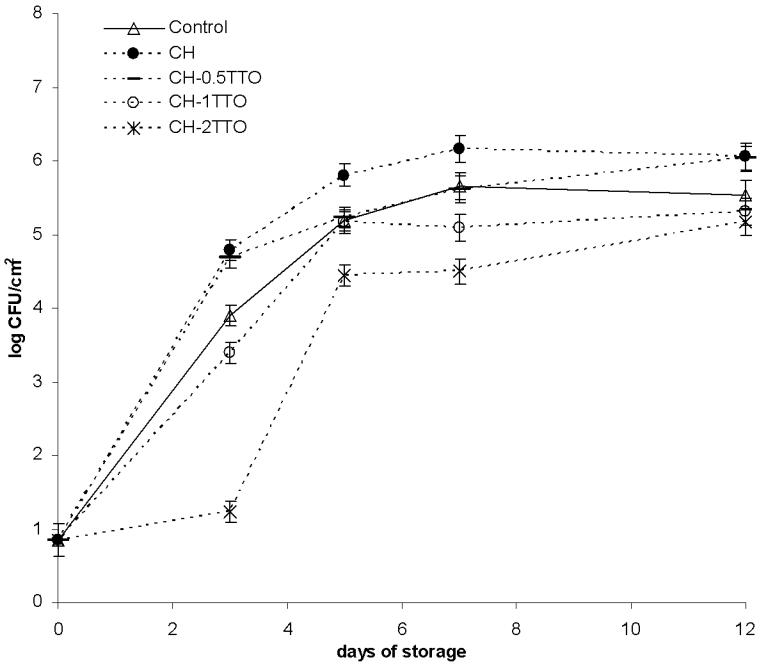

Figure 6. Effect of CH and CH-TTO composite films on the growth and survival of *Penicillium italicum* on PDA medium stored at 20ºC. Mean values and 95% LSD intervals for each sample time.

This effectiveness was evaluated through the analysis of the growth (or survival) of a determined infection level of *Penicillium italicum* (10^5 spores/ mL) following the methodology described above. Thus, the growth of fungus was followed by counts immediately after the inoculation and periodically during the storage period of PDA plates.

CH films did not show antifungal effect for the assayed times. Previous studies have demonstrated that the antimicrobial effect of chitosan depends on the type of microorganism, being mainly effective against bacteria and also against some moulds and yeast (Tharanathan and Kittur, 2003). Nevertheless, Roller and Covill (1999) found some microorganisms resistant to the antimicrobial activity of chitosan (*Aspergillus flavus, Cladosporium cladosporioides* and *Penicillium aurantiogriseum*). Zivanovic et al. (2005) did not observe inhibition of any of the tested bacteria by the chitosan films, which was attributed to the immobilization of chitosan molecules within the film. This phenomenon could partially affect the antimicrobial activity of CH compounds.

The TTO composite films delayed the fungal growth of *P. italicum* (in comparison to the control), which was dependent on the TTO concentration. At low TTO levels, no antimicrobial effect was observed. Only when TTO-CH ratio was higher than 1, a moderate inhibition of fungus growth was detected. The level of reduction of the *Penicillium* population in CH-2TTO films observed during the first three days of storage was especially remarkable, reaching a fungal reduction of 3 logs in comparison with the control plates.

Nevertheless, the inhibition level of the composite films decreased throughout storage time. Considering that the antimicrobial activity of TTO has been probed with very low concentrations in the liquid phase (0.5% v/v, data are no published), the observed behavior could be explained by the availability level of active antimicrobial compounds against the fungi agent. Numerous studies have demonstrated that these compounds are more effective in reducing microbial growth when incorporated into a film or gel and applied to the product surface than when applied on the surface via spray solution or directly added to the product (Cutter and Siragusa, 1996, 1997; Outtara et al., 2000, Sebti and Coma, 2002; Sebti et al., 2003; Kristo et al., 2008) because of the active substances can evaporate or diffuse into the medium. The antimicrobial effect of TTO was limited to the first

three days surely due to the high concentration of the active compounds kept on the agar medium surface. After this period of time, the rate of the microbial growth increased as the progression of the release of the antimicrobial compounds leads to a low availability of the active substances on the surface, where the contamination is prevalent (Kristo et al., 2008; Outtara et al, 2000).

The obtained result is interesting because of no previous studies about antimicrobial activity of CH-TTO composite films have been found to the best of our knowledge.

3.2.5.2. *Listeria monocytogenes*

Growth curves of *Listeria monocytogenes* in control TSANaCl plates and in TSANaCl plates coated with the different films are show in Figure 7.

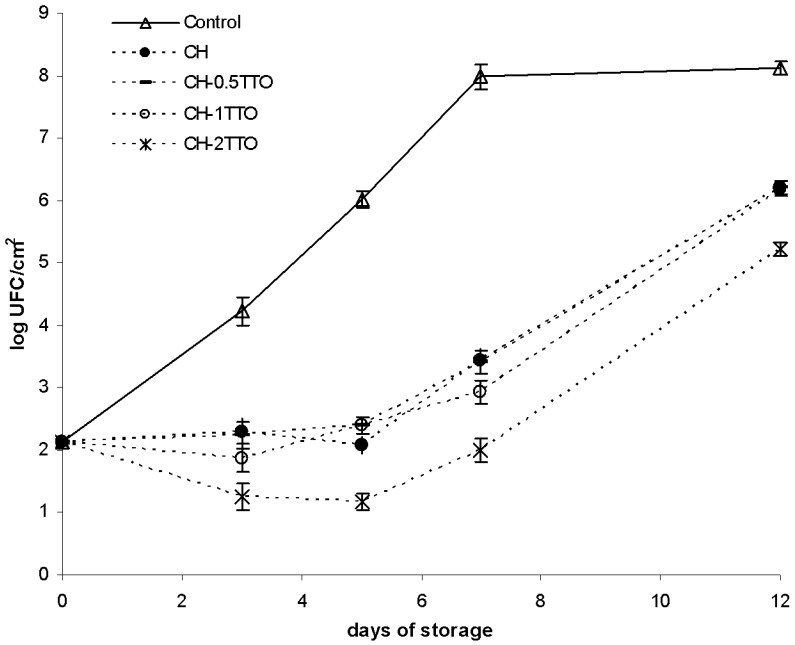

Figure 7. Effect of CH and CH-TTO composite films on the growth and survival of *Listeria monocytogenes* on TSANaCl medium stored at 10ºC. Mean values and 95% LSD intervals for each sample time.

L.monocytogenes population increased from 2 to 8 logs UFC/cm^2 at the end of the storage period. CH films without essential oil presented a significant antimicrobial activity and a complete inhibition of the microbial growth was observed during the first fifth days at 10ºC. Pathogen population did not exceed 6 logs UFC/cm^2 at the end of the storage period. Previous studies (Kristo et al., 2008; Beverlya et al., 2008) observed similar antimicrobial activity of pure CH films against *Listeria monocytogenes* strains. The incorporation of TTO in the ratio CH:TTO 1:2 improved the antimicrobial properties of CH films, but no significant effect was observed for lower TTO concentrations (CH:TTO ratios of 1:0.5 and 1:1). Zivanovic et al. (2005) also observed that the incorporation of oregano essential oil into CH matrix improves the antilisteria effect of CH.

The reduction of volatile compounds concentration (which also contribute to the total antimicrobial activity of EO) during the film drying process and the time required for the microbial experiments could also partially explain the lost of antimicrobial effectiveness of the composite films over time for both tested microorganisms.

4. CONCLUSION

CH is a good polymer matrix for entrapping TTO oil which can be used in different applications. The physical properties of the initial emulsions reveal that CH molecules adsorb on the oil-water interface, thus contributing to the emulsion stability. Nevertheless, the film microstructure reflects the fact that flocculation and creaming of oil droplets occurred during film drying, but to differing extents depending on the TTO-CH ratio. These phenomena contribute to the roughness of the film surface, decreasing the film gloss in line with the TTO concentration. The incorporation of TTO, at a ratio of CH:TTO of 1:2 in the polysaccharide matrix, reduced the water vapour permeability by 40 %. Likewise, the films' resistance to

break was notably reduced by TTO incorporation due to the presence of discontinuities in the film matrix that affect its mechanical response. Only the composite films with TTO:CH ratios higher than 1 showed a limited antifungal effectiveness against *Penicillium*, which was notably reduced after 3 days of storage. Nevertheless, CH films presented a significant antimicrobial activity against *Listeria monocytogenes* and the incorporation of TTO in the CH:TTO ratio of 1:2 improved the antibacterial properties of these films, showing a complete inhibition of the microbial growth during the first fifth days at 10ºC.

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PHYSICAL PROPERTIES OF EDIBLE CHITOSAN FILMS CONTAINING BERGAMOT ESSENTIAL OIL AND THEIR INHIBITORY ACTION ON *PENICILLIUM ITALICUM*

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ABSTRACT

Chitosan-based films containing bergamot essentials oil (BO) at 0.5,1,2 and 3% w/w were prepared to evaluate their physical and antifungal properties. Film-forming dispersions (FFD) were also characterised in terms of rheological properties, particle size distribution and ζ-potential. In order to study the impact of the incorporation of BO into the chitosan (CH) matrix, water vapour permeability (WVP), mechanical and optical properties of the dry films were evaluated. Furthermore, the antifungal effectiveness of CH-BO composite films against *Penicillium italicum* was studied. Results showed that incorporation of BO provoked a decrease in the water vapour permeability, this reduction being around 50% when using a BO-CH ratio of 3:1. Concerning mechanical and optical properties, CH-BO composite films were less resistant to break, less deformable and less glossy. The load parameters (TS and EM) decreased more than 50% and the percentage of elongation at break was also dramatically reduced from 22% to 5%, as compared with the pure chitosan films. CH-BO composite films showed a significant inhibitory effect on the growth of *P. italicum*, which depended on the BO concentration. Chitosan films with the maximum bergamot oil content (3:1 BO-CH ratio) led to a total inhibition of the fungus growth during the first five days at 20ºC. Although the antifungal effectiveness of the films decreased throughout the storage time, a significant reduction of 2 logs units as compared with the control remained possible, after 12 days at 20°C, using the highest BO content.

Keywords: *Penicillium italicum*, water vapour permeability, mechanical properties, particle size distribution, ζ-potential.

1. INTRODUCTION

To design proper edible antimicrobial films to be used in food preservation can be considered as one of the major challenges for food technologists in the next few years. In the future, these films will be tailor made to solve some specific problems for a given product. It is important to know the influence of different factors on their properties in order to design the most suitable film for a determined use and functionality.

Among the active biomolecules, chitosan has a great potential for a wide range of food applications due to its biodegradability, biocompatibility, antimicrobial activity, non-toxicity and film forming capacity (Li et al., 1992; Tharanathan et al., 2003; Arvanitoyannis, 1999). Chitosan based films have been proven to present moderate oxygen barrier properties and good carbon dioxide barrier properties but high water vapour permeability, due to their hydrophilic nature (Butler et al., 1996). Usually, hydrophobic compounds, such as lipids, are incorporated into this type of hydrophilic hydrocolloid films to improve their water barrier properties. One possibility is the use of essential oils (EO), as hydrophobic constituents, which have also been demonstrated to present potential antimicrobial activity against a wide variety of bacteria, moulds and yeast (Fisher and Phillips, 2006). Generally, phenolic and terpene compounds are major contributors to these antimicrobial properties. The specific advantage of EO appears to be the synergistic effects of their compounds as evidenced in the greater activity when applied as natural EO, as compared with the sum of the effects of the individual substances (Duke and Beckstrom-Sternberg, 1992).

Recently, the application of citrus essentials oils to food preservation has received increased attention because not only do they lend themselves to use in food products but are also generally recognized as safe at flavouring concentrations (GRAS). These factors made them into very promising compounds to be used as a

natural alternative to chemical-based preservatives, in line with the changes in legislation and consumer trends (Brul and Coote, 1999).

Bergamot oil is a citrus oil (from Citrus bergamia), whose major chemical compounds are volatile, such as limonene (32-45%) and linalool (around 10.23%) (Svoboda and Greenaway, 2003; Moufida and Marzouk, 2003). The antimicrobial efficiency of BO, and its components, linaool and citral, has been found to be effective against *Campylobacter jejuni, Escherichia Coli* O157, *Listeria monocytogenes, Bacillus cereus, Staphylococcus aureus, Arcobacter butzleri* and *Penicillium digitatum* (Fisher and Phillips, 2008), among others, both when oil is applied directly and when in contact with the oil vapour. The mechanisms by which essential oils bring about their antimicrobial effect are not clear but there are a number of proposed mechanisms (Holley and Patel, 2005): terpenes have the ability to disrupt and penetrate not only the lipid structure of the cell membrane, but also the mitochondrial membrane, leading to the denaturing of proteins and the destruction of cell membrane, cytoplasmatic leakage, cell lysis and eventually, cell death.

In composite chitosan-essential oil based films, the possible presence of interactions between the active antimicrobial agent and the chitosan should be taking into account, as they could affect the efficiency of the antimicrobial response. Chitosan might interact with terpens which are the major components of essential oils, mainly by weak interactions such as hydrogen bonding (Mayachiew et al., 2010). Hossseini et al. (2009) found that the intensity of these interactions depended on the nature of the essential oil. These authors showed that the incorporation of essential oils into chitosan films led to a loose in the compactness of the film structure, this effect being more accused with cinnamon essential oil in comparison with thyme and clove oils. These interactions could affect the release of the added antimicrobial agent (Hosseini et al., 2009) and so, the antimicrobial response of the composite films.

On the other hand, films and coatings should be designed to fulfill a number of requirements, such as to have proper mechanical properties, good appearance (adequate gloss and transparency) and water and gas barrier properties. Thus, knowing how different factors influence these physical film properties is relevant to be able to improve and optimize the film functionality. Among these factors, the stability related properties of the film forming dispersions (FFD), such as rheological behavior, particle size and distribution and ζ-potential of the dispersed lipid particles, play an important role in the properties of lipid-hydrocolloid composite films. The control of the FFD properties could allow us to design films with determined functional properties (McClements, 2007)

The aim of this work was to evaluate how the functionality of chitosan based films was affected by the incorporation of different ratios of bergamot essential oil, through the analysis of different physical and structural properties of the FFD and films. The antifungal properties of the films against *Penicillium italicum* were also evaluated. This fungus is one of the major causes of citrus fruit decay (blue mold) and films containing citrus essential oils could be used, without any impact on the fruit flavor, to prevent the microbial growth.

2. MATERIALS AND METHODS

2.1 Materials

High molecular weight chitosan (CH) with a deacetylation degree of 82.7% (Batch 10305DD, Sigma-Aldrich Química, Madrid, Spain), 98% glacial acetic acid (Panreac, Barcelona, Spain) and bergamot essential oil (BO), supplied by Herbes del Molí (Alicante, Spain) were used to prepare the film-forming dispersions.

2.2. Preparation of film forming dispersions

Chitosan (1% w/w) was dispersed in an aqueous solution of glacial acetic acid (0.5% w/w) at 25ºC. After stirring overnight, bergamot essential oil (BO) was added to chitosan (CH) solution to reach a final concentration of 0, 0.5, 1, 2 and 3% (w/w). CH-BO mixtures were emulsified at room temperature (25ºC) using a rotor-stator homogenizer (Ultraturrax DI 25 basic-Yellowline, Janke & Kunkel, Staufen, Germany) at 13,500 rpm for 4 minutes. These emulsions were vacuum degasified at room temperature (25ºC) with a vacuum pump (Diaphragm vacuum pump, Wertheim, Germany). Sample nomenclature was CH-nBO, the n value being the ratio BO:CH in the film or FFD.

2.3 Characterization of the film-forming dispersions

The density of the FFD was measured by means of a digital densimeter DA-110M, (Mettler Toledo, Barcelona, Spain). A pH-meter C831 (Consort, Tumhout, Belgium) was used to determine the pH of the FFD at 20ºC.

2.3.1. ζ- potential measurements

In order to perform ζ- potential measurements, FFD were diluted to a droplet concentration of 0.02% BO using an aqueous solution of glacial acetic acid (0.5%w/w). ζ-potential was determined by using a Zetasizer nano-Z (Malvern Instruments, Worcestershire, UK). The Smoluchowsky mathematical model was used to convert the electrophoretic mobility measurements into ζ-potential values.

2.3.2. Particle size measurements

Particle size analysis of the FFD was carried out by using a laser diffractometer (Mastersizer 2000, Malvern Instruments, Worcestershire, UK). The samples were diluted in deionised water at 2,000 rpm until an obscuration rate of 10% was obtained. The Mie theory was applied by considering a refractive index of 1.52 and

absorption of 0.1 for BO. Three samples of each FFD were measured in quintuplicate.

2.3.3. Rheological behaviour

The rheological behaviour of FFD was analysed in triplicate at 25ºC by means of a rotational rheometer (HAAKE Rheostress 1, Thermo Electric Corporation, Karlsruhe, Germany) with a sensor system of coaxial cylinders, type Z34DIN Ti. Rheological curves were obtained after a stabilization time of 5 minutes at 25ºC. The shear stress (σ) was measured as a function of shear rate ($\dot{\gamma}$) from 0 to 512 s^{-1}, taking 5 minutes to reach the maximum shear rate and another 5 minutes to attain zero shear rate. The power law model (Eq. 1) was applied to determine the consistency index (K) and the flow behaviour index (n). Apparent viscosities were calculated at 100 s^{-1}.

$$\sigma = K \cdot \dot{\gamma}^{n} \qquad \text{(Eq. 1)}$$

2.4. Preparation of films

A casting method was used to obtain films. FFD were poured onto a framed and levelled polytetrafluorethylene (PTFE) plate (ϕ = 15 cm) and were dried in atmospheric conditions (25ºC, 60% relative humidity) for 48 hours. Film thickness was controlled by pouring the amount of FFD that will provide a surface density of solids in the dry films of 56 g/m^2 in all formulations. Dry films were peeled off the casting surface and preconditioned in desiccators at 20ºC and 54.4% relative humidity (RH) prior to testing. A hand-held digital micrometer (Palmer - Comecta, Spain, ± 0.001 mm) was used to measure film thickness at three different points of the same sample at least.

2.5. Water vapour permeability

Water vapour permeability (WVP) was measured in dry film discs (ϕ = 7 cm), which were equilibrated at 54.4% RH and 20ºC, according to the "water method" of the ASTM E-96-95 (ASTM, 1995), using Payne permeability cups (Elcometer SPRL, Hermelle /s Argenteau, Belgium). Deionised water was used inside the testing cup to achieve 100% RH on one side of the film, while an oversaturated magnesium nitrate solution was used to control the RH on the other side of the film. During WVP testing, the side of the film in contact with the PTFE plate was placed in contact with that part of the test cup having the highest RH. This situation tries to simulate the case of a film applied on the wet surface of a fresh cut vegetable or fruit. A fan placed on the top of the cup was used to reduce resistance to water vapour transport. Water vapour transmission rate measurements (WVTR) were performed at 20ºC. To calculate WVTR, the slopes of the steady state period of the curves of weight loss as a function of time were determined by linear regression. For each type of film, WVP measurements were replicated three times and WVP was calculated according to Villalobos et al. (2006).

2.6. Mechanical properties

Mechanical properties were measured by using a Texture Analyser TA-XT-plus (Stable Micro Systems, Surrey, UK), with a 50 N load cell equipped with tensile grips (A/TG model). Sample films, previously equilibrated at 54.4% RH and 20ºC, were cut into 25.4 mm wide and 100 mm long strips, according to the ASTM D-882 standard (ASTM, 2001). Grip separation was set at 50 mm and cross-head speed was 50 mm/min. Tensile strength (TS) and percentage of elongation (% E) at break, and elastic modulus (EM) were evaluated in eight samples from each type of film.

2.7. Optical properties

Gloss was measured using a flat surface gloss meter (Multi-Gloss 268, Minolta, Langenhagen, Germany) at an angle of 60º with respect to the normal to the film surface, according to the ASTM standard D523 (ASTM, 1999). Prior to gloss measurements, films were conditioned in desiccators at 20ºC and 54.4% RH. Gloss measurements were performed over a black matte standard plate and were taken in quintuplicate. Results were expressed as gloss units, relative to a highly polished surface of standard black glass with a value close to 100.

The transparency of the films was determined through the surface reflectance spectra in a spectrocolorimeter CM-3600d (Minolta Co, Tokyo, Japan) with a 10 mm illuminated sample area. Measurements were taken from three samples in each formulation by using both a white and a black background. The transparency was determined by applying the Kubelka-Munk theory for multiple scattering to the reflection spectra. As each light flux passes through the layer, it is affected by the absorption coefficient (K) and the scattering coefficient (S). Transparency (K/S) was calculated, as indicated by Hutchings (1999), from the reflectance of the sample layer on a white background of known reflectance and on an ideal black background.

2.8. Microbiological analysis

2.8.1. Fungal strain

Stock culture of *Penicillium italicum* (CECT 2294), supplied by Colección Española de Cultivos Tipos (CECT, Burjassot, Spain), was kept frozen (-25ºC) in Potato Dextrose Broth (Scharlab, Barcelona, Spain) supplemented with 30% glycerol (Panreac, Barcelona, Spain). The fungus was inoculated on Potato Dextrose Agar (PDA) and incubated at 25ºC until sporulation. The inoculums' concentration was adjusted by means of a haemocytometer at 10^5 spores per mL.

2.8.2. Antifungal effectiveness of films

The methodology followed for the determination of antimicrobial effectiveness of films was adapted from Kristo et al. (2008).

Aliquots of PDA (20 g) were poured into Petri dishes. After solidification of the culture medium, diluted spore solution was inoculated on the surface and different test films (containing or not antimicrobial substance) of the same diameter as the Petri dishes were placed on the inoculated surface. Inoculated uncoated PDA was used as control. Plates were then covered with parafilm to avoid dehydration and stored at 20ºC for 12 days. *Penicillium italicum* counts on PDA plates were examined immediately after the inoculation and periodically during the storage period.

The agar was removed aseptically from Petri dishes and placed in a sterile plastic bag with 100 ml of tryptone phosphate water (Scharlab, Barcelona, Spain). The bag was homogenized for 2 minutes in a Stomacher blender (Bag Mixer 400, Interscience). Serial dilutions were made and then poured onto PDA. Plates were incubated for 5 days at 25ºC before colonies were counted. All tests were run in triplicate.

2.9. Statistical analysis

Results were analysed by multifactor analysis of variance with 95% significance level using Statgraphics®Plus 5.1. Multiple comparisons were performed through 95% Least Significant Difference intervals (LSD).

3. RESULTS AND DISCUSSION

3.1. Characterization of the film-forming dispersions

3.1.1. Density, ζ-potential and particle size distribution.

Density and ζ-potential values of the different FFD are reported in Table 1. The incorporation of bergamot oil (BO) led to the expected decrease in the density of

the film-forming dispersions (FFD). The values found for chitosan FFD were in the range of those reported by other authors at the same pH (Vargas et al., 2009) and of those predicted on the basis of the FFD composition, taking into account density of CH: ~1450 kg/m^3 (Park et al., 2004) and the value for bergamot oil: 897 Kg/m^3 kg. The pH of FFD was around 4.3 at ambient temperature and did not vary significantly ($p<0.05$) with the incorporation of BO.

Table 1. Density (ρ), Ostwald de Waale rheological parameters, apparent viscosity (η_{ap}), mean particle size (d_{43}) and ζ-potential values of film forming dispersions at 25°C. Mean values and standard deviation.

FFS	ρ (Kg/m^3)	$0 \leq \dot{\gamma} \leq 512$ s^{-1} n	k (Pa·s)^n	η_{ap} (100 s^{-1}) (Pa·s)	r^2*	d_{43} (μm)	ζ (mV)
CH	1004.69 (0.14)^a	0.785 (0.007)^a	0.58 (0.02)^a	0.217 (0.002)^a	0.975	-	101 (3)^a
CH-0.5BO	1003.888 (0.005)^b	0.777 (0.002)^b	0.554 (0.116)^a	0.179 (0.019)^b	0.996	7.1(0.4)^a	82 (3)^b
CH-1BO	1002.55 (0.05)^c	0.7936 (0.0116)^{ac}	0.38 (0.04)^b	0.150 (0.008)^c	0.997	15.1(0.4)^b	80.3 (1.4)^{bc}
CH-2BO	1001.26 (0.17)^d	0.82 (0.02)^{cd}	0.29 (0.06)^{bc}	0.139 (0.015)^{cd}	0.997	22.1(0.2)^c	77.60 (1.02)^c
CH-3BO	1000.319 (0.107)^e	0.842 (0.019)^d	0.24 (0.05)^c	0.122 (0.015)^d	0.997	28.6(0.4)^d	73.9 (1.8)^d

$^{a, b, c, d, e}$ Different superscripts within a column indicate significant differences among formulations ($p<0.05$).
*correlation coefficient of the fitting.

The typical average particle size distributions of these FFD are plotted in Figure 1. Particle size distributions were bimodal except for the formulation containing the lowest BO content, which showed a monomodal trend towards smaller particles.
The increase in BO content significantly ($p<0.05$) increased the mean particle size and decreased the ζ-potential of the particles ($p<0.05$) when compared to the values of the CH dispersion. Thus, the increase of BO content led to bigger

droplets with lower electrical net charge. This is also reflected in Table 1, where the mean diameter was expressed as volume-length diameter (d_{43}). The reduction of the electrical net charge of CH-BO particles (decrease in ζ-potential) when BO content increased could be explained by the presence of electrostatic interactions between CH and BO components. At this pH, the amino group of chitosan (pKa NH^{+3} /NH_2 ≈ 6.5) is positively charged and could be partially neutralized through the interactions at the O/W interface with some polar groups of bergamot oil components, such as alcohols (linaool, methanol, isopropanol, etc). To corroborate this assumption, an aqueous dispersion of BO (1% v/w) was prepared in distilled water and pH (4.2); particle size and ζ-potential were determined. In the absence of chitosan, BO droplets showed negative charge (ζ-potential = -61 ± 3 mV) and showed a monomodal distribution, with a significantly lower mean particle size (d_{43}= 3.67 ± 0.05 μm) than that reported for CH–1BO FFD. Negative charge is usual in oil dispersions due to the predominant adsorption of the negative ions present in the aqueous system. Therefore, the hypothesis for the structure of CH–BO system is that chitosan chains are adsorbed on the BO droplets, leading to bigger, positively charged particles. This theory could also explain the presence of bimodal distributions when there is a considerable amount of BO (over 0.5%) as there is not enough chitosan to cover all oil particles, leading to some degree of droplet flocculation (bridging flocculation) . Nevertheless, the steric stabilization promoted by the chitosan interfacial adsorption and viscosity of the system ensures the stability of the emulsified system (Roland et al., 2003).

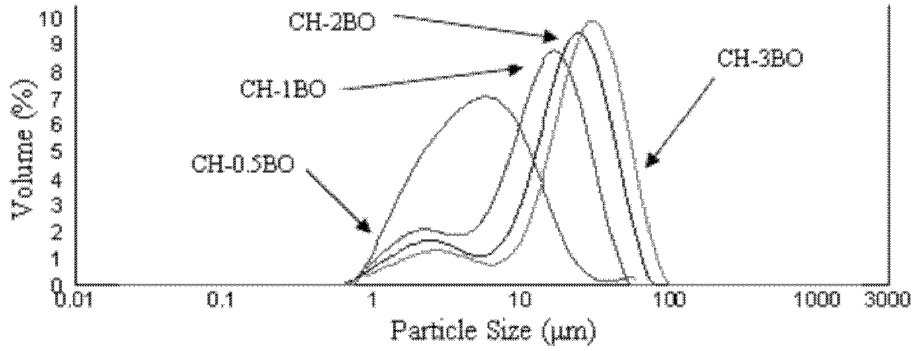

Figure 1. Particle size distribution of CH-BO film-forming dispersions.

3.1.2. Rheological characterization.

All FFD showed a shear-thinning behaviour ($n<1$) and no thyxotropic effects were observed from the comparison of the up and down curves. Rheological data were fitted to the Ostwald de Waale model and the model parameters are shown in Table 1, together with the apparent viscosity (η_{ap}) values at a shear rate of 100 s^{-1}. Rheological parameters and apparent viscosity at 100 s^{-1} for pure chitosan FFD are in the order of those found by No et al. (2006), Rodriguez et al. (2002) and Vargas et al. (2009) in chitosan solutions and chitosan-oleic acid dispersions respectively.

The apparent viscosity values significantly ($p<0.05$) decreased when the BO content increased, which was not how oil in water emulsions are expected to behave, as viscosity usually increases when the concentration of the dispersed phase increases. Nevertheless, in this case there are two contributions to the rheological behaviour of the BO emulsions: the viscosity imparted by CH molecules dissolved in the aqueous phase and the viscosity imparted by dispersed BO droplets. As deduced from droplet size and ζ- potential values, CH molecules adsorb on BO droplets, thus reducing their viscous contribution in the continuous

phase. In this sense, the increase in the BO ratio led to a greater reduction not only of the CH available in the aqueous phase but also of the particle charge, while increasing the mean particle size. All these effects can explain the viscosity decrease when BO ratio increased in the emulsion. Droplet charge influences the rheology due to electroviscous effects (Pal, 1996; Larson, 1999; Rubio-Hernández et al., 2004). So, the particles with a higher charge will lead to more viscous systems. This can be explained by the greater attraction of the cloud of counter ions surrounding the charged droplets, which makes the droplet movement more difficult and by the greater effective diameter of the droplets as they cannot get so close together due to electrostatic repulsions (McClements, 2005).

3.2. Characteristics of the films

3.2.1. Mechanical behaviour

Table 2 shows the mechanical characterization of the obtained films, in terms of percentage of elongation at break (E%), tensile strength (TS) and elastic modulus (EM), equilibrated at 54.4% and 20ºC. TS indicates the maximum tensile stress that the film can sustain, E% is the maximum change in length of a test specimen before breaking, and EM is a measure of the stiffness of the film. Values of mechanical properties obtained for CH films agreed with those found by other authors when using similar chitosan-acetic acid ratio (Zivanovic et al., 2005: TS 105 MPa, E% 5%) but differed from those obtained in other works (Vargas et al., 2009: TS 12 MPa, E% 17%; Srinivasa et al., 2007: TS 39.10 MPa, E% 10.84%). The source of chitosan, the acid medium used to dissolve the polymer and the experimental conditions (pH, concentration, RH used during equilibration of the film, presence of emulsifiers..) can explain the observed differences.

Table 2. Elongation (E), tensile strength (TS), elastic modulus (EM), water vapour permeability (WVP) (100/54.4 RH gradient), equilibrium water content (We) estimated with GAB parameters (a_w=0.77) and film thickness of CH and CH-BO composite films at 20°C. Mean values and standard deviation.

Film	E (%)	TS (MPa)	EM (MPa)	WVP ($g.Pa^{-1}.s^{-1}.m^{-1}$) x 10^{11}	We (g H_20/g d.m.)	Thickness (μm)
CH	22 (5)[a]	113 (20)[a]	2182 (277)[a]	124 (12)[a]	0.235	52(2)[a]
CH-0.5BO	7 (4)[b]	65 (10)[b]	766 (205)[b]	130.2 (0.3)[a]	0.189	56(2)[a]
CH-1BO	5.5 (0.7)[b]	63 (21)[b]	799 (163)[b]	86 (5)[b]	0.174	41(2)[b]
CH-2BO	6 (2)[b]	50 (8)[bc]	747 (225)[b]	92 (9)[b]	0.137	36(3)[c]
CH-3BO	1.7 (0.4)[b]	22 (8)[c]	682 (196)[b]	65 (2)[c]	0.123	32(2)[c]

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

The incorporation of the BO dispersed phase leads to softer, less resistant to break and less stretchable films, almost regardless of the BO concentration added, since no significant differences were found between mechanical parameters of the different composite films. The load parameters (TS and EM) decreased more than 50% and the percentage of elongation at break was also dramatically reduced from 22% to 5%, in comparison with the pure chitosan films. This could be explained by discontinuities in the polymer matrix introduced by the BO incorporation and by changes in the polymer chain interactions when oil components are present, which lead to a weak mechanical response. Srinivasa et al. (2008) and Vargas et al. (2009) found similar behavior when analyzing the mechanical properties of chitosan films blended with different fatty acids. On the other hand, Zivanovic et al. (2005) also found that the tensile strength decreased when introducing some essential oils into CH films but no changes in the elongation percentage were found. This different behavior can be attributed to the type of chitosan (solvent and molecular weight) and particular interactions with the essential oil components which, in turn, are affected by relative humidity, the presence of surfactants, temperature, etc.

3.2.2. Water vapour permeability

The water vapour permeabilities (WVP) of the films at 100/54.4 RH gradient and 20ºC are also reported in Table 2. The RH gradient was chosen to simulate the environmental conditions of coatings applied to fruit or vegetables stored at 20ºC. The thickness of pure and composite films is reported in Table 2. The composite films are not as thick as the pure CH films, suggesting the existence of a different arrangement of components (CH surrounding oil droplets), as commented on above. In pure CH or composite films containing low amounts of BO, the hydration layers of polymer will contribute to increase the film thickness, giving rise to a swelling effect and, subsequently, thicker films. Nevertheless, when the polymer ratio is reduced a smaller number of hydration water layers will be present and so film thickness is reduced.

Lipid compounds are known to enhance the water barrier properties of polymer based films due to their hydrophobic nature and to decrease the water sorption capacity of the films. This can be observed in Table 2, where the values of the equilibrium water content of the films are shown. Nevertheless, due to the greater polarity of essential oil components, this trend was only significant ($p<0.05$) in CH-BO composite films when the BO ratio in the film was higher than 0.5. The WVP value decreased by 50% when using a BO-CH ratio of 3:1. The WVP values for the 100/54.4 RH gradient were in the range of those reported by Park and Zhao (2004) and Vargas et al. (2009) (160 10^{-11} g Pa^{-1} $s^{-1}m^{-1}$ at 5ºC for pure chitosan) in chitosan-oleic acid films. No correlation was found between the WVP values and the equilibrium water content of the films (Table 2). A linear decrease of WVP as a function of the BO ratio in the film was observed (Figure 2) which is coherent with the reduction of the hydrophilic phase (polysaccharide) ratio where water molecules diffuse preferentially. The oil phase introduces an increase in the tortuosity factor for water transfer in the matrix, thus increasing the distance travelled by water molecules diffusing through the film. The tortuosity factor is

higher when the oil phase ratio increases or when the oil particle size is reduced (Pérez-Gagó et al., 2001).

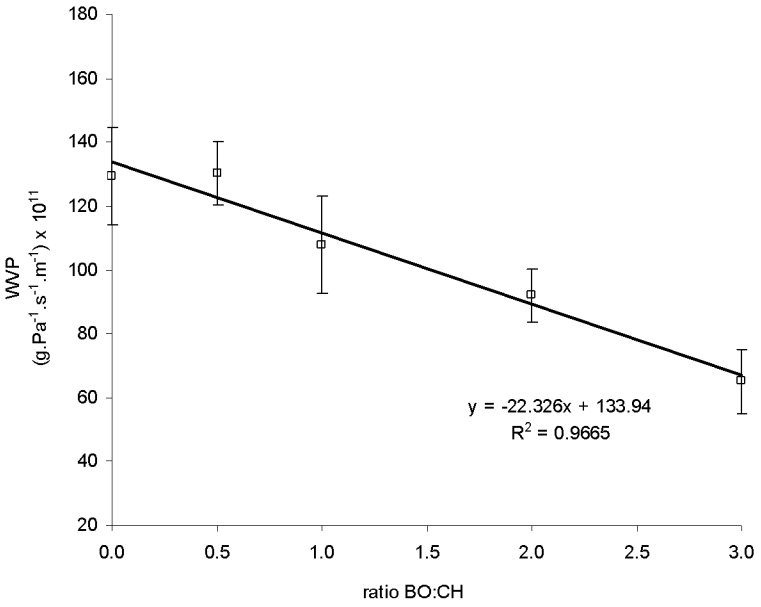

Figure 2. Water vapour permeability of films at 20ºC (100/54.4 RH gradient) as a function of the BO ratio in the film matrix. Mean values and 95% LSD intervals.

3.2.3. Optical properties

The optical properties of the films were evaluated through their gloss and transparency since these properties have a direct impact on the appearance of the coated product. Film transparency was evaluated through the Kubelka–Munk K/S coefficient (Figure 3a) at 450 nm, defined as the ratio between light absorption and scattering. Usually, an increase in K/S can be assumed as an increase in transparency (Hutchings, 1999). The gloss values of the films were measured at an incidence angle value of 60º. As can be observed in Figure 3, relevant changes in the transparency and gloss of the films were detected due to BO incorporation into

the CH matrix. BO-CH composite films become less opaque and less glossy and are hardly affected by the amount of BO. The different transparency level is related with the internal structure of the films. Chitosan films are considered to be constituted by densely packed chains which make them opaque. This structure is altered by the presence of the oil dispersed phase, where the lipid droplets are interrupting the continuous matrix of chitosan chains, leading to more open structure and so films become more transparent. This effect has been also observed in CH films containing oleic acid (Vargas et al., 2009).

On the other hand, the gloss of the films is linked to the morphology of their surface. In general, the smoother the surface, the glossier they are (Ward and Nussinovitch, 1996). The decrease of the gloss of the composite films could be related to the presence of discontinuities in the film matrix surface (oil dispersed droplets), which increase the surface roughness and lead to a decrease in the specular reflectance in the air-film interface. Similar behavior was found by Sánchez-González et al. (2009) in HPMC-TTO composite films.

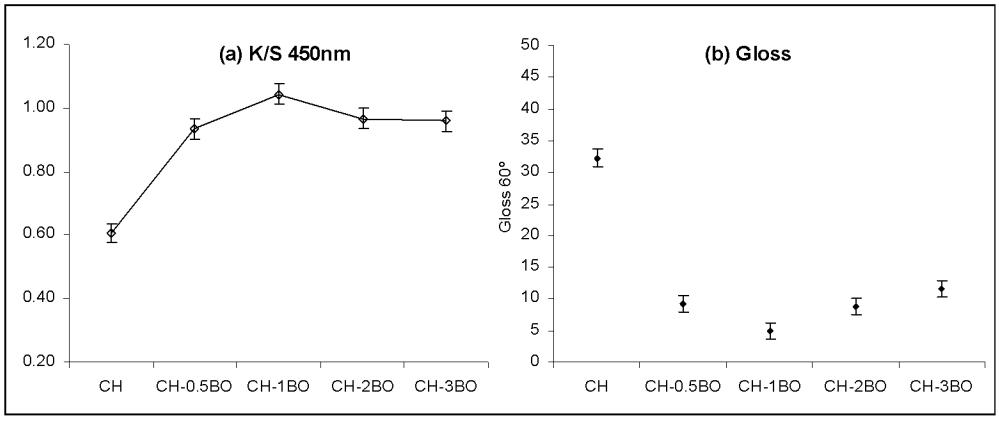

Figure 3. (a) K/S values at 450 nm and (b) Gloss at 60º of CH and CH-BO composite films at 54.4% relative humidity and 20ºC. Mean values and 95% LSD intervals.

3.2.4. Antifungal activity

The possible antifungal effect against *Penicillium italicum* at 20ºC of CH and composite films was determined on PDA medium and is shown in Figure 4.

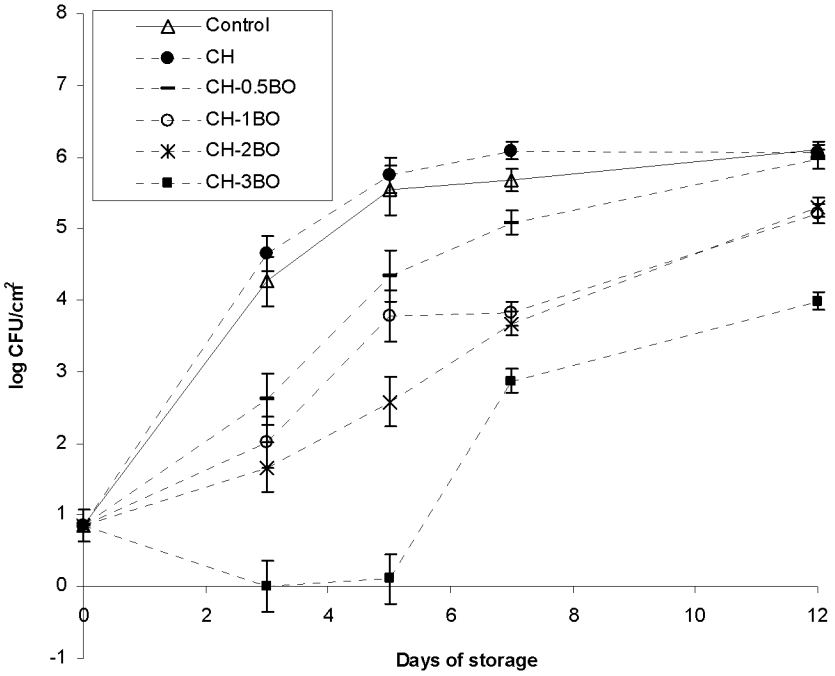

Figure 4. Effect of CH and CH-BO composite films on the growth and survival of *Penicillium italicum* on PDA medium stored at 20ºC. Mean values and 95% LSD intervals.

Although some evaporation of BO components can occur during the preparation and drying of the films, the initial BO concentration in the FFD was considered for discussion of the obtained results, by assuming that proportional losses will occur in the films containing different ratios of BO. Pure chitosan films, with no BO, served as a control to determine the potential antimicrobial effect of chitosan films per se, but CH films were found not to be effective against this fungus. Chitosan

has been proven to have antimicrobial properties against numerous groups of microorganisms, mainly bacteria, but also against some moulds and yeasts (Tharanathan and Kittur, 2003). El Ghaouth et al. (1992) observed a fungistatic effect of chitosan against *Botrytis* and *Rhizopus* rather than a fungicidal. Nevertheless, some microorganisms have been shown to be insensitive to chitosan activity. In this sense, Roller and Covill (1999) found, in similar film studies, that the growth of *Aspergillus flavus*, *Cladosporium cladosporioides* and *Penicillium aurantiogriseum* was not affected by the presence of chitosan, either. Neither did Zivanovic et al. (2005) observe any inhibition of the tested bacteria (*Escherichia coli* and *Listeria monocytogenes*) by the chitosan films.

The composite films showed an inhibitory effect on the growth of *P. italicum*, which depended on the BO concentration. The films containing the highest BO content (3:1 BO-CH ratio) exhibited the strongest power of inhibition. No fungal growth was observed throughout the first 5 storage days. Du Plooy et al. (2009) also observed an inhibitory effect of different essential oils against *Penicillium digitatum* in *in vitro* and *in vivo* assays. Nevertheless, the power of inhibition of the composite films decreased throughout the storage time. This behavior has also been observed by other authors and has been explained by taking into account the changes in the availability of the antimicrobial compounds throughout time. In this sense, Cutter and Siragusa (1997), Outtara et al. (2000), Sebti and Coma (2002), Sebti et al. (2003) and Kristo et al. (2008) observed that these antimicrobial compounds were more effective when applied on the product surface as a component of a film or gel than when sprayed on as a solution or directly spread on the product. This is because the active substances can evaporate or diffuse more easily into the culture medium if they are not embedded in a polymeric matrix. So, the antimicrobial effect of BO could be limited by the diffusion of the active compounds into the agar medium, decreasing their availability on the medium

surface where the microorganisms are predominantly located, thus increasing the rate of microbial growth (Kristo et al., 2008; Outtara et al., 2000).

4. CONCLUSION

The incorporation of bergamot essential oil into chitosan films offers the possibility not only of imparting antimicrobial activity against *Penicillium italicum*, but also of improving the film's water vapour barrier properties. The most intense, longest-lasting antimicrobial effect against *Penicillium italicum* was obtained in films with the maximum bergamot oil content (3:1 BO-CH ratio), which showed a reduction of 2 logarithm units, as compared with the control film, at the end of the storage. So, these films could be used to extend the shelf life of fruits, by controlling moisture losses and fungal decay, with no notable changes in the fruit appearance. Nevertheless, in vivo studies are necessary to take the possible interactions between the film constituents and the food matrix into account.

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Capítulo II

Efecto del tipo de matriz y de aceite sobre las propiedades fisicoquimicas y el poder antimicrobiano de los films

ANTIMICROBIAL ACTIVITY OF POLYSACCHARIDE FILMS CONTAINING ESSENTIAL OILS

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Food Control, (enviado)

ABSTRACT

Antimicrobial films were prepared by incorporating different concentrations of bergamot (BO), lemon (LO) and tea tree (TTO) essential oils, into chitosan (CH) and hydroxypropylmethylcellulose (HPMC) films. Their antibacterial effectiveness against *Listeria monocytogenes*, *Escherichia coli* and *Staphylococcus aureus* was studied at 10ºC during a storage period of 12 days. HPMC-EO and CH-EO composite films present a significant antimicrobial activity against the three pathogens considered. The nature and amount of the essential oils (EO), the structure of the film and the possible interactions which exist between the polymers and active constituents of EO affected the antimicrobial activity of the films. In all film matrices, TTO exhibited the highest antimicrobial activity. A complete inhibition of microbial growth was observed for CH or HPMC-TTO films for *E. Coli*, HPMC-TTO for *L. monocytogenes* and HPMC-BO for *S. aureus*.

Keywords: chitosan, hydroxypropylmethylcellulose, *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*.

1.INTRODUCTION

Cases of food poisoning are on the rise in Europe; in 2007 a total of 5609 food-borne outbreaks were reported by European Union Member States (The EFSA Journal 2009). This increasing problem is emphasized by changes in legislation and consumer trends that request preservative free, safe, but mildly processed foods (Brul and Coote, 1999). To ensure the safety and quality of minimally processed and ready-to-eat food products, alternative technologies to thermal aggressive treatments, such as high hydrostatic pressure or pulsed electric field, are being considered. However, these alternatives could be less effective than conventional thermal treatments and sometimes pathogenic microbial growth is not eliminated (Valero and Francés, 2006). Moreover, problems of the microbial recontamination of the food surface could take place during the post-processing steps leading to recontamination. The combination of emerging processing technologies with active antibacterial technologies appears to be a good alternative to lengthen the shelf- life and improve the quality and safety of minimally processed foods. Since microbial recontamination on the food surface exists during the steps of the process, in order to guarantee food safety active food packaging could be more interesting than applying antibacterial technologies directly to the food (Ouatarra et al., 2000).

The incorporation of essential oils (EO) to edible films as natural bactericides might be an interesting option. In vitro studies have shown EO to have antibacterial properties against *Listeria monocytogenes, Salmonella typhimurium, Escherichia coli, Bacillus cereus* and *Staphylococcus aureus* at very low levels (Burt, 2004).

Likewise, the costs of applying essential oils and problems of intense aroma can be minimised by using this promising alternative. The mechanisms of action of EO have not been clearly identified but they seem to be related with the hydrophobic nature of the terpens (Burt 2004; Bakkali et al., 2008).

The potential benefits of the uses of essential oil of *Melaleuca alternifolia*, also known as tea tree oil (TTO), are widely known. What is remarkable is how it can be used pharmaceutically; it is reputed to have several medicinal properties including antibacterial, antifungal, antiviral, anti-inflammatory and analgesic properties (Carson et al., 1998). The TTO is a complex mixture of terpen hydrocarbons and tertiary alcohols and its composition is regulated by an international standard which sets levels of 14 components (Hammer et al., 2006). The main compounds responsible for the antimicrobial activity are terpinen-4-ol and 1.8-cineole.

Limited research has been carried out on citrus EO in terms of their use as antimicrobials in the food industry; it has been shown that they have potential bactericidal properties not only against yeast, moulds and spore forming bacteria, but also against food-poisoning bacteria (Deans and Ritchie, 1987). Limonene is one of the major components of citrus oils, with concentrations ranging from 88% to 95% of lemon oil, although levels in bergamot are lower, with concentrations ranging from 32% to 45% (Fisher and Phillips, 2006).

Cellulose derivatives are interesting film forming compounds, as they are odourless, tasteless and biodegradable (Krochta and Mulder-Johnston, 1997). In addition, their application cost is low. Hydroxypropylmethylcellulose (HPMC) presents excellent film-forming properties (Nisperos-Carriedo, 1994; Villalobos et al., 2006), with very efficient oxygen, carbon dioxide and lipid barriers. However, HPMC films are highly permeable to water vapour, which is an important drawback that limits their application (Krochta and Mulder-Johnston, 1997), since an effective control of moisture transfer is a desirable property.

Another biopolymer with excellent film forming ability is chitosan (Li et al., 1992). This non-toxic compound, obtained by the deacetylation of chitin, a structural component present in the shell of some crustaceans, presents antimicrobial properties.

The aim of this work is to analyse the effect of essential oil incorporation on the antimicrobial properties of chitosan and HPMC based films. The effectiveness of composite films against three food-borne pathogens, *Escherichia coli, Listeria monocytogenes* and *Staphylococcus aureus*, was evaluated at 10ºC during a storage period of 12 days.

2. MATERIALS AND METHODS

2. 1. Materials

Hydroxypropylmethylcellulose (HPMC, E464, Methocel Food grade, Dow Chemical Company, Midland, USA), high molecular weight chitosan (CH) with a deacetylation degree of 82.7% (Batch 10305DD, Sigma-Aldrich Química, Madrid, Spain), 98% glacial acetic acid (Panreac, Barcelona, Spain) and essential oils of lemon, bergamot and tea tree, supplied by Herbes del Molí (Alicante, Spain), were used to prepare the film-forming dispersions (FFD).

2.2. Preparation of the film-forming dispersions

Chitosan (1% w/w) was dispersed in an aqueous solution of glacial acetic acid (0.5%w/w) at 25ºC. Following overnight agitation, essential oils (EO) were added to the chitosan (CH) solution in the ratios indicated in Table 1.

Hydroxypropylmethylcellulose 1% wt was dispersed in deionised water at 80ºC. After the dissolution of the polysaccharide, EO were added in the ratios indicated in Table 1.

Both CH-EO and HPMC-EO mixtures were emulsified at room temperature using a rotor-stator homogenizer (Ultraturrax DI 25 basic-Yellowline, Janke & Kunkel, Staufen, Germany) at 13,500 rpm for 4 minutes. These emulsions were vacuum degasified at room temperature using a vacuum pump (Diaphragm vacuum pump, Wertheim, Germany).

Table 1. Concentration of components in aqueous film-forming dispersions (FFD)

Sample	HPMC (%p/p)	CH (%p/p)	Acetic acid (%v/p)	EO (% p/p)
HPMC	1	-	-	-
HPMC-0.5EO	1	-	-	0.5
HPMC-1EO	1	-	-	1
HPMC-2EO	1	-	-	2
HPMC-3EO	1	-	-	3
CH	-	1	0.5	-
CH-0.5EO	-	1	0.5	0.5
CH-1EO	-	1	0.5	1
CH-2EO	-	1	0.5	2
CH-3EO	-	1	0.5	3*

* This EO concentration was only used for films where no oil phase separation occurred in the film forming dispersion.

2.3. Preparation of films

FFD were poured onto a framed and levelled Petri dish plate (ϕ = 8.5 cm) and subsequently dried under atmospheric conditions. Film thickness was controlled by pouring the amount of FFD that will provide a surface density of solids in the dry films of 56 g/m^2 in all formulations. Dry films were peeled off from the casting surface and preconditioned in desiccators at 10ºC and at 57.4% relative humidity (RH) prior to testing.

2.4. Microbiological analysis

Stock culture of *Eschericia coli* (CECT515), *Listeria monocytogenes* (CECT 932) and *Staphylococcus aureus* (CECT 240) supplied by the Spanish Type Culture Collection (CECT, Burjassot, Spain), were kept frozen (-25ºC) in Tryptone Soy

Broth (TSB, Scharlab, Barcelona, Spain) supplemented with 30% glycerol (Panreac, Barcelona, Spain). Cultures were then regenerated by transferring a loopful of each bacteria into 10 mL of TSB and incubated at 37ºC overnight. A 10 µl aliquot from each overnight culture was again transferred into 10 mL of TSB and grown at 37ºC to the end of the exponential phase of growth. Subsequently, these appropriately diluted cultures were then used for the inoculation of the agar plates in order to obtain a target inoculum of 10^2 UFC/cm^2.

The methodology was adapted from Kristo et al. (2008). Tryptone Soy Agar (International Diagnostics, UK) with 3% NaCl (Sigma-Aldrich GmbH, Germany) was used as a model solid food system (TSANaCl). Aliquots of TSANaCl (20 g) were poured into Petri dishes. After the culture medium solidified, properly diluted overnight cultures from each strain were inoculated on the surface and different test films (containing or not antimicrobial substance) of the same diameter as the Petri dishes were placed onto the inoculated surface. Inoculated and uncoated TSANaCl Petri dishes were used as control. Plates were then covered with parafilm to avoid dehydration and stored at 10ºC for 12 days. Microbial counts on TSANaCl plates were examined immediately following the inoculation and periodically during the storage period. To this end, the agar was removed aseptically from the Petri dishes and placed in a sterile plastic bag with 100 ml of tryptone phosphate water (Scharlab, Barcelona, Spain). The bag was homogenized for 2 minutes in a Stomacher blender (Bag Mixer 400, Interscience). Serial dilutions were made and then poured onto TSA. Plates were incubated at 37ºC for 24 hours before colonies were counted. All tests were run in triplicate.

2.5. Statistical analysis

Results were analysed by means of a multifactor analysis of variance with 95% significance level using Statgraphics®Plus 5.1. Multiple comparisons were performed through 95% Least Significant Difference intervals (LSD).

3. RESULTS

3.1. *Escherichia coli*

Growth curves of *E.coli* in control TSANaCl plates and in TSANaCl plates coated with the different films are shown in figure 1. No significant differences were observed between the growth of *E.coli* on control TSANaCl plates and plates coated with HPMC film during the storage period. *E.coli* population increased from 2 to 8.5 logs UFC/cm^2 after the 12 days. Wu et al. (2004) also observed the absence of antimicrobial activity of cellulose films against *E.coli* strain. Growth data indicated that HPMC-EO composite films were effective at reducing microbial growth. This reduction increased when the essential oil concentration in the HPMC film rose.

The most effective essential oil is the tea tree oil. In fact, HPMC films with more than 1% of TTO completely inhibited pathogen growth for the first 7 days of storage. At the end of the storage, only HPMC films with the highest concentration of TTO (2%) could maintain a complete inhibition of the microbial growth, whereas a significant, but rather less important, increase (approximately 1 log UFC/cm^2) of *E.coli* counts was observed for 1% of essential oil. Even if a complete inhibition of the microbial growth was not observed when 0.5% of TTO was incorporated, *E.coli* counts were significantly reduced after the third day of storage, as compared to the control sample, and microbial population did not exceed 4 logs UFC/cm^2 at the end of the 12 days.

Concerning HPMC-BO composite films, differences among films with different BO concentration were not significant at the end of the storage period when a microbial reduction of more than 3 logs, as compared with the control plates, was observed. With the highest concentration of BO (2%), a total inhibition of the pathogen growth occurred during the first 3 days of the storage period.

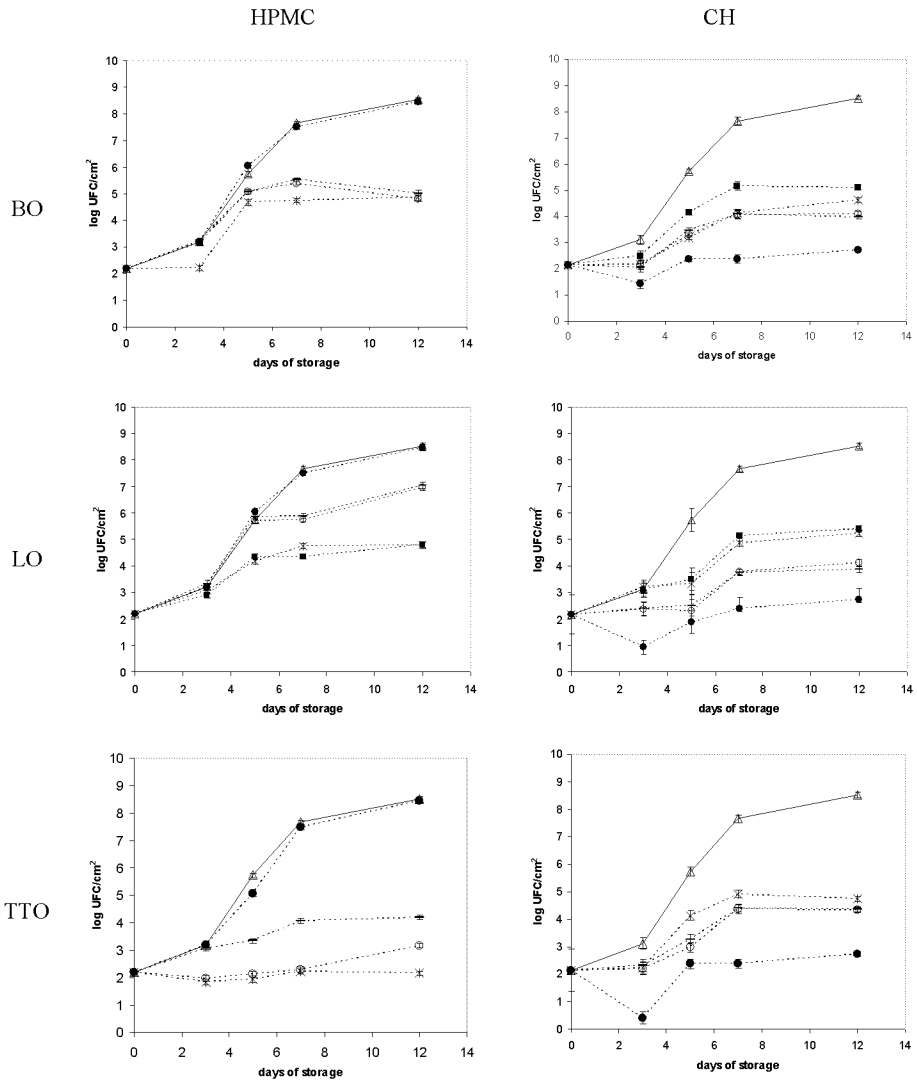

Figure 1. Effect of different films on the growth and survival of *Escherichia coli* on TSANaCL medium stored at 10ºC. Mean values and 95% LSD intervals for each time (Δ control, • Polymer, _ Polymer-0.5EO, ○ Polymer-1EO, * Polymer-2EO, ■ polymer-3EO).

HPMC-LO composite films showed a less marked antimicrobial activity for the lowest LO concentration levels (0.5 and 1%). A reduction of the *E.coli* counts, with respect to the control, was observed after the third day of storage for the highest LO (2 and 3%) and after the fifth day for 0.5 and 1%. At the end of the storage period, a pathogen reduction, as compared to the control plates, of about 1.5 and 4 logs was reached for the lowest and highest LO concentrations, respectively.

The obtained results agree with those reported by Moreira et al. (2005), when analysing the activities against different *E.coli* strains of plant essential oils. Their results demonstrated that both *Melaleuca alternifolia* and *Citrus limonum* essential oils showed bactericidal activities against *E.coli*, but the minimum inhibitory concentration (MIC) value obtained for LO was higher than those found for TTO (2.5 and 0.5 ml/100 ml, respectively). According to our results, when TTO was incorporated into the HPMC matrix, it was also more effective than citrus oil at reducing *E.coli* growth.

CH films without essential oil presented a significant antimicrobial activity against *E.coli*, showing a complete inhibition of the microbial growth during the tested storage period. Pathogen population did not exceed 2 logs UFC/cm^2 throughout the 12 storage days. Previous studies also showed the antimicrobial activity of chitosan films (Wu et al., 2004). Other authors report that the incorporation of the essential oils enhanced the antimicrobial efficiency of chitosan against different strains (*Escherichia coli, Listeria monocytogenes, Staphylococcus 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). Nevertheless, in this study, when CH films were tested against *E.coli*, the incorporation of essential oils (TTO, BO and LO) into the films led to a significant decrease of their antimicrobial effectiveness. This can be attributed to the dilution effect of CH when EO is present (see Table 2), thus being less available for microorganisms, and it reflects the milder antimicrobial effect of the EO compounds as compared to

CH, despite the fact that their antimicrobial effect has been proved in the HPMC films as well as in previous works (Moreira et al., 2005). The antimicrobial reduction of EO could be due to the bonding of the active compounds in the CH network through the strong interactions with the charged polymer chains which made their access to the microorganisms difficult.

In the case of CH-TTO composite films, a very mild effect of the TTO concentration on the growth of *E.coli* was observed. After 12 days, CH-TTO composite films provoked a microbial reduction of about 4 logs with respect to the control plates, compared to the 6 log reduction observed when pure CH films were used.

A similar loss of activity occurred when LO and BO were incorporated into the CH matrix and this effect was promoted when the EO concentration increased in the film, especially for LO. At the end of the storage period, an inhibition of about 4.5 logs was obtained with the lowest concentrations of LO (0.5, 1%) and BO (0.5,1,2%), whereas for the highest levels of BO (3%) and LO (2 and 3%) the loss of activity was greater (only 3 logs reduction as compared to the control). The dilution effect of CH and its impact on the antimicrobial activity of the CH films were ratified by the EO concentration effect observed in LO and BO.

3.2. *Listeria monocytogenes*

The antimicrobial effect against Listeria monocytogenes at 10ºC of CH, HPMC, CH-EO and HPMC-EO composite films was determined on TSANaCl medium and shown in Figure 2. Pure HPMC films were not effective at reducing *Listeria monocytogenes* growth, since no significant differences were observed in microbial growth with respect to the control TSANaCl plates. *Listeria monocytogenes* population increased from 2 to 8 logs UFC/cm^2 at the end of the storage period.

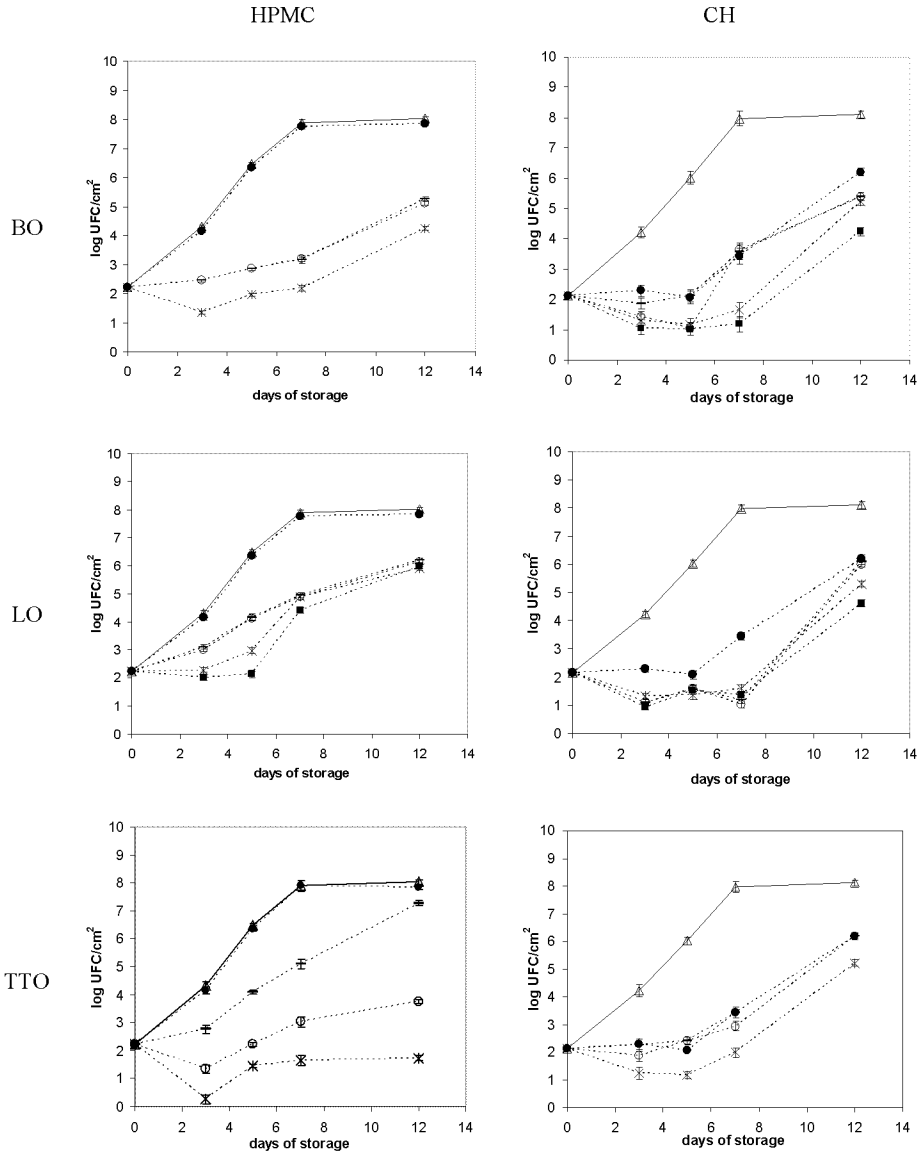

Figure 2. Effect of different films on the growth and survival of *Listeria monocytogenes* on TSANaCL medium stored at 10ºC. Mean values and 95% LSD intervals for each time (Δ control, • Polymer, _ Polymer-0.5EO, ○ Polymer-1EO, * Polymer-2EO, ■ polymer-3EO).

TTO incorporated into the HPMC matrix was more effective than citrus oils at the same concentration. Following a storage period of 12 days, films with 2% of TTO reduced the microbial growth with respect to the control to 6.5 logs whereas only 2 and 4 logs were reduced with the same level of LO or BO, respectively. At the highest concentration (2%), TTO incorporation into the HPMC matrix led to a reduction of the initial pathogen population throughout the entire storage period and a complete inhibition of growth was observed with 1% TTO until the fifth day of storage, giving rise to a microbial reduction of approximately 4.5 logs after 12 storage days. As expected, the antimicrobial effect is less marked with 0.5% TTO where a microbial reduction of approximately 2-3 logs, as compared to the control plates, was observed during the first 7 days of storage.

Although, as commented on above, HPMC-LO composite films presented a less marked antimicrobial activity than HPMC-TTO films, the highest levels of lemon essential oil (2 and 3%) led to a complete inhibition of *Listeria monocytogenes* growth for 5 days, reaching a reduction of approximately 2 logs at the end of the storage period. At this final point, the antimicrobial activity was equivalent for all concentrations of LO and the pathogen population did not exceed 6 logs UFC/cm^2.

HPMC-BO composite films were more effective at controlling *Listeria monocytogenes* growth than HPMC-LO films. During the first 3 days, a complete inhibition of microbial growth was observed with the three BO concentrations, 0.5, 1 and 2%. At the end of storage, the pathogen population did not exceed 4 and 5 logs UFC/cm^2 for 2% and both 0.5 and 1% of BO, respectively.

CH films without essential oil showed a significant antimicrobial activity against *Listeria monocytogenes*; a complete inhibition of the microbial growth was observed during the first five days and the pathogen population did not exceed 6 logs UFC/cm^2 at the end of the storage period. Previous studies also reported the antimicrobial activity of CH against *Listeria monocytogenes* strains (Kristo et al., 2008; Beverlya et al., 2008). The incorporation of essential oils into the CH matrix

slightly improved the antimicrobial effect of CH, as was previously reported by Zivanovic et al. (2005) for oregano essential oil. In the cases of TTO and BO, this was more intense when the EO concentration increased, but for LO no effect of the EO concentration was observed.

3.3. *Staphylococcus aureus*

Growth curves of *S.aureus* on control TSANaCl plates and TSANaCl plates coated with the different films are shown in figure 3. *S.aureus* grew slowly; the population only increased approximately from 2 to 3 logs UFC/cm^2 at the end of the storage period. This can be explained by the culture conditions and, more specifically, the incubation temperature. The Food Safety and Inspection Service (FSIS) reported that *S.aureus* can survive and grow slowly at various refrigeration temperatures (4.4, 10 and 15.5ºC) (Lim and Mustapha, 2007). This observation agrees with our results and with that reported in previous studies (Lim and Mustapha, 2007; Millette et al., 2007; Fernandez-Saiz et al., 2010). HPMC films were not effective at reducing *Staphylococcus aureus* growth, since no significant differences appeared with respect to the microbial growth on control TSA-NaCl plates. Wu et al. (2004) also observed the absence of antimicrobial activity of cellulose films against *S.aureus* strain.

EO incorporation into HPMC films reduced the pathogen growth. Bergamot essential oil seems to be the most effective antimicrobial compound tested. During the first 3 days the highest concentrations of BO (1 and 2%) led to a microbial reduction of the initial population in the plates and, although the antimicrobial effectiveness slightly decreased afterwards, the pathogen population remained lower than the initial level of infection after 12 days. In the presence of 0.5% BO, a complete inhibition of microbial growth was observed during the entire storage period.

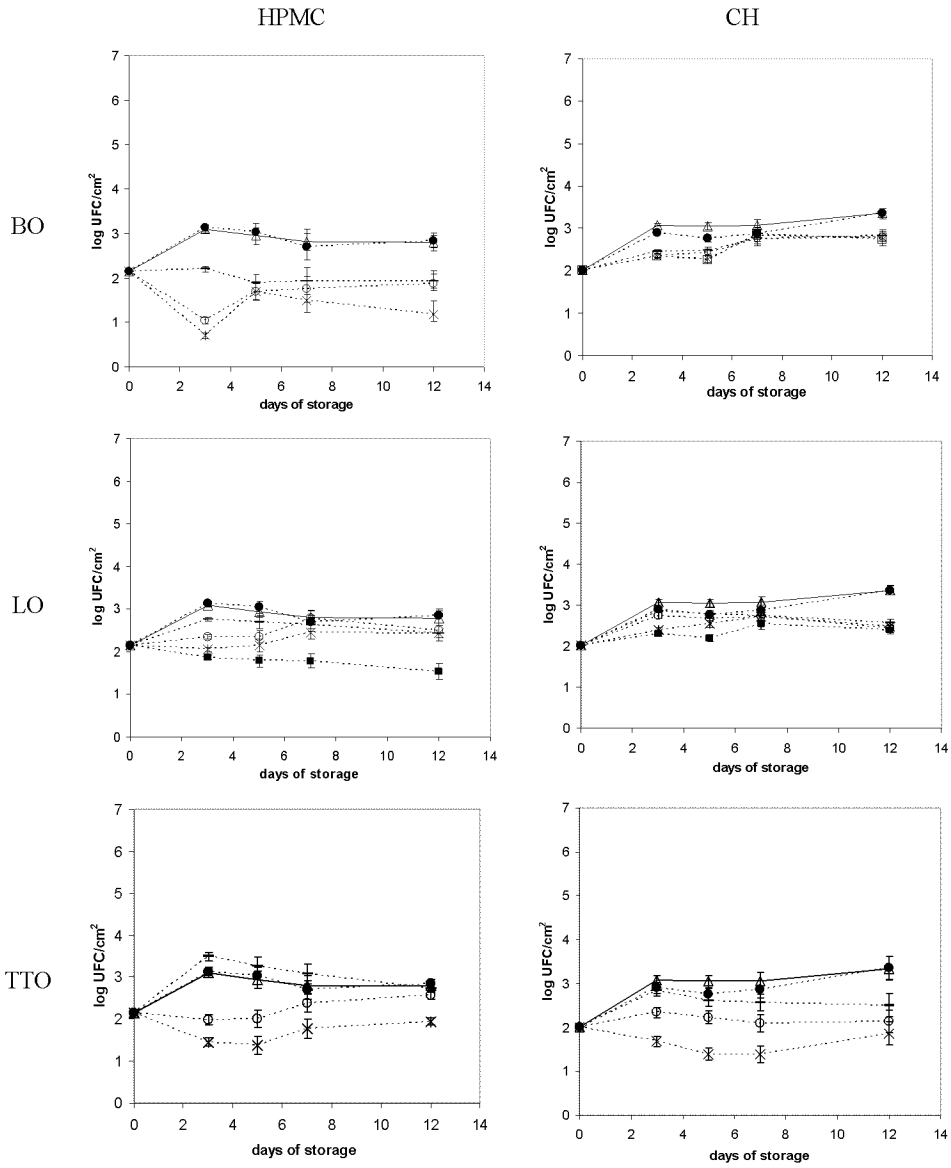

Figure 3. Effect of different films on the growth and survival of *Staphylococcus aureus* on TSANaCL medium stored at 10ºC. Mean values and 95% LSD intervals for each time (Δ control, • Polymer, _ Polymer-0.5EO, ○ Polymer-1EO, * Polymer-2EO, ■ polymer-3EO).

The incorporation of LO into HPMC films was less effective, but with the highest concentration of LO (3%) a reduction of 1 log with respect to the control plate was observed throughout the whole storage period.

HPMC-TTO composite films showed a similar antimicrobial activity to those of HPMC-citrus essential oil composite films. Up to 1% of TTO, films did not show antimicrobial effectiveness, as compared to control plates, but with the highest TTO concentration (2%), a microbial reduction of approximately 1 log was exhibited during the entire storage period.

Pure CH films did not show antimicrobial activity against *S.aureus*, since no significant differences were observed during the complete storage period with respect to the control plates, but the EO incorporation into CH films reduced the pathogen growth. The antimicrobial effectiveness of CH-citrus essential oils was more limited than in HPMC composite films and very small differences appeared as a function of the essential oil concentration used. Throughout the whole storage period, the incorporation of LO or BO supposed a microbial reduction of approximately 1 log. However, CH-TTO composite films were more effective at controlling *Staphylococcus aureus* growth than CH-citrus EO films. During the storage period, a complete inhibition of microbial growth was observed with the two highest TTO concentrations (1 and 2%). The maximum amount of TTO exhibited a pathogen reduction of almost 2 logs with respect to the control plates.

3.4. Discussion

To understand antimicrobial effectiveness of HPMC-EO and CH-EO composite films, several factors must be considered. Parameters concerning microorganisms such as inoculum sizes, bacteria physiological state, and storage conditions (temperature, culture media) are important to explain the antimicrobial activity of chitosan, essential oils and different films. Fernandez-Saiz et al. (2009) pointed out that the sensitivity of *S.aureus* seems to be higher when bacteria were inoculated in

the mid-log phase and microbial reduction was significantly more pronounced compared to the late-log phase or the stationary phase. In the present study, the influence of all these factors was not evaluated, the bacteria were inoculated at stationary phase, the storage conditions were fixed and only one level of infection was considered.

In this study, LO, BO and TTO incorporated into CH or HPMC films were effective at inhibiting or reducing pathogen growth. Nevertheless, antimicrobial activity of EO varied as a function of the type of bacteria, the nature of the essential oil and the characteristics of the film matrix where they were included. Although EO were effective against the three pathogens considered, they were more effective, at the same concentration, against *L.monocytogenes* than *E.coli* when incorporated into HPMC films. Some previous studies (Smith-Palmer et al., 2001) have also found that Gram-positive bacteria were more sensitive to EO than Gram-negative bacteria, due to the relatively impermeable outer membrane that surrounds Gram-negative bacteria. In some cases, antimicrobial effectiveness of EO decreased throughout storage, which could be explained by the evaporation of volatile compounds responsible for the antimicrobial activity and/or by the migration of EO components into the agar medium. Essential oils contain around 85-99% volatile and 1-15% non-volatile components. The antimicrobial activity of these natural compounds is essentially due to a complex mixture of terpens which constitute the volatile fraction (Fisher and Phillips, 2008). The selective activity of the EO compounds is reflected in the obtained results since the response of each microorganism was different for each EO. Likewise, interactions of these compounds with the film matrix also have a great impact on the antimicrobial activity. In fact, both for HPMC and CH, the influence of the nature and the amount of the essential oil differ with the pathogen considered. Concerning *E.coli*, the nature of the essential oil incorporated into CH or HPMC matrix was more relevant than the concentration of EO in the film to determine the antimicrobial

effect. During the first days of the storage period, the concentration of the EO, rather than the type of EO, was the most relevant parameter in the inhibition of *S.aureus* growth. Nevertheless, after 12 days, this tendency changed and the nature of the incorporated EO became more significant. Regarding *L.monocytogenes*, results differed depending on the nature of the polysaccharide. The amount of EO incorporated into the HPMC matrix was more relevant than the nature of the EO, whereas for CH films this only occurred during the last days of storage.

The polymer which constitutes the film matrix is also relevant in defining the antimicrobial properties. In the present study, chitosan and HPMC behaved very differently. The first polymer, electrically charged, showed antimicrobial activity itself and Liu et al. (2004) and Li et al. (2010) described the mechanisms. According to these authors, chitosan increased the permeability of the outer and inner membranes and ultimately disrupted bacterial cell membranes, releasing the cellular content. In all likelihood, this damage was caused by the electrostatic interaction between $-NH^{3+}$ groups of chitosan acetate and carbonyl and phosphoryl groups of phospholipid components of cell membranes. Different factors, such as the concentration, degree of acetylation or the molecular weight of chitosan and the incorporation of acetic acid, affect the antimicrobial activity of the polymer. When the concentration was higher than 200 ppm, acetic acid showed a significant antimicrobial activity against *E.coli* strains (Liu et al., 2006). In the present study, a part of the antimicrobial activity observed with chitosan films could be attributed to the residual amount of this compound in the film, since some of the acetic acid incorporated in the FFD evaporates during film drying. According to Brody et al. (2004), the antimicrobial effect of chitosan occurred without any migration of active agents. As chitosan is in a solid form, only microorganisms in direct contact with the active sites of the polymer are inhibited because chitosan cannot diffuse through the adjacent agar media (Coma et al., 2002). In the present study, bacteria are inoculated on the plate surface, so chitosan is in direct contact with

microorganisms. Incorporating antimicrobial agents, such as essential oils, into chitosan edible films can improve the antimicrobial efficiency of the film, as the diffusion of the oil compounds would compensate the non-migrated antimicrobial power of CH. Nevertheless, this was only observed for the Gram positive bacteria tested, *Listeria monocytogenes* and *Staphylococcus aureus*, which can be explained because CH is less effective against these pathogens if compared with essential oils. Previous studies show that EO were active against Gram-positive rather than Gram-negative bacteria (Burt, 2004) and, generally, the latter were more sensitive to CH (Devlieghere et al., 2004; Liu et al., 2004). *Escherichia coli*, as opposed to the other microorganisms studied, is classified among Gram-negative bacteria and a more effective antimicrobial activity of pure CH films has been observed.

Cellulose and these derivatives, as opposed to chitosan, do not show antimicrobial activity and so, only the action of the EO compounds can be observed, since the polymer acts only as a neutral support releasing the active compounds to the surface where the pathogens were plated. In this sense, the particular interactions of EO compounds and the polymer matrix can also play an important role. For the same concentration of a particular EO incorporated into HPMC or CH films, significant differences were observed in the behaviour of each pathogen under consideration. The reduction growth of the studied pathogens was more heavily influenced by the type of matrix than by the nature of the essential oil. Chitosan could bind with terpens which are the major components of essential oils (Ravi Cumar et al., 2004). These interactions could limit the release of antimicrobial compounds. The rate of microbial growth rose in line with a progression in the release of the antimicrobial compounds which leads to a scarce availability of the active substances on the surface, where the contamination is prevalent (Kristo et al., 2008; Outtara et al., 2000).

Mayachiew et al. (2010) studied the effect of the drying method, particularly the drying temperature, on the antimicrobial activities of chitosan films. The

antimicrobial activity, swelling and functional group interaction of the CH films with galangal extract were found to be affected by the drying methods and conditions. Ambient drying and low-temperature hot air drying led to films with the highest antimicrobial activity.

Following 12 days at 10ºC, the effectiveness of the films tends to decrease significantly which can be attributed to the film's destructuration. After the films were placed on the inoculated surface of TSANaCl, the CH and HPMC hydrophilic matrices absorbed water, which induced changes in the film structure. At the end of storage, films were completely dissolved and, consequently, the EO compounds were liberated and they were able to evaporate or diffuse into the agar medium.

4. CONCLUSION

HPMC-EO and CH-EO composite films, containing BO, LO or TTO, showed a significant antimicrobial activity against the three pathogens studied (*E.coli, L.monocytogenes, S.aureus*). In all film matrices, TTO exhibited the highest antimicrobial activity. A complete inhibition of microbial growth was observed for CH films or HPMC-2TTO for *E. Coli*, HPMC-2TTO for *L. monocytogenes* and HPMC-2BO for *S. aureus*. The nature and amount of the EO, the EO-polymer ratio in the film and the possible interactions between the polymer and the active compounds of EO play an important role in the film's antimicrobial activity. When the polymer has intense antimicrobial activity (such as chitosan against Gram negative bacteria) the incorporation of EO reduced this activity due to the effective reduction of the available polymer concentration. Nevertheless, the antimicrobial activity is enhanced when the EO is more active than the polymer, such as in the case of CH films against Gram positive bacteria (case of *Listeria monocytogenes*). When the polymer did not show antimicrobial activity, the antimicrobial effect of the EO generally increased as the ratio of EO-polymer rose in the matrix.

Nevertheless, the effect of the EO ratio is affected by the interactions of the active compounds in the film matrix, which determine their diffusion rate to the infected surface.

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EFFECT OF ESSENTIAL OILS ON PROPERTIES OF FILM FORMING EMULSIONS AND FILMS BASED ON HYDROXYPROPYLMETHYLCELLULOSE AND CHITOSAN

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ABSTRACT

Film forming dispersions (FFD) and films, prepared by incorporating different concentrations of bergamot (BO), lemon (LO) and tea tree (TTO) essential oils into hydroxyproplymethylcellulose (HPMC) and chitosan (CH) were obtained and their physico-chemical properties were characterised. Results showed that the increment of essential oil (EO) content promoted significant changes in the size and surface charge of the FFD particles. As regards the film properties, the higher the EO content, the lower the water vapour permeability and the moisture sorption capacity. In general, the addition of EO into the HPMC or CH matrix leads to a significant decrease in gloss, transparency, tensile strength and elastic modulus of the composite films. Discriminant analyses of obtained data revealed that the polymer type was the main factor which defined the FFD and composite film behaviour. For a given polymer, although both the nature and concentration of the EO influenced FFD behaviour, the nature played a more important role. In film properties, the discriminant analyses did not reveal different groups associated to the different nature or concentration of the essential oils, although composite films with BO appeared to differ slightly from the rest.

Keywords: tea tee oil, bergamot oil, lemon oil, water vapour permeability, mechanical properties, particle size distribution, ζ-potential.

1. INTRODUCTION

In the next few years one of the major challenges for food technologists is the design of active food packaging. This technology appears to be a promising alternative with an increasing amount of applications due to its advantages over traditional packaging systems. The use of edible films and coatings as carriers of active substances has been suggested as an interesting option (Cuq, Gontard and Gontard, 1995; Han, 2000). Essential oil compounds, which have a well documented antimicrobial activity against spoilage microorganisms, foodborne and postharvest pathogens (Burt, 2004; Bakkali et al., 2008) are of great potential use in bioactive coatings. The mechanisms of action of essential oils (EO) have not been clearly identified but they seem to be related with the hydrophobic nature of the different terpens (Burt, 2004; Bakkali et al., 2008).

The specific advantage of EO appears to be the synergistic effects of their compounds as evidenced in the greater activity when applied as natural EO, as compared with the sum of the effects of the individual substances (Duke and Beckstrom-Sternberg, 1992). The components of EO are important as their qualitative and quantitative composition determines the characteristics of the oils, which, in turn, could have an effect on their antimicrobial potential (Dugo et al., 2000). The typical composition of lemon, bergamot and tea tree oil, which are the EO used in this work, is reported in table 1. Limonene is one of the major components of citrus oils with concentrations from 88% to 95% in lemon oil, although levels in bergamot are lower with concentrations ranging from 32% to 45% (Fischer and Phillips, 2008). The composition of the essential oil of Melaleuca alternifolia, also known as tea tree oil (TTO), is quite different. TTO is a complex mixture of terpens, hydrocarbons and tertiary alcohols and its composition for determined uses is regulated by an international standard which sets levels of 14 components (Hammer et al., 2006). Its main compound is terpinen-4-ol (around

40%), which is responsible for its antimicrobial activity (Cox et al., 2001; Terzi et al., 2007).

Table 1. Typical composition of essential oils (principal volatile compounds).

Component	Composition (%)		
	Tea tree oil [a]	Bergamot oil [b]	Lemon oil [b]
Terpinen-4-ol	40.1	0.00	0.00
γ-Terpinene	23.0	-	-
α-Terpinene	10.4	0.23	0.46
1,8-Cineole	5.1	-	-
Terpinolene	3.1	-	-
ρ-Cimene	2.9	5.62	1.75
α-Pinene	2.6	1.39	0.27
α-Terpineol	2.4	0.00	1.30
Limonene	1.0	72.88	78.84
Butylacetate	-	4.97	1.47
Linalool	0.00	10.23	0.02
Valencene	-	0.00	3.34

[a] Brophy et al. (1989)
[b] Moufida and Marzouk (2003)

Among the biopolymers used to obtain films/coatings, cellulose derivatives are interesting film forming compounds, as they are odourless, tasteless and biodegradable (Krochta and Mulder-Johnston, 1997). Hydroxypropylmethylcellulose (HPMC) presents a great potential for a wide range of food applications due to its biocompatibility, non-toxicity, low cost and excellent film forming capacity (Nisperos-Carriedo, 1994; Villalobos, Hernández-Muñoz and Chiralt, 2006). HPMC films are very efficient oxygen, carbon dioxide and lipid barriers. However, they are highly permeable to water vapour, which is an important drawback that limits its application (Krochta and Mulder-Johnston, 1997), since an effective control of moisture transfer is one of the most desirable properties of the films. While the incorporation of essential oils into HPMC

matrices could confer antimicrobial properties to the films, it may contribute to reduce water vapour permeability due to their hydrophobic nature.

Among the bioactive macromolecules, chitosan has a great potential for a wide range of food applications due to its biodegradability, biocompatibility, antimicrobial activity, non-toxicity and film forming capacity (Li et al., 1992; Tharanathan et al., 2003). Chitosan based films have been proven to present moderate oxygen barrier properties and good carbon dioxide barrier properties but high water vapour permeability, due to their hydrophilic nature (Butler et al., 1996). Combined antimicrobial effects have been described for chitosan films containing essential oils (Sánchez-González, 2010) while their water barrier properties were also improved when these hydrophobic compounds were incorporated in composite films.

Films and coatings must be designed to fulfil a number of requirements, such as to have proper mechanical properties, good appearance (adequate gloss and transparency) and adequate water and gas barrier properties. In composite films containing lipids in a biopolymer matrix, the microstructure plays a very important role (Villalobos et al., 2005; Fabra, Talens and Chiralt, 2009) in these properties, which, in turn are greatly affected by the structural properties and stability of the film forming dispersions (FFD). The stability of FFDs is affected by their particle size and distribution, rheological behavior, and ζ-potential of the dispersed lipid particles (McClements, 2007).

Although edible coatings with EO have been previously studied (Sánchez-González et al., 2009; Zinoviadou, Koutsoumanis and Biliaderis, 2009; Pranoto, Rakshit and Salokhe, 2005), a comparative study of the effect of different EO (of different composition) in differing polymer matrices has not been published.

The aim of this work is to evaluate how the nature of the EO (lemon, bergamot and tea tree oil) and the EO:polymer ratio affect the properties of HPMC and chitosan based films, through the characterization of the stability related parameters

(particle size, rheological behavior and ζ potential) of the FFDs and the physical properties (barrier, mechanical and optical) of the films.

2. MATERIALS AND METHODS

2. 1. Materials

Hydroxypropylmethylcellulose (HPMC, E464, Methocel Food grade, Dow Chemical Company, Midland, USA), high molecular weight chitosan (CH) with a deacetylation degree of 82.7% (Batch 10305DD, Sigma-Aldrich Química, Madrid, Spain), 98% glacial acetic acid (Panreac, Barcelona, Spain) and essential oils (bergamot, lemon, tea tree oil) supplied by Herbes del Molí (Alicante, Spain) were used to prepare the film-forming dispersions.

2.2. Preparation of the film-forming dispersions

Hydroxypropylmethylcellulose 1% wt was dispersed in deionised water at 80 ºC. Chitosan (1% w/w) was dispersed in an aqueous solution of glacial acetic acid (0.5% w/w) at 25ºC. After the dissolution of the polysaccharides, essential oils (EO) were added to polymer solutions to reach a final concentration of 0, 0.5, 1 and 2% (w/w). HPMC-EO and CH-EO mixtures were emulsified at room temperature using a rotor-stator homogenizer (Ultraturrax DI 25 basic-Yellowline, Janke & Kunkel, Staufen, Germany) at 13,500 rpm for 4 minutes. These emulsions were vacuum degasified at room temperature with a vacuum pump (Diaphragm vacuum pump, Wertheim, Germany).

2.3. Characterization of the film-forming dispersions

The density of the FFD was measured by means of a digital densimeter DA-110M, (Mettler Toledo, Barcelona, Spain). A pH-meter C831 (Consort, Tumhout, Belgium) was used to determine the pH of the FFD at 20ºC.

2.3.1. ζ- potential measurements

In order to perform ζ- potential measurements, FFD were diluted to a droplet concentration of 0.02% EO using deionised water. ζ-potential was determined by using a Zetasizer nano-Z (Malvern Instruments, Worcestershire, UK). The Smoluchowsky mathematical model was used to convert the electrophoretic mobility measurements into ζ-potential values.

2.3.2. Particle size measurements

Particle size analysis of the FFD was carried out by using a laser diffractometer (Mastersizer 2000, Malvern Instruments, Worcestershire, UK). The samples were diluted in deionised water at 2,000 rpm until an obscuration rate of 10% was obtained. The Mie theory was applied by considering a refractive index of 1.52 and absorption of 0.1 for essential oils. Three samples of each FFD were measured in quintuplicate.

2.3.3. Rheological behaviour

The rheological behaviour of FFD was analysed in triplicate at 25ºC by means of a rotational rheometer (HAAKE Rheostress 1, Thermo Electric Corporation, Karlsruhe, Germany) with a sensor system of coaxial cylinders, type Z34DIN Ti.

Rheological curves were obtained after a stabilization time of 5 minutes at 25ºC. The shear stress (σ) was measured as a function of shear rate ($\dot{\gamma}$) from 0 to 512 s^{-1}, taking 5 minutes to reach the maximum shear rate and another 5 minutes to attain zero shear rate.

The power law model (Eq. 1) was applied to determine the consistency index (K) and the flow behaviour index (n). Apparent viscosities were calculated at 100 s^{-1}.

$$\sigma = K \cdot \dot{\gamma}^{n} \qquad \text{(Eq. 1)}$$

2.4. Preparation of films

A casting method was used to obtain films. FFD were poured onto a framed and levelled polytetrafluorethylene (PTFE) plate (ϕ = 15 cm) and were dried in atmospheric conditions. Film thickness was controlled by pouring the amount of FFD that will provide a surface density of solids in the dry films of 56 g/m^2 in all formulations. Dry films were peeled off the casting surface and preconditioned in desiccators at 20 ºC and 54.4 % relative humidity (RH) prior to testing. A hand-held digital micrometer (Palmer - Comecta, Spain, ± 0.001 mm) was used to measure film thickness at three different points of the same sample at least.

2.5. Characterization of the films

2.5.1. Water vapour permeability

Water vapour permeability (WVP) was measured in dry film discs (ϕ = 7 cm), which were equilibrated at 54.4 % RH and 20 ºC, according to the "water method" of the ASTM E-96-95 (ASTM, 1995), using Payne permeability cups (Elcometer SPRL, Hermelle /s Argenteau, Belgium). For each type of film, WVP measurements were replicated three times and WVP was calculated following the methodology described by Sánchez-González et al. (2009), at 20ºC and 54.4-100 % relative humidity gradient. The equilibrium moisture content of the films at a_w 0.75 (intermediate value in the RH gradient) was determined from the weight loss when drying the equilibrated films in a vacuum oven at 70ºC.

2.5.2. Mechanical properties

Mechanical properties were measured in films equilibrated at 54.4 % RH at 20ºC by using a Texture Analyser TA-XT-plus (Stable Micro Systems, Surrey, UK), with a 50 N load cell equipped with tensile grips (A/TG model) according to Sánchez-González et al. (2009).Tensile strength (TS) and percentage of elongation

(% E) at break, and elastic modulus (EM) were evaluated in eight samples from each type of film.

2.5.3. Optical properties

Gloss was determined by using a gloss meter (Multi-Gloss 268, Minolta, Langenhagen, Germany) at an incidence angle of 60º, following the ASTM standard D523 (ASTM, 1999) in films previously equilibrated at 20 ºC and 54.4 % RH. Gloss measurements were carried out in quintuplicate over a black matte standard plate. Results were expressed as gloss units, relative to a highly polished surface of standard black glass with a value close to 100.

The transparency of the films was determined through the surface reflectance spectra in a spectrocolorimeter CM-3600d (Minolta Co, Tokyo, Japan) with a 10 mm illuminated sample area. Measurements were taken from three samples in each formulation by using both a white and a black background. Film transparency was evaluated through the internal transmittance Ti (0-1, theoretical range) (Hutchings, 1999), by applying the Kubelka-Munk theory for multiple scattering to the reflection data (Sánchez-González et al., 2009). Values of Ti at 450 nm were used to compare samples.

2.6. Statistical analysis

Results were analysed by multifactor analysis of variance and discriminant analysis with 95% significance level using Statgraphics®Plus 5.1.

3. RESULTS AND DISCUSSION

3.1. Characterization of the film-forming dispersions

Density, pH and ζ-potential values of the different film-forming dispersions (FFD) are reported in Tables 3 and 4, for HPMC and CH FFDs. Only for the highest

essential oil (EO) concentrations (2%) was a significant decrease in the density of the HPMC FFD observed. Citrus oils presented a more marked effect on density of the FFD in comparison with tea tree oil (TTO). The same tendency was observed for CH FFD density whose values were in the range of those reported by other authors at the same pH (Vargas et al., 2009). Concerning the pH of CH FFD, values were around 4.3 at room temperature and did not vary significantly ($p<0.05$) with the incorporation of EOs, since, at the low pH value of CH dispersion, no dissociation of the weak acid EO components occurs. However, the incorporation of EO into HPMC led to a significant decrease of pH due to the acid nature and dissociation in the aqueous solution of some of the EO components, such as has been reported in a previous study (Sánchez-González et al., 2009).

Concerning the ζ-potential values of the HPMC FFD, they presented slight negative values (between -2.5 and -7 mV). These values were markedly lower than those corresponding to the aqueous dispersion of EO (1% wt) in absence of HPMC (Table 2) (ζ-potential values of 1% TTO, BO and LO measured in water were -31 ± 2 mV, -61 ± 3 mV, and -49 ± 3 mV, respectively). Modified cellulose derivatives have been shown to present interfacial activity and can adsorb on EO droplets, thus modifying the electrophoretic mobility plane and so the ζ-potential values (Huang et al., 2001). So, results point to the fact that the HPMC chains are adsorbed on the surface of EO droplets, leading to an increase in their effective size, with a lower electrical net charge on the surface surrounding the adsorbed polymer layer. Particle sizes showed monomodal distributions whose mean size values are shown in tables 2 and 3 for EO dispersions without and with polymer, respectively. As can be deduced from these tables, except for TTO, particles showed significantly lower mean size values when there is no polymer in the system. In general, the increment of EO did not affect the ζ-potential values of the particles, except for the HPMC-BO dispersions. In this case, the highest EO content led to more negatively charged particles.

Concerning CH FFD, the same trends commented on above were observed when EOs were added to the pure CH dispersion: the mean particle size significantly ($p < 0.05$) increased and the ζ-potential of the particles decreased ($p<0.05$), leading to bigger droplets with lower electrical net charge. In this case, the electrostatic interactions between CH and EO compounds at the pH of the FFD (4.30) will contribute to the reduction of the electrical net charge, as was described in previous studies (Vargas et al., 2009). The stability of the emulsified system was ensured by the steric stabilization promoted by the CH interfacial adsorption and the high value of ζ-potential (significantly higher than +30 mV) (Roland et al., 2003).

In both HPMC and CH dispersions, the increase in EO content significantly ($p < 0.05$) increased the mean particle size, although this effect was more intense in CH dispersions. Significant differences were observed as a function of the nature of the EO, depending on the type of polymer. FFD of HPMC with TTO presented the lowest particle sizes and those with LO the highest values, whereas in CH dispersions, the values of FFD with LO and TTO, at a determined EO ratio, were closer and, at high OE ratio, lower than those with BO.

With regard to the rheological characteristics, all FFD showed a shear thickening behaviour and no thyxotropic effects were observed from the comparison of the up and down curves. So, rheological data were fitted to the Ostwald de Waale model. Tables 3 and 4 show the flow and consistency indexes, together with the apparent viscosity (η_{ap}) values at a shear rate of 100 s^{-1} for HPMC and CH FFDs. The values of the correlation coefficient were in all cases around 0.98.

Rheological parameters and apparent viscosity at 100 s^{-1} for pure HPMC dispersions agreed with those reported by Chen (2007). As expected, the addition of EO to the HPMC dispersion promoted slight but significant changes in the rheological pattern: the consistency index (k) increased whereas the flow index (n) and the apparent viscosity decreased ($p<0.05$). So, EO incorporation made the fluid systems less viscous and less shear thinning than the pure HPMC solution. This

behaviour is coherent with the adsorption of the HPMC molecules on the droplet surface, which contributes to reduce their viscous contribution in the continuous phase while droplets are more stable and less sensitive to changes promoted by the shear forces. Neither the concentration nor the nature of the essential oil significantly affected ($p>0.05$) the rheological parameters and the viscosity of the FFD.

For CH FFD, rheological parameters and apparent viscosity at 100 s^{-1} for pure chitosan are in the order of those found by No et al. (2006) and Vargas et al. (2009) for this polymer. The incorporation of EO promoted similar changes to those commented on above for HPMC FFD, in agreement with the polymer adsorption on the droplet surface. Nevertheless, a significant decrease in viscosity and consistency index (k) ($p<0.05$) was observed for LO and BO when their concentration exceeded 1% in the FFD. These results could be explained by the significant reduction of the particle charge or ζ-potential, which also contributes to the system viscosity by reducing the electroviscous effects (McClements, 2005).

Table 2. EO characterization: ζ-potential, density (ρ) and particle size (d_{43} and d_{32}) values at 25 °C. Mean values and standard deviation.

EO	ζ (mV)	ρ (Kg/m^3)	d_{43} (μm)	d_{32} (μm)
TTO	-30.6 (1.5)^a	896.988 (0.3)^a	4.8 (0.4)^a	2.60 (0.08)^a
BO	-61 (3)^b	896.957 (0.2)^a	3.66 (0.05)^b	2.83 (0.04)^b
LO	-49 (3)^c	896.605 (0.3)^a	3.94 (0.04)^c	3.6 (0.4)^c

$^{a, b, c}$ Different superscripts within a column indicate significant differences among formulations ($p < 0.05$).

Table 3. FFD HPMC characterization: pH, ζ-potential, density (ρ), Ostwald de Waale model parameters, apparent viscosity (η_{ap} at 100 s^{-1}) and particle size (d_{43} and d_{32}) values at 25 °C. Mean values and standard deviation.

FFS	pH	ζ (mV)	ρ (Kg/m^3)	$0 \leq \dot{\gamma} \leq 512$ s^{-1}				d_{43} (μm)	d_{32} (μm)
				n	k (Pa·s)^n	r^2	η_{ap} (Pa·s)		
HPMC	7.87 (0.06)^a	-3.4 (0.6)^a	1002.5 (0.3)^a	1.162 (0.006)^a	0.00209 (0.00009)^a	0.985	0.00441 (0.00006)^a	-	-
HPMC-0.5BO	6.04 (0.02)^b	-2.5 (0.4)^b	1001.5 (0.9)^a	1.028 (0.002)^b	0.00362 (0.00008)^b	0.970	0.00413 (0.00006)^b	3.68 (0.02)^a	2.721 (0.017)^a
HPMC-1BO	5.38 (0.05)^c	-5.1 (0.2)^c	1002.6 (0.3)^a	1.029 (0.002)^b	0.00351 (0.00016)^b	0.998	0.00415 (0.00014)^b	4.39 (0.08)^b	3.31 (0.04)^b
HPMC-2BO	4.71 (0.02)^d	-5.6 (0.2)^c	999.10 (0.14)^b	1.0297 (0.0008)^b	0.0038 (0.0002)^b	0.999	0.0044 (0.0003)^b	4.9 (0.2)^c	3.73 (0.15)^c
HPMC-0.5LO	7.29 (0.05)^e	-7.0 (0.5)^d	1002.0 (0.8)^a	1.0265 (0.0012)^b	0.00367 (0.00003)^b	0.970	0.00415 (0.00004)^b	3.8 (0.3)^a	2.75 (0.15)^{ag}
HPMC-1LO	6.39 (0.04)^f	-6.9 (0.5)^d	1002.5 (0.3)^a	1.0271 (0.0012)^b	0.00367 (0.00003)^b	0.974	0.00416 (0.00004)^b	4.7 (0.3)^d	3.43 (0.13)^d
HPMC-2LO	5.35 (0.05)^c	-7.3 (0.4)^d	999.16 (0.14)^b	1.0247 (0.0016)^b	0.0036 (0.0004)^b	0.975	0.00431 (0.00014)^b	5.52 (0.14)^e	4.08 (0.05)^e
HPMC-0.5TTO	6.97 (0.02)^g	-2.76 (0.09)^b	1002.4 (0.3)^a	1.029 (0.005)^b	0.00360 (0.00003)^b	0.984	0.00413 (0.00009)^b	3.30 (0.05)^f	2.00 (0.03)^f
HPMC-1TTO	5.74 (0.02)^h	-2.7 (0.3)^b	1002.3 (0.2)^a	1.024 (0.002)^b	0.00366 (0.00004)^b	0.983	0.00417 (0.00014)^b	4.13 (0.12)^g	2.803 (0.012)^g
HPMC-2TTO	4.70 (0.02)^d	-2.72 (0.08)^b	1000.74 (0.05)^c	1.025 (0.006)^b	0.00383 (0.00004)^b	0.986	0.00429 (0.00009)^b	4.6 (0.3)^d	3.29 (0.15)^b

$^{a, b, c,d,e,f,g,h}$ Different superscripts within a column indicate significant differences among formulations ($p < 0.05$).

Table 4. FFD CH characterization: pH, ζ-potential, density (ρ), Ostwald de Waale model parameters, apparent viscosity (η_{ap} at 100 s^{-1}) and particle size (d_{43} and d_{32}) values at 25 °C. Mean values and standard deviation.

FFS	pH	ζ (mV)	ρ (Kg/m^3)	$0 \leq \gamma^{\&} \leq 512\ s^{-1}$				d_{43} (μm)	d_{32} (μm)
				n	k (Pa·s)^n	r^2	η_{ap} (Pa·s)		
CH	4.28 (0.02)^{a}	100 (3)^{a}	1004.69 (0.13)^{a}	0.785 (0.007)^{b}	0.58 (0.02)^{a}	0.975	0.216 (0.002)^{a}	-	-
CH-0.5BO	4.31 (0.03)^{a}	82 (3)^{c}	1003.888 (0.005)^{b}	0.777 (0.002)^{a}	0.554 (0.116)^{b}	0.997	0.179 (0.019)^{cd}	7.0 (0.4)^{a}	3.9 (0.2)^{a}
CH-1BO	4.25 (0.02)^{bc}	80.3 (1.4)^{cde}	1002.55 (0.05)^{d}	0.7936 (0.0116)^{b}	0.38 (0.04)^{de}	0.996	0.150 (0.008)^{e}	15.0 (0.4)^{b}	6.5 (0.2)^{b}
CH-2BO	4.24 (0.02)^{c}	77.60 (1.02)^{ef}	1001.26 (0.17)^{f}	0.82 (0.02)^{bc}	0.29 (0.06)^{ef}	0.997	0.139 (0.015)^{e}	22.1 (0.2)^{c}	8.53 (0.17)^{c}
CH-0.5LO	4.29 (0.02)^{a}	80 (2)^{cd}	1004.407 (0.002)^{a}	0.83 (0.02)^{c}	0.36 (0.07)^{de}	0.984	0.169 (0.017)^{d}	4.2 (0.2)^{d}	3.03 (0.04)^{d}
CH-1LO	4.29 (0.02)^{a}	79 (2)^{cde}	1003.438 (0.002)^{c}	0.877 (0.013)^{d}	0.25 (0.02)^{f}	0.983	0.145 (0.012)^{e}	9.47 (0.13)^{e}	4.55 (0.07)^{e}
CH-2LO	4.28 (0.03)^{ab}	76 (2)^{f}	1002.538 (0.002)^{d}	0.86 (0.02)^{d}	0.25 (0.06)^{f}	0.986	0.146 (0.019)^{e}	18.6 (0.6)^{f}	6.8 (0.2)^{f}
CH-0.5TTO	4.29 (0.02)^{a}	87 (3)^{b}	1004.62 (0.13)^{a}	0.817 (0.014)^{c}	0.47 (0.03)^{d}	0.970	0.202 (0.002)^{b}	5.72 (0.16)^{g}	2.87 (0.09)^{g}
CH-1TTO	4.30 (0.02)^{a}	86 (2)^{b}	1003.5 (0.2)^{bc}	0.816 (0.008)^{bc}	0.47 (0.02)^{cd}	0.973	0.203 (0.002)^{bc}	9.87 (0.08)^{h}	4.38 (0.16)^{h}
CH-2TTO	4.30 (0.02)^{a}	78 (3)^{def}	1002.0 (0.3)^{e}	0.807 (0.006)^{b}	0.501 (0.005)^{bc}	0.975	0.201 (0.002)^{b}	14.7 (0.3)^{i}	6.2 (0.3)^{i}

$^{a, b, c,d,e,f,g,h,i}$ Different superscripts within a column indicate significant differences among formulations ($p < 0.05$).

By considering all the determined properties of the FFDs, a discriminant analysis was carried out in order to analyse the different degrees to which the kind of polymer, the kind of EO or the EO concentration contribute to the differences in FFD behaviour. The discriminant plot obtained by considering all the properties characterised in the FFDs of both polymers, is shown in Figure 1a in terms of functions F1 and F2 which explains 96.4% of total variance (92.2% explained by F1). Samples were clearly separated in two groups, depending on the polymer, which indicates that, more than EO (kind and amount), the kind of polymer significantly contributes to modify the characteristics of the FFD. This suggests that the properties of the aqueous continuous phase and the induced particle charge, where the polymer plays an important role, greatly influence the FFD behaviour.

Taking this result into account, similar statistical analyses were performed separately for CH and HPMC FFDs to evaluate the main factor determining the properties of FFDs: the EO nature or concentration. Discriminant plots are shown in Figures 1b and 1c. F1 and F2 functions explain 99.5 and 97 % of total variance for HPMC and CH FFDs, respectively. For both polymers, the different samples were clearly separated according to the EO type and its concentration. Function 1 separates samples with different EOs whereas the effect of concentration appeared clearly differentiated by F2. Taking into account that F1 explains 93 or 82.5 % of variance for HPMC and CH FFDs, respectively, it may be said that differences in the behaviour of FFDs of a determined hydrocolloid are mainly due to the type of EO. From values of standardized coefficients of F1, it is possible to conclude that the differences among dispersions were mainly caused by the mean particle size (d_{43} and d_{32}) and rheological properties. In terms of F2, the variables responsible for differences were the consistency index and apparent viscosity, although this aspect is less relevant since a small percentage of variance was explained by F2 (6.5% and 14.5% for HPMC and CH FFDs, respectively).

In conclusion, it is the nature of EO compounds more than the EO concentration which induces greater differences in the behaviour of the FFD of both HPMC and CH, which are mainly explained by the particle size distribution reached under determined homogenization conditions. So, the interactions of EO compounds with the polymer and the aqueous media determined the interfacial adsorption of the polymer that affected particle size, viscosity and surface charge.

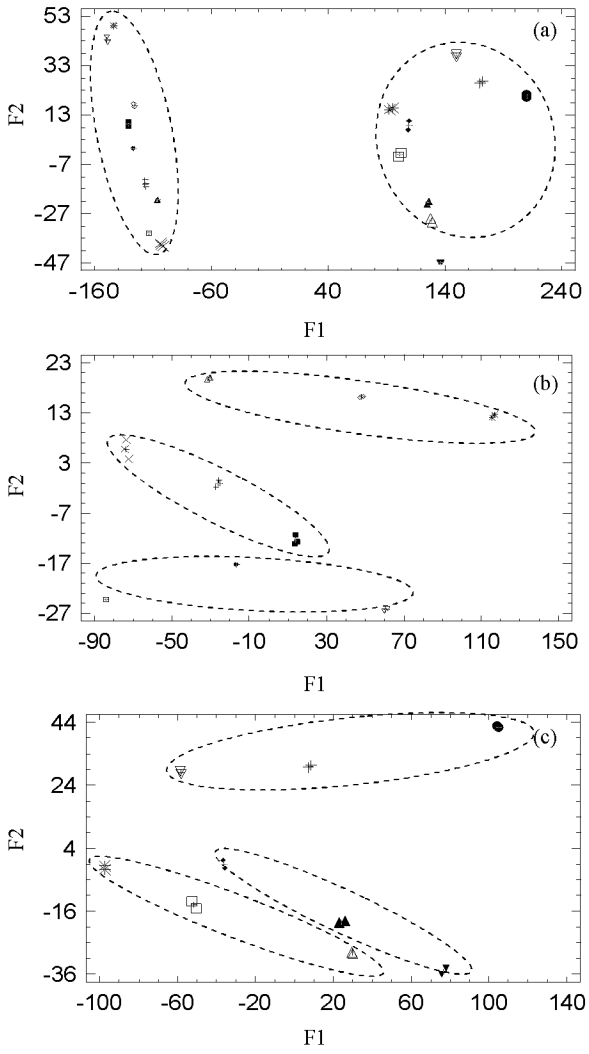

Figure 1. Plot of discriminant functions obtained from data of both HPMC-EO, CH-EO FFD (a); HPMC-EO FFD (b) and CH-EO FFD (c) (■ HPMC-0.5BO, + HPMC-1BO, × HPMC-2BO, ∗ HPMC-0.5LO, ◊ HPMC-1LO, Δ HPMC-2LO, ∇ HPMC-0.5TTO, • HPMC-1TTO, □ HPMC-2TTO, ♦ CH-0.5BO, ▲ CH-1BO, ▼ CH-2BO, ∗ CH-0.5LO, □ CH-1LO, Δ CH-2LO, ∇ CH-0.5TTO, + CH-1TTO, • CH-2TTO).

3.2. Characteristics of the films

Tables 5 and 6 show the physical properties characterized in the CH and HPMC films. Mechanical properties (in samples equilibrated at 54.4% and 20ºC) were measured in terms of the percentage of elongation at break (E%), tensile strength (TS) and elastic modulus (EM). TS represents the film's resistance to elongation or its stretching capacity and EM is a measure of the stiffness of the film. The values of mechanical properties obtained for pure HPMC and CH films agreed with those found by other authors (De Moura et al., 2009; Sánchez-González et al., 2009; Zivanovic, Chi and Draughon, 2005). As can be deduced from Tables 5 and 6, pure CH films were mechanically more resistant to fracture and more stretchable (greater TS, EM and E% values) than pure HPMC films. The mechanical response of the films from both polymers presented similar trends when the EO was incorporated into the matrix, although the changes were more pronounced when using CH matrix. Within the concentration range under consideration, the addition of EO led to a decrease not only in the elastic modulus, but also in the tensile strength and deformation at break. In CH films, these changes in the mechanical parameters provoked by the EO concentration were only significant at the highest EO concentration levels, whereas in the case of HPMC, only the TS parameter varied significantly when the EO concentration increased. These effects coincide with the results reported by other authors when adding essential oil to a chitosan matrix (Pranoto, Rakshit and Salokhe, 2005; Zivanovic, Chi and Draughon, 2005). The presence of structural discontinuities in the polymer network, provoked by the incorporation of the lipid dispersed phase, explains the smaller degree of film stretchability (lower E% values) and resistance to break (lower TS values). This response was usually observed in many composite films. The nature of the EO did not significantly affect the mechanical behaviour of HPMC films. However, in CH films and at a determined level of EO ratio, BO generally induces a greater

decrease of the film elastic modulus, stretchability and resistance to break than LO and TTO, which is more accused at low concentrations.

The thicknesses of pure HPMC, CH and composite films are reported in Tables 5 and 6. Composite films were not as thick as pure films ($p<0.05$) and the thickness was even more reduced when the EO concentration increased. This result suggests that possible losses of oil could occur during film drying which reduce the total amount of solids contributing to the film thickness.

The water vapour permeabilities (WVP) of the films at 100/54.4 RH gradient and 20ºC are also reported in Tables 5 and 6. WVP values were in the range of those reported by other authors for films based on HPMC (Sebti, Ham-Pichavant and Coma, 2002; Villalobos, Hernández-Muñoz and Chiralt, 2006; Sánchez-González et al., 2009) and CH (Park and Zhao, 2004; Vargas et al., 2009), respectively. Regardless of the type of polymer, WVP values showed a decrease in line with the increase in the EO concentration, this being more significant ($p<0.05$) when the EO ratio increased. This behaviour is expected, as an increase in the hydrophobic compound fraction usually leads to an improvement in the water barrier properties of films. Concerning composite films, at low levels of essential oil (up to HPMC-EO ratio of 1:1), the nature of the oil did not significantly affect WVP. However, when incorporating higher EO concentrations (2%), citrus oils (BO and LO) showed better water vapour barrier properties than TTO. The incorporation of 2% BO or LO caused a reduction of about 50% in the WVP values, whereas only 20% reduction was obtained with the same TTO concentration. The greater hydrophobic nature of the main component of both citrus oils (limonene) as compared with the major component of TTO (α-terpineol) (Jordan, 1999) could explain the observed differences. On the contrary, the WVP reduction in CH composite films was less affected by the nature of the EO (around 30-40% in all cases), probably due to the greater hygroscopic nature of the chitosan matrix, reflected in the higher equilibrium moisture contents as compared with HPMC films (Tables 5 and 6).

The greater moisture content and its impact on the molecular mobility may mask the different effect of each EO on the WVP of CH films.

The gloss and transparency of the films are relevant properties since they have a direct impact on the appearance of the coated product. Film transparency was evaluated through the internal transmittance, Ti (0-1, theoretical range). An increase in Ti can be assumed as an increase in transparency (Hutchings, 1999). Ti values at λ = 450 nm of the HPMC, CH and HPMC-EO, CH-EO composite films were reported in Tables 5 and 6. In general, significant differences were observed associated with the nature and amount of the essential oil. Ti values were significantly lower in films incorporating the highest amounts of EO. The composite films were more opaque than pure CH and HPMC films. These results coincide with those found in previous studies (Sánchez-González et al., 2009; Vargas et al., 2009). This phenomenon is related with the light scattering provoked by lipid droplets (with a different refractive index) distributed throughout the film network. Regarding the nature of the oil, BO films were the least transparent (lower Ti values) probably due to a selective absorption of some components of this EO (at 668 nm), leading to lower transmittance values.

Gloss values of the films measured at incidence angle values of 60º were reported in Tables 5 and 6. The addition of EO to the HPMC and CH matrix led to a decrease of the gloss, especially for citrus oils, regardless of EO concentration. For TTO, a smaller gloss reduction was observed, which, in this case, was dependent on the EO concentration. Gloss reduction in composite films containing lipids was also observed by different authors (Trezza and Krochta, 2000; Villalobos et al., 2005; Sánchez-González et al., 2009). The gloss of the films is related with the surface morphology reached during film drying. In general, the smoother the surface, the higher the gloss (Ward and Nussinovich, 1996). In this sense, the decrease in gloss with the incorporation of EO could be explained by an increase of the surface roughness of the composite films. This roughness appears as a

consequence of the migration of droplets or aggregates to the top of the film during film drying, which leads to surface irregularities. Flocculation and creaming of oil droplets occurred during film drying and this effect on gloss seems more intense in citrus oils (BO and LO) than in TTO. This could be related with the greater stability of the TTO emulsions reflected in the smaller droplets and narrower particle size distribution deduced from the smaller difference between d_{43} and d_{32} values.

Table 5. Properties of HPMC and HPMC-EO composite films: Elongation (E), tensile strength (TS), elastic modulus (EM), thickness, water vapour permeability (WVP), equilibrium moisture content at aw=0.75 (We), internal transmittance (Ti) and gloss values. Mean values and standard deviation.

Film	E (%)	TS (MPa)	EM (MPa)	Thickness (μm)	WVP ($g.Pa^{-1}.s^{-1}.m^{-1}$) x 10^{11}	We (g H_20/g d.m.)	Ti (λ=450 nm)	Gloss (60°)
HPMC	7.9 (0.6)^{a}	56 (7)^{a}	643 (74)^{a}	61.6 (0.6)^{a}	71 (7)^{a}	0.073	0.851 (0.006)^{ab}	50 (5)^{a}
HPMC-0.5BO	5.1 (0.8)^{b}	54 (5)^{a}	473 (180)^{a}	46.5 (0.7)^{b}	65 (5)^{ab}	0.089	0.820 (0.007)^{d}	11 (2)^{b}
HPMC-1BO	4.7 (0.5)^{b}	47 (5)^{a}	420 (107)^{a}	35.3 (0.8)^{c}	52 (6)^{b}	0.057	0.826 (0.014)^{d}	11 (2)^{b}
HPMC-2BO	2.9 (0.7)^{b}	39 (3)^{b}	444 (75)^{a}	31.1 (1.7)^{d}	f31 (4)^{c}	0.062	0.789 (0.035)^{e}	11 (2)^{b}
HPMC-0.5LO	6.0 (0.6)^{b}	54 (3)^{a}	515 (230)^{a}	40.6 (0.4)^{e}	68 (5)^{a}	0.072	0.849 (0.005)^{ab}	11.9 (0.3)^{b}
HPMC-1LO	4.5 (0.9)^{b}	50 (1)^{a}	459 (13)^{a}	32.8 (1.5)^{d}	65 (7)^{a}	0.058	0.857 (0.002)^{a}	11.8 (0.8)^{b}
HPMC-2LO	3.9 (0.4)^{b}	40 (4)^{b}	397 (139)^{a}	25.6 (2.8)^{f}	41 (6)^{c}	0.053	0.842 (0.005)^{bc}	8.2 (1.2)^{b}
HPMC-0.5TTO	7.6 (0.4)^{b}	42 (6)^{b}	696 (104)^{a}	32.8 (1.2)^{d}	70 (5)^{a}	0.061	0.863 (0.003)^{a}	24 (3)^{c}
HPMC-1TTO	4.2 (0.4)^{b}	35 (2)^{c}	542 (160)^{a}	27.5 (1.6)^{f}	66 (4)^{ab}	0.057	0.859 (0.002)^{a}	20 (2)^{d}
HPMC-2TTO	4.2 (0.2)^{b}	34 (5)^{c}	365 (124)^{a}	22.3 (1.2)^{g}	57.3 (1.5)^{b}	0.056	0.83 (0.02)^{cd}	16 (3)^{e}

$^{a, b, c, d, e, f, g}$ Different letters in the same column indicate significant differences among formulations ($p < 0.05$).

Table 6. Properties of CH and CH-EO composite films: Elongation (E), tensile strength (TS), elastic modulus (EM), thickness, water vapour permeability (WVP), equilibrium moisture content at aw=0.75 (We), internal transmittance (Ti) and gloss values. Mean values and standard deviation

Film	E (%)	TS (MPa)	EM (MPa)	Thickness (μm)	WVP ($g.Pa^{-1}.s^{-1}.m^{-1}$) x 10^{11}	We (g H_20/g d.m.)	Ti (λ=450 nm)	Gloss (60°)
CH	22 (5)^{a}	113 (20)^{a}	2182 (277)^{a}	52.0 (1.7)^{a}	129 (10)^{ab}	0.235	0.801 (0.013)^{a}	32 (5)^{a}
CH-0.5BO	7 (4)^{b}	65 (10)^{de}	766 (205)^{cd}	55 (2)^{a}	130 (1)^{a}	0.189	0.761(0.012)^{c}	9 (3)^{c}
CH-1BO	5.5 (0.7)^{b}	63 (21)^{de}	799 (163)^{cd}	41 (2)^{b}	108 (15)^{b}	0.174	0.746 (0.014)^{de}	4.9 (1.2)^{b}
CH-2BO	6 (2)^{b}	50 (8)^{b}	747 (225)^{b}	36 (3)^{b}	92 (9)^{bc}	0.137	0.744 (0.012)^{e}	8.8 (1.5)^{c}
CH-0.5LO	18.1 (0.8)^{c}	94 (9)^{f}	1534 (185)^{e}	41 (2)^{b}	101.9 (1.5)^{b}	0.163	0.764 (0.015)^{e}	17 (2)^{de}
CH-1LO	14.6 (0.4)^{c}	57 (7)^{d}	1466 (160)^{e}	36.0 (1.4)^{b}	88 (4)^{bc}	0.140	0.765 (0.009)^{c}	15 (2)^{d}
CH-2LO	6.4 (0.2)^{b}	37 (3)^{c}	954 (113)^{d}	36 (3)^{b}	77 (3)^{cd}	0.110	0.782 (0.009)^{c}	9.9 (1.8)^{c}
CH-0.5TTO	20 (8)^{c}	74 (15)^{e}	1447 (308)^{e}	39 (3)^{b}	99 (4)^{b}	0.209	0.757 (0.018)^{b}	28 (5)^{a}
CH-1TTO	17 (6)^{c}	72 (12)^{e}	1419 (322)^{e}	38 (3)^{b}	100.1 (1.3)^{b}	0.159	0.764 (0.009)^{cd}	19 (2)^{e}
CH-2TTO	8 (2)^{b}	54 (5)^{d}	652 (157)^{c}	24.3 (1.2)^{c}	74.7 (1.8)^{d}	0.134	0.789 (0.013)^{c}	5.7 (1.2)^{b}

$^{a, b, c, d, e, f}$ Different letters in the same column indicate significant differences among formulations ($p < 0.05$)

By considering all the properties measured in the films (WVP, optical and mechanical properties), a discriminant analysis was performed in order to analyse how much the kind of polymer, the kind of EO or the EO concentration contribute to the different behaviour of the films. The discriminant plot is shown in Figure 2a in terms of functions F1 and F2 which explains 80 % of total variance (64% being explained by F1). Again, different samples were clearly separated as a function of the polymer type. So, similar statistical analyses were performed separately for CH-EO and HPMC-EO composite films to evaluate what the main factor causing differences was: the EO nature or its concentration. Discriminant plots are shown in Figures 2b and 2c. Functions F1 explain 64 and 60% of total variance for CH and HPMC films, respectively. Regardless of the type of polymer, the discriminant F1 function did not group samples in terms of the nature of essential oils, whereas F2 (which explain 22 and 23 % of variance for CH and HPMC flms, respectively) separated the films containing BO from the others. From values of standardized

coefficients of F1and F2, it is possible to conclude that the variables which caused the greatest differences among films were water vapour permeability and internal transmittance (Ti). So, differences in film behaviour must be mainly attributed to the polymer that forms the continuous matrix, whereas the incorporation of different EO or ratios did not impart clearly differentiated behaviour, except when BO is used as samples appear as an independent group.

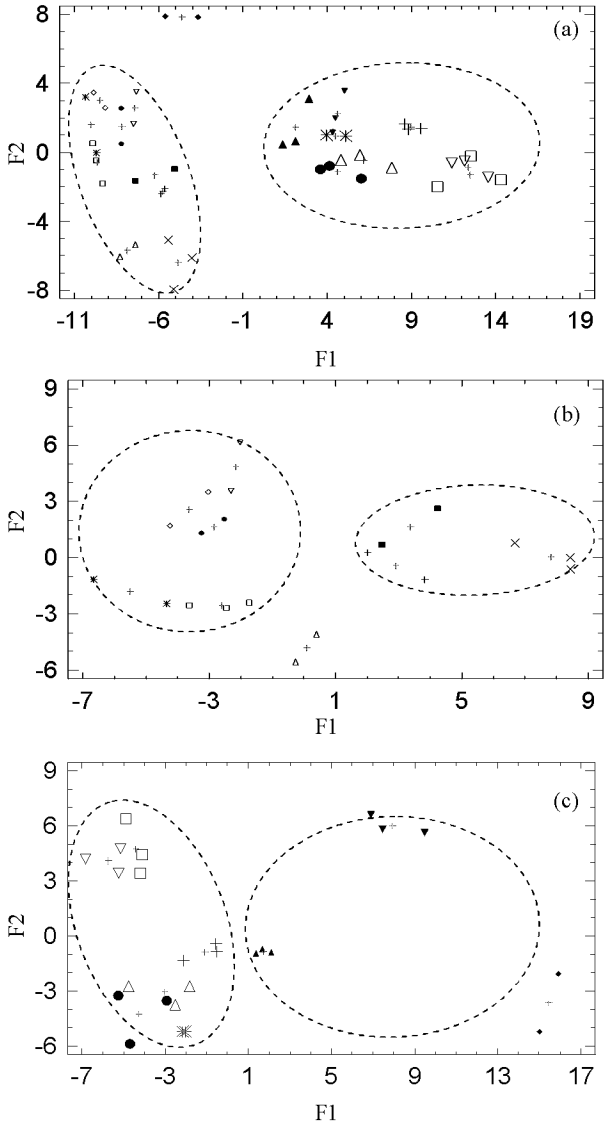

Figure 2. Plot of discriminant functions obtained from data of both CH-EO, HPMC-EO composite films (a); HPMC-EO films (b) and CH-EO films (c) (■ HPMC-0.5BO, + HPMC-1BO, × HPMC-2BO, ∗ HPMC-0.5LO, ◊ HPMC-1LO, Δ HPMC-2LO, ∇ HPMC-0.5TTO, • HPMC-1TTO, □ HPMC-2TTO, ♦ CH-0.5BO, ▲ CH-1BO, ▼ CH-2BO, ∗ CH-0.5LO, □ CH-1LO, Δ CH-2LO, ∇ CH-0.5TTO, + CH-1TTO, • CH-2TTO).

4. CONCLUSION

HPMC and CH are good polymer matrices for entrapping essential oils (EO). The incorporation of EO led to significant changes in the properties of film-forming dispersions and films. Different EOs promote changes in particle size and size distribution and in ζ-potential. HPMC and CH contribute to the emulsion stability by adsorption on the oil droplet surface, although the film gloss data reflected that flocculation and creaming of oil droplets occurred during film drying. The higher the EO content, the lower the water vapour permeability. In both HPMC and CH matrices, the addition of EO led to a significant decrease in gloss, transparency and tensile strength and elongation at break of the composite films. Discriminant analyses revealed that the type of polymer is the main factor inducing differences in both FFD and film behaviour. For films of both polymers, both concentration and nature of EO contribute in the differentiation of groups of film-forming dispersions associated with their behaviour, the nature of the oil playing a more important role. As regards film properties, discriminant analyses did not reveal clearly different groups associated with the nature or concentration of essential oil, although composite films with BO were differentiated from the rest.

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Capítulo III

Estudio de difusión de los compuestos activos de los films y de la perdida de volatiles durante el secado

STUDY OF THE RELEASE OF LIMONENE PRESENT IN CHITOSAN FILMS ENRICHED WITH BERGAMOT OIL IN FOOD SIMULANTS

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ABSTRACT

Chitosan films enriched with different concentrations of bergamot oil (BO) were obtained and the migration of limonene, the major oil component, to five liquid food simulants (water, 10% ethanol, 50% ethanol, 95% ethanol, isooctane) was studied at 20ºC. The release kinetics of limonene from chitosan matrix was described using Fick's second law of diffusion. Diffusion coefficients (D) were determined for each BO concentration and food simulant.

The results show that limonene diffusion is major in ethanol 95%. Concerning the other food simulants, limonene release remains less important. Composite films remain intact with isooctane CH-BO and no release of limonene is observed. Polarity of simulant and migrant is a key factor to explain these results. For a given limonene concentration diffusion coefficient decreases with film thickness increase. With ethanol 95% a linear relationship was observed between limonene concentration and D value.

Keyword: citrus oil, diffusion coefficient, polarity, solubility.

1. INTRODUCTION

Active food packaging appears as a promising technology with increasing applications due to their advantages over traditional methods. It's an interesting alternative to the use of chemical preservatives. Natural antimicrobial and antioxidant agents can be incorporate into biodegradable materials to increase shelf life and quality of food products.

Different biopolymers have been largely studied as polymeric matrix. Among polysaccharides, chitosan presents excellent film forming ability (Li, Dunn, Grandmaison & Goosen, 1992). This non-toxic compound, obtained by deacetylation of chitin, a structural component present in the shell of some crustaceans, presents interesting antimicrobial properties.

Citrus essential oils (EO) appear as interesting natural compounds with great potential use in foodstuffs preservation. *In vitro* studies have revealed significant antimicrobial effects of these natural compounds. Fisher and Phillips (2006) reported the effectiveness of oils and vapours of lemon, sweet orange and bergamot against 5 common foodborne pathogens, *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* O157 and *Campylobacter jejuni*. Viuda-Martos, Ruiz-Navajas, Fernández-López and Pérez-Ávarez (2008) evaluated the effect of lemon, mandarin and orange oils on the growth of moulds associated with food spoilage. Orange and mandarin essential oils were the most effective against *Aspergillus niger* and *Aspergillus flavus* respectively. Even if the essential oils mechanism of action is not clearly described, it seems that the antimicrobial activity is essentially due to their hydrophobicity. Terpens, the major compounds of essential oils, have the ability to disrupt and penetrate the lipid structure of the cell wall bacteria, leading to denaturing of proteins and destruction of cell membrane (Turina, Nolan, Zygadlo & Perillo, 2006).

Previous *in vitro* studies reported promising results with chitosan-essential oils composite films. The incorporation of essential oil into chitosan films offers the possibility not only of imparting antimicrobial activity, but also of improving films physicochemical properties. A decrease in water vapour permeability was reported by several authors (Sánchez-González, González-Martínez, Chiralt & Cháfer, 2010a; Sánchez-González, Cháfer, Chiralt & González-Martínez, 2010b; Zivanovic, Chi & Draughon, 2005).

Determination of active compounds diffusion rates will be interesting to design efficiency active packaging. But release kinetics of antimicrobial substances from biodegradable films to food products was little explored. Antimicrobial release is dependant of the food product characteristics. For this reason it can be interesting to evaluate substance migration in different food-simulating solvents. EFSA recommendations in terms of food simulants are contained in the European Commission Directive 97/48/EC. Aqueous and acidic foodstuffs are well simulated by distilled water and 10% - 50% ethanol. Concerning fatty foodstuffs the choice of the food simulant is more complicated. Food oil was recommended by European directive but migrations measurement in oil is a technical challenge due to the numerous components present. For this reason it can be interesting to use substitute fatty simulant as isooctane or 95% ethanol (McCort-Tipton & Pesselman, 2000).

The aim of this work is to study the behaviour of chitosan-bergamot oil composite films in five different liquid food simulants (water, 10% ethanol, 50% ethanol, 95% ethanol, isooctane). The kinetic of limonene release, the major component of the bergamot essential oil previously incorporated into chitosan films, was described using Fick's second law. The influence of the essential oil concentration as well as the nature of the food simulant was evaluated.

2. MATERIALS AND METHODS

2. 1. Materials

Medium molecular weight chitosan (CH) (Batch MKB130566, Sigma-Aldrich Chemical Co., St Louis, USA), 98% glacial acetic acid (Sigma-Aldrich Chemical Co., St Louis, USA) and bergamot essential oil supplied by Herbes del Molí (Alicante, Spain) were used to prepare the film-forming dispersions.

Isooctane (2,2,4-Trimethylpentane, Sigma-Aldrich Chemical Co., St Louis, USA) and 95% ethanol pure or diluted in distilled water (Sigma-Aldrich Chemical Co., St Louis, USA) were used as liquid food simulants.

2.2. Preparation of the film-forming dispersions

Chitosan (1 % w/w) was dispersed in an aqueous solution of glacial acetic acid (0.5%w/w) at 25ºC. After the dissolution of the polysaccharide, bergamot essential oil (BO) was added to CH solution to reach a final concentration of 0.5, 1, 2 and 3% (w/w). CH-BO mixtures were emulsified at room temperature using a rotor-stator homogenizer (Ultraturrax DI 25 basic-Yellowline, Janke & Kunkel, Staufen, Germany) at 13,500 rpm for 4 minutes.

2.3. Preparation of films

A casting method was used to obtain films. FFD were poured onto a framed and levelled Teflon Petri dishes (90x110 mm, Welch, USA) and were dried 48 hours in atmospheric conditions. Film thickness was controlled by pouring the amount of FFD that will provide a surface density of solids in the dry films of 56 g/m^2 in all formulations. A manual micrometer (Messmer, London, England, ± 0.002 mm) was used to measure film thickness at ten different points of the same sample at least.

2.4. Kinetic of release and Diffusion coefficients determination

2.4.1. Kinetic of release

Pieces of films (8 cm^2) were introduced into vials containing 2 ml of food simulants. Some characteristics of these solvents were reported in Table 1. The quantity of limonene release in the food simulant was analysed by headspace chromatography each day periodically during 4 days at 20ºC. A calibration curve was constructed for peak area against limonene quantity with a limonene standard solution.

All tests were run in triplicate.

Table 1. Physico-chemical properties of food simulants and migrant (limonene).

Name	pH	Polarity[1]
Distilled water	3.99 (0.08)[a]	16.00*
Ethanol 10 %	3.87 (0.02)[b]	14.40*
Ethanol 50 %	4.04 (0.02)[a]	10.82*
Ethanol 95 %	5.73 (0.03)[c]	8.80*
Isooctane	7.19 (0.02)[d]	0*
Limonene	-	0.97*

[a,b,c,d] Different letters in the same column indicate significant differences among formulations ($p < 0.05$).
[1]Hansen polarity
*Data from Molecular Modeling Pro

2.4.2. Chromatographic analyses

The measurements were made on a Perichrom Sarl model PR 2100 automatic Headspace Sampler on the Gas Chromatograph with flame ionization detector. The volume of the headspace vial was 11 ml. A fused silica capillary column (Sigma Aldrich Co., USA) was employed (SUPELCOWAX 10, 60 m x 0.32 mm).

The carrier gas was N_2 at a flow rate of 1 ml/min. The analysis was performed using the following temperature program: oven temperature from 60 to 150ºC at the rate of 3ºC.min^{-1}, and isotherm at 150ºC during 10 min. Injector and detector temperatures were held both at 250ºC.

2.4.3. Diffusion coefficients determination

Fick's second law was considered to study the diffusion mechanism of limonene, the major compound of bergamot essential oil, from chitosan to liquid food simulants.

The diffusion coefficient of limonene (D) was determined from the experimental data using a relationship derived from the Fick's second law for a plane sheet with constant boundary condition and uniform initial concentration (Crank, 1975) (Eq.2).

$$\frac{M_t}{M_\infty} = 1 - \frac{8}{\pi^2}\sum_{n=0}^{\infty}\frac{1}{(2n+1)^2}\exp\left[\frac{-2(n+1)^2\pi^2 Dt}{L^2}\right] \qquad (2)$$

Where Mt and M∞ are the mass of limonene released from the film at time t and at infinite time respectively. L is the thickness of the film.

For short times (Mt/M∞ < 2/3), equation 2 can be simplify to the equation 3 and D can be determined with equation 4 (Crank, 1975).

$$\frac{Mt}{M\infty} = 4\left(\frac{Dt}{4L^2\pi}\right)^{\frac{1}{2}} \quad (3)$$

$$D = \left(\frac{S \times L}{2}\right)^2 \times \pi \quad (4)$$

Where S is the slope of a plot of Mt/M∞ against $t^{1/2}$.

2.5. Statistical analysis

Results were analysed by multifactor analysis of variance with 95% significance level using Statgraphics®Plus 5.1.

3. RESULTS AND DISCUSSION

3.1. Estimation of limonene lost during film formation

Approximately 94% of bergamot oil is composed by limonene. Limonene loss versus film formation time was reported in terms of percentage in Table 2. Active compound losses are included between 39 and 99%, for the various amount of BO incorporated in the film forming solution. Monedero, Hambleton, Talens, Debeaufort, Chiralt and Voilley (2010) equally reported important aroma losses during film formation. In fact, more than 50% of *n*-hexanal incorporated in soy protein isolate-lipid films was lost.

Losses of limonene and bergamot oil increased with the ratio CH:BO. Polymer quantity decreased with the addition of BO, films structure was less open. The continuous matrix of chitosan chains was interrupted by oil droplets presence (Sánchez-González, Cháfer, Chiralt and González-Martínez, 2010b). Essential oil is less retained in the matrix. These changes in the film structure could explain major limonene losses for higher levels of BO incorporated.

Table 2. Thickness, theoretical and experimental limonene content of CH-BO composite films and percentage of limonene lost during film drying.

Film	Thickness (µm)	Theoretical limonene concentration (µl/cm²)	Experimental limonene concentration (µl/cm²)	Limonene loss (%)
CH-0.5BO	42 (3)[a]	1.962	1.185	39
CH-1BO	42 (2)[a]	2.974	1.018	65
CH-2BO	32 (2)[b]	3.925	0.086	97
CH-3BO	20 (2)[c]	4.461	0.033	99

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

3.2. Kinetic of release

Limonene release was studied as function of time in five different food simulating liquids. Other terpenes present in bergamot oil diffused but in minor concentrations, detection was not possible with the sensibility of headspace chromatography equipment.

According to Buonocore, Del Nobile, Panizza, Corbo and Nicolais (2003) the release of an active compound from a polymeric network takes place in several steps. First water molecules diffuse from the outer solution to the polymer matrix leading to network weakening. These changes in the film structure allow the diffusion of the active compound through the polymer matrix into the outer solution until achieve a thermodynamic equilibrium. Thus, limonene released is dependent of different factors as liquid migration to chitosan matrix, polymer solubility and diffusion of the active compound through polymer matrix to food simulating liquid. This last phenomenon is not only due to a mass transfer but it's

the result of different factors as the specific interactions between the volatile compound and the matrix.

Limonene release kinetics for the different liquid food simulants were reported in Figure 1.

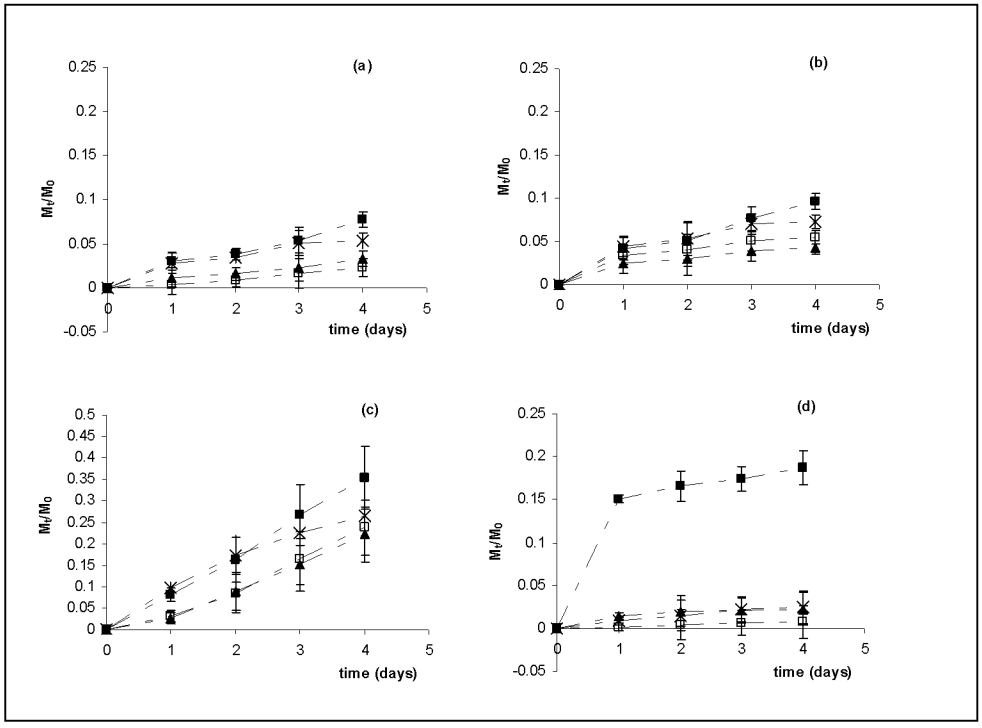

Figure 1. Limonene release kinetics at 20ºC for different CH-BO composite films (▲ CH-0.5BO, □ CH-1BO, × CH-2BO, ■ CH-3BO) in food simulants: (a) Ethanol 10%, (b) Ethanol 50%, (c) Ethanol 95%, (d) Distilled water. Mean values and 95% LSD intervals.

Diffusion of active compound was not observed when CH-BO composite films were in contact with isooctane whatever the essential oil concentration incorporated. A possible explanation can be the pH of isooctane, around 7. Previous studies related with chitosan reported that structure of the film remains stable for pH above 6.0. Partial dissolution and/or structural changes are observed

for pH below 6.0 (Qin, Li, Xiao, Liu, Zhu and Du, 2006). So, in our conditions, the polymeric system remained intact during storage, preventing limonene release.

Concerning water, ethanol 10% and ethanol 50%, the release rate was generally low and the highest level was observed during the first day. Significant decrease of limonene release rate was observed in the following days and the quantity released remains constant after 2-3 days of storage at 20ºC.

The maximum limonene diffusion was observed for ethanol 95%. In this case the liberation rate is rather constant during all the storage period at 20ºC. This observation is in line with expected results. Limonene, a cyclic terpene, presents a major solubility in ethanol than water. Aqueous solubility of limonene is limited (Li, Perdue, Pavlostathis and Araujo, 1998): 41 µM. Limonene release increased with ethanol concentration in the food simulant. To explain these results polarity of solvents and limonene should be taken into account. Physico-chemical properties of food simulants and migrant were reported in Table 1. Molecular polarity is dependent on the difference in electronegativity between constituent atoms of the molecule. Non-polar molecules in contrast with polar compounds present an equal sharing of electrons between different atoms. The non-polar nature of limonene (polarity value near zero) explains its greater affinity for ethanol compared to distilled water. Limonene presents a greater affinity for the food simulant with increasing ethanol content.

A possible explanation for differences observed between limonene release rates is the stability of the film structure. Unlike what happens in presence of ethanol, with distilled water film's structure is weakened. This phenomenon allows an easier release of limonene and the equilibrium is reached more quickly.

Limonene release kinetic was described by means of Fick`s Second Law. The proposed model described satisfactory the experimental data. As expected the

quantity of limonene released increases during the storage period, however the equilibrium is not always reached after four days.

3.3. Diffusion coefficients determination

Diffusion coefficients (D) for all films studied in five food simulants were reported in Table 3. D values were shown dependent on the liquid food simulant nature. Molecular mobility of limonene within the polymeric network and film structure appeared as important factors to take account.

D values are of the same order for ethanol 10 and 50%, around 10^{-14} cm^2. s^{-1}. However significant differences were observed with other simulants tested. Diffusion coefficients were greater with ethanol 95%, the order of 10^{-13}-10^{-12} $cm^2.days^{-1}$ and lower with distilled water and isooctane where phenomenon of diffusion is not possible. As commented above limonene polarity and solubility in the food simulant are determinant to explain diffusion phenomenon. The affinity of limonene for ethanol is superior to that of the same compound for distilled water. Moreover the solubility of biopolymer in acidic aqueous solution leads to a weakening of the polymeric network and limonene release was easier.

Differences in terms limonene quantity incorporated in the chitosan matrix have only one reliable effect on the D values in ethanol 95%. Concerning other food simulants tested, no significant differences were observed between D values of CH-BO composite films studied.

Table 3. Diffusion coefficients of CH-BO composite films at 20°C.

Food simulant	Film	D ($cm^2.s^{-1}$) x 10^{14}	r^2
Ethanol 10%	CH-0.5BO	1.603	0.908
	CH-1BO	1.493	0.958
	CH-2BO	1.692	0.943
	CH-3BO	1.868	0.900
Ethanol 50%	CH-0.5BO	1.462	0.980
	CH-1BO	1.922	0.975
	CH-2BO	2.125	0.939
	CH-3BO	2.614	0.934
Ethanol 95%	CH-0.5BO	150.720	0.984
	CH-1BO	174.439	0.970
	CH-2BO	64.632	0.997
	CH-3BO	65.018	0.984
Isooctane	CH-0.5BO	0	-
	CH-1BO	0	-
	CH-2BO	0	-
	CH-3BO	0	-
Distilled water	CH-0.5BO	0.276	0.934
	CH-1BO	0.130	0.967
	CH-2BO	0.606	0.985
	CH-3BO	1.082	0.992

Film thickness occurs equally in the phenomenon of limonene release. Experimental data showed a decrease of film thickness with an increase of essential oil concentration. But for the same thickness and different BO amount (CH:BO

ratios 1:0.5 and 1:1), limonene release remained more important for films enriched with the higher EO concentration. This result is in line with previous studies. Mastrometteo, Barbuzzi, Conte and Del Nobile (2009) described a slower release of active compounds (thymol) across the zein polymeric matrix with an increase of film thickness.

3.4. Influence of the essential oil concentration incorporated in the chitosan matrix on diffusion coefficient

D values of limonene for CH-BO composite films in ethanol 95% were plotted versus limonene concentration present in the film after drying (Figure 2).

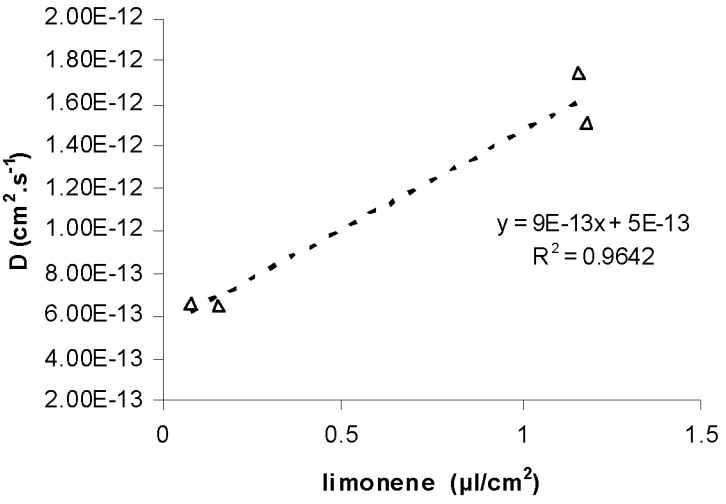

Figure 2. Diffusion coefficients against limonene concentration incorporated in chitosan matrix.

Due to the significant losses of volatile compounds observed during the film drying, initially measured concentration of limonene was used instead of theoretic data. A linear relationship was observed. Diffusion coefficients increase with the

limonene quantity present in the chitosan matrix. This result is in line with previous studies. Del Nobile, Conte, Incoronato and Panza (2008) reported thymol diffusion coefficient increases with thymol concentration incorporated in zein based films.

4. CONCLUSION

When the ratio CH:BO increases, losses of limonene and bergamot oil are higher. This observation questions the incorporation of important quantities of essential oil into biodegradable films. An optimum ratio can be found between initial incorporation of essential oil and retention during drying process and storage.

Concerning limonene release in food simulants, it's a complex phenomenon which involved different factors as film structure, solvent and migrant polarity and solubility. Diffusion coefficients are major in ethanol 95%, certainly due to the affinity of limonene for ethanol. A linear relationship was observed between D values in ethanol 95% and BO concentration.

For possible future applications of these films based on chitosan and bergamot oil, antimicrobial effectiveness will certainly be improved by a contact between active packaging and food product.

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Capítulo IV

Aplicación de recubrimientos a base de polisacáridos y aceites esenciales

EFFECT OF HYDROXYPROPYLMETHYLCELLULOSE AND CHITOSAN COATINGS WITH AND WITHOUT BERGAMOT ESSENTIAL OIL ON QUALITY AND SAFETY OF COLD STORED GRAPES

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ABSTRACT

Biodegradable coatings based on hydroxypropylmethylcellulose (HPMC) or chitosan (CH) with and without bergamot essential oil are applied to table grapes, cv. Muscatel, in order to find environmentally friendly, healthy treatments with which to better preserve fresh fruit quality and safety during postharvest cold storage. The physicochemical properties (weight loss, ºBrix, total phenols, antioxidant activity, colour and texture), respiration rates and microbial counts of samples were determined throughout cold storage. The coatings were observed to have a significant effect on the development of variables, with the different effect of essential oil addition as a function of the polysaccharide matrix being especially remarkable. Although the incorporation of essential oil implied smaller weight losses and greater antimicrobial effect, it also led to browner samples when using CH. Chitosan coatings containing bergamot oil were more effective than pure CH and HPMC coatings at inhibiting respiration rates. All the coatings improved the mechanical resistance of the samples at the end of storage. The most recommendable coating for muscatel table grapes is the CH containing bergamot oil since, despite only contributing slightly to the sample colour, this showed the highest antimicrobial activity and the greatest control of the respiration rates with a reasonably good control of water loss during storage.

Keywords: chitosan, hydroxypropylmethylcellulose, bergamot essential oil, coatings, edible, grape.

1. INTRODUCTION

In the last few years, the conventional production systems of fruit and vegetables have been characterized by an excessive use of chemical compounds during pre- and post-harvest treatments. Nevertheless, new consumer trends and the subsequent legislative changes demand healthier, environmentally friendly food production systems. Table grapes are traditionally treated with different chemical products like SO_2 to control their main post-harvest pathology; the gray mould caused by *Botritys cinerea*. Likewise, storing table grapes presents severe problems such as weight and firmness losses and colour changes. Muscatel cultivar is a very fruity grape that retains its grape flavor in wine. It is used for wine making (from dry and full-bodied to a heavy dessert wine), but also raisins and table grapes. It is characterised by a delicate, particular aroma and sweet taste which made it very appreciated in the Mediterranean Region. Berry colour is white, ranging from light green to amber, depending on the degree of ripening. The berries have a pronounced sweet floral aroma and high concentrations of antioxidant flavonoids (Salazar and López, 2006). Its commercialization as table grape has suffered a decrease because of its not so homogenous appearance and the difficulties to maintain its quality during cold storage (high rate of water loss and browning). Treatments that improve its overall quality during the cold storage-commercialization period would be needed to be developed.

New alternatives, such as the use of modified atmospheres (Artés-Hernández et al., 2006) and thermal treatments combined or not with the application of natural or low toxicity compounds in edible coatings (Serrano et al., 2006) are being investigated and applied in order to obtain safer, more natural and healthier fruit. In this sense, natural, low toxicity compounds represent a promising alternative to the chemical-based preservatives. Among these compounds, essential oils have proven to be good antimicrobial agents (Kalemba and Kunicka, 2003) and

nowadays are being widely used in the pharmaceutical, sanitary, cosmetic, agricultural and food industries. For the food industry, the main problems of essential oils centre around their high volatility, price, odor-flavor and/or biological effects (Bakkali et al., 2008). Incorporating them into edible composite coatings could improve some of these problems, while at the same time permitting the preservation of the required standards (attributes) of quality and safety of fresh vegetables.

In edible formulation of films/coatings, different polysaccharide matrices, such as cellulose derivative materials, have been widely used. Over the last few years, chitosan has been used as a matrix for entrapping different active compounds to be used for medical or food purposes (Aider, 2010; Jayakumar et al., 2010; Dutta et al., 2009). Some studies have revealed that chitosan based treatments were shown to protect grapes from gray mold (caused by *Botrytis cinerea*) (Romanazzi, 2010; Romanazzi et al., 2007; Xu et al., 2007) and to improve its quality attributes in pre and postharvest applications (Meng et al., 2008)

The incorporation of minor constituents, such as essential oils, into the polymer matrices can notably change and/or improve antimicrobial and some physicochemical properties like mechanical, colour or water vapour barrier, as has been described for composite films (Sánchez-González et al., 2009, 2010a, 2010b) and for coatings in fruit application (Bosquez-Molina et al., 2010; Du Plooy et al., 2009). In this case, coating application can also affect the consumer sensory appreciation and an adequate design of the composition, including the antimicrobial agents, will be considered, taking the compatibility of the compound with each food application into account.

Bergamot oil (B) is qualified as an essential oil of intermediate-low odor and flavor intensity. B is citrus oil (extracted from Citrus bergamia), whose major chemical compounds are volatiles, such as limonene (32-45%) and linalool (around 10.23%) (Moufida and Marzouk, 2003; Svoboda and Greenaway, 2003). The antimicrobial

efficiency of B and its components, linaool and citral, against different moulds and, mainly, bacterias (Fisher and Phillips, 2008) has been proven. Previous studies into the characterization of different films enriched with essential oils have also demonstrated the key role of the polymer matrix in the functional properties of composite films (Sánchez-González et al., 2009, 2010a, 2010b; Zivanovic et al., 2005), but there is little data dealing with its influence when applied to the fruit surface.

The aim of this work was to analyse the effect of two different polysaccharide matrices, hydroxypropylmethylcellulose and chitosan, containing or not bergamot essential oil on the development of the physicochemical properties, respiration rate and microbial counts of organic table grapes, cv. Muscatel, throughout 22 storage days at 1-2ºC.

2. MATERIALS AND METHODS

2.1. Raw materials

Organically grown table grapes (Vitis vinifera cv. Muscatel) were harvested in Pinos (Alicante, Spain) and immediately transported to the laboratory, washed in 10 mL/L sodium hypochlorite solution to remove residuals prior to coating, drained and dried at room temperature. The grapes, selected without signs of mechanical damage or fungal decay, were standardized in small clusters with grapes that were homogeneous in size, shape and colour.

To obtain film-forming dispersions, high molecular weight hydroxypropylmethylcellulose (H) (Methocel® E15 Food Grade, The Dow Chemical Company, Midland, USA), chitosan (CH), with a deacetylation degree of 82.7% (CAS Number 9012-76-4, Sigma–Aldrich, EEUU), glacial acetic acid and bergamot essential oil (B) (Herbes del Moli, Alicante, Spain) were used.

2.2. Methodology of film-forming dispersions

Chitosan (1%, w/v) was dispersed in an aqueous solution of glacial acetic acid (1%, v/v), at 40ºC (v/v) for 12 hours, whereas hydroxypropylmethylcellulose (1%, w/v) was dispersed in distilled water at 80ºC for 2 hours and stirred overnight at room temperature. After stirring, a 2% (w/v) concentration of bergamot essential oil (B) was added to each polymer solution and emulsified, by using a rotor stator homogenizer ultraturrax (DI25 Yellow Line, IKA®, Germany), at 13,500 rpm for 4 min and then degasified at room temperature by means of a vacuum pump (Diaphragm vacuum pump, Wertheim, Germany). These film-forming dispersions were named CH, CH-B, H and H-B, respectively.

2.3. Application of the coatings

Selected clusters of 12-15 grapes were dipped in the film-forming dispersions for 1 min. Afterwards, they were hung up and dried at room temperature under natural convection for 2-3 hours and then cold stored in perforated PET trays in an incubator (EC-1400-HR, Radiber S.A., Spain) at 1-2ºC and 85-90% R.H.

2.4. Grape characterization

Three different clusters for each time/treatment were characterized as to the different properties described below, at different cold storage times (3, 5, 8, 12 and 22 days). After each cold period, samples were placed under room temperature conditions for 2 days before the analyses, to simulate market operations.

2.4.1. Total soluble solids and pH

Seedless grapes were ground using a rotor stator ultraturrax at 13,500 rpm for 1 min. Total soluble solids were measured by a refractometer (3T, Atago Co., Ltd., Japan) and measurements of pH were taken by means of a pH-meter (GLP21+, Crison Instruments, Spain). Both analyses were carried out in triplicate, at 20ºC.

2.4.2. Total phenols

Total phenols were extracted according to the method described by Tomás-Barberán et al. (2001). 35 g of seedless grapes, with 40 mL of methanol plus 10 mL of HCl 6 N and 4.2 mg of NaF, to inactivate polyphenol oxidases and prevent phenolic degradation, were ground using an ultraturrax at 9,500 rpm for 5 min. Then, the homogenate was centrifuged at 10,000 rpm, 4ºC for 10 min to obtain the supernatant. Total phenols were quantified by using the method reported by Selvendran and Ryden (1990) and Benzie and Strain (1999), based on the Folin-Ciocalteu method. 250 µL of supernatant was mixed in a volumetric flask of 25 mL with 15 mL of Milli-Q water, plus 1.25 mL of Folin-Ciocalteu reagent for 8 min. Then, 3.75 mL of 7.5% Na_2CO_3, plus the required amount of Milli-Q water, were added. After mixing, the volumetric flasks were incubated in darkness for 2 hours at room temperature. Absorbance was measured at 765 nm by using a spectrophotometer (Helios Zeta UV-VIS, Thermo Fisher Scientific, UK). The total phenolic content was expressed as mg of gallic acid equivalent per gram of sample, using a standard curve range of 0-800 mg/mL of gallic acid (Sigma-Aldrich, Germany). These analyses were carried out in triplicate.

2.4.3. Antioxidant activity

Antioxidant activity was assessed using the free radical scavenging activity of the samples evaluated with the stable radical 2,2-Diphenyl-1-picrylhydrazyl (DPPH), according to the method described by Sánchez-Moreno et al. (2003). 10 g of seedless grapes with 10 mL of methanol were ground using an ultraturrax at 9,500 rpm for 5 min. Then, the homogenate was centrifuged for 10 min at 10,000 rpm and 4ºC to obtain the supernatant. 0.1 mL of supernatant was added to 3.9 mL of DPPH solution (0.03 g DPPH/L methanol; DPPH, Sigma-Aldrich, Germany; Methanol, Panreac, Spain). Absorbance (A) at 515 nm was measured by using a spectrophotometer (Helios Zeta UV-VIS, Thermo Fisher Scientific, UK) at 10 s

intervals until the reaction reached a plateau (time at the steady state). The percentage of DPPH was calculated as the ratio of the Asample minus Acontrol with respect to Acontrol. This analysis was carried out in triplicate.

2.4.4. Weight loss

Weights of coated and uncoated grape clusters were controlled at different storage times. Cumulative weight losses were expressed as a percentage loss of the initial weight (storage time=0).

2.4.5. Measurement of mechanical properties

The mechanical properties were measured by using a texture analyser (TA-XTplus, Stable Micro Systems, UK) with a 50 kg load cell, using a 75 mm diameter cylindrical probe. Grapes from each cluster (fifteen per treatment and each time of storage) were placed longitudinally with the peduncle on the left of the texture analyser and 50% compressed at a speed of 2 mm/s. Force and distance at the failure point were used as mechanical parameters. Mechanical parameters were measured at 3, 5, 8 and 22 days of cold storage.

2.4.6. Colour measurement

Colour was measured using a spectrocolorimeter (CM-3600d, Minolta Co., Japan) with a 10 mm diameter window. Measurements were taken in the different grapes (15 of 3 different clusters) for each treatment, throughout the whole storage period. To avoid the effects of heterogeneity in the fruit, measurements were always taken in the same previously marked sample zone in the grape. CIE-L*a*b* coordinates, hue (h^*_{ab}) and chrome (C^*_{ab}) (CIE, 1986) were obtained from the reflection spectra of the samples using D65 illuminant/10º observer.

2.4.7. Respiration rate

In order to measure the respiration rate, a closed system was used (Castelló et al., 2006). At each sample time during storage, grape clusters (each one about 150-200 g) were placed in 0.655 L hermetic glass jars with a septum in the lid for sampling gas in the headspace at different times. These samples were not used for further analysis. Gas sampling was carried out every 30 min for 10 h by means of a needle connected to the gas analyser. O_2 and CO_2 contents were measured using an O_2 and CO_2 meter (Checkmate 9900, PBI Dansensor, Denmark).

This headspace gas analyser is based on an electrochemical sensor to record the 0_2 content and a mini-IR spectrophotometer to record CO_2 content (Rocculi et al., 2005). Experimental points were considered in the time range where a linear relationship was observed between gas concentration and time. This means that no changes in the respiration pathway of the samples occurred in this period and so changes in the headspace composition did not produce notable alterations in their metabolism. Respiration rate (RRi, $mg.kg^{-1}.h^{-1}$) of the samples in terms of CO_2 generation and O_2 consumption was determined from the slope of the fitted linear equation, as described by Fonseca et al. (2002).

Respiration rates at time 0 were only determined in the uncoated samples, whereas, they were measured for all treatments at 3, 5, 8, 12 and 22 days of cold storage. These analyses were carried out in triplicate.

2.4.8. Microbiological analysis

Total aerobic mesophilic microorganisms, yeast and mould populations were evaluated periodically throughout storage. In sterile conditions, 10 g of sample was homogenized for 2 min with 90 mL of tryptone phosphate water (Scharlab, Spain) using a stomacher blender (Bag Mixer 400, InterScience, USA). Serial dilutions of fruit homogenates were poured in plate count agar (PCA, Scharlab, Spain) and chloramphenicol glucose agar (CGA, Scharlab, Spain) for enumerated mesophilic

aerobic bacteria (ISO 4833, 2003) and yeasts and moulds (ISO 7954, 1987). PCA and CGA plates were incubated respectively at 30ºC for 48 hours and at 25ºC for 5 days. Microbial counts were carried out at 3, 5, 8 and 19 days of cold storage. All tests were run in duplicate.

2.4.9. Statistical analysis

Results were analysed by a multifactor analysis of variance with 95% significance level using Statgraphics® Plus 5.1. Multiple comparisons were performed through 95% LSD intervals.

3. RESULTS AND DISCUSSION

3.1. Changes in weight loss, pH, soluble solids and mechanical properties

Figure 1 shows the changes in weight loss both for uncoated samples and those coated with the different formulations throughout the cold storage time. Weight loss occurred mainly during the first 3 days of storage and was more pronounced for the control samples and those coated with a pure CH coating than for the samples with coatings containing B oil which showed the smallest weight losses. The effect of storage time led to no significant, observable differences. So, with the exception of the pure CH coating, the rest provided a significant water vapour barrier, showing lower weight losses than the uncoated samples. In both matrices, the addition of B oil improved this property, as may be expected from its hydrophobic nature. These results are coherent with the values of the water vapour permeability (WVP) of the isolated films reported in previous studies, where CH films exhibited greater WVP than HPMC films (Vargas et al., 2008) and, in both cases, a significant reduction of this parameter was obtained when an essential oil was incorporated (Sánchez-González et al., 2009, 2010a, 2010b).

The constancy of the weight loss throughout the storage period could be related

with the reduction of the process driving force throughout the storage time. The sample water loss leads to a decrease in the sample water activity (solute concentration) and so it becomes closer to the water activity value in the chamber atmosphere (relative humidity/100), which implies that the system is near the equilibrium conditions and no notable water losses occur. The uncoated samples experienced an acceleration of the weight loss on the last day of storage, which can be attributed to an increase in the fruit's metabolic activity, associated to the tissue senescence at long storage times, which is slowed down after coating application. Previous studies have reported the effectiveness of polysaccharide coatings as a water barrier in citric fruit and its enhancement by the incorporation of lipids (Du Plooy et al., 2009; Rojas-Argudo et al., 2009; Valencia-Chamorro et al., 2009).

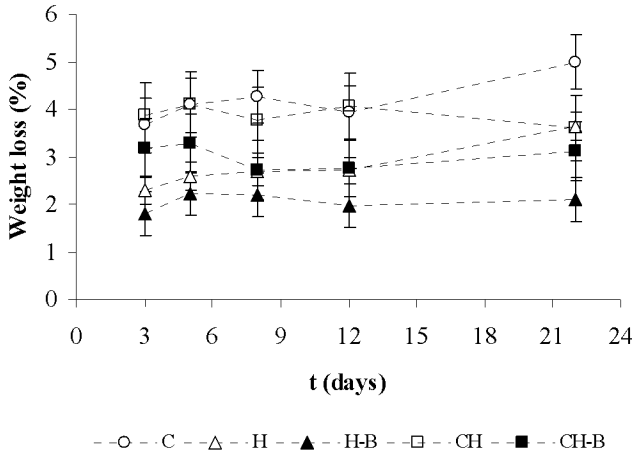

Figure 1. Weight loss of the different samples as a function of the cold storage time. (Mean values and LSD intervals).

Figure 2 shows the soluble solid content of the different samples as a function of storage time. A significant effect of storage time was detected, which was reflected

in an increase of the values throughout the first 8 days and a subsequent, sharp decrease, depending on the sample type. At the end of storage, a notable dispersion of °Brix values was observed for the different samples, with the greatest values being those of uncoated samples, followed by those coated with composite coatings containing B oil. This behaviour seems to reflect a progressive ripening of the grapes during the first storage period, as has been described by other authors for different grape cultivars (Meng et al., 2008; Valero et al., 2006; Valverde et al., 2005), whereas physiological disorders associated with senescence led to a divergent development of the different samples. In this sense, the coatings containing B oil led the samples to behave in a similar way to the control.

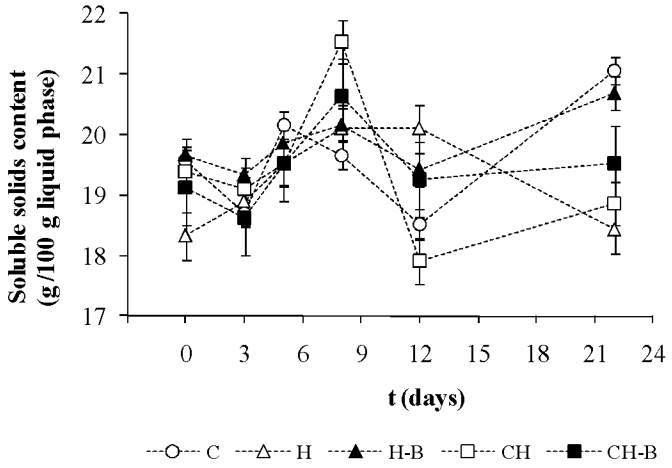

Figure 2. Soluble solids concentration (g/100 g fruit liquid phase) of different samples as a function of the cold storage time. Mean values and LSD intervals.

The pH values did not show significant changes during storage, although there were differences among the samples. Those coated with CH showed slightly greater mean values (3.7) than the other ones (3.6), but these can be attributed to the natural variability of the product and not to the treatment. Due to the nature of

fruit organic acids, the usual decrease in fruit acidity (Vargas et al., 2006) did not provoke any notable changes in pH.

Table 1 shows the mean values of force at failure (F) for the different cold storage times, for uncoated and different coated samples.

Table 1. Force at break of the different samples a function of the cold storage time. Mean values and standard deviation.

t (days)	F (N)				
	C	H	H-B	C-H	CH-B
0	16.8 (0.8)^{b,x}	20 (2)^{a,y}	18 (2)^{a,x}	25 (2)^{c,z}	24 (1)^{c,z}
3	22 (1)^{d,y}	18 (1)^{a,x}	18.4 (0.6)^{a,x}	18 (1)^{a,x}	19 (1)^{a,x}
5	19.2 (0.8)^{c,x}	19.6 (0.6)^{a,x}	19.5 (0.3)^{a,x}	23 (1)^{b,y}	19 (1)^{ab,x}
8	19 (2)^{c,xy}	23 (1)^{b,z}	19 (1)^{a,x}	22.2 (0.7)^{b,z}	20 (1)^{b,y}
22	14.62 (0.24)^{a,x}	24 (2)^{b,w}	22 (2)^{b,z}	21.6 (0.4)^{b,yz}	19 (1)^{ab,y}

a-d: Different superscripts within a column indicate significant differences among storage times ($p<0.05$)
x-w: Different superscripts within a file indicate significant differences among samples ($p<0.05$)

An ANOVA revealed that storage time and coating type led to differences in the mechanical response; the latter being more significant. Before storage, samples coated with CH (with and without B oil) showed a greater resistance to break which could be due to the greater mechanical resistance to failure of CH compared to HPMC films, as described in previous studies (Sánchez-González et al., 2009, 2010a). In this sense, the weakening effect of B oil on the polymer resistance was also observed for coated grapes. This result indicates that the compression response of the grapes is greatly affected by the mechanical resistance of the skin and coating. Although there are fluctuations of the F values of the different samples throughout the storage period, which may be due to the natural variability of the product, at the end of the period a significantly greater loss of firmness was observed in the uncoated samples compared to the rest of the coated samples. It is

remarkable that samples coated with pure HPMC, followed by those coated with pure CH, are the ones that showed the greatest firmness. The obtained values of the fruit firmness are in agreement with the weight loss results. The greater the water loss, the lower the fruit turgor and so, the lower the values of the compression force. In this sense, the water vapour barrier property of coatings has a positive effect on maintaining the fruit texture. A loss of firmness during postharvest cold storage was observed for other grape varieties (Valverde et al., 2005), strawberry (Vargas et al., 2006; del Valle et al., 2005; Mali and Grossmann, 2003), apple (Moldao-Martins et al., 2003) and sweet cherry (Yaman and Bayindirh, 2002), both uncoated or coated with different edible coatings, although, in general, coatings contribute to better maintain the fruit texture.

3.2. Antioxidants, phenols and colour properties

Observations of the phenol content and antioxidant activity of the samples showed up no significant differences. The phenol content sharply significantly decreased from 121 to 82 mg/100 g sample during the first 3 cold storage days for all the treatments, regardless of the coating treatment, and a progressive, slow decay occurred afterwards (reaching a value of 68 mg/100 g sample in the last control) as has been previously observed in grapes (Meng et al., 2008; Valero et al., 2006) and in other non-climateric fruit, such as strawberry (Ferreyra et al., 2007), coherent with the natural phenol content decay that occurs in grape maturation and postharvest stages. The activity of phenylalanine ammonia-lyase (PAL) is key in the phenol compound accumulation in grapes and this activity decreases in the maturation and postharvest stages (Meng et al., 2008). Nevertheless, since non-red colour compounds (mainly carotenoids such as xanthophylls and flavonols such as quercetin (Jackson, 2008) are present in this white cultivar, this did not lead to a decrease in grape colour, as will be commented on below.

The antioxidant capacity of the samples sharply increased ($p<0.05$) during the first

3 storage days (from 51 to 68%), but afterwards hardly increased at all, regardless of the treatment, which could be attributed to the development of Maillard compounds in line with the development of the brown colour (Karadeniz et al., 2000). Enzymatic browning could also contribute to the formation of antioxidant compounds, since an increase in PPO and POD activities has been observed in grapes during postharvest storage (Meng et al., 2008). Phenolic acids (cynnamic and benzoic, esterified or not with tartaric acid) are mainly present in white grapes. These compounds are highly oxidative, producing brown compounds that also show antioxidant activity. Previous works dealing with the oxidative process of salad tomato during ripening also revealed changes in oxidative and antioxidative parameters (Jimenez et al., 2002). The levels of the aqueous-phase antioxidants increased during the ripening process and this increase was associated with significant changes in their redox status, becoming more reduced as ripening progressed.

Figure 3 shows the development of different colour coordinates for the different coated and uncoated samples. Luminosity (L*) and hue (h*ab) decreased with storage time, which reflects the development of sample browning. Luminosity values were significantly higher in the samples coated with CH, whereas the rest of the coatings did not lead to any detectable differences. These higher L* values of the CH samples can be explained by the notably higher film opacity that inhibits the light absorption of the grape surface, thus softening its colour.

The decrease of hue was mainly indicative of sample browning. The samples coated with pure polymers showed the highest hue values throughout the entire storage period, whereas the CH coatings containing B oil slightly increased the sample redness (lower hue values) due to the fact that the natural colour of the oil is more clearly expressed in this coating. It is remarkable that there is a practically parallel development of hue in the different samples, which reflects the fact that the impact of the film on the grape appearance affects grape colour but does not seem

to inhibit browning. The chroma values hardly change throughout the storage time, although there were differences between the samples. The coatings with pure polymers, especially HPMC, led to the less saturated grape colour, whereas the incorporation of B oil into CH coatings increased the colour saturation of the grapes due to the contribution of the coating to the selective light absorption.

The detected changes in the total phenols and the development of brown compounds caused changes on the visible or measured grape colour. Some authors (González-Barrio et al., 2005) attribute the appearance of brown colour during storage of white grape to the degradation of chlorophyll b to pheophytins (brownish pigments) through the increase in the chlorophyllase activity. Likewise, studies into the enzymatic activity of grapes during cold storage reflect a great increase in polyphenoloxidase (PPO) and peroxidase (POD) activities (Meng et al., 2008), which explains both the decrease of phenol content as well as the development of the brown colour in this white grape cultivar. Non enzymatic browning has been also described as responsible for brown colour development (Karadeniz et al., 2000).

Grape coatings improve the product appearance since lower values of hue are shown throughout the complete storage period, which means that they mitigate the brown appearance. Moreover, pure CH coating provoked an increase of the fruit luminosity. Coatings of CH contain B oil was the only treatment that seems to enhance the brownish aspect of grapes slightly.

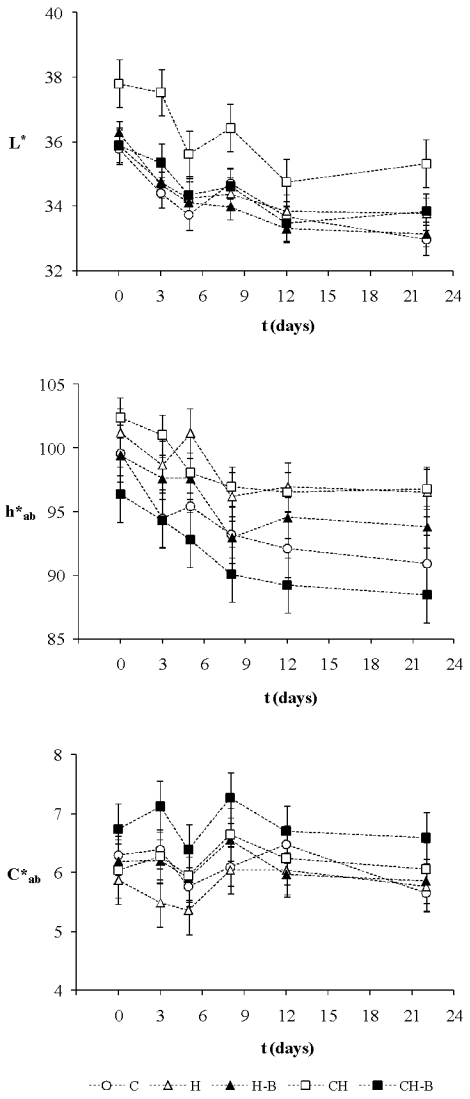

Figure 3. Development of luminosity (L*), hue (h^*_{ab}) and chroma (C^*_{ab}) of the different samples as a function of the cold storage time (mean values and LSD).

3.3. Respiration rates

The effect of film application on the fruit respiration rate was evaluated through O_2 consumption and CO_2 generation. The respiration quotient (RQ) was also calculated. These values for non-stored, uncoated grapes, expressed as mg $kg^{-1}h^{-1}$, were 20.2±0.2 and 36.0±0.4 for O_2 consumption and CO_2 generation, respectively, while the respiration quotient was 1.19±0.09, near 1, which reflects the normal respiration pathway of the fruit.

Figure 4 reflects the development of the respiration rates of the different samples as a function of the cold storage time. Coatings of CH containing B oil inhibited both O_2 consumption and CO_2 generation throughout all the storage time which can be associated with lower gas permeability values of these films. The HPMC coating also inhibited respiration rates, but only during the first 8 storage days, which could be attributed to the progressive hydration of the film with the subsequent loss of the gas barrier power. The incorporation of B oil into the HPMC matrix did not significantly modify the respiration rates, in contrast with the behaviour observed for the CH matrix. The different interactions among the film components lead to different film microstructure and transport properties. In this sense, the highly charged chains of CH seemed to develop strong interactions with B oil compounds, which greatly modified the gas barrier properties. At the end of storage, no significant differences in the CO_2 generation were observed for the different samples, all values being close to the control. The increase in the respiration rate at long storage times is coherent with the increase in the metabolic activity of the samples, related with the tissue senescence and cell breakdown.

Different authors reported a good correlation between the respiration rates and the sample weight loss (Fallik et al., 2005; Porat et al., 2005; Valverde et al., 2005). A reduction of the respiration rates provoked by the coatings has also been described for other cultivars of grapes (Valverde et al., 2005) and other fruit such as avocado (Maftoonazad and Ramaswamy, 2005) and sweet cherry (Alonso and Alique,

2004). Nevertheless, as compared with other grape cultivars and fruit, very low respiration rates were observed throughout cold storage for uncoated and coated samples.

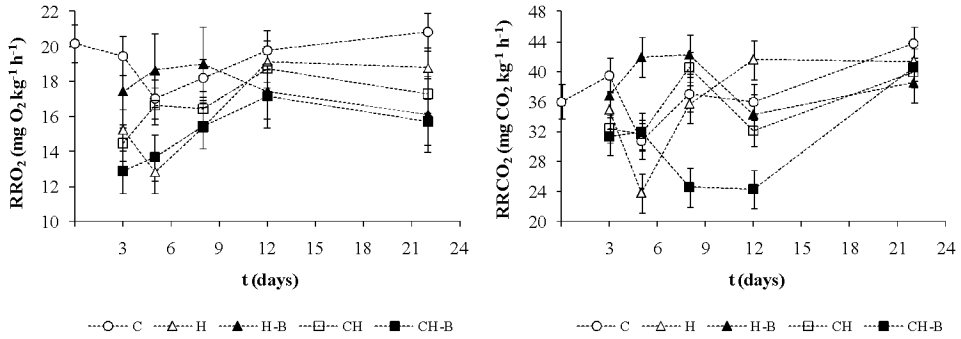

Figure 4. Development of respiration rates in terms of O_2 consumption (RRO_2) and CO_2 generation ($RRCO_2$) of the different samples as a function of the cold storage time. (Mean values and LSD intervals).

As a consequence of the changes in the O_2 and CO_2 respiration pathways provoked by coatings and storage time, the respiration quotients (RQ) showed the expected increase throughout storage in all cases. This increase was more marked for the samples coated with CH, HPMC, and CH containing B oil (RQ near 1.7 at the end of storage), whereas it maintained lower values for the control samples and those coated with HPMC containing B oil (RQ near 1.5 at the end of storage). This indicates that anaerobic pathways were developed mainly in those samples coated with films with low oxygen permeability.

3.4. Microbiological analysis

Figure 5 shows the microbiological counts for coated and uncoated grapes throughout storage. These counts for non-stored, uncoated grapes, were 0.17±0.02 and 0.175±0.013 logs UFC/g for moulds and yeasts and mesophiles, respectively.

It is remarkable that, for both uncoated and coated fruit, the safety of grapes was maintained till 19 days of cold storage. The protection role of coatings was relatively low, but let the effect of initial washing be enhanced and the microbial growth be maintained below 0.4 logs UFC/g (maximum value required by law), as is shown in figure 5. As regards moulds and yeasts, coatings with CH and B oil reduced the initial counts of the samples and coatings with HPMC and B oil inhibited growth throughout the whole storage period. Coatings without B oil inhibited growth till 9 storage days, but at the end of storage the values of log UFC were similar to the control sample. As for mesophiles, a reduction of the initial population was observed till 9 storage days for CH and coatings containing B oil. At the end of storage, lower counts than in the control samples were obtained in both cases, especially in samples coated with CH-B, where the sample initial counts were hardly reached. The B oil enhanced the antimicrobial activity of the pure CH coatings as was clearly observed in both moulds and yeasts and mesophiles counts.

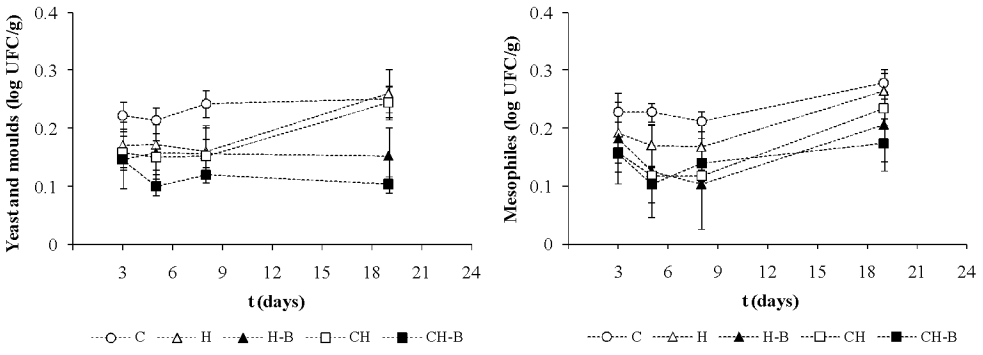

Figure 5. Development of the growth of molds and yeasts and mesophiles in the different samples as a function of the cold storage time. (Mean values and LSD intervals).

4. CONCLUSIONS

Edible coatings obtained from HPMC and chitosan with and without bergamot essential oil are a good alternative for grape preservation, improving weight losses and fruit firmness, at the same that slightly reduced respiration rates. CH coatings containing bergamot oil produced the most effective antimicrobial activity, and showed the greatest inhibition of the respiration rates in terms of both O_2 consumption and CO_2 generation. Although the coatings did not seem to reduce the rate of grape browning during storage, they softened the colour development, thus improving the product appearance. Taking into account the overall results obtained, the most recommendable coating for Muscatel table grape is the CH containing bergamot oil since, despite only contributing slightly to the sample colour, it showed the highest antimicrobial activity and the greatest control of the respiration rates with a reasonably good control of water loss during storage.

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IV. CONCLUSIONES

- La incorporación de aceites esenciales en formulaciones formadoras de recubrimiento (FFR) a base de quitosano o hidroxipropilmetilcelulosa supuso cambios significativos en las propiedades de las mismas. Para los dos polímeros estudiados, las FFR presentaron un comportamiento reológico pseudoplástico, independiente del tiempo. En las FFR a base de quitosano, el aumento del contenido en aceite estuvo acompañado de un descenso significativo en la viscosidad aparente. El contenido en aceite esencial afectó de forma significativa a la distribución de tamaño de partícula y potencial-ζ de las FFR. El menor potencial-ζ de las FFR a base de quitosano apunta hacia la existencia de interacciones de tipo electrostático entre el polímero y los aceites esenciales. En cuanto a las FFR a base de hidroxipropilmetilcelulosa y tween 85, el aumento del contenido de aceite esencial de árbol de té no supuso cambios notables en cuanto a las propiedades reológicas de las FFR pero si se observó un aumento significativo de la carga eléctrica neta de las partículas, lo que puede ser atribuido a una mayor cantidad de surfactante adsorbido en la interfase.

- Las propiedades fisco-químicas de los films a base de quitosano o hidroxipropilmetilcelulosa se vieron afectadas de forma significativa por la incorporación de los aceites esenciales. Esta incorporación supuso una mejora de las propiedades barrera al vapor de agua, con una disminución de la capacidad de sorción. En cuanto a las propiedades ópticas de los films, se observó una disminución del brillo y la translucidez. En las propiedades antimicrobianas evaluadas destacan de forma significativa las correspondientes a los films a base de quitosano-aceite esencial de bergamota y quitosano-aceite esencial de árbol de té frente a las cepas *Penicillium italicum* y *Listeria monocytogenes,* respectivamente. En este sentido, en los films de quitosano enriquecidos con un 3% de aceite esencial de bergamota se alcanzó una inhibición total del crecimiento del hongo durante los cinco primeros días del período de almacenamiento.

- El efecto del tipo de matriz, naturaleza y concentración de aceite esencial sobre las propiedades de los recubrimientos, realizado por análisis estadístico multivariable, puso de relieve la mayor influencia del tipo de matriz sobre todas las propiedades fisicoquímicas analizadas, tanto en los FFR como en los film aislados. Además se pudo concluir que para un tipo de polímero determinado, el tipo de aceite esencial incorporado y el nivel son los factores que más contribuyen, respectivamente, en las propiedades de las FFR y de los films secos aislados.

- Los films diseñados a base de polisacáridos (hidroxipropilmetilcelulosa y quitosano) y aceites esenciales (árbol de té, limón, bergamota) presentaron propiedades antimicrobianas significativas frente a las tres cepas estudiadas (*Escherichia coli, Staphylococcus aureus, Listeria monocytogenes*). En los dos tipos de matriz, el aceite esencial de árbol de té mostró la mayor efectividad antimicrobiana. En estos films, se observó una inhibición completa del crecimiento de la cepa Gram negativa, *E.coli*, en cuanto a las dos bacterias Gram positiva, *L.monocytogenes* y *S.aureus*, se obtuvieron los mejores resultados en los films de hidroxipropilmetilcelulosa - aceite esencial de árbol de té y hidroxipropilmetilcelulosa - aceite esencial de bergamota, respectivamente.

- En cuanto a los mecanismos difusionales de los componentes activos de los aceites esenciales, las pérdidas de compuestos volátiles fueron significativas durante el proceso de secado de los films. Estas fueron mayores a medida que aumentó la cantidad de aceite esencial incorporada en el film.

Este análisis mostró la complejidad de estos mecanismos en alimentos modelos, dependientes de varios factores como la estructura del film, la polaridad y solubilidad del alimento modelo y del migrante. Los resultados mostraron que la difusión del limoneno, compuesto mayoritario del aceite esencial de bergamota, es mayor en etanol al 95%. Se observó una relación lineal entre los valores de los

coeficientes de difusión en etanol al 95% y el contenido del film de quitosano en aceite esencial. Para un nivel de aceite esencial determinado, el coeficiente de difusión disminuye con el aumento del espesor del film.

- La aplicación de recubrimientos a base de quitosano y hidroxipropilmetilcelulosa, enriquecidos o no con aceite esencial de bergamota, son una buena alternativa para la conservación de uvas, variedad *moscatel*. Estos recubrimientos supusieron cambios significativos en algunas de las propiedades fisicoquímicas del fruto como las perdidas de peso y firmeza. Los mejores resultados se obtuvieron con los recubrimientos de quitosano y aceite esencial de bergamota ya que, además de presentar una capacidad antimicrobiana significativamente mayor, consiguieron reducir la tasa respiratoria y un interesante efecto barrera frente a las pérdidas de agua.

V. ANEXOS