TESIS DOCTORAL

Quitosano como matriz biopolimérica para el desarrollo de envases activos antimicrobianos de alimentos

Laura Higueras Contreras

Valencia, Octubre de 2015

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Ciència dels Aliments

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GOBIERNO DE ESPAÑA | MINISTERIO DE CIENCIA E INNOVACIÓN | CSIC

Dra. Pilar Hernández Muñoz, Científica Titular del Instituto de Agroquímica y Tecnología de los Alimentos,

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CERTIFICAN:

Que **Dª Laura Higueras Contreras**, licenciada en Ciencia y Tecnología de los Alimentos, ha realizado bajo nuestra dirección en el Instituto de Agroquímica y Tecnología de los Alimentos (IATA – CSIC) el trabajo titulado **"Quitosano como matriz biopolimérica para el desarrollo de envases activos antimicrobianos de alimentos"**, y presenta esta memoria que constituye su Tesis para optar al título de Doctor por la Universitat de València.

Y para que conste a efectos oportunos firman el presente certificado en Paterna, el _____ de _____de 2015.

Firmado: Dra. Pilar Hernández Muñoz

Firmado: Dra. Gracia López Carballo

Firmado: Dr. Rafael Gavara Clemente

A mis padres
A vosotros

"Investigar es ver lo que todo el mundo ya ha visto y pensar lo que nadie ha pensado todavía"

Albert Szent-Györgyi (1893-1986)

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Resumen

En esta Tesis se ha abordado el desarrollo de películas renovables y activas de quitosano con agentes antimicrobianos de diversa naturaleza para su aplicación en el envasado activo de alimentos. Las películas de quitosano se obtuvieron mediante la técnica de extensión y evaporación del solvente, y las sustancias bioactivas fueron incorporadas mediante diferentes metodologías.

En primer lugar, el sólido etil-N^{α}-dodecanoil-*L*-arginato (LAE) se incorporó previamente a la formación de la película de quitosano. Las películas con LAE liberaron completamente el agente activo en un simulante alimentario acuoso en varias horas, a diferentes temperaturas. Las películas de quitosano, que no fueron neutralizadas, presentaron actividad antimicrobiana frente a microorganismos presentes en pechugas de pollo frescas. Esta actividad antimicrobiana se incrementó al incorporar el LAE a la matriz, reduciendo satisfactoriamente los microorganismos presentes en el alimento para el control del deterioro microbiano de pollo fresco envasado.

En segundo lugar, se incorporó el precursor nitrato de plata a la solución filmógena de quitosano, para generar *in situ* nanopartículas de plata durante la neutralización de las películas de acetato de quitosano. Las películas desarrolladas presentaron una actividad antibacteriana *in vitro* mantenida en el tiempo. Las nanopartículas de plata pudieron actuar como reservorios de iones de plata liberados sostenidamente en un entorno acuoso.

El tercer mecanismo para incorporar el agente antimicrobiano fue el anclaje reversible del volátil cinamaldehído a películas preformadas de quitosano mediante la formación de una base de Schiff. Este enlace covalente fue hidrolizado tras la aplicación de diversos tratamientos de temperatura/tiempo habituales en la conservación de alimentos en un medio acuoso, liberándose el cinamaldehído. Las películas iminoderivadas aumentaron la seguridad microbiológica de la leche. La leche con el cinamaldehído liberado fue aceptada sensorialmente.

El último método empleado se basó en la incorporación de monoterpenos antimicrobianos, principalmente carvacrol, en películas de quitosano con coadyuvantes mediante la inmersión de las películas

preformadas en el agente volátil en estado líquido. La presencia conjunta de hidroxipropil-β-ciclodextrinas, glicerol y agua en las películas de quitosano dio lugar a una elevada capacidad de sorción de carvacrol de carácter lipófilo. La liberación del carvacrol presente en las películas compuestas fue activada por la humedad relativa ambiental. Este carvacrol liberado de las películas presentó efectividad antimicrobiana en fase vapor cuando fue incorporado en un sistema de envasado para pechugas de pollo fresco. La capacidad de retención de las películas se pudo modular en función de su formulación y de la estructura química del monoterpeno sorbido. Las películas con dichos monoterpenos presentaron capacidad antimicrobiana en fase vapor *in vitro*, y pueden emplearse en el diseño de envases activos adaptados al alimento aplicado.

Resum

En aquesta Tesi s'ha abordat el desenvolupament de pel·lícules renovables i actives de quitosà amb agents antimicrobians de diversa naturalesa per a la seua aplicació en l'envasat actiu d'aliments. Les pel·lícules de quitosà es van obtindre mitjançant la tècnica d'extensió i evaporació del solvent, i les substàncies bioactives van ser incorporades mitjançant diferents metodologies.

En primer lloc, el sòlid etil-N^{α}-dodecanoil-*L*-arginat (LAE) es va incorporar prèviament a la formació de la pel·lícula de quitosà. Les pel·lícules amb LAE van alliberar completament l'agent actiu en un simulant alimentari aquós en diverses hores, a diferents temperatures. Les pel·lícules de quitosà, que no van ser neutralitzades, van presentar activitat antimicrobiana enfront de microorganismes presents en pits de pollastre fresques. Aquesta activitat antimicrobiana es va incrementar en incorporar el LAE a la matriu, reduint satisfactòriament els microorganismes presents en l'aliment per al control de la deterioració microbiana de pollastre fresc envasat.

En segon lloc, es va incorporar el precursor nitrat de plata a la solució filmògena de quitosà, per a generar *in situ* nanopartícules de plata durant la neutralització de les pel·lícules d'acetat de quitosà. Les pel·lícules desenvolupades van presentar una activitat antibacteriana *in vitro* mantinguda en el temps. Les nanopartícules de plata van poder actuar com a reservoris d' ions de plata alliberats sostingudament en un entorn aquós.

El tercer mecanisme per incorporar l'agent antimicrobià va ser l'ancoratge reversible del volàtil cinamaldèhid a pel·lícules preformades de quitosà mitjançant la formació d'una base de Schiff. Aquest enllaç covalent va ser hidrolitzat després de l'aplicació de diversos tractaments de temperatura/temps habituals en la conservació d'aliments en un medi aquós, alliberant-se el cinamaldèhid. Les pel·lícules iminoderivades van augmentar la seguretat microbiològica de la llet. La llet amb el cinamaldèhid alliberat va ser acceptada sensorialment.

L'últim mètode emprat es va basar en la incorporació de monoterpens antimicrobians, principalment carvacrol, en pel·lícules de quitosà amb coadjuvants mitjançant la immersió de les pel·lícules preformades en l'agent volàtil en estat líquid. La presència conjunta de hidroxipropil-β-

ciclodextrines, glicerol i aigua en les pel·lícules de quitosà va donar lloc a una elevada capacitat de sorció de carvacrol de caràcter lipòfil. L'alliberament del carvacrol present en les pel·lícules compostes va ser activada per la humitat relativa ambiental. Aquest carvacrol alliberat de les pel·lícules va presentar efectivitat antimicrobiana en fase vapor quan va ser incorporat en un sistema d'envasat per a pits de pollastre fresc. La capacitat de retenció de les pel·lícules es va poder modular en funció de la seua formulació i de l'estructura química del monoterpè absorbit. Les pel·lícules amb aquests monoterpens van presentar capacitat antimicrobiana en fase vapor *in vitro*, i poden emprar-se en el disseny d'envasos actius adaptats a l'aliment aplicat.

Abstract

This PhD dissertation focuses on the development of renewable active chitosan films with a variety of antimicrobial agents for active food packaging applications. Chitosan films were obtained by the solvent-casting technique. Bioactive substances were incorporated using various methodologies.

The solid ethyl-N^{α}-dodecanoyl-*L*-arginate (LAE) was incorporated prior to the formation of the chitosan film. The LAE was completely released by the films in an aqueous food simulant over several hours at various temperatures. The chitosan films, which were not neutralized, showed antimicrobial activity against microorganisms on fresh chicken breasts. This antimicrobial activity was increased by the incorporation of LAE in the matrix, successfully reducing common food spoilage microorganisms to control microbial deterioration of packaged fresh chicken.

Silver nitrate precursor was incorporated into the film-forming chitosan solution to generate silver nanoparticles *in situ* during neutralization of the chitosan acetate films. The films showed *in vitro* antibacterial activity maintained over time. Silver nanoparticles could act as silver ion reservoirs for sustained release of silver ions in an aqueous environment.

The third mechanism for incorporating the antimicrobial agent was reversible anchorage of volatile cinnamaldehyde to preformed chitosan films by the formation of a Schiff base. This covalent bond was hydrolysed after application of various common food preservation temperature/time treatments in an aqueous medium, releasing the cinnamaldehyde. The imino-chitosan films increased the microbiological safety of milk. Milk with the released cinnamaldehyde was sensorially accepted.

The last method employed was based on the incorporation of antimicrobial monoterpenes, especially carvacrol, in chitosan films with adjuvants by immersing the preformed films in the volatile liquid agent. The combined presence of hydroxypropyl-β-cyclodextrin, glycerol and water in the chitosan films resulted in a high capacity for sorption of lipophilic carvacrol. Release of the carvacrol present in the composite film was activated by the environmental relative humidity. Carvacrol released from the films presented antimicrobial effectiveness in vapour phase when it was

incorporated into a packaging system for fresh chicken breasts. It was possible to tailor the retention capacity of the films by varying the film formulation and the chemical structure of the sorbed monoterpene. Films with these monoterpenes showed *in vitro* antimicrobial activity in vapour phase. Thus they can be applied in the design of active packages adapted to food products.

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Abreviaturas y símbolos

ADN	ácido desoxirribonucleico
AE	aceite esencial
ARN/ARNm	ácido ribonucleico/ácido ribonucleico mensajero
ATR-FTIR	espectroscopía de infrarrojos por transformada de Fourier con reflexión total atenuada
CD	ciclodextrina
CE	Comunidad Europea
CEE	Comunidad Económica Europea
CS	quitosano/chitosan
Da	dalton
EIA	*US Energy Information Administration*
EFSA	*European food safety authority*
EPA	*US Environmental Protection Agency*
EVOH	copolímero de etileno y alcohol vinílico
FAO	*United Nations Food and Agriculture Organization*
FDA	*US Food and Drug Administration*
G	glicerol
GFAAS	absorción atómica de horno de grafito
GSFA	*Government Superannuation Fund Authority*
GRAS	*Generally Recognized As Safe*
HP-βCD	hidroxipropil-β-ciclodextrina
IDA	Ingesta Diaria Admisible
JECFA	*Joint Expert Committee on Food Additives*
LAE	etil-N^{α}-dodecanoil-*L*-arginato
OECD/SIDS	*The Organisation for Economic Co-operation and Development/Screening Information Dataset*
OMS/WHO	Organización Mundial de la Salud/*World Health Organization*
PA	poliamida
PE	polietileno
PG	propilenglicol
PLA	poliácido láctico
PP	polipropileno
PS	poliestireno
PVOH	polialcohol vinílico
RH	*relative humidity*/humedad relativa
SCENIHR	*Scientific Committee on Emerging and Newly Identified Health Risks*
SEM	microscopía electrónica de barrido
TEM	microscopía electrónica de transmisión
TGA	análisis termogravimétrico
UE	Unión Europea/*European Union*
UNEP	*United Nations Environment Programme*
USDA	*US Department of Agriculture*

Prólogo

La presente Tesis Doctoral se estructura en siete secciones. En la sección **INTRODUCCIÓN** se presenta el contexto de esta Tesis, describiendo los aspectos fundamentales sobre los que se va a trabajar. En la sección **OBJETIVOS** se proponen esquemáticamente los objetivos generales y los diferentes objetivos específicos de esta Tesis Doctoral. A continuación, en la sección **JUSTIFICACIÓN Y ESQUEMA DE TESIS** se expone una breve y cohesiva descripción de los capítulos y los artículos que los conforman. La siguiente sección denominada **CAPÍTULOS** está constituida por cuatro apartados que incluyen los antecedentes y el estado de la cuestión, y las publicaciones científicas que ha originado esta investigación. En la sección **DISCUSIÓN GENERAL**, se examinan e integran los resultados de cada apartado, resaltando los más notables. En la sexta sección, se presentan las **CONCLUSIONES** más relevantes. Y finalmente en la sección **ANEXOS** se adjunta una extensa revisión bibliográfica de materiales biopoliméricos para el envasado antimicrobiano de alimentos y las siete publicaciones a las que esta Tesis Doctoral ha dado lugar.

1. INTRODUCCIÓN

Alrededor de un tercio de los alimentos producidos en el mundo para el consumo humano (aproximadamente 1300 millones de toneladas anuales) se pierde o desperdicia. Esto significa que enormes cantidades de los recursos destinados a la producción de alimentos se utilizan en vano, y que las emisiones de gases de efecto invernadero causadas por su producción (5%) son emisiones evitables (FAO, 2012; UNEP, 2011, 2013).

Los alimentos se deterioran con el tiempo, fundamentalmente por la acción de organismos vivos (bacterias, levaduras, hongos, insectos, roedores o el propio hombre), la acción físico-química del entorno (temperatura, humedad relativa (RH), oxígeno, radiaciones) y la actividad biológica del propio alimento (enzimas, respiración, estrés o daño físico). Este deterioro implica la merma de las características organolépticas, del valor nutritivo y seguridad microbiológica del alimento, llevando finalmente a la pérdida del mismo.

Por ello, a lo largo de la historia el hombre ha desarrollado una serie de tecnologías y procesos de diferente naturaleza y complejidad para la conservación de alimentos como la modificación de la temperatura (cocción, refrigeración), la conservación química basada en la disminución del pH o de la actividad de agua (acidificación, adición de azúcares, salazones, curado, ahumado, secado al sol), conservación biológica (fermentaciones), inmersión en grasa (orza) o aceite, o adición de especias. Para proteger, contener y almacenar estos alimentos tratados se desarrollaron los envases.

Los primeros envases se elaboraron con materiales naturales disponibles en el momento como hojas, calabazas o conchas, y más tarde cestas de caña, botas de vino, cajas y barriles de madera, jarrones y ánforas de barro, bolsas tejidas, etc. Con el tiempo, los materiales fueron cada vez más elaborados como es el caso del vidrio, metal y papel, o ya de plástico a partir de principios del siglo XX (Berger, 2002).

En la actualidad, el desarrollo de la tecnología del envasado es fundamental para reducir las pérdidas de alimentos y para garantizar la seguridad alimentaria, así como la mejora del comercio mundial de alimentos, que es la clave para el desarrollo económico de las diferentes economías (FAO, 2014; Marsh y Bugusu, 2007). La industria del envase es el tercer mayor sector industrial, por detrás únicamente de la industria

alimentaria y petroquímica. De toda la industria mundial del envase, el envase alimentario y de bebidas representa el 69% (FAO, 2014).

La principal función del envase es contener y proteger a los alimentos frente a factores ambientales como microorganismos, contaminantes químicos, oxígeno y vapor de agua, extendiendo su vida útil y mejorando su seguridad. Además, el envase tiene otras funciones importantes como comodidad y conveniencia, el *marketing*, y la comunicación e información al consumidor. El envase debe facilitar determinados tratamientos industriales, el almacenamiento, transporte y distribución, así como el consumo del alimento (envases unitarios, familiares) (Brody *et al.*, 2008; Fernández-Álvarez, 2000; Marsh y Bugusu, 2007). Por otra parte, el envase es la imagen de la empresa productora, por lo que un diseño adecuado y atractivo es fundamental como reclamo publicitario. Asimismo, es el medio de comunicación entre la empresa productora y el consumidor, ya que contiene mucha información (denominación, lista de ingredientes, alérgenos, cantidad neta del alimento, fecha de duración mínima o fecha de caducidad, condiciones especiales de conservación y/o de utilización, nombre o la razón social y la dirección del operador o del importador, modo de empleo e información nutricional) obligada por la legislación (UE, 2011d). La trazabilidad es otra función primordial del envase, ya que se han desarrollado diversos sistemas para el seguimiento de la distribución y compra, como son código de producto universal, los códigos de barras o los códigos QR (Brody *et al.*, 2008; Marsh y Bugusu, 2007).

Sin embargo, en los últimos años debido al desarrollo tecnológico y los cambios en el estilo de vida de los consumidores, se han desarrollado sistemas de envasado de alimentos con nuevas funciones como es el envase activo.

1.1. Envasado activo

Los sistemas convencionales de envasado de alimentos están diseñados para contener y proteger a los alimentos de forma pasiva, es decir que no interactúan con el alimento, actuando únicamente como una barrera física e inerte entre el alimento y su entorno, y tratando de retrasar o bloquear los efectos negativos del entorno en el alimento. Sin embargo, desde hace algunos años ha surgido un nuevo concepto de envase, el

denominado envase activo que, además de desarrollar las funciones de un envase tradicional, tiene la capacidad de interaccionar con el producto y/o con su entorno para mejorar uno o más aspectos de su calidad y/o seguridad (Catalá y Gavara, 2001; Rooney, 1995). En el envasado activo, el sistema alimento-envase-entorno actúa de forma coordinada para prolongar la vida útil de los alimentos, mejorar la aceptabilidad organoléptica, la calidad y seguridad alimentaria y/o facilitar su procesado y consumo (Catalá y Gavara, 2001; Fernández-Álvarez, 2000). "El envase activo está diseñado para incorporar intencionadamente agentes activos que pueden ser liberados en el alimento envasado o en su entorno, o pueden absorber sustancias del alimento o de su entorno" (CE, 2004, 2009).

Las interacciones beneficiosas creadas entre el alimento y el envase pueden basarse en la regulación del contenido en gases (oxígeno, dióxido de carbono, etileno, etc.); en el control de la humedad (aditivos antivaho, absorbentes, etc.); en la liberación de aromas, saborizantes y/o la retención de compuestos no deseados (naringina y limonina, mercaptanos); en la liberación de compuestos con propiedades antioxidantes y/o antimicrobianas (Brody *et al.*, 2008).

Respecto a los aspectos legislativos referentes al envase activo, cabe decir que los envases deben cumplir con la normativa referida a todos aquellos materiales que vayan a entrar en contacto con el alimento, Reglamento 135/2004 (CE, 2004), que incluye las limitaciones específicas y globales de migración de las sustancias desde el material. Además de respetar el Reglamento 450/2009 específico de envases activos (CE, 2009), que completa los principios generales definidos en el reglamento anterior y contiene una lista positiva de sustancias autorizadas que podrán utilizarse en los materiales activos. Asimismo, deberán cumplir la legislación específica del material de que se componga el envase (Dainelli *et al.*, 2008; Restuccia *et al.*, 2010). Por otra parte, todas las sustancias activas que migren al alimento son consideradas aditivos alimentarios (CE, 2008a) o aromas (CE, 2008b), por lo que deberá cumplir la legislación vigente; así como la referente al etiquetado (UE, 2011d).

La comercialización de los envases activos comenzó en Japón y Australia en los años ochenta, sin embargo su empleo en Europa y EEUU ha estado más restringido por una legislación más estricta y un menor

conocimiento del consumidor de las ventajas y eficacia de estos sistemas, y su impacto económico y medioambiental (De Kruijf *et al.*, 2002). Actualmente, América del Norte, y en menor medida Europa, suponen el principal mercado para la tecnología de envasado activo. En los EEUU, en 2010, el envasado en atmósfera modificada, y el envasado activo e inteligente conjuntamente representaron el 5% del mercado total de envases. Sin embargo, se prevé que el mercado de envases activos e inteligentes en los EEUU supere el 8% anual del mercado total de envases y los 3.5 millones de $ en 2017 (Freedonia, 2014).

Una forma de envases activos, que están siendo objeto de diversos estudios a nivel tecnológico y suscitando gran interés industrial, son los envases activos antimicrobianos, los cuales están destinados a controlar el crecimiento microbiano en el alimento envasado. En el **Anexo A** se recogen algunos ejemplos de compuestos antimicrobianos incorporados en diversas matrices poliméricas aplicadas en el envasado activo de alimentos.

1.1.1. Envasado activo antimicrobiano

Las infecciones e intoxicaciones alimentarias son enfermedades ocasionadas por la ingesta de alimentos en mal estado debido a la presencia de microorganismos patógenos o sus toxinas. La presencia de estos microorganismos en los alimentos no suelen ser detectables en apariencia pero pueden producir trastornos gastrointestinales graves. En 2012 en Europa, se notificaron 5363 brotes de origen alimentario (siendo España el quinto país que más brotes comunicó), afectando a 55453 personas, resultando 5118 hospitalizaciones y 41 muertes (EFSA, 2014).

Además del problema de salud pública que pueden suponer algunos microorganismos al desarrollarse en los alimentos, es importante el control del crecimiento microbiano ya que es una de las principales causas del deterioro de los alimentos, lo que supone un enorme problema económico, ambiental y ético, en todo el mundo.

Por otra parte, en la actualidad los consumidores demandan alimentos fáciles de preparar, con mayor información, seguros pero que estén libres de aditivos, y que hayan sufrido un procesamiento mínimo conservando su frescura y sus propiedades organolépticas y nutritivas. Por ello, en los últimos años se están desarrollando tecnologías alternativas a los métodos

de conservación tradicionales más agresivos como puedan ser los tratamientos térmicos para la inactivación microbiana. Estas nuevas tecnologías pretenden mantener las propiedades originales del alimento, ahorrar energía y ser más respetuosas con el medioambiente, y a la vez ser efectivas contra los microorganismos patógenos y alterantes. Sin embargo, estas alternativas más leves suelen ser también menos eficaces y pueden no eliminar todos los microorganismos alterantes y patógenos a diferencia de los tratamientos térmicos convencionales. Así, para la aplicación de estas tecnologías de procesamiento emergente que pueden no ser suficiente por ellas mismas, una estrategia adecuada es su combinación en lo que se conoce como tecnologías de barreras (*hurdle technology*) (Leistner y Gorris, 1995). En este sentido, el envasado activo presenta un gran potencial para ser aplicado en combinación con otras tecnologías de conservación de alimentos no térmicas.

Los envases activos antimicrobianos incorporan agentes antimicrobianos que inhiben o retardan el desarrollo de los microorganismos en la superficie del alimento o del envase (Appendini y Hotchkiss, 2002). En muchos alimentos, la mayor carga microbiana se encuentra en la superficie del mismo, debido principalmente a la recontaminación o contaminación cruzada de la superficie de los alimentos ya tratados. Este problema se viene solucionando mediante la aplicación de sustancias antimicrobianas por inmersión o pulverización. Sin embargo, la aplicación directa de estos agentes antimicrobianos en la superficie del alimento presenta el inconveniente de poder ser neutralizados rápidamente, interaccionar con componentes del alimento (lípidos o proteínas) o difundir hacia el interior del alimento, precisando mayor cantidad del agente. El resultado es el encarecimiento del producto, además de una posible modificación sensorial y consecuente rechazado por el consumidor. Por lo que los envases activos antimicrobianos pueden ser una alternativa adecuada al empleo de estos tratamientos en superficie, ya que pueden liberar de forma sostenida el agente antimicrobiano en la superficie del alimento manteniendo concentraciones adecuadas en ella y durante períodos prolongados de tiempo (Appendini y Hotchkiss, 2002; Coma, 2008; Ouattara *et al.*, 2000).

Por todo lo mencionado, y dado el gran potencial que presenta el envasado activo de alimentos frente al envasado tradicional, la presente

Tesis Doctoral se ha focalizado en el desarrollo y caracterización de materiales poliméricos con actividad antimicrobiana para su aplicación en el diseño de envases activos para alimentos.

1.2. Biopolímeros

El gran uso de los plásticos convencionales ha hecho que se planteen serios problemas como: 1) incremento en el consumo de derivados del petróleo, ya que más del 99% de los plásticos provienen de recursos fósiles, cada vez más escasos, caros y con precios variables según el mercado; 2) el elevado consumo de energía en su fabricación; 3) el agotamiento de los vertederos, y 4) la contaminación del medio ambiente, porque el 62.2% de los residuos plásticos son envases (PlasticsEurope, 2013) y hay acumulación de materiales difíciles de gestionar, acumulación de los aditivos empleados en su fabricación que permanecen largo tiempo en el entorno y producción de productos tóxicos por su combustión. Esto no quiere decir que el agotamiento del petróleo significará el fin de los plásticos convencionales, ya que sólo el 2% del consumo del petróleo se utiliza para la producción de plásticos (EIA, 2013) y algunos como las poliolefinas ya pueden ser sintetizadas a partir de recursos renovables. Sin embargo, como respuesta a todos problemas mencionados y a la demanda de los consumidores y de las autoridades reguladoras de unos materiales más sostenibles, alternativamente se pueden emplear, siempre que sea posible, biopolímeros para el desarrollo de plásticos.

Los biopolímeros provienen de recursos renovables y la mayor parte son biodegradables, es decir que son susceptibles de ser convertidos en agua, dióxido de carbono, metano, compuestos inorgánicos, o biomasa, incluso emplearse para enriquecer los suelos como compost (Siracusa *et al.*, 2008; Song *et al.*, 2009). Por otro lado, la obtención de estos materiales a partir de los excedentes y desechos industriales, agrícolas o de la pesca, podría abaratar los costes de producción y facilitar la eliminación de residuos procedentes de la industria alimentaria.

Los polímeros elaborados a partir de biomasa o biopolímeros pueden dividirse en tres categorías basándose en el método de producción (Petersen *et al.*, 1999; Srinivasa y Tharanathan, 2007):

1. Polímeros extraídos directamente a partir de materiales naturales (modificados o no), como son: polisacáridos (almidón, alginato, carragenato, celulosa, goma guar, pectinas, quitosano); proteínas (albúmina, caseína, colágeno, gelatina, gluten de trigo, proteína de soja, proteínas de suero de leche, zeína); o bien lípidos (ácidos grasos, acilgliceroles, ceras). Son de origen fundamentalmente vegetal, aunque también marino.

2. Polímeros sintetizados químicamente a partir de monómeros de biomasa, como es el poliácido láctico (PLA).

3. Polímeros producidos por microorganismos o bacterias modificadas genéticamente, como son los poliésteres polihidroxialcanoatos (poli-3-hidroxibutirato, poli-3-hidroxivalerato); o los polisacáridos (celulosa bacteriana, curdlan, goma xantana o pululano).

Así, los biopolímeros suelen ser biodegradables, mientras que todos los materiales biodegradables no necesariamente tienen que ser biomateriales (Weber *et al.*, 2002); el polialcohol vinílico es biodegradable pero no proviene de la biomasa, mientras que la poliamida (PA) 9 y PA 11 provienen del ácido oleico y el aceite de ricino respectivamente, pero no son biodegradables (Siracusa *et al.*, 2008).

Si bien los biopolímeros presentan numerosas ventajas, también tienen problemas como su rendimiento, procesamiento y coste, sobre todo los polímeros extraídos directamente de la biomasa (Alonso *et al.*, 2011; Petersen *et al.*, 1999). El coste medio de obtención actual de los biopolímeros es de 2-5 €/kg, frente a los 1.2 €/kg de los petropolímeros, aproximadamente (Song *et al.*, 2009). La capacidad de producción mundial de materiales basados en biopolímeros ascendió a más de 1.5 millones de toneladas en 2012 y se prevé que aumente a más de 6.2 millones de toneladas para el año 2017 (EuropeanBioplastics, 2013). El incremento en la producción de estos materiales facilitará la reducción de su precio (Alonso *et al.*, 2011; Petersen *et al.*, 1999).

Cuando se piensa en el empleo de biopolímeros para el envasado de alimentos, hay que tener presente que algunos de ellos presentan diversas desventajas respecto a sus propiedades funcionales frente a los polímeros convencionales. Así por ejemplo, los polisacáridos y proteínas son en general materiales muy hidrófilos y como consecuencia sus propiedades mecánicas y barrera varían en gran medida con la RH, además de presentar grandes

dificultades a la hora de ser procesados por vía seca mediante las técnicas usuales empleadas en plásticos convencionales. Si bien estos materiales podrían emplearse en aplicaciones específicas, como en alimentos con baja actividad de agua o que no necesitan un envase de alta barrera, o incluso convertir este inconveniente en una ventaja en el caso de alimentos frescos que se benefician del intercambio gaseoso (Siracusa *et al.*, 2008; Weber *et al.*, 2002). O bien, se pueden desarrollar sistemas multicapa con materiales que suplan sus carencias (Weber *et al.*, 2002) o diseñar derivados de los biopolímeros que mejoren estas características (Petersen *et al.*, 1999).

Muchos de estos biopolímeros (hidrogeles como polisacáridos y proteínas), debido a su capacidad filmógena y solubilidad en medios acuosos y/o etanólicos, son los principales constituyentes de las películas y recubrimientos comestibles (Campos *et al.*, 2011; Debeaufort *et al.*, 1998), o recubrimientos de otros materiales como papel, cartón u otros polímeros, demostrando ser excelentes sistemas portadores y liberadores de compuestos activos. Esta última propiedad ha hecho que se piense en ellos para el desarrollo de envases activos basados en la liberación de sustancias. En el **Anexo A** se señalan diversos ejemplos de envases activos antimicrobianos que emplean este tipo de biopolímeros. Además de por las ventajas ya descritas, estos polímeros naturales se caracterizan por una amplia disponibilidad, baja toxicidad, biocompatibilidad y su susceptibilidad a la degradación enzimática. Entre este tipo de biopolímeros destacan los glicopolímeros, como el quitosano, que presenta una baja inmunogenicidad, frente a otros biopolímeros de origen proteico (Bhattarai *et al.*, 2010).

1.2.1. Quitosano

El quitosano (CS) (**Figura 1.1**) es una familia de aminopolisacáridos catiónicos lineales basados mayoritariamente en unidades de glucosamina (2-amino-2-desoxi-β-*D*-glucosa) unidas mediante enlaces glicosídicos β-(1→4) y obtenido de la *N*-desacetilación parcial de la quitina. El grado de acetilación suele ser inferior al 30-40%, aunque para que el CS sea considerado como tal debe alcanzar al menos un grado de desacetilación del 50% (Rinaudo, 2006; Shahidi y Abuzaytoun, 2005).

La quitina, el segundo polímero natural más abundante de la tierra después de la celulosa, es un biopolímero lineal de alto peso molecular de

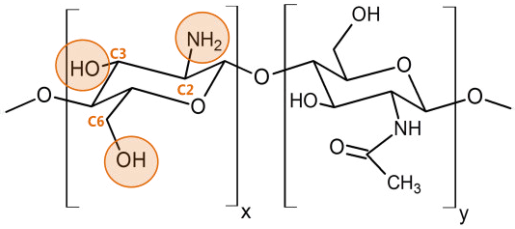


Figura 1.1. Estructura química del CS.

poli β-(1→4)-2-acetamido-2-desoxi-β-*D*-glucosa (*N*-acetil-*D*-glucosamina). La estructura química de la quitina es la misma que la celulosa, con el grupo hidroxilo en posición C2 sustituido por un grupo acetamido (Dutta *et al.*, 2004; Kurita, 2006). Anualmente, alrededor de 10^{11} toneladas de quitina son producidas por la biomasa (Tharanathan y Kittur, 2003). La quitina se encuentra como componente estructural y de soporte: en el exosqueleto de animales invertebrados como los artrópodos (insectos, arácnidos o crustáceos), anélidos, moluscos (cefalópodos), celentéreos o braquiópodos; en paredes celulares de hongos (micelios y esporas) y de levaduras (Kurita, 2006; Rinaudo, 2006; Tharanathan y Kittur, 2003), aunque la principal fuente industrial de la quitina son las gambas, langostinos y cangrejos. La obtención del CS se muestra en la **Figura 1.2**.

El proceso industrial de obtención del CS más empleado es el método químico, resultando cadenas de diferentes pesos moleculares y grados de desacetilación que determinan las propiedades y funcionabilidad del CS (Shahidi y Abuzaytoun, 2005). Así, se puede adquirir comercialmente CS de bajo (50000-190000 Da), medio (190000-310000 Da) y alto peso molecular (310000-375000 Da). En la presente Tesis Doctoral se ha trabajado con CS de bajo peso molecular con un 75-85% de desacetilación.

A diferencia de la quitina, el CS es soluble en soluciones ácidas de ácidos orgánicos, con una solubilidad limitada en ácidos inorgánicos, y casi insoluble a pH alcalino y neutro. Su pK_a se encuentra en torno a 6.3 (pK=6.2-6.8, según autores). Así, en disolución ácida (pH<6), los grupos amino en la posición C2 del polímero se protonan resultando un polisacárido soluble cargado positivamente (Agulló *et al.*, 2003; Rinaudo, 2006).

El CS, debido a la presencia de grupos amino, posee una gran capacidad quelante, por lo que puede adsorber iones metálicos, formar micelas con tensioactivos, unirse a proteínas o a ADN (Rinaudo, 2006).

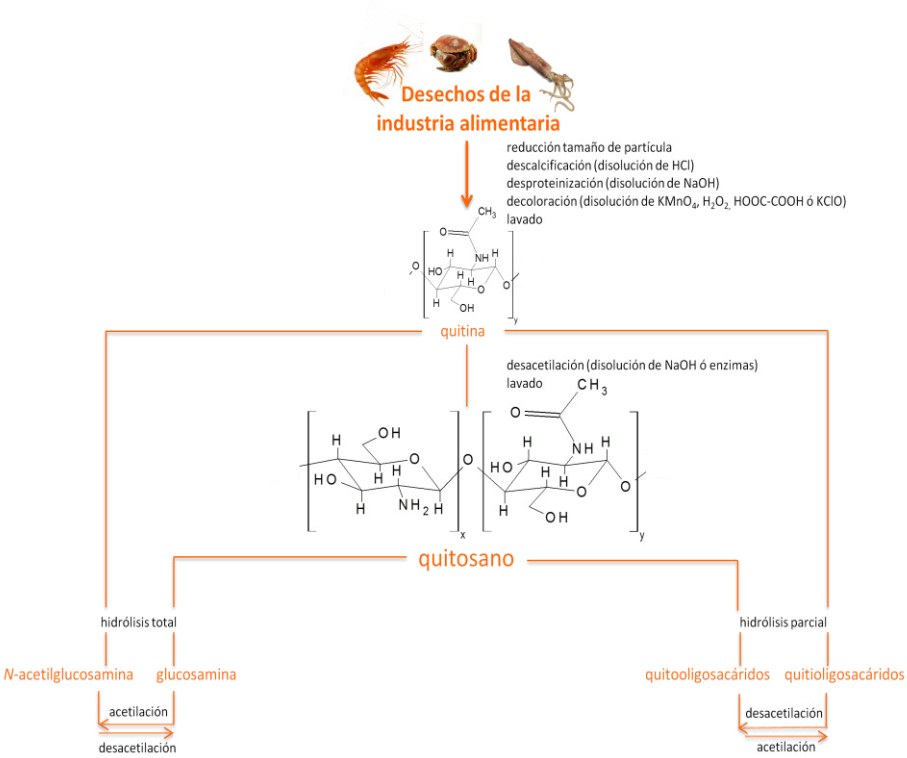


Figura 1.2. Obtención de la quitina, CS y sus derivados. Figura basada en Shahidi *et al.* (1999).

Estas cargas positivas del CS también le confieren su actividad antimicrobiana frente a bacterias, hongos y levaduras (Agulló *et al.*, 2003; No *et al.*, 2007; Shahidi *et al.*, 1999). El mecanismo de acción de la actividad antimicrobiana del CS es diferente según se trate de bacterias gram positivas o negativas, debido a sus diferencias estructurales (Kong *et al.*, 2010); aunque el mecanismo de acción aún hoy es confuso, e incluso contradictorio según el estudio de diversos autores. El CS interactúa electrostáticamente con las cargas negativas de la membrana plasmática celular bacteriana, desestabilizándola, alterando su permeabilidad, seguido de su adhesión al ADN celular, inhibiendo la síntesis de ARNm y proteínas, e impidiendo su replicación (Coma, 2012; Dutta *et al.*, 2009; Shahidi *et al.*, 1999). En el caso de las gram positivas, puede que el CS forme una membrana polimérica en la superficie celular inhibiendo la entrada de nutrientes a la célula bacteriana o interaccione con las cargas negativas de los grupos fosforilo de ácidos teicoicos y teicurónico. Cuando se trata de gram negativas, el CS puede

competir con el lipopolisacárido y proteínas de la superficie por los cationes divalentes que estabilizan la membrana externa alterando la función celular o provocando incluso la lisis celular, aunque actualmente se acepta más que el CS tiene actividad bacteriostática más que bacteriocida. Además, el CS de menor peso molecular penetra en la célula bacteriana por difusión alterando el metabolismo celular (Dutta *et al.*, 2009; Kong *et al.*, 2010; No *et al.*, 2007). Existen varios factores que afectan a la capacidad antimicrobiana del CS: su grado de desacetilación, peso molecular, concentración, y el pH del medio (Dutta *et al.*, 2009; Kong *et al.*, 2010). Por otra parte, existen diferentes propuestas para el mecanismo antimicrobiano frente a hongos: interfiriendo en su crecimiento, activando respuestas defensivas (acumulación de quitinasas, síntesis de inhibidores de peptidasas, lignificación, etc.), quelando diversas sustancias, e inhibiendo la esporulación y la germinación (Agulló *et al.*, 2003; Dutta *et al.*, 2009; Kong *et al.*, 2010).

Como se observa en la **Figura 1.1**, además del grupo amino, el CS posee un grupo hidroxilo secundario en la posición C3 y un grupo hidroxilo primario en la posición C6 haciendo de éste un polímero muy versátil (Prashanth y Tharanathan, 2007; Shahidi *et al.*, 1999). De este modo, los grupos hidroxilo originan reacciones inespecíficas, generalmente eterificación y esterificación/sulfatación, mientras que el grupo amino causa reacciones específicas como la *N*-carboxialquilación, formación de bases de Schiff, sustitución enzimática, etc. Así se pueden introducir diversos grupos para diseñar un derivado del CS para una determinada aplicación (Kurita, 2006; Prashanth y Tharanathan, 2007; Rinaudo, 2006).

La versatilidad y peculiares características del CS y sus derivados permiten su amplia aplicación en diversas áreas tecnológicas **(Tabla 1.1)**: medicina, farmacia, dietética, cosmética, biotecnología e investigación, agricultura y medio ambiente, tratamiento de aguas e industria de los alimentos, papelera, textil, fotográfica y de baterías.

El CS fue aprobado como producto GRAS en EEUU para la alimentación animal (1983) y para la purificación del agua. En Japón el CS fue aprobado como aditivo alimentario en 1983 y como ingrediente funcional en 1992, y en Corea como aditivo alimentario en 1995 (Kong *et al.*, 2010; No *et al.*, 2007; Shahidi y Abuzaytoun, 2005). La FDA (2011) determinó el estado GRAS únicamente para el CS producido por el hongo *Aspergillus niger*

Tabla 1.1. Aplicaciones del CS y sus derivados (Dutta *et al.*, 2004; Rinaudo, 2006; Shahidi y Abuzaytoun, 2005; Srinivasa y Tharanathan, 2007).

medicina	• anticoagulante, antitrombótico • piel artificial • reparación de cartílago • membranas de diálisis • hemostático • lentes de contacto • implantes dentales
cosmética	• cremas • geles • lociones • esmalte de uñas • dentífricos • champúes y acondicionadores de cabello
farmacia	• diluyente • inmunoadyuvante • sistemas de liberación de principios activos • microcápsulas • microesfera • nanopartículas
dietética y nutrición	• anticolesterolemiante • hipotrigliceridemiante • fibra soluble y prebiótico
industria alimentaria	• espesante • emulsionante • estabilizante de color y aroma • antioxidante y antimicrobiano • clarificación y desacidificación de zumos y bebidas fermentadas • floculación y recuperación de residuos sólidos • productos alimenticios con valor añadido
agroquímica	• fertilizante • biopesticida • descontaminación
industria del envasado	• películas y recubrimientos comestibles • agente activo antimicrobiano y antioxidante
biotecnología e investigación	• transmisión de genes por vías no víricas • biosensores • inmovilización enzimática • cromatografía
tratamiento de aguas	• floculante • quelante de metales, pesticidas y bifenilos policlorados • adsorción de colorantes
otras	• industria textil: agente antiestático • industria papelera: aditivo • fotografía: agente fijador de color • baterías: conductor

permitiendo su uso como aditivo alimentario directo secundario en la producción de bebidas alcohólicas. Además, ha habido diversos intentos fallidos por parte de la empresa Primex Ingredients, ASA para aprobar el CS procedente de camarón (*Pandalus borealis*) como aditivo alimentario en carnes y aves de corral (FDA (2002, 2005b, 2013)), aunque ya se comercializa

como un suplemento dietético en más de 50 países en todo el mundo, incluida la UE, los EEUU y Japón.

Según el informe "*Chitin and chitosan: a global strategic business report*" (de *Global Industry Analysts, Inc.*), tras la aprobación de todas las patentes pendientes, el mercado mundial de quitina y CS crecerá muy rápidamente, llegando a 63000 y 21000 millones de $ respectivamente en 2015. La glucosamina, seguida del CS, son los derivados más reclamados de la quitina. La principal aplicación del CS es el tratamiento de agua, seguido de su aplicación en agroquímica. El mercado mundial de CS se estimó en 13700 toneladas para 2010 y se esperan 21400 toneladas para el año 2015 (Cosgrove, 2010).

Por una parte, 30% de los pescados y productos marinos se desperdician a lo largo de la cadena (FAO, 2012). Por otra, los residuos de las plantas procesadoras de mariscos suponen un problema medioambiental dada su acumulación por su lenta velocidad de degradación. Así, actualmente se puede obtener CS en grandes cantidades a partir de quitina procedente de estos desechos y excedentes (Healy *et al.*, 1994; Srinivasa y Tharanathan, 2007). Y la reutilización de estas materias primas para obtener biomateriales destinados a la conservación de alimentos puede reducir la acumulación de desechos y su impacto medioambiental.

1.3. Los hidrogeles como sistemas poliméricos de liberación sostenida

El desarrollo de sistemas antimicrobianos basados en la liberación sostenida del agente activo es un área de interés en diversos sectores tecnológicos aparte del sector alimentario, como son el sector agrícola, textil, médico y farmacéutico. En la actualidad, se están realizando grandes esfuerzos en el desarrollo de mecanismos de liberación sostenida con el fin de mantener una determinada concentración del compuesto activo en el alimento envasado durante un período de tiempo determinado (Coma, 2008). Otro factor a tener presente es el mecanismo por el cual el hidrogel va a desarrollar su actividad, es decir, liberar el agente activo que contiene al medio externo. Existen diferentes mecanismos en respuesta a determinados estímulos ambientales: físicos (temperatura, ultrasonidos, luz, electricidad,

tensión mecánica), químicos (pH, fuerza iónica) y biológicos (enzimas, biomoléculas) (Bajpai *et al.*, 2008; Gupta *et al.*, 2002; Kim *et al.*, 2009).

Los hidrogeles, tanto sintéticos (polialcohol vinílico) como naturales (polisacáridos como el CS), han sido estudiados para el desarrollo de vehículos portadores y liberadores de agentes activos basados en sistemas poliméricos (Bhattarai *et al.*, 2010; Gupta *et al.*, 2002). Los hidrogeles son redes poliméricas tridimensionales que presentan la característica de absorber grandes cantidades de agua debido a la presencia en su estructura de grupos polares (-OH, $-NH_2$, $-CONH_2$, -COOH…). Así, son capaces de contener el agente activo en seco y liberarlo en respuesta a un ambiente húmedo, ya que la humedad provoca el hinchamiento del polímero debido a un incremento en su volumen en agua, lo que facilita la difusión del agente activo a través de la matriz polimérica (Bajpai *et al.*, 2008; Bhattarai *et al.*, 2010; Gupta *et al.*, 2002). Asimismo, los hidrogeles se pueden formular bajo diversa formas físicas, incluyendo micro- y nanopartículas, recubrimientos y películas (Bhattarai *et al.*, 2010).

En la presente Tesis Doctoral se ha trabajado con dos matrices capaces de formar hidrogeles: principalmente CS, del que ya se han descrito sus principales características y propiedades, y polialcohol vinílico.

1.3.1. Quitosano

El CS es la base de muchos materiales poliméricos y puede presentarse formando películas, recubrimientos, hidrogeles, fibras, cápsulas o nanopartículas; y encontrarse entrecruzado o no, solo o mezclado formando materiales compuestos, etc. (Dutta *et al.*, 2009; Prashanth y Tharanathan, 2007; Rinaudo, 2006).

Los mecanismos del CS implicados en la conservación de los alimentos, que permiten su uso como película comestible son varios: 1) capacidad antimicrobiana cuando se encuentra protonado; 2) antioxidante; 3) control de la transferencia de humedad entre el alimento y el medio debido a que presenta valores moderados de permeabilidad al agua; 4) reducción de la presión parcial de oxígeno por ser una excelente barrera a la transmisión de éste cuando existe baja RH; 5) control de la tasa de respiración; 6) control de la maduración enzimática en frutas por retardar la producción de etileno; 7) control de la liberación de sustancias

antimicrobianas, antioxidantes, nutrientes y aromas, y finalmente 8) inversión osmótica (Shahidi *et al.*, 1999).

Las películas obtenidas a partir de CS se caracterizan por ser transparentes, ligeramente amarillentas, de suave superficie, flexibles, cohesivas, de gran resistencia mecánica comparable a las de muchos polímeros comerciales, hidrófilas, inocuas, biocompatibles, biodegradables (Butler *et al.*, 1996; Shahidi *et al.*, 1999; Tharanathan y Kittur, 2003) y adecuadas para distintos grupos de alimentos (No *et al.*, 2007). Como otras películas basadas en polisacáridos, estas propiedades se ven mermadas cuando las películas de CS se someten a elevada RH, ya que interaccionan con el agua al ser tan hidrófilas (Dutta *et al.*, 2009; No *et al.*, 2007).

Las películas de CS se pueden obtener mediante distintos métodos (Donhowe y Fennema, 1994; Krajewska, 2005), siendo el del *casting* o extensión y evaporación del solvente el más empleado para la obtención de películas comestibles a base de biopolímeros (Campos *et al.*, 2011; Dutta *et al.*, 2009). Para ello, en primer lugar se disuelve el polímero en una disolución acuosa a pH ligeramente ácido (pH < 6.5) para después reformar la cadena polimérica en una matriz o hidrogel. Esto se logra por extensión de la solución filmógena y evaporación del disolvente, creándose enlaces de hidrógeno e hidrofílicos intercatenarios y/o entrecruzamiento electrolítico o iónico (Butler *et al.*, 1996). Estos entrecruzamientos y la presencia de los grupos hidroxilo y amino permiten al CS formar hidrogeles (Bhattarai *et al.*, 2010). Una vez evaporado el disolvente, la película generada está constituida por la sal de CS, por lo que para estabilizar la película en agua, evitar su disolución y la pérdida de sus propiedades debe ser sometida a un proceso de neutralización con una base (Vojdani y Torres, 1990). Por este motivo, las películas de CS así elaboradas pierden su capacidad antimicrobiana, ya que los grupos amino no se encuentran protonados y no interacciona con los microorganismos (Foster y Butt, 2011; Ouattara *et al.*, 2000).

Las películas de CS están siendo ampliamente estudiadas en el desarrollo de sistemas portadores y de liberación controlada de agentes activos antimicrobianos en el envasado activo de alimentos (**Anexo A**).

1.3.2. Polialcohol vinílico

El polialcohol vinílico (PVOH) es un polímero sintético e hidrófilo proveniente de la hidrólisis controlada total o parcial del poliacetato de vinilo (PVA) en presencia de un catalizador alcalino, tal como se observa en la **Figura 1.3**.

Dependiendo de la cantidad de grupos acetato capaces de ser hidrolizados, el PVOH se clasifica comercialmente como de alta (~99%), media (~88%) y baja (~78%) hidrólisis, siendo éste el porcentaje molar de unidades repetitivas con grupos hidroxilo. Este grado de hidrólisis, propiedad más importante del PVOH, define sus propiedades químicas y físicas, como son la solubilidad en agua y la actividad superficial. Dado que la solubilidad en agua es debida a los grupos hidroxilo, el grado de solubilidad se puede modificar mediante el control del grado de hidrólisis. También resulta de importancia el peso molecular del polímero, el cual se suele relacionar con la viscosidad de las soluciones acuosas que forma (Barrera *et al.*, 2007; Goodship y Jacobs, 2005). El efecto del peso molecular y grado de hidrólisis sobre diferentes propiedades del PVOH se muestra en la **Figura 1.4**.

El PVOH es un polímero inodoro y no tóxico (DeMerlis y Schoneker, 2003; Goodship y Jacobs, 2005), con un gran potencial tecnológico como material biodegradable. Se ha demostrado que varios microorganismos, ubicuos en sistemas sépticos, vertederos, compostaje o suelo, son capaces de degradar el PVOH mediante procesos enzimáticos. Una combinación de actividades oxidasa e hidrolasa, degradan el PVOH en ácido acético. En este caso, también el grado de hidrólisis y su solubilidad afecta a su tasa de biodegradación (DeMerlis y Schoneker, 2003; Dorigato y Pegoretti, 2012). Por otra parte, el PVOH se puede obtener a partir de gas natural como materia prima renovable en lugar de derivados del petróleo (Dorigato y Pegoretti, 2012).

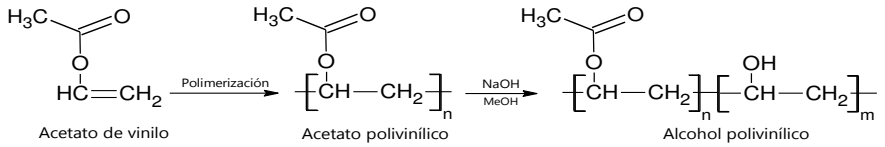


Figura 1.3. Obtención de PVOH.

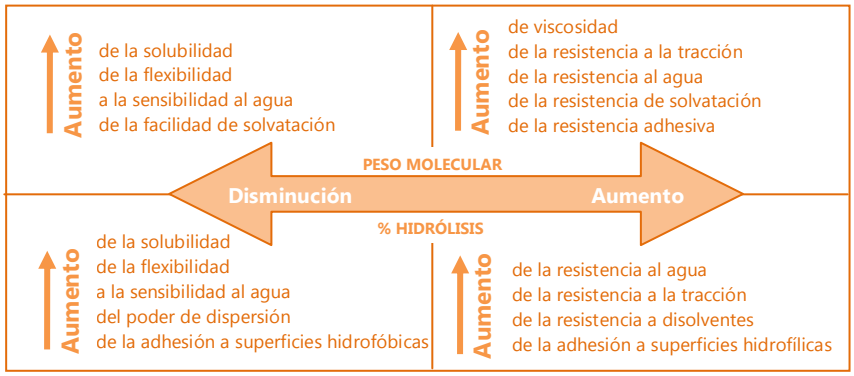


Figura 1.4. Efecto del grado de hidrólisis y peso molecular sobre propiedades de interés en el PVOH.

El PVOH es un polímero semicristalino, y posee excelentes propiedades adhesivas, emulsionantes y filmógenas. Presenta fuertes fuerzas intermoleculares creadas por puentes de hidrógeno entre los grupos hidroxilo de diferentes cadenas lo que confiere un elevado grado de cohesión y una reducción del volumen libre. Las películas de PVOH pueden ser extraídas u obtenidas por disolución en agua, *casting* o extensión y evaporación del solvente; presentan gran flexibilidad y resistencia mecánica, químicamente son resistentes a grasas, aceites, y solventes orgánicos, y solubles en agua. Las películas de PVOH son excelentes barrera al oxígeno en seco, consecuencia de su mencionada cristalinidad y cohesión interna. Sin embargo, estas propiedades dependen de la humedad, que actúa como plastificante reduciendo su resistencia mecánica y barrera al oxígeno (Dorigato y Pegoretti, 2012; Goodship y Jacobs, 2005).

Su principal uso se encuentra en la industria de plásticos, adhesivos, pinturas, textil, agricultura y farmacéutica. Se emplea como: excipiente de medicamentos; lentes de contacto blandas y emoliente oftálmico; hilo quirúrgico; bolsas para la ropa sucia industrial y hospitalaria; guantes para la protección química; etiquetas y sellos rehumedecibles; espesante de pinturas y lacas; adhesivo y apresto en telas; pelotas de golf; formador de películas para químicos, fertilizantes, herbicidas, desinfectantes, tintes, colorantes, escaladores, cosméticos, etc.

Sin embargo, debido a su difícil procesado y su extrema sensibilidad al agua, el PVOH tiene pocas aplicaciones como material de envase. Se emplea como recubrimientos en papel o películas solubles en agua, siendo esta última la principal aplicación en el campo de envases de alimentos. Este polímero está autorizado por la FDA para su uso en contacto con alimentos. El PVOH con un grado de hidrólisis de 86.5 a 89.0% está aceptado como aditivo alimentario con INS n.º 1203 (*Codex Alimentarius*) (FAO/OMS, 2004). El PVOH está aprobado como un diluyente en mezclas de aditivos colorantes para la coloración de los huevos con cáscara (21 CFR 73.1 (b) (2), para su uso en productos de embalaje de carne y en el envasado de productos de aves de corral por la División de Inspección de Carnes y Aves del USDA (DeMerlis y Schoneker, 2003).

1.4. Coadyuvantes para la obtención de las películas

En el desarrollo de sistemas de liberación sostenida a partir de hidrogeles, suelen emplearse coadyuvantes con la finalidad de conseguir una mayor capacidad de carga y modular su posterior liberación. Entre dichos coadyuvantes cabe destacar los plastificantes (Karbowiak *et al.*, 2006) y las ciclodextrinas (Hoare y Kohane, 2008).

1.4.1. Plastificantes

Los plastificantes son aditivos cuya función principal es mejorar la extensibilidad y procesabilidad de polímeros mediante la reducción de la temperatura de transición vítrea. La Unión Internacional de Química Pura y Aplicada (IUPAC) definió plastificante como una "sustancia o material incorporado en un material (por lo general un plástico o un elastómero) para aumentar su flexibilidad, manejabilidad o distensibilidad". Los plastificantes son resinas o líquidos de bajo peso molecular no volátiles, que intercalándose entre las cadenas de los polímeros forman enlaces secundarios, reducen las interacciones intermoleculares polímero-polímero y la cohesión de la matriz polimérica, proporcionando un mayor volumen libre y movilidad de las cadenas, resultando una estructura fácilmente deformable y menos frágil. Las interacciones entre el plastificante y el polímero son de carácter débil, porque algunos plastificantes pueden volatilizarse o migrar de

la matriz polimérica (Rahman y Brazel, 2004; Sothornvit y Krochta, 2005; Vieira *et al.*, 2011).

El uso de plastificantes es necesario para mejorar las propiedades mecánicas de películas formadas a partir de proteínas o polisacáridos. Los polioles (glicerol (G), etilenglicol, propilenglicol (PG), dietilenglicol y trietilenglicol) se han estudiado como plastificantes para estos polímeros biodegradables (Rahman y Brazel, 2004; Vieira *et al.*, 2011).

Entre los plastificantes más empleados destaca el G, con la ventaja de ser considerado como GRAS por la FDA y aditivo alimentario con INS n.º 422 por la GSFA. Existen numerosos estudios que lo consideran un excelente plastificante de películas hidrófilas, dada su gran capacidad para retener agua, que a su vez también actúa de plastificante universal y ubicuo. Los plastificantes en general, y el G en particular, mejoran la extensibilidad de las películas y la capacidad de tomar agua, sin embargo reducen su resistencia mecánica, elasticidad y propiedades barrera al vapor de agua, oxígeno, aromas y grasas/aceites, y migra a menudo a partir de las películas con alta sensibilidad frente a la RH (Gontard *et al.*, 1993; Rahman y Brazel, 2004; Srinivasa *et al.*, 2007). Hay autores que apuntan que otro mecanismo de acción de los plastificantes es facilitar la incorporación de agua, y que es ésta la que ejerce la acción (Karbowiak *et al.*, 2006). Sin embargo, su acción es a corto plazo puesto que el agua se evapora con facilidad (Sothornvit y Krochta, 2005). Igualmente el PG es bastante empleado como agente plastificante (Smits *et al.*, 2003; Suyatma *et al.*, 2005; Vanin *et al.*, 2005), considerado GRAS y aditivo alimentario con INS n.º 1520.

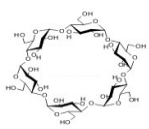
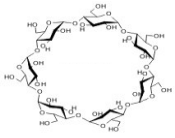
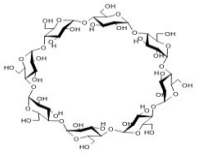
Es razonable suponer que los plastificantes empleados como coadyuvantes en biopolímeros deben ser también preferentemente biodegradables (Rahman y Brazel, 2004; Vieira *et al.*, 2011). Así, existe un gran interés en el desarrollo y mejora de plastificantes biodegradables, al igual que se desarrollan materiales biodegradables y a partir de recursos renovables. Así, tanto el G es biodegradable bajo condiciones aeróbicas y anaeróbicas de acuerdo con OECD 301 (OECD/SIDS, 2002), como el PG (OECD/SIDS, 2001).

1.4.2. Ciclodextrinas

Las ciclodextrinas (CD) son una familia de oligosacáridos cíclicos constituidas habitualmente por seis, siete u ocho unidades de *D*-glucopiranosa unidas por enlaces α-(1→4), denominándose α-CD, β-CD y γ-CD, respectivamente. Las CD se obtienen durante la degradación enzimática del almidón mediante la enzima ciclomaltodextrin-glucosiltransferasa (Del Valle, 2004; Kurkov y Loftsson, 2013; Szejtli, 1998). Las CDs presentan una estructura molecular troncocónica hueca con una cavidad interior de volumen específico. Las principales propiedades de las CDs son el diámetro de su cavidad y su solubilidad. En la **Tabla 1.2** se muestra la estructura y características de las CDs naturales.

En la molécula de CD, en torno al borde superior y más ancho, se hallan los hidroxilos secundarios de C2 y C3; en el borde inferior se halla el hidroxilo primario de C6, que es más estrecho debido a la rotación libre de este grupo que reduce el diámetro efectivo de la cavidad (Astray *et al.*, 2009; Del Valle, 2004). El átomo de oxígeno del grupo hidroxilo del C2 de la unidad glucopiranósica forma un enlace por puente de hidrógeno con el protón del grupo hidroxilo del C3 de la unidad adyacente (Szejtli, 1998). El

Tabla 1.2. Estructura química y características de las CDs naturales (Del Valle, 2004; Szejtli, 1998).

Propiedades	α-CD	β-CD	γ-CD
			
Número de unidades de glucopiranosa	6	7	8
Peso molecular (g/mol)	972	1135	1297
Solubilidad en agua a 25 ℃ (g/100mL)	14.5 ± 0.4	1.85 ± 0.4	23.2 ± 0.4
Forma del cristal	placas hexagonales	paralelogramos monoclínicos	prismas cuadrangular
Altura (Å)	7.9 ± 0.1	7.9 ± 0.1	7.9 ± 0.1
Diámetro exterior (Å)	14.6	15.4	17.5
Diámetro interior (Å)	4.7-5.3	6.0-6.5	7.5-8.3
Volumen interior ($Å^3$)	174	262	427
Hidrólisis por α-amilasa de *A. oryzae*	despreciable	lenta	rápida
Moléculas de agua en interior de cavidad	6	11	17

interior del cono está revestido por los hidrógenos de C3 y C5, y los oxígenos glucosídicos. Los pares electrónicos no compartidos de los puentes de oxígeno glucosídicos hacen que haya una elevada densidad electrónica y le confieren cierto carácter de base de Lewis al interior de la CD. Por todo ello, las CDs se caracterizan por tener una cavidad interior relativamente apolar y un exterior polar, como se muestra en la **Figura 1.5** (Del Valle, 2004; Szejtli, 1998).

Las CDs son estables en disoluciones neutras y básicas, pero se degradan lentamente en pH ácido (Del Valle, 2004; Kurkov y Loftsson, 2013). El punto de fusión de las CDs nativas no está bien definido, pero en general, comienzan a descomponerse a partir de 197 ℃ y en atmósfera inerte 252-400 ℃ (Trotta *et al.*, 2000). Debido a la presencia de estos grupos hidroxilo en los extremos de la cavidad, las CDs son solubles en agua y son insolubles en la mayoría de los solventes orgánicos comunes, y su solubilidad es el resultado de la capacidad de interacción de dichos grupos hidroxilo con el medio acuoso. En el caso de la β-CD, se establecen siete enlaces por puentes de hidrógeno formando un cinturón en la cara secundaria y origina una estructura rígida, estable y difícil de solvatar, lo que explica en cierta medida la menor solubilidad en agua (Astray *et al.*, 2009; Szejtli, 1998). La solubilidad de las CDs naturales, especialmente de la β-CD, se puede aumentar por la formación de derivados mediante ataque nucleofílico a los grupos hidroxilo (Del Valle, 2004). En el presente trabajo, se han empleado las hidroxipropil-β-CDs (HP-βCD) que presentan una mayor solubilidad en agua que su CD nativa, la β-CD.

Debido a su peculiar estructura cónica hueca de superficie externa hidrófila y cavidad interior hidrofóbica, las CDs pueden hospedar distintos compuestos formando complejos de inclusión (**Figura 1.6**). Una o dos moléculas huésped pueden ser atrapadas por una, dos o tres CDs, siendo la

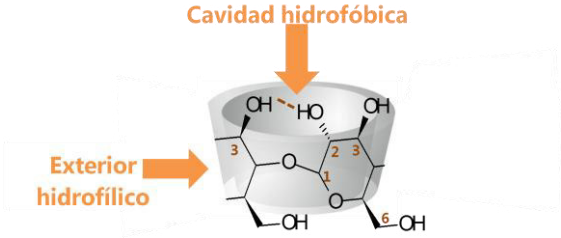


Figura 1.5. Representación esquemática de la estructura química de las CDs.

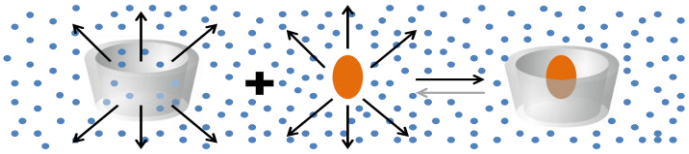


Figura 1.6. Representación esquemática de la formación de complejos de inclusión. Los puntos azules representan moléculas de agua; el óvalo naranja, la molécula hidrófoba huésped.

estequiometría más usual la 1:1. Esta inclusión puede ser completa o parcial si la molécula sustrato es de mayor dimensión respecto al interior de la CD. Por una parte, la formación del complejo de inclusión depende de la compatibilidad estérica del sustrato y la cavidad interna de la CD. Por otra, depende de las interacciones termodinámicas entre los diferentes componentes del sistema (CD, sustrato, solvente). Las principales fuerzas impulsoras de la formación de complejos son: el desplazamiento de moléculas de agua de elevada entalpía de la cavidad apolar de la CD; la formación de puentes de hidrógeno entre las moléculas de agua que salen de la CD y el resto de moléculas de agua del medio; disminución de la repulsión entre el sustrato apolar y el medio acuoso; aumento de las interacciones hidrofóbicas entre el sustrato y la cavidad de CD, y disminución de la tensión en el anillo de CD. Las fuerzas de Van der Waals, interacciones hidrofóbicas y los puentes de hidrógeno mantienen la CD y el sustrato unidos (Astray *et al.*, 2009; Del Valle, 2004; Szejtli, 1998). Cabe resaltar la estabilidad de los complejos formados por las β-CDs con los mono- y sesquiterpenos (Szente y Szejtli, 1988).

Debido a su capacidad para establecer enlaces covalentes o no covalentes con otras CDs, las CDs en disolución pueden autoagregarse y formar complejos supramoleculares como catenanos, rotaxanos, polirotaxanos y tubos (Del Valle, 2004; He *et al.*, 2008), agregados tipo micelar (Del Valle, 2004; He *et al.*, 2008; Messner *et al.*, 2010), perlas, nanofibras o nanocápsulas (Kurkov y Loftsson, 2013). Por otra parte, se pueden formar complejos ternarios polímero hidrosoluble/CD/principio activo, que pueden aumentar la estequiometría 1:1 entre la CD y el principio activo (Kurkov y Loftsson, 2013).

Las CDs han sido muy estudiadas y empleadas desde su descubrimiento en 1891 por A. Villiers (Del Valle, 2004; Kurkov y Loftsson, 2013; Szejtli, 1998). La formación de los complejos de inclusión provoca

variaciones reversibles en las propiedades fisicoquímicas del sustrato: solubilización o precipitación de principios activos, estabilización de sustancias volátiles, protección contra la oxidación, luz o calor. Por ello, las CDs y sus derivados son ampliamente empleadas en biotecnología, protección ambiental, química analítica, industria textil, etc. Y destaca su aplicación en la tecnología de los alimentos y envasado, farmacología, cosmética y aseo personal para: 1) la estabilización de aromas, vitaminas o aceites esenciales; 2) la eliminación de aromas, sabores indeseables u otros compuestos como grasas; 3) la solubilización de colorantes y vitaminas, y 4) la liberación controlada de ciertos constituyentes alimentarios (Astray *et al.*, 2009; Del Valle, 2004).

Las CDs son productos biocompatibles, seminaturales, producidos a partir de un material natural y renovable como es el almidón, por una conversión enzimática relativamente simple. Se producen en miles de toneladas por año mediante tecnologías respetuosas con el medio ambiente. Por lo que, el coste inicialmente alto de las CDs ha descendido hasta niveles aceptables para la mayoría de aplicaciones industriales (Szejtli, 1998).

Las CDs son consideradas atóxicas por vía oral. No se absorben en el tracto intestinal alto (<3%) debido a su exterior hidrofílico, sino que se degradan parcialmente debido al ácido estomacal y la mayor parte de las CDs ingeridas o los subproductos de la acción de la α-amilasa pancreática son metabolizadas por la microbiota bacteriana del colon (Kurkov y Loftsson, 2013). Entre las CDs, la β-CD (sustancia GRAS (2001), aceptada por la FAO/OMS (1995) como aditivo alimentario con INS n.º 459) es la más accesible, la más barata y, generalmente, la más utilizada en las industrias farmacéutica y alimentaria con una ingesta diaria admisible (IDA) de 5 mg/kg/día en alimentos (Del Valle, 2004; Kurkov y Loftsson, 2013). Aunque de momento, no se ha encontrado nada especificado por parte de la FDA y FAO/OMS para derivados como las HP-βCD.

1.5. Agentes antimicrobianos

En la presente Tesis Doctoral, se ha trabajado con los siguientes agentes antimicrobianos: etil-N^{α}-dodecanoil-*L*-arginato, nanopartículas de plata, y componentes de aceites esenciales, como son el carvacrol y el cinamaldehído.

1.5.1 Etil-N^{α}-dodecanoil-*L*-arginato

El monohidrocloruro de etil-N^{α}-dodecanoil-*L*-arginato, etil lauroil arginato o éster etílico de N^{α}-lauroil-*L*-arginina (LAE) es un derivado del ácido láurico, *L*-arginina y etanol, con propiedades tensioactivas y actividad antimicrobiana (Bakal y Díaz, 2005; Rodríguez *et al.*, 2004). La estructura química del LAE se muestra en la **Figura 1.7**.

Se trata de un sólido blanco, que se puede disolver en agua a 20 °C más de 247 g/kg, con un punto de fusión entre 50.5 y 58 °C y se descompone a temperaturas superiores a 107 °C. Su coeficiente de reparto aceite-agua es menor a 0.1, por lo que la molécula se localiza principalmente en la fracción acuosa, más susceptible a la contaminación microbiana. El LAE muestra estabilidad química a un pH entre 3 y 7, y mantiene su actividad antimicrobiana dentro de este intervalo (Bakal y Díaz, 2005).

Las propiedades antimicrobianas del LAE se deben a su acción como tensioactivo catiónico. De este modo, el LAE altera la membrana externa y citoplasmática de las bacterias gram negativas, y la membrana celular y el citoplasma de las bacterias gram positivas, desnaturalizando las proteínas. Estas alteraciones producen perturbaciones en el potencial y permeabilidad de membrana, la estructura celular y los procesos metabólicos, inhibiendo la proliferación celular y perdiendo la viabilidad celular (Bakal y Díaz, 2005; Rodríguez *et al.*, 2004). El LAE se caracteriza por tener un amplio espectro y elevada eficacia antimicrobiana contra bacterias gram negativas, gram positivas, hongos y levaduras, a bajas concentraciones (Bakal y Díaz, 2005; Infante *et al.*, 1997; Rodríguez *et al.*, 2004).

El LAE se metaboliza en el tracto gastrointestinal humano rápidamente dando lugar a compuestos habituales en la dieta humana, como son el ácido láurico y los aminoácidos ornitina y arginina que, a su vez, se metabolizan en CO_2 y urea. Esta característica otorga al LAE un importante grado de

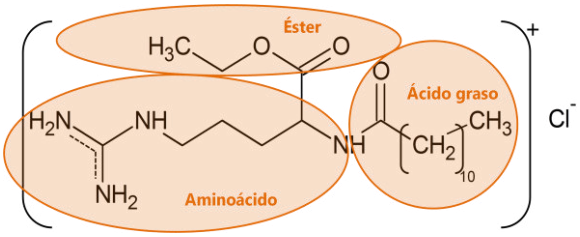


Figura 1.7. Estructura química del LAE.

seguridad, que ha sido demostrado en diferentes estudios toxicológicos desarrollados en los últimos años (Hawkins *et al.*, 2009; Ruckman *et al.*, 2004). Además, debido a su estructura simple y natural a partir de fuentes renovables -aminoácidos y aceites vegetales- es biodegradables y de baja ecotoxicidad (Infante *et al.*, 1997).

El LAE fue sintetizado por un grupo de investigación del CSIC en 1984 (Bakal y Díaz, 2005). Su preparación y aplicación están descritas como patente española (García Domínguez *et al.*, 1983) y como patente europea (Contijoch Mestres *et al.*, 2001; Urgell Beltrán y Seguer Bonaventura, 2003a, b, c). El LAE está siendo empleado como un innovador conservante en alimentación, productos farmacéuticos y cosméticos (Bakal y Díaz, 2005; Infante *et al.*, 1997). Su baja toxicidad, alta actividad antimicrobiana y el hecho de no modificar las características organolépticas del producto original, convierten al LAE en una valiosa herramienta para controlar o prevenir el crecimiento microbiano en los alimentos.

El empleo de LAE como conservante de alimentos es de hasta 225 mg/kg. El LAE ha sido declarado sustancia GRAS para su uso como agente antimicrobiano en más de 20 alimentos, incluyendo aves de corral entre otras carnes (FDA, 2005a). La EFSA (2007) ha establecido para el LAE una IDA 0 - 0.5 mg/kg de peso corporal. Más recientemente, el Comité mixto FAO/OMS (2009) evaluó el LAE (INS n.º 243), estableciendo una IDA de 0 - 4 mg/kg peso corporal. Las posibles aplicaciones de LAE son: semiconservas de carne y pescado, embutidos, bebidas no alcohólicas, zumos, quesos, legumbres y cereales procesados, platos preparados, aperitivos, postres lácteos, salsas y condimentos, rellenos y coberturas, etc.

En el **Anexo A** se recogen algunos ejemplos de bioenvases antimicrobianos con LAE estudiados en alimentos.

1.5.2. Nanopartículas de plata

El carácter bactericida y bacteriostático de la plata ha sido conocido empíricamente desde tiempos remotos. Su uso fue disminuyendo por la aparición de los antibióticos, a pesar de continuar siendo el principal tratamiento para quemaduras e infecciones cutáneas. Sin embargo actualmente, se está recuperando el uso de la plata como agente antimicrobiano debido al aumento de las resistencias de ciertos

microorganismos patógenos a los antibióticos (Rai *et al.*, 2009; Wei *et al.*, 2009).

La plata iónica posee un amplio espectro biocida, mientras que en estado metálico es inerte. Los iones de plata son capaces de unirse a los grupos funcionales donadores de electrones como fosfatos, hidroxilos, imidazoles, indoles, aminas y tioles principalmente de: 1) proteínas y enzimas, alterando la estructura y función de diferentes orgánulos de los microorganismos como la pared celular y membrana externa, por tanto la cadena respiratoria, y la permeabilidad e integridad celular; 2) ADN, ARN y proteínas ribosomales, interfiriendo en la traducción de proteínas e inhibiendo la síntesis de ATP, y 3) catalizan especies reactivas de oxígeno (Dallas *et al.*, 2011; De Azeredo, 2013; Gibbins y Warner, 2005; Rai *et al.*, 2009). Por otra parte, el empleo abusivo de éste y todos los biocidas puede llevar al desarrollo de resistencias en los microorganismos. Y se han descrito resistencias a metales pesados como la plata, aunque raras y esporádicas y en menor medida que frente a antibióticos (Rai *et al.*, 2009; Silver, 2003; Silver *et al.*, 2006). El mecanismo de acción inespecífico y con diferentes dianas de la plata, hace menos probable que los microorganismos desarrollen resistencias como ocurre con los antibióticos convencionales, ya que implicaría mutaciones simultáneas (Gibbins y Warner, 2005).

La plata iónica como agente antimicrobiano ha sido empleada tradicionalmente en forma de sal como el nitrato de plata. Sin embargo, una alternativa son las nanopartículas de plata metálica, ya que se ha comprobado que son más eficaces contra bacterias, virus y otros microorganismos eucariotas que sus homólogas de cobre, titanio, magnesio u oro (Rai *et al.*, 2009). Un nanomaterial es "un material natural, accidental o fabricado que contenga partículas, sueltas o formando un agregado o aglomerado y en el que el 50% o más de las partículas en la granulometría numérica presente una o más dimensiones externas en el intervalo de tamaños comprendido entre 1 nm y 100 nm" (UE, 2011a). Se piensa que las nanopartículas metálicas presentan un mecanismo de acción similar al de los iones de plata, pero con mayor efecto antimicrobiano debido a su elevada superficie específica y mayor penetrabilidad (Dallas *et al.*, 2011). Ciertos autores señalan como fundamental la presencia de iones plata en la superficie de la nanopartícula para ejercer su actividad antimicrobiana. Por

otro lado, las nanopartículas de plata constituyen un depósito de iones plata que pueden ser liberados con el tiempo a través de la oxidación de la plata metálica de la superficie de la nanopartícula en presencia de humedad (Gibbins y Warner, 2005; Wei *et al.*, 2009).

Algunas aplicaciones de las nanopartículas de plata se han resumido en la **Tabla 1.3**, y diferentes estudios que incorporan nanopartículas de plata en envases biopoliméricos alimentarios pueden observarse en el **Anexo A**.

Sin embargo, el uso de la nanotecnología en el sector alimentario es restringido. El riesgo potencial de los nanomateriales en la salud del hombre así como en el medio ambiente es algo desconocido (Dowling, 2004; Sharma *et al.*, 2009). Y hay que tener presente que además de estas fuentes intencionales o sistemas nanotecnológicos descritos, las nanopartículas se encuentran en: 1) las fuentes naturales como el polvo volcánico, en la mayoría de las aguas naturales (0.2–0.3 µg/L), suelos y sedimentos, alimentos (10–100 µg/kg), en menor medida en el aire (<ng/m^3), y 2) las fuentes accidentales como subproductos de combustión y cocción, emisión de vehículos, pilas de combustibles y diferentes procesos industriales (OMS, 2003; Smita *et al.*, 2012).

Actualmente no existe una legislación específica sobre la nanotecnología, salvo en algunos colorantes, filtros UV en productos

Tabla 1.3. Aplicaciones de las nanopartículas de plata (Rai *et al.*, 2009; Sharma *et al.*, 2009; Silver *et al.*, 2006). También se puede consultar: http://www.nanotechproject.org/inventories/silver/.

medicina y farmacia	• liberación sostenida a partir de materiales médicos • resinas en odontología • recubrimiento de catéteres e instrumental quirúrgico • injertos vasculares • vendajes y apósitos para quemaduras (Acticoat® by Smith & Nephew)
cosmética	• protectores solares • desodorantes
industria alimentaria	• recipientes y utensilios de cocina antimicrobianos • superficies antimicrobianas en las plantas de procesado de alimentos • preparados higienizantes de frutas y verduras (Microdyn®, Méjico) • desinfección de agua y filtros agua (ej. Brita Company®, Australia)
industria del envasado	• aplicado en pollo
industria textil	• calcetines; ropa y calzado deportivo • alfombras • textiles para hospitales
electrodomésticos	• lavadoras: Sharp®, Japón; Samsung Electronics fabricadas por Nanogist, EEUU; Daewoo Electronics®, Chile • frigoríficos: Daewoo Electronics®, fabricado por Nanogist • lavavajillas: Hitachi®, Japón
otras	• superficies antimicrobianas en inodoros (Toto®, Japón) • filtros de aire

cosméticos y medicamentos que sí tienen en cuenta el tamaño de partícula (CE, 2012). El REACH (CE, 2006) no contiene disposiciones que se refieran de manera explícita a las nanopartículas. La EFSA (2011) publicó una Guía de Orientación para evaluar los riesgos de las aplicaciones de la nanociencia y de las nanotecnologías en los alimentos y en la cadena alimentaria. Sin embargo, actualmente aún no se disponen de los resultados de esa evaluación para la regulación de la introducción de estos materiales en el mercado (Frejo *et al.*, 2011). El Reglamento 1935/2004 y el posterior 10/2011 específico para materiales en contacto con alimentos y de envasado, no incluye ninguna indicación particular para el uso de los compuestos de plata en envases y establece la prohibición de incorporar nanopartículas a materiales plásticos que estén en contacto con alimentos debido a la falta de información existente. El Reglamento 450/2009 sobre envases activos e inteligentes señala que el elemento activo tiene que ser identificado, con la información adecuada sobre los usos permitidos y su cantidad máxima. Y el Reglamento 1169/2011 sobre la información alimentaria facilitada al consumidor incluye la definición de "nanomaterial artificial" y obliga a etiquetar todos los ingredientes presentes en forma de nanomateriales artificiales. Además, señala que los nanomateriales artificiales deberán indicarse claramente en la lista de ingredientes, seguidos de la palabra "nano" entre paréntesis (Ávalos *et al.*, 2013).

La EPA (1992) y la OMS (2003, 2008) sugieren que los niveles de plata en agua potable se sitúen por debajo de 0.1 mg/L para evitar el problema de salud denominado argiria (irreversible coloración azul-grisácea de piel y mucosas). La EFSA (2006) y SCENIHR (2014), Comité Científico asesor de la CE, concluyeron que no hay problemas de seguridad para el consumidor si la migración total de iones de plata no supera el límite específico de migración de 0.05 mg/kg en alimentos ó 0.05 mg/L en agua, aunque no se especifica para la nanoplata. La plata es considerada aditivo alimentario (UE, 2011c), pero se trata de un colorante restringido a coberturas de confitería y repostería, chocolate para decoración y licores.

Sin embargo, en la actualidad la aplicación de nanopartículas de plata en materiales nanocompuestos para generar superficies antimicrobianas es de gran interés puesto que presenta varias ventajas frente a otros antimicrobianos. La incorporación de nanopartículas de plata genera

polímeros más estables puesto que no se evaporan como otros aditivos orgánicos, resisten altas temperaturas, no suelen afectar a las propiedades intrínsecas de los polímeros, les confieren un amplio espectro antimicrobiano y puede aportar propiedades conductoras a los nanomateriales.

1.5.3. Aceites esenciales y sus componentes

En las últimas décadas se ha puesto de relieve el potencial de los extractos vegetales, y en especial de algunos aceites esenciales (AE), como agentes antimicrobianos naturales. Tradicionalmente, los AE, y los componentes o derivados de éstos han sido utilizados en la industria alimentaria como aromatizantes en la preparación de bebidas y alimentos. Sin embargo estos compuestos, debido a su capacidad antimicrobiana y antioxidante presentan gran potencial como conservantes naturales atendiendo así la demanda de los consumidores por productos saludables sin aditivos artificiales añadidos (Burt, 2004; Dorman y Deans, 2000).

Los AE son sintetizados como metabolitos secundarios por algunos vegetales, como respuesta a situaciones de estrés, siendo almacenados en células secretoras, cavidades, canales, células epidérmicas o tricomas glandulares (Bakkali *et al.*, 2008; Holley y Patel, 2005). Se caracterizan por ser líquidos volátiles a temperatura ambiente (generalmente, con pesos moleculares por debajo de 300 Da), y poseer un fuerte aroma. Su densidad suele ser inferior a la del agua (excepto, los AE de sasafrás, clavo o canela). Son liposolubles y solubles en los disolventes orgánicos habituales (Bakkali *et al.*, 2008). Los AE se extraen habitualmente por el método de destilación por arrastre con vapor o extracción mecánica por presión en frío. También, se pueden emplear fluidos supercríticos, disolventes orgánicos o grasas, pirogenación o microondas, pero muchos autores consideran que al producto de extracción por estos últimos métodos no se le puede denominar AE (Bakkali *et al.*, 2008; Burt, 2004).

Generalmente, los AE poseen notables propiedades antimicrobianas frente a un amplio espectro de organismos como bacterias, hongos y levaduras, virus, insectos, plantas… Los AE más destacados por su elevada efectividad son los procedentes de clavo, orégano, romero, tomillo, albahaca, hierba limón o citronela, salvia, mostaza, canela y vainilla (Bakkali *et al.*, 2008; Burt, 2004; Holley y Patel, 2005). Sin embargo, su mecanismo de acción no

está bien definido. Considerando la gran variedad de compuestos químicos presentes en los AE, es muy probable que su actividad antimicrobiana no sea atribuible a un mecanismo específico sino a la acción combinada de varios de ellos sobre distintas localizaciones de la célula (Burt, 2004). Por una parte, la hidrofobicidad de estos AE permite su unión a los lípidos de la membrana celular y mitocondrial bacteriana, distorsionando las estructuras y haciéndolas más permeables, provocando la salida de iones y el contenido celular; por lo que, se inhibe el transporte de electrones, la translocación de proteínas o la cascada de fosforilación. Además, los componentes de los AE pueden interaccionan con las ATPasas presentes en la membrana citoplasmática (Burt, 2004; Tiwari *et al.*, 2009). Por otra parte, los componentes de los AE a bajas concentraciones afectan a enzimas para la producción energética, mientras que a concentraciones mayores pueden causar la desnaturalización proteica. Igualmente, pueden actuar sobre las proteínas de la membrana citoplasmática e incluso atravesar las porinas y acceder al periplasma de las bacterias gram negativas (Bakkali *et al.*, 2008; Burt, 2004; Dorman y Deans, 2000; Lambert *et al.*, 2001). Además, debido a este mecanismo inespecífico, no se han descrito resistencias o adaptaciones significativas a los AE a concentraciones biocidas, a diferencia de los antibióticos (Bakkali *et al.*, 2008).

Los AE son muy empleados como aromatizantes en alimentación, en cosmética y perfumería (dentífricos, loción para después del afeitado, perfumes), en productos farmacéuticos y fitosanitarios (preparados para masajes, repelente de insectos) (Burt, 2004). Asimismo, existe una gran cantidad de estudios dedicados al empleo de los AE y sus componentes como conservantes de alimentos, y como agentes activos en el diseño de envases antimicrobianos para alimentos, como se puede observar en el **Anexo A**.

Sin embargo, éstos pueden ser tóxicos si se emplean en elevadas concentraciones. Otros inconvenientes son su impacto en las propiedades organolépticas del alimento y posible rechazo a nivel sensorial por parte del consumidor, y el posible enmascaramiento de olores que se desarrollan durante la alteración de los alimentos. Por otra parte, en ocasiones, la reducción de la actividad antimicrobiana de los AE, cuando son empleados en alimentos en comparación con los resultados obtenidos *in vitro*, hace que

sea necesario el empleo de niveles superiores a los aceptados organolépticamente (Burt, 2004; Gutiérrez *et al.*, 2008; Holley y Patel, 2005). De modo que, la utilización de los AE debe ser evaluada y controlada para no alcanzar los niveles que puedan dar lugar a estos problemas. Todo ello hace que el empleo de los AE se contemple en alimentos tradicionalmente especiados y de composición sencilla o se emplee en la tecnología de barreras o *hurdle technology* (Bagamboula *et al.*, 2004; Burt, 2004; Tiwari *et al.*, 2009). Así, hay casos citados en los que los AE empleados a muy bajas concentraciones son efectivos microbiológicamente y aceptados sensorialmente (Chi *et al.*, 2006; Du *et al.*, 2012).

La FDA (2014b) en la última revisión mantiene que las especias y condimentos, y sus AE y componentes de éstos pueden considerarse sustancias GRAS (21CFR182.10, 21CFR182.20, 21CFR182.40, 21CFR182.50). En Europa, las sustancias aromatizantes ya están legisladas (CE, 2008b), y existe una lista de estas sustancias que no representan un riesgo para la salud de los consumidores (UE, 2012, 2013, 2014).

Los AE son mezclas muy complejas que presentan una gran variabilidad en calidad, cantidad y composición en función de factores climáticos y estacionales, composición del suelo, tejido del que se extrae, la edad, ciclo vegetativo y estrés de la planta... (Bakkali *et al.*, 2008; Burt, 2004). Los AE pueden componerse de más de sesenta componentes individuales; y los componentes principales pueden constituir hasta el 85%, mientras que otros componentes están presentes sólo como trazas (Burt, 2004). Esta variabilidad y heterogeneidad hacen necesario el uso de AE estandarizados o preparados, o bien componentes de AE aislados y purificados (Delaquis *et al.*, 2002).

Los constituyentes de los AE pertenecen, de manera casi exclusiva, a dos grupos caracterizados por orígenes biogenéticos distintos: el grupo de los terpenoides y el grupo de los compuestos aromáticos derivados del fenilpropano, que son mucho menos frecuentes. Dos componentes de los AE que destacan por su elevada capacidad antimicrobiana, y que han sido empleados en este trabajo, son el carvacrol y el cinamaldehído.

1.6.3.1. Carvacrol

El carvacrol (2-metil-5-(1-metiletil)-fenol), como se muestra en la (**Figura 1.8)**, es un compuesto fenólico (alcohol monoterpénico monocíclico) constituyente de diversos AE, como los de orégano (*Origanum vulgare, Thymus capitatus, Lippia graveolens*) y tomillo (*Thymus vulgaris*) (De Vincenzi *et al.*, 2004).

Se ha demostrado la bioactividad del carvacrol frente a un amplio espectro de microorganismos patógenos, incluyendo bacterias gram positivas y negativas, hongos y levaduras, parásitos (Suntres *et al.*, 2015). El modo de acción del carvacrol no se conoce completamente, pero su carácter hidrófobo permite su acumulación en la membrana citoplasmática, aumentando la fluidez y permeabilidad, desintegrando la membrana externa de bacterias gram negativas, liberando lipopolisacárido y aumentando la permeabilidad de la membrana citoplasmática al ATP. El carvacrol interactúa con la membrana celular de las bacterias gram positivas y negativas, distorsionándola e incrementando su permeabilidad. Se produce la disrupción de la fuerza protón motriz, alterando el flujo de electrones, provocando el desacople de protones y la salida de K^+, alterando el transporte activo, inhibiendo la generación de ATP y produciendo la muerte celular (Burt, 2004; Holley y Patel, 2005; Lambert *et al.*, 2001).

Según algunos autores, la estructura fenólica del carvacrol y del timol (**Figura 1.8**) es responsable de sus propiedades antibacterianas, de modo que podrían actuar como transportador de H^+ al interior celular y de K^+ al exterior. Se ha demostrado que si se encuentra bloqueado el grupo hidroxilo del grupo fenólico del carvacrol, como ocurre en el éster metílico o el anillo bencénico se encuentra saturado como en el mentol (**Figura 1.8**), su actividad disminuye considerablemente (Ben Arfa *et al.*, 2006; Burt, 2004; Holley y Patel, 2005). Sin embargo, otros autores apuntan que el grupo

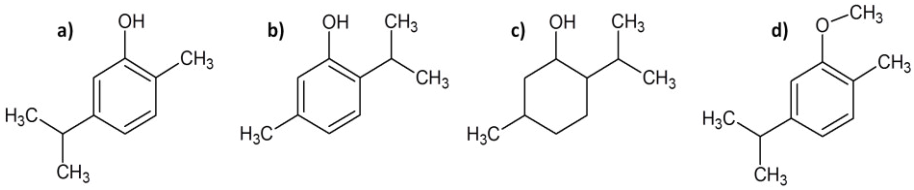


Figura 1.8. Estructuras de: a) carvacrol, b) timol, c) mentol y d) éster metílico del carvacrol.

hidroxilo sólo contribuye a esta acción biocida, pero que no es esencial (Veldhuizen *et al.*, 2006).

Como se puede observar en el **Anexo A**, son diversos los estudios que emplean el carvacrol como agente activo para la obtención de películas antimicrobianas para su aplicación en envases alimentarios. Esta gran cantidad de trabajos tiene que ver con el reconocimiento del carvacrol como sustancia segura por la FAO/OMS (2001b) (n.º de la JECFA 710), como aditivo alimentario permitido para adición directa a alimentos de consumo humano (21CFR172.515) por la FDA (2014a) y su reciente inclusión en la lista de sustancias aromatizantes (UE, 2012).

1.6.3.2. Cinamaldehído

El cinamaldehído (3-fenil-2-propenal), cuya estructura química se muestra en la **Figura 1.9** es un compuesto fenilpropanoide constituyente del AE de la corteza del árbol de la canela y otras especies de su género (*Cinnamomum verum, C. cassia*), encontrándose generalmente en un 65-95% de la composición total del AE, y dotándolo de su sabor y olor característicos (Cocchiara *et al.*, 2005; OMS, 1999). El cinamaldehído ha demostrado tener un amplio espectro de acción antimicrobiana (Bickers *et al.*, 2005).

Debido a su estructura e hidrofobicidad, el cinamaldehído puede pasar a través de las porinas de la membrana externa de las bacterias gram negativas, sin la desintegración de la membrana ni la depleción del ATP (Holley y Patel, 2005). El cinamaldehído actúa inhibiendo la producción de enzimas intracelulares, lo que provoca el deterioro de la pared (Holley y Patel, 2005). Al acceder al citoplasma puede afectar a diferentes orgánulos, como las mitocondrias (Usta *et al.*, 2002).

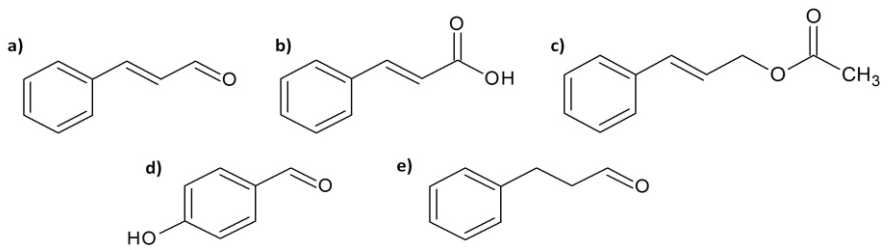


Figura 1.9. Estructura de: a) cinamaldehído, b) ácido cinámico, c) acetato de cinamilo, d) 4-hidroxibenzaldehído y e) 3-fenilpropionaldehído.

La elevada electronegatividad del grupo aldehído conjugado con un doble enlace carbono-carbono (**Figura 1.9**) parece ser la principal responsable de la actividad antimicrobiana del cinamaldehído (Dorman y Deans, 2000; Holley y Patel, 2005). Tales compuestos electronegativos pueden interferir en los procesos biológicos que involucran la transferencia de electrones y reaccionar con compuestos nitrogenados como las proteínas y ácidos nucleicos, e inhibir el crecimiento de los microorganismos (Dorman y Deans, 2000). La relevancia de este grupo aldehído se ve reflejada en que el cinamaldehído tiene mayor efecto antimicrobiano que el ácido cinámico y el acetato de cinamilo (**Figura 1.9**), mientras que la importancia de la deslocalización electrónica en la cadena carbonada más larga se demuestra porque el cinamaldehído es más eficaz que el 4-hidroxibenzaldehído y 3-fenilpropionaldehído (**Figura 1.9**) (Chang *et al.*, 2001).

El AE de canela es comúnmente utilizado en la industria de alimentos y fragancias debido a su aroma característico, pero ha sido en los últimos años cuando ha despertado un gran interés por su actividad antimicrobiana. Por ello, el cinamaldehído también se ha empleado como agente activo para la obtención de películas antimicrobianas para alimentos, como se muestra en el **Anexo A**.

El cinamaldehído fue reconocido como sustancia segura (n.º de la JECFA 656) por la FAO/OMS (2001a). La dosis sin efecto adverso observable se ha establecido en 620 mg/kg de peso corporal y día, mientas que la estimación de su ingesta como aromatizante es de 42 µg/kg de peso corporal, por lo que no existe preocupación en cuanto a la seguridad en los niveles actuales de ingestión como aditivo alimentario (FAO/OMS, 2001a). El cinamaldehído se metaboliza en el organismo a ácido cinámico y se elimina por vía urinaria como glicina, glucuronato del ácido cinámico o minoritariamente como conjugado del glutatión para formar derivados del ácido mercaptúrico (Bickers *et al.*, 2005). La FDA (2014c) considera que el cinamaldehído es sustancia GRAS para su uso como sustancia aromatizante sintética y adyuvante (21CFR182.60), y ha sido incluida en la lista de sustancias aromatizantes (UE, 2012).

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2. OBJETIVOS

2.1. Objetivo general

El objetivo principal de la presente Tesis Doctoral es el estudio del biopolímero quitosano como matriz portadora y liberadora de agentes activos para el desarrollo de películas antimicrobianas y su aplicación como envase activo antimicrobiano.

2.2. Objetivos específicos

Para lograr este objetivo general se plantean los siguientes objetivos específicos:

- Obtener películas de quitosano mediante la técnica de extensión y evaporación del solvente, con o sin coadyuvante, y en las que se incluyan diferentes agentes antimicrobianos incorporados en la matriz empleando diferentes metodologías.

- Caracterizar el efecto de la incorporación de los agentes activos, y en su caso de los coadyuvantes, en las propiedades funcionales más relevantes de las películas activas desarrolladas.

- Estudiar los diferentes mecanismos de liberación de los diversos agentes activos incorporados en las películas.

- Estudiar la capacidad antimicrobiana *in vitro* de los agentes activos y las películas obtenidas frente a microorganismos patógenos modelo.

- Evaluar la eficacia de las películas desarrolladas en alimentos reales y su posible empleo en el diseño de envases activos antimicrobianos para alimentos.

3. JUSTIFICACIÓN Y ESQUEMA DE TESIS

Actualmente, el interés por los sistemas de envasado antimicrobiano de alimentos está aumentando. Por una parte, el sistema físicamente aísla el alimento y evita la contaminación con nuevos microorganismos. Por otra, la presencia de agentes antimicrobianos permite la inhibición o retardo del crecimiento de los microorganismos. Estas acciones conjuntas permiten el control de microorganismos patógenos que pueden suponer un problema de salud pública y de microorganismos alterantes responsables del deterioro de los alimentos.

Por otra parte, se pretende el desarrollo de materiales más sostenibles a partir de recursos renovables, y a partir de excedentes y residuos de la industria alimentaria, agrícola y de la pesca. Con ello, el propósito es reducir la acumulación de desechos, aportándoles un valor añadido. Entre estos materiales destacan los biopolímeros, que pueden emplearse en el envasado activo de alimentos. Asimismo, estos biopolímeros, además de ser muchos de ellos comestibles, debido a sus características intrínsecas suelen ser "multifuncionales" a diferencia de los plásticos convencionales, que suelen ser más inertes y con ciclos de vida más largos.

Entre estos biopolímeros destaca el CS principalmente debido a su elevada disponibilidad, y su carácter no tóxico y biodegradable. Entre las diferentes propiedades del CS, es de destacar su gran versatilidad y la presencia de distintos grupos funcionales que le confieren la capacidad de desarrollar diferentes sistemas portadores y liberadores de agentes activos. Asimismo, este material "pseudonatural" de naturaleza polisacárida y comestible tiene capacidad filmógena, de manera que puede aplicarse como película o recubrimiento en el envasado activo de alimentos. Además, cuando sus grupos amino se encuentran protonados el CS tiene propiedades quelantes y antimicrobianas.

La incorporación de agentes antimicrobianos en recubrimientos y películas para el envasado de alimentos, así como en las superficies de procesado de alimentos, presenta ventajas frente a su aplicación directa sobre el alimento o en su seno: 1) se reduce la pérdida de agente activo por neutralización, interacción con componentes del alimento o dilución en el seno del alimento; 2) presenta una elevada superficie de contacto, debido a que la contaminación microbiana se produce principalmente en la superficie de los alimentos; 3) el contacto del agente activo y la superficie del alimento

se produce en el envasado de éste, después de una potencial contaminación de la superficie por la manipulación del alimento, lo que se suma a la acción protectora del envase que evita posteriores contaminaciones, y 4) se pueden incorporar al envase sustancias activas lábiles, que entrarían en contacto con el alimento durante el envasado, que puede ser posterior al tratamiento agresivo del alimento. De esta manera, se pueden adicionar menores cantidades de antimicrobiano, reducir el posible impacto en las propiedades organolépticas del alimento, además de aumentar la seguridad microbiológica del producto y su aceptación por el consumidor.

En la actualidad, se están desarrollando sistemas de envasado basados en matrices poliméricas, naturales o artificiales, que incorporan agentes activos y que mediante fenómenos de transferencia de masa se pueden ceder de manera gradual y controlada a la superficie del alimento durante el tiempo de almacenaje y distribución (Coma, 2008). El dispositivo activo puede formar parte de la pared del envase o bien estar incluido en la etiqueta, tapa, en forma de almohadilla, separadores, pero siempre evitando que sea apreciado por el consumidor como un elemento extraño, o que pueda ser ingerido accidentalmente. Por otro lado, es fundamental tener presente el mecanismo por el cual se produzca la liberación del agente activo por parte del polímero cuando sea requerido, normalmente durante el almacenamiento del producto envasado. Son varios los factores que pueden desencadenar la actividad del polímero.

El agente activo puede ser no volátil o volátil, en el primer caso es necesario el contacto del polímero con el alimento. Mientras que los agentes activos volátiles pueden liberarse al espacio de cabeza del envase sin la necesidad de que exista contacto directo, lo cual es muy adecuado a la hora de envasar alimentos sólidos. Cabe decir que existen otros tipos de polímeros antimicrobianos, aquellos cuya capacidad antimicrobiana es inherente debido a su estructura química, o polímeros que han sido modificados y en los cuales se ha inmovilizado de forma irreversible la molécula antimicrobiana. En la presente Tesis Doctoral se han desarrollado sistemas activos basados en matrices de CS capaces de liberar agentes antimicrobianos de diferente naturaleza: 1) un sólido, como el LAE, previamente incorporado a la formación de la película; 2) iones plata que se liberan desde la matriz y proceden de nanopartículas de plata creadas

previamente durante el proceso de formación de la película; 3) volátiles anclados de forma reversible una vez obtenida la película, y 4) volátiles incorporados en la película tras su procesado. De acuerdo con ello, la Tesis se ha dividido en cuatro capítulos.

El **capítulo 1** titulado **Desarrollo de películas de quitosano con etil-*N*$^{\alpha}$-dodecanoil-*L*-arginato y su aplicación en el envasado activo antimicrobiano de alimentos** consta del artículo científico: "**Development of a novel antimicrobial film based on chitosan with LAE (ethyl-*N*$^{\alpha}$-dodecanoyl-*L*-arginate) and its application to fresh chicken"**. En este trabajo se han desarrollado películas de CS mediante la técnica de extensión y evaporación del solvente, e incorporando LAE al 1, 5 y 10%. Su liberación se monitorizó en un simulante alimentario acuoso a 4 y 28 ºC. Previamente al estudio de la actividad antimicrobiana de las películas, se determinó la concentración mínima inhibitoria y la concentración mínima biocida del agente antimicrobiano frente a determinados microorganismos modelo (bacterias, hongos y levaduras) en medio líquido y sólido. Finalmente, se evaluó la eficacia de las películas en el envasado de pechugas de pollo fileteadas, estudiándose su actividad frente a bacterias mesófilas, psicrófilas, *Pseudomonas* spp., coliformes, ácido-lácticas, sulfuro-productoras, hongos y levaduras tras 2, 6 y 8 días de almacenamiento en condiciones de refrigeración.

El **capítulo 2: Desarrollo de películas híbridas de quitosano con nanopartículas de plata formadas *in situ***, comprende el artículo científico denominado **"Silver ions release from antibacterial chitosan films containing *in situ* generated silver nanoparticles"**. En este capítulo se hace uso del conocimiento sobre el empleo de nanopartículas de plata como agentes portadores y liberadores de iones plata, las propiedades acomplejantes y la capacidad reductora del polímero CS. Con ello, se desarrolla una metodología en la que las nanopartículas son creadas *in situ* en la película de CS. El nitrato de plata se emplea como precursor de las nanopartículas, incorporándose en la disolución filmógena. Las nanopartículas se obtienen durante la neutralización de las películas de acetato de CS con hidróxido de sodio. La ligera capacidad reductora y

quelante de iones del CS, sumadas a la acción del anión hidróxido, favorecen la reducción de la plata y la formación de las nanopartículas sin tener que recurrir a reductores fuertes y más tóxicos. Así, se añadieron a la disolución de CS diferentes concentraciones de nitrato de plata empleado como precursor (0.1, 0.2, 0.5, 1 y 1.5% de plata). Las películas elaboradas por extensión y evaporación del disolvente, se neutralizaron con hidróxido de sodio a dos temperaturas (22 y 37 ºC) para determinar el efecto de la temperatura de neutralización en la forma y tamaño de las nanopartículas. La evidencia de la formación de nanopartículas de plata se evaluó mediante microscopía electrónica de transmisión (TEM), espectroscopía UV-visible y difracción de rayos X. Las películas nanocompuestas se caracterizaron mediante la determinación de su color y análisis termogravimétrico (TGA). Se determinó la actividad antimicrobiana de las películas frente a *Escherichia coli* y *Staphylococcus aureus* en medio de crecimiento líquido diluido y sin diluir. Asimismo, se estudió si la actividad antimicrobiana de las películas se mantenía a lo largo del tiempo para demostrar si dichas nanopartículas podrían actuar como nanoreservorios de iones plata de liberación sostenida. La liberación se cuantificó mediante espectrometría de absorción atómica de horno de grafito (GFAAS).

El **capítulo 3: Desarrollo de películas de quitosano con compuestos volátiles antimicrobianos anclados mediante un enlace covalente reversible y su aplicación en el envasado activo de alimentos** está constituido por el artículo científico **"Reversible covalent immobilization of cinnamaldehyde on chitosan films via Schiff base formation and their application in active food packaging"**. En este capítulo, se desarrollan películas de CS en las que se encuentra anclado el compuesto volátil cinamaldehído a través de la formación de una base de Schiff, creando un enlace covalente, el cual es reversible mediante hidrólisis permitiendo la liberación de cinamaldehído. La formación de la base de Schiff se llevó a cabo en una disolución etanólica acidificada de cinamaldehído en la que se introducen las películas de CS neutralizadas obtenidas por extensión y evaporación del solvente. De todos los estudios realizados anteriormente, se eligieron las condiciones de trabajo (tiempo, temperatura, concentración) en las que se obtuvo un mayor rendimiento de

la reacción cuantificado mediante análisis elemental. La evidencia del anclaje se confirmó mediante espectroscopía de infrarrojos por transformada de Fourier con reflexión total atenuada (ATR-FTIR). Se procedió a la caracterización de las películas en relación a algunas de sus propiedades funcionales como sorción de agua, ángulo de contacto y propiedades ópticas. Se estudió el efecto de la temperatura simulando diferentes tratamientos térmicos de conservación en la liberación del volátil en medio líquido. Se determinó la capacidad antimicrobiana de las películas en ensayos *in vitro* en medio líquido frente a dos modelos bacterianos *Escherichia coli* y *Staphylococcus aureus*. Finalmente se validó su aplicación como sistema activo en leche pasteurizada inoculada con *Listeria monocytogenes* durante su almacenamiento en condiciones de refrigeración, y su aceptación sensorial.

El **capítulo 4** denominado **Desarrollo de películas de quitosano con hidroxipropil-β-ciclodextrinas y otros agentes coadyuvantes, con adaptable capacidad de sorción y liberación de compuestos volátiles antimicrobianos y su aplicación en el envasado activo de alimentos** consta de cuatro artículos científicos. En este capítulo se estudia cómo la presencia de CD junto con plastificantes y humedad pueden modular la capacidad de sorción de volátiles antimicrobianos naturales procedentes de AE en películas de CS. En este capítulo también se estudia la liberación de dichos compuestos simulando el espacio de cabeza de un envase y diferentes RH, y finalmente la aplicación de uno de los sistemas generados.

En el primer artículo científico de este apartado, **"Preparation and characterization of chitosan/HP-β-cyclodextrins composites with high sorption capacity for carvacrol"**, se detalla el desarrollo de las películas compuestas a partir de materiales procedentes de recursos renovables y con carácter biodegradable como son el CS y las CD. Para ello, se fija el contenido en HP-βCD respecto al CS en 1:1 (p/p) y se varía el contenido en G (0, 20 y 35% (g/100 g de CS). Las películas se obtuvieron por extensión y evaporación del disolvente y se caracterizaron físico-químicamente (propiedades ópticas, mecánicas, barrera a gases y vapores, sorción de vapor de agua, morfológicas y TGA). Tras la caracterización se estudió el efecto de las HP-βCD, G y contenido en agua en la capacidad de sorción de carvacrol,

para ello las muestras se acondicionaron a diferentes RH (53, 75 y 90%) y se sumergieron en el líquido volátil a 23 ºC hasta alcanzar el equilibrio de sorción. El contenido en carvacrol se determinó mediante desorción térmica acoplada a cromatografía de gases.

Asimismo, se determinó la capacidad antimicrobiana en fase vapor de todas las películas formuladas después de su inmersión en carvacrol frente a *Escherichia coli* y *Staphylococcus aureus*, así como su capacidad antibacteriana en el tiempo en función del carvacrol remanente en la película. Por otro lado, se evaluaron las cinéticas de sorción y desorción de las películas que retuvieron un mayor contenido en carvacrol. Así como el efecto de la cantidad y tipo la dextrina incorporada a la formulación en la capacidad de sorción de carvacrol. Todo ello se recoge en el artículo científico: **"Incorporation of hydroxypropyl-β-cyclodextrins into chitosan films to tailor loading capacity for active aroma compound carvacrol"**.

"Antimicrobial packaging of chicken fillets based on the release of carvacrol from chitosan/cyclodextrin films" constituye el tercer artículo de este capítulo. En él, la formulación que presentó una mayor sorción de carvacrol se incluyó en un sistema de envasado de filetes de pollo fresco para el control del crecimiento microbiano. Para ello, los filetes se envasaron en barquetas de polipropileno/copolímero de etileno y alcohol vinílico (PP/EVOH/PP) selladas con una tapa de aluminio autoadhesivo. Diferentes tamaños de la película desarrolladas se adhirieron en la cara interna de la tapa y se almacenaron a 4 ºC durante 9 días. Se estudió la cantidad de carvacrol presente en la bandeja, tapa, espacio de cabeza y alimento, así como el carvacrol remanente en la película. Se evaluó la capacidad antimicrobiana del envase frente a microorganismos endógenos (bacterias mesófilas, psicrófilas, *Pseudomonas* spp., Enterobacteriaceae, ácido-lácticas, hongos y levaduras) presentes en el pollo. Dichos estudios se llevaron a cabo el día en el que se envasaron las pechugas, y tras 3, 6 y 9 días de almacenamiento en condiciones de refrigeración.

En la cuarta publicación del presente capítulo titulada **"Effect of HP-β-cyclodextrins and coadjuvants on the sorption capacity of hydrophilic polymer films for naturally occurring antimicrobial monoterpene alcohols"** se trabaja con las películas que mayor capacidad de sorción de carvacrol han mostrado, éstas son CS con HP-βCD plastificadas con 35% G y

acondicionadas a 75% RH, y se estudia el efecto de la estructura química en la sorción de otros monoterpenos cíclicos y acíclicos, y monoterpenos fenólicos de origen natural presentes en AE (*m*-cumenol, *o*-cumenol, carvacrol, guayacol, isoeugenol, mirtenol, nerol, carveol, dehidrocarveol e isopulegol). Previamente a los estudios de sorción se determinó la actividad antimicrobiana *in vitro* en fase vapor de todos los compuestos frente a *Escherichia coli* y *Staphylococcus aureus*. Se estudió el efecto del polímero y del plastificante empleado en la capacidad de sorción, para ello se eligió el polímero PVOH, y PG como plastificante, siempre manteniendo el mismo contenido en humedad que en las películas de CS.

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4. CAPÍTULOS

4.1. Capítulo 1. Desarrollo de películas de quitosano con etil-*N*$^{\alpha}$-dodecanoil-*L*-arginato y su aplicación en el envasado activo antimicrobiano de alimentos

4.1.1. Estado de la cuestión

El LAE es una novedosa molécula empleada como aditivo conservante que ha sido aceptada por diferentes instituciones sanitarias oficiales. El LAE es un tensioactivo que destaca por su elevada efectividad a bajas concentraciones y amplio espectro antimicrobiano. Este compuesto es metabolizado por el organismo, generando arginina y ornitina, por lo que se considera un producto seguro que no presenta toxicidad en humanos. El LAE es hidrófilo, estable y activo a temperaturas por debajo de 107 ℃ y pH comprendido entre 3-7. Éste puede ser aplicado en diferentes alimentos sin alterar sus propiedades organolépticas.

En los últimos años, se ha demostrado la elevada efectividad del LAE frente a microorganismos patógenos y alterantes, siendo mayoritariamente aplicado en la superficie de productos cárnicos. Así, Bakal y Díaz (2005) demostraron la efectividad del agente activo frente a patógenos como *Listeria monocytogenes* y a bacterias ácido-lácticas alterantes de las características organolépticas en jamón curado y cocido. El conservante fue empleado en solución acuosa para la inmersión de los productos, el resultado fue un aumento de su vida útil en condiciones de refrigeración. El efecto antimicrobiano del LAE frente a *L. monocytogenes* también ha sido estudiado, pulverizándolo en una solución acuosa en la superficie del alimento o del envase justo antes del envasado al vacío de diversos alimentos como jamón (Luchansky *et al.*, 2005; Stopforth *et al.*, 2010; Taormina y Dorsa, 2009a) o salchichas (Martin *et al.*, 2009; Porto-Fett *et al.*, 2010; Taormina y Dorsa, 2009b) conservados a baja temperatura. Sommers *et al.* (2010) testaron el LAE en salchichas frente a otros patógenos como *Salmonella* y *Staphylococcus aureus*, además de frente a *L. monocytogenes* y posteriormente en *L. innocua* (Sommers *et al.*, 2012). Benli *et al.* (2011) estudiaron su efectividad en carcasas de aves frente a *Salmonella enterica*. Asimismo el LAE se muestra eficaz en lácteos y derivados. Woodcock *et al.* (2009), Soni *et al.* (2010), Soni *et al.* (2012), Serio *et al.* (2012) y Ma *et al.* (2013) probaron la eficacia del LAE en productos lácteos. El LAE también se

ha probado en zumo de manzana como coadyuvante para la reducción del tiempo y la temperatura aplicados en la pasteurización del zumo por pulsos eléctricos. Por otra parte, el LAE se ha utilizado en la conservación de productos del mar. Por ejemplo, Guo *et al.* (2013) recubrieron gambas cocidas con CS incorporando LAE antes de su congelación para el control del crecimiento de *Listeria*. Kang *et al.* (2014) y Soni *et al.* (2014) añadieron LAE sobre salmón ahumado conservado bajo vacío y refrigeración como listericida. Incluso ya existen patentes en las que se incluye el LAE en una mezcla para la desinfección de superficies de frutas, verduras o piezas de carne (Coughlin, 2010).

No obstante, pocos trabajos se han encontrado en los que el antimicrobiano sea aplicado en pollo. Sharma, Ates, Joseph, Nannapaneni*, et al.* (2013), Sharma, Ates, Joseph, Soni*, et al.* (2013) y Oladunjoye *et al.* (2013) emplearon el LAE en pechugas y carne picada de pollo o pavo para la reducción del crecimiento de *Salmonella*. El LAE también redujo el crecimiento de *Campylobacter jejuni* en pechugas de pollo (Nair *et al.*, 2014). En muchos de estos estudios, la concentración probada de LAE no disminuyó satisfactoriamente el crecimiento de la carga microbiana, por lo que se recurre a la estrategia de combinar distintos agentes antimicrobianos. Otra estrategia sería emplear el LAE como agente antimicrobiano incorporado en materiales poliméricos que permitan su liberación sostenida en la superficie del alimento y su empleo en el diseño de envases activos. En este sentido, ya se han nombrado las ventajas de incorporar el agente antimicrobiano en el material que conforma el envase respecto a su aplicación directa en la superficie del alimento. Sin embargo, la información disponible sobre la aplicación de LAE en películas poliméricas y la evaluación de su efectividad en alimentos es limitada. Muriel-Galet *et al.* (2012) han demostrado con éxito la efectividad de incorporar LAE en películas de EVOH, éstas inhibieron el crecimiento de *L. monocytogenes* y *Salmonella enterica* en una leche infantil almacenada en condiciones de refrigeración. Theinsathid *et al.* (2012) aplicaron un recubrimiento de PLA con LAE en jamón cocido loncheado confirmando su actividad antimicrobiana frente a *L. monocytogenes* y *Salmonella* Typhimurium. Jin *et al.* (2013) desarrollaron exitosamente recubrimientos de PLA o CS con LAE para la cáscara de huevo y frente a *Salmonella*. Posteriormente, se ha desarrollado una película de PLA

recubierta de CS incorporando LAE y un recubrimiento de CS con LAE siendo eficaces frente a *Listeria* y *Salmonella* en derivados cárnicos (Guo, Jin, Wang, *et al.*, 2014; Guo, Jin y Yang, 2014). Otero *et al.* (2014) desarrollaron una película de politereftalato de etileno recubierta con LAE y demostraron su capacidad para inhibir el crecimiento de *Escherichia coli* O157:H7 en queso. En un trabajo posterior de Muriel-Galet *et al.* (2015) aplicaron películas de EVOH con LAE en caldo de pollo y derivados de pescado demostrando su eficacia frente a la carga total microbiana y bacterias patógenas inoculadas previamente. Además, existen algunas patentes que incluyen el LAE como agente antimicrobiano en matrices extruidas para su aplicación en productos alimenticios como carne roja fresca (Ebner, 2012).

Sin embargo, debido a la escasez de trabajos existentes en relación a la aplicación de LAE en películas de CS, uno de los objetivos de esta Tesis Doctoral, fue estudiar el comportamiento de este biopolímero como matriz portadora de dicho agente activo. Cabe decir que el empleo de películas de CS incorporando el tensioactivo LAE, ambos de carácter catiónico, evita interacciones electrostáticas, acomplejación, posible formación de precipitados en la solución formadora de película, además de la pérdida de actividad antimicrobiana de la película resultante debido a la incapacidad de liberar el LAE, aunque estas hipótesis deben de ser demostradas. En este sentido, se han realizado diversos estudios con polisacáridos aniónicos y LAE en solución acuosa observándose una pérdida de la actividad antimicrobiana de este último (Loeffler *et al.*, 2014) y formación de agregados insolubles dependiendo del tipo de biopolímero (Bonnaud *et al.*, 2010).

En la presente Tesis Doctoral se han obtenido películas de CS y LAE, estudiado su liberación y eficacia sobre un alimento cárnico fresco como son los filetes de pechuga de pollo, ya que son muy limitados los estudios realizados sobre la eficacia del LAE en este tipo de producto.

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4.1.2. Artículo científico 1.

Development of a novel antimicrobial film based on chitosan with LAE (ethyl-N^{α}-dodecanoyl-*L*-arginate) and its application to fresh chicken

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Resumen

Se han desarrollado películas de quitosano (CS) con el agente antimicrobiano etil-N^{α}-dodecanoil-*L*-arginato (LAE) para su aplicación en el envasado de alimentos. Las películas fueron obtenidas por extensión y evaporación de la solución filmógena que contenía 1, 5 o 10% de LAE y 20% de glicerol. Se determinaron las propiedades ópticas, la liberación del LAE y la capacidad antimicrobiana de las películas desarrolladas. También se determinó la concentración mínima inhibitoria (MIC) y la concentración mínima biocida (MBC) del LAE. Se obtuvieron películas de CS con LAE transparentes y uniformes, sin discontinuidades ni partículas visibles, y sin diferencias visuales con las películas de CS sin LAE. En contacto con un simulante alimentario acuoso, este agente se liberó completamente en unas pocas horas a 4 y 28 ºC siguiendo un comportamiento fickiano. La actividad antimicrobiana de las películas frente a mesófilos, psicrófilos, *Pseudomonas* spp., coliformes, bacterias ácido-lácticas y bacterias sulfuro-productoras, hongos y levaduras fue evaluada a los dos, seis y ocho días para su aplicación en pechugas de pollo fileteadas. Las películas fueron activas frente a bacterias, hongos y levaduras, en medio líquido y sólido. Las películas de CS originaron una reducción logarítmica entre 0.47-2.96, mientras que las películas CS-5%LAE produjeron 1.78-5.81 reducciones logarítmicas. Estos resultados indican que la incorporación de LAE en sistemas de envasado basados en CS contribuye relevantemente a la capacidad antimicrobiana para mejorar la estabilidad de los productos de aves de corral frescos.

Palabras clave

Películas de quitosano, LAE, envasado antimicrobiano, envasado activo, liberación de agentes antimicrobianos, envasado de aves de corral.

Abstract

Chitosan (CS) films incorporating the antimicrobial compound ethyl-N^{α}-dodecanoyl-*L*-arginate (LAE) were developed for food packaging applications. Cast chitosan films were made with 1, 5 or 10% LAE and 20% glycerol in the film forming solution. Optical properties, release of LAE and antimicrobial activity of developed films was determined. The minimum inhibitory concentration (MIC) and the minimum biocide concentration (MBC) of LAE were determined. CS films with LAE were transparent and uniform, without discontinuities or visible particles and no visual differences could be perceived between CS and CS-LAE films. When in contact with an aqueous food simulant, the agent was fully released following a Fickian behavior in a few hours at 4 and 28 ℃. Antimicrobial activity of films against mesophiles, psychrophiles, *Pseudomonas* spp., colifoms, lactic acid bacteria, hydrogen sulfide-producing bacteria, yeast and fungi, was evaluated at two, six and eight days for its application on chicken breast fillets. Films were active against bacteria, yeasts and fungi in liquid and solid media. CS films evidenced antimicrobial activity in the range 0.47-2.96 log reductions, while CS-5%LAE film produced 1.78-5.81 log reduction. Results highlighted that LAE incorporation in a chitosan-based packaging structure may provide a relevant antimicrobial activity that could improve the stability of fresh poultry products.

Keywords

Chitosan films, LAE, antimicrobial packaging, active packaging, antimicrobial release, poultry packaging.

1. Introduction

The incidence of foodborne diseases associated with microbial pathogens is widespread and represents a threat to public health, and a challenge for the food industry (López-Carballo *et al.*, 2008). Considerable efforts are underway to find effective treatments to control recontamination of meat and poultry products to enhance their safety and quality. In this sense, applications of antimicrobial films and coatings to food have received considerable attention in recent years because they can act as protective barriers against microbiological contamination (Cagri *et al.*, 2004; Suppakul *et al.*, 2003). Direct addition of antimicrobial agents into meat formulations may result in partial inactivation of the active substances by interaction with product constituents and is, therefore, expected to have only limited effect on surface microbiota (Siragusa *et al.*, 1999; Torres *et al.*, 1985). Also, direct application of antimicrobial agents onto meat surfaces by dipping or spraying, has been shown to be inefficient, due to the rapid diffusion of the active substances within the bulk of food (Ouattara *et al.*, 2000b; Siragusa *et al.*, 1999; Torres *et al.*, 1985). In contrast, an antimicrobial active film can be developed to deliver a continued and gradual release of an antimicrobial agent during the storage and distribution of food packaging, thus providing an effective prevention of surface contamination of foods (Aymerich *et al.*, 2008; Marcos *et al.*, 2007). In this case it is possible to reduce the amount of active agent required, satisfying the demand of consumers for fewer additives.

One of the most innovative antimicrobial agent is ethyl-N^{α}-dodecanoyl-*L*-arginate hydrochloride (LAE). It is a synthetically derivative of lauric acid, *L*-arginine and ethanol (Infante *et al.*, 2004; Infante *et al.*, 1997; Ruckman *et al.*, 2004), which is notable for its antimicrobial effectiveness resulting from its chemical structure and surfactant properties (Brandt *et al.*, 2010; Pinazo *et al.*, 1999; Rodríguez *et al.*, 2004). LAE's antimicrobial properties are due to its action as cationic surfactant on cytoplasmic membrane and the outer membrane of Gram-negative, and cell membrane and cytoplasm of Gram-positive denaturation proteins. These changes produce disturbances in membrane potential, resulting cell growth inhibition and loss of viability (Infante *et al.*, 1997; Kanazawa *et al.*, 1995; Luchansky *et al.*, 2005; Rodríguez *et al.*, 2004; Tattawasart *et al.*, 2000).

LAE is characterized by a broad spectrum (Infante *et al.*, 1997) and high antimicrobial efficiency against Gram-negatives, Gram-positives, fungi and yeasts, with a low-dose application (Infante *et al.*, 1984; Rodríguez *et al.*, 2004). In addition, LAE has a low oil–water equilibrium partition coefficient (K_{OW} b 0.1), which means that it tends to concentrate in the aqueous phase, where most bacterial action occurs (Ruckman *et al.*, 2004). On top of that, LAE shows chemical stability and antimicrobial activity in a range of pH 3-7 (Asker *et al.*, 2011). With these properties, LAE used in lower concentrations than other agents presents a significant advantage over other food preservatives created for the same applications on the market (Asker *et al.*, 2011; Weiss *et al.*, 2007).

LAE is primarily and rapidly metabolized and hydrolyzed chemically in the human body to natural endogenous compounds present in the human diet (i.e. the amino acid arginine, which in turn is metabolized to CO_2 and urea, and lauric acid and ornithine) (Asker *et al.*, 2011; Ruckman *et al.*, 2004). This property gives LAE an important degree of security. As a result, LAE has been classified as GRAS (generally recognized as safe) and approved for food safety and quality; the USDA (United States Department of Agriculture) has approved its use in meat and poultry products, but is currently not approved in dairy products (OMS, 2009; Theinsathid *et al.*, 2012). To date, the use of LAE as an antimicrobial agent in food products has been well reported by different studies (Luchansky *et al.*, 2005; Martin *et al.*, 2009; Soni *et al.*, 2010; Taormina and Dorsa, 2009).

Thus due to its low toxicity, low modification of food organoleptic characteristics and considerable antimicrobial activity, LAE is becoming a product widely used in the field of conservation of pharmaceuticals, cosmetics and food. However, limited information is available related to its antimicrobial activity when LAE is applied via packaging system (Bonnaud *et al.*, 2010).

CS, a biodegradable and biocompatible polymer obtained from biomass with excellent film-forming properties, has attracted great interest for technological applications in several areas such as pharmacy, medicine, agrochemistry and packaging. The hydrophilic nature of CS has aroused interest as a sustained carrier when the release of the retained active compound is required under a moisture environment.

The aim of this study, therefore, was to prepare and characterize LAE-CS antimicrobial films for food packaging applications. LAE release from CS films was also assessed. The efficacy of the antimicrobial films was monitored both by *in vitro* microbiological tests and by performing storage trials on samples of chicken breast fillets.

2. Materials and methods

Chitosan (CS), acetic acid and glycerol (G) were supplied by Sigma (Barcelona, Spain). LAE (69.3% purity) in maltodextrin (commercialized as Mirenat-D) was gently provided by Vedeqsa Grupo LAMIRSA (Barcelona, Spain).

2.1. Films preparation

First, a 1.5% CS (w/w) solution in an aqueous 0.5% (w/w) acetic acid solution was prepared and filtered to eliminate impurities. LAE (0.1443 g of Mirenat-D) was diluted in 50 mL of Milli-Q water to obtain a stock of 2000 µg/mL. Different amounts of this stock were added to the film forming solution to obtain films with 1, 5 or 10% LAE (g of LAE/100 g of dry CS). Considering purity, actual LAE concentrations were 0.693%, 3.465% and 6.930%, respectively. Also solutions without LAE were prepared and used to produce control films. All films were plasticized with 20% G (g G/100 g of dry CS). Films were prepared by casting on a flat polystyrene (PS) tray under controlled environmental conditions (36 h, 40.0±1.5 ℃ and 20±9% RH).

2.2. Optical properties

The colour of the CS films was measured with a CR-300 Minolta Chroma meter® (Minolta Camera Co., Ltd., Osaka, Japan). Film samples were placed on a white standard plate; results were expressed in accordance with the CIELAB system with reference to illuminant D65 and a visual angle of 10°. The measurements were performed through a 6.4-mm-diameter diaphragm containing an optical glass, monitoring L*, a*, b*, chroma (C^*_{ab} = $(a^{*2} + b^{*2})^{1/2}$), hue (h_{ab} = arctan (b* / a*)) and total colour difference (ΔE_{ab}^* = $((\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2)^{1/2}$). Tests were done in triplicate in eight different positions for each film sample.

2.3. LAE release from films

Samples of 1 cm^2 of CS film with 1 (average weight 0.0110±0.0005 g), 5 (0.0115±0.0005 g) and 10% (0.0120±0.0005 g) of LAE previously measured in weight and thickness (55±5 µm) were immersed in 10 mL of MilliQ water and conditioned at 28 ºC or 4 ºC. The amount of LAE released from film was determined by using an Agilent 1100 HPLC equipped with a UV detector (204.16 nm) using a Zorbax Eclipse XDB® C18 column, 4.6 × 150 mm, 5 µm (Agilent, Barcelona, Spain), a mobile phase of acetonitrile/water acidified with TFA (0.1%) (50:50), at 1.0 mL/min and 10 µL injection volume. All samples were conditioned at 28±1 ºC before injection.

2.4. Strains

The microorganisms tested were *Escherichia coli* CECT (Spanish Type Culture Collection, Valencia) 434, *Staphylococcus aureus* MIM (Microbiologia Industriale, Milano) 178, *Listeria monocytogenes* CECT 934, *Salmonella enterica* CECT 4300 and *Pseudomonas putida* ATCC (American Type Culture Collection) 12633; *Candida utilis* CCY (Czeck Collection of Yeast) 29.38.1, *Saccharomyces cerevisiae* var. *ellipsoideus* NCYC (National Collection of Yeats Cultures) 2959 and *Torulopsis pinus* IMAP (Istituto Microbiologia Agraria Perugia) 4543; *Aspergillus niger* MIM 28, *Penicillium chrysogenum* MIM 29 and *Cladosporium cladosporioides* MIM 259.

Tryptic soy agar (TSA) and malt extract agar (MEA) were employed as solid media, and peptone water (PW, 0.1%), tryptone soy broth (TSB) and malt extract broth (MEB) as liquid media. All media were supplied by Scharlab, Barcelona, Spain.

Bacteria strains were stored in TSB with 20% glycerol at -80 ºC until needed. For experimental use, stock cultures were maintained by regular subculture on TSA slants at 4 ºC and transferred monthly. Yeast and fungi strains were stored in MEB with 25-30% glycerol at -20 ºC. For experimental use, stock cultures were maintained by regular subcultures on MEA slants at 4 ºC and transferred monthly.

Cell suspension of O.D. (600 nm): 0.400-0.500 in PW from a fresh slant was made, providing a concentration of 10^7-10^8 cells/mL for bacteria, 10^5-10^6 cells/mL for yeasts and 10^4-10^5 spores/mL for fungi. Measures were taken employing a Jenway Mod. 6705 UV/Vis Spectrophotometer.

2.5. LAE antimicrobial activity

The minimum inhibitory concentration (MIC) and minimum biocide concentration (MBC) of LAE were determined. First, for the determination of the MIC and MBC for bacteria and yeasts in liquid medium, cell suspension of microorganisms (100 µL) was inoculated in 10 mL of culture medium with different amounts of LAE and incubated for 24-48 h at 28 ºC. LAE tested concentrations were 0, 2, 4, 8, 16, 24, 32 and 40 µg/mL. Turbidity was determined after 24 and 72 h of incubation employing a Jenway Mod. 6705 UV/Vis Spectrophotometer. MIC was determined as the lowest LAE concentration able to inhibit microbial growth. To determine MBC, all tubes showing no growth were subcultured (1 mL) on agar; MBC is defined as the lowest LAE concentration from which no colonies growth was observed. Tests were performed in triplicate.

On the other hand, to determine the MIC and MBC of LAE for bacteria, yeasts and fungi in solid medium, 12 mL TSA or MEA was pour plated in presence of 3 mL of serial LAE dilutions, made up in sterile Milli-Q water from a 2000 µg/mL LAE stock. LAE tested concentrations were 0, 2 and 4, and from 8 to 360 µg/mL in a stepwise of 8 µg/mL. Solidified culture media were spread surface inoculated with 3 µL of cell suspension and incubated at 28 ºC, 24-48 h for bacteria and yeasts and 5 days for fungi. Tests were performed in triplicate.

2.6. Antimicrobial activity of LAE-chitosan films

Antimicrobial activity of films against bacteria and yeasts in liquid medium was also determined. A 1 cm^2 film sample (pre-weighed) and 100 µL of the microorganism suspension were added to 10 mL of culture medium and incubated at 28 ºC for 24 h. Ten-fold dilution series were made in peptone water and plated on solid medium. Colonies were counted after incubation at 37 ºC for 48 h. Tests were performed in triplicate.

Moreover, the antimicrobial activity of the films against bacteria, yeasts and fungi on agar was tested. Bacteria suspension (100 µL) was plated on TSA. 1 cm^2 pre-weighed films of CS-1% LAE, CS-5% LAE and CS-10% LAE were placed on the medium surface. Plates were incubated at 28 ºC for 24 h and the diameter of the resulting bacterial inhibition zone around the film was measured. Tests were performed in triplicate.

Yeast suspension (300 µL) was pour plated in 15 mL MEA. Preweighed films were placed on the surface of solidified medium and plates incubated 24 h at 28 ℃. Tests were performed in triplicate.

As regards fungi, 10 µL of the previous spore suspension was spread on the surface of 15 mL of solidified MEA plates. Pre-weighed films were placed on the inoculated plates and incubated for 5 days at 28 ℃. Tests were performed in triplicate.

2.7. Antimicrobial activity of LAE-CS films on chicken breast fillets

Skinless chicken breast fillet was purchased in a local store. Slices (ca. 25 g and 63 cm^2 of surface) were individually prepared for each tested periods, i.e. day of receipt (t_0), after 2 days (t_2), the suggested day as the expiration date (t_6) and 2 days after expiration date (t_8). Slices were wrapped with CS or CS-5%LAE films to achieve intimate contact between film and meat, and externally wrapped with PE film. A negative control sample for each time was also prepared without CS wrapping. Samples were stored at 4 ℃. At appropriate times, samples were transferred aseptically and weighed in a sterile Stomacher bag, diluted with 25 mL PW (Scharlab, Barcelona, Spain) and blended in Stomacher (IUL S.L., Barcelona) for 6 min. Ten-fold dilution series in PW of the obtained suspensions were made and plated on selective solid media: TSA (mesophiles and psychrophiles), MEA (yeasts and fungi), *Pseudomonas* agar base (*Pseudomonas* spp.), VRBLA (Violet Red Bile Agar) (coliforms), MRS agar (lactic acid bacteria) and Lyngby Iron agar (hydrogen sulfide-producing bacteria). Colonies were counted after incubation at 30 ℃ for 24 h for mesophiles, 10 ℃ for 10 d for psychrophiles, 30 ℃ for 5 d for yeasts and fungi, 25 ℃ for 24 h for *Pseudomonas*, 37 ℃ for 24 h for coliforms, 25 ℃ for 5 d for lactic acid bacteria and 20 ℃ for 3 d for hydrogen sulfide-producing bacteria. Tests were performed in triplicate.

2.8. Data analysis

Statistical analysis of the results was performed with SPSS commercial software (SPSS Inc., Chicago, IL, USA). A one-way analysis of variance was carried out for data. Differences between means were assessed on the basis of confidence intervals using the Tukey-*b* test at a level of significance of $P \leq 0.05$. Data were represented as average±standard deviations. Data were

plotted using the SigmaPlot 10.0 software (Systat Software Inc., Richmond, CA). The release of LAE data was analyzed using the Regression Wizard Tool of the SigmaPlot 10.0 software.

3. Results and discussion

3.1. Optical properties

CS films with LAE were transparent, flexible and uniform, without discontinuities or visible particles and with an average thickness of 50±3 μm. As shown in **Table 4.1.1**, the presence of LAE in the polymer matrix increased significantly the chroma of films with respect to the control sample, the concentration of LAE having a non-significant effect. However, the hue was not significantly different. So the differences were not visually perceptible between control CS film and LAE-incorporated films since ΔE_{ab}^* < 1 colour differences were not obvious for the human eye (Bodart *et al.*, 2008).

3.2. LAE release from films

LAE release from the tested films into water was studied at 28 ºC, temperature selected for the antimicrobial activity tests, as well as at 4 ºC, habitual temperature for refrigerated storage. **Figure 4.1.1** shows the results obtained at both temperatures. For better comparison, results were presented as the ratio between the amount of LAE released into the aqueous media at time t and that obtained at equilibrium (M_t/M_∞) which in both cases was in coincidence with the theoretical value estimated considering full extraction. As can be seen in the figure, both tests provided similar profiles which could be described as that of an exponential function growing to a maximum which was achieved at ca. 10 h at 28 ºC and at 15 h at 4 ºC.

Table 4.1.1. Colour parameter values of CS films with different amounts of LAE and plasticized with 20% G.

LAE (%)	L*	a*	b*	C_{ab}^*	h_{ab}	ΔE_{ab}^*
0	87.7 ± 0.3^a	-2.4 ± 0.1^a	10.7 ± 0.5^a	11.0 ± 0.5^a	102.8 ± 0.4^a	
1	87.6 ± 0.5^a	-2.6 ± 0.1^b	11.6 ± 0.9^b	11.9 ± 0.9^b	102.8 ± 0.6^a	0.9 ± 0.6
5	87.8 ± 0.3^a	-2.6 ± 0.1^b	11.5 ± 0.5^b	11.7 ± 0.5^b	102.6 ± 0.2^a	0.9 ± 0.3
10	88.0 ± 0.3^a	-2.6 ± 0.1^b	12.0 ± 0.7^b	12.3 ± 0.7^b	102.4 ± 0.4^a	0.7 ± 0.5

$^{a–b}$ Different letters in the same column indicate a statistically significant difference ($P \leq 0.05$) comparing the different amounts of LAE in the matrix.

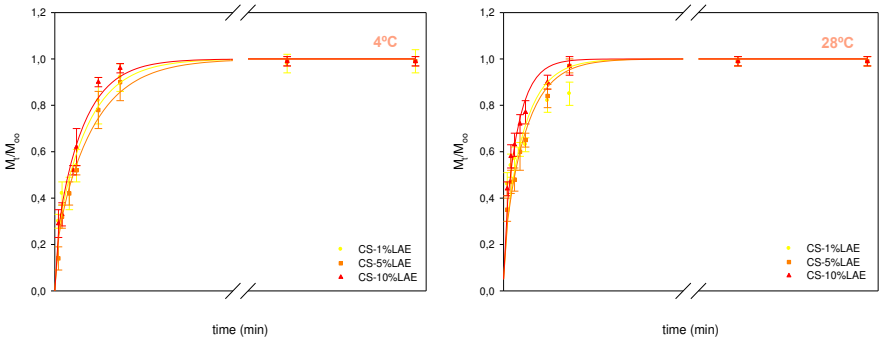

Figure 4.1.1. Normalized release of LAE (M_t/M_∞) from CS films into water versus time of exposition at 4 and 28 ºC. Dots are experimental data and lines represent theoretical curves obtained with **Eq. 1** and *D* values included in **Table 4.1.2**.

When exposed to wet environments, CS, as an hydrophilic polymer, is able to gain a large amount of water that swells the polymer, increases chain flexibility, reduces interchain cohesion energy and consequently, increases the kinetics of mass transport processes within the polysaccharidic matrix. As a consequence of the plasticizing process, the diffusion of substances within the CS matrixes is altered and the process might not follow Fickian behaviour. Nevertheless, the size of the water molecule is so small compared to LAE's molecule that the water sorption in the matrix and its relaxation can be considered to occur before any relevant amount of LAE has been released. This assumption has been successfully applied in the mass transport of other substances from hydrophilic polymeric materials (López-Carballo *et al.*, 2005; López de Dicastillo *et al.*, 2011).

To characterize the kinetics of LAE release from CS films, the solution to Fick's law considering that the film was immersed in a homogenous media so that the agent was released by both surfaces, and that any release by the film edges could be neglected, was used. According to Crank (1975), the equation that describes the evolution of release of an agent from a film of uniform thickness (ℓ) and constant diffusion coefficient (*D*) is:

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

results and the diffusion coefficient values were estimated by curve fitting to the experimental values using the Regression Wizard Tool of the SigmaPlot

10.0 software. The *D* values and the theoretical curves have been included in **Table 4.1.2** and **Figure 4.1.1**. As can be seen, there was a good agreement between theoretical data and experimental results as revealed by the regression coefficient values also included in figure. Results evidenced that the release of LAE accelerates with temperature, as expected since molecular diffusion was an activated process. Also, the values obtained showed an effect of LAE concentration on the release kinetics. The higher the concentration, the slower process which could be attributed to an antiplasticizing effect of the agent on the matrix. This effect has been observed in other mass transport processes when the polymer/substance interactions improve polymer cohesion (López-Rubio *et al.*, 2006). In absolute values, the diffusion of acetic and propionic acids in CS was in the 10^{-12} m^2/s range, much higher than the results obtained in this work, as should be expected from the high size of the LAE molecule (Ouattara *et al.*, 2000a). LAE diffusion in hydrophilic EVOH was estimated in the 10^{-14} m^2/s range, in agreement with these results (Gavara, data not published).

3.3. LAE antimicrobial activity

MICs and MBCs of LAE against bacteria, yeasts and fungi determined on solid and liquid media were included in **Table 4.1.3**. MIC values were 8 µg/mL for Gram-positive bacteria and 16 µg/mL for Gram-negative bacteria either on solid or in liquid medium. MIC values for yeasts ranged from 4 to 16 µg/mL in liquid medium and 32 to 104 µg/mL on solid medium. MIC values for fungi were found between 24 and 120 µg/mL on solid medium. Similar MIC values have also been reported in the literature (Infante *et al.*, 1997; Oh and Marshall, 1992; Rodríguez *et al.*, 2004). LAE MBC values were16

Table 4.1.2. Values of the parameters describing LAE release from CS matrices into water, according to the **Eq. 1.**

T (ºC)	4 ºC		28 ºC	
LAE (%)	10^{14}·*D* (m^2/s)	R^2	10^{14}·*D* (m^2/s)	R^2
1	8.9 ± 0.8^{c}	0.97	13.4 ± 1.5^{b}	0.89
5	7.1 ± 0.5^{b}	0.99	11.7 ± 0.7^{ab}	0.98
10	5.4 ± 0.4^{a}	0.99	10.6 ± 0.8^{a}	0.97

[a–c] Different letters in the same column indicate a statistically significant difference ($P \leq 0.05$) comparing the different amounts of LAE in the matrix.

Table 4.1.3. Minimum inhibitory concentration (MIC, µg/mL) and minimum biocide concentration (MBC, µg/mL) of LAE against the selected microorganisms in liquid and solid media.

Microorganisms	Species	Liquid medium		Solid medium	
		MIC	MBC	MIC	MBC
Gram-positives	*Listeria monocytogenes*	8	16	8	16
	Staphylococcus aureus	8	16	8	16
Gram-negatives	*Escherichia coli*	16	24	16	24
	Pseudomonas putida	16	24	16	24
	Salmonella enterica	16	24	16	24
Yeasts	*Candida utilis*	16	24	104	120
	Saccharomyces cerevisiae	16	24	104	120
	Torulopsis pinus	4	8	32	48
Fungi	*Aspergillus niger*	-	-	24	320
	Cladosporium cladosporioides	-	-	24	80
	Penicillium chrysogenum	-	-	120	280

µg/mL for Gram-positive bacteria and 24 µg/mL for Gram-negative bacteria either on solid or in liquid medium. These values were 8–24 µg/mL in liquid medium and 48–120 µg/mL on solid medium for yeasts, and 80–320 µg/mL for fungi on solid medium.

Results highlighted a slightly higher sensitivity of Gram-positive bacteria than Gram-negative bacteria. This behaviour, observed for other antimicrobial compounds (Canillac and Mourey, 2001; Delaquis *et al.*, 2002), was mainly due to the presence of an outer membrane surrounding cell wall in Gram-negative bacteria that restricts the diffusion of hydrophobic compounds through its lipopolysaccharide covering, among other defence mechanisms (Nikaido and Vaara, 1985; Russell, 1995). Furthermore, in general, these values show a greater sensibility of bacteria than yeasts and fungi against biocides also reported in many references (McDonnell and Russell, 1999; Russell, 2003).

3.4. Antimicrobial activity of LAE-CS films

Antimicrobial activity of LAE-CS films was tested against the selected microorganisms in liquid and solid media. **Table 4.1.4** presents antimicrobial effectiveness of 1 cm^2 of LAE films against bacteria and yeasts in liquid media. It is noteworthy that there was some inhibition of bacteria (ca. 0.3 log units) by CS film, due the cationic nature of the non-neutralized polysaccharide film (Shahidi *et al.*, 1999). Films with 1% of LAE increased the bacterial growth inhibition to 1–1.5 log reductions. Relevant reductions were

observed on samples containing CS-5%LAE and CS-10%LAE films. The growth of Gram-negative bacteria was reduced 2.5 logs by CS-5%LAE films and 4.8 logs by CS-10%LAE films. The inhibition caused in the growth of Gram-positive bacteria was even larger, 4 and 6 log reductions for the CS-5%LAE and CS-10%LAE films, respectively. In the case of yeast, CS film produces a large inhibition (5 logs), whereas the presence of LAE in films produced total growth inhibition. There was probably a synergistic effect of LAE and CS, both known to be yeasts inhibitors (Dai *et al.*, 2010; Shahidi *et al.*, 1999). **Table 4.1.5** shows antimicrobial activity of 1 cm^2 of LAE-films against bacteria, yeasts and fungi on solid media. It is noteworthy to mention that CS films inhibition only occurred in the area of direct contact with the film since there was no diffusion of antimicrobial agents from film, while those with LAE evidenced a halo of growth inhibition around the film due to LAE migration. Significantly, LAE films produced a greater inhibition against Gram-positive than Gram-negative bacteria. A slight inhibitory effect against yeasts was observed for CS-5%LAE and CS-10%LAE films, nevertheless the amount of LAE migrated from CS-1% LAE films could have not been enough to produce inhibition. No growth inhibition against fungi was observed around any of the tested films because the migrated concentration could have not been sufficient. As can be seen in **Table 4.1.5**, there was no difference between the halos produced by 5 to 10%. For this reason, film with CS-5%LAE was selected for studying its antimicrobial activity in food.

3.5. Antimicrobial activity of LAE-CS films on chicken breast fillets

Figure 4.1.2 shows the efficacy of CS and CS-5%LAE on microorganisms present in fresh chicken breast fillets. In general, there was an increase in microbial growth as time increases until it reached the expiry date stated on the product itself. At t_0 counts were low, as expected in samples of chicken breast fillets without skin.

A large growth reduction (>4 log) for mesophiles, psychrophiles and *Pseudomonas* spp. after 2, 6 and 8 days was evident in chicken wrapped with CS-5%LAE film. A much lower reduction was produced by control CS films, from 0.5 log after 2 days and 1.70 log at 8 days, presumably due to CS antimicrobial activity (Shahidi *et al.*, 1999). Similar results were observed

Table 4.1.4. Antimicrobial effectiveness of LAE films against the selected microorganisms in liquid medium at 28 ℃ for 24 h. Values expressed as logarithm of colony forming units (log CFU/mL) and log reduction value (LRV in brackets).

Microorganisms	Species	Control	CS (LRV)		CS-1%LAE (LRV)		CS-5%LAE (LRV)		CS-10%LAE (LRV)	
Gram-positives	*Listeria monocytogenes*	9.0 ± 0.1^{d}	8.7 ± 0.1^{d}	(0.3)^{A}	8.1 ± 0.1^{c}	(0.9)^{A}	4.9 ± 0.1^{b}	(4.1)^{B}	2.7 ± 0.1^{a}	(6.3)^{B}
	Staphylococcus aureus	9.0 ± 0.1^{d}	8.6 ± 0.3^{d}	(0.4)^{A}	8.0 ± 0.1^{c}	(1.0)^{A}	4.5 ± 0.1^{b}	(4.5)^{B}	2.9 ± 0.1^{a}	(6.1)^{B}
Gram-negatives	*Escherichia coli*	8.9 ± 0.1^{d}	8.6 ± 0.1^{d}	(0.3)^{A}	7.5 ± 0.1^{c}	(1.4)^{B}	6.3 ± 0.1^{b}	(2.6)^{A}	4.1 ± 0.1^{a}	(4.8)^{A}
	Pseudomonas putida	9.1 ± 0.1^{d}	8.7 ± 0.1^{d}	(0.4)^{A}	7.5 ± 0.1^{c}	(1.6)^{B}	6.5 ± 0.3^{b}	(2.6)^{A}	4.4 ± 0.1^{a}	(4.7)^{A}
	Salmonella enterica	8.9 ± 0.1^{d}	8.6 ± 0.2^{d}	(0.3)^{A}	7.4 ± 0.1^{c}	(1.5)^{B}	6.5 ± 0.1^{b}	(2.4)^{A}	4.8 ± 0.1^{a}	(4.1)^{A}
Yeasts	*Candida utilis*	7.7 ± 0.1^{c}	2.5 ± 0.1^{b}	(5.2)^{B}	Total inhibition^{aC}		Total inhibition^{aC}		Total inhibition^{aC}	
	Saccharomyces cerevisiae	7.8 ± 0.1^{c}	2.7 ± 0.2^{b}	(5.1)^{B}	Total inhibition^{aC}		Total inhibition^{aC}		Total inhibition^{aC}	
	Torulopsis pinus	7.6 ± 0.1^{c}	2.3 ± 0.1^{b}	(5.3)^{B}	Total inhibition^{aC}		Total inhibition^{aC}		Total inhibition^{aC}	

$^{a–d}$ Different letters in the same row indicate a statistically significant difference ($P \leq 0.05$) comparing the control samples and the different amounts of LAE in the matrix.
$^{A–C}$ Different letters in the same column indicate a statistically significant difference ($P \leq 0.05$) comparing the inhibition effectivity of a given sample against the different microorganisms.

Table 4.1.5. Antimicrobial activity of LAE-films against the selected microorganisms on solid medium at 28 ℃ and for 24 h for bacteria and yeasts, and 5 d for fungi. Zones of growth inhibition (mm) showing antimicrobial activity; film size 10 mm, plate diameter 90 mm. Values for zone of growth inhibition are presented as mean±SD (n = 3).

Microorganisms	Species	CS	CS-1%LAE	CS-5%LAE	CS-10%LAE
Gram-positives	*Listeria monocytogenes*	10.1 ± 0.1^{a}	23.8 ± 0.3^{bC}	26.9 ± 0.2^{cE}	27.5 ± 0.5^{cE}
	Staphylococcus aureus	10.0 ± 0.1^{a}	21.4 ± 0.4^{bC}	25.5 ± 0.4^{cD}	25.6 ± 0.4^{cD}
Gram-negatives	*Escherichia coli*	10.0 ± 0.1^{a}	12.3 ± 0.2^{bB}	14.1 ± 0.3^{cC}	14.3 ± 0.5^{cB}
	Pseudomonas putida	10.0 ± 0.1^{a}	11.5 ± 0.2^{bB}	14.0 ± 0.3^{cC}	14.6 ± 0.3^{cB}
	Salmonella enterica	10.0 ± 0.1^{a}	11.9 ± 0.2^{bB}	14.1 ± 0.2^{cC}	14.2 ± 0.4^{cB}
Yeasts	*Candida utilis*	10.0 ± 0.1^{a}	10.0 ± 0.1^{aA}	13.5 ± 0.9^{bC}	14.5 ± 0.7^{bB}
	Saccharomyces cerevisiae	10.0 ± 0.1^{a}	10.0 ± 0.1^{aA}	11.0 ± 0.8^{bB}	14.0 ± 0.9^{cB}
	Torulopsis pinus	10.1 ± 0.1^{a}	10.1 ± 0.1^{aA}	15.3 ± 0.5^{bC}	18.8 ± 0.8^{cC}
Fungi	*Aspergillus niger*	10.0 ± 0.1^{a}	10.0 ± 0.1^{aA}	10.0 ± 0.1^{aA}	10.1 ± 0.1^{aA}
	Cladosporium cladosporioides	10.0 ± 0.1^{a}	10.0 ± 0.1^{aA}	10.0 ± 0.1^{aA}	10.1 ± 0.1^{aA}
	Penicillium chrysogenum	10.0 ± 0.1^{a}	10.0 ± 0.1^{aA}	10.1 ± 0.1^{aA}	10.1 ± 0.1^{aA}

$^{a–d}$ Different letters in the same row indicate a statistically significant difference ($P \leq 0.05$) comparing the control samples and the different amounts of LAE in the matrix.
$^{A–E}$ Different letters in the same column indicate a statistically significant difference ($P \leq 0.05$) comparing the inhibition effectivity of a given sample against the different microorganisms.

against coliforms and hydrogen sulfide-producing bacteria, where CS-5%LAE film halved growth inhibition caused by CS alone. As regards lactic acid bacteria, CS films originated 0.93±0.2 log reduction at all tested times, while films with 5% LAE evidenced a log reduction range between 1.78 at t_2, and 3.44 at t_8. In the case of fungi and yeasts obtained data were not conclusive.

Results summarize that CS films had antimicrobial activity in the range of 0.47-2.96 log reduction dependent of time and bacterial group studied. On the other hand, incorporation of LAE in the matrix (CS-5%LAE film) increased antimicrobial activity to 1.78-5.81 log reduction. Therefore, the composite CS-LAE presents excellent potential for packaging of fresh poultry products.

4. Conclusions

This work provides an example of active food packaging, in which 1, 5 or 10% LAE was incorporated in a CS matrix. Films were found continuous, flexible and transparent, and able to release the antimicrobial agent when exposed to highly humid media. When films were immersed in water, LAE release follows a Fickian process, reaching full release within the first 15 h. LAE diffusion coefficient in the CS matrix was estimated in the 10^{-14} m^2/s range, decreasing with temperature and with LAE concentration. The addition of LAE to CS significantly increased its antimicrobial activity towards diverse microorganisms. Films of CS-5%LAE reduced satisfactorily the microbiota commonly found in fresh chicken, therefore presenting excellent potential for packaging of fresh poultry products. Future work will indicate whether these antimicrobial packaging systems will be effective to improve the safety and to extend the shelf-life of other raw and processed food products.

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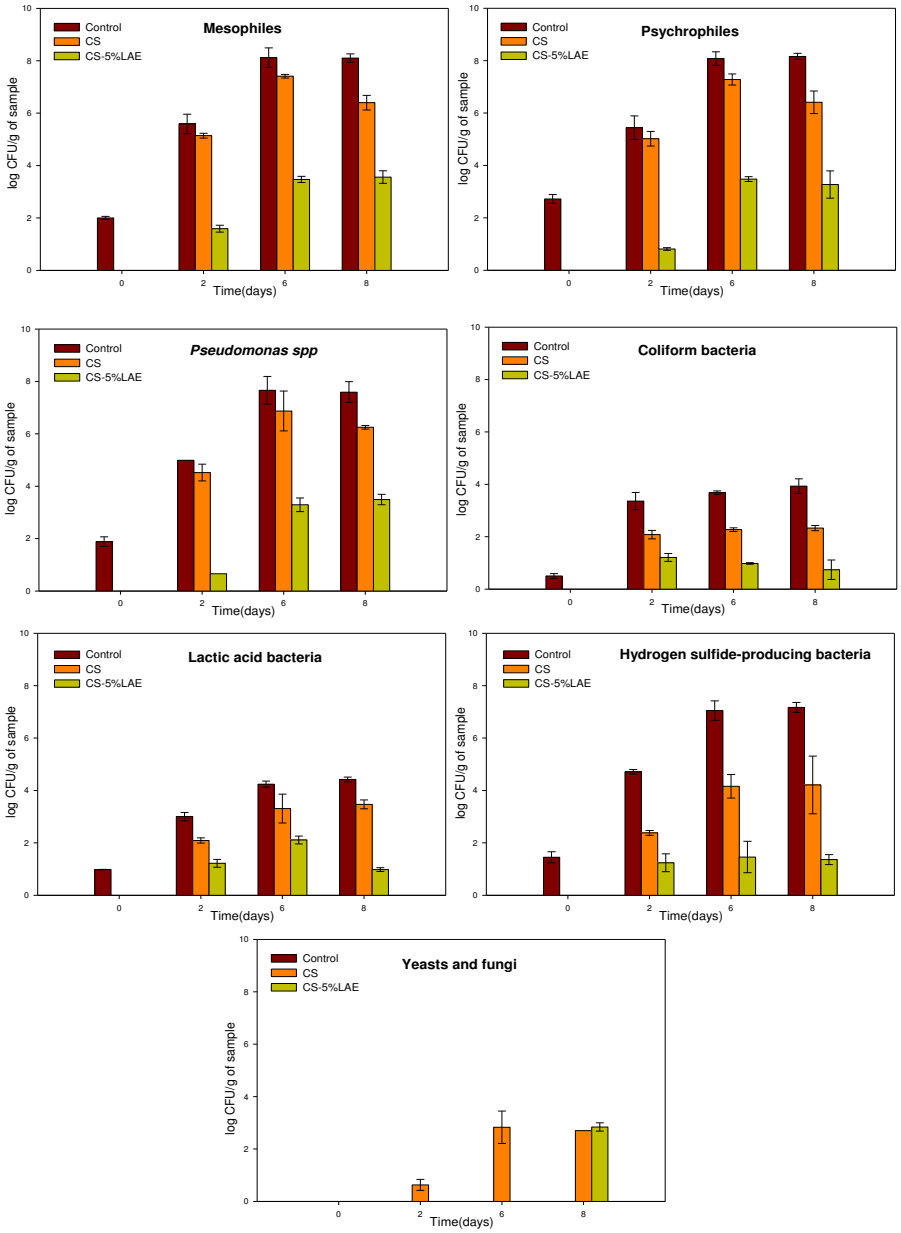

Figure 4.1.2. Growth reduction of indigenous spoilage microbiota in chicken breast fillets at 2, 6 and 8 d of storage and 4 ºC in absence and presence of CS and CS-5%LAE films.

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4.2. Capítulo 2. Desarrollo de películas híbridas de quitosano con nanopartículas de plata formadas *in situ*

4.2.1. Estado de la cuestión

Desde la antigüedad la plata iónica ha sido empleada como biocida debido a su elevada efectividad y amplio espectro de acción. Actualmente el interés por la plata está en auge dada la mayor frecuencia de microorganismos cada vez más resistentes al extenso conjunto de agentes antimicrobianos disponibles, sobre todo en el ámbito de la medicina y de la conservación de alimentos.

Actualmente los iones de plata se están incorporando en materiales inorgánicos, siendo el intercambio iónico el método más empleado. Generalmente se utilizan materiales porosos como, por ejemplo, las zeolitas (Demirci *et al.*, 2014) que liberan lentamente iones de plata y pueden incorporarse a otros materiales formando superficies antimicrobianas. La FDA acepta varios de estos dispositivos como sustancias seguras para entrar en contacto con los alimentos. Asimismo, estos portadores cargados con plata se pueden incorporar en materiales poliméricos a partir de los cuales liberarse: zeolitas en poliuretano (Kamışoğlu *et al.*, 2008), polietileno (PE) (Boschetto *et al.*, 2012) o PLA (Fernández *et al.*, 2010), filosilicatos en policaprolactona o zeína (Incoronato *et al.*, 2010) o CS (Lavorgna *et al.*, 2014) y fosfato de sodio y circonio en policaprolactona (Duan *et al.*, 2007) o polietersulfona (Huang *et al.*, 2012). Sin embargo, esta tecnología precisa elevadas cantidades de sales de plata, por lo que aumenta el coste económico y ambiental del material.

En la actualidad, las nanopartículas de plata suponen una excelente alternativa, y están siendo ampliamente estudiadas y empleadas en la fabricación de polímeros nanocompuestos para el desarrollo de nuevos materiales con propiedades específicas. Aunque las nanopartículas de plata son de por sí antimicrobianas, éstas también pueden actuar como reservorios de iones plata permitiendo su liberación sostenida en el tiempo. De este modo, la superficie de las nanopartículas de plata metálica se oxida a iones de plata en medio acuoso y con el tiempo las nanopartículas se disuelven liberando los iones de plata antimicrobianos en el medio líquido. Por lo tanto, otra manera de crear materiales nanocompuestos poliméricos

antimicrobianos es la inmovilización de nanopartículas en una matriz polimérica capaz de liberar los iones de plata oxidada. Existen numerosas investigaciones, patentes y aplicaciones comerciales en relación con las nanopartículas de plata incluidas en matrices poliméricas. Sin embargo, su aplicación en el desarrollo de envases antimicrobianos alimentarios está restringida debido a cuestiones legislativas por la cantidad de plata que puede migrar.

Generalmente, la formación de las nanopartículas de plata en solución requiere una sal precursora y soluble, un agente reductor y un agente estabilizador. La elección de cada uno de ellos, así como del método de obtención determinará las características, propiedades y estabilidad de las nanopartículas de plata que se generen. Si bien es posible la formación de nanopartículas de plata mediante la reducción de su forma iónica a través de métodos foto-, sono- y electroquímicos, físicos y biológicos etc., el método más utilizado es la vía húmeda mediante reducción química con agentes reductores tales como borohidruro de sodio, citrato, dimetilformida, hidracina, ascorbato, hidrógeno elemental, etc. Siendo el nitrato de plata, la sal precursora más empleada (Sau y Rogach, 2010; Sharma *et al.*, 2009; Tolaymat *et al.*, 2010). Inicialmente se reduce el ion de plata Ag^{+}, que permite la formación de átomos de Ag^{0} libres. A continuación, se agregan en pequeños grupos de 2-10 átomos (*clusters*) en los que coexisten átomos e iones plata. Finalmente, estos núcleos crecen, se reducen completamente y pueden dar lugar a la formación de partículas coloidales de plata (Ershov, 1999; Sau y Rogach, 2010). Esto, en la bibliografía se describe como el enfoque *bottom-up*, en el que las unidades más pequeñas (iones, átomos y moléculas) se ensamblan para formar estructuras nanométricas (**Figura 4.2.1**); en contraposición al enfoque *top-down* que parte de entidades más grandes y reduce su tamaño hasta obtener el rango nanométrico (Sau y Rogach, 2010; Tolaymat *et al.*, 2010).

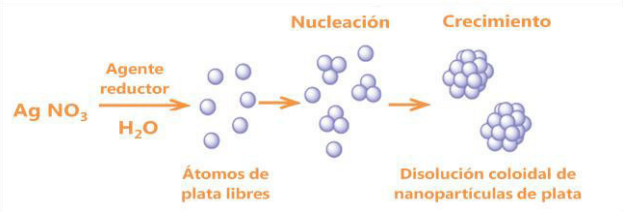

Figura 4.2.1. Esquema de la generación de nanopartículas de plata.

Los agentes estabilizadores más comunes son los tensioactivos y los polímeros, que ejercen su acción al unirse a la superficie de la partícula (Sharma *et al.*, 2009). Existen diferentes métodos tanto físicos como químicos para preparar dichos compuestos poliméricos, siendo el más común la dispersión de nanopartículas preparadas previamente (síntesis *ex situ*) en la matriz polimérica en solución. Sin embargo, este método puede producir una distribución y tamaño no homogéneos de las partículas en el polímero debido a su aglomeración en el medio acuoso. Para evitar esto se pueden generar las nanopartículas metálicas *in situ,* mediante la disolución y la reducción de las sales metálicas en el polímero. Otra alternativa para conseguir una dispersión homogénea de las nanopartículas en la matriz polimérica sería mediante la polimerización y la reducción del metal de forma simultánea (De Azeredo, 2013; Sharma *et al.*, 2009).

Se han publicado diversos estudios sobre la incorporación de nanopartículas de plata libres en diversas matrices poliméricas con el objeto de conferirles actividad antimicrobiana, como en: PA (Damm *et al.*, 2007; Damm *et al.*, 2008; Perkas *et al.*, 2007), polietersulfona (Basri *et al.*, 2011; Basri *et al.*, 2010), PP (Yeo y Jeong, 2003), PE (Dehnavi *et al.*, 2013; Sánchez-Valdes *et al.*, 2009; Zapata *et al.*, 2011), PS (Nassar y Youssef, 2012), poliuretano (Jain y Pradeep, 2005; Sheikh *et al.*, 2009; Triebel *et al.*, 2011), polivinilpirrolidona (An *et al.*, 2008); o bien en PVOH (Galya *et al.*, 2008; Hong *et al.*, 2006). Así como también en matrices biopoliméricas, como se recogen en el **Anexo A**.

La actividad antimicrobiana de las películas nanocompuestas con iones o nanopartículas de plata dependerá de varios factores tales como la cristalinidad y la capacidad de hinchamiento en agua del polímero, ya que la matriz tiene que estar plastificada para facilitar la difusión. Otros factores están relacionados con el tipo de nanomaterial empleado para soportar los iones o nanopartículas, así la liberación de iones en zeolitas o de nanopartículas metálicas será diferente. Por ejemplo, las nanopartículas de plata ofrecen más estabilidad y una liberación de iones de plata más lenta, mientras que las zeolitas con plata tiene un efecto antimicrobiano superior en tiempos cortos. También hay que considerar el tamaño y la forma de la partícula. Las partículas más pequeñas tienen una mayor superficie específica para liberar iones de plata. Por su parte, las nanopartículas de plata esféricas

son más reactivas debido a su alta densidad atómica y por tanto una liberación de iones más rápida.

Existen diversos trabajos en los que se desarrollan películas nanocompuestas de nanopartículas de plata y CS. Algunos trabajos incluyen nanopartículas de plata en una matriz de CS (Huang *et al.*, 2011; Lu *et al.*, 2008; Pinto *et al.*, 2012; Potara *et al.*, 2011; Rhim *et al.*, 2006), pero en estos casos la obtención de las nanopartículas son generadas previamente. Otros autores han obtenido nanopartículas de plata en la disolución de CS, pero empleando agentes reductores fuertes como el borohidruro de sodio (Huang *et al.*, 2004). Por otra parte, existen diversos ejemplos de métodos de obtención de nanopartículas de plata en el seno de la matriz de CS en una sola etapa, y en condiciones suaves, respetando el medio ambiente. Así, Murugadoss y Chattopadhyay (2008) y Sanpui *et al.* (2008) desarrollaron un método de obtención de nanocompuestos de CS/plata, en el que el CS actúa como reductor de la plata iónica en presencia de hidróxido de sodio a 95 ℃ en medio acuoso. De forma similar procedieron Hoang *et al.* (2010), Regiel *et al.* (2013), Thomas *et al.* (2009) Wei y Qian (2008), y Wei *et al.* (2009), ya que añadieron la disolución de nitrato de plata a la disolución acética de CS, empleando una temperatura de entre 30-100 ℃ para la formación de las nanopartículas. Algunos de éstos, además, obtuvieron películas a partir de esta disolución mediante extensión y evaporación del disolvente, que fueron secadas a 60 ℃ y neutralizadas con hidróxido de sodio. Sin embargo, la etapa de formación de nanopartículas de plata se realiza a elevada temperatura y en disolución; mientras que en nuestro caso la máxima temperatura empleada es de 40 ℃ y las nanopartículas de plata se forman principalmente durante la neutralización de la película ya conformada, etapa necesaria para la obtención de películas de CS insolubles. Un trabajo similar es el de Tankhiwale y Bajpai (2010), en el que sumergen la película de CS en la solución de nitrato de plata y posteriormente en una solución de citrato trisódico, y en el que las nanopartículas de plata se formaron en la propia película. Otro similar es el de Li *et al.* (2010), aunque en este caso se incluyó óxido de zinc en la composición y la sonicación como etapa adicional necesaria para la obtención del nanocompuesto.

En la presente Tesis Doctoral, las nanopartículas de plata se han generado *in situ* en películas de CS, incluyendo nitrato de plata como

precursor en la formulación de las películas. Las nanopartículas se generaron durante la neutralización de las películas con hidróxido de sodio. Además, el CS es un agente reductor débil en condiciones alcalinas, lo que refuerza la acción reductora del hidróxido. Esa metodología constituye una alternativa a las empleadas hasta ahora, donde comúnmente las partículas se generaban en la solución formadora de películas mediante la incorporación de agentes reductores tóxicos como el borohidruto de sodio u otros no tóxicos como la glucosa. El CS es un excelente quelante de iones plata debido a la presencia en su estructura de grupos amino, contribuyendo también a ello aunque en menor medida los grupos hidroxilo. Gracias a estos grupos, es posible generar nanopartículas controlando su tamaño y evitando su agregación (Dallas *et al.*, 2011; Murugadoss y Chattopadhyay, 2008; Twu *et al.*, 2008). Esta vía alternativa de obtención de las nanopartículas de plata tiene otras ventajas: 1) no emplean disolventes orgánicos, ya que la matriz de CS se disuelve y neutraliza en agua; 2) no se generan subproductos tóxicos; 3) no emplea agentes reductores fuertes ni estabilizadores tóxicos; 4) se generan en el seno de una matriz biodegradable que proviene del material de desecho de la industria alimentaria, renovable y no tóxica, por lo que se reduce el impacto medioambiental, y 5) se emplean moderadas y bajas temperaturas, lo que supone menor gasto energético. Todas ellas, cuestiones clave entre los doce principios de la denominada Química Sostenible (Anastas y Eghbali, 2010; Dallas *et al.*, 2011; Sharma *et al.*, 2009).

En el trabajo realizado en la presente Tesis Doctoral también se han caracterizado las películas resultantes y las nanopartículas generadas, además de estudiarse la actividad antimicrobiana de las películas y la liberación de iones plata en el tiempo, para demostrar si las películas en medio líquido liberan gradualmente plata iónica suministrando una acción antimicrobiana mantenida en el tiempo.

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4.2.2. Artículo científico 2.

Silver ions release from antibacterial chitosan films containing *in situ* generated silver nanoparticles

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Resumen

Este estudio tiene como objetivo desarrollar películas antimicrobianas que consisten en quitosano y nanopartículas de plata homogéneamente distribuidas en la matriz polimérica. Las nanopartículas se generaron *in situ* durante la neutralización de las películas de acetato de quitosano con hidróxido de sodio. La temperatura de neutralización y la concentración de plata en la película fueron dos factores cruciales de la forma y el tamaño de las nanopartículas. Las películas neutralizadas presentaron actividad antimicrobiana frente a *Escherichia coli* y *Staphylococcus aureus* en medio de cultivo líquido. Sin embargo, la eficacia de las películas fue considerablemente mayor en los medios de cultivo diluidos. Además, no se encontraron diferencias significativas en la capacidad antimicrobiana de películas que incorporan diferentes cantidades de plata o en la cantidad de plata que emigró en los medios líquidos después de 18 h de inmersión de la película. Las películas neutralizadas mantuvieron su actividad tras 1 mes inmersas en agua desionizada, lo cual puede atribuirse a la liberación sostenida de iones de plata, y por lo tanto la eficacia a lo largo del tiempo.

Palabras clave

Síntesis, nanopartículas de plata, quitosano, actividad antimicrobiana.

Abstract

This study aims to develop antimicrobial films consisting of chitosan (CS) and silver nanoparticles that are homogeneously distributed throughout the polymer matrix. Nanoparticles were generated *in situ* during the neutralization of the chitosan acetate film with sodium hydroxide. The temperature of neutralization and the concentration of silver in the film were crucial determinants of the shape and size of the nanoparticles. Neutralized films exhibited antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* in liquid growth media. However, the effectiveness of the films was considerably greater in diluted growth media. Furthermore, no significant differences were found either in the antimicrobial capacities of films incorporating different amounts of silver or in the amount of silver that migrated into the liquid media after 18 h of immersion of the film. Neutralized films maintained their activity after 1 month of immersion in deionized water, which can be attributed to the slow sustained release of silver ions and thus efficacy over time.

Keywords

Synthesis, silver-based nanoparticles, chitosan, antimicrobial activity.

1. Introduction

Silver ions have long been recognized as an effective biocide against a broad spectrum of microorganisms, their inhibition mechanism being the subject of considerable research (Musarrat *et al.*, 2010; Petrus *et al.*, 2011; Yamanaka *et al.*, 2005). Ionic silver can exert its antimicrobial action in several ways. It has been reported to complex with the thiol groups of enzymes and proteins, altering their structure and function (Liau *et al.*, 1997). Silver ions have also been found to bind with DNA and cause structural changes in the cell envelope and cytoplasmic membrane of bacteria (Kim and Ahn, 2008; Yakabe *et al.*, 1980). Ionic silver has been used as an antimicrobial in the form of salts, and more recently it has been incorporated into inorganic materials such as zeolites, zirconium phosphate, and glass (Guerra *et al.*, 2012; Huang *et al.*, 2012). A new approach is the development of silver nanoparticles that can act as nanoreservoirs for the delivery of silver ions, ensuring their availability in the substrate over time. There is a great variety of chemical and physical processes for the synthesis of silver nanoparticles, most of them involving the formation of colloidal nanoparticles or their incorporation in other materials (Rai *et al.*, 2009; Sharma *et al.*, 2010). In this regard, increasing attention is being paid to the incorporation of silver nanoparticles in polymer matrices for the development of antimicrobial films and coatings. The antimicrobial capacity of the composites formed will depend on the physical and chemical properties of the nanoparticles and also the silver ion release properties of the carrier matrix. There is also great interest in the use of synthetic and natural hydrophilic polymers and hydrogels as carriers of silver. These materials absorb large amounts of water, owing to the presence in their structure of polar groups (−OH, $-NH_2$, $-CONH_2$, −COOH...). Hydrophilic polymers are capable of swelling in a moist environment, thus facilitating the diffusion of the active agent through the polymer matrix and its release to the medium in contact with the polymer.

CS is a biodegradable and biocompatible polymer obtained from biomass and possesses excellent film-forming properties, which have made it of great interest for technological applications in several areas such as pharmacy, medicine, agrochemistry, and packaging. The hydrophilic nature of CS has aroused interest in its use as a sustained release carrier when the release of the retained active compound is required in a moist environment.

Inclusion of silver-based nanoparticles as an antimicrobial nanofiller in a CS matrix could be done directly or by using silver nitrate as a precursor. CS is soluble in aqueous solution and has the ability to bind many metal ions, including silver, via chelation with the amine groups. CS has also been reported to be a mild reducing agent used for reduction of silver ions, and it is frequently employed as an ion capping agent to control the growth of nanoparticles and avoid their aggregation (Murugadoss and Chattopadhyay, 2008).

The aim of this study, therefore, was to develop films for the slow, sustained release of silver ions, consisting of CS polymer as the carrier matrix filled with silver-based nanoparticles synthesized *in situ*. The resulting films were characterized and the antimicrobial activity was tested in liquid growth media. The ability of the carrier system to exert antimicrobial activity over time was also studied.

2. Materials and methods

2.1. Synthesis of chitosan/silver-based nanoparticle films

Low molecular weight chitosan (CS, MW 50–190 kDa, 75–85% deacetylated) from shrimp shells, sodium hydroxide (ACS reagent, ≥97.0%, pellets), and silver nitrate (ACS reagent, ≥99.0%) were obtained from Aldrich Chemical Co., Inc., Milwaukee, WI, USA. A 1.5% (w/w) CS solution was prepared in 0.5% (w/w) acetic acid and stirred at 40 °C for 1 h. After the solution had cooled to room temperature, silver nitrate, previously diluted with a small amount of distilled water, was added to the solution and left shaking, protected from light, until complete dissolution. Several CS solutions with different silver nitrate concentrations were prepared, corresponding to silver concentrations of 0.1, 0.2, 0.5, 1, and 1.5% (g/100 g CS). Films were formed by casting on PS plates and dried at 37 °C with a RH of 22% for 48 h. The CS acetate films were neutralized with sodium hydroxide to make them insoluble in water at a pH above the pK_a of CS. Hydroxyl ions also accelerate the reduction reaction of silver ions and the formation of silver-based nanoparticles by increasing the reducing power of CS.

For this purpose, the films were immersed in a solution of 0.1 M sodium hydroxide for 20 h in a thermostatic chamber and protected from light. The effect of the neutralization temperature on the formation of nanoparticles was studied by assaying two temperatures, 22 and 37 ℃. After neutralization, the films were washed with deionized water and dried in an oven at 37 ℃. Finally, the films were stored in a glass desiccator at 22 ℃ and 0% RH prior to use.

2.2. Characterization of chitosan/silver-based nanoparticle films

2.2.1. Film colour

The colour of neutralized CS film was measured using a Konica Minolta CM-3500d spectrophotometer set to D65 illuminant/10° observer. Film specimens were measured against the surface of a standard white plate, and the CIELAB colour space was used to obtain the colour coordinates L* (lightness) [black (0) to white (100)], a* [green (−) to red (+)], and b* [blue (−) to yellow (+)]. The colour was expressed using the polar coordinates L*C*h°, and ΔE*, where L* is the same as above, C* is chroma, h° is hue angle, and $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. Eight measurements were taken of each sample, and three samples of each film were measured.

2.2.2. Thermogravimetric analysis (TGA)

TGA of CS films neutralized at 37 ℃ was carried out using a Mettler Toledo TGA/ SDTA/851. Samples of approximately 10 mg were heated from room temperature to 800 ℃ at 10 ℃/min and held at an isotherm for 3 min. Nitrogen was used as the carrier gas. The TGA data were plotted as the weight percentage versus temperature, and the decomposition temperature was obtained from the first derivative of weight loss curve (DTG).

2.2.3. UV−Visible spectroscopy

The particles generated in CS films neutralized at 37 ℃ were characterized by testing their optical absorption with an Agilent 8453 UV−vis diode array spectrophotometer.

2.2.4. Transmission electron microscopy (TEM)

The morphology of the nanoparticles generated in CS films neutralized at 37 ℃ was studied using a JEOL-1200 EX transmission electron

microscope. The morphology of the nanoparticles generated in films neutralized at 37 ℃ was studied using a JEOL-1200 EX transmission electron microscope at an acceleration voltage of 100 kV. TEM specimens were cut from films using a Porter−Blum MT-2B ultramicrotome equipped with a diamond knife to give around 70 nm thick sections and placed on 300 mesh copper grids. Images were obtained with a digital micrograph acquisition software (Gatan, Inc., 2007).

2.2.5. X-ray diffraction (XRD)

The XRD patterns of CS films neutralized at 37 ℃ were recorded using a Bruker AXS D500 spectrometer with a Bragg−Brentano geometry at a wavelength of 1.5406 (corresponding to the peak Cu Kα). X-ray diffractograms were recorded in a diffraction angle (2θ) range of 5−80° using a step size of 0.02° and an exposure time of 2 s.

2.3. Antimicrobial activity of films neutralized at 37 ℃

Staphylococcus aureus ATCC 12600 and *Escherichia coli* ATCC 25922 were obtained from the Spanish Type Culture Collection (Valencia, Spain). Strains were stored in tryptone soy broth (TSB, Scharlab, Barcelona, Spain) with 20% G at −80 ℃ until needed. For experimental use, the stock cultures were maintained by regular subculture on tryptone soy agar (TSA, Scharlab) slants at 4 ℃ and transferred monthly. In a first step a loopful of each strain was transferred to 10 mL of TSB and incubated at 37 ℃ for 18 h to obtain early stationary phase cells. Cell cultures of each microorganism in stationary phase, with an optical density of 0.9 at 600 nm, were diluted in TSB and incubated at 37 ℃ until an optical density of 0.2 at 600 nm (10^5 CFU/mL) was reached. Tubes with 10 mL of Mueller−Hinton broth (MHB, Scharlab) and 1:125 diluted MHB were inoculated with 100 µL of the exponential phase culture of each microorganism. Only films neutralized at 37 ℃ were tested. Previously, a set of films were first autoclaved to study the effect of moist heat at >100 ℃ on their antimicrobial activity. This study was done because some silver ions could remain in the matrix after the formation of nanoparticles and hydrothermal treatment could lead to chemical reduction and possibly modify their antimicrobial activity. Samples weighing 0.25 g were then cut into 1.5 cm^2 pieces and added to each tube. A control film of

neutralized CS without the incorporation of silver nitrate was used as a blank in each experiment. The tubes were then incubated at 37 ℃ for 18 h. Depending on the turbidity of the tubes; serial dilutions with peptone water were made and plated in Petri dishes with 15 mL of TSA culture medium. Colonies were counted after incubation at 37 ℃ for 18 h.

2.4. Antimicrobial activity of films neutralized at 37 ℃ in liquid media over time

Three sets of experiments were carried out with films having 0.2 and/or 1.5% (w/w) of silver and neutralized at 37 ℃ to study the antimicrobial capacity of the films over time. The first experiment studied how the immersion time of the film in the growth medium prior to inoculation with bacteria affects its antimicrobial activity. For this purpose, 0.25 g of film was immersed in 10 mL of 1:125 diluted MHB for 0, 2, 10, 24, 48, 168, or 360 h, after which bacteria were inoculated in the tubes and the antimicrobial activity was evaluated as described above.

In the second experiment, we determined the antimicrobial activity of the culture medium in which the film was previously immersed. To do this, 0.25 g of film was immersed in 10 mL of 1:125 diluted MHB, the old culture medium being replaced every 24 h with fresh medium. The antimicrobial capacity of the replaced culture medium was assayed at 1, 3, 5, 10, 15, and 30 days. After 30 days, the antimicrobial capacity of the film was also tested in fresh culture medium as described above.

In the third experiment, films were immersed in 200 mL of sterile distilled water for a month. During this period the water was periodically refreshed to avoid microbial contamination. The antimicrobial activity of the films was evaluated at 1 and 30 days; films were put in tubes with 10 mL of 1:125 diluted MHB and inoculated with *S. aureus*. The antimicrobial activity of the films was studied as described previously.

2.5. Release of silver into the liquid culture medium

Migration of silver from films neutralized at 37 ℃ to the culture growth medium was studied by immersion of 0.25 g of films of different silver concentrations comprising 0.1, 0.2, 0.5, 1, or 1.5% (g/100 g CS) in 10 mL of 1:125 diluted MHB for a period of time at 37 ℃, incubation conditions

resembling those of microorganism growth. After this time, the media were diluted with 0.5% HNO_3 and the concentration of silver in the samples was quantified by graphite furnace atomic absorption spectroscopy (GFAAS) with a longitudinal AC Zeeman (Analyst 600, Perkin-Elmer, Madrid, Spain) equipped with a transversely heated graphite atomizer and a built-in, fully computer-controlled AS-800 autosampler (Perkin-Elmer). The furnace program [temperature (℃)/ramp time (s)/hold time (S)] employed for silver determination was as follows: drying (90 ℃/10 s/20 s; 120 ℃/10 s/20 s; 130 ℃/5 s/40 s; 300 ℃/5 s/5 s); pyrolysis (500 ℃/10 s/20 s); cooling (20 ℃/10 s/20 s); atomization (1400 ℃/0 s/5s); cleaning (2450 ℃/1 s/5 s). Twenty microliters of sample with 10 µL of matrix modifier (0.05 mg of Pd and 0.003 mg of $Mg(NO_3)_2$) was injected. Triplicate analyses of three independent samples were performed for each defined time.

3. Results and discussion

3.1. *In situ* synthesis of silver-based nanoparticles in a chitosan film

Films were successfully developed, based on silver nitrate as a nanoparticle precursor and CS acting as a polymer carrier and mild silver ion reducing agent. The amino groups of CS serve as ligands to complex silver ions at near-neutral pH. To a lesser extent, this polymer can also form complexes with hydroxyl groups. This makes CS a chelating polymer with excellent adsorption capacities for silver ions in the preparation for the formation of silver nanoparticles. This polysaccharide has also been described as a weak reducing agent owing to the presence in their structure of the organic compounds aldehyde, ketone, and alcohol. The presence of sodium hydroxide accelerated the reduction rate of silver ions (Singh *et al.*, 2009) and, thus, the formation of silver nanoparticles in the film. However, the coexistence of elementary silver and silver oxide nanoparticles is expected, owing to the presence of sodium hydroxide.

Treatment with sodium hydroxide allows neutralization of amino groups in CS film, promoting the integrity of the resulting films in aqueous medium at a pH above the pK_a of CS. This less toxic method for the synthesis of nanoparticles avoids the employment of commonly used toxic reducing agents.

3.2. Chitosan/silver-based nanoparticles films

Films neutralized at 37 ℃ were successfully developed with nominal concentrations of silver between 0.1 and 1.5%. Higher concentrations of silver greatly increased the viscosity of the film-forming solution, giving rise to the formation of a gel. The films were homogeneous to the naked eye, and their thickness ranged between 55 and 65 μm. **Figure 4.2.2** shows a photograph of films with different concentrations of silver, neutralized at 22 or 37 ℃. The incorporation of silver nitrate produced films with a different color after the neutralization step. It can be observed that transparency decreased and the films acquired a reddish brown tone as the concentration of silver and the neutralization temperature increased. Colour coordinates of the films are shown in **Table 4.2.1**.

CS−silver nitrate films neutralized at 22 ℃ experienced an increase in chroma (C*) and colour difference (ΔE) and a decrease in hue angle (h°) and lightness (L*) compared to un-neutralized films. As the concentration of silver increased, the films experienced a decrease in C*, h°, lightness, and ΔE. These changes were more acute for films neutralized at 37 ℃, indicating a greater conversion of silver nitrate into silver-based nanoparticles. The C* values of the films neutralized at 37 ℃ also increased with silver; however, C* values of 1 and 1.5% silver films were lower than the control and films became opaque.

Because a higher neutralization temperature ensures a greater conversion of silver nitrate into silver-based nanoparticles, studies of the thermal stabilities of the films and their antimicrobial activities, along with characterization of the nanoparticles formed in the films, were undertaken only with those neutralized at 37 ℃.

3.3. Thermogravimetric analysis

Figure 4.2.3 shows the effect of silver content in 37 ℃ neutralized CS films on the first derivative of weight loss curves (DTG). The initial thermal decomposition of films neutralized at 37 ℃ happened at a slightly higher temperature than the film prepared without silver. It can be seen in the DTG_{max} curves that the maximum decomposition temperature of CS appeared at 288 ℃, whereas for film with silver-based nanoparticles the peak

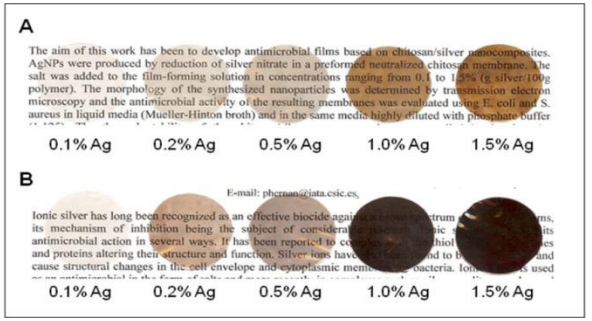

Figure 4.2.2. CS/silver-based nanoparticle films with different concentrations of silver: (A) neutralized at 22 ℃; (B) neutralized at 37 ℃.

Table 4.2.1. Colour parameters of CS films with different concentrations of silver neutralized at 22 ℃ and 37 ℃.

	Ag (%)	L*	a*	b*	C*	h°	ΔE*
Control	0.0	94.27 ± 0.82^{a}	-0.61 ± 0.05^{a}	7.10 ± 0.71^{a}	7.13 ± 0.71^{a}	94.91 ± 0.60^{a}	
	0.1	88.71 ± 0.44^{b}	-0.62 ± 0.03^{a}	5.82 ± 0.19^{b}	5.85 ± 0.18^{b}	96.11 ± 0.44^{b}	5.71 ± 0.39^{a}
	0.2	75.10 ± 1.47^{c}	2.28 ± 0.84^{b}	12.57 ± 0.96^{c}	12.78 ± 1.09^{c}	79.72 ± 1.03^{c}	20.14 ± 1.66^{b}
Films neutralized at 22 ℃	0.5	70.84 ± 2.11^{d}	4.40 ± 0.86^{c}	15.50 ± 1.14^{d}	16.11 ± 1.22^{d}	74.15 ± 0.76^{d}	25.39 ± 2.32^{c}
	1.0	43.01 ± 1.28^{e}	7.50 ± 0.09^{d}	21.00 ± 0.85^{e}	22.30 ± 0.89^{e}	70.35 ± 1.14^{e}	53.73 ± 1.31^{d}
	1.5	41.43 ± 1.97^{e}	10.31 ± 1.72^{e}	26.00 ± 1.73^{f}	27.97 ± 1.98^{f}	68.38 ± 0.58^{f}	57.17 ± 1.42^{e}
Control	0.0	94.27 ± 0.82^{a}	-0.61 ± 0.05^{a}	7.10 ± 0.71^{a}	7.13 ± 0.71^{a}	94.91 ± 0.60^{a}	
	0.1	86.06 ± 0.36^{b}	0.13 ± 0.15^{b}	8.21 ± 0.81^{b}	8.21 ± 0.81^{b}	89.08 ± 1.04^{b}	8.32 ± 0.43^{a}
	0.2	64.06 ± 0.83^{c}	2.75 ± 0.83^{c}	15.90 ± 0.47^{c}	16.14 ± 0.49^{c}	80.19 ± 1.35^{c}	31.65 ± 0.91^{b}
Films neutralized at 37 ℃	0.5	55.37 ± 1.92^{d}	4.81 ± 0.55^{d}	14.00 ± 0.69^{d}	14.80 ± 0.71^{d}	71.04 ± 2.02^{d}	39.88 ± 1.85^{c}
	1.0	31.32 ± 0.92^{e}	2.00 ± 0.53^{e}	6.03 ± 0.48^{e}	6.35 ± 0.51^{e}	71.64 ± 1.99^{d}	63.02 ± 0.91^{d}
	1.5	26.66 ± 0.74^{f}	1.00 ± 0.12^{f}	4.18 ± 0.31^{f}	4.29 ± 0.30^{f}	76.53 ± 2.01^{e}	67.69 ± 0.73^{e}

$^{a-f}$ Values within a column followed by a different lower-case letter are significantly different from each other comparing different amounts of silver in the films neutralized at 22 ℃ or 37 ℃ (Tukey's adjusted analysis of variance $P<0.05$).

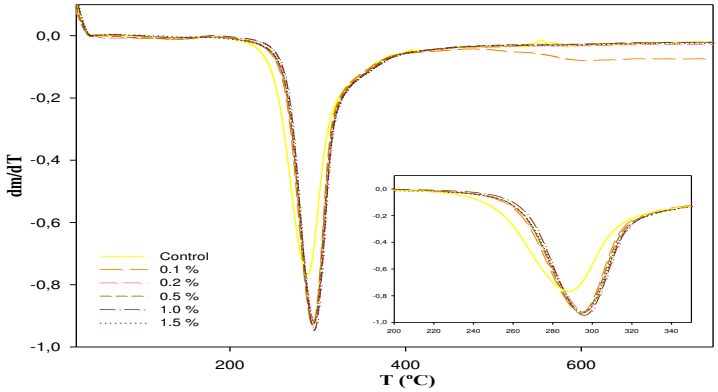

Figure 4.2.3. Effect of silver content in 37 ℃ neutralized CS films on the first derivative of weight loss (DTG) curves (dm/dT = first derivative of weight loss *vs.* time).

shifted to a higher temperature, indicating that silver-based nanoparticles increase the thermal stability of the films.

3.4. Transmission electron microscopy studies

TEM was used to study the size and shape of the nanoparticles generated in films with 0.1, 0.2, 0.5, 1, and 1.5% of silver. Panels A and B of **Figure 4.2.4** show TEM images of neutralized CS films with silver concentrations of 0.2 and 1.5%, respectively. The formation of nanoparticles with a spherical morphology and sizes ≤5 nm, distributed homogeneously throughout the polymer matrix, was observed for films having 0.1−0.5% of silver.

Films possessing silver concentrations of 1 and 1.5% displayed the formation of spherical nanoparticles of 5−10 nm in size and a second population of round-shaped, anisotropic nanoparticles with diameters ranging from 30 to 50 nm. These larger nanoparticles are probably aggregates formed from smaller ones.

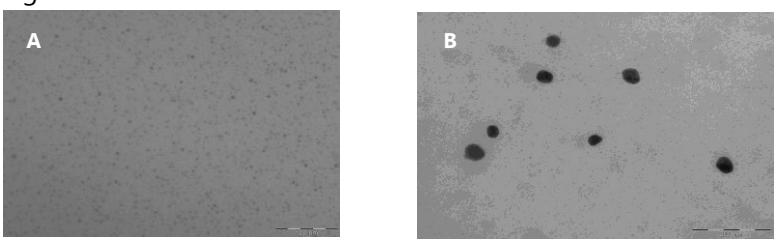

Figure 4.2.4. TEM images of films containing (A) 0.2% and (B) 1.5% of silver and neutralized at 37 ℃.

3.5. UV−visible analysis

Figure 4.2.5 shows the UV−visible spectra of CS films incorporating various concentrations of silver. The figure depicts two spectral bands at 355 and 454 nm. Under conditions in which one-electron reduction of metal ions occurs predominantly, the subsequent aggregation of the resulting atoms and ions gives more or less complex small clusters and then quasimetallic particles. These species are associated with the presence of bands around 350 nm (Ershov, 1999).

The conduction electrons on the surface of metallic nanoparticles undergo a collective oscillation when stimulated with incident light. This oscillation is known as a surface plasmon resonance (SPR) and results in strong scattering and absorption properties. Silver nanoparticles have a characteristic surface plasmon band around 400 nm in the UV−visible spectrum. SPR of spherical silver nanoparticles is responsible for the band appearing at 454 nm in **Figure 4.2.5** for films neutralized at 37 °C. It can be observed that this band became stronger, with a large asymmetrical broadening, as the silver concentration in the film increased. This band shape has been associated with the deviation of nanoparticles from a perfect spherical shape and an increase in size distribution, which has been previously observed in TEM studies. The presence of a shoulder at 570 nm can be observed, a phenomenon that has also been reported by other authors. According to Mie's theory, small spherical nanoparticles should exhibit a single surface plasmon band, whereas anisotropic particles should

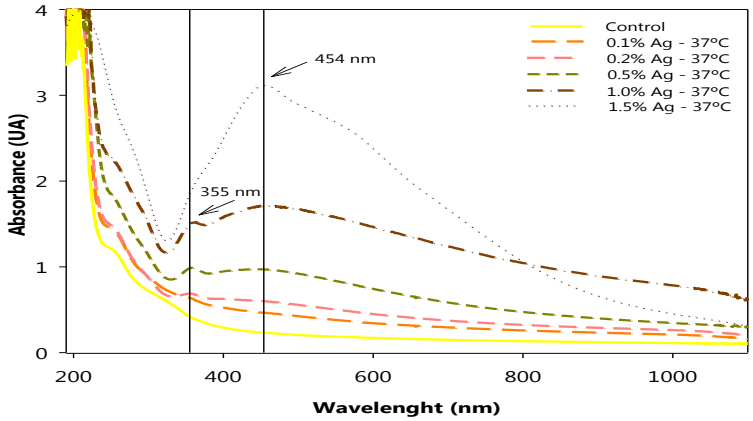

Figure 4.2.5. UV−visible spectrum of CS films with 0, 0.1, 0.2, 0.5, 1 and 1.5% of silver neutralized at 37 °C.

exhibit more than one band, depending on their shape (Pal *et al.*, 2007). Spherical metallic nanoparticles give a symmetrical intense band close to 400 nm, whereas the presence of silver oxide in metallic nanoparticles gives broader, less intense bands that shift to red (Yin *et al.*, 2002). In this work, the absence of well-defined bands could also be associated with the formation of silver oxide during neutralization with sodium hydroxide. The typical band associated with the SPR of silver nanoparticles is not observed in the UV−visible spectra of films with 0.1 and 0.2% of silver, which is probably due to the formation of small nanoparticles greatly dispersed in the matrix.

3.6. Structural characterization

The crystal structure of CS films neutralized at 37 ℃ was determined by XRD. **Figure 4.2.6** shows the XRD patterns of films incorporating different amounts of silver and neutralized at 37 ℃ and also the powder diffraction pattern of metallic silver and silver oxide. The typical XRD pattern of commercial silver nanoparticle powder (diameter < 100 nm) includes four diffraction peaks at 2θ of 38.4°, 44.5°, 64.7°, and 77.6°, corresponding respectively to the [111], [200], [220], and [311] planes of the facecentered cubic (fcc) structure of silver. The powdered silver oxide sample possesses a simple cubic structure with diffraction peaks at 2θ of 32.7°, 38°, 54.7°, and 65.2°, assigned to the reflections from the [111], [200], [220], and [311] planes.

As **Figure 4.2.6** shows, the XRD pattern of the neutralized CS film has a broad peak at 2θ of 20°, indicating low levels of crystallinity, and this peak was also observed in films neutralized at 37 ℃. Only one diffraction peak of

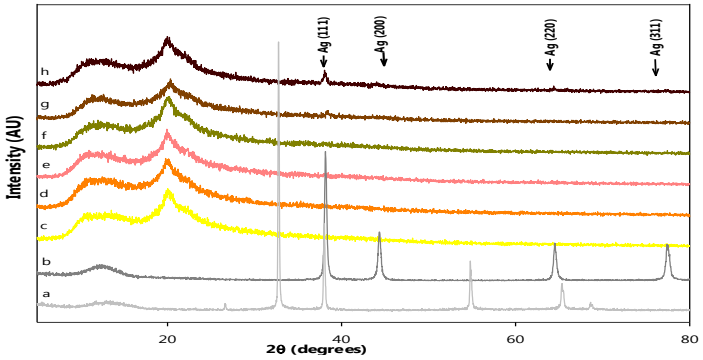

Figure 4.2.6. X-ray diffraction spectra: (a) silver oxide; (b) silver; (c) CS neutralized at 37 ℃; (d−h) CS neutralized at 37 ℃ with 0.1% (d), 0.2% (e), 0.5% (f), 1% (g), and 1.5% (h) of silver.

low intensity, at 2θ = 38.04°, was found in films containing 1.5% of silver. This diffraction peak might be associated with the [111] plane of metallic silver or the [200] plane corresponding to powdered silver oxide, because their positions are very close. However, the greatest intensity peak, at 2K = 32.7°, corresponding to the [111] reflection plane of powdered silver oxide, was not observed. In fact, faint, diffuse peaks associated with the crystalline structure of metallic silver can hardly be seen. The shape of these peaks suggests the presence of small crystalline silver nanoparticles.

3.7. Antimicrobial activity of films neutralized at 37 ℃

The antimicrobial capacity of the resulting films neutralized at 37 ℃ was evaluated against S. *aureus* and *E. coli*. It is well known that CS with deprotonated amino groups does not exhibit antimicrobial activity (Shahidi *et al.*, 1999). In this work, neutralized CS films without silver acted as a control. Thus, the antimicrobial properties of the films were expected to be due to the release of silver ions from the nanoparticles embedded in the CS matrix, which acted as a support for silver-based nanoparticles.

Table 4.2.2 shows the antimicrobial capacity of films neutralized at 37 ℃ with a silver concentration ranging from 0.1 to 1.5% against the pathogen microorganisms *S. aureus* and *E. coli*, using MHB or 1:125 diluted MHB as culture medium. It is noteworthy that the antimicrobial capacity of the films did not change after autoclaving. Some authors have reported that hydrothermal treatments such as autoclaving can produce nanoparticles from silver ions (Lu *et al.*, 2011; Zou *et al.*, 2007). In our experiment, nanoparticles were supposed to be generated during the neutralization step, without free silver ions remaining in the matrix.

When MHB was used, films containing 0.1% silver produced reductions of 0.62 log for *S. aureus* and 0.88 against *E. coli*, whereas for higher silver concentrations the growth of microorganisms was reduced by about 2 log. The antimicrobial activity of the films increased significantly when microbiological tests were carried out in 1:125 diluted MHB, giving an approximate reduction of 3 log cycles of viable cells for each microorganism tested. The lower antimicrobial capacity of the films in MHB compared with the films in diluted MHB might be due to excess proteins in the culture

Table 4.2.2. Antimicrobial activity of non-autoclaved and autoclaved (AUTO-) CS films neutralized at 37 ℃ against *S. aureus* and *E. coli* in Mueller Hinton Broth (MHB) and diluted MHB (1:125).

Bacterial count (Log CFU/mL)	Ag (%)	MHB	AUTO-MHB	MHB (1:125)	AUTO-MHB(1:125)
S. aureus	Control	8.34 ± 0.37[a]	8.34 ± 0.37[a]	7.45 ± 0.23[a]	7.45 ± 0.23[a]
	0.1	7.65 ± 0.52[b]	7.49 ± 0.21[b]	4.78 ± 0.69[b]	4.97 ± 0.03[b]
	0.2	5.74 ± 0.40[c]	6.52 ± 0.17[c]	4.50 ± 0.17[b]	4.74 ± 0.10[b]
	0.5	6.44 ± 0.24[c]	6.32 ± 0.06[c]	4.25 ± 0.26[b]	4.60 ± 0.12[b]
	1.0	5.79 ± 0.40[c]	6.35 ± 0.11[c]	4.29 ± 0.13[b]	4.53 ± 0.29[b]
	1.5	6.25 ± 0.19[c]	6.20 ± 0.20[c]	4.28 ± 0.21[b]	4.31 ± 0.45[b]
E. coli	Control	8.16 ± 0.14[a]	8.16 ± 0.14[a]	7.31 ± 0.54[a]	7.31 ± 0.54[a]
	0.1	7.28 ± 0.09[b]	7.42 ± 0.14[b]	4.90 ± 0.00[b]	5.10 ± 0.25[b]
	0.2	6.78 ± 0.02[c]	6.29 ± 0.03[c]	4.32 ± 0.32[b]	4.28 ± 0.44[b]
	0.5	6.26 ± 0.05[c]	5.33 ± 0.06[c]	3.57 ± 0.30[b]	4.12 ± 0.30[b]
	1.0	6.44 ± 0.08[c]	5.52 ± 0.04[c]	3.79 ± 0.70[b]	3.89 ± 0.05[b]
	1.5	6.02 ± 0.19[c]	6.18 ± 0.36[c]	3.89 ± 0.29[b]	4.08 ± 0.19[b]

[a-c] Values within a column followed by a different lower-case letter are significantly different from each other comparing different amounts of silver in the films against *S. aureus* or *E. coli* (Tukey's adjusted analysis of variance $P<0.05$).

media chelating the released silver ions. This could decrease the availability of free silver ions to exert antimicrobial activity.

Although we observed a slight tendency for the antimicrobial activity of the films to increase as their silver concentration increased, no significant differences were found in the antimicrobial capacity of films incorporating different amounts of silver ranging from 0.1 to 1.5% in MHB diluted to 1:125. This behaviour might be due to a similar quantity of silver ion migrating to the medium. To confirm this hypothesis, the silver concentration in diluted MHB after 18 h of being in contact with films incorporating different amounts of silver was evaluated by GFAAS. The release of silver proved to be similar in all of the samples. The silver concentration in the medium ranged from 135 to 150 µg/L, and there were no differences ($P \geq 0.05$) in migration values between films of different silver concentrations. Although the films released a similar amount of silver after 18 h of contact with the test environment, the kinetics of silver ion migration may differ between films and thus affect their antimicrobial activity. This might explain slight but nonsignificant differences in the antimicrobial effectiveness of the films.

At present, the mechanism of action of silver is not clear. When silver ions are inside the bacterial cell, this causes condensation of the DNA molecule, which loses its ability to replicate, thus affecting cell viability (Feng *et al.*, 2000). The silver ions also interact with thiol groups of proteins, causing bacterial enzyme inactivation (Liau *et al.*, 1997). The entry of silver ions through cell walls can cause deposition of proteins in cells (Feng *et al.*, 2000). Other authors claim that silver ions affect only the membrane surface, activating a bacterial defence mechanism (Morones *et al.*, 2005). The presence of silver ions has an antimicrobial effect, but some authors also suggest that small nanoparticles might be bactericidal (Kong and Jang, 2008; Lok *et al.*, 2006; Sondi and Salopek-Sondi, 2004). The mechanism of action by which silver nanoparticles have an antimicrobial effect is mainly due to their adhesion to the cell membrane, altering its permeability and attacking the respiratory chain (Kvitek *et al.*, 2008), but they can also penetrate inside bacteria and release silver ions, which interact with thiol groups and/or phosphates of compounds such as bacterial DNA or protein. Silver nanoparticles show a clear antimicrobial capacity compared with silver salts because of their high specific surface, which allows a greater area of contact

with microorganisms. Studies show that the antibacterial effect of silver nanoparticles depends on their size, with those between 1 and 10 nm presenting a more direct interaction with bacteria (Morones *et al.*, 2005).

In this work, we did not expect migration of nanoparticles from the film, given their confinement in the CS matrix and the difficulty for them to diffuse through the polymer. However, generation of silver ions on the surface of the nanoparticles embedded in the CS matrix is expected. Diffusion of silver ions through the polymer matrix has been probed, this being encouraged by the hydrophilic nature of CS. Swelling of CS by water facilitates the mobility of the polymer chains and therefore the transport of ions through the matrix to the release media.

Table 4.2.2 shows that the films had a slightly higher antimicrobial capacity against *E. coli* than against *S. aureus*. Studies by TEM energy dispersive X-ray showed that the morphological changes that occur in the internal structure of the cell are similar in both types of bacteria, such that it loses its ability to replicate and proteins are inactivated by interaction with silver ions (Feng *et al.*, 2000). However, although similar morphological changes were observed in both organisms, they were less marked in *S. aureus*, which is attributed to differences in the cell wall of the two microorganisms. In this case *S. aureus* has a more effective defence system than *E. coli* because Gram-positive cell walls have a thicker peptidoglycan layer than Gram-negative cell walls. It has been reported in the bibliography that the peptidoglycan cell wall has a greater capacity to protect the cell from the penetration of silver ions and small nanoparticles into the cytoplasm (Rai *et al.*, 2009).

3.8. Antimicrobial activity of films neutralized at 37 ℃ in liquid media over time

Three studies were carried out to determine the transfer of silver to the media. In the first study, we studied how the immersion time of the film in the growth medium prior to inoculation with bacteria affects its antimicrobial activity. For this purpose, films with a silver concentration of 0.2% were immersed in 1:125 diluted MHB and stored in an incubator at 37 ℃ for 1, 2, 10, 24, 48, 168 and 360 h prior to inoculation of the microorganism. **Table 4.2.3** shows the antimicrobial activity of diluted MHB

Table 4.2.3. Antimicrobial activity of diluted MHB (1:125) which has been in contact with films incorporating 0.2 % of silver for several times.

S. aureus	
Time (h)	**Bacterial count (Log CFU/mL)**
0	7.02 ± 0.30[a]
1	3.63 ± 0.13[b]
2	3.70 ± 0.71[b]
10	3.22 ± 0.17[b]
24	3.50 ± 0.06[b]
48	3.50 ± 0.16[b]
168	3.36 ± 0.09[b]
360	3.19 ± 0.32[b]

[a-b] Values within a column followed by a different lower-case letter are significantly different from each other (Tukey's adjusted analysis of variance $P<0.05$).

(1:125) against *S. aureus* after being in contact with 0.2% silver films neutralized at 37 ℃ as a function of the immersion time. As can be seen, the antimicrobial effect of the films did not vary over time. After 24 and 360 h in contact with the culture medium, films showed log reductions of 3.38 and 3.80 log, respectively. According to the migration values obtained for these films, after 10 h of immersion in the medium, the amount of total silver (both ionic and elemental) in the medium increased with time: levels of 58, 60, 57, 135, 170, 256, and 354 µg/L silver were found in the growth medium after 1, 2, 10, 24, 48, 168, and 360 h, respectively. However, the antimicrobial capacities of the films did not change. These results could be explained by the fact that the amount of free silver ions available in the medium would be lower than the amount of total silver. Some of the migrating silver ions might be reduced to elemental silver over time or might not be available because of their interaction with proteins present in the medium.

In the second study, films with 0.2 and 1.5% silver were immersed in 1:125 diluted MHB, the medium being replaced with fresh medium every 24 h to avoid possible saturation of the system. The medium was collected at 1, 3, 5, 10, 15, and 30 days, and the antimicrobial activity was evaluated against *S. aureus*. After 30 days, the antimicrobial activity of the films was also evaluated. **Table 4.2.4** shows that the antimicrobial capacity of the culture medium where 0.2 or 1.5% silver films were immersed produced a reduction of 3.5 log, and this activity was maintained throughout the 30 days of the

Table 4.2.4. Antimicrobial activity of diluted MHB (1:125) which has been in contact with films containing 0.2 or 1.5% silver for 24 h being the medium replaced with fresh medium every 24 h for a total period of 30 days; and antimicrobial activity of these films after 30 days.

Bacterial count (Log CFU/mL)		
Control	7.39 ± 0.27[a]	
Time (days)	**0.2 % Ag**	**1.5 % Ag**
1	4.53 ± 0.31[b]	4.28 ± 0.21[b]
3	4.84 ± 0.26[b]	3.88 ± 0.13[b]
5	4.52 ± 0.41[b]	3.54 ± 0.65[b]
10	4.95 ± 0.95[b]	4.14 ± 0.76[b]
15	4.62 ± 0.45[b]	4.02 ± 0.01[b]
30	4.74 ± 0.35[b]	4.28 ± 0.33[b]
30 film	3.63 ± 0.24[c]	2.87 ± 0.04[c]

[a-c] Values within a column followed by a different lower-case letter are significantly different from each other (Tukey's adjusted analysis of variance $P<0.05$).

test. Films with 0.2 and 1.5% silver were tested after 30 days and produced an inhibition of 3.8 and 4.5 log, respectively. The antimicrobial activity of the films was slightly higher than that of the liquid. This could be explained by assuming that the films exert antimicrobial activity by the release of silver ions to the medium but also by direct contact of the film surface containing ionic silver with the microorganism. It is worth noting that the antimicrobial activity of 1.5% silver films which were in contact with the medium for 30 days was almost 1 log higher than that of fresh films. This result shows that the release of silver ions from the film had not slowed after 30 days. In addition, the immersion of 1.5% film in liquid medium for 30 days might promote the formation of a large amount of silver ions on the surface of the nanoparticles embedded in the CS matrix compared with the fresh films that were tested, giving rise to a greater migration of silver ions.

The third study was conducted to verify the long-term antimicrobial capacity of the films after immersion in liquid medium; 0.2 and 1.5% silver films neutralized at 37 ℃ were immersed in an excess of sterile distilled water at a temperature of 22 ℃. The water was replaced with fresh water every 3 days. The antimicrobial capacity of the films was tested at day 1 and after 1 month, and the results are shown in **Table 4.2.5**. The antimicrobial capacity of the films after 1 month of immersion in water remained constant for films containing 0.2% silver, whereas the films with a higher silver concentration showed a slight increase in activity. These results are similar to

Table 4.2.5. Antimicrobial effect of films neutralized at 37 °C with 0.2 and 1.5 % silver against *S. aureus* tested in diluted MHB (1:125) after different immersion times in distilled water.

Bacterial count (Log CFU/mL)		
Control	7.39 ± 0.27[a]	
Time (days)	**0.2 % Ag**	**1.5 % Ag**
1	4.50 ± 0.17[b]	4.28 ± 0.21[b]
30	4.46 ± 0.23[b]	3.31 ± 0.29[c]

[a-b] Values within a column followed by a different lower-case letter are significantly different from each other (Tukey's adjusted analysis of variance $P<0.05$).

those obtained in the experiment described above for the antimicrobial activity of 0.2 and 1.5% silver films after immersion in 1:125 diluted MHB for 30 days. Although the experimental conditions were different, both experiments show that films are capable of releasing silver ions after immersion in liquid medium and maintain their effectiveness over time. It is noteworthy that films with a greater silver concentration slightly increased their antimicrobial activity after 30 days of immersion in water compared with 0.2% silver films. This result might indicate that, although initially the films neutralized at 37 °C tested had similar antimicrobial activity, the films with a greater number of nanoparticles may have a greater number of silver ions available to exert their antimicrobial activity over time. These silver ions would be released after oxidation of silver from the surface of the nanoparticles over time.

In this work, a methodology using compounds of low toxicity has been developed to obtain silver-based nanoparticles embedded in a CS film. Silver nitrate was used as a precursor for the synthesis of nanoparticles, and sodium hydroxide accelerated the reduction rate of silver ions and the formation of nanoparticles during the neutralization step. However, the coexistence of elementary silver and silver oxide is expected, owing to the presence of sodium hydroxide. TEM images showed the formation of spherical nanoparticles in films incorporating 0.1 and 0.2% of silver and neutralized at 37 °C. Films with 0.5 to 1.5% silver presented two populations of nanoparticles, one comprising spherical nanoparticles of 5−10 nm in size and a second population of round-shaped, anisotropic nanoparticles with diameters ranging from 30 to 50 nm. Films neutralized at 37 °C incorporating different amounts of silver ranging from 0.2 to 1.5% released similar amounts of total silver to the culture medium after 18 h of immersion and

showed similar antimicrobial activity. The release of silver to the culture medium from films containing 0.2% silver neutralized at 37 ℃ was monitored for 360 h and showed an increase in the release of silver after the first 10 h of contact. However, the antimicrobial activity did not change. This could be explained by conversion of ionic silver to elemental silver or interaction with the proteins present in the culture medium. It has been shown that CS is capable of acting as a carrier of silver nanoparticles, allowing slow, extended release of silver ions in a liquid medium for 30 days and maintaining their antimicrobial activity. Moreover, films with a higher silver concentration can exert their antimicrobial activity for longer as they have a larger reservoir of silver ions in the form of silver-based nanoparticles. The developed films could be used in several fields such as medicine, pharmacy, and food packaging when a long-term antimicrobial effect is desired.

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4.3. Capítulo 3. Desarrollo de películas de quitosano con compuestos volátiles antimicrobianos anclados mediante un enlace covalente reversible y su aplicación en el envasado activo de alimentos

4.3.1. Estado de la cuestión

El cinamaldehído es un aldehído aromático α,β-insaturado componente del AE de canela con un amplio espectro antimicrobiano y aceptado como aromatizante. El cinamaldehído ha sido incluido en matrices biopoliméricas en diferentes estudios como agente activo para el envasado antimicrobiano de distintos grupos de alimentos (**Anexo A**). En muchos de estos trabajos (**Tabla 4.3.1**), dicho aldehído es incorporado a la disolución filmógena, previamente a la formación de la película, y debido a su elevada volatilidad se pierde en gran parte durante la etapa de evaporación del disolvente (Ben Arfa *et al.*, 2007). Una estrategia para disminuir las pérdidas de la biomolécula es formar complejos de inclusión con compuestos coadyuvantes como pueden ser las CDs (Brasil *et al.*, 2012; Sipahi *et al.*, 2013). No obstante, este proceso limita la cantidad de principio activo que se puede incluir en los polímeros, ya que los complejos de inclusión suelen presentar una estequiometria 1:1 ó 1:2. Otra forma de reducir las pérdidas del volátil durante el secado de las películas es mediante encapsulación en nanoliposomas (Makwana *et al.*, 2014). Sin embargo, existen otras estrategias más ventajosas que consisten en anclar químicamente el cinamaldehído a la

Tabla 4.3.1. Diversos biopolímeros con cinamaldehído para el envasado antimicrobiano de alimentos.

Biopolímero	Referencia
proteínas de soja	Ben Arfa *et al.* (2007); Gamage *et al.* (2009)
puré de manzana	Mild *et al.* (2011)
CS	Ouattara *et al.* (2000)
proteínas de soja/ proteínas de suero de leche/PVOH	Ouattara *et al.* (2001)
puré de manzana puré de hibisco puré de zanahoria	Ravishankar *et al.* (2012); Ravishankar *et al.* (2009)
alginato de calcio	Raybaudi-Massilia *et al.* (2008)
alginato de calcio/puré de manzana	Rojas-Graü *et al.* (2007)
celulosa	Sanla-Ead *et al.* (2012)

matriz mejorando las propiedades del biopolímero a la vez que le confieren capacidad antimicrobiana, como se observa en los trabajos de Balaguer *et al.* (2011a, b), Balaguer, López-Carballo, *et al.* (2013), Balaguer, Borne, *et al.* (2013) y Balaguer *et al.* (2014) en los que se modifican químicamente gliadinas con cinamaldehído o en el de Soliman *et al.* (2014) en el que se modifica zeína con el mismo fin.

El cinamaldehído, debido a su estructura, presenta una elevada reactividad. Su grupo carbonilo (C=O) proporciona un lugar de reacción para la adición nucleofílica, debido principalmente a la capacidad del oxígeno para aceptar una carga negativa. Por ello, es posible lograr la unión covalente del cinamaldehído con el CS, dando como resultado la formación de compuestos iminoderivados del CS del tipo *N*-acilado mediante la formación de la base de Schiff.

En la bibliografía, se han descrito diversas bases de Schiff formadas entre el CS y diferentes aldehídos, y más concretamente entre el CS y el cinamaldehído (Babu y Kannan, 2012; Badawy y Rabea, 2013, 2014; Han y Lin, 2012; Le y Dupuis, 2009). Sin embargo, en la mayor parte de estos casos la base de Schiff se emplea como puente entrecruzante, producto intermedio a cuaternizar, grupo protector en alguna etapa intermedia de otro proceso, modificación para desarrollar derivados quelantes para la sorción de metales, en la determinación de los grupos amino libre del CS...

Otros autores han estudiado la síntesis y caracterización de la base de Schiff a partir de diversos aldehídos, como se observa en la **Tabla 4.3.2**. No obstante, en dichos trabajos, la base de Schiff no forma parte de una matriz polimérica ni se estudia su reversibilidad para la liberación del agente antimicrobiano.

Muzzarelli y Ilari (1994) formaron la base de Schiff entre varios aldehídos (*o*-vainillina, vainillina, siringaldehído, veratraldehído, 3,4-dihidroxibenzaldehído) y el CS en disolución, obteniendo a continuación películas de características similares a la lignina al incorporar grupos metoxifenilo. Hirano y Hayashi (2003) prepararon, mediante la formación de una base de Schiff en disolución, fibras de CS modificado con cinamaldehído, 2-hexilcinamaldehído, jasminal, citronelal, citral, heptanal, *n*-nonanal, 1-decanal y 10-undecanal, como nuevos biomateriales perfumados

Tabla 4.3.2. Bases de Schiff sintetizadas y caracterizadas a partir de CS y diversos aldehídos.

Aldehído	Referencia
acetaldehído	Kurita *et al.* (2002)
4-anisaldehído	Tirkistani (1998)
benzaldehído y derivados	Tirkistani (1998)
cinamaldehído	Marin *et al.* (2014)
4-dimetilaminobenzaldehído	Tirkistani (1998)
formaldehído	Hirano *et al.* (1977); Kurita *et al.* (2002); Singh *et al.* (2006)
furfural	Hongliang *et al.* (2010)
glutaraldehído	Hirano *et al.* (1977)
pentanal	Kurita *et al.* (2002)
salicilaldehído	Dos Santos *et al.* (2005a, b); Guinesi y Cavalheiro (2006a, b)
vainillina y derivados	Sashikala y Syed Shafi (2014)

para su aplicación en distintos campos tales como filtros de aire, cosméticos y textiles. Sin embargo, en estos trabajos el aldehído se añadió a la disolución de CS lo que conduce, en muchos casos, a la gelificación o la disminución de la capacidad filmógena del CS (Muzzarelli y Ilari, 1994). Incluso Marin *et al.* (2012) apuntan que el rendimiento de la formación de la imina en la disolución acuosa ácida es bajo (1-12%) y rápidamente reversible, y obtienen elevados rendimientos de formación de la base de Schiff entre el CS en forma de hidrogel y el cinamaldehído (90%). Por esta razón, en la presente Tesis Doctoral se ha desarrollado una metodología en la que inicialmente se obtienen películas de CS que posteriormente se sumergen en una disolución etanólica del aldehído para que se forme el iminoderivado con la finalidad de obtener rendimientos elevados en la formación de la base de Schiff.

Moore y Roberts (1981) impregnaron películas de CS con los aldehídos: serie homóloga de acetaldehído a decanal; isobutiraldehído, isovaleraldehído, pivalaldehído; benzaldehído, salicilaldehído, *o*-, *m*-, y *p*-tolualdehído, *o*- y *p*-nitrobenzaldehído; y *p*-dimetilaminobenzaldehído. Pero, no estudiaron la potencial capacidad antimicrobiana de los biocompuestos y su aplicación para la conservación de alimentos. Hirano *et al.* (1999) anclaron formaldehído, glioxal, propional, butanal, benzaldehído, 2-hidroxibenzaldehído y vainillina a fibras ya preformadas de CS,

comprobando que las fibras mantenían el aroma tras 6 meses en un ambiente húmedo. Las fibras resultantes fueron estables en agua y soluciones acuosas alcalinas, pero inestables y solubles en soluciones acuosas ácidas. Sin embargo, no determinaron la liberación de dichos aldehídos a partir de las fibras, como consecuencia de la hidrólisis de la base de Schiff, no estudiaron la actividad antimicrobiana, ni lo aplicaron en alimentos.

Por otra parte, se han encontrado varios trabajos en los que se demuestran las propiedades antimicrobianas de las bases de Schiff desarrolladas (Guo *et al.*, 2007; Jin *et al.*, 2009, 2010; Kumar *et al.*, 2014; Mohamed y Fekry, 2011; Porras *et al.*, 2009; Wang *et al.*, 2012). Aunque, en todos ellos, la formación de la base de Schiff se produce en disolución y los estudios de la capacidad antimicrobiana se realizaron sobre los iminoderivados en forma de polvo, y no de película. Por otra parte, Marin *et al.* (2013), Marin *et al.* (2015) y Stroescu *et al.* (2015) obtuvieron iminoquitosanos a partir de varios aldehídos (benzaldehído, cinamaldehído, citral, salicilaldehído, vainillina…) para desarrollar películas antimicrobianas frente *Escherichia coli*, *Staphylococcus aureus* y *Candida albicans*, aunque como en los casos anteriores la formación del enlace imino se lleva a cabo en disolución.

En el presente trabajo se desarrollan y caracterizan matrices activas basadas en el anclaje de cinamaldehído en una película de CS preformada mediante la formación de una base de Schiff. El agente activo antimicrobiano puede ser liberado como respuesta a diferentes condiciones debido a la reversibilidad de la base de Schiff. Las películas desarrolladas se aplican como envase activo en leche y se estudia su efecto a nivel sensorial.

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4.3.2. Artículo científico 3.

Reversible covalent immobilization of cinnamaldehyde on chitosan films via Schiff base formation and their application in active food packaging

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Resumen

En este trabajo, el cinamaldehído fue reversiblemente anclado a películas de quitosano mediante la formación de un grupo imino. Esta base de Schiff se sintetizó en fase sólida, empleando películas de quitosano neutralizado inmersas en una solución etanólica al 95% (v/v) y acidificada en la que estaba disuelto el aldehído. El grado de sustitución (%) del grupo amino por cinamaldehído fue próximo al 70%. La espectroscopía de infrarrojos por transformada de Fourier con reflexión total atenuada (ATR-FTIR) reveló la formación de la base de Schiff entre el quitosano y el cinamaldehído. La hidrólisis del enlace imino y posterior liberación del cinamaldehído se estudiaron después que las películas fueron sometidas a diferentes tratamientos de temperatura/tiempo simulando los métodos de conservación de alimentos. La cantidad del aldehído que se mantuvo unido covalentemente a las películas se monitorizó por ATR-FTIR, y el grado de sustitución se determinó mediante análisis elemental. También se evaluaron ángulo de contacto y los parámetros de color de las películas de cinamaldehído-imina-quitosano, y de éstas sometidas a los diferentes tratamientos. Las propiedades antimicrobianas de películas de quitosano-base de Schiff se probaron *in vitro* frente a *Staphylococcus aureus* y *Escherichia coli*, y en leche inoculada con *Listeria monocytogenes*. La actividad antimicrobiana varió dependiendo del tratamiento aplicado, y por tanto del grado de hidrólisis del enlace imino y cinamaldehído liberado. Las películas de quitosano-base Schiff sometidas a diferentes tratamientos de tiempo/temperatura inhibieron el crecimiento de *L. monocytogenes* durante 12 días bajo condiciones de refrigeración y pueden extender la vida útil microbiológica de estos productos. El análisis sensorial de la leche en contacto con las películas mostró que el olor a canela no causa ningún rechazo entre los consumidores potenciales. Estas nuevas películas podrían ser utilizadas en el diseño de envases antimicrobianos alimentarios y en otras áreas tecnológicas donde se requieran sistemas de liberación sostenida.

Palabras clave

Quitosano, cinamaldehído, base de Schiff, hidrólisis, liberación, película antimicrobiana.

Abstract

In this work, cinnamaldehyde was reversibly anchored to chitosan (CS) films via imino-covalent bonding. The Schiff base was synthesized in solid phase employing neutralized chitosan films immersed in acidified 95% (v/v) ethanolic solution in which the aldehyde was dissolved. The substitution degree (%) of cinnamaldehyde to the amine group was close to 70%. Attenuated total reflectance–Fourier transform infrared spectroscopy (ATR-FTIR) analysis revealed the formation of the chitosan-cinnamaldehyde Schiff base. The hydrolysis of the imino bond and subsequent release of cinnamaldehyde were studied after the films had been subjected to different combinations of temperature/time treatments simulating food preservation methods. The amount of aldehyde that remained covalently attached to the films was monitored by ATR-FTIR, and the substitution degree was determined by elemental analysis. Surface contact angle and colour parameters of cinnamaldehyde-imine-chitosan films and these films subjected to different treatments were also evaluated. The antimicrobial properties of chitosan-Schiff base films were tested *in vitro* against *Staphylococcus aureus* and *Escherichia coli* and in milk inoculated with *Listeria monocytogenes*. The antimicrobial activity varied depending on the treatment applied and consequently the degree of imino bond hydrolysis achieved and cinnamaldehyde released. Films of Schiff base-chitosan derivative subjected to different time/temperature treatments inhibited the growth of *L. monocytogenes* for 12 days under refrigeration conditions, which may extend the microbiological shelf life of such products. Sensory analysis of milk in contact with the films showed that a cinnamon smell does not cause any rejection among potential consumers. These novel films could be used in the design of antimicrobial food packaging and in various other technological areas where sustained release systems are required.

Keywords

Chitosan, cinnamaldehyde, Schiff base, hydrolysis, release, antimicrobial films.

1. Introduction

CS is a natural, biocompatible, biodegradable, biorenewable and biofunctional polysaccharide that is finding attractive applications in several industrial areas. CS produces highly transparent grease-resistant films with excellent barrier properties to gases and aroma compounds. These properties make CS a good candidate for use in food packaging applications, providing a barrier layer to other polymer films and porous materials such as fibre-based paper (Gällstedt and Hedenqvist, 2006). In addition, amino and hydroxyl groups of CS can be used to modify the polymer chemically in order to provide it with new properties and widen its technological applications. Moreover, CS has been extensively studied as a carrier and system for sustained release of active compounds, and in this regard, it has been applied in a great variety of technological areas, such as agrochemistry, pharmacy, biomedicine, textiles, and active food packaging (Sashiwa and Aiba, 2004). The development of antimicrobial materials and their application in the design of active packaging is arousing considerable expectation in the food industry, since food safety is an area of great concern. Regarding CS films, many studies in the literature have endeavoured to use the antimicrobial properties of protonated CS polymer to form films for active food packaging applications. However, protonated films are water soluble, which limits their applications, whereas neutralized films lack antimicrobial properties. Other studies have focused on the development of antimicrobial films for food packaging applications by incorporating antimicrobial compounds in the film forming solution prior to film formation.

Usually, the active molecule is incorporated during film formation, but this process has certain drawbacks when working with volatile compounds because of the loss of volatiles during film processing.

In order to overcome this drawback, recent studies have reported the incorporation in CS of previously encapsulated essential oils (Abreu *et al.*, 2012; Higueras *et al.*, 2013; Hosseini *et al.*, 2013). There are some studies devoted to the synthesis of Schiff base from CS and the potential antimicrobial activity of the derivatives obtained (Dos Santos *et al.*, 2005; Guinesi and Cavalheiro, 2006; Guo *et al.*, 2007; Jin, 2010; Wang *et al.*, 2012). However, none of these studies focus on the reversibility of the Schiff base and its effect on the antimicrobial results obtained. Hydrolysis of the imino

bond (C=N) formed in the Schiff base can be promoted by temperature, UV light, pH, etc. or combinations of them (Huang *et al.*, 2001; Kirdant *et al.*, 2011; Li *et al.*, 2013). The formation of a reversible Schiff base in the backbone of polymer films could have great potential in the design of sustained-release systems.

Cinnamaldehyde is a naturally occurring aromatic α,β-unsaturated aldehyde derived from cinnamon and approved by the FAO/WHO Expert Committee on Food Additives (JECFA) for use as a food-flavouring agent, and it is the main component of cinnamon bark extract (Cocchiara *et al.*, 2005). Cinnamaldehyde is a well-known natural antimicrobial compound, active against a wide spectrum of food-borne pathogens (Holley and Patel, 2005). However, the organoleptic effect of essential oils is one of the most important factors that limit their application as antimicrobial agents to real food products, even though their antimicrobial efficiency has been widely described in *in vitro* tests (Belletti *et al.*, 2008). Therefore, any food application of this agent should consider the potential sensory impact, which could result in non-acceptance by the consumer.

The aim of this study was, firstly, to obtain and characterize homogeneous, transparent cinnamaldehyde-imino-chitosan films and to study the release of cinnamaldehyde via hydrolysis of the imino bond as affected by several combinations of time/temperature treatments. The antimicrobial properties of the films were then studied in vitro and in vivo against foodborne pathogens. The sensory impact of the films when applied to a food product was also evaluated.

2. Materials and methods

2.1. Materials

Low molecular weight chitosan with a degree of acetylation of 15–25% and sodium peroxide were supplied by Sigma (Barcelona, Spain). Trans-cinnamaldehyde and acetic acid were provided by Aldrich (Steinheim, Germany). Sodium hydroxide and ethanol 96% (v/v) were purchased from Panreac (Barcelona, Spain) and hydrochloric acid 37% from Merck (Darmstadt,Germany). Ortho-phosphoric acid/sodium hydroxide pH 3 buffer and potassium dihydrogen phosphate/disodium hydrogen phosphate pH 7

buffer were purchased from Scharlab (Barcelona, Spain). Water was obtained from a Milli-Q Plus purification system (Millipore, Molsheim, France).

2.2. Film preparation

2.2.1. Chitosan films

A 1.5% CS (w/w) solution was solubilised in 0.5% (w/w) acetic acid aqueous solution and filtered to eliminate impurities. CS acetate films with 55±5-µm average thickness were obtained by casting on PS plates dried at 37 ℃ for 48 h and 22% RH. CS acetate films were neutralized with 0.1 M sodium hydroxide for 24 h at 37 ℃ to make them insoluble in water. After neutralization, the CS films were washed with deionised water and dried at 37 ℃. The films were cut into 1.7×1.7-cm samples and stored in amber glass desiccators prior to use at 23 ℃ and with P_2O_5 to achieve a RH close to zero in order to avoid the presence of moisture since this can promote Schiff base hydrolysis during the storage and consequently the release of cinnamaldehyde from the films.

2.2.2. Schiff base formation

A cinnamaldehyde solution was prepared by adding 4 g of cinnamaldehyde to 75 mL of ethanol 96% (v/v). Then, neutralized CS films (2 g) were immersed in the solution and kept in a shaking bath at 60 ℃ for 24 h. After that time, the samples were washed by dipping and shaking them in ethanol 96% (v/v) for 24 h at room temperature, and this process was performed three times. Finally, chitosan-cinnamaldehyde Schiff base polymer films (CScin) were stored in an amber glass desiccator with P_2O_5 at 23 ℃ until use. The film thickness was measured individually with a digital Mitutoyo micrometre (Metrotec, San Sebastian, Spain).

2.2.3. Treatment of CScin films at different combinations of temperature/time treatments

With the purpose of promoting hydrolysis of the imino bond in the CScin films and thus the release of cinnamaldehyde, films were subjected to different combinations of temperature/ time treatments simulating different food preservation processes. For this purpose, film samples (0.25 g) were

placed in a glass vial with 10 mL of Mueller Hinton broth (MHB) (Scharlab, Barcelona, Spain) buffer solution at pH 7 or pasteurized whole milk. The vials were then subjected to different treatments: (a) 30 min at 4 ℃ in a cooling chamber to simulate refrigeration conditions; (b) 30 min at 65 ℃, 15 min at 72 ℃ or 10 min at 95 ℃ in a thermostatic bath with agitation to simulate pasteurization treatments; and (c) 5 min at 121 ℃ in an autoclave to simulate retorting processes.

2.3. Characterization of CScin films before and after imino bond hydrolysis

2.3.1. Elemental analysis

The substitution degree (%) of cinnamaldehyde to the amino group in the CS films was determined by measuring the C/N ratio of dry samples. The analysis was performed with a CHNS-O elemental analyser (CE Instruments EA 1110, Thermo Fisher Scientific, Waltham, MA, USA). Samples were analysed in triplicate. Results are expressed as average value±standard deviation.

2.3.2. Optical properties

Colour coordinates of the various CS-based films were measured with a CR-300 Minolta Chroma meter® (Minolta Camera Co. Ltd., Osaka, Japan). The film samples were placed on a standard white plate; the results were expressed in accordance with the CIELAB system with reference to illuminant D65 and a visual angle of 10°. The measurements were performed through a 6.4-mm-diameter diaphragm containing an optical glass, monitoring L* (lightness), a*, b* and calculating chroma ($C^*_{ab}=(a^{*2}+b^{*2})^{1/2}$) and hue ($h_{ab}$=arctan (b*/a*)). Films were measured in triplicate, and eight measurements were taken at different locations of each sample.

2.3.3. Water uptake and dimensional stability

Film specimens (1.7×1.7 cm) comprising CS and CScin films subjected to different treatments were immersed in aqueous solution buffered at pH 3 or pH 7 for 24 h at 23 ℃. After that time, the films were removed from the water, wiped off with a paper towel and immediately weighed (final wet

weight, w_w^f). The samples were replaced in the desiccator until they reached a constant weight (final dry weight, w_d^f). The percentage of water uptake (ΔW) was calculated as follows:

$$\Delta W\ (\%) = \frac{W_w^f - W_d^f}{W_d^f} \cdot 100 \quad (1)$$

The dimensional stability of the films was calculated similarly by measuring the film surface area after removing the film from the water and drying it. The increase was taken as the dimensional stability of the film in buffered solution. The experiment was performed in triplicate.

2.3.4. Contact angle (CA)

After the films had been neutralized (CS films), modified (CScin films) and subjected to different treatments, they were left under pressure between two glass sheets for 48 h to increase film flatness and then stored for 48 h in a desiccator with P_2O_5. The CA was measured using an OCA 15EC goniometer (DataPhysics Instruments GmbH, Filderstadt, Germany). A 2-µL water droplet was dispensed onto the sample surface, and the drop image was recorded for 2 min. The CA at 60 s was estimated by using the SCA20 embedded software module. The experiment was performed in triplicate.

2.3.5. Attenuated total reflectance–Fourier transform infrared spectroscopy (ATR-FTIR)

The films were analysed by ATR-FTIR. Dry samples were placed in a Golden Gate single reflection diamond ATR accessory (Teknokroma, Barcelona, Spain), and the spectra were recorded with a Bruker Tensor 27 FTIR spectrometer (Bruker Española S.A., Barcelona, Spain). The resolution was 4 cm^{-1} in the range of 4000 to 600 cm^{-1} and 128 scans were recorded per test. Results were recorded in triplicate and analysed with the OPUS v.2.06 software.

2.4. Imino bond hydrolysis and cinnamaldehyde release

After each temperature/time treatment, the amount of cinnamaldehyde released in MHB owing to hydrolysis of the CS-cinnamaldehyde Schiff base was determined. For this purpose, immediately after each treatment, the liquid medium was transferred to a quartz cuvette,

and the amount of cinnamaldehyde was measured by UV–vis spectroscopy at 221 nm using an Agilent 8453 UV–visible spectrophotometer (Agilent, Barcelona, Spain).

Additionally, the release of cinnamaldehyde was determined by measuring specific migration from the polymer into ethanol 50%, a fatty food simulant specified in European law (EC Regulation 10/2011). A 1.7×1.7-cm film sample was placed in a glass vial with 7 mL of ethanol 50%, and the vial was closed tightly with a PTFE septum and aluminium caps to constitute a sample. After the various treatments ('Treatment of CScin films at different combinations of temperature/time treatments' section), yielding three vial samples per treatment and exposure time (5, 10, 15 and 30 min and 1, 8, 24 and 48 h), the vials were opened and the content of cinnamaldehyde in the liquid quantified by UV–vis spectroscopy at 221 nm. Three vial samples per treatment and exposure time were analysed.

2.5. Antimicrobial assays

2.5.1. Culture strains

Staphylococcus aureus CECT 86, *Escherichia coli* CECT 434 and *Listeria monocytogenes* CECT 934 were obtained from the Spanish Type Culture Collection (Valencia, Spain). Strains were stored in Tryptone Soy Broth (TSB, Scharlab, Barcelona, Spain) with 20% G at −80 ℃ until needed. For experimental use, the stock cultures were maintained by regular subculture on agar Tryptone Soy Agar (TSA, Scharlab, Barcelona, Spain) slants at 4 ℃ and transferred monthly.

2.5.2. In vitro *antimicrobial activity of CScin films against* S. aureus *and* E. coli

Before analysis, a loopful of each strain was transferred to 10 mL of TSB and incubated at 37 ℃ for 18 h to obtain early stationary phase cells. Cell cultures of each microorganism in stationary phase, with an optical density of 0.9 at 600 nm, were diluted in TSB and incubated at 37 ℃ until exponential phase with an optical density of 0.2 at 600 nm (10^5 colony forming units (CFU)/mL). A sample of 0.25 g of CScin film was placed in contact with 10 mL of MHB and subjected to different temperature/time

treatments (as described above). Neutralized CS films were used as controls and also underwent these treatments. After each treatment, the liquid medium was recovered and was allowed to reach room temperature. Then, 100 µL of cell culture in exponential phase (10^5 CFU/mL) was added and the tubes were incubated at 37 ºC for 18 h. Depending on the turbidity of the tubes, serial dilutions with peptone water were carried out and plated in Petri dishes with 15 mL of TSA culture medium. Colonies were counted after incubation at 37 ºC for 18 h. The result was expressed in log of CFU per millilitre. All analyses were carried out in triplicate.

2.5.3. Antimicrobial assays in milk

The antimicrobial activity of the films was tested in commercial pasteurized cows' milk. For this purpose, the procedure described in '*In vitro* antimicrobial activity of CScin films against *S. aureus* and *E. coli*' section was followed, using milk instead of MHB and inoculating *L. monocytogenes* in exponential phase. Sterilized tubes with 10 mL of milk were inoculated in sterilized conditions with 100 µL of *L. monocytogenes* in exponential phase (10^5 CFU/mL). The tubes were then kept at 4 ºC for 12 days, and antimicrobial assays were performed on days 3, 6 and 12. Serial dilutions with peptone water were made and plated in PALCAM Listeria selective agar (Merck, Darmstadt, Germany). Plates were incubated at 37 ºC for 48 h. All experiments were carried out in triplicate.

2.6. Sensory analysis

Sensory tests on commercial pasteurized cow milk that had been exposed to the films and subjected to the time/temperature treatments were carried out on the 3rd, 6th and 12th days by an untrained panel (44 judges). The tests were done in a standardized test room (ISO 8589-2007). Samples of milk were placed in hermetic sealed transparent tubes and identified by three-digit codes. The panel members were asked to smell the sample and describe the intensity of the perceived cinnamon aroma and preference in terms of smell. The odour intensity was indicated on a 1 to 5 scale in which 1 was the lowest cinnamon odour intensity and 5 the most intense. For the preference test, the samples were ordered from 1 to 5, 1 to assign the greatest acceptance of sample and 5 the lowest. Data analysis was

performed with the Compusense® five program, release 5.0 (Compusense Inc., Guelph, Ontario, Canada).

2.7. Data analyses

Statistical tests were performed using the SPSS® Statistics computer program, version 19.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance was carried out. Differences between pairs of means were assessed on the basis of confidence intervals using the Tukey-*b* test. Moreover, comparisons between two samples were analysed by Student's t test. The level of significance was $P \leq 0.05$. The data are represented as average± standard deviation. The data were analysed and plotted using the SigmaPlot 10.0 software (Systat Software Inc., Richmond, CA, USA).

3. Results and discussion

The unmodified CS films were transparent, without discontinuities and with an average thickness of 55±5 µm. **Figure 4.3.1** shows the formation of CS-cinnamaldehyde Schiff base. Covalent bonding of the volatile aldehyde to the backbone of the polymer stabilizes the molecule, avoiding losses during processing and storage of the polymer film. Since the compound is reversibly attached to the polymer, it can be liberated through hydrolysis of the imino bond. Bond cleavage in aqueous or humid media can be promoted by several factors, such as light, pH, temperature, etc. Therefore, when the film is used to package food, the packaged food processing treatments and/or the conditions inside the package during storage can activate or extend the release of the molecule to exert its effect. In this connection, Schiff base can act as a stimuli-responsive linker, providing a means of obtaining antimicrobial release systems with potential applications in active packaging of foods.

Synthesis of Schiff base derivative was confirmed by ATR-FTIR and the substitution degree was evaluated. To the naked eye, the films maintained their transparency and acquired a light yellow colour owing to the presence of a conjugated double bond after Schiff base formation. CScin films were subjected to various food preservation processes consisting of different temperature/time combinations, after which the films were characterized and the cinnamaldehyde released was evaluated.

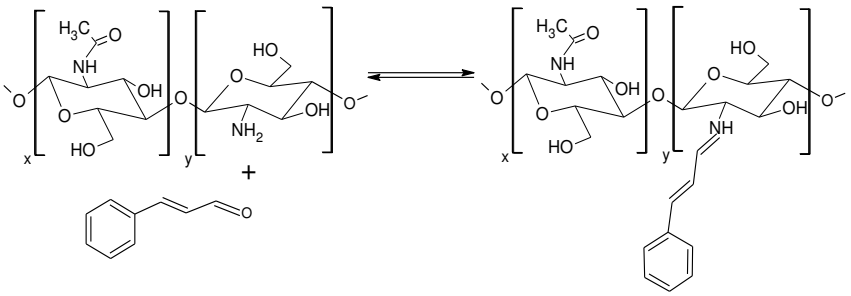

Figure 4.3.1. Nucleophilic addition of amino group of CS backbone to carbonyl group of cinnamaldehyde and formation of Schiff base.

3.1. Characterization of chitosan films modified with cinnamaldehyde

3.1.1. Elemental analysis

The elemental composition of CS films before and after Schiff base formation, and after the various preservation processes, is shown in **Table 4.3.3**. The degree of acetylation (*DA*) for the untreated sample was calculated with the following equation (Kasaai *et al.*, 1999):

$$DA = \frac{\left(C/N\right) - 5.145}{6.861\text{-}5.145} \cdot 100 \qquad (2)$$

where (*C/N*) is the carbon/nitrogen ratio. The degree of acetylation of CS was 20.3%, which was in agreement with the value given by the supplier for low molecular weight CS (15–25% degree of acetylation).

The degree of substitution (*DS*), defined as the ratio of Schiff base units in relation to free amino groups in CS, was estimated following the work of Inukai *et al.* (1998):

$$DS = \frac{\left(C/N\right) - \left(C/N\right)_0}{n} \qquad (3)$$

where (*C/N*) is the carbon-to-nitrogen ratio of the CS derivative, $(C/N)_0$ is the carbon-to-nitrogen ratio of CS and *n* is the number of carbon introduced into the modified CS.

Table 4.3.3 shows the *DS* (in percentage) of CS films after Schiff base linkage and of those films after being subjected to different temperature/time treatments. The *DS* value indicated a high conversion of amino groups into imine linkages. After the films had been subjected to

Table 4.3.3. Elemental analysis and substitution degree (%) of films.

Films	N (%)	C (%)	H (%)	Substitution degree (%)
CS	7.33 ± 0.07^{d}	40.22 ± 0.07^{a}	7.18 ± 0.04^{d}	
CScin	6.03 ± 0.04^{b,c}	53.93 ± 0.08^{e}	6.64 ± 0.01^{a}	72.11 ± 0.20^{a}
CScin-4℃/30min	5.58 ± 0.30^{a}	51.48 ± 0.19^{b}	6.77 ± 0.04^{c}	65.46 ± 0.59^{b}
CScin-65℃/30min	5.76 ± 0.27^{a,b}	52.73 ± 0.41^{c}	6.80 ± 0.05^{b}	65.09 ± 0.78^{b}
CScin-72℃/15min	5.97 ± 0.09^{a,b,c}	53.07 ± 0.11^{c,d}	6.75 ± 0.03^{c}	59.32 ± 0.28^{c}
CScin-95℃/10min	6.15 ± 0.16^{b,c}	51.82 ± 0.45^{b}	6.92 ± 0.06^{b}	53.55 ± 0.66^{d}
CScin-121℃/5min	6.29 ± 0.06^{c}	53.37 ± 0.03^{d,e}	6.95 ± 0.09^{b}	52.17 ± 0.17^{e}

$^{a-e}$ Different letters in the same column indicate a statistically significant difference ($P \leq 0.05$).

different treatments, the *DS* values experienced a reduction, which was more acute for films treated at greater temperatures. It is noteworthy that even after a retorting-like process, more than 50% of bonded cinnamaldehyde remained in the film, implying a large reservoir of cinnamaldehyde.

3.1.2. Optical properties

Table 4.3.4 shows the film colour coordinates, L*, a* and b*, and the chroma (C_{ab}*) and hue (h_{ab}) of CS and CScin films and also CScin films subjected to different temperature/time treatments. The formation of an unsaturated Schiff base system (C=C−C=N) from α,β-unsaturated cinnamaldehyde caused the films to acquire a vivid yellow colour, as shown by a decrease in the hue and an increase in the chroma values. This has also been reported for other α,β-unsaturated aldehydes, such as citral (Jin *et al.*, 2009).

The hue of the CScin films decreased significantly when subjected to thermal treatments of 95 ℃ for 10 min and 121 ℃ for 5 min, acquiring an orange-red colour. These changes in colour can be explained by the formation of Maillard reaction products when CS with amine and ketone

Table 4.3.4. Colour parameters of films.

Films	L*	a*	b*	C_{ab}*	h_{ab}
CS	91.5 ± 0.2^{a}	-1.9 ± 0.1^{a}	9.0 ± 0.2^{a}	9.2 ± 0.2^{a}	101.8 ± 0.1^{a}
CScin	74.2 ± 0.4^{b}	13.4 ± 0.1^{b}	86.6 ± 1.2^{b}	87.6 ± 1.2^{b}	81.2 ± 0.1^{b}
CScin-4℃/30min	73.5 ± 0.2^{b}	14.4 ± 0.3^{b}	87.7 ± 0.1^{b}	88.8 ± 0.3^{b}	80.7 ± 0.2^{b,c}
CScin-65℃/30min	74.0 ± 0.9^{b}	14.5 ± 1.0^{b}	88.2 ± 1.5^{b}	89.4 ± 1.3^{b}	80.7 ± 0.7^{b,c}
CScin-72℃/30min	72.2 ± 1.5^{c}	16.2 ± 0.5^{c}	90.4 ± 1.1^{c}	91.9 ± 1.0^{c}	79.9 ± 1.0^{c}
CScin-95℃/10min	69.1 ± 0.8^{d}	20.2 ± 1.4^{d}	94.0 ± 1.1^{d}	96.2 ± 0.8^{d}	77.9 ± 0.9^{d}
CScin-121℃/5min	53.2 ± 0.8^{e}	38.7 ± 0.6^{e}	73.3 ± 0.2^{e}	82.9 ± 0.1^{e}	62.2 ± 0.4^{e}

$^{a-e}$ Different letters in the same column indicate a statistically significant difference ($P \leq 0.05$).

groups is exposed to high temperatures. Regarding the chroma values, no differences were observed between the CScin films and those subjected to low-temperature treatments (4 and 65 ºC). The C_{ab}* of films treated at higher temperatures, 72 ºC for 15 min, 95 ºC for 10 min and 121 ºC for 5 min, increased significantly. Films treated at 72 and 95 ºC showed a higher colour intensity than CScin films. However, this parameter decreased for films treated at 121 ºC for 5 min. This behaviour could be related to a higher release of cinnamaldehyde from the film during the treatment as a consequence of Schiff base hydrolysis in combination with Maillard formation products.

The L* (lightness) parameter was used to measure the transparency of the films. CS films have high transparency, which was significantly reduced after Schiff base formation. This decrease became greater as the temperature of the treatment increased. A decrease in the L* parameter was also observed after heating CS films, which had reduced lightness and acquired a vivid yellow colour. Retorted CS film presented the following colour parameters: L*=88.6±0.8; a*=2.38±0.1; b*=23.65±1.2; C_{ab}*=23.7±1.1; and h_{ab}=84.2±0.4 (results not included in **Table 4.3.4**).

3.1.3. Water uptake and dimensional stability

CS is a hydrophilic polymer that presents a high water retention capacity. Therefore, the effect of grafting cinnamaldehyde on CS films on the water sorption and dimensional stability of the resulting films and also of films subjected to preservation treatments were studied. For this purpose, films were immersed in two buffered media at pH 3 and 7 for 24 h. The results are shown in **Table 4.3.5**. The pK_a of CS is 6.5–6.3; at lower pH values, protonation of amino groups increases water uptake and swelling properties of the film until complete protonation of amino groups is achieved and the film dissolves. As the results in **Table 4.3.5** show, CS films absorb a great amount of water at pH 3 since high ionization of amino groups produces electrostatic repulsion between polymer segments, allowing film swelling and large water gain, which subsequently practically doubled their surface area. Grafting of cinnamaldehyde to CS films did not modify water uptake and dimensional stability when parameters were measured at pH 3. At this pH, a high degree of protonation is achieved. Acidic pHs favour Schiff base

Table 4.3.5. Water uptake and dimensional stability of films.

Films	pH 3		pH 7	
	Water uptake (%)	Area increase (%)	Water uptake (%)	Area increase (%)
CS	231.7 ± 1.6[a]	119.9 ± 3.4[a]	155.8 ± 0.9[a]	99.8 ± 1.9[a]
CScin	235.2 ± 6.2[a]	116.7 ± 5.3[a]	36.9 ± 0.9[b]	15.4 ± 1.6[b]
CScin-4ºC/30min	237.1 ± 6.1[a]	110.7 ± 5.9[a]	29.1 ± 0.4[b]	15.8 ± 1.1[b]
CScin-65ºC/30min	166.8 ± 13.6[b]	88.3 ± 10.9[b]	30.1 ± 1.2[b]	17.8 ± 3.6[b]
CScin-72ºC/15min	165.6 ± 10.3[b]	81.3 ± 14.3[b]	31.4 ± 1.5[b]	17.8 ± 4.1[b]
CScin-95ºC/10min	167.3 ± 13.6[b]	78.3 ± 10.4[b]	31.3 ± 1.5[b]	17.7 ± 3.1[b]
CScin-121ºC/5min	91.5 ± 14.2[c]	52.4 ± 6.7[c]	32.1 ± 1.6[b]	17.9 ± 4.6[b]

[a-c] Different letters in the same column indicate a statistically significant difference ($P \leq 0.05$).

hydrolysis, releasing cinnamaldehyde and providing more free amino groups for protonation. These two facts could explain the absence of differences between CS, CScin and CScin 4 ºC for 30 min films. CScin films subjected to thermal treatment had reduced water uptake capacity and increased dimensional stability. The water uptake and area of films treated at 65 ºC for 30 min, 72 ºC for 15 min and 95 ºC for 5 min were reduced by around 30 and 65%, respectively, whereas films treated at 120 ºC for 5 min experienced a reduction in water uptake of approx. 68% and the area decreased by 79%. These results may be due to thermal crosslinking of the CS matrix, giving rise to a more compact film structure (Ji and Shi, 2013).

The films presented much lower water sorption values at pH 7 than in acidic conditions. Water uptake of neutralized CS films immersed in buffered solution at pH 7 was reduced by approx. 33% with respect to the same films immersed in acidic solution, whereas surface area was reduced by approx. 17% (**Table 4.3.5**). The decrease in water uptake is due to the unprotonated state of the amino groups (pH>pK_a of CS). When cinnamaldehyde was attached to amino groups of CS films, water uptake and surface area were reduced by approx. 75 and 35%, respectively, compared with the values for CS films immersed in the same buffer solution. It is worth noting that several authors have also shown the properties of cinnamaldehyde as a crosslinking agent for proteins, increasing the strength and reducing the moisture barrier and water holding properties of the films obtained (Balaguer *et al.*, 2011a, b). No significant differences were observed between water sorption and dimensional stability of CScin films and those subjected to thermal treatments. The results showed that at pH 7, hydrolysis is not favoured and

primary amino groups forming imino bonds are not available for interaction with water molecules or thermal crosslinking. It is worth mentioning that this study was also conducted at 48 h with relevant results. At pH 3, the integrity of the films was lost and therefore the samples could not be handled or measured. When the experiment was carried out at pH 7, no differences were found between films immersed in buffer solution for 24 or 48 h.

3.1.4. CA

Hydrophobicity of modified CS films was analysed by determination of water CA. The water CA of neutralized CS films was 78.3±2.1° (**Table 4.3.6**), similar to the result reported by Vallapa *et al.* (2011). The CA is defined as the angle between the surface of a liquid (in this work, water) and the tangent line at the point of contact with the substrate. The value of the CA depends mainly on the relationship between the adhesive forces between the liquid and the solid and the liquid cohesive forces. CS films were modified with a hydrophobic molecule, cinnamaldehyde, and therefore higher CA and lower wettability would be expected. After grafting of cinnamaldehyde to the films, the surface became more hydrophobic since the CA experienced a slight increase, with a value of 82.73±0.59°, but the differences were not significant. The CA of films subjected to thermal treatments experienced a reduction, which increased slightly with treatment temperature. Thus, films treated at 121 ℃ for 5 min presented CA values close to those of neutralized CS films. This was probably because, as the temperature of the treatment increases, more cinnamaldehyde is lost from the film surface and it becomes more hydrophilic. However, the CA differences were not statistically significant.

It should be pointed out that covalently anchored cinnamaldehyde to CS films barely changed the CA values, but swelling properties of the films

Table 4.3.6. Contact angle of films.

Films	CA(M)[°]
CS	78.03 ± 2.01[a]
CScin	82.73 ± 0.59[b]
CScin-4℃/30min	82.63 ± 2.56[b]
CScin-65℃/30min	81.17 ± 2.12[ab]
CScin-72℃/15min	81.06 ± 0.89[ab]
CScin-95℃/10min	79.54 ± 0.78[a]
CScin-121℃/5min	78.18 ± 2.54[a]

[a-b] Different letters in the same column indicate a statistically significant difference ($P \leq 0.05$).

were considerably modified. This difference may be due to the nature of the measurements made. Polymer chain relaxation due to water intake occurs during swelling. Chemical (cinnamaldehyde) or physical (temperature) crosslinking gives rise to a more compact polymer matrix and from a physical point of view water molecules will be more difficult to enter. By contrast, CA only provides information about the hydrophobicity of the film surface.

3.1.5. ATR-FTIR

ATR-FTIR spectra were recorded from the various films obtained in this work. **Figure 4.3.2** shows the FTIR-ATR spectra of samples of CS and CScin before and after the washing procedure. The spectra have been maximized with respect to the CS peak at 1,025 cm^{-1}. In comparison with the CS spectra, the CScin film spectra presented distinctive features at 690, 751, 1451 and 1492 cm^{-1}, which correspond to the phenolic group of cinnamaldehyde. The 1660-cm^{-1} peak, corresponding to the stretching of the C=O bond, shows that the aldehyde group is present in the unwashed sample. However, this band appears as a shoulder in the washed sample, indicating that the free cinnamaldehyde is practically eliminated after washing. In both washed and unwashed CScin films, there is a strong band at 1633 cm^{-1}, which is assigned to the stretching of the imine group (C=N) of the Schiff base. Also, part of the cinnamaldehyde bonded to the CS is also released, probably because of a partial reversion of the Schiff base reaction.

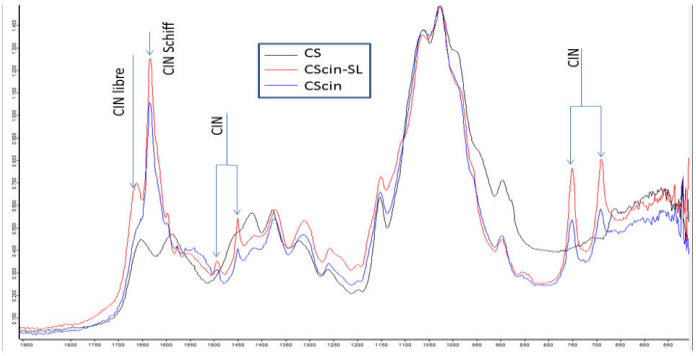

Figure 4.3.2. ATR-FTIR spectra of CS and CS modified with cinnamaldehyde before (CScin-SL) and after washing (CScin).

After washing, the films were exposed to different temperature/time treatments. **Figure 4.3.3** compares the ATR-FTIR spectra for the various samples, including pure CS, using the 1025-cm^{-1} band as reference. During the treatments, there is a partial release of cinnamaldehyde because of the reversibility of the reaction. Nevertheless, there is a large percentage of cinnamaldehyde still anchored to the CS matrix even after the most severe treatment (121 ºC for 5 min).

3.2. Release of cinnamaldehyde

Two experiments were performed to evaluate the cinnamaldehyde released by the films exposed to the different treatments: (a) release to MHB during the treatments and (b) release to 50% ethanol during treatments and until 1 h afterwards. The main aim of the second experiment was to study the effect that changing the medium to one more compatible with cinnamaldehyde had on the amount released from the film. A further aim was to ascertain whether, after the temperature/time treatment, the films continued releasing cinnamaldehyde to the medium. The results of the two experiments are presented together in **Figure 4.3.4**.

In the first test, films were immersed in MHB liquid culture medium and subjected to several preservation treatments. Immediately afterwards, the films were removed and the liquid was analysed by UV–vis spectroscopy. Five cinnamaldehyde solutions in MHB were also analysed for calibration. As can be seen in **Figure 4.3.4**, the concentrations of cinnamaldehyde in the

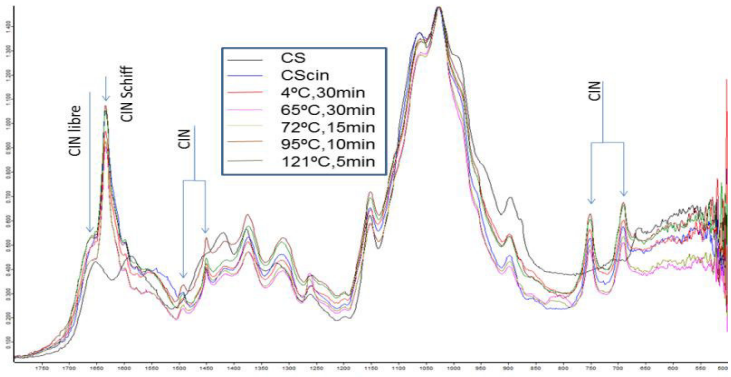

Figure 4.3.3. ATR-FTIR spectra of CS and CScin films after the different preservation treatments.

liquid medium increased with the treatment temperature applied to the films. At refrigeration temperature, the release was significantly lower than when moderate thermal treatments were applied. No differences were observed between samples processed at 65 ºC for 30 min and at 95 ºC for 10 min. The severe retorting process resulted in a greater release of the agent into the liquid medium.

The second experiment was carried out on a fatty food stimulant, ethanol 50%, which simulates alcoholic foods with an alcohol content of above 20%, and oil-in-water emulsions in accordance with the conditions set out in Regulation 10/2011/EC Commission Regulation (EU) of 14 January 2011 on plastic materials and articles intended to come into contact with food. After temperature/time treatments, the films were stored at room temperature, and liquid aliquots were extracted at several times during 1 h (48 h for the refrigerated sample).

As **Figure 4.3.4** reveals, the release profile of cinnamaldehyde over time depended on the treatment. CScin films processed at low temperature present a very low release (ca. 1 mg/L) after the treatment, but the amount released increases with time, reaching ca. 70 mg/L after 48 h. The treatments at 65, 72 and 95 ºC yielded much higher release values, 280 mg/L, without differences between treatments. It should also be noted that the cinnamaldehyde released does not change significantly with time during the storage period studied, indicating that probably all the free cinnamaldehyde present in the matrix owing to hydrolysis of the imino bond was released during the treatments. The films subjected to the sterilization treatment released the highest concentration of cinnamaldehyde, with values of ca. 700 mg/L after treatment. However, the concentration of the agent presented a decreasing trend during storage. Since the measured concentration indicates the cinnamaldehyde molecules that had already moved out of the film, a rebuild of the Schiff base is certainly unexpected. Most probably, the decrease in concentration was due to condensation of the volatile on the walls and septum of the vial and even cinnamaldehyde sorption in the film caused by a change in the partition equilibrium constant of cinnamaldehyde with temperature.

Another important feature is the large difference in the amount of cinnamaldehyde released in each liquid medium. Other authors have

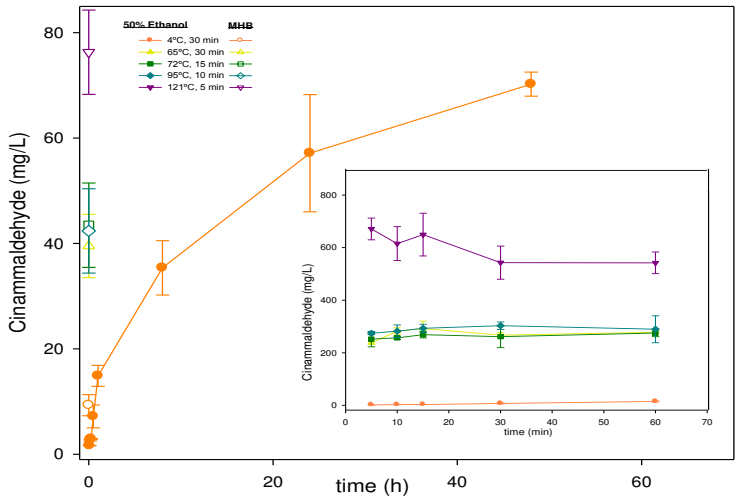

Figure 4.3.4. Cinnamaldehyde released from films into MHB during different temperature/time treatments (empty symbols) and time evolution of cinnamaldehyde released into ethanol 50% at 23 ℃ after applying preservation treatments (full symbols).

reported the importance of the solvent along with the temperature with regard to the reversibility of the Schiff base (Huang *et al.*, 2001; Mohamad, 2013; Muhamad, 2011). MHB is an aqueous medium buffered at pH 7 which causes film swelling and therefore increases the diffusion rate of any substance through the matrix. This effect explains the higher concentration of agent observed in MHB at 4 ℃. However, after the thermal treatments (65 ℃ and above), the release into the water-ethanol medium was greater. This might be caused by the higher solubility of cinnamaldehyde in this simulant.

The release results show that the films were activated by temperatures ≥65 ℃, reaching high concentrations of cinnamaldehyde in the medium. Films stored at refrigeration temperature produce a sustained release over time. The data obtained indicated that the films developed in this work can be used as a reservoir capable of sustained release of cinnamaldehyde over time and as a coadjuvant of preservation treatments.

3.3. Antimicrobial activity

3.3.1. In vitro *study*

The antimicrobial activity of the films was studied against a Gram-positive bacterium, *S. aureus*, and a Gram-negative bacterium, *E. coli*. First,

the *in vitro* effectiveness of the films exposed to various preservation treatments in MHB liquid medium (pH=7) was determined.

Figure 4.3.5 shows the antimicrobial activity of CScin films before and after preservation treatments. CS is a known antimicrobial agent: positively charged amino groups interact with the negatively charged membrane of bacteria, altering the permeability and disrupting DNA replication (Coma *et al.*, 2002; Zivanovic *et al.*, 2005). However, the results showed that the prepared CS film did not present substantial antimicrobial activity, as was to be expected, since the CS films were neutralized and, subsequently, the amino groups were not protonated (Foster and Butt, 2011; Shahidi *et al.*, 1999).

All the CScin films subjected to the various preservation treatments showed antimicrobial activity against the two microorganisms that were tested. Generally, Gram-negative bacteria are more resistant to essential oils than Gram-positive bacteria. The outer membrane of Gram-negative bacteria is highly impermeable to hydrophobic molecules like those found in essential oils, and they can work on different targets on the bacteria (Nazzaro *et al.*, 2013). However, the mechanisms of action of essential oil components and thus their antimicrobial activity against Gram-positive and Gram-negative bacteria depend on their chemical structure. Antibacterial effects of essential oils are commonly associated to interactions with the cell

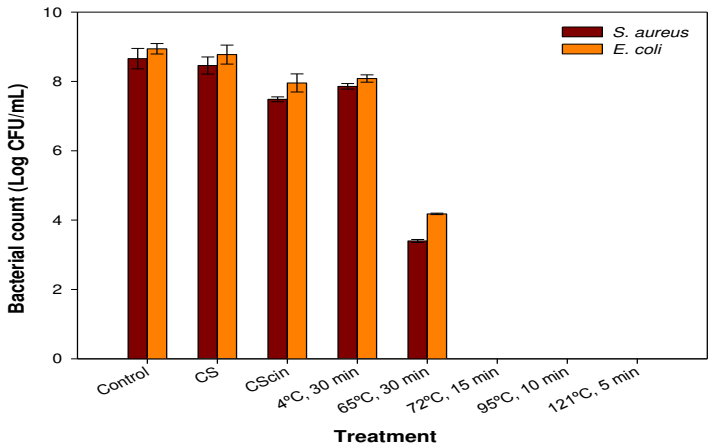

Figure 4.3.5. Antimicrobial activity of CS, CScin and CS-Schiff base films subjected to various combinations of temperature/time treatments against *S. aureus* and *E. coli*.

membrane, although the precise mechanisms remain unclear. The experimental pieces of evidence on the action mechanisms of cinnamaldehyde are contradictory and support both membrane interaction and inhibition of specific cellular processes or enzymes (Gill and Holley, 2004).

CScin and CScin films subjected to a storage temperature of 4 ºC for 30 min showed reduced antimicrobial activity (1 log reduction). These results are in agreement with the release study described in the previous section (**Figure 4.3.4**). Films not activated by thermal treatment released cinnamaldehyde very slowly owing to the slow reversibility of the Schiff base at neutral pH accompanied by low temperature. After treatment at 65 ºC for 30 min, CScin films showed a large log reduction of 5.66±0.04 against *S. aureus* and of 4.76±0.02 against *E. coli*. It was also observed that the films treated at 72 ºC for 15 min, 95 ºC for 10 min and 121 ºC for 5 min produced a bactericidal effect. Therefore, the antimicrobial activity is related to the active agent released during the different treatments. Films subjected to higher temperatures presented a higher degree of Schiff base hydrolysis and consequently released more cinnamaldehyde, increasing their antimicrobial capacity.

The results of the antimicrobial study show that these films can be very effective when subjected to a thermal treatment. At low temperatures, cinnamaldehyde-imino-chitosan films presented extended stability with very slow agent release. At mild and sustained heat treatments (hot filling or mild pasteurization), the release is high enough to produce substantial inhibition of microbial growth. More severe heat treatments for short times are much more effective, with the films even providing a bactericidal effect.

3.3.2. Study of the antimicrobial capacity of CScin films applied to inoculated food

Once the *in vitro* effectiveness of the CScin films had been verified, their antimicrobial activity was examined in refrigerated and pasteurized whole milk with a fat content of 3.6%. CScin films were immersed in milk and subjected to different preservation treatments, after which the milk was inoculated with *L. monocytogenes* and then kept at 4 ºC for 12 days, and the antimicrobial assays were performed on days 3, 6 and 12. *L. monocytogenes*

is a microorganism that is able to grow at low temperatures (Doyle and Beuchat, 2007). Previous studies on the thermal behaviour of *L. monocytogenes* in foods showed that the mean minimum growth temperature was 1.1 ℃ (Junttila *et al.*, 1988) and that this microorganism can survive pasteurization (Fleming *et al.*, 1985; Lovett *et al.*, 1987). The characteristics of refrigerated milk (pH close to neutrality, large presence of nutrients) might also have favoured an increase in the viable counts of *L. monocytogenes* (Muriel-Galet *et al.*, 2012).

As **Figure 4.3.6** shows, the more severe the temperature treatment was, the greater was the reduction of bacterial growth, in good correlation with the data obtained in the in vitro assays and in the release tests. CScin films treated at 4 ℃ for 30min yielded a log reduction of 1.34 at 3 days, 0.81 at 6 days and 0.52 at 12 days. Activation of the films by higher temperatures resulted in more efficient antimicrobial activity. Thus, CScin films treated at 95 ℃ for 10 min showed a log reduction of 4.15±0.02 at 3 days, 3.41±0.02 at 6 days and 3.87±0.07 after 12 days.

It was not possible to inoculate the samples treated at 121 ℃ for 5 min because the milk was coagulated after treatment. There are two possible reasons for this effect. It is documented that certain aromatic compounds, such as cinnamaldehyde, may cause conformational changes in proteins by binding (Damodaran and Kinsella, 1980; Kuhn *et al.*, 2006). Combinations of temperature and the aromatic aldehyde can lead to denaturation of milk proteins and subsequent unfolding and aggregation forming a gel. This

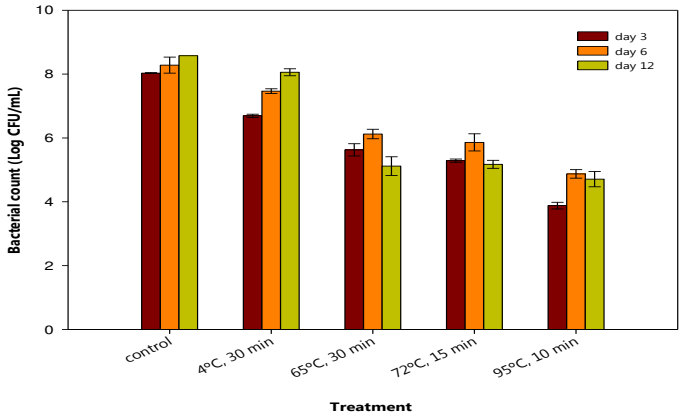

Figure 4.3.6. Antimicrobial effect of CS film modified with cinnamaldehyde and subjected to different preservation treatments against *L. monocytogenes* in pasteurized milk.

treatment could also produce hydrolysis of CS and migration to the medium, causing milk coagulation due to the coagulation and flocculation properties of CS (Renault *et al.*, 2009).

The lower antimicrobial activity of the films observed when applied to milk (**Figure 4.3.6**) compared with that in MHB medium (**Figure 4.3.5**) can be explained because the use of the optimal culture medium for the microorganism magnifies any effect. Moreover, milk is a complex food matrix which may interfere with the antimicrobial agent, requiring higher concentrations to achieve the same effect (Gutiérrez *et al.*, 2008). Similar differences between in vivo and in vitro antimicrobial activity of antimicrobial agents and antimicrobial films have been reported previously (Belletti *et al.*, 2008; Burt, 2004; Muriel-Galet *et al.*, 2012).

L. monocytogenes is an important pathogenic microorganism involved in cases of septicaemia and meningitis, especially in children, the elderly and those with immunosuppression induced by drugs or diseases. However, there are also cases of listeriosis in apparently healthy children and adults. In pregnant women, it can cause abortions or premature death of the foetus. Therefore, the films developed in this work could improve the safety of products susceptible to contamination with microorganisms such as *L. monocytogenes* and could also extend the commercialization period, an important advantage for a product with a shelf life of only 3 days under refrigeration.

3.4. Sensory analysis

The use of essential oils in food may have a significant sensory impact that could result in non-acceptance by the consumer. For this reason, a sensory analysis was carried out by a panel of judges with the aim of determining whether the content of active component migrated to pasteurized milk modifies its aroma appreciably and, if so, to determine whether this odour is accepted by consumers. The tests were conducted on the 3rd, 6th and 12th days of refrigerated storage at 4 ℃. The samples were evaluated by a minimum of 40 random non-expert judges.

Friedman analysis indicated significant differences in the intensity of cinnamon odour perceived and acceptability, since in all cases, the value of F exceeded the threshold level of significance of $P \leq 0.001$.

According to the intensity of cinnamon odour after 3 days of storage, there were no significant differences among the milk samples treated with CScin films, but the differences with respect to the control sample were significant (**Figure 4.3.7**). The same results were obtained in the tests carried out on the 6th and 12th days.

Preference was evaluated with a score from 1 to 5, 5 being the worst score. Samples with cinnamaldehyde were preferred to the control, without any significant differences among them after 3 and 12 days of storage. Sensory analysis showed that panellists perceived the presence of cinnamaldehyde in the milk exposed to CScin films. Nevertheless, the panellists preferred the milk samples in contact with CScin films at the 3 days tested. Fresh pasteurized milk is a product whose shelf life is very short, 2–3 days once opened. The use of the films developed may not only increase the safety of such products and subsequently lengthen their shelf life owing to their antimicrobial activity but additionally provide a flavour that has high acceptance by the consumer.

4. Conclusions

In this work, CS-cinnamaldehyde Schiff base films intended for use in antimicrobial food packaging were developed. CS was selected as a support matrix for the covalent binding of cinnamaldehyde via nucleophilic addition to free amino groups. The degree of substitution achieved was ca. 70%.

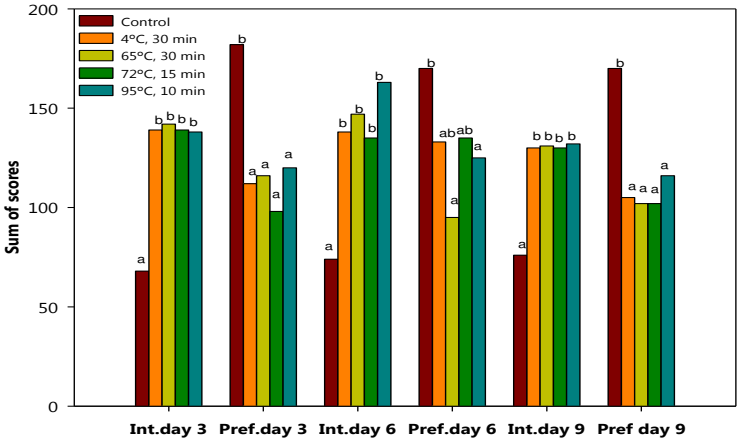

Figure 4.3.7. Values of the parameters of odour intensity and preference.

CS-derived Schiff base can be hydrolyzed under several temperature/time treatments in different liquid media. The amount of cinnamaldehyde released and the antimicrobial activity of the films depended on the treatment applied and the liquid medium composition. The effectiveness of the films increased as the temperature of the treatment increased and thus the amount of cinnamaldehyde released. Although the release of the agent caused a perceptible cinnamon aroma in milk, the sensory panel considered this effect as positive, treated milk being preferred to the control sample.

The present study shows that CS-Schiff base polymer has great potential for use in the development of stimuli-responsive active volatile compound release systems. These systems can be applied in the design of new active packages to increase food safety.

Acknowledgments

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4.4. Capítulo 4. Desarrollo de películas de quitosano con hidroxipropil-β-ciclodextrinas y otros coadyuvantes, con adaptable capacidad de sorción y liberación de compuestos volátiles antimicrobianos y su aplicación en el envasado activo de alimentos

4.4.1. Estado de la cuestión

Existe una creciente demanda por parte de los consumidores por alimentos más saludables y libres de aditivos artificiales. Una alternativa es el empleo de conservantes de origen natural como son los extractos vegetales y AE. En la actualidad se conocen aproximadamente 3000 tipos diferentes de AE de los cuales más de 300 son empleados como aromatizantes. Las especias y hierbas aromáticas se han empleado empíricamente en remedios caseros y en la conservación de alimentos desde la antigüedad. Actualmente se sabe que diferentes extractos vegetales y AE, poseen actividad antimicrobiana y podrían emplearse como antimicrobianos naturales en los alimentos. Entre estos compuestos destaca el carvacrol, monoterpeno fenólico y componente del AE del orégano o del tomillo, que posee una elevada actividad antimicrobiana de amplio espectro. Por ello, los AE o sus componentes activos son interesantes en el desarrollo de envases antimicrobianos. Los biopolímeros, como el CS, han sido ampliamente utilizados como matrices portadoras de éstos (**Anexo A**).

No obstante, la incorporación libre de estos compuestos en la matriz polimérica genera elevadas pérdidas durante el procesado de la película, mayoritariamente durante la etapa de secado debido a su elevada volatilidad, siendo necesario adicionar altas concentraciones de agente en la solución formadora de película para que una vez procesada la película, quede una cantidad residual capaz de conferir actividad antimicrobiana a la misma. Así, en diversos trabajos se han encontrado pérdidas del 39-99% del carvacrol adicionado inicialmente, en función de los distintos métodos de obtención de las películas de CS y de las condiciones en las que se procesan (Chi *et al.*, 2006; Kurek *et al.*, 2012; Kurek *et al.*, 2013; López-Mata *et al.*, 2013; Perdones *et al.*, 2014). Estudios previos llevados a cabo en nuestro laboratorio demostraron pérdidas similares (99.8, 98.6 y 98%) cuando se adicionó a la disolución de CS un 100% de carvacrol respecto a la materia seca, en películas plastificadas con 0, 20 y 35% de G, respectivamente, y

secadas en cámara 36h, 40.0±1.5 ºC y 20±9% HR. Por lo que, hay que recurrir a otras técnicas para incluir el volátil en la matriz o a coadyuvantes para un uso más racional y eficaz de estos agentes antimicrobianos. En el presente trabajo, los volátiles se incluyen después de la formación de la película de CS por inmersión en el líquido volátil para evitar pérdidas del compuesto antimicrobiano durante la etapa de secado de la película. Hasta la fecha no se han descrito trabajos similares que empleen dicha técnica. Sin embargo, Ouédraogo *et al.* (2013) presentaron un estudio con algunas similitudes al desarrollado en la presente Tesis Doctoral, ya que determinaron el efecto de la estructura de los monoterpenos *α*-pineno, citronelal, carvona y terpinen-4-ol en su retención en distintas matrices celulósicas tras la impregnación de las matrices con los monoterpenos diluidos en acetona o hexano. Misharina *et al.* (2006) examinaron las interacciones de diferentes compuestos aromáticos en agua con CS, carragenano y almidón.

Por otro lado, es limitado el porcentaje de compuestos ligeramente hidrófobos, como los componentes de AE, que se puede incorporar en películas hidrófilas, como las formadas de CS. Asimismo, hay que tener presente que exista cierta afinidad química entre el compuesto a incorporar y la matriz polimérica, para favorecer una buena dispersión en la película pero sin que inhiba su liberación (Chalier *et al.*, 2007; Kurek *et al.*, 2012). Como se expone en la **Introducción** de la presente Tesis Doctoral, el uso de diversos coadyuvantes, como las CD, pueden aumentar la estabilidad y compatibilidad entre el CS y los agentes activos lipófilos. Así, las CD se pueden emplear para disminuir las pérdidas de estas sustancias inestables y volátiles mediante la formación de complejos de inclusión. De esta manera, estas pequeñas biomoléculas pueden microencapsularse en las CD, y éstas a su vez se pueden incluir libremente en la matriz polimérica (Ayala-Zavala *et al.*, 2008; Brasil *et al.*, 2012; Kayaci y Uyar, 2012; Moreira *et al.*, 2014; Sipahi *et al.*, 2013), o bien anclar al polímero para que se dé un efecto acumulativo en la capacidad de formación de complejos de inclusión (Aoki *et al.*, 2003). Debido a la reversibilidad del complejo, estas moléculas aromáticas se puede liberar de forma controlada desde el sistema (Ciobanu *et al.*, 2013; Kfoury *et al.*, 2014). Sin embargo, en estos casos, la cantidad incluida en la matriz es pequeña, ya que está limitada al volumen interior total de las CD. Por ello, se puede recurrir a otros mecanismos distintos a la formación de complejos de

inclusión, como los agregados autoensamblados que se producen con elevadas concentraciones de CD, para aumentar la capacidad de retención de estas sustancias bioactivas (Kurkov y Loftsson, 2013; Messner *et al.*, 2010; Van de Manakker *et al.*, 2009).

Además, con el propósito de mejorar el procesado y manipulación de las películas, se puede incluir un plastificante hidrófilo compatible con la matriz, como el G, que es un aditivo alimentario y biodegradable. Asimismo se ha demostrado que el G facilita una mayor retención de estos compuestos volátiles, debido a que es capaz de interponerse en las cadenas interrumpiendo las fuerzas intercatenarias (Kurek *et al.*, 2012). Igualmente, se podría pensar que otros plastificantes con el mismo mecanismo de acción, como el agua, realizarían una acción similar.

El objetivo de crear películas antimicrobianas con agentes volátiles que conformen el envase o parte de él, es que pueda difundir hacia el espacio de cabeza, donde se acumule en estado vapor, y ejerza su acción antimicrobiana en la superficie del alimento, e incluso difundir en el alimento y realizar una acción en profundidad, sin necesidad de contactar con el alimento. Idealmente, la matriz polimérica actúa como reservorio desde el que se libera de forma sostenida el agente. De esta manera, se asegura una concentración eficaz de antimicrobiano en el espacio de cabeza que impida la proliferación de microorganismos en la superficie del alimento, precisándose cantidades menores de agente activo (Buonocore *et al.*, 2003; Ouattara *et al.*, 2000). Además, la liberación del volátil desde la matriz polimérica puede ser activada mediante diferentes estímulos, como la temperatura y la RH (Chalier *et al.*, 2009; Kurek *et al.*, 2014). Así, hidrogeles, como el CS, podría hincharse al sorber el agua presente en el espacio de cabeza del envase, plastificarse y facilitar la difusión y liberación de moléculas atrapadas en la matriz, como podrían ser los agentes volátiles (Buonocore *et al.*, 2003; Mascheroni *et al.*, 2011).

Por otra parte, cabe destacar que el carvacrol posee una elevada actividad antimicrobiana en estado vapor (Becerril *et al.*, 2007; Ben Arfa *et al.*, 2006; Burt *et al.*, 2007; Nostro *et al.*, 2009). Existen diversos trabajos publicados en los que el carvacrol actúa en estado vapor, tras ser incorporado en distintas matrices como PP (Ramos *et al.*, 2013; Ramos *et al.*, 2012), PP y PE/EVOH (Gutiérrez *et al.*, 2010; López *et al.*, 2007) y EVOH

(Cerisuelo *et al.*, 2013; Cerisuelo *et al.*, 2012; Muriel-Galet *et al.*, 2012; Muriel-Galet *et al.*, 2013). No obstante, hasta lo que se ha podido indagar, únicamente Ávila-Sosa *et al.* (2012) y Kurek *et al.* (2013) incorporan el AE de orégano o carvacrol, en películas de CS y prueban su eficacia en estado vapor frente a microorganismos alterantes o patógenos; aunque sólo realizan estudios *in vitro*, no lo aplican en el envasado activo de alimentos.

Es de destacar, que la mayor parte de los trabajos existentes en la bibliografía, relacionados con la incorporación de líquidos volátiles naturales con propiedades antimicrobianas, hacen referencia a la cantidad de compuesto que se incorpora inicialmente en la disolución filmógena, y no a la cantidad real remanente en las películas ya elaboradas y secas, y la concentración alcanzada en el espacio de cabeza del envase. Algunos autores han evaluado la acción antimicrobiana de diversos AE y sus principales constituyentes en fase vapor estableciendo las concentraciones mínimas inhibitorias en el espacio de cabeza contenido en el recipiente de ensayo (Tyagi *et al.*, 2012). Otros grupos de investigación han estudiado minuciosamente la cinética y los parámetros de equilibrio del carvacrol en filetes de salmón envasado en bandejas selladas térmicamente con una tapa de PP/EVOH/PP con carvacrol en su núcleo, proponiendo un modelo matemático para describir y simular el funcionamiento del sistema de envasado y predecir su comportamiento bajo diferentes condiciones (Cerisuelo *et al.*, 2013). Por otra parte, Gutiérrez *et al.* (2010) demuestran *in vitro* que el comportamiento cinético de los AE y sus componentes es de gran relevancia en las propiedades antimicrobianas del envase activo. Sin embargo, no se han encontrado trabajos en los que se estudie en profundidad el reparto de la cantidad de volátil añadido inicialmente en cada uno de los componentes del sistema de envasado que protege al alimento. Sólo estudios parciales como el de Chi *et al.* (2006) en el cual determinaron el carvacrol en películas de CS y en las lonchas de mortadela antes y tras 5 días de contacto con la película colocada como separador. Kurek *et al.* (2013) determinaron la capacidad antimicrobiana de películas de CS con carvacrol en relación a su coeficiente de partición, evaluando la cantidad de carvacrol en las películas y en la fase gaseosa, pero sin ser aplicado a ningún alimento. Passarinho *et al.* (2014) evaluaron la concentración de AE de orégano en pan tras ser envasado junto con bolsitas que contenían el agente antimicrobiano.

Rodríguez *et al.* (2008) analizaron la cantidad de AE de canela en el pan envasado con papel recubierto de parafina con dicho AE para el control del crecimiento de *Rhizopus stolonifer* durante el almacenamiento, pero no se realiza un análisis del AE en el resto del sistema de envasado a lo largo del tiempo. Por lo tanto, es necesario un conocimiento más profundo del comportamiento termodinámico de estos activos volátiles en el sistema de envasado y en el propio alimento para determinar la eficacia y validez de los sistemas activos antimicrobianos en alimentos específicos.

Al igual que el carvacrol, otros constituyentes individuales volátiles de los AE, presentan una elevada capacidad antimicrobiana en estado vapor pudiéndose adaptar el volátil al alimento al que se aplica, en función de su carácter organoléptico para una mayor aceptación de los productos envasados y en función de riesgo microbiológico (López *et al.*, 2005; Nedorostova *et al.*, 2009). La capacidad de retención de sistemas poliméricos con CD y la actividad antimicrobiana de otros monoterpenos distintos al carvacrol puede estar determinada por la estructura química de estos volátiles.

Por todo lo descrito, en el presente trabajo se ha desarrollado y caracterizado un sistema polímero/CD/plastificante capaz de retener volátiles naturales antimicrobianos en función de la formulación de la película. Seguidamente se ha estudiado la cinética de liberación del agente como respuesta a estímulos como la RH del entorno para poder ser aplicado en estado vapor en el envasado antimicrobiano de alimentos. Posteriormente, con las películas obtenidas se ha desarrollado y estudiado la eficacia de un envase activo para la conservación de pechugas de pollo.

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4.4.2. Artículo científico 4.

Preparation and characterization of chitosan/HP-β-cyclodextrins composites with high sorption capacity for carvacrol

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Resumen

El objetivo de este trabajo fue diseñar nuevos sistemas basados en polímeros que presentan una capacidad de carga de carvacrol ajustable dependiendo de la formulación de la película. Para este propósito, se desarrollaron películas biocompuestas empleando quitosano (CS) como matriz polimérica e hidroxipropil-β-ciclodextrinas (HP-βCDs) como un adyuvante para aumentar la sorción de carvacrol en la matriz polimérica. Se investigaron la morfología, propiedades ópticas, mecánicas y de barrera de las películas obtenidas, y se evaluó su capacidad de sorción de carvacrol. Los biocompuestos resultaron muy transparentes y de elevada resistencia mecánica y barrera a la humedad. La sorción de carvacrol se vio muy afectada por el contenido de humedad y glicerol (G) de las biocompuestos. Los valores más altos de sorción se lograron para materiales compuestos con un 35% de glicerol y acondicionados a 75% RH, reteniendo un 216% carvacrol (g/100 g de materia seca). Estos resultados indican que la inclusión de carvacrol en las películas puede estar ocurriendo por mecanismos distintos de la formación de complejos de inclusión.

Palabras clave

Quitosano, hidroxipropil-β-ciclodextrinas, biocompuesto, propiedades funcionales, sorción de carvacrol.

Abstract

The aim of this work was to design new polymer-based systems exhibiting an adjustable loading capacity of carvacrol depending on the film formulation. For this purpose, biocomposite films were developed employing chitosan (CS) as the polymer matrix and hydroxypropyl-β-cyclodextrins (HP-βCDs) as an adjuvant to improve the sorption of carvacrol in the polymer matrix. The morphology, optical, mechanical and barrier properties of the resulting films were investigated, and the sorption capacity of carvacrol evaluated. Biocomposites resulted highly transparent with higher mechanical resistance and moisture barrier properties. Sorption of carvacrol was greatly affected by the humidity and glycerol (G) content of the biocomposites. The highest sorption values were achieved for composites incorporating 35% glycerol and conditioned at 75% RH these composites retained 216% carvacrol (g/100 g dry matter). These results indicate that inclusion of carvacrol in the films could be occurring by mechanisms other than formation of inclusion complexes.

Keywords

Chitosan, hydroxypropyl-β-cyclodextrins, biocomposite, functional properties, sorption of carvacrol.

1. Introduction

CS has been widely studied as a polymer for the design of reservoir delivery systems for the slow release of active compounds over an extended period of time. These regulated delivery systems are effective in minimizing the amount of compound used for a specific application and thus improve efficacy and reduce possible side effects associated with the use of large amounts of bioactives (Pedro *et al.*, 2009; Sivakumar *et al.*, 2002). CS employed as a delivery system can find applications in a variety of technological areas, such as agrochemistry, pharmacy, biomedicine, textiles and food packaging. The development of antimicrobial materials and their application in the design of active packaging is creating considerable expectation in the food industry, since food safety is an area of great concern. Although there are many studies in the literature that focus on the use of CS films as antimicrobials in contact with food, the use of CS films for the release of active compounds has received much less attention.

Because of their antimicrobial properties, many plant extracts and essential oils have found applications as natural preservatives. In this regard, carvacrol (5-isopropyl-2-methylphenol) is a constituent of essential oils of oregano and thyme, with known antifungal, insecticidal, antitoxigenic and antiparasitic activities (Burt, 2004; Veldhuizen *et al.*, 2006). Carvacrol is categorized as GRAS (Generally Regarded as Safe) by the FDA (Food and Drug Administration) for food. Volatile active compounds can be added to films to achieve a more effective and rational use of them. This step is especially problematic since a large amount of the compounds is lost or inactivated during processing and the remaining amount in the polymer is not enough to exert its effects on the food. In addition, the active compound must be chemically compatible with the polymer matrix to allow good dispersion in the film but not inhibit its release (Chalier *et al.*, 2007; Kurek *et al.*, 2012). CS films have been loaded with active volatile compounds for several purposes (Abdollahi *et al.*, 2012; Altiok *et al.*, 2010). However, loading of volatiles in a CS matrix presents several difficulties. Its incorporation as an additive into the water-based CS film forming solution is challenging because of general limited aqueous solubility of volatiles and the inevitable partial loss of the compound by evaporation during the casting and drying of the film. In order to overcome these problems, Presence of cyclodextrins

in the CS matrix could improve compatibility between the polymer matrix and the agent. Moreover, the volatile could be loaded after film casting to avoid agent losses during the film drying step.

Cyclodextrins (CDs) are donut like oligosaccharides with hydrophobic cavities and hydrophilic outer surface. They are widely used as excipients in pharmacy to solubilise lipophilic drugs by means of inclusion complex. However, non-inclusion based aspects of CDs are being studied and their importance to solubilisation by formation of self-assemble aggregates or surfactant like effects.

The aim of this work was to develop CS films with a selective carvacrol loading capacity. For this purpose, biocomposites based on the addition of HP-βCD, a highly water-soluble CD derivative, into the CS film-forming solution were prepared and their physico-chemical properties studied, including morphology and optical, mechanical and barrier properties. The carvacrol loading capacity of the films was also studied as a function of the water and G content.

2. Materials and methods

2.1. Materials

Carvacrol (kosher >98%) and low-molecular-weight chitosan (CS) were supplied by Sigma (Barcelona, Spain). Hydroxypropyl-β-cyclodextrin (HP-βCD, CAVASOL® W7-HP) was supplied by Wacker Ibérica (Barcelona, Spain). Glycerol (G) and acetic acid were purchased from Aldrich (Barcelona, Spain).

2.2. Film preparation

First, a 1.5% CS (w/w) solution in an aqueous 0.5% (w/w) acetic acid solution was prepared and filtrated to eliminate impurities. Pure CS films were prepared by casting, that is, pouring a suitable amount of the solution into a flat PS tray and allowing it to dry under controlled environmental conditions (36 h, 40.0±1.5 ℃ and 20±9% RH). G-plasticized films were produced by adding G at 20% or 35% (g G/100 g dry matter) to the film-forming solution.

Chitosan/hydroxypropyl-β-cyclodextrin composites (CS–CD) were obtained by adding HP-βCD to the CS solution in a 1:1 proportion (w/w) with

respect to CS, stirring at 1500 rpm and 37 ℃ until complete dissolution and submitting the solution to the casting process. Plasticized CS–CD films were prepared by adding G at 20% or 35% (g G/100 g dry matter) to the film forming solution.

2.3. Film thickness

The film thickness of each sample was individually measured using a digital micrometre (Mitutoyo Manufacturing Co. Ltd., Tokyo, Japan) with a sensitivity of 1 μm. Five readings were taken for each sample, one at the sample centre and four measurements around the perimeter. Average thickness of the films was 55±5 μm.

2.4. Optical properties

The colour of the films was measured with a CR-300 Minolta Chroma meter® (Minolta Camera Co., Ltd., Osaka, Japan). The film samples were placed on a white standard plate; the results were expressed in accordance with the CIELAB system with reference to illuminant D65 and a visual angle of 10°. The measurements were performed through a 6.4-mm-diameter diaphragm containing an optical glass, monitoring L*, a*, b*, chroma ($C^*_{ab}=(a^{*2}+b^{*2})^{1/2}$) and hue ($h_{ab}$=arctan (b*/a*)). The samples were measured in triplicate by eight measurements in different locations for each film sample.

The apparent opacity was evaluated (Agilent 8453 UV-visible spectrophotometer (Agilent, Barcelona, Spain)) as the integrated area under the curve, which was calculated using UV-WIN-Lab software and expressed as the product of absorbance value (*A*) and wavelength (nm). Samples were measured in triplicate.

2.5. Morphology

Films were fractured under liquid nitrogen and the cross-section surface morphology studied by field emission (FE) scanning electron microscopy (SEM) using a HITACHI S-4100 unit equipped with a secondary electron (SE) detector and an EMIP 3.0 image capture system (HITACHI, Madrid, Spain). Samples were coated under vacuum with gold–palladium in a

sputter coating unit and their fracture surface was investigated. Images were captured at 10 kV, at a distance of 14 cm, with 1000× magnification.

2.6. Thermogravimetric analysis (TGA)

TGA of films was carried out using a Mettler Toledo TGA/SDTA/851 (Columbus, OH, USA). Samples of approximately 10 mg were heated from room temperature to 900 ℃ at 10 ℃ /min and held at an isotherm for 3 min under a nitrogen atmosphere. The TGA data were plotted as weight per cent versus temperature and the decomposition temperature was measured from the first derivative of weight per cent versus temperature (DTGA).

2.7. Moisture content

Samples (0.4–0.5 g) of each film were cut into pieces and placed on aluminium plates. They were placed in desiccators containing saturated solutions of magnesium nitrate 6-hydrate (Sigma, Barcelona, Spain), sodium chloride (Scharlau, Barcelona, Spain) and barium chloride 2-hydrate (Fluka, Madrid, Spain) in a chamber conditioned at 23±1 ℃ in order to maintain a RH of 53.0±0.5, 75.0±0.5 and 90.0±3.0%, respectively (ASTM, 2007). These values were confirmed by direct RH measurements with hygrometers (Hygrodynamics, Newport-Scientific Inc. Jessup, MD, USA). After reaching weight equilibrium, in approximately 2 weeks, they were weighed and placed in desiccators with phosphorus pentoxide (Sigma, Barcelona, Spain) for dehydration for 2 more weeks. The tests were done in triplicate.

2.8. Barrier properties

2.8.1. Water vapour permeability (WVP)

WVP tests were carried out at two RH gradients (0/53% and 0/75%) and 23±1 ℃ using permeability cups (Elcometer, Manchester, England) in accordance with ASTM E96/E96M-10 for flexible films (ASTM, 2010c). To ensure the necessary RH, the cups were stored in desiccators containing salt solutions: magnesium nitrate 6-hydrate and sodium chloride for 53% and 75% RH, respectively. The cups were weighed daily, and the plot of the weight increase vs. time provided the water vapour transmission rate. These

values were then divided by the water pressure gradient and multiplied by the sample thickness to obtain the water vapour permeability value.

2.8.2. Oxygen permeability

The oxygen permeation rates of the materials were determined at 50 and 75% RH and 23±1 ℃ using an OXTRAN Model 2/21 ML Mocon (Lippke, Neuwied, Germany) based on the ASTM standard (ASTM, 2010b). The film samples were previously conditioned at the RH of the experiment. After conditioning the samples in the OXTRAN cells for 6 h, the transmission values were determined every 45 min until constant.

2.9. Mechanical properties

A Mecmesin MultiTest 1-í universal test machine (Landes Poli Ibérica, S.L., Barcelona, Spain) equipped with a 100-N static load cell was used to evaluate the maximum tensile strength (σ_m), percentage of elongation at break (ε_b) and Young's modulus (E) of the films according to ASTM D882-09 18 (ASTM, 2010a). Films were conditioned at 53 and 75% RH for one week before testing. Sample films were cut into 25.4 mm × 130 mm strips. Grip separation was set at 100 mm and cross-head speed at 25 mm/min. Twenty replicates from each sample were tested.

2.10. Conditioning and immersion in carvacrol

Circular film samples 55 mm in diameter were stored in glass desiccators at 0 (with phosphorus pentoxide to dry films), 53.0±0.5, 75.0±0.5 and 90.0±3.0% RH (ASTM, 2007) in a temperature-controlled room at 23±1 ℃. After reaching equilibrium water sorption, the films were immersed in carvacrol the necessary time to achieve equilibrium.

2.11. Sorption of carvacrol

The analysis of the concentration of carvacrol retained in the materials was performed by thermal desorption coupled to gas chromatography using a Dynatherm Thermal Desorber Model 890/891 (Supelco, Teknokroma, Barcelona, Spain) connected in series to the column of an HP5890 gas chromatograph Series II Plus (Agilent Technologies, Barcelona, Spain) via a heated transfer line. A cut piece of the film was cleaned with a paper tissue

to remove any excess of volatile compound on the film surface and then inserted into an empty desorption tube (11.5 cm×0.39 cm I.D.). The tube was placed in the desorber chamber, which was immediately sealed. Conditions for desorption were as follows: desorption temperature, 210 ℃; transfer line, 230 ℃; desorption time, 7 min; He desorption flow, 8.15 mL/min. The GC was equipped with a TRB5 (30 m, 0.32 mm, 0.25 μm) column (Teknokroma, Barcelona, Spain) and a flame ionization detector. The chromatographic conditions were: 260 ℃ detector temperature, 7 min at 45 ℃, heating ramp to 220 ℃ at 18 ℃/min, and 1 min more at 220 ℃. After the analysis, the film sample was recovered from the desorption tube and weighed on an analytical balance (Voyager V11140 model, Ohaus Europe, Greifensee, Switzerland).

2.12. Data analysis

Statistical analysis of the results was performed with SPSS commercial software (SPSS Inc., Chicago, IL, USA). A two-way analysis was applied to compare the effect of different amounts of G in the same matrix CS or CS–CD. Additionally one-way analysis of variance was carried out for the other data. Differences between means were assessed on the basis of confidence intervals using the Tukey-*b* test at a level of significance of $P \leq 0.05$. The data are represented as average±standard deviations. The data were analyzed and plotted using the SigmaPlot 10.0 software (Systat Software Inc., Richmond, CA, USA).

3. Results and discussion

3.1. Optical properties

The influence of the presence or absence of HP-βCDs and the different proportions of G added to the film-forming solution on the colour parameter values (L*, C^*_{ab}, h_{ab}, a*, b*) are presented in **Table 4.4.1**. In all the materials, the high values of L* (>94) are indicative of high lightness, while slightly negative values of a* and positive b* are indicative of a yellow-green colour. The addition of G did not significantly affect the colour coordinates ($P>0.05$) within the G range tested. However, when 50% of the CS content in the film was replaced by HP-βCD, significant changes in colour parameters were

Table 4.4.1. Colour parameter values of CS films and chitosan/hydroxypropyl-β-cyclodextrin composite (CS–CD) plasticized with different concentrations (%) of G and transparency parameters obtained from transmittance (T%) in the UV-visible region.

Materials films	COLOUR				
	L*	a*	b*	C^*_{ab}	h_{ab}
CS	94.5 ± 0.1^{a,x}	-1.31 ± 0.08^{a,x}	10.9 ± 0.4^{a,x}	10.9 ± 0.5^{a,x}	96.9 ± 0.2^{a,x}
CS-20G	94.3 ± 0.8^{a,x}	-1.41 ± 0.15^{a,x}	11.3 ± 0.7^{a,x}	11.4 ± 0.7^{a,x}	97.1 ± 0.4^{a,x}
CS-35G	94.7 ± 0.4^{a,x}	-1.32 ± 0.09^{a,x}	10.7 ± 0.4^{a,x}	10.8 ± 0.4^{a,x}	97.0 ± 0.3^{a,x}
CS–CD	95.5 ± 0.2^{a,y}	-0.27 ± 0.06^{a,y}	5.9 ± 0.3^{a,y}	5.9 ± 0.3^{a,y}	92.7 ± 0.5^{a,y}
CS–CD-20G	95.5 ± 0.2^{a,y}	-0.33 ± 0.05^{a,y}	5.9 ± 0.2^{a,y}	5.9 ± 0.3^{a,y}	93.3 ± 0.3^{a,y}
CS–CD-35G	95.3 ± 0.3^{a,y}	-0.48 ± 0.07^{a,y}	6.2 ± 0.3^{a,y}	6.2 ± 0.3^{a,y}	93.4 ± 0.4^{a,y}
Materials films	**TRANSPARENCY**				
	Opacity (AU x nm)	***T* (%) at 280 nm**		***T* (%) at 325 nm**	**Average *T* (%) (400-800nm)**
CS	31.8 ± 1.6^{n}	12.7 ± 1.4^{m}		22.9 ± 1.8^{m}	83.4 ± 0.8^{m}
CS–CD	26.6 ± 1.7^{m}	36.5 ± 1.7^{n}		44.9 ± 1.3^{n}	85.8 ± 0.3^{m}

[a] No statistically significant differences between means (P>0.05) were found by Tukey's test when comparing different amounts of G in the same matrix CS or CS-CD.
Different letters (x and y) in the same column indicate a statistically significant difference (P≤0.05) comparing CS and CS–CD.
Different letters (m and n) in the same column indicate a statistically significant difference (P≤0.05) comparing CS and CS–CD.

observed, with higher values of L* and lower values of a*, b*, C^*_{ab} and h_{ab}. These results suggest that HP-βCDs dilute the CS matrix, reducing the colour and increasing the lightness of the composite films.

Both films had high transmittance, greater than 80% in the visible region, indicative of transparent films. The addition of HP-βCDs to the films resulted in higher transmittance for wavelengths between 190 and 600 nm and a decrease in opacity, as the values of these parameters in **Table 4.4.1** show. These results correlated well with the L* values and were indicative of the previously mentioned dilution effect caused by the addition of HP-βCDs to the film matrix.

3.2. Morphology

In a visual inspection, the CS–CD composite films were homogeneous, with no observed phase separation and with smooth surfaces and high transparency. SEM images (not shown) indicate compact surfaces, smooth and homogeneous, without pores or discontinuities, indicating a good miscibility of the three components, biopolymer, oligosaccharide and plasticizer. No differences were observed between samples, which indicates

high dispersion and solubilisation of the HP-βCDs in the CS matrix. Films formed without plasticizer showed a similar morphology, without pores or cracks.

3.3. Thermogravimetric analysis

Dry samples were analyzed by TGA to determine the thermal stability of the samples and to detect potential interactions between components. **Figure 4.4.1** shows the weight loss with temperature (dm/dT derivative) for selected samples including pure HP-βCD and CS. As can be seen, CS–CD composites presented two main features at temperatures close to those of their two components. The addition of HP-βCDs to CS appeared to produce a delay of ca. 6 ℃ in CS thermal degradation and an advance of 10 ℃ in HP-βCD degradation. These observations are indicative of a certain degree of interaction between the two components. The addition of 35% G to the CS

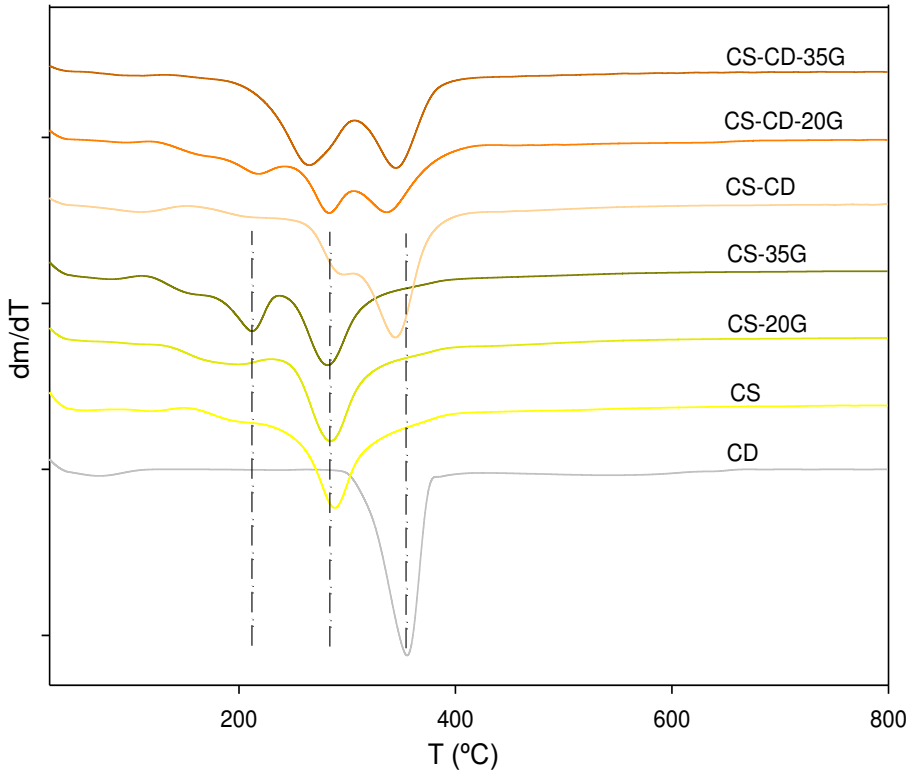

Figure 4.4.1. Temperature derivative of sample mass obtained by TGA for selected composites and components.

matrix reduced the temperature at which maximum weight loss was measured from 289 to 282 ℃ (285 ℃ with 20% G, thermogram not shown). A previous transition at 212 ℃ is due to the degradation of G. Finally, the composite films plasticized with 20% G showed the three transitions which could be expected from the addition rule. In contrast, the sample with 35% G presented only two transitions, as can be seen in **Figure 4.4.1**. The transition assigned to the HP-βCDs appeared at the same temperature as in the unplasticized film. However, a new intermediate feature appeared between those of CS and G, indicating that the two compounds degrade and volatilize together.

3.4. Moisture content

The properties and behaviour of the composites were expected to be dependent on environmental humidity, as occurs with most hydrocolloid-based films. The water gained by the composites was measured at three RHs: 53, 75 and 90%. **Table 4.4.2** resumes the results for various films and components, including bibliographic data for G (Bell and Labuza, 2000). HP-βCDs showed the lowest water gain at all the humidities tested. Plain CS films had higher water affinity than HP-βCDs. The CS–CD composite films had intermediate values which are in agreement with the additivity principle. G was the component with the highest water uptake and therefore the plasticized films had higher water uptake values than the corresponding unplasticized samples, as shown in the table. This effect was more noticeable at 75 and 90% RH. Compared with the results derived from the application of the additivity principle, water sorption by the plasticized composites was below the theoretical value in all cases. This is interpreted as a result of

Table 4.4.2. Water vapour uptake by films and components at 23±1 ℃ and various RH (%).

Materials films	53% RH	75% RH	90% RH
Glycerol (G)	27.00[a]	82.00[a]	215.00[a]
Hydroxypropyl-β-cyclodextrin (HP- βCD)	9.30 ± 0.07	11.30 ± 0.30	28.70 ± 0.13
CS	16.98 ± 0.60	28.60 ± 0.30	45.95 ± 0.50
CS-20G	18.10 ± 0.20	33.10 ± 0.90	64.20 ± 1.50
CS-35G	18.90 ± 0.60	37.70 ± 1.60	75.50 ± 0.40
CS–CD	12.25 ± 0.25	19.30 ± 0.20	39.04 ± 0.30
CS–CD-20G	13.20 ± 0.30	28.80 ± 0.10	61.00 ± 0.40
CS–CD-35G	13.70 ± 0.30	34.80 ± 0.20	70.00 ± 0.35

[a] Data obtained from Bell and Labuza (2000).

interaction between the G and the film matrix, especially with CS, which reduces the ability of the compound to retain water and limits the accessibility of water molecules in the matrix. This result is also in agreement with the information gathered from the thermal analysis.

3.5. Barrier properties

Permeability to gases and vapours is one of the most important properties of materials with potential application in food packaging design. In this work, permeability to water vapour and to oxygen were measured at 23±1 ℃ and at two RH conditions.

3.5.1. Water vapour permeability (WVP)

The effect of the various matrix components and the humidity gradient on the resistance to permeation of water vapour was evaluated for the films and the values are presented in **Table 4.4.3**. The images show various features. First, G in the films gave rise to greater values of permeability to water vapour through both CS- and CS–CD-based materials. As a plasticizer, G reduces the fragility of the polymer material by reducing interchain interactions. G interacts via hydrogen bonds with the NH_2, C=O and −OH substituents of the macromolecules (Brown *et al.*, 2001; Quijada-Garrido *et al.*, 2007), thus increasing chain flexibility and mobility, which leads to lower resistance to the diffusion of permeants. The higher the concentration of G, the more noticeable is the increase in water vapour permeability for a given sample and humidity gradient.

The presence of HP-βCDs in the matrix resulted in an improvement in the water vapour barrier provided. The presence of HP-βCDs appeared to produce an antiplasticizing effect on the film, as has been reported after the incorporation of fillers in polymers. Polymer–CD interactions and the structural rigidity of HP-βCDs could create steric hindrance and decrease segmental mobility, restricting diffusivity of the permeant through the CS matrix. Moreover, as can be seen in **Table 4.4.2**, the addition of HP-βCDs also reduces the water uptake of the matrices. Thus, it might be expected that a reduction in diffusion and in the solubility coefficient would result in a decrease in the permeability values of the biocomposites.

Finally, increasing the humidity gradient yielded higher permeability values for the plain CS and the CS–CD films with or without G. This effect can be correlated to the known water plasticization of hydrocolloids (Caner *et al.*, 1998; Wiles *et al.*, 2000). As humidity increases, water uptake also increases, and so does the plasticization of biopolymer chains.

The effects of HP-βCDs and G on the CS films were also affected by the humidity gradient. The barrier improvement caused by the addition of HP-βCDs is more noticeable at the greater gradient (75% RH), as could be derived from the lower water content observed in the composite samples in comparison with the content in plain CS films. On the other hand, the plasticizing effect of G was less perceptible in the WVP values measured at 75% RH, since at high humidity the water uptake plasticizes the polymer film, mimicking the G effect.

3.5.2. Oxygen permeability

Table 4.4.3 shows the oxygen permeability values measured for CS- and CS–CD-based films at 23±1 ℃ and 50 and 75% RH. CS films provided a high barrier to permeation of oxygen in dry and intermediate humidity conditions, but this property worsened with environmental humidity, as happens with other polymers with high cohesive energy density but also high affinity for water, such as EVOH or PVOH (Gällstedt and Hedenqvist, 2006; Kjellgren *et al.*, 2006; Mensitieri *et al.*, 2011). A similar effect was caused by the addition of G. The presence of humidity and G plasticized the polymer matrix of CS and CS–CD films and consequently caused a large deterioration in the oxygen permeability.

Contrasting with the effect observed in water permeability, the addition of HP-βCDs produced a significant increase in oxygen permeability ($P \leq 0.05$), which was noticeable in all composites and conditions. The cavities of HP-βCDs might be used as channels for diffusion, which would explain the results obtained. The huge deterioration in the oxygen permeability determined for the CS–CD-35G sample is noteworthy; the barrier worsened by a factor of 100,000. The obtaining of a loose polymer network owing to the G content might be responsible for this mass transport behaviour.

3.6. Mechanical properties

Tensile strength, elongation at break and modulus of elasticity were determined (**Table 4.4.4**). The effect on these properties of the presence of HP-βCDs in CS films and the effect of the humidity and the concentration of G in the films were evaluated.

As shown, the addition of HP-βCDs to the CS matrix produced changes in its mechanical properties. In the absence of G, the tensile strength and modulus of elasticity of CS and CS–CD films did not differ significantly at any of the humidities tested. However, the elongation at break was significantly reduced ($P \leq 0.05$) by the incorporation of HP-βCDs at both 53% and 75% RH. This reduction was more acute in films conditioned at 75% RH. Thus, HP-βCDs act as fillers for the CS matrix, decreasing the strain of the films. It has been reported that fillers produce a rapid decrease in the elongation at break of polymers, especially if there is good adhesion between the phases.

Regarding the effect of moisture, both matrices, CS and CS–CD, showed a decrease in tensile strength and modulus of elasticity when films were conditioned at 75% RH, which can be explained by the plasticizing effect of water on the polymer matrix. The elongation at break of CS films rose with humidity, but the effect of humidity on the elongation at break of the CS–CD composites was much less acute.

G produced a decrease in tensile strength and modulus of elasticity in both, CS and CS–CD matrices, and higher values of elongation, effects which were more marked for films having a greater content of G. All these effects were predictable, since G acts in any of these matrices as a plasticizer, reducing interchain interactions and cohesion. The addition of this plasticizer had a secondary effect, which was the increment in water content of the matrices at any humidity because of the high hydrophilicity of G, as already shown in **Table 4.4.2**. Since both water and G produced the same effect on the matrices, their effect on mechanical properties was cumulative, the materials being less brittle and more deformable for a greater content in either G or humidity.

Regarding the effect of G in the elongation at break of CS or CS–CD films, **Table 4.4.4** shows that contents exceeding 20% G did not affect this

Table 4.4.3. Water vapour permeability of CS and CS–CD films obtained with 0–53% and 0–75% humidity gradient at 23±1 ℃ and oxygen permeability of CS and CS–CD films obtained at 50% and 75% RH at 23±1 ℃.

Materials films	Water vapour permeability ($\times 10^{-11}$ g·m/(m^2·s·Pa))		Oxygen permeability (cc·m/(m^2·s·Pa))	
	0–53% RH	0–75% RH	50% RH	75% RH
CS	$2.49 \pm 0.13^{a,y,m}$	$5.77 \pm 0.37^{a,y,n}$	$(2.80 \pm 0.12)10^{-14\ a,x,m}$	$(9.27 \pm 0.06)10^{-14\ a,x,n}$
CS-20G	$2.57 \pm 0.12^{a,y,m}$	$6.93 \pm 0.62^{b,y,n}$	$(4.98 \pm 0.05)10^{-14\ b,x,m}$	$(5.40 \pm 0.90)10^{-13\ b,x,n}$
CS-35G	$5.29 \pm 0.22^{b,x,m}$	$11.11 \pm 0.92^{c,x,n}$	$(1.15 \pm 0.01)10^{-13\ c,x,m}$	$(1.25 \pm 0.16)10^{-12\ c,x,n}$
CS–CD	$2.16 \pm 0.03^{a,x,m}$	$3.49 \pm 0.34^{a,x,n}$	$(4.51 \pm 0.13)10^{-14\ a,y,m}$	$(1.04 \pm 0.10)10^{-13\ a,y,n}$
CS–CD-20G	$2.35 \pm 0.06^{b,x,m}$	$5.96 \pm 0.38^{b,x,n}$	$(7.98 \pm 0.15)10^{-14\ b,y,m}$	$(7.17 \pm 0.79)10^{-13\ b,y,n}$
CS–CD-35G	$6.61 \pm 0.10^{c,y,m}$	$10.37 \pm 1.38^{c,x,n}$	$(2.43 \pm 0.09)10^{-8\ c,y,m}$	$(1.45 \pm 0.25)10^{-7\ c,y,n}$

Different letters in the same column (a–c) indicate a statistically significant difference ($P \leq 0.05$) comparing different amounts of G in the same matrix CS or CS–CD.
Different letters in the same column (x–y) indicate a statistically significant difference ($P \leq 0.05$) comparing CS and CS–CD.
Different letters in the same rows (m–n) indicate a statistically significant difference ($P \leq 0.05$) comparing different RHs of analysis for the same matrix and G content.

Table 4.4.4. Tensile strength, modulus of elasticity and elongation at break of CS and CS–CD films with different G contents at 53 and 75% RH and 23±1 ℃.

Materials films	Tensile strength (MPa)	Elongation at break (%)	Modulus of elasticity (MPa)	Tensile strength (MPa)	Elongation at break (%)	Modulus of elasticity (MPa)
	53% RH			75% RH		
CS	$57.47 \pm 3.25^{b,x,n}$	$16.39 \pm 4.23^{a,y,m}$	$1635.54 \pm 150.63^{c,x,n}$	$46.45 \pm 3.32^{c,x,m}$	$40.58 \pm 7.01^{a,y,n}$	$1297.0 \pm 121.7^{c,x,m}$
CS-20G	$40.00 \pm 4.74^{a,y,n}$	$57.04 \pm 7.59^{b,x,m}$	$609.00 \pm 70.06^{b,y,n}$	$25.46 \pm 3.46^{b,y,m}$	$54.15 \pm 3.53^{b,x,m}$	$149.2 \pm 38.0^{b,y,m}$
CS-35G	$31.00 \pm 7.24^{a,y,n}$	$56.92 \pm 5.27^{b,x,m}$	$175.95 \pm 52.61^{a,y,n}$	$19.40 \pm 3.26^{a,y,m}$	$53.52 \pm 5.21^{b,x,m}$	$28.7 \pm 4.6^{a,y,m}$
CS–CD	$58.00 \pm 3.88^{c,x,n}$	$5.09 \pm 0.79^{a,x,m}$	$1855.28 \pm 160.00^{c,x,n}$	$44.32 \pm 3.27^{c,x,m}$	$6.19 \pm 0.73^{a,x,m}$	$1440.8 \pm 128.5^{c,x,m}$
CS–CD-20G	$19.91 \pm 1.19^{b,x,n}$	$55.21 \pm 4.27^{b,x,m}$	$198.00 \pm 61.00^{b,x,n}$	$13.21 \pm 1.29^{b,x,m}$	$54.41 \pm 5.24^{b,x,m}$	$46.6 \pm 7.4^{b,x,m}$
CS–CD-35G	$7.87 \pm 2.17^{a,x,m}$	$56.61 \pm 7.39^{b,x,m}$	$14.36 \pm 2.10^{a,x,n}$	$5.00 \pm 1.91^{a,x,m}$	$55.33 \pm 5.08^{b,x,m}$	$8.7 \pm 0.7^{a,x,m}$

Different letters in the same column (a–c) indicate a statistically significant difference ($P \leq 0.05$) comparing different amounts of G in the same matrix CS or CS–CD.
Different letters in the same column (x–y) indicate a statistically significant difference ($P \leq 0.05$) comparing CS and CS–CD.
Different letters in the same rows (m–n) indicate a statistically significant difference ($P \leq 0.05$) comparing different RHs of conditioning for the same matrix and G content.

property at 53 and 75% RH. Apparently, the integrity of the matrices is damaged when films are elongated more than 50% of their initial length. G and humidity produced a similar effect on the tensile strength and modulus of elasticity of both CS and CS–CD films. However, the decline of these properties was more pronounced in the CS–CD composites, indicating a greater plasticizing capacity. It is worth highlighting that the amount of G related to the CS polymer in the composite was double the amount in the plain film.

3.7. Sorption of carvacrol

Samples of CS and CS–CD composite films, prepared with and without G and conditioned at various RHs, were immersed in carvacrol for three months. After this prolonged exposure to carvacrol, the films did not break or lose their integrity, they were easy to handle and showed an apparently good mechanical resistance. Differences within samples were evident by visual inspection, since initially colourless composites acquired a yellow/green colour depending on the amount of carvacrol sorbed (**Figure 4.4.2**). **Table 4.4.5** shows the sorption of carvacrol by CS and CS–CD films plasticized with different amounts of G (0, 20 and 35%) and conditioned at various RHs (dry film, 53, 75 and 90%).

For the CS films the sorption of carvacrol did not exceed 1%, reflecting the low affinity of this hydrophilic polymer for carvacrol. The presence of water and G in the CS matrix significantly affected the carvacrol retention capacity. It was observed that, in the films conditioned at a given RH and thus having a fixed water content, sorption of carvacrol increased when the G content increased from 0 to 35% (g/100 g dry matter). G-unplasticized films and films with 35% G conditioned at 90% RH retained less carvacrol than those conditioned at 53 and 75% RH. On the one hand, water and G have a

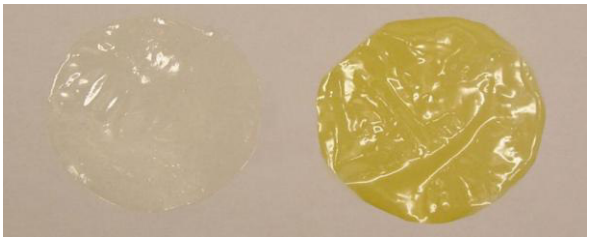

Figure 4.4.2. CS–CD-35G composites conditioned at 75% RH, before (left) and after (right) in contact with carvacrol for three months.

Table 4.4.5. Sorption equilibrium of carvacrol in CS and CS–CD films at 23±1 ℃.

Materials films	% Sorption of carvacrol (g/100 g dry matter)			
	0% RH	53% RH	75% RH	90% RH
CS	$0.08 \pm 0.01^{a,x,m}$	$0.17 \pm 0.01^{a,x,o}$	$0.19 \pm 0.03^{a,x,o}$	$0.11 \pm 0.01^{a,x,n}$
CS-20G	$0.09 \pm 0.02^{a,x,m}$	$0.23 \pm 0.03^{b,x,n}$	$0.36 \pm 0.01^{b,x,o}$	$0.27 \pm 0.03^{b,n}$
CS-35G	$0.47 \pm 0.07^{b,x,m}$	$0.96 \pm 0.04^{c,x,o}$	$0.92 \pm 0.07^{c,x,o}$	$0.68 \pm 0.07^{c,n}$
CS–CD	$0.26 \pm 0.02^{a,y,m}$	$0.40 \pm 0.01^{a,y,n}$	$0.43 \pm 0.03^{a,y,n}$	$9.97 \pm 1.12^{y,o}$
CS–CD-20G	$0.34 \pm 0.02^{b,y,m}$	$6.13 \pm 0.39^{b,y,n}$	$56.84 \pm 3.52^{b,y,o}$	-
CS–CD-35G	$4.50 \pm 0.26^{c,y,m}$	$133.27 \pm 16.93^{c,y,n}$	$216.00 \pm 22.00^{c,y,o}$	-

Different letters (a–c) in the same column indicate a statistically significant difference ($P \leq 0.05$) comparing different amounts of G in the same matrix CS or CS–CD.
Different letters (x and y) in the same column indicate a statistically significant difference ($P \leq 0.05$) comparing CS and CS–CD with the same G content.
Different letters (m–o) in the same rows indicate a statistically significant difference ($P \leq 0.05$) comparing different RHs of conditioning for the same matrix and G content.

positive effect on the sorption of carvacrol; these compounds act as plasticizers, decreasing polymer-polymer interactions and increasing chain mobility and free volume in the polymer matrix, facilitating sorption of carvacrol. However, the presence of a high water content in the polymer matrix makes the films more polar reducing their affinity for the non-polar phenolic compound carvacrol.

It is worth noting that only CS–CD films without G could be conditioned at 90% RH, since when G was added the films conditioned at that humidity were very sticky and could not be handled. In general, water and G have a similar effect in CS matrices incorporating HP-βCDs. However, retention of carvacrol in the composites ranged from 0.26% for non-plasticized dry film to 216% for films containing 35% G and conditioned at 75% RH, the sorption amount being dependent on the water and G content in the films.

It was expected that HP-βCDs would promote the sorption of carvacrol, owing to their ability to form inclusion complexes with non-polar molecules. Theoretically, assuming the formation of 1:1 or 1:2 β-CD:carvacrol complexes (Locci *et al.*, 2004; Ravi and Divakar, 2001) the carvacrol content of the composites would range between 5 and 10% (g carvacrol/100 g dry matter). As shown in **Table 4.4.5**, HP-βCDs enhanced the retention of carvacrol in the biocomposites compared to plain films. Biocomposites without G conditioned at 53 and 75% RH or in a dry environment, and dry biocomposites containing 20% G presented significant increases in carvacrol gain with respect to CS films, but carvacrol retention was below 1%.

Biocomposites containing 35% G and conditioned at 53 or 75% RH and those having 20% G and conditioned at 53% RH retained high levels of carvacrol, 133, 216 and 57% carvacrol (g/100 g dry matter), respectively, carvacrol sorption in these biocomposites being much greater than the theoretical sorption expected. Assuming that carvacrol is able to form 1:1 and 2:1 inclusion complexes (guest:HP-βCD), the theoretical amount of carvacrol expected to be sorbed by the film would be <10% (g carvacrol/100 g dry matter). Since amounts of carvacrol above 10% were found in some films, an alternative mechanism to the formation of inclusion complexes must be taking place. These results suggest that sorption of carvacrol in CS–CD films largely plasticized by G and water could be occurring by mechanisms other than formation of inclusion complexes with HP-βCDs. It has been reported that CDs are able to self-assemble to form nanosized complex aggregates, and aggregation happens rapidly with CD concentration (Messner *et al.*, 2010). Given that the composition of the biocomposites is CS:βCDs 1:1, HP-βCDs occupy a considerable volume in the polymer matrix, which could be forming nanoparticles. Furthermore, a great increase in the free volume of the film is expected as a result of the presence of G and water.

4. Conclusions

Films having good transparency and moderate mechanical properties and permeability to water and oxygen have been developed incorporating 1:1 HP-βCDs into a CS matrix. These hydrophilic films are capable of retaining different amounts of the non-polar, volatile compound carvacrol. Sorption depends on the degree of plasticization of the film by G and water, thus it is possible to tailor the amount of the volatile agent in the film. Due to the antimicrobial properties of carvacrol, the film developed could be used as a sustained release device in food packaging, pharmaceutical and agrochemistry applications.

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4.4.3. Artículo científico 5.

Incorporation of hydroxypropyl-β-cyclodextrins into chitosan films to tailor loading capacity for active aroma compound carvacrol

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Food Hydrocolloids (2015) 43, 603–611

Resumen

Se han desarrollado películas de quitosano (CS) con hidroxipropil-β-ciclodextrinas (HP-βCD) y glicerol (G) capaces de modular la capacidad de carga y la liberación de carvacrol. Las películas fueron obtenidas por extensión y evaporación del disolvente y acondicionadas a diferentes humedades relativas (RH) antes de su inmersión en carvacrol líquido. La incorporación de las ciclodextrinas en la matriz de quitosano aumentó ligeramente sorción de carvacrol y fue necesario utilizar glicerol y agua como coadyuvantes para el control de carga de las películas con el volátil. Se encontró buena correspondencia entre la retención de carvacrol y plastificación de la película de glicerol y agua. Se evaluó la cinética de sorción carvacrol de las películas se evaluó a 25 ℃. Los coeficientes de difusión de sorción variaron desde $0.011x10^{-14}$ m^2/s para las películas que incorporaban el 35% de glicerol acondicionadas al 0% HR a $1.9x10^{-14}$ m^2/s para las películas que incorporan 35% de glicerol acondicionadas al 75% de RH. La liberación de carvacrol de las películas se ve muy afectada por RH. Las películas mostraron actividad antimicrobiana contra *Staphylococcus aureus* y *Escherichia coli* después de 20 días de almacenamiento a 25 ℃ y 43% de RH ambiental. Estas películas podrían ser empleadas en el diseño de sistemas de liberación de volátiles activos.

Palabras clave

Quitosano, hidroxipropil-β-ciclodextrinas, carvacrol, carga y liberación, películas antimicrobianas.

Abstract

Chitosan (CS) incorporating hydroxypropyl-β-cyclodextrins (HP-βCD) and glycerol (G) films capable of modulating loading capacity and release of carvacrol have been developed. Films were obtained by casting and conditioned at different relative humidities (RH) prior to immersion in liquid carvacrol. Incorporation of cyclodextrins in the chitosan matrix slightly increased sorption of carvacrol and it was necessary to use glycerol and water as coadjuvants to control loading of the films with the volatile. Good agreement was found between carvacrol retention and plasticization of the film by glycerol and water. The kinetics of carvacrol sorption by the films was evaluated at 25 ºC. Diffusion coefficients of sorption varied from $0.011x10^{-14}$ m^2/s for films incorporating 35% glycerol and conditioned at 0% RH to $1.9x10^{-14}$ m^2/s for films incorporating 35% glycerol and conditioned at 75% RH. Release of carvacrol from the films was greatly affected by RH. The films showed antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* after 20 days of storage at 25 ºC and 43% environmental RH. These films could be useful in the design of systems for delivering active volatiles.

Keywords

Chitosan, hydroxypropyl-β-cyclodextrins, carvacrol, loading and release, antimicrobial films.

1. Introduction

Delivery systems based on polymers capable of carrying and delivering a continuous supply of biologically active molecules into a specific environment have become of increasing interest in recent years. These systems are able to reduce the amount of active agent required for treatment by maintaining an effective concentration in the system applied over a certain period of time (Ouattara *et al.*, 2000). There is a great need for these devices in diverse technological applications encompassing multidisciplinary areas such as biomedicine, pharmacology, agriculture, packaging, food technology, textiles and the cosmetic industry for the entrapment and delivery of drugs, enzymes, nutraceuticals, agrochemicals, flavours and fragrances, biocides, etc. Currently, most of the recently developed delivery systems consist of natural and synthetic polymers, polymer blends, and composites of organic and inorganic materials that form membranes, capsules or micelles, depending on the application required. Issues concerning biodegradability, biocompatibility and non-toxicity of the materials used for the development of carrier systems need to be considered. Renewable polymers are being widely investigated as delivery vehicles because most of them fulfil the aforementioned requirements.

CS polymer (poly β-(1, 4)*N*-acetyl-*D*-glucosamine) has been intensively studied during recent decades (Dutta *et al.*, 2012; López-Carballo *et al.*, 2013; Valencia-Chamorro *et al.*, 2011). It is a natural cationic linear aminopolysaccharide obtained from partial *N*-deacetylation of chitin. CS is receiving a great deal of attention in biomedicine and pharmacology for the delivery of drugs (Ramya *et al.*, 2012). CS can also act as a carrier for sustained release and delivery of compounds other than drugs which are of interest in foods, personal care, agriculture, etc. (Kumar *et al.*, 2004; Prabaharan and Mano, 2006; Zhang *et al.*, 2009).

Antimicrobial carriers are of great interest in the area of food packaging, and great efforts are being made to develop effective antimicrobial food packaging systems (Appendini and Hotchkiss, 2002; Suppakul *et al.*, 2003). Antimicrobial food packaging technologies which are based on the incorporation of active volatiles in polymer matrices do not require the film be in contact with the food product to be active. In this case,

the volatile is released to the headspace of the package and exerts its activity when contact with the food surface.

There is a wide range of volatile compounds derived from plants or forming part of the aroma profile of fruits presenting biocide properties which could be applied in the design of antimicrobial carriers since most of them are generally recognized as safe (GRAS) and are used as food flavouring or seasoning agents. However, volatiles can be lost to some extent during entrapment or encapsulation in the polymer matrix, which consequently decreases the retention process. Therefore, it would be of great interest to develop suitable carriers with a high entrapment capacity and sustained release properties for volatile compounds. In addition, the release of the volatile from the polymer matrix can be triggered by different stimuli such as the moisture present in the headspace of the package; in this respect, the hydrophilic nature of the polymer and the RH of the headspace are major factors controlling the release kinetics of the agent.

Cyclodextrins (CDs) are cyclic oligosaccharides consisting of a three-dimensional structure forming a truncated cone with a hydrophobic cavity and a hydrophilic outer surface. CDs are widely used as excipients in pharmacy to solubilise lipophilic molecules by means of inclusion complexes. However, non-inclusion aspects of CDs are being studied, such as solubilisation by formation of selfassembled aggregates or surfactant-like effects. In recent years, CDs and their derivatives have been used as building blocks for the development of a wide variety of polymeric networks and assemblies with a higher drug loading capacity. They have been blended with polymers in the design of nano/microparticles, and micelles, for the sustained release and targeted delivery of bioactive substances (Van de Manakker *et al.*, 2009).

The aim of this work was to develop novel films by blending CS with HP-βCD (a water soluble CD derivative) in order to improve the capacity of CS film to be loaded with carvacrol, a naturally occurring phenolic volatile with antimicrobial properties which is a major component of thyme and oregano essential oils. The loading capacity and sorption kinetics of carvacrol were studied as a function of film formulation, and the release of carvacrol was evaluated at different RH. The antimicrobial activity of the films was tested *in vitro* in vapour phase against pathogen bacteria *Staphylococcus*

aureus and *Escherichia coli*. Finally, the antimicrobial activity of the films was monitored over time.

2. Materials and methods

2.1. Materials

Carvacrol (kosher > 98%), dimethyl sulfoxide (99.9% ACS Reagent grade), phosphorus pentoxide, magnesium nitrate 6-hydrate, glycerol (G), acetic acid and low molecular weight chitosan (CS) were supplied by Sigma (Barcelona, Spain). Sodium chloride, potassium carbonate and barium chloride 2-hydrate, were supplied by Fluka (Madrid, Spain). Hydroxypropyl-β-CDs (HP-βCDs, CAVASOL ® W7-HP) were provided by Wacker Fine Chemicals, S.L. (Barcelona, Spain). Maltodextrins (MDs) from maize starch, Biochemika, 10, was supplied by Sigma–Aldrich (Madrid, Spain).

2.2. Film preparation

A flowchart with the preparation of chitosan/hydroxypropyl-β-cyclodextrin (CS:CD) films is shown in **Figure 4.4.3**. CS (1.5% w/w) was solubilised in 0.5% (w/w) acetic acid solution and filtrated to eliminate impurities. Films were prepared by casting, pouring a suitable amount of the film-forming solution into a flat PS tray and allowing it to dry under controlled environmental conditions (36 h, 40.0±1.5 ℃ and 20±9% RH). CS:CD films were obtained by adding HP-βCDs to the CS solution in 1:1 weight ratio of HP-βCDs to CS, and G at 0% (CS:CD-0G), 20% (CS:CD-20G) and 35% (CS:CD-35G) (g G/100 g dry matter) was added to the film-forming solution while stirring at 1500 rpm and 37 ℃ until complete dissolution. Films were obtained by casting as described above. After peeling the films from the tray, they were plasticized with different amounts of water, for which purpose samples 550 mm in diameter and 55±5 mm in thickness were stored in glass desiccators with phosphorus pentoxide to achieve humidities close to 0%, to dry the films, or at humidities of 52.9±0.2 and 75.3±0.1 RH, using saturated salt solutions (ASTM, 2007) in a temperature-controlled room at 25±1 ℃ until moisture equilibrium was reached. Films were named as (CS:CD-xxGxxRH), depending on the amount of G and the RH at which they were conditioned prior to being loaded with carvacrol.

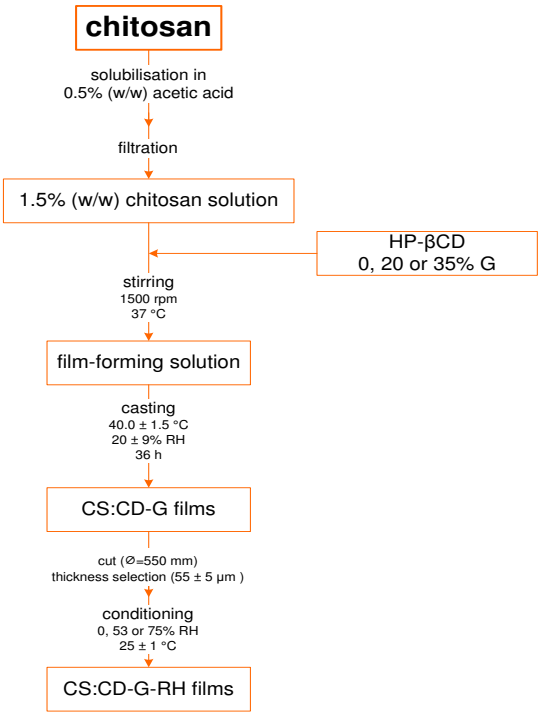

Figure 4.4.3. Flowchart of film preparation.

To study how the amount of HP-βCDs blended with CS affects sorption of carvacrol by the resulting films, specimens were prepared at 1:2, 1:0.5 and 1:0.25 weight ratio and compared with 1:1 CS:CD proportion; film samples of 550 mm in diameter and 55 ± 5 mm in thickness were plasticized with 35% G and conditioned at 75% RH at room temperature as described above prior to immersion in carvacrol.

CS film samples of similar size and thickness as mentioned above and plasticized with 35% G were also mixed with MDs in a 1:1 (w/w) proportion and conditioned at three different humidities as described above, and carvacrol sorption properties studied.

2.3. Optical properties of the films before and after immersion in carvacrol

The colour of the films before and after immersion in carvacrol was measured with a CR-300 Minolta Chroma meter® (Minolta Camera Co., Ltd., Osaka, Japan). The film samples were placed on a white standard plate; the results were expressed in accordance with the CIELAB system with reference

to illuminant D65 and a visual angle of 10º. The measurements were performed through a 6.4-mm-diameter diaphragm containing an optical glass, monitoring L*, a*, b*, chroma ($C^*_{ab}=(a^{*2}+b^{*2})^{1/2}$) and hue ($h_{ab}$=arctan (b*/a*)). The samples were measured in triplicate by eight measurements in different locations for each film sample.

2.4. Loading and release of carvacrol

After being conditioned at different RH to achieved the desired water content, film samples with various matrix compositions as described in "Film preparation" section were immersed in liquid carvacrol at 25 ºC and the amount of the compound sorbed in the film was measured over time until sorption equilibrium was reached. For that, after a determined period of time depending of the film composition, a piece of the film was cleaned with a paper tissue to remove any excess of carvacrol on the film surface and then inserted into an empty desorption tube (11.5 x 0.39 cm I.D.) for thermal desorption. The release of carvacrol from the films was evaluated as a function of time at 25 ºC and at three RH: 43.2±0.4, 52.9±0.2, and 90±2%. For this purpose, a flow of air of 200 mL/min was bubbled in a saturated salt solution to provide the desired RH (ASTM, 2007), and passed through a hermetically closed 500-mL container where the film sample was placed. The release of carvacrol from the films was calculated by analysing the amount of carvacrol remaining in the film by thermal desorption.

2.5. Analysis of carvacrol in a film

The amount of carvacrol in a film was determined by thermal desorption coupled to gas chromatography using a Dynatherm Thermal Desorber Model 890/891 (Supelco, Teknokroma, Barcelona, Spain) connected in series to the column of an HP5890 gas chromatograph Series II Plus (Agilent Technologies, Barcelona, Spain) via a heated transfer line. The desorption tube containing the film sample was placed in the desorber chamber, which was immediately sealed. Conditions for desorption were as follows: desorption temperature, 210 ºC; transfer line, 230 ºC; desorption time, 7 min; He desorption flow, 8.15 mL/min. The GC was equipped with a TRB5 (30 m, 0.32 mm, 0.25 mm) column (Teknokroma, Barcelona, Spain) and a flame ionization detector. The chromatographic conditions were: 260 ºC

detector temperature, 7 min at 45 ℃, heating ramp to 220 ℃ at 18 ℃/min, and 1 min more at 220 ℃. After the analysis, the film sample was recovered from the desorption tube and weighed on an analytical balance (Voyager V11140 model, Ohaus Europe, Greifensee, Switzerland).

2.6. Antimicrobial assays

2.6.1. Bacterial strains and growth conditions

Two model microbial strains were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain): the Gram-positive bacterium *S. aureus* CECT 86 and the Gram-negative bacterium *E. coli* CECT 434. The strains were stored in Mueller Hinton Broth (MHB, Scharlab, Barcelona, Spain) with 20% G at –80 ℃ until needed. For experimental use, the stock cultures were maintained by regular subculture on Tryptone Soy Agar (TSA, Scharlab, Barcelona, Spain) slants at 4 ℃ and transferred monthly. In the first step, a loopful of each strain was transferred to 10 mL of Tryptone Soy Broth (TSB, Scharlab, Barcelona, Spain) and incubated at 37 ℃ overnight to obtain early stationary phase cells.

2.6.2. Determination of minimal inhibitory concentration of carvacrol in vapour phase

The microatmosphere method was selected to carry out an antimicrobial test in which no direct contact between the device containing the volatile and the agar medium is necessary for the former to exert its activity. In this method, the volatile compound migrates from the carrier (filter paper, film) into the headspace of the system, thus becoming available to contact the growth medium and the microorganism. 100 mL of a bacterium suspension containing approximately 10^7 CFU/mL was spread over the surface of 90-mm-diameter Petri dishes containing approximately 15 mL of solid culture TSA medium. Decreasing quantities of carvacrol were dissolved in dimethyl sulfoxide (DMSO), and a final volume of 10 mL of the carvacrol solution was added to 25-mm-diameter sterilized filter paper. Blanks prepared by adding 10 mL of DMSO to 25-mm-diameter sterile filter disks showed that the DMSO did not have any effect against any of the microorganisms tested. Once the Petri dishes and lids had been assembled,

the units were sealed with Parafilm™ to reduce leakage of the volatile agent and incubated at 37 ℃ for 24 h. At the end of the incubation period, the antimicrobial activity was determined by measuring the diameter in millimetres of the zone below the filter papers where there was no microbial growth. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of active compound that yields inhibition of microorganism (Delaquis *et al.*, 2002; Hammer *et al.*, 1999). Each assay was performed in triplicate.

2.6.3. Antimicrobial activity of the films

The procedure to determine the antimicrobial activity of the films was similar to that described above. In this case, films of the same size as the filter papers and loaded with carvacrol were placed in the lid of the inoculated Petri dishes. Each assay was performed in triplicate.

2.7. Data analysis

Statistical analysis of the results was performed with SPSS commercial software (SPSS Inc., Chicago, Illinois, USA). A two-way analysis was applied to compare the effect of different amounts of G in the same CS or CS:CD matrix. Additionally, one-way analysis of variance was carried out for the other data. Differences between means were assessed on the basis of confidence intervals, using the Tukey-*b* test at a significance level of $P \leq 0.05$. The data are represented as average±standard deviation. The data were analysed and plotted using the SigmaPlot 10.0 software (Systat Software Inc., Richmond, California, USA).

3. Results and discussion

CS:CD films prepared at a weight ratio of 1:1 were homogeneous, with no phase separation visible to the naked eye, easy to handle and highly transparent. After immersion in carvacrol, the films maintained their integrity and did not break when handled.

3.1. Colour properties

Table 4.4.6 shows colour parameters of films incorporating 0, 20 and 35% G and conditioned at different RH before and after being immersed in carvacrol. Before immersion in carvacrol the incorporation of G at 20 or 35%

Table 4.4.6. Colour parameters of CS:CD (1:1 weight ratio) films.

% Glycerol	L*	a*	b*	C^*_{ab}	h_{ab}
Prior to immersion in carvacrol					
0	95.5 ± 0.2^{a,wx}	-0.27 ± 0.06^{a,w}	5.9 ± 0.3^{a,w}	5.9 ± 0.3^{a,w}	92.7 ± 0.5^{a,w}
20	95.5 ± 0.2^{a,w}	-0.33 ± 0.05^{a,w}	5.9 ± 0.2^{a,}	5.9 ± 0.3^{a,x}	93.3 ± 0.3^{a,x}
35	95.3 ± 0.3^{a,w}	-0.48 ± 0.07^{b,x}	6.2 ± 0.3^{a,z}	6.2 ± 0.3^{a,z}	93.4 ± 0.4^{a,x}
After immersion in carvacrol 0% RH					
0	95.6 ± 0.8^{a,wx}	- 0.32 ± 0.03^{a,w}	5.9 ± 0.3^{a,w}	5.9 ± 0.3^{a,w}	93.2 ± 0.3^{a,w}
20	95.8 ± 0.7^{a,w}	- 0.32 ± 0.03^{a,w}	5.7 ± 0.4^{a,x}	5.7 ± 0.4^{a,x}	93.1 ± 0.4^{a,x}
35	95.6 ± 0.5^{a,w}	- 0.33 ± 0.02^{a,w}	5.9 ± 0.5^{a,y}	5.9 ± 0.6^{a,y}	93.1 ± 0.6^{a,x}
After immersion in carvacrol 53% RH					
0	95.5 ± 0.5^{a,wx}	- 0.29 ± 0.04^{a,w}	5.8 ± 0.4^{b,w}	5.9 ± 0.4^{b,w}	92.9 ± 0.3^{b,w}
20	95.6 ± 0.9^{a,w}	- 0.35 ± 0.06^{a,w}	5.9 ± 0.4^{b,x}	5.9 ± 0.4^{b,x}	93.4 ± 0.4^{b,x}
35	91.9 ± 0.6^{b,x}	- 3.76 ± 0.07^{b,y}	24.7 ± 0.9^{a,x}	24.4 ± 1.2^{a,x}	98.7 ± 0.4^{a,w}
After immersion in carvacrol 75% RH					
0	96.8 ± 0.4^{a,w}	- 0.23 ± 0.05^{a,w}	5.7 ± 0.5^{c,w}	5.7 ± 0.5^{c,w}	92.5 ± 0.4^{b,w}
20	93.6 ± 0.8^{b,x}	- 3.03 ± 0.23^{b,x}	19.6 ± 0.9^{b,w}	19.8 ± 0.6^{b,w}	99.2 ± 0.5^{a,w}
35	91.3 ± 0.8^{c,x}	- 4.51 ± 0.14^{c,z}	27.2 ± 0.6^{a,w}	27.5 ± 0.5^{a,w}	99.5 ± 0.5^{a,w}

$^{a-c}$ Different letters in the same column indicated significant differences ($P \leq 0.05$) when comparing films incorporating different content of G and conditioned at the same RH.
$^{w-z}$ Different letters in the same column indicated significant differences ($P \leq 0.05$) when comparing films conditioned at different RH prior immersion in carvacrol and incorporating the same amount of G.

did not significantly ($P > 0.05$) modify the colour parameters of the films when compared with those prepared without G. Moreover, there were no significant differences in colour parameters ($P > 0.05$) of G plasticized films conditioned at different RH (data not shown). After immersion in carvacrol, films plasticized with G at 20 or 35% and conditioned at 75% RH, and films plasticized with 35% G and conditioned at 53% RH acquired a vivid yellow-green colour, increasing their chroma and hue, and slightly decreasing their lightness. These changes are related to the amount of carvacrol that the films are capable of retaining. **Figure 4.4.4** shows the sorption equilibrium of carvacrol in films incorporating different percentages of G and conditioned at various RH. According to the results obtained, changes in colour parameters were not significant for films retaining less than 6% of carvacrol.

3.2. Sorption properties

3.2.1. Sorption equilibrium of carvacrol in films

As **Figure 4.4.4** shows, the amount of carvacrol retained in the films depended on the level of plasticization by G and moisture. Carvacrol

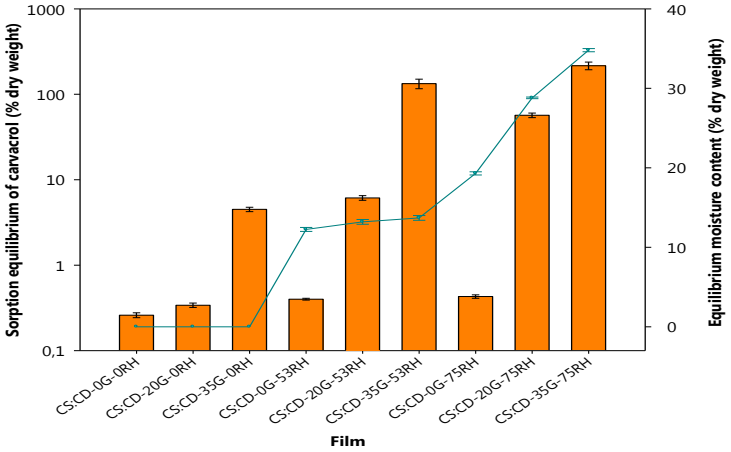

Figure 4.4.4. Sorption equilibrium of carvacrol in CS:CD films with a 1:1 (w/w) blend ratio, incorporating several percentages of G and conditioned at different RH prior to immersion in the solvate and measured at 25 ℃. Figure also shows the equilibrium moisture content of the films at 25 ℃ before being immersed in carvacrol.

retention in films with a fixed amount of G increased as the RH at which they were conditioned increased; water acts as a plasticizer for CS:CD films, enhancing their carvacrol loading capacity. It can also be observed that at a fixed RH carvacrol sorption increased with the G content in the film. The highest carvacrol loading capacity was achieved by films plasticized with 35% G and conditioned at 75% RH, followed by the same films conditioned at 53% RH, and films incorporating 20% G and conditioned at 75% RH; the sorption of carvacrol in these films was 216.3±22.1, 133.3±16.9 and 56.8±3.5% (g carvacrol/g dry film), respectively. In spite of having lower water content (**Figure 4.4.4**), films incorporating 35% G and conditioned at 53% RH absorbed a greater amount of carvacrol than films incorporating 20% G and conditioned at 75% RH. When G was not incorporated in the CS:CD matrix, sorption of carvacrol did not exceed 0.45% (g carvacrol/g dry film), regardless of the RH at which they were conditioned; whereas the maximum amount of carvacrol sorbed by films stored under dry conditions was 4.5%, corresponding to films plasticized with 35% G. These observations highlight the fact that G plays a crucial role in the sorption of carvacrol. Thus, the use of HP-βCD together with G and moisture is required to control the loading of carvacrol in a CS matrix. It is worth pointing out that previous studies (Higueras *et al.*, 2013) showed that CS films without HP-βCD did not

retain more than 1% of carvacrol, whatever the amount of G and water incorporated in the matrix. Incorporation of HP-βCD in the CS matrix slightly increased the sorption of carvacrol compared with plain CS films, and the use of water and G as coadjuvants was necessary to control the loading of the films with carvacrol. Kurek *et al.* (2014) studied the effect of various additives on the retention of carvacrol during the processing of CS films by casting and found that carvacrol retention was directly correlated with the incorporation of G and nanoclays into the matrix; moreover, retention of carvacrol was also favoured by blending CS with gum arabic because of the formation of coacervates which encapsulate carvacrol and prevent its evaporation from the matrix during the drying of the films.

CDs are commonly used to solubilise lipophilic molecules, β-CDs are capable of forming 1:1 and 1:2 host/guest complexes with carvacrol (Locci *et al.*, 2004; Ravi and Divakar, 2001). In this work, the maximum percentage of carvacrol that could be held in CS films incorporating HP-βCD was <10% (g carvacrol/100 g dry matter). When water and G were present, the amount of carvacrol retained in the films exceeded this percentage, so mechanisms other than the formation of inclusion complexes participate in the sorption of carvacrol. In the last few years, a growing body of research has shown that CDs can act as building units able to self-assemble into aggregates driven by CD–CD H-bonds, and these aggregates can act as solubilizers. The size of these aggregates tends to grow with increasing concentration of CDs, and aggregates up to several micrometres in diameter have been reported (Messner *et al.*, 2010). Water-soluble polymers contribute to the stabilization of these aggregates through formation of CD-polymer hydrogen bonds (Ribeiro *et al.*, 2003). Formation of CD aggregates could enhance the loading of amphiphilic molecules such as carvacrol by plasticized CS films that otherwise present a very low sorption potential. Thus, formation of CD aggregates could explain the high carvacrol sorption values found for some of the films developed in the present work.

Moreover, the effect of HP-βCDs concentration in a CS film on carvacrol sorption capacity was also studied. **Figure 4.4.5** shows the sorption equilibrium of carvacrol in CS films plasticized with 35% G and conditioned at 75% RH and incorporating HP-βCDs in CS:CD weight ratios of 1:0, 1:0.25, 1:0.5, 1:1 and 1:2. The sorption of carvacrol greatly depended on the HP-

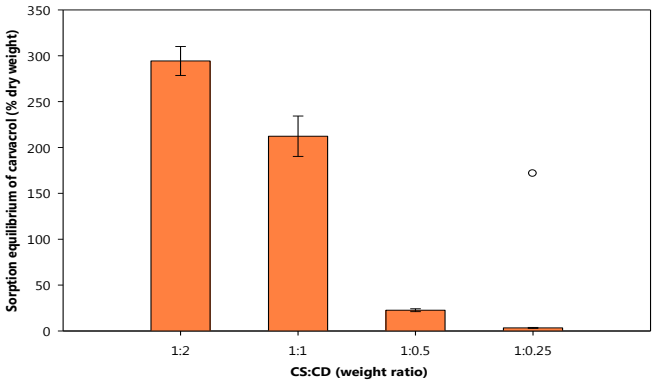

Figure 4.4.5. Effect of HP-βCD content in a CS:CD-35G-75RH film on the sorption capacity of carvacrol at 25 ℃.

βCDs content in the film. The highest sorption value was obtained for the 1:2 formulation. However, although 1:2 films were plasticized with G and water, they were very brittle and difficult to handle because of the high CD content incorporated into the CS matrix, which exerts an anti-plasticizing effect.

Maltodextrins (MDs) are non-cyclic oligosaccharides consisting of linear and branched amylose and amylopectin degradation products. These starch derivatives can form complexes with hydrophobic molecules, host-guest complexation being the most common. MDs conformation goes from flexible coil to helix as the dextrose equivalent (DE) number decreases. The inside of the helical structure is hydrophobic, as in CDs, but more flexible than the cavity of them, which means less steric hindrance. As in the case of CDs, in addition to hydrophobic interactions MDs participate in hydrogen-bonding with guest molecules (Garnero *et al.*, 2013). In an attempt to establish the effect of the molecular shape of oligosaccharides on the carvacrol sorption capacity of CS films, non-cyclic oligosaccharide MDs were incorporated in the CS matrix as an alternative to HP-βCDs and carvacrol sorption properties were studied. **Figure 4.4.6** shows the sorption equilibrium of carvacrol in films formulated with CS and MDs at a ratio of 1:1 by weight, plasticized with 35% G, and conditioned at 0, 53 and 75% RH prior to immersion in carvacrol. In no case did sorption of carvacrol exceed 1.5%. These results show the effect of the molecular geometry of low molecular weight starch derivatives incorporated in CS films on the carvacrol loading capacity.

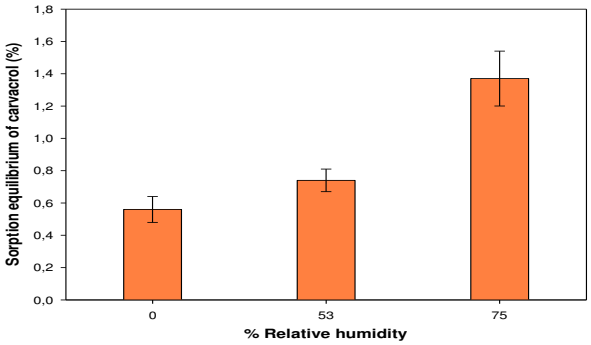

Figure 4.4.6. Carvacrol sorption capacity by films of CS incorporating MDs (1:1 weight ratio). Films were plasticized with 35% G and conditioned at 0, 53 and 75% RH prior to immersion in carvacrol.

3.2.2. Kinetics of sorption of carvacrol in CS:CD films

Figure 4.4.7 shows the kinetics of sorption of carvacrol in 1:1 CS:CD films plasticized with 35% G and conditioned at 0, 53 or 75% RH, and films plasticized with 20% G and conditioned at 75% RH. The plots represent the sorption of liquid carvacrol into the films versus time. It can be observed that the equilibrium times varied among films, depending on their G and water content. Sorption equilibrium was achieved faster for films which presented a greater level of plasticization, i.e. films incorporating 20 or 35% G and conditioned at 75% RH, whereas it took longer to reach sorption equilibrium for films conditioned under dry conditions or at 53% RH before being immersed in carvacrol.

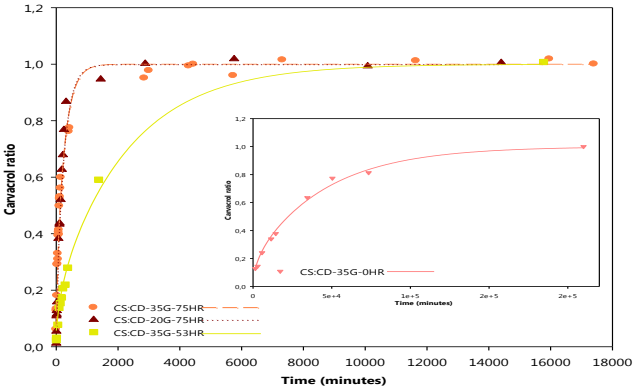

Figure 4.4.7. Comparison between experimental sorption curves (symbols) of liquid carvacrol in CS:CD films with a 1:1 (w/w) blend ratio at 25 ℃ and modelled curves (continuous line) obtained from **Equation (2)**. Inset graph: Experimental data and predicted values (line) for CS:CD-35G-0RH films.

According to Alfrey *et al.* (1966), the diffusion of a sorbate in a polymer sheet can be classified as Fickian (Case I) or non-Fickian (anomalous, Case II and Super Case II) depending on the solvent diffusion and polymer relaxation rates. Diffusion categories can be distinguished by the shape of the sorption uptake curve of a polymer-penetrant system:

$$\frac{M_t}{M_\infty} = kt^n \qquad (1)$$

where M_t is the mass of solute uptake at time t, and M_∞ is the mass of solute uptake when the polymer has reached equilibrium, k is a constant and n a diffusion exponent which denotes the type of diffusion mechanism. If the exponent n is equal to 0.5 the diffusion is Fickian and the solvent diffusion rate is slower than the polymer relaxation rate; n equal to 1 refers to Case II type diffusion, with the solvent diffusion rate faster than the polymer relaxation process. A value of n between 0.5 and 1 refers to anomalous diffusion, which happens when the diffusion and relaxation rates are comparable. Super Case diffusion occurs for $n>1$. Experimental sorption uptake curves plotted in **Figure 4.4.7** were fitted to the power law described by **Equation 1**, and the mass transport mechanism was evaluated by calculating parameter n. The values of n were between 0.5 and 0.6 (**Table 4.4.7**). Therefore the mathematical model based on the one-dimensional solution of Fick's second law of diffusion in a plane sheet was applied to the experimental sorption uptake data. This model considers the diffusion coefficient independently of the concentration of the sorbed compound. Assuming the initial/boundary conditions:

$$t = 0 \qquad 0 < x < L \qquad c = c_0$$
$$t > 0 \qquad x = 0, \qquad x = L \qquad c = c_\infty$$

where c_0 is the initial concentration of sorbate in the polymer ($c_0 = 0$) and c_∞ is the concentration of the sorbate in both surfaces of the plane sheet, which is assumed to be constant throughout the experiment, the solution under these conditions is:

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

where L is the thickness of the film (m) and D the diffusion coefficient (m^2/s) (Crank, 1975). **Figure 4.4.7** shows that sorption uptake data were well fitted

Table 4.4.7. Power law parameters and diffusion coefficients of carvacrol in CS:CD films at 25 ℃.

Film	K (min^{-n})·10^2	n	R^2	D (m^2/s) ·10^{14}	R^2
CS:CD-35G-0RH	0.26 ± 0.09[c]	0.52 ± 0.04	0.988	0.0110 ± 0.0005[c]	0.990
CS:CD-35G-53RH	0.98 ± 0.19[b]	0.56 ± 0.03	0.987	0.22 ± 0.02[b]	0.992
CS:CD-35G-75RH	3.37 ± 0.82[a]	0.55 ± 0.05	0.951	1.90 ± 0.08[a]	0.988
CS:CD-20G-75RH	2.86 ± 0.85[a]	0.59 ± 0.06	0.988	2.10 ± 0.13[a]	0.981

[a-c] Different letters in the same column indicated significant differences ($P \leq 0.05$).

by **Equation 2**. **Table 4.4.7** shows values of diffusion coefficients for films plasticized with 35% G and conditioned at 0, 53 and 75% RH prior to immersion in carvacrol, and films plasticized with 20% G and conditioned at 75% RH. The diffusion coefficient of carvacrol in films incorporating 35% G and conditioned at different RH increased as the water content increased. Diffusion coefficients of films conditioned at 0 and 53% RH differed by one order of magnitude, and also of films conditioned at 53 and 75% RH. Water has the ability to plasticize and swell hydrophilic polymers such as CS, increasing chain mobility and interchain distance. As the RH at which the films were conditioned increased, they experienced a successive plasticization and swelling by water molecules, giving rise to a looser polymer matrix, which enhanced the diffusion of carvacrol. Films conditioned at 75% RH are expected to be greatly plasticized by water and an increase in the amount of G from 20 to 35% did not greatly affect the diffusion coefficient.

3.3. Desorption kinetics of carvacrol

The release of carvacrol vapour from 1:1 CS:CD films plasticized with 35% G and conditioned at 75% RH (CS:CD-35G-75RH) prior to immersion in the volatile solvent was evaluated at 25 ℃ and under different RH: 43, 53 and 90%. **Figure 4.4.8** shows the normalized experimental curves of release of carvacrol vapour from these films. As in the sorption process described in 3.2 section, the one-dimensional solution of Fick's second law of diffusion in a plane sheet for the boundary conditions in a desorption process:

$$t = 0 \qquad 0 < x < L \qquad c = c_0$$
$$t > 0 \qquad x = 0, \qquad x = L \qquad c = 0$$

The solution under these conditions is:

$$M_t = M_\infty \left(\frac{8}{\pi^2} \sum_{n=0}^{\infty} \left[\frac{1}{(2n+1)^2} exp \left\{ \frac{-\pi^2 \cdot D \cdot (2n+1)^2 \cdot t}{L^2} \right\} \right] \right) \qquad (3)$$

The theoretical curves modelled with **Equation 3** for a desorption process are also shown in **Figure 4.4.8**. The rate of carvacrol release largely depended on the RH to which the films were exposed. It is known that the diffusion of small molecules in hydrophilic polymers such as CS strongly depends on the moisture content of the matrix (Chalier *et al.*, 2009; Mascheroni *et al.*, 2011); at intermediate and high RH hydrophilic materials absorb moisture, which triggers the release of volatiles entrapped in their structure. Moisture acts as a plasticizer, thereby governing the rate of volatile loss. The release of carvacrol was greatly accelerated when films were exposed to high RH conditions (90%) compared to those exposed to intermediate RH (43 and 53%). All the desorption curves had a two-step pattern. In the first step there was a fast release of carvacrol, which was more acute for films conditioned at 90% RH, followed by a slower release of the remaining volatile. Films conditioned at 90% RH released 96% of the carvacrol in the first 34 h, and took approx. 8 days to release 99% of the initial amount. Films conditioned at 53% RH had released 94% of the initial amount of carvacrol after 15 days, and 1.5% still remained in the film after 23 days. Films exposed to 43% RH released 95% of the carvacrol content in 22 days, and after 62 days the residual amount of volatile was 2.6%.

Carvacrol diffusion coefficients were calculated for films exposed to different RH by fitting experimental release kinetics data to **Equation 2** and the values obtained are shown in **Table 4.4.8**. Increasing the RH at which the

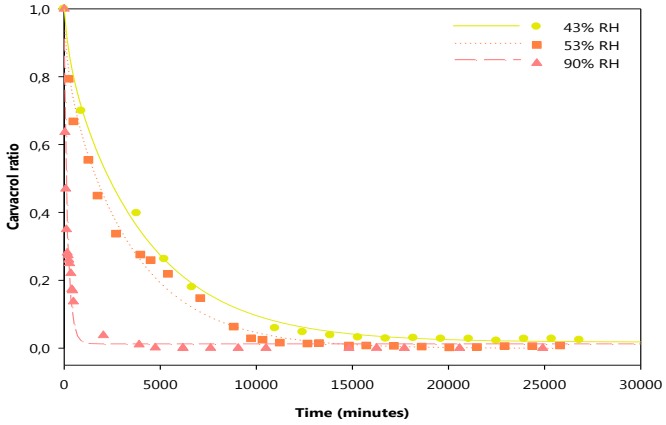

Figure 4.4.8. Experimental (symbols) and theoretical (continuous line) normalized time desorption curves of carvacrol from CS:CD-35G-75RH film measured at 25 ºC as a function of environmental RH.

Table 4.4.8. Diffusion coefficient of carvacrol in CS:CD-35G-75RH film at different RH and 25 ℃.

% RH	D (m^2/s) $\cdot 10^{15}$	R^2
43	1.21 ± 0.04[c]	0.998
53	1.45 ± 0.08[b]	0.988
90	25.3 ± 2.1[a]	0.968

[a-c] Different letters in the same column indicated significant differences ($P \leq 0.05$).

desorption was carried out led to an increase in diffusivity of carvacrol. Films exposed to 90% RH experienced a rapid loss of carvacrol, having a diffusion coefficient one order of magnitude greater than that obtained for films exposed to intermediate RH. Films conditioned at 43 or 53% RH had diffusion coefficients with the same order of magnitude. Diffusion coefficient values were of the same order of magnitude as those obtained by Mascheroni *et al.* (2011) at 30 ℃ in wheat gluten coated paper containing 15% (wt) of carvacrol, where the diffusion coefficients ranged from 0.143 x 10^{-14} to 2.8 x 10^{-14} m^2 s^{-1} for RH ranging between 60 and 100%. Chalier *et al.* (2009) studied the diffusivity of carvacrol at 30 ℃ in soy protein coated paper, where the diffusion coefficients ranged from 0.02 to 1.4 x 10^{-14} m^2 s^{-1} for RH varying between 60 and 100%. Kurek *et al.* (2014) evaluated the diffusion coefficient of carvacrol in CS films measured at RH >96%, finding values of 3.8 x 10^{-15} at 20 ℃ and 5.5 x 10^{-13} at 37 ℃.

3.4. Antimicrobial activity

3.4.1. Antimicrobial activity of carvacrol vapour against S. aureus *and* E. coli

The antimicrobial activity of carvacrol was tested against *S. aureus* and *E. coli* using the microatmosphere method as described in Materials and Methods, and the MIC (minimal inhibitory concentration) was determined. **Table 4.4.9** shows that the minimum dose of carvacrol found to produce inhibition zones on agar was 1 mg. The inhibition zone diameters produced by disks with carvacrol were 15 and 13 mm for *S. aureus* and *E.coli*, respectively. The inhibition zones increased as the amount of carvacrol added to the filter paper disk increased; thus, 5 mg of carvacrol produced an inhibition zone of 44 mm against *S. aureus* and *E. coli*.

Table 4.4.9. Antimicrobial activity produced by carvacrol in vapour phase against *S. aureus* and *E. coli*.

Carvacrol (mg)	*S. aureus* Inhibition zone (mm)	*E. coli* Inhibition zone (mm)
5.00	44	44
2.00	29	30
1.50	20	21
1.25	18	18
1.00	15	13
0.75	-	-
0.50	-	-

Several studies regarding the antimicrobial activity of essential oils against food spoilage microorganisms and foodborne pathogens agree on their slightly greater activity against Gram-positive bacteria compared to Gram-negative bacteria (Burt, 2004). This has been attributed to differences in the cell wall of the two types of microorganism, since the outer membrane of Gram-negative bacteria restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering (Vaara, 1992). On the other hand, some studies suggest that Gram-positive bacteria are more resistant than Gram-negative bacteria to the antibacterial properties of essential oils (Zaika, 1988). However, other authors have not obtained evidence for a general greater effectiveness of essential oils against Gram-positive or Gram-negative bacteria, and the results found depend on the essential oil tested (Dorman and Deans, 2000). With regard to the antimicrobial activity of carvacrol, the major component of oregano and thyme oil, a similar antibacterial activity against *E. coli* and *S. aureus* was found in the present work; these results are in agreement with other studies (Ben Arfa *et al.*, 2006; Griffin *et al.*, 1999).

3.4.2. Study of the antimicrobial activity of films loaded with carvacrol

The antimicrobial properties of carvacrol loaded 1:1 CS:CD films plasticized with 20 or 35% G and conditioned at different RH values were tested against *S. aureus* and *E. coli*.

Table 4.4.10 shows the carvacrol content of the films and the diameter of the inhibition zone of *S. aureus* and *E. coli*. It is worth mentioning that the antimicrobial activity of carvacrol loaded CS films having different

amounts of G and water incorporated into the matrix but without the incorporation of HP-βCDs was also evaluated. The retention capacity of these films was less than 1% carvacrol and none of them exerted antimicrobial activity in vapour phase. The addition of HP-βCDs to the CS matrix increased sorption of carvacrol and therefore changed the antimicrobial capacity. No inhibition zone was observed for carvacrol loaded 1:1 CS:CD films without G and conditioned at 0, 53 and 75% RH prior to immersion in carvacrol, since the amount of carvacrol in the film did not reach the minimum inhibitory dose of 1 mg. However, there was observed a reduction in growth density across the Petri dish for films without G and conditioned at 53 and 75% RH, the amount of carvacrol retained by these films was next to 0.6 mg. Dry CS:CD films plasticized with 20% G retained less than 0.4 mg of carvacrol, thus they did not also experience any antimicrobial activity. CS:CD films plasticized with 35% G and conditioned at 75% and 53% RH, and CS:CD films plasticized with 20% G and conditioned at 75% RH possessed the greatest content in carvacrol (>60 mg) and produced complete inhibition of bacterial growth in the Petri dish (85 mm diameter), whereas the inhibition halo was reduced to 79 mm and 76 mm against *S. aureus* and *E. coli* respectively for dry films plasticized with 35% G and around 19 mg carvacrol content. An inhibition halo of 67 mm was observed for CS:CD films plasticized with 20% G, conditioned at 53% RH and having around 11 mg of carvacrol. In conclusion, the major antimicrobial activity was exerted by CS:CD films

Table 4.4.10. Antimicrobial activity of CS:CD films loaded with carvacrol against *S. aureus* and *E. coli*.

Film	*S. aureus*		*E. coli*	
	Carvacrol (mg)	Inhibition zone (mm)	Carvacrol (mg)	Inhibition zone (mm)
CS:CD-0G-0RH	0.124	-	0.124	-
CS:CD-0G-53RH	0.567	(slight decrease in growth density)	0.581	(slight decrease in growth density)
CS:CD-0G-75RH	0.626	(marked decrease in growth density)	0.605	(marked decrease in growth density)
CS:CD-20G-0RH	0.315	-	0.340	-
CS:CD-20G-53RH	11.6	67	11.1	67
CS:CD-20G-75 RH	61.4	85	61.1	85
CS:CD-35G-0RH	19.3	79	18.4	76
CS:CD-35G-53RH	84.3	85	80.8	85
CS:CD-35G-75RH	153	85	146	85

possessing adequate combination of G content and RH conditioning, which allowed to be loaded with a higher content of carvacrol (≥60 mg) and produce bactericide effect against Gram positive and Gram negative bacteria.

3.4.3. Antimicrobial activity of films during long-term storage

CS:CD-35G-75RH films, which showed the greatest capacity for sorption of carvacrol, were chosen to follow their antimicrobial capacity over time. For this purpose, the antimicrobial activity in vapour phase of films submitted to desorption at 25 ºC and 43% RH was monitored for 20 days (every 2 days for 2 weeks and then after 8 days). **Figure 4.4.9** shows the effect of time on the amount of carvacrol remaining in the film and the inhibition zone created against *E. coli* and *S. aureus*. As expected, the inhibition halo experienced a reduction as the carvacrol content in the film decreased over time. The initial amount of carvacrol in the film was 146 mg, producing total inhibition of bacterial growth. A rapid reduction of carvacrol in the films was observed during the first week of storage, giving rise to almost total growth inhibition on the agar plate. Thus, the amount of carvacrol in the film was reduced by 93% after 8 days of storage, and being the inhibition zone reduced to 64 and 66 mm in diameter for *S. aureus* and *E. coli* respectively. After that, film experienced a slow release of carvacrol; at

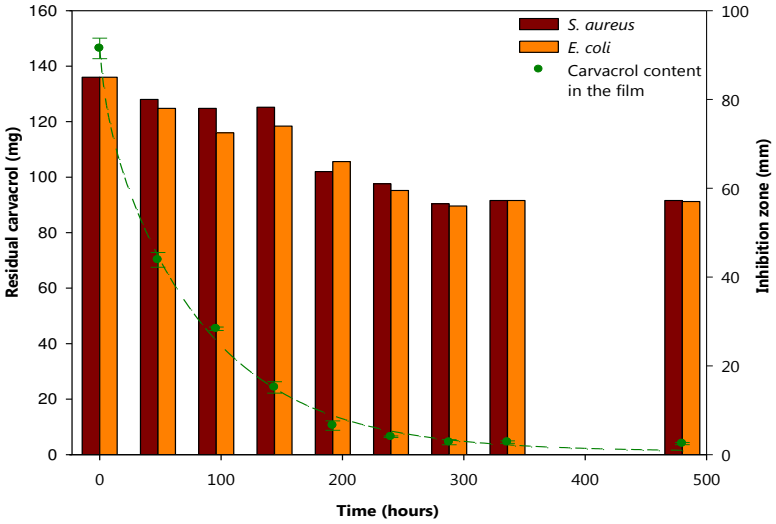

Figure 4.4.9. Carvacrol remaining in CS:CD-35G-75RH film at 25 ºC and 43% RH and inhibition halo produced against *S. aureus* and *E. coli* over time.

the middle of the storage period, the carvacrol content in the film was 6.5 mg having inhibition zones of around 60 mm. After 12 days of storage, the carvacrol content retained in the film was 4.5 mg, and the inhibition zones produced were 57 and 56 mm in diameter for *E. coli* and *S. aureus*, respectively. After 20 days of storage, the amount of carvacrol remaining in the film did not change greatly, this was found to be 4mg and the inhibition zone against the bacteria tested was similar to the 12th day.

It is worth pointing out that the inhibition zone created by the film disk containing 4.5 mg of carvacrol was slightly greater than that produced by a filter paper of similar dimensions and incorporating 5 mg of carvacrol when the two samples (film and filter paper) were submitted to the same microatmosphere test conditions (37 ºC for 24 h and >99% RH). Because of the high RH reached in the Petri dish and the temperature used in the antimicrobial experiment, it was expected that the small amount of carvacrol incorporated in the paper (5 mg) and remaining in the film (4.5 mg) would be released in 24 h. Slight differences in inhibition could be related to carvacrol release behaviour from these two materials during the antimicrobial test, which could affect microbial growth.

4. Conclusions

This work shows that it is possible to control the loading of hydrophobic antimicrobial carvacrol in hydrophilic CS. For this purpose, the polymer was blended with HP-βCDs and G and water were incorporated as coadjuvants. Depending on the amount of these compounds in the CS matrix, the films can be loaded with different input amounts of carvacrol. Moreover, the release rate of carvacrol from the films depends greatly on the environmental RH. The films developed have antimicrobial properties and can be active for an extended period of time. They could be applied in the design of active packages to inhibit microbial growth on the surface of solid foods. Because of the volatile properties of carvacrol, direct contact of the film with the food would not be required, since the moisture present in the package triggers and controls release of the compound entrapped in the film.

Acknowledgments

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4.4.4. Artículo científico 6.

Antimicrobial packaging of chicken fillets based on the release of carvacrol from chitosan/cyclodextrin films

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Resumen

Las películas de quitosano/ciclodextrina (CS:CD) con carvacrol fueron obtenidas por extensión y evaporación del disolvente y acondicionadas a 23 ℃ y una humedad relativa del 75% antes de ser sumergidas en carvacrol líquido hasta que se alcanzó el equilibrio de sorción. En un trabajo anterior, se estudió la actividad antimicrobiana *in vitro* de estas películas. En este trabajo, se utilizaron películas activas para inhibir el crecimiento microbiano en pechugas de pollo fileteadas envasadas. Las muestras de películas de CS:CD cargadas con carvacrol, de diferentes tamaños y por tanto con diferentes cantidades de agente antimicrobiano, fueron pegadas a la tapa de aluminio empleadas para sellar tarrinas de PP/EVOH/PP con 25 g de filetes de pollo. Estas muestras fueron almacenadas durante 9 días a 4 ℃. Los envases fueron sellados herméticamente y se confirmó que presentaban una barrera infinita a carvacrol. Se analizó el reparto del agente antimicrobiano en el sistema alimentario/embalaje. Estos dispositivos liberaron rápidamente un gran porcentaje de la carga del antimicrobiano, cantidades que fueron obtenidas por el recubrimiento adhesivo de la tapa y especialmente por los filetes de pollo. Éstos últimos fueron la principal fase sorbente, con concentraciones medias que oscilan entre 200 y 5.000 mg/kg durante el período de almacenamiento. Se analizó la microbiota de los filetes de pollo frescos envasados -mesófilos, psicrófilos, *Pseudomonas* spp, enterobacterias, bacterias ácido-lácticas, y levaduras y hongos- y monitorizó durante el almacenamiento. Se observó una inhibición microbiana general, aumentando con el tamaño del dispositivo activo. La inhibición con un dispositivo de 24 cm^2 osciló desde 0.3 reducciones logarítmicas frente a bacterias de ácido-lácticas a las 1.8 logs frente a levaduras y hongos. Sin embargo, la gran cantidad de antimicrobiano que fue absorbido o reaccionado con el filete provocó un deterioro sensorial inaceptable. Estos altos valores de sorción se deben probablemente a la gran compatibilidad química entre las proteínas de pollo y carvacrol.

Palabras clave

Quitosano, hidroxipropil-β-ciclodextrinas, carvacrol, envasado activo antimicrobiano, liberación controlada, pollo.

Abstract

Chitosan/cyclodextrin films (CS:CD) incorporating carvacrol were obtained by casting, and conditioned at 23 ºC and 75% relative humidity prior to being immersed in liquid carvacrol until they reached sorption equilibrium. In a previous work, the *in vitro* antimicrobial activity of these films was studied. In this work, active films were used to inhibit microbial growth in packaged chicken breast fillets. Samples of CS:CD films loaded with carvacrol, of different sizes and thus with different quantities of antimicrobial agent, were stuck to the aluminium lid used to seal PP/EVOH/PP cups containing 25 g of chicken fillets. These samples were stored for 9 days at 4 ºC. The packages were hermetically sealed and it was confirmed that they provided an infinite barrier to carvacrol. The partition of the antimicrobial agent within the food/packaging system was analysed. The antimicrobial devices rapidly released a large percentage of the agent load, amounts that were gained by the adhesive coating of the lid and especially by the chicken fillets. The latter were the main sorbent phase, with average concentrations ranging between 200 and 5000 mg/Kg during the period of storage. The microbiota of the packaged fresh chicken fillets -mesophiles, psychrophiles, *Pseudomonas* spp., enterobacteria, lactic acid bacteria and yeasts and fungi- were analysed and monitored during storage. A general microbial inhibition was observed, increasing with the size of the active device. Inhibition with a 24 cm^2 device ranged from 0.3 log reductions against lactic acid bacteria to 1.8 logs against yeasts and fungi. However, the large amount of antimicrobial that was sorbed or that reacted with the fillet caused an unacceptable sensory deterioration. These high sorption values are probably due to a great chemical compatibility between chicken proteins and carvacrol.

Keywords

Chitosan films, hydroxypropyl-β-cyclodextrins, carvacrol, antimicrobial active packaging, controlled release, chicken.

1. Introduction

Antimicrobial packaging systems have received special attention in recent years because they can provide an important obstacle in the so called "hurdle" technologies that are being implemented to commercialize fresher food products. In these systems, interactions between the food, the surrounding headspace and the package walls or independent devices are responsible for growth inhibition or death of pathogenic and/or spoilage microorganisms (Appendini and Hotchkiss, 2002). Although, exceptionally, this beneficial activity can be carried out by direct contact of the microorganisms with the package surface (Conte *et al.*, 2008; Muriel-Galet *et al.*, 2013; Zi-Xuan *et al.*, 2012), the mechanism of activity in most packaging systems is based on mass transport processes through the packaging/food interface, and especially on the release of antimicrobial substances. The active agent can be incorporated within a suitable polymeric matrix from which it is released following diffusion mechanisms and accumulated into the food package system following thermodynamic principles. Therefore, compatibility between the agent and the various system components and diffusion from the polymeric vehicles are key parameters, and the efficiency and validity of an active packaging has to be tested for the specific product.

Recently, a biopolymeric device consisting of a combination of two biodegradable, renewable materials, CS and HP-βCD, and a known natural volatile antimicrobial compound, carvacrol, was developed and characterized. In that report, the film demonstrated a great capacity for sorption of carvacrol. The kinetics of release in ideal conditions were also measured and the *in vitro* antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* was tested (Higueras *et al.*, 2013; Higueras *et al.*, 2015).

In this work, a package containing this device was designed and used as an active package containing fresh chicken fillets. The partition of carvacrol among the different components of the food/active package/environment system was studied and the effect of the active packaging on the chicken microbiota was analysed.

2. Materials and methods

2.1. Materials

Carvacrol (kosher >98%), glycerol (G), acetic acid and low molecular weight chitosan (CS) were supplied by Sigma (Barcelona, Spain). hydroxypropyl-β-cyclodextrin (HP-βCD, CAVASOL® W7-HP) were provided by Wacker Fine Chemicals, S.L. (Barcelona, Spain).

2.2. Film preparation

A 1.5% CS (w/w) solution in an aqueous 0.5% (w/w) acetic acid solution was prepared and filtered to eliminate impurities. G plasticized chitosan/hydroxypropyl-β-cyclodextrin films were obtained by adding HP-βCD to the CS solution in a 1:1 proportion (w/w) with respect to CS and G at 35% (g G/100 g dry matter) and stirring at 1500 rpm and 37 ºC until complete dissolution. Films were obtained by casting, i.e. pouring a suitable amount of the solution into a flat PS tray and allowing it to dry under controlled environmental conditions (36 h, 40.0 ± 1.5 ºC and 20±9% RH). After peeling the films from the tray, samples measuring 550 mm in diameter and 55±5 µm in thickness (using a digital micrometer (Mitutoyo Manufacturing Co. Ltd., Tokyo, Japan)) were stored in glass desiccators at 75.0±0.5% RH (ASTM, 2007) in a temperature-controlled room at 23±1 ºC for at least two weeks, the time required by the samples to reach moisture equilibrium. Some of these films were used as control samples (CS:CD-Control). Others (CS:CD-Active) were immersed in carvacrol for at least 3 weeks, the time required to reach equilibrium. The analysis of the carvacrol sorbed by the samples was performed by thermal desorption and subsequent gas chromatographic analysis as described below. A more detailed description of the manufacturing procedure can be found elsewhere.

2.3. Packaging of chicken breast fillets

Samples of chicken breast (25 g) were placed at the bottom of polypropylene (PP)/ethylene-vinyl alcohol copolymer (EVOH)/PP trays measuring 156 cm^3. The trays were sealed with adhesive aluminium foil (Miarco, Valencia, Spain) and stored at 4 ºC for 0, 3, 6 and 9 days. CS:CD-

Active films measuring 0.24, 4.8 and 24 cm^2 were stuck to the centre of the tray lid and constituted the small, medium and large samples, respectively. A negative control sample for each time was also prepared with CS-Control. Packages without chicken containing a medium size CS:CD-Active films were prepared too.

2.4. Quantification of carvacrol concentration

The analysis of the initial concentration of carvacrol retained in the materials was performed by thermal desorption coupled to gas chromatography using a Dynatherm Thermal Desorber Model 890/891 (Supelco, Teknokroma, Barcelona, Spain) connected in series to the column of an HP5890 gas chromatograph Series II Plus (Agilent Technologies, Barcelona, Spain) via a heated transfer line. A sample of the CS:CD-Active film was cleaned with a paper tissue to remove any excess of volatile compound on the film surface and then immediately inserted into an empty desorption tube (11.5 × 0.39 cm I.D.). The tube was placed in the desorber chamber, which was then closed. Conditions for desorption were as follows: desorption temperature, 210 ℃; transfer line, 230 ℃; desorption time, 7 min; He desorption flow, 8.15 mL/min. The GC was equipped with a TRB5 (30 m, 0.32 mm, 0.25 µm) column (Teknokroma, Barcelona, Spain) and a flame ionization detector. The chromatographic conditions were: 260 ℃ detector temperature, 7 min at 45 ℃, heating ramp to 220 ℃ at 18 ℃/min, and 1min more at 220 ℃. After the analysis, the film sample was recovered from the desorption tube and weighed on an analytical balance (Voyager model V11140, Ohaus Europe, Greifensee, Switzerland). The desorber-GC was calibrated by measuring other polymeric (polyethylene and polypropylene) film samples with known amounts of carvacrol (measured independently by gravimetry).

The same procedure was used to determine the carvacrol concentration during chicken storage within each phase of the food package/system. Briefly, samples of tray, lid, film or chicken were cut and desorbed following the previous procedure. Every package was tested three times and three packages were analysed per sample.

The carvacrol concentration in the package headspace was analysed by sampling 500 µL of gas with a gas-tight Hamilton syringe through an

adhesive septum stuck to the package wall. The gas sample was immediately analysed by using a Model 6850 GC (Agilent Technologies) equipped with a semi-capillary RTX-1301 (30 m, 0.53 mm, 0.5 µm) column (Restek, Teknokroma, Barcelona, Spain). The chromatographic conditions were: 3.8 mL/min He, oven at 150 ℃ for 11 min, injector and detector temperatures 220 ℃ and 250 ℃, respectively. The GC was previously calibrated by injections of a known amount of carvacrol in hexane.

2.5. Antimicrobial activity of carvacrol-CS films on chicken breast fillets

At selected times, chicken samples were transferred aseptically and weighed in a sterile Stomacher bag, diluted with 25 mL of 0.1% peptonated water (Scharlab, Barcelona, Spain) and blended in a Stomacher (IUL S.L., Barcelona) for 6 min. Ten-fold dilution series of the suspensions obtained were made in peptonated water and plated using the following selective media (Scharlab, Barcelona, Spain) and culture conditions: tryptone soy agar for total aerobic bacteria, incubated at 30 ℃ for 48 h, and also for total aerobic psychrophiles, incubated at 10 ℃ for 10 days, King B agar for *Pseudomonas* spp., incubated at 25 ℃ for 48 h, violet red bile glucose agar for enterobacteria, incubated at 37 ℃ for 24 h, MRS agar for lactic acid bacteria, incubated at 25 ℃ for 5 days, and malt extract agar for yeasts and fungi, incubated at 28 ℃ for 5 days. Tests were performed in triplicate.

2.6. Data analysis

Statistical analysis of the results was performed with SPSS commercial software (SPSS Inc., Chicago, Illinois, USA). Differences between means were assessed on the basis of confidence intervals, using the Tukey-*b* test at a level of significance of $P \leq 0.05$. The data are represented as average±standard deviation.

3. Results and discussion

CS:CD-Control samples were prepared first. They were transparent, practically colourless and without discontinuities. After the immersion in carvacrol for 3 weeks, the samples were transparent and had an intense yellow colour. The initial carvacrol content of the samples was 2.3±0.4 g/g of dried film (HP-βCD and CS). These high uptakes were in agreement with

previous studies (Higueras *et al.*, 2013). Nevertheless, the carvacrol content in each sample was determined individually prior to use.

3.1. Quantification of carvacrol concentration in the packaging/headspace/chicken system

Fresh chicken breast fillets are commonly packaged in trays closed with a thermosealable lid, and distributed and commercialized under refrigeration. The materials used for the design of these trays are variable (polyethylene terephthalate (PET), expanded polystyrene (PS) or PP laminated or coextruded to a polyamide/polyethylene (PA/PE or EVOH/PP…)), but, since the product is normally preserved in a modified atmosphere, materials that provide a medium to high barrier to oxygen and carbon dioxide are commonly employed. These materials present this barrier because of a combination of properties that might include high crystallinity, low free volume, adequate chain rigidity and high interchain cohesive energy. With these properties, the materials also provide a high to very high barrier to organic compounds such as food aroma components, vitamins, fats or organic acids (Gavara and Catalá, 2002).

To resemble real behaviour, a very high barrier package consisting of a tray and a lid was selected for this study. The thermoformed tray was obtained from a PP/EVOH/PP coextruded sheet with an initial thickness of 750 µm as measured in the top flat sealing area. To assess the structure and dimensions of the layers along the tray profile, a sample was cut diagonally, immersed in an aqueous solution of cresol red to tint the EVOH layer and observed under magnifying lenses. The average thickness of the PP layers was 200±20 µm and the 10 µm EVOH layer was located in the centre of the structure. With this composition, it was evident that the EVOH layer was well protected and that, in terms of aroma barrier, the tray could be considered as an infinite barrier. Nevertheless, the aroma scalping ability of polyolefins is known and therefore a portion of the added carvacrol was expected to be sorbed in the most internal PP layer of the tray. Instead of a thermosealable lid, an aluminium foil tape was used to close the package hermetically, to guarantee an infinite barrier to the antimicrobial agent, and to attach the antimicrobial device, that is, the CS:CD-Active film. However, the adhesive

coating could also be involved in scalping processes and therefore it was considered in the study.

Besides the packaging and, of course, the device, carvacrol was also partitioned in the package headspace and in the chicken fillet. These two phases were also analysed.

Figure 4.4.10 presents the concentration of carvacrol in the different parts of the packaging/headspace/chicken system. As can be seen in **Figure 4.4.10a**, the CS:CD-Active film device partially released the agent to the other packaging phases. Large differences were observed between the sample without chicken (medium empty) and the samples containing chicken. After three days the small device had released ca. 98% of the initial content, reaching 99% after the 9 days of storage. The medium and large devices released 90% after three days and slightly above 95% after 9 days, without differences between samples. In contrast, the control sample only released 35% after 3 days and this release percentage was maintained

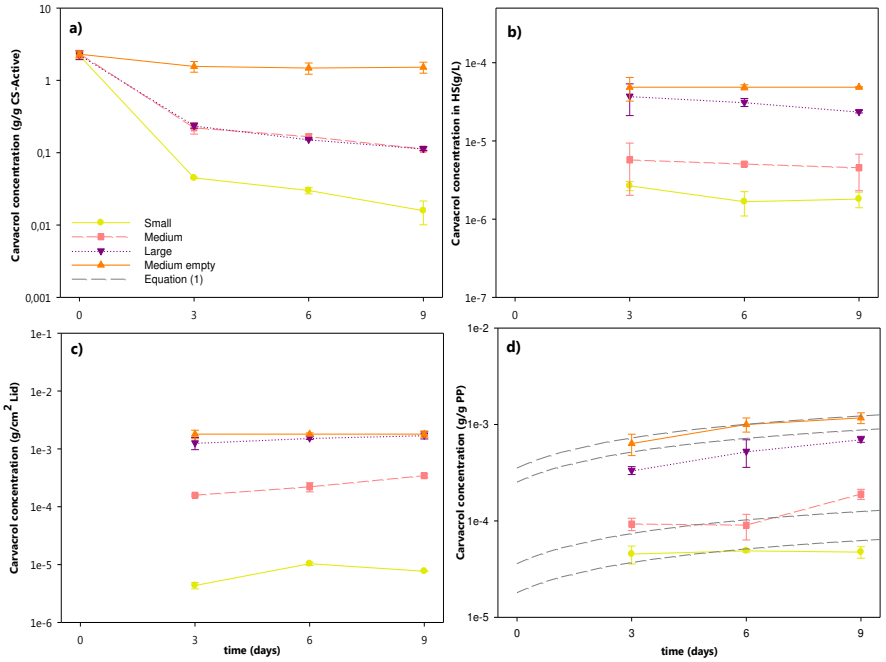

Figure 4.4.10. Evolution of carvacrol concentration during product storage for the three device sizes and for control packages without chicken in the different package parts: (a) CS:CD-Active, b) headspace (HS), c) package lid and d) the PP inner layer of the tray. Values are expressed as mean and standard deviation (n= 9).

throughout the 9-day storage. This large difference between the sample without chicken and food packaged samples is obviously due to the presence of the meat fillet. A great chemical compatibility between chicken proteins and carvacrol could explain the showed differences.

Figure 4.4.10b shows the concentration of carvacrol in the headspace. As can be seen, the concentration in the sample without chicken is nearly $5{\cdot}10^{-5}$ g/L. This concentration is close to that at saturation ($6{\cdot}10^{-5}$ g/L). This high concentration could be expected since the original device (obtained by immersion in carvacrol) should have activity=1. The samples containing chicken showed much lower headspace concentrations: the smaller the device, the lower the carvacrol concentration, differences being significant between samples. The concentration measured in the sample with the smallest active film was ca. $1.5 \cdot 10^{-6}$ g/L, that is, the carvacrol activity in the vapour phase was below 0.03.

Figure 4.4.10c plots the evolution of carvacrol concentration in the package lid. Initially, no scalping due to the lid was expected since it consists mainly of aluminium foil. However, analysis revealed that the adhesive coating was absorbing considerable amounts of carvacrol. As the figure shows, the surface concentration increased with the device size and with the time of exposure.

Finally, **Figure 4.4.10d** shows the average concentration measured in the tray walls. Even though sampling was made by cutting the tray and analysing the complete PP/EVOH/PP, the results are expressed as if all the carvacrol was accumulated in the internal 200 µm PP layer. The high barrier imposed by EVOH and the short exposure period validate this hypothesis. As in the other regions of the package, the carvacrol concentration increased with the size of the active device. The mass transport of carvacrol through PP was fully characterized in a recent paper (Cerisuelo *et al.*, 2012). Considering the partition coefficient for carvacrol between PP and air at 23 °C ($H_{PP/air}$) and the diffusion coefficient of carvacrol in PP ($D_C{}^{P}$), and assuming that the concentration of carvacrol in the headspace was maintained constant (C_C^{HS}), the predicted concentration was estimated by using the solution to Fick's laws for a film sample that sorbs a penetrant through one of its surfaces (Crank, 1975):

$$C_c^P = \frac{2 \cdot C_{c\,O}^P}{\pi^2} \sum_{\nu=1}^{\infty} \frac{1}{\left(\nu - \frac{1}{2}\right)^2} \cdot e^{\frac{-\pi^2 \cdot \left(\nu - \frac{1}{2}\right)^2 \cdot D_c^P \cdot t}{L_P^2}} \quad (1)$$

The average concentration of carvacrol in the polymer (C_C^P) is a function of time (t), the carvacrol concentration in the polymer at equilibrium ($C_C^P{}_O$)=($H_{PP/air}$·C_C^{HS}) and the diffusion coefficient. With all these assumptions, the predicted concentration of carvacrol in the tray was calculated and the results are also plotted in **Figure 4.4.10d**. As can be seen, the predictions are within the same order of magnitude despite the assumptions just mentioned. Although, because of the time scale of the figure and the log scale of the Y-axis, the predicted values appear to be reaching constancy, the predicted release after 9 days was less than 20% of the maximum release that would be expected when equilibrium had been reached. This result is in agreement with the hypothesis of a tray with an infinite barrier.

To have amore visual idea of the partition of carvacrol in the various package regions, the total amount of carvacrol contained in each part of the package was calculated and the results are presented as a percentage of the total amount in **Figure 4.4.11**. As can be seen, the carvacrol release was practically complete after the first three days. Interestingly, the lid was the package constituent that retained most carvacrol, which is especially relevant for the largest size. The carvacrol scalping in PP was significant but much reduced, with ca. 2% of the total amount at the end of the storage period. Finally, the carvacrol present in the headspace could be negligible (1 per million) in all cases when it is compared with the other components of the packaging system.

As mentioned at the beginning of the discussion section, the package was designed to fully retain all the carvacrol initially inserted in the food/packaging system. This was confirmed when the package without chicken was analysed. As can be seen in **Figure 4.4.11d**, the package retained nearly 97% of the initial content, in agreement with the infinite barrier approach. Therefore the percentage of carvacrol that is not present in the package component should necessarily be assigned to sorption in the chicken fillet, as represented in **Figure 4.4.11**. Assuming that the agent was homogeneously distributed in the food matrix, the concentration of carvacrol

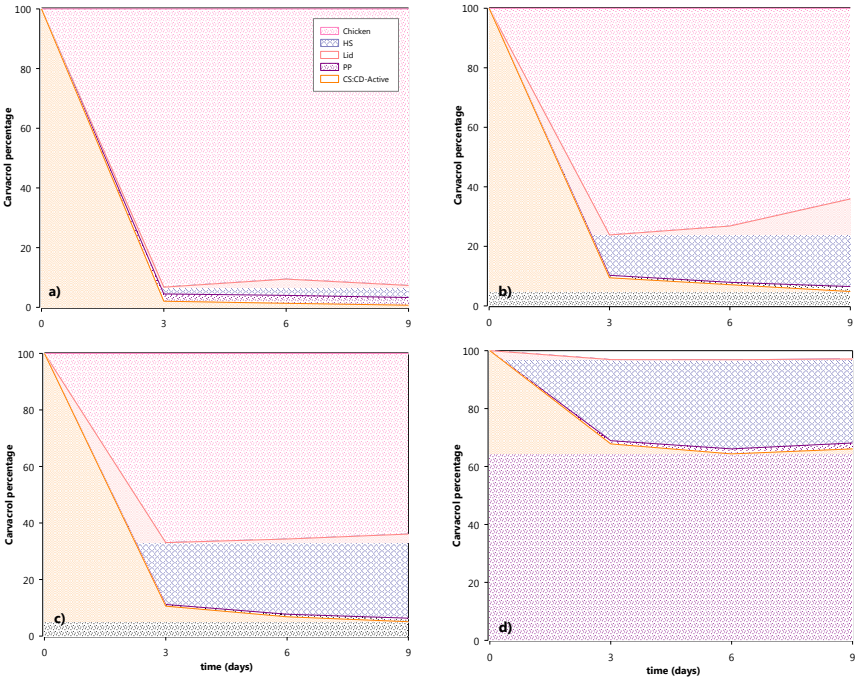

Figure 4.4.11. Portion of carvacrol percentage present in each phase of the product/package system for the three device sizes and the empty tray with the medium device: (a) small, b) medium, c) large and d) empty tray.

in the chicken fillet would be 200±20, 900±100 and 4500±200 mg/Kg, for the small, medium and large devices, respectively. To check the actual concentration in chicken meat, samples were cut from different areas of the fillet and analysed by thermal desorption and GC, as described in the experimental section. Large differences were observed between samples taken from the upper surface and those taken from the fillet bulk. The concentration in the upper surface was between 5 and 10-fold higher than in the bulk or in the surface in contact with the tray bottom.

Carvacrol is widely used as an active agent in food packaging (Ben Arfa *et al.*, 2007; Du *et al.*, 2008; Kavoosi *et al.*, 2013; López-Mata *et al.*, 2013; Nostro *et al.*, 2012; Ramos *et al.*, 2012). This may be because carvacrol was recognized as safe by the Joint FAO/WHO (2001) as a food additive permitted for direct addition to food for human consumption (synthetic flavouring substances and adjuvants) with no safety concern; and it has recently been included in the list of flavouring substances (EFSA, 2012).

3.2. Antimicrobial activity of CS:CD-Active films on chicken breast fillets

Fresh poultry products are very perishable because of their rich nutritional composition, high pH (5.5–6.5) and water activity (0.98–0.99). Thus, these products are very susceptible to microbial contamination and development of a wide variety of spoilage microorganisms and food-borne pathogens (Aymerich *et al.*, 2008). In order to solve this problem, four types of packages were prepared and filled with 25 g of chicken breast: small, medium and large samples with different sizes of CS:CD-Active and a medium sample of CS-Control (without active component). **Figure 4.4.12** shows the effect of the different packages on chicken breast microbiota. There was an increase in microbial growth as time increased until it reached the expiry date stated on the product. The present mesophile, enterobacteria and lactic acid bacteria counts are in agreement with those reported by other authors (Rokka *et al.*, 2004; Voidarou *et al.*, 2011).

In general, the small film did not produce a relevant microbial growth reduction, especially at shorter exposure times. After 9 days of exposure, the growth of fungi and yeasts, mesophiles and enterobacteria was reduced by 0.38, 0.37 and 0.33 log/g, respectively. The medium film did not produce relevant inhibition at short times. The growth reduction improved with exposure time, reaching a log reduction ranging between 0.63 and 0.89 for all microorganisms after 9 days (except for lactic acid bacteria, where the reduction was 0.33). The large film after 3 days produced an inhibition of 0.26 to 0.40 log, while after 9 days inhibition was 1.13 to 1.77 log for all groups of microorganisms. Again, lactic acid bacteria were the microorganism least affected by carvacrol, with 0.16 and 0.64 log reduction after 3 and 9 days. This is in agreement with the literature, since it has been observed that lactic acid bacteria were the most resistant to carvacrol while fungi and yeasts were the most sensitive (Holley and Patel, 2005).

Sánchez (2011) reported that values equal to or lower than 4.84 log CFU/g of the total count of aerobic bacteria and equal to or lower than 3.70 log CFU/g for enterobacteria can be accepted for fresh chicken. Wehr (1982) considered the acceptability threshold to be 6.70 and 3 log CFU/g for aerobic bacteria and enterobacteria, respectively. Sofos (1994) indicated as common enterobacteria counts between 3 and 4 log CFU/cm^2 on chicken

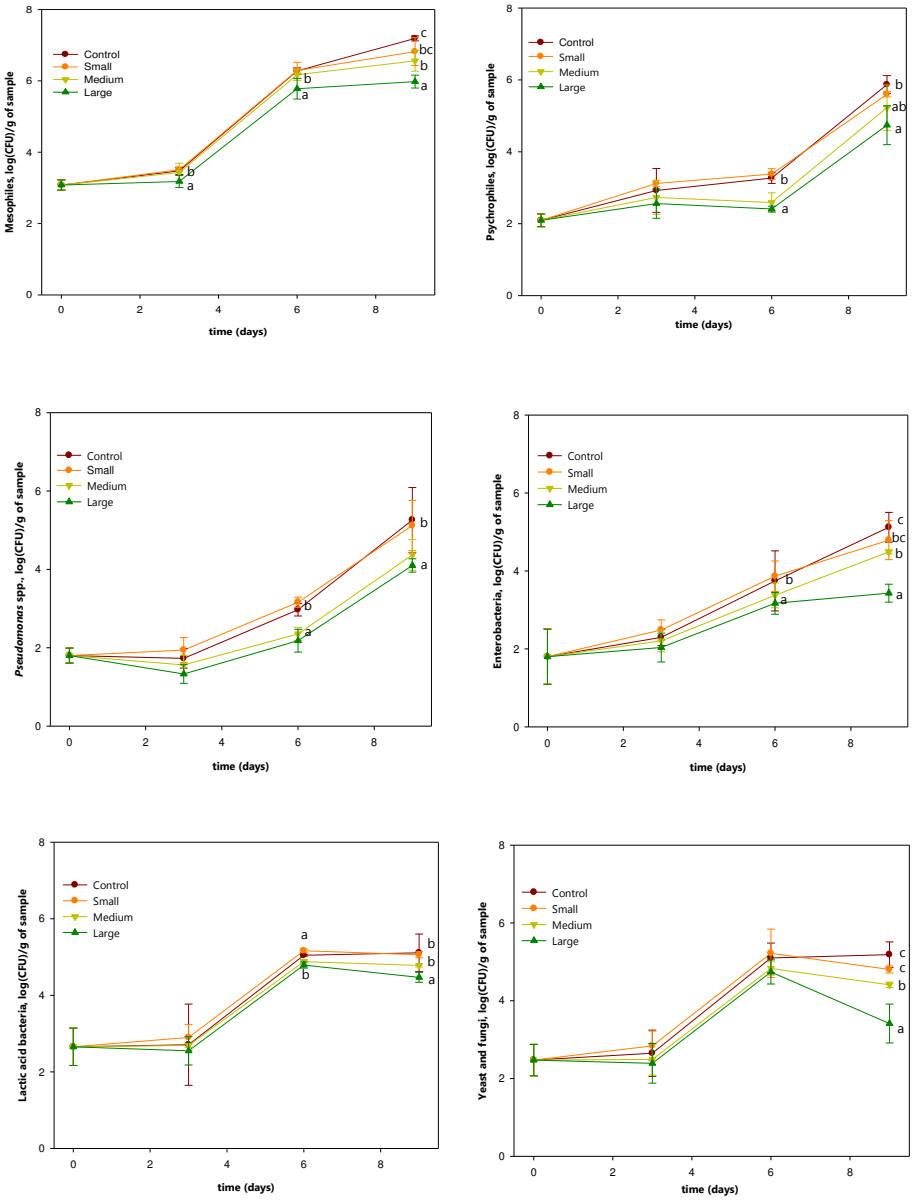

Figure 4.4.12. Evolution of chicken fillet microbiota packaged with control and active packages during 9 days of storage. Different letters (a, b, c) indicate significant differences among samples at a specific storage time (Turkey's adjusted analysis of variance $P<0.05$). Values are expressed as mean and standard deviation (n = 3).

surface. Differences can be explained by the type of food processing. In this work, small, medium and large CS:CD-Active films maintained mesophilic bacteria counts within the limit suggested by other authors during storage time (Rokka *et al.*, 2004; Wehr, 1982). Large samples also limited the growth of enterobacteria, counts of which remained between log 2 and log 3 during the 9-day storage.

The small size CS:CD-Active film did not produce an efficient microbial inhibition even though the total amount of carvacrol present in that device was slightly greater than the observed MIC (1 mg) of carvacrol in headspace tests (Higueras *et al.*, 2015). One important reason is that chicken tends to sorb carvacrol from the headspace, keeping the actual headspace concentration below the MIC. Actually, the carvacrol activity was 40 times lower than that at saturation. Moreover, the role of food proteins in protecting microorganisms from carvacrol action has been reported (Gutiérrez *et al.*, 2008). Therefore the antimicrobial efficiency of essential oils and their components is diminished by their interaction with proteins, and only the free fraction of carvacrol would exert antimicrobial activity (Veldhuizen *et al.*, 2007).

In this regard, only the CS:CD-Active films of medium and large size would provide a sufficient amount of free carvacrol to produce inhibition, and consequently the growth of microorganisms would be more affected by the exposure time. However, large quantities of carvacrol would be required to obtain this effective quantity of carvacrol in the headspace, probably exceeding the acceptable sensory threshold for the consumer, which may limit its use (Burt, 2004; Gutiérrez *et al.*, 2009; Škrinjar and Nemet, 2009). Previous studies carried out in *in vitro* conditions showed that medium CS:CD-Active film stuck to the lid of a Petri dish produced a bactericidal effect against *S. aureus* and *E. coli* (Higueras *et al.*, 2015). The lower antimicrobial activity of the films observed for chicken compared with the activity assayed in laboratory conditions may be due to the complexity of the food matrix and interaction of components with carvacrol; in addition, the use of the optimal culture medium for the microorganism in *in vitro* assays magnifies any effect (Burt, 2004).

The initial working plan included a sensory test with consumers, which, of course, was cancelled because of the unacceptable carvacrol content of

the product. Nevertheless, the authors of this work decided to check the aroma of the fresh fillet stored in the small active package after the nine days of storage, and, in fact, the effect on the aroma of the fresh fillets was perceptible but acceptable. A further consideration is that the process of cooking would produce an appreciable loss of antimicrobial agent. Samples of chicken breast were cooked in the oven (270 ºC, 20 min) and tested by the authors. The aroma of the baked fillets was acceptable and they could be distinguished from a control sample by their 'warmly pungent' odour, as described in the literature, an aroma that is readily accepted by consumers (Burt, 2004; Chi *et al.*, 2006). However, in the mouth, a bitter flavour and a dry texture were observed. Scramlin *et al.* (2010) reported that oregano oil (carvacrol is the major component of this essential oil) can reduce lipid oxidation and extend the shelf life of meat products but tends to develop off flavours, causing lower acceptability.

4. Conclusions

Materials containing carvacrol were developed as antimicrobial devices for active packaging of food products and were tested in the packaging of fresh chicken. CS:CD-Active films produced an antimicrobial effect that depended on the size of the film and the storage time: the larger the active device, the greater the microbial inhibition observed, which became more evident at longer exposure times. However, an increase in the device size produced an increase in the concentration of carvacrol retained in the chicken, which affected sensory attributes, hampering its application for this product in the present form. Further studies are needed to optimize the films developed, such as its application with other hurdle technologies such as modified atmosphere packaging or with other food products.

Acknowledgments

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4.4.5. Artículo científico 7.

Effect of HP-β-cyclodextrins and coadjuvants on the sorption capacity of hydrophilic polymer films for naturally occurring antimicrobial monoterpene alcohols

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Submitted to *Carbohydrate Polymers*

Resumen

Las películas de quitosano (CS) con hidroxipropil-β-ciclodextrinas (HP-βCD) en proporción 1:1 en peso y plastificadas con glicerol (G) o polietilenglicol (PG) fueron preparadas mediante extensión y evaporación del solvente y acondicionadas a diferentes humedades relativas (RH) para alcanzar un contenido de agua similar. Se estudiaron las propiedades de sorción de las películas de diversos monoterpenos con estructura fenólica o no fenólica lineal, o ciclohexilalcohol después de su inmersión en los líquidos volátiles. En general, las películas presentaron una considerable capacidad de retención de los compuestos monofenólicos, con valores que van desde 430% para el *m*-cumenol al 106% para el guayacol, dependiendo de la formulación de la película. Estos valores fueron dos órdenes de magnitud mayores que los de los compuestos sin el grupo fenol. La afinidad por los compuestos monofenólicos disminuyó de las películas plastificadas con el hidrófilo PG, mientras que no se observaron cambios en la retención de los monoterpenos no fenólicos. La retención de compuestos monofenólicos disminuyó considerablemente cuando se sustituyó el CS por el alcohol polivinílico (PVOH), excepto el caso del isoeugenol. Por último, se evaluó la actividad antimicrobiana de los monoterpenos y de las películas con ellos *in vitro* mediante el método de la microatmósfera frente *E. coli* y *S. aureus*. El presente estudio muestra que las HP-βCD y el nivel de plastificación alcanzado por películas hidrófilas se pueden utilizar para regular selectivamente la capacidad de carga y de sorción de compuestos antimicrobianos origen natural.

Palabras clave

Quitosano, alcohol polivinílico, hidroxipropil-β-ciclodextrinas, películas, sorción selectiva, actividad antimicrobiana.

Abstract

Chitosan (CS) films filled with hydroxypropyl-β-cyclodextrins (HP-βCD) at a 1:1 weight ratio and plasticized with glycerol (G) or propylene glycol (PG) were prepared by casting and conditioned at different relative humidities (RH) to achieve a similar water content. Sorption properties of the films for various monoterpene compounds with phenolic or non-phenolic linear or cyclohexyl alcohol structures were studied after their immersion in the volatile liquids. In general, the films presented a considerable capacity to retain monophenolic compounds, with values ranging from 430% for *m*-cumenol to 106% for guaiacol, depending on the film formulation; these values were two orders of magnitude higher than those of compounds without the phenol group. The affinity for monophenolic compounds decreased in films plasticized with hydrophilic PG, whereas no changes were observed in the retention of non-phenolic monoterpenes. Replacement of CS with polyvinyl alcohol (PVOH) polymer considerably decreased the retention of monophenolic compounds, with the exception of isoeugenol. Finally, the antimicrobial activity of monoterpenes and films loaded with them was evaluated *in vitro* by the microatmosphere test against *E. coli* and *S. aureus*. The present study shows that HP-βCD and the plasticization level achieved by hydrophilic films can be used to regulate loading capacity and sorption selectivity of naturally occurring antimicrobial compounds.

Keywords

Chitosan, polyvinyl alcohol, hydroxypropyl-β-cyclodextrins, films, sorption selectivity, antimicrobial activity.

1. Introduction

Nowadays consumers demand natural products that undergo minimal processing and are free of synthetic preservatives. However, these products often have a short microbiological shelf life, which makes it necessary to find new alternatives. In this regard, hurdle technology employs combined treatments and their synergies to preserve food more efficiently. Active packaging technologies combined with the use of naturally occurring preservatives could be an approach to hurdle technology for the preservation of minimally processed foods.

Among the antimicrobial agents used as food preservatives, there is a growing tendency to employ natural compounds from plant extracts and essential oils (Burt, 2004; Lang and Buchbauer, 2012). Essential oils and their components are considered food additives and classified by the JECFA (Joint FAO/WHO Expert Committee on Food Additives) as flavourings. These compounds have a great potential to be used as active agents in the development of antimicrobial active packaging technologies, which are a complementary method for increasing the microbial safety of packaged foods.

Antimicrobial food packaging technologies which are based on the incorporation of active volatiles in polymer matrices do not require the film to be in contact with the food product to be active. In this case, the volatile is released to the headspace of the package and exerts its activity when it comes into contact with the food surface, inhibiting or retarding microbial growth (Buonocore *et al.*, 2003; Cutter, 2002). The release of the volatile from the polymer matrix can be triggered by various stimuli, such as the humidity present in the headspace of the package, with the hydrophilic nature of the polymer and the level of humidity of the headspace being major factors that control the release kinetics of the agent.

In this respect, hydrogels are hydrophilic polymers of either natural or synthetic origin with a great potential to be used as sustained-release carriers of active molecules. Hydrogels are responsive to changes in environmental humidity; depending on their composition, they can absorb a certain amount of moisture and consequently swell to different extents, facilitating release of the entrapped molecule. Thus these polymers can

retain the volatile compound in a dry environment and release it when they are moisture-activated.

CS and PVOH are biodegradable hydrophilic polymers with excellent film-forming properties. CS is a natural linear cationic biopolymer produced by partial deacetylation of chitin. PVOH is a synthetic polymer obtained from controlled hydrolysis of polyvinyl acetate. Solid dispersions based on CS and PVOH polymers have been extensively employed in the form of thin films, nanoparticles, capsules, tablets, etc. as carrier systems for sustained release of drugs (Goodship and Jacobs, 2009; Kumar *et al.*, 2004; Rinaudo, 2006). CS and PVOH can be modified to improve the drug-loading capacity of the carrier and to modulate the release kinetics of entrapped compounds. These polymers have been blended with other natural or synthetic polymers of various polarities and molecular weights, nanofibres and nanoclays, mixed with plasticizers, and crosslinked in order to control the kinetics of the release (Islam and Yasin, 2012; Jiang *et al.*, 2012; Kumar, 2000; Lavorgna *et al.*, 2010; Muhd Julkapli *et al.*, 2011; Rahman *et al.*, 2010; Yu *et al.*, 2011; Zuber *et al.*, 2013).

In the design of polymer systems capable of retaining and releasing antimicrobial volatile organic compounds it is necessary that a minimal amount of volatile be entrapped in the film to provide it with antimicrobial properties. The major drawback in the development of these systems is that a high percentage of the active agent is evaporated or inactivated during film processing. The alternative method of absorption for loading the volatile into the formed film has low efficacy. This is currently due to the fact that most organic volatile compounds are hydrophobic and thus have low compatibility with hydrophilic films (Balaguer *et al.*, 2012). Kurek *et al.* (2012) recently studied how the composition of the film-forming solution and process parameters affect the retention of volatile liquid carvacrol. They found that G and gum arabic were the most effective additives to improve retention of carvacrol, whereas the effect of nanoclays and emulsifiers was weak.

β-CDs are cyclic oligosaccharides composed of seven glucopyranose units with a truncated cone shape characterized by a hydrophilic external surface and a hydrophobic cavity. This unique structure enables CDs to form inclusion complexes, entrapping all or part of a "guest" molecule inside their

cavities, and presenting potential interest as agents to retain or release entrapped substances. However, enhancement of the solubility of hydrophobic compounds by non-inclusion aspects of CDs is currently being studied, such as solubilisation by formation of self-assembled aggregates or surfactant-like effects (Messner *et al.*, 2010).

The present work studies the effect of incorporating water-soluble derivatives of CDs (HP-βCD) and low molecular weight hydrophilic plasticizers (G, water and PG) on the sorption capacity of films based on CS polymer for several naturally occurring monoterpenes. Sorption properties of those films which absorbed a greater amount of sorbate were compared with those of films formed by replacing CS with synthetic PVOH as the polymer matrix. Previously, the antibacterial activity of the volatile liquids was tested against *S. aureus* and *E. coli* using the microatmosphere method of Kellner and Kober (1954). Finally, the antimicrobial activity of the resulting films was tested by the same method.

2. Materials and methods

2.1. Chemicals

Low molecular weight chitosan (CS) was supplied by Sigma (Barcelona, Spain). Polyvinyl alcohol (PVOH, Gohsenol type AH-17, saponification degree 97–98.5% mol and viscosity 25–30 mPa·s) was obtained from The Nippon Synthetic Chemical Co. (Osaka, Japan). Hydroxypropyl-β-cyclodextrins (HP-βCD, CAVASOL® W7-HP) were supplied by Wacker Ibérica (Barcelona, Spain). Carvacrol (kosher >98%), L-carveol >95% mixture of *cis* and *trans*, dihydrocarveol kosher >96%, isopulegol >99%, isoeugenol >98% mixture of *cis* and *trans*, nerol kosher >97%, guaiacol and dimethyl sulfoxide 99.9% ACS reagent (DMSO) were supplied by Sigma (Barcelona, Spain). *meta*-Cumenol ≥97% and *ortho*-cumenol ≥98% were purchased from Fluka (Madrid, Spain). *R*-Myrtenol >95%, glycerol (G), propylene glycol (PG) and acetic acid were obtained from Aldrich (Barcelona, Spain). Sodium nitrite, sodium chloride, potassium chloride and barium chloride dehydrate were supplied by Sigma-Aldrich (Madrid, Spain). Peptone Water (PW, 0.1%), Tryptone Soy Agar (TSA) and Tryptone Soy Broth (TSB) were supplied by Scharlau (Barcelona, Spain).

2.2. Film preparation

Films based on CS were prepared from 1.5% (w/w) CS solution dissolved in 0.5% (w/w) acetic acid, stirred at 40 ℃ for 1 h and filtered to eliminate impurities. For films based on PVOH, a 4% (w/w) PVOH solution was prepared in distilled water and stirred at 85–90 ℃ for 2 h. CDs were added to the film-forming solution in a 1:1 proportion (w/w) with respect to CS or PVOH, and stirred at 37 ℃ until complete dissolution. Then, G or PG plasticizer was added at the corresponding % [(g plasticizer/100 g dry matter (polymer + CD)] to the film-forming solution. Films were formed by casting on PS plates and dried at 37 ℃ and 40% relative humidity (RH) for 36 h. Film thickness was measured using a digital micrometer (Mitutoyo Manufacturing Co. Ltd., Tokyo, Japan) with a sensitivity of 1 µm. Five measurements were taken for each sample, one at the sample centre and four around the perimeter. The average thickness of the films was 55 ± 5 µm. With the aim of comparing sorption properties of films obtained using different plasticizers and polymers but having a similar water content, prior to immersion in various volatile liquids, various film samples (0.4–0.5 g) 550 mm in diameter and 55 ± 5 µm in thickness were placed on aluminium plates and stored at 23 ± 1 ℃ in glass desiccators containing saturated salt solutions to achieve the desired water content (ASTM, 2007). RH values achieved in each desiccator were confirmed by direct RH measurements with hygrometers (Hygrodynamics, Newport-Scientific Inc., Jessup, MD, USA). After reaching moisture sorption equilibrium, the water content of the films was measured. For this purpose, the films were weighed and dried with phosphorus pentoxide (Sigma, Barcelona, Spain) until constant weight was reached. The tests were done in triplicate. Finally, the following films with the same moisture contents were obtained: CS:CD films with 35% of G conditioned at 75% RH (CS:CD-35G-75RH), CS:CD films incorporating 50% of G conditioned at 65% RH (CS:CD-50G-65RH), CS:CD films with 35% of PG conditioned at 90% RH (CS:CD-35PG-90RH) and PVOH:CD films with 35% of G conditioned at 84% RH (PVOH:CD-35G-84RH).

2.3. Study of miscibility of monoterpene compounds with the plasticizers G and PG

Pyrex tubes were filled with 3 mL of volatile liquid and 3 mL of the plasticizer studied, and the solution was homogenized and allowed to stand at room temperature (23 ℃). The solutions were observed for liquid-liquid phase separation. The experiments were done in triplicate.

2.4. Sorption method for loading monoterpenes into CS and PVOH films

Films with different matrix compositions as described in section 2.2. were immersed in different pure volatile liquids at 23 ℃ and the amount of the compound sorbed in the film was measured over time until sorption equilibrium was reached.

2.5. Determination of monoterpene sorbed in a film

The amount of volatile liquid in a film was determined by thermal desorption coupled to gas chromatography using a Dynatherm Thermal Desorber Model 890/891 (Supelco, Teknokroma, Barcelona, Spain) connected in series to the column of an HP5890 gas chromatograph Series II Plus (Agilent Technologies, Barcelona, Spain) via a heated transfer line. A cut piece of the film was cleaned with a paper tissue to remove any excess of volatile compound on the film surface and then inserted into an empty desorption tube (11.5 × 0.39 cm I.D.). The tube was placed in the desorber chamber, which was immediately sealed. Conditions for desorption were as follows: desorption temperature, 210 ℃; transfer line, 230 ℃; desorption time, 7 min; He desorption flow, 8.15 mL/min. The GC was equipped with a TRB5 (30 m, 0.32 mm, 0.25 μm) column (Teknokroma, Barcelona, Spain) and a flame ionization detector. The chromatographic conditions were: 260 ℃ detector temperature, 7 min at 45 ℃, heating ramp to 220 ℃ at 18 ℃/min, and 1 min more at 220 ℃. After the analysis, the film sample was recovered from the desorption tube and weighed on an analytical balance (Voyager V11140 model, Ohaus Europe, Greifensee, Switzerland).

2.6. Antimicrobial assays

2.6.1. Bacterial strains and growth conditions

Two model microbial strains were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain): the Gram-positive bacterium *Staphylococcus aureus* CECT 86 and the Gram-negative bacterium *Escherichia coli* CECT 434. The strains were stored in Mueller Hinton Broth (MHB, Scharlab, Barcelona, Spain) with 20% G at −80 °C until needed. For experimental use, the stock cultures were maintained by regular subculture on TSA slants at 4 °C and transferred monthly. In the first step, a loopful of each strain was transferred to 10 mL of TSB and incubated at 37 °C overnight to obtain early stationary phase cells.

2.6.2. Determination of minimal inhibitory dose of each volatile liquid in vapour phase

The microatmosphere method was selected to carry out an antimicrobial test in which no direct contact between the device containing the volatile and the agar medium is necessary for the former to exert its activity. In this method, the volatile compound migrates from the carrier (filter paper, film) to the headspace of the Petri dish, thus becoming available to contact the growth medium and the microorganism. For this purpose, 100 µL of a bacterium suspension containing approximately 10^7 colony forming units (CFU)/mL was spread over the surface of 90-mm-diameter Petri dishes containing approximately 15 mL of solid culture TSA medium. Decreasing quantities of each volatile liquid were dissolved in DMSO, and a final volume of 10 µL of the solution was added to 25-mm-diameter sterilized filter paper which was placed in the centre of the Petri lid. Blanks were prepared by adding 10 µL of DMSO to 25-mm-diameter sterile filter disks, having previously checked that the DMSO did not have any antibacterial effect against any of the microorganisms tested. Once the Petri dishes and lids had been assembled, the units were sealed with Parafilm™ to reduce leakage of the volatile agent and incubated upside down at 37 °C for 24 h. At the end of the incubation period, the antimicrobial activity of the volatile liquids was determined by measuring the diameter of the zone on the surface of the agar where there was no microbial growth. The minimum inhibitory dose is

defined as the lowest amount of active compound that yields inhibition of microbial growth on the agar surface. Each assay was performed in triplicate.

2.6.3. Antimicrobial activity of the films

The procedure for determining the antimicrobial activity of the films was similar to that described above. In this case, films which were of the same size as the filter papers and loaded with the compound were placed on the centre of the Petri lid. After the incubation period, the diameter of the resulting inhibition zone was measured. Each assay was performed in triplicate.

2.7. Data analysis

The data are represented as average ± standard deviation. The data were graphically plotted with SigmaPlot software (Systat Software Inc., Richmond, CA, USA).

3. Results and discussion

Films based on CS or PVOH as the polymer matrix incorporating CD at a 1:1 weight ratio and plasticized with G or PG were successfully obtained by casting. To the naked eye the films were homogeneous, with smooth surfaces, very transparent and easy to handle. The thickness of the films was about 55 ± 5 µm.

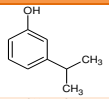
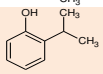
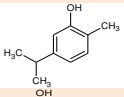
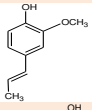
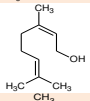
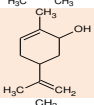
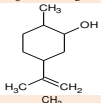
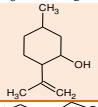
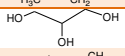
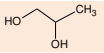
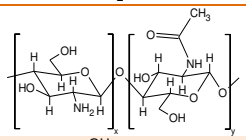
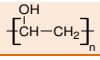
3.1. Equilibrium moisture content of the films

A study was made of the effect of incorporating CD, and the polarity and amount of the plasticizer added (G or PG), on the sorption properties of CS films for various monoterpene compounds with phenolic or non-phenolic linear or cyclohexyl alcohol structures. Moreover, in order to investigate the effect of the hydrophilicity of the polymer matrix on the sorption behaviour of the films, CS was replaced with PVOH polymer. This synthetic polymer presents great affinity for water but is less hydrophilic than CS. Prior to immersion in the volatile liquids, the various films were conditioned at the appropriate RH to obtain a similar water content in all of them. In general, the water content of the films studied ranged between 32 and 35% (g/g dry film).

CS:CD-35G films conditioned at 75% RH retained 33.9 ± 0.8% of water. The water content of CS:CD-50G films conditioned at 65% RH was 32.9 ± 1.7%. As expected, an increase in the G content increased the water retention capacity, so the RH at which they were conditioned was lower. When G was replaced with PG in the CS:CD-35PG films, they had to be conditioned at 90% RH to reach a water content of 33.9 ± 1.4%, which can be explained by the less hydrophilic nature of PG and its lower capacity to retain water. **Table 4.4.11** shows some physico-chemical parameters of the various plasticizers used in this work. They include log P, which is the octanol/water partition coefficient and provides a quantitative measurement of the hydrophobic nature of a compound; it can be observed that PG has a higher log P than G. **Table 4.4.11** also shows the Hildebrand solubility parameter (δ), which is defined as the square root of the cohesive energy density of a substance (Hildebrand and Scott, 1949). This parameter provides a numerical estimate of the degree of miscibility of components of a system; it is expected that greater interactions will take place among components that have closer solubility parameters. Since the solubility parameter is related to the heat of vaporization, this parameter can be theoretically calculated for non-volatile compounds by group contribution methods. However, these methods have limitations when it comes to predicting interactions among molecules in which hydrogen bonding and electrostatic interactions are predominant. In this regard, **Table 4.4.11** also shows that the value of the Hildebrand solubility parameter of G is greater than that of PG (33.5 vs. 30.1 $MPa^{1/2}$), so it would be expected to have a greater affinity for water (δ = 47.9 $MPa^{1/2}$).

When PVOH was used as the polymer matrix in the PVOH:CD-35G formulation, the films had to be conditioned at a higher RH than the corresponding films made with CS (84% vs. 75% RH, respectively) to achieve a similar water content (32.4 ± 1.7%). The solubility parameter is frequently used to study compatibility in polymer–plasticizer, polymer–drug and polymer–aroma systems. With regard to the polymer–water system studied in the present work, the experimental Hildebrand solubility parameter of PVOH ranges from 25.8 to 29.1 $MPa^{1/2}$ compared with 38 $MPa^{1/2}$ for CS, whereas δ for water is 47.9 $MPa^{1/2}$, which is in accordance with the lower moisture sorption of PVOH films and the greater RH required to make their water content equal to that of CS films. A further factor to be taken into

Table 4.4.11. Physico-chemical properties and molecular structure of volatile liquids plasticizers, and polymers.

Name	Molecular structure	Molecular mass	[a]Log P	Vapour pressure (Pa)	δ ($MPa^{1/2}$)
m-cumenol		136.19	2.82	6.67	24.1[b]
o-cumenol		136.19	2.82	14.93	24.1[b]
carvacrol		150.22	3.28	4.00	23.6[b]
guaiacol		124.14	1.19	23.86	26.5[b]
isoeugenol		164.20	2.45	0.67	24.3[b]
myrtenol		152.23	3.22	2.40	21.0[b]
nerol		154.25	3.28	1.73	20.9[b]
carveol		152.23	2.55	1.60	19.7[b]
dihydrocarveol		154.25	2.92	2.40	19.3[b]
isopulegol		154.25	2.92	13.20	19.3[b]
glycerol		92.09	-1.84	<0.01	33.5[b]
propylene glycol		76.09	-1.05	27.20	30.1[b]
water	H_2O	18.02	-1.38	3263.01	47.9[c]
CS		50 - 190 KDa	-	<0.01	38[b]
PVOH		-	-	-	from 25.8 to 29.1[d]

Note: molar volume and log P predicted from ACD/Labs.

[a] log P: hydrophobicity of the molecule expressed as the logarithm of octanol/water partition coefficients and estimated using software ACD/Labs 12.0-ChemSketch.

[b] Hildebrand solubility parameter estimated according to Fedors, in van Krevelen and te Nijenhuis (2009).

[c] Solubility parameter of water from Grulke (1989).

[d] Literature experimental Hildebrand solubility parameter values in van Krevelen and te Nijenhuis (2009).

account is that PVOH is a semicrystalline polymer, which also limits its water sorption capacity.

3.2. Miscibility studies

Plasticizers are low-molecular compounds, chemically compatible with the polymer to be plasticized, which at appropriate concentrations impart flexibility and facilitate film handling. In a plasticization process, the plasticizer molecules are accommodated in the polymer matrix by disrupting intermolecular forces between polymer chains, spacing them apart and increasing the free volume, thus acting as diluents. Plasticization of hydrophilic polymer matrices is commonly carried out by polyols, among which G and PG are commonly used for this purpose. Most plasticizers employed in polysaccharide films, such as polyols, are frequently employed in the flavouring industry as solvents and liquid supports for flavours. The presence of hydroxyl groups in G make it a good solvent for many ingredients used in pharmaceutical preparations and flavour compounds. G can behave as a binder of relatively polar volatile compounds such as alcohols, aldehydes, ketones and organic acids by forming hydrogen bonds, even acetals, ketals and esters. PG is used as a plasticizer to improve the flexibility and processability of polymers, and is extensively used in the food industry as a flavour solvent carrier. PG is miscible with water, alcohol and many flavour compounds, and can also interact chemically or physically with them. As commented above, **Table 4.4.11** shows some physico-chemical parameters of the plasticizers used in this work, including δ and log P. It can be observed that PG has a higher log P than G, whereas the δ of G is higher than that of PG. These two parameters indicate the greater hydrophilicity and capacity to retain water of G, a ubiquitous plasticizer for hydrophilic polymers, whereas, owing to its less hydrophilic nature, PG would be expected to have a higher affinity to the hydrophobic volatile liquids present in essential oils.

3.3. Sorption of monoterpenes in the films

Chemical compatibility between volatile liquids and the film polymer matrix will determine sorption properties, and the presence in the film of low molecular weight compounds such as residual solvents and plasticizers can

also affect the sorption behaviour. Therefore, before studying the effect of G and PG on the sorption properties of the films for the volatile liquids appearing in **Table 4.4.11**, miscibility studies between the plasticizers and those compounds were carried out. It was found that there was complete miscibility of the various volatile liquids in PG, resulting in a single liquid layer. Complete miscibility was also observed for G and *o*-cumenol, *m*-cumenol and guaiacol. Carvacrol and G were partially miscible, and complete immiscibility was observed for the remaining compounds. The miscibility studies showed that PG has greater compatibility with all the compounds tested, whereas the greater polarity of G limits its compatibility with them. This feature could modify the sorption properties of the films, depending on the plasticizer used.

Table 4.4.11 shows the solubility parameters of monoterpenes. It is expected that the closer the parameters for a sorbent and a polymer, the greater the sorption affinity will be. Hydrophilic components of the films - CS, PVOH, G, PG and water (38, 25.8–29.1, 33.5, 30.1 and 47.9, respectively) - had high values of δ compared with those of the monoterpenes. This means that phenolic monoterpenes with δ values closer to those of the film components (ranging from 23.6 to 26.5 $MPa^{1/2}$) are expected to be retained in the films to a greater extent than cyclic and linear non-phenolic monoterpene alcohols with lower solubility parameters, comprised between 19.3 and 21 $MPa^{1/2}$.

Absorption of volatile liquids in a film will be affected by chemical affinity between the sorbate and the film components. Sorption properties of the films for the various compounds are shown in **Table 4.4.12**. A common feature for all the films formulated was that phenolic monoterpenes were sorbed in greater amounts than non-phenolic cyclic and linear monoterpenes. The presence of the benzene ring increases affinity of the molecule for the film, owing to the affinity between the double bonds of benzene and polar groups of the film (hydroxyl, amino, acetamido and carbonyl groups of the chain end). Sorption of phenols increased in the following order: *m*-cumenol > *o*-cumenol > carvacrol > guaiacol > isoeugenol). *m*-Cumenol was sorbed to a slightly greater extent than *o*-cumenol, one possible explanation for which is stearic hindrance of the

Table 4.4.12. Sorption capacity of films based on CS or PVOH incorporating HP-βCD in 1:1 weight ratio, and plasticized with G or PG for monoterpenes at 23 °C (%, g/100 g dry film).

	CS:CD-35G-75RH	CS:CD-50G-65RH	CS:CD-35PG-90RH	PVOH:CD-35G-84RH
m-cumenol	455.06 ±18.88	372.38 ± 18.85	269.94 ± 13.38	274.27 ± 44.19
o-cumenol	419.67 ± 21.99	339.40 ± 17.95	227.28 ± 18.42	226.37 ± 8.68
carvacrol	230.11 ± 18.74	224.80 ± 9.85	179.62 ± 3.09	128.23 ± 2.09
guaiacol	193.22 ± 1.32	184.89 ± 7.95	106.59 ± 3.16	85.70 ± 7.48
isoeugenol	12.02 ± 6.72	12.59 ± 3.28	11.50 ± 2.04	13.56 ± 2.56
myrtenol	3.48 ± 0.93	2.16 ± 0.58	2.26 ± 0.97	2.27 ± 0.11
nerol	3.09 ± 0.79	2.48 ± 0.06	2.43 ± 0.79	2.39 ± 0.51
carveol	2.37 ± 0.74	2.41 ± 0.08	2.50 ± 0.40	2.39 ± 0.06
dihydrocarveol	1.27 ± 0.09	1.85 ± 0.08	1.91 ± 0.20	2.05 ± 0.27
isopulegol	0.30 ± 0.74	0.52 ± 0.03	0.28 ± 0.01	0.92 ± 0.19

isopropyl group, hindering hydrogen bonding through hydroxyl groups of *o*-cumenol and the hydrophilic matrix of CS or PVOH. In fact, swelling and loss of dimensional stability were observed in CS:CD-35G films loaded with *m*-cumenol. With regard to carvacrol, the incorporation of a methyl group in the 3-isopropylphenol structure increased the hydrophobicity of the molecule as given by log P (see **Table 4.4.11**), decreasing its affinity for the film and its absorption in the bulk matrix. Substitution of isopropyl in *o*-cumenol by the methoxyl group in guaiacol produced a decrease in sorption values in spite of having a lower log P than carvacrol and *ortho*- and *meta*-cumenol, on the basis of which greater affinity for the highly hydrophilic matrices would be expected. As in the case of *o*-cumenol, it might be thought that the methoxyl group in the *ortho* position in phenol could hinder formation of hydrogen bridges with polar groups of the polymer matrix. However, although the isopropyl substituent in *o*-cumenol is bulkier than the methoxyl group in guaiacol, the former is sorbed to a greater extent. Guaiacol has the ability to form intramolecular hydrogen bonds, thus competing with the formation of hydrogen bonds with the polymer matrix and limiting its retention in the polymer. Glemza *et al.* (1998) observed suppression in the uptake of *o*-methoxyphenol in acrylic ester sorbent compared with the *meta*- and *para*- isomers owing to the formation of intramolecularly hydrogen-bonded species. With respect to isoeugenol, the presence of the propylene substituent in the 2-methoxyphenol structure engenders the hydrophobicity of the molecule, which has a log P close to isopropylphenol; however, its sorption in the polymers was low, an order of magnitude lower than the other phenols studied.

A remarkable depletion was observed in the sorption of cyclic and linear non-phenolic monoterpenes, with values ranging from 0.25 to 3.5% (g/100 g dry matter). Bicyclic myrtenol and linear nerol (with a log P similar to carvacrol) together with the more hydrophilic carveol had the highest sorption values among the non-phenolic compounds, whereas isopulegol had the lowest sorption among all the monoterpenes studied. Positional isomers dihydrocarveol and isopulegol have a slightly higher log P than carveol (2.92 vs. 2.55). The cyclohexene ring in carveol increased polarity and sorption affinity for the films compared with the cyclohexane ring of dihydrocarveol, whereas the sorption of positional isomer isopulegol decreased considerably, which might be due to the isopropenyl substituent next to the hydroxyl group.

It can be concluded that the presence of a benzene group in the molecular structure of monoterpenes allowed high sorption values and plasticization of the films. Plasticization by benzene of hydrophilic pervaporation membranes has been reported in the literature (Villaluenga and Tabe-Mohammadi, 2000).

In a comparison of the sorption properties of the films formulated with CS:CD possessing approximately the same water content and differing in the amount of G, greater sorption values were observed for *ortho*- and *meta*-cumenol in CS:CD films with 35% G. In a previous study regarding the sorption capacity of CS films blended with CD and different amounts of G and water, it was demonstrated that sorption is almost suppressed in unplasticized films, requiring the presence of CD and plasticization by G and water to retain carvacrol (Higueras *et al.*, 2013). In that work, it was concluded that G enhances sorption of carvacrol more than water does. In the present study, it was observed that, at a fixed water content of 33–34% (g/g dry film), increasing the G content from 35 to 50% did not affect the sorption of phenolic monoterpenes with the exception of *ortho*- and *meta*-cumenol, which reduced their sorption. This might be related to an excess of film plasticization, restricting the very high sorption values of cumenol isomers. In an unpublished previous study it was found that, for a lower water content in the films (around 15% when CS:CD films were conditioned at 53% RH), the content of sorbed carvacrol increased with the amount of G in the film, giving sorption values of 6.13, 133.27 and 300% when plasticized

with 20, 35 and 50% G, respectively. Therefore, an increase in the plasticization level of the film facilitates the entry of sorbate molecules. However, this behaviour was not observed for CS:CD films when the G content increased from 35 to 50%, but it must be taken into account that in the present case the water content of the films was much higher (34 vs. 15% approx.), thus making the films much more hydrophilic.

When 35% G was replaced with PG in the CS:CD films, maintaining the same water content, the sorption of cumenol isomers, carvacrol and guaiacol decreased, whereas the sorption of the other compounds was not modified (**Table 4.4.12**). Owing to the miscibility of PG with monoterpenes, one might expect greater sorption of the volatile liquids. The lower capacity of PG to plasticize the polymer matrix compared with G might explain these results.

As stated above, it was demonstrated that sorption was favoured when the polymer film was plasticized by G, but an excess of water used as plasticizer caused an increase in film hydrophilicity, reducing affinity for less hydrophilic compounds. In this regard, **Figure 4.4.13** shows the effect of water on the sorption of carvacrol in CS:CD films plasticized with 35% PG. Water acts as a plasticizer for hydrophilic CS-based films, favouring the entry of sorbates. However, an excess of water suppressed sorption of carvacrol.

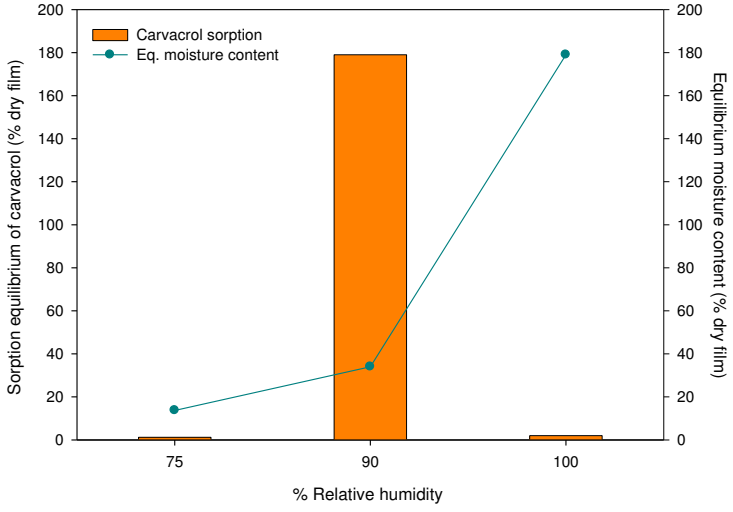


Figure 4.4.13. Effect of RH on equilibrium moisture content and sorption equilibrium of carvacrol in CS:CD-35PG.

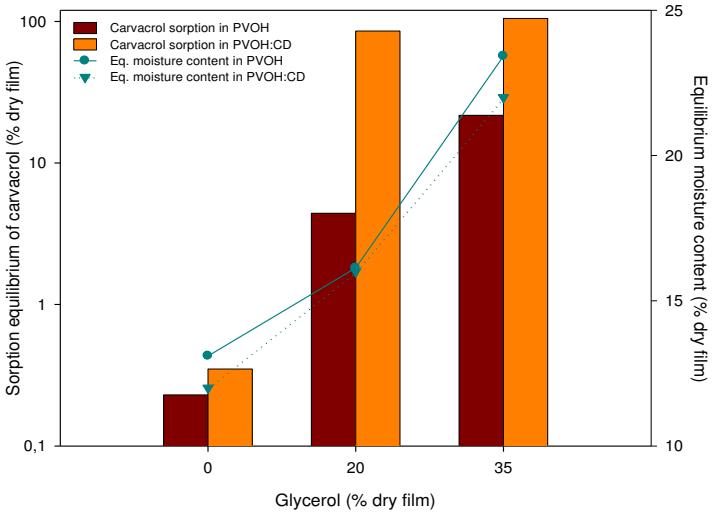


Figure 4.4.14. Effect of incorporating HP-βCD and G on equilibrium moisture content and sorption equilibrium of carvacrol in PVOH films conditioned at 75% RH.

In the studies carried out with PVOH, it is noteworthy that sorption was negligible when CDs were not incorporated in the films (**Figure 4.4.14**); there are studies reporting that the addition of β-CDs enhances the solubility of xylene and benzene in PVOH membranes (Chen *et al.*, 2000; Peng *et al.*, 2006). **Figure 4.4.14** shows that the content of plasticizer exerts a favourable effect on the sorption capacity of the films. This effect was also observed with the water content in the films (**Figure 4.4.15**), as described for CS; however, a high water content in the film decreased its sorption capacity. Sorption of cumenol isomers, carvacrol and guaiacol was lower in the PVOH:CD-35G films than in the films with CS as the polymer matrix. Owing to its high hydrophilicity, G has a greater capacity to plasticize more polar CS compared with PVOH, which might explain the lower sorption values obtained for phenolic monoterpenes with the exception of isoeugenol. Moreover, PVOH has a certain degree of crystallinity, which restricts sorption. Similar sorption values were found for isoeugenol and non-phenolic monoterpenes in the CS:CD-35G and PVOH:CD-35G films; the less hydrophilic nature of PVOH tended to increase values of 4.4 and 23% for films plasticized with 20 and 35% G, respectively, whereas carvacrol sorption in CS films without CD was lower than 1%, irrespective of their water and G contents (Higueras *et al.*, 2013).

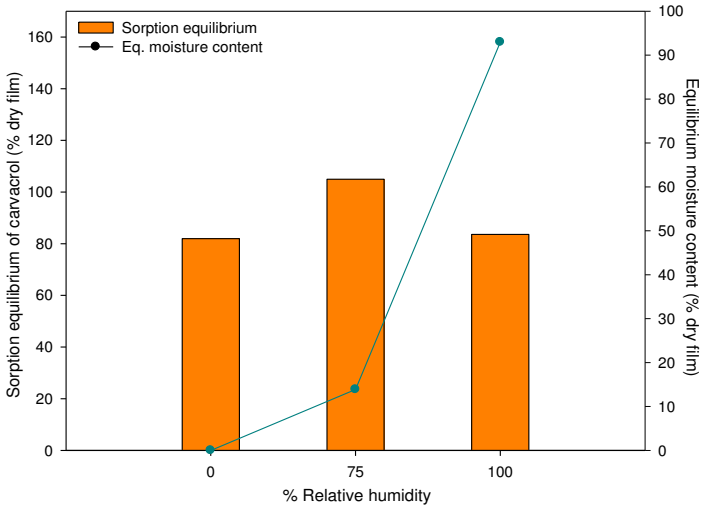


Figure 4.4.15. Sorption equilibrium of carvacrol and equilibrium moisture content of PVOH:CD-35G films conditioned at different RH.

3.4. Antimicrobial capacity of monoterpenes in vapour phase

The minimum inhibitory dose of monoterpenes against *S. aureus* and *E. coli*, evaluated in vapour phase, is given in **Table 4.4.13**. Carvacrol showed the greatest antimicrobial activity against both microorganisms, with 0.9 mg of carvacrol being needed to produce clear inhibition of growth on agar its sorption ability for more hydrophobic compounds as compared with CS. In fact, **Figure 4.4.14** shows that the affinity between the PVOH films without CD and carvacrol was greater than for CS films, reaching carvacrol sorption plates, followed by *ortho*- and *meta*-cumenol. However, it was necessary to use more than 1 mg of these compounds to see a clear effect, and the inhibition halo created by *o*-cumenol was larger (29 vs. 19 mm). The greater vapour pressure of *o*-cumenol compared with that of *m*-cumenol (**Table 4.4.11**) might explain the higher activity in vapour phase; the greater antimicrobial activity of *ortho*- and *meta*- isomers compared with *para*-isomers of some drugs is reported in the literature (Biava *et al.*, 1999). Phenolic compounds present in essential oils have been found to be good inhibitors of target bacteria (Kuhn *et al.*, 2006). Ultee *et al.* (2002) stated that the capacity of the hydroxyl group in phenol to release its proton and the presence of a delocalized electron system corresponding to the benzene ring, which to some extent stabilizes the phenoxide ion formed, is responsible for the high antimicrobial activity of this molecule. These features give carvacrol greater antimicrobial activity compared with other

Table 4.4.13. Minimum amount of volatile liquid required to produce growth inhibition in vapour phase against *S. aureus* and *E. coli*.

Compound	(mg)
m-cumenol	1.4
o-cumenol	1.4
carvacrol	1.0
guaiacol	22.0
isoeugenol	2.6
myrtenol	3.2
nerol	2.4
carveol	2.4
dihydrocarveol	2.0
isopulegol	8.0

compounds with a similar structure but with a cyclohexanol rather than phenol group, such as menthol, which has a very weak antimicrobial activity (Ben Arfa *et al.*, 2006). The presence of acetate (carvacryl acetate) or methyl ether (carvacrol methyl ether) groups instead of the hydroxyl group in carvacrol makes the molecule a proton acceptor but incapacitates the release of a proton from these groups, so they are inefficient as antimicrobials. Like carvacrol, cumenol isomers contain a hydroxyl group and a system of delocalized electrons in the benzene ring possessing a strong antimicrobial activity. Compared with carvacrol, the absence of the methyl group in cumenol isomers decreased its antimicrobial activity. A decrease in the hydrophobicity of the molecule resulting from the removal of aliphatic substituents may affect its interaction with protein and lipid components of the bacterial membrane. Log P of cumenols is considerably lower than that of carvacrol (2.82 vs. 3.28). In spite of its high vapour pressure and the presence of the phenol group, the very low antimicrobial activity of guaiacol can be attributed to its lower hydrophobicity compared to cumenol isomers and carvacrol; moreover, the presence of a methoxyl group in *ortho* position gives rise to intramolecular hydrogen bonding with the hydroxyl group (Knauth and Sabbah, 1990), which could depress its capacity to release its proton and thus inhibit its antimicrobial activity. The presence of the propenyl chain in isoeugenol increased its hydrophobicity with respect to guaiacol, and it had a higher log P (2.45 vs. 1.19) and greater antimicrobial activity. The antimicrobial capacity of isoeugenol was lower than that of molecules with an isopropylphenol structure and also than that of dihydrocarveol, carveol and nerol. In a comparison of monoterpenes with

similar structures but possessing a cyclohexanol or phenol group, molecules with a methylisopropylphenol structure (cumenol isomers and carvacrol) showed higher activity than compounds with a methylisopropenylcyclohexanol (dihydrocarveol, isopulegol) or methylisopropenylcyclohexenol (carveol) structure. This highlights the above-mentioned importance of the phenol group in the antimicrobial activity of the molecule.

Isoeugenol, nerol and carveol showed similar antimicrobial activity, whereas it was slightly higher for dihydrocarveol and lower for myrtenol. With regard to the compounds derived from *para*-methylisopropenylcyclohexanol, isopulegol and dihydrocarveol, the microorganisms presented lower sensitivity against isopulegol than against dihydrocarveol, the only difference between them being the hydroxyl group position. Thus, 8.0 mg of isopulegol was needed compared with 2.0 mg of dihydrocarveol to produce inhibition of bacterial growth.

Lipophilicity has been used as the descriptor with the strongest influence on antimicrobial activity owing to the great affinity of lipophobic compounds for cell membrane (Dambolena *et al.*, 2012). This property is specially considered for compounds with log P between 3 and 4. However, in the present study, monoterpenes with similar log P values exerted different antimicrobial activities. These results indicate that other factors besides hydrophobicity are involved, such as the presence and hydrogen-donating ability of compounds with a phenol chemical structure.

3.5. Antimicrobial capacity of films loaded with monoterpenes

Table 4.4.14 shows the antimicrobial capacity in vapour phase against *S. aureus* and *E. coli* of CS:CD films plasticized with G or PG and PVOH:CD films plasticized with G after reaching sorption equilibrium in the volatile liquids. Antimicrobial activity was measured as the inhibition growth halo produced on the agar surface. A clear positive relationship was found between the bacterial growth inhibition zone and the amount of volatile retained in the film. All the films that retained a greater amount of antimicrobial compound than that corresponding to the minimal inhibition dose exerted antimicrobial activity. This shows that the formulated films based on CS or PVOH possess an excellent capacity to release the retained

Table 4.4.14. Antimicrobial activity of films (25 mm diameter surface) against *S. aureus* and *E. coli* after loading with monoterpenes, measured by the microatmosphere method.

S. aureus	CS:CD-35G-75RH		CS:CD-50G-65RH		CS:CD-35PG-90RH		PVOH:CD-35G-84RH	
	Amount (mg)	Zone of inhibition (mm)	Amount (mg)	Zone of inhibition (mm)	Amount (mg)	Zone of inhibition (mm)	Amount (mg)	Zone of inhibition (mm)
m-cumenol	327.1	>85	275.4	>85	226.2	>85	169.3	>85
o-cumenol	347.5	>85	243.7	>85	222.8	>85	101.5	>85
carvacrol	146.5	>85	184.9	>85	194.9	>85	147.9	>85
guaiacol	106.0	71	121.3	79	96.4	70	194.2	80
isoeugenol	18.7	54	24.1	63	22.2	59	27.0	67
myrtenol	5.7	44	3.9	10	4.5	29	3.3	10
nerol	5.4	40	3.8	30	3.0	12	3.2	30
carveol	3.5	27	4.4	34	4.3	32	4.6	35
dihydrocarveol	3.0	24	2.8	20	3.4	20	4.0	40
isopulegol	1.9	-	0.8	-	2.9	-	1.7	-

E. coli	CS:CD-35G-75RH		CS:CD-50G-65RH		CS:CD-35PG-90RH		PVOH:CD-35G-84RH	
	Amount (mg)	Zone of inhibition (mm)	Amount (mg)	Zone of inhibition (mm)	Amount (mg)	Zone of inhibition (mm)	Amount (mg)	Zone of inhibition (mm)
m-cumenol	282.9	>85	297.1	>85	244.0	>85	206.7	>85
o-cumenol	272.2	>85	211.3	>85	193.2	>85	182.8	>85
carvacrol	120.2	>85	208.3	>85	219.6	>85	152.0	>85
guaiacol	136.0	64	144.2	81	114.6	72	190.1	77
isoeugenol	20.6	50	27.5	65	25.4	63	17.9	51
myrtenol	6.3	40	3.6	20	3.6	20	3.2	8
nerol	5.4	37	3.8	24	2.9	8	3.0	25
carveol	4.7	22	4.4	29	0.5	-	3.4	28
dihydrocarveol	5.0	24	3.2	17	3.9	20	3.2	36
isopulegol	1.8	-	0.8	-	2.9	-	1.2	-

volatile liquid in the agar medium. The antimicrobial activity of the films observed against *S. aureus* and *E. coli* was similar.

The films with cumenols and carvacrol produced complete inhibition of bacterial growth against both microorganisms. Films loaded with guaiacol produced almost complete microbial inhibition; in spite of its low antimicrobial activity, this result is in accordance with the greater amount of compound sorbed compared with isoeugenol and non-phenolic monoterpenes. Isoeugenol produced inhibition zones covering about 60–80% of the surface of the Petri dish. Although this compound presented antimicrobial capacity similar to nerol, carveol, dihydrocarveol and myrtenol, it was sorbed in greater amounts than them. The antimicrobial activity of isopulegol was lower than that of all the other compounds studied except guaiacol, and it had the lowest sorption values of all the films tested. Consequently, films with isopulegol did not show antimicrobial activity; a higher amount of compound in the films would be needed to produce an inhibitory effect, and in the present case the isopulegol content in the films was less than 8 mg, the amount of compound required to produce an inhibition effect (**Table 4.4.13**). Except for isopulegol, the amount of antimicrobial in the films was in all cases greater than the amount required to cause a microbial inhibitory effect in vapour phase. Apparently, the antimicrobial activity of the films with myrtenol, nerol, dihydrocarveol and carveol was similar, owing to the similar sorption values and similar antimicrobial activity of these compounds, although slight differences were found. For example, CS:CD-50G films with 3.9 mg of myrtenol produced an inhibition zone of 10 mm, while the film with 3.8 mg of nerol produced an inhibition zone of 30 mm, the minimal dose necessary to produce inhibition being 3.2 mg for myrtenol and 2.4 mg for nerol.

4. Conclusions

The present study shows that HP-βCD together with low molecular weight plasticizers G and PG and moisture are capable of regulating the sorption capacity of hydrophilic CS films for various monoterpene alcohols. The sorption affinity for monoterpene phenolic compounds was dramatically higher than that for compounds possessing a cyclohexanol structure or linear alcohols. This was associated with the greater polarity of the benzene

ring and its affinity for CS polar groups. It was also found that sorption properties of positional isomers differed slightly, which might be related to phenolic or cyclohexanol substituents impeding interactions between the hydroxyl group and polar groups in CS. Increasing the affinity of the plasticizer for monoterpene alcohols did not increase sorption properties. On the contrary, sorption values were lower for phenolic compounds. This indicated that the plasticizer plays a more important role in swelling the polymer matrix than in increasing film affinity for sorbates. When PVOH was employed as the polymer matrix the sorption behaviour of the films for monoterpenes was similar, but the sorption values achieved for phenolic compounds were lower than those of CS:CD films. In spite of its less polar nature, the less pronounced plasticizing effect of G on PVOH and its crystallinity might be responsible for these findings. All the monoterpene alcohols presented antimicrobial activity against the bacteria assayed, whereas the activity of the loaded films was directly related to the volatile content.

The films that were developed could be used for various purposes, including acting as carriers and systems for release of naturally occurring compounds for active packaging applications.

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5. DISCUSIÓN GENERAL

La presente Tesis Doctoral ha tenido como objetivo principal el desarrollo y caracterización de películas de CS en las que se incorporaron agentes antimicrobianos para su aplicación en el envasado activo de alimentos. Esta memoria ha sido estructurada en cuatro capítulos, correspondiendo cada uno de ellos a diferentes mecanismos de incorporación y liberación de agentes antimicrobianos de diversa naturaleza en una matriz de CS. Todo ello, se muestra resumido en la **Tabla 5.1**.

La matriz seleccionada para el desarrollo de los sistemas de envasado antimicrobiano fue el CS, debido a sus diversas y ventajosas propiedades. El CS se caracteriza por su elevada disponibilidad, y por ser biodegradable y comestible. A causa de su estructura policatiónica, el CS presenta capacidad antimicrobiana. La presencia de grupos funcionales reactivos, hacen del CS un polímero muy versátil y funcional. Asimismo, tiene capacidad de formar películas e hidrogeles, por lo que el CS puede ser empleado como matriz biopolimérica para el envasado activo de alimentos donde sea preciso la incorporación y liberación controlada de sustancias activas desde su seno.

Tabla 5.1. Películas antimicrobianas de CS.

Sistema	Modo de incorporación del agente antimicrobiano	Estado del agente antimicrobiano en la matriz de CS	Mecanismo de acción del sistema	Aplicación
CS/LAE	Sólido, incorporación a la disolución filmógena de CS	Libre en la matriz	Migración al medio líquido o sólido	Pechuga de pollo
CS/nanopartículas de plata	Precusor sólido, incorporación a la disolución filmógena de CS	Creación *in situ* de las nanopartículas. Libre en la matriz	Migración al medio líquido	No aplicada en alimentos
CS/cinamaldehído	Líquido, anclaje mediante la formación de una base de Shiff a la película de CS	Anclado a la matriz	Reversión del enlace covalente en presencia de agua y bajo temperatura. Migración al medio líquido	Leche pasteurizada
CS/carvacrol	Líquido, incorporación a la película compuesta de CS mediante inmersión	Libre en la matriz, acomplejado con CD o agregados de CD	Migración al espacio de cabeza	Pechuga de pollo

De entre las diferentes formas de incorporar el agente activo en la matriz polimérica, la más común es durante la formación de la película. Este método es muy útil cuando el agente activo no es volátil ni termolábil, de forma que durante la etapa de secado no hay pérdidas. Por otro lado, la película ha de estar en contacto directo con el alimento envasado para que el agente pueda ser liberado efectivamente. Siguiendo este mecanismo de acción, se desarrollaron películas de CS con LAE. Las películas con el agente activo se caracterizaron por ser flexibles y transparentes, y sin cambios visibles de color. Debido a la capacidad del CS de formar hidrogeles y a la presencia del G, las películas obtenidas se hincharon en presencia de agua y liberaron el LAE. Cabe destacar que el carácter catiónico, tanto del CS cuando se encuentra protonado, como del LAE, permitió la liberación del agente tensioactivo, ya que no se produjeron interacciones electrostáticas ni formación de complejos. Esta liberación se vio incrementada con el aumento de temperatura y menores concentraciones de LAE. Además, la liberación del LAE pudo extenderse 10 h a 28 ℃ y 15 h a 4 ℃ hasta el agotamiento de la película, a lo cual influyó directamente el elevado tamaño de la molécula de LAE.

Asimismo, se probó la eficacia antimicrobiana de las películas de CS con 5% LAE para el envasado activo de pollo fresco. En este caso, se evidenció la capacidad antimicrobiana inherente del polímero, ya que las películas de CS no fueron neutralizadas, siendo de esperar que parte de sus grupos amino se mantuvieran protonados después del procesado de la película. La actividad antimicrobiana se vio incrementada significativamente por la presencia de LAE en la matriz biopolimérica. El recuento de bacterias aerobias mesófilas se utiliza como índice de la calidad higiénica de la carne, con niveles aceptables entre $5x10^5$ y $5x10^6$ CFU de aerobios por gramo de carne separada mecánicamente (CE, 2005; Fàbregas y Toca, 1997). En las muestras control de pechugas de pollo analizadas, el recuento de bacterias mesófilas se encontró por debajo de este nivel hasta el día de fecha de caducidad (sexto día) indicada por el proveedor, a partir de la cual ya se sobrepasó. Por su parte, las muestras envasadas con las películas de CS efectuaron una ligera inhibición del recuento microbiano total. Sin embargo, las muestras envasadas con las películas con CS y LAE mantuvieron estos niveles por debajo de 10^4 incluso tras 8 días desde la recepción de la materia prima en el comercio, alargando así la vida útil del producto. Por otra parte, los principales microorganismos causantes de alteración pertenecen al género *Pseudomonas*, siendo las pseudomonas psicrótrofas la flora predominante sobre su superficie en canales de aves almacenadas a baja temperatura. Cuando su número llega a 10^8-10^9 UFC/g de carne, aparecen

los olores desagradables, y cuando sobrepasan de 10^9 UFC/g se forma el limo (Fàbregas y Toca, 1997; ICMSF, 2005). Las muestras de pollo control alcanzaron estos niveles el día indicado como la fecha de caducidad. Mientras que las muestras de pollo envasadas con películas de CS produjeron una cierta reducción del recuento de bacterias psicrótrofas y *Pseudomonas*, disminuyéndolo por debajo de 10^7; cuando las películas incorporaron LAE esta disminución fue mucho más significativa ($<10^4$). Asimismo, las Enterobacteriaceae, coliformes y *E. coli* valoran el grado de contaminación de origen fecal (Fàbregas y Toca, 1997), estableciéndose legislativamente niveles entre 50-500 CFU de *E. coli* por gramo de carne separada mecánicamente (CE, 2005). Se observa que las muestras de pollo en el día de recepción en el comercio no sobrepasaron estos niveles, tal como se exige en la legislación. La acción de las películas de CS mantuvo los recuentos de coliformes en niveles aceptables, y las películas de CS con LAE los disminuyeron por debajo de los esos niveles. Por otra parte, la película de PE de baja densidad con la que se envolvió externamente, entorpecería la entrada de oxígeno, lo que podría justificar el predominio de *Pseudomonas*. En envasados más impermeables, se acumula CO_2 inhibiendo el crecimiento de *Pseudomonas* y desplazando el crecimiento mayoritario hacia una carga microbiana menos oxígeno-dependiente como las bacterias ácido-lácticas y las sulfuro-productoras, predominando *Shewanella putrefaciens* entre éstas últimas (ICMSF, 2005). Las películas probadas redujeron los niveles tanto de las bacterias ácido-lácticas como de las sulfuro-productoras, por lo que este sistema de envasado podría también aplicarse en sistemas de envasado al vacío o con materiales menos permeables. En el caso de hongos y levaduras, los datos obtenidos no son concluyentes.

Estos resultados demuestran que las películas de CS con LAE pueden emplearse potencialmente en el diseño del envasado activo antimicrobiano de pollo. Además, probablemente pueda ser empleado para otros alimentos, ya que como se expone en el estado de la cuestión del **capítulo 1**, el LAE ya ha sido satisfactoriamente aplicado frente a diversos microorganismos y en distintos grupos de alimentos puesto que se trata de un aditivo de baja toxicidad y que no altera las propiedades organolépticas del producto en el que se aplica.

El siguiente sistema antimicrobiano desarrollado consistió en la formación de películas de CS con nanopartículas de plata. En este caso, se empleó una sal inorgánica de plata, nitrato de plata, como agente precursor de las nanopartículas de plata. Esta sal se incorporó en la solución formadora de las películas, y las nanopartículas se obtuvieron durante la neutralización

de las películas de CS con hidróxido de sodio. La matriz de CS actuó como agente quelante de los iones plata de la sal, como agente reductor débil en condiciones alcalinas, estabilizante, soporte y portador de las nanopartículas formadas y generó la liberación sostenida en el tiempo de los iones plata que se formaron en la superficie de las nanopartículas tras su oxidación. La formación de las nanopartículas en la matriz de CS permitió una liberación más lenta de los iones plata. Cabe resaltar que el hidróxido de sodio empleado como agente reductor es el habitualmente utilizado en este proceso de neutralización de las películas de CS, en el que se aumenta el pH del medio por encima del pK_a del CS y se estabilizan las películas en medios acuosos. Por lo que, se evita recurrir a otros reductores más tóxicos empleados comúnmente en la síntesis de nanopartículas. Esta cuestión, entre otras expuestas en el estado de la cuestión del **capítulo 2**, hace que esta síntesis *in situ* de nanopartículas de plata se enmarque en la denominada Química Sostenible.

La incorporación de nitrato de plata produjo películas de CS con diferente color, tras la neutralización. Éstas presentaron una mayor coloración marrón-rojiza con el incremento de la concentración de plata incluida en las películas y de la temperatura de neutralización de las mismas. De los resultados obtenidos, se deduce que la temperatura de neutralización de la película es un factor importante. De las temperaturas de neutralización probadas (22 y 37 ℃), se seleccionó la mayor temperatura ya que se observó una mayor conversión del precursor (nitrato de plata) a nanopartículas de plata, pasando a ser estas películas neutralizadas a 37 ℃ el objeto principal del estudio.

Mediante TEM, espectroscopía UV-visible y difracción de rayos X, se evidenció la formación de nanopartículas de plata en las películas de CS neutralizadas a 37 ℃. Mientras, que el TGA indicó que las películas de CS con nanopartículas de plata neutralizadas a 37 ℃ son más termoestables que las películas de CS.

Otro factor a tener en cuenta, es la concentración de plata en las películas. Se observó, mediante TEM y espectroscopía UV-visible, que las películas de CS neutralizadas a 37 ℃ con menor concentración de plata añadida, presentaron partículas esféricas, de ≤ 5 nm y homogéneamente distribuidas en el matriz polimérica. A mayores concentraciones, se observaron dos poblaciones de partículas de distinto diámetro. Una población se caracterizó por ser esférica con diámetro de 5-10 nm, mientras que otra fue algo más irregular con diámetro de 30-50 nm.

Por otra parte, se probó la capacidad antimicrobiana de las películas de CS con diferentes cantidades de plata y neutralizadas a 37 ℃ en medio

de cultivo líquido diluido y sin diluir. Puesto que las películas de CS fueron neutralizadas y sus grupos amino desprotonados, éstas perdieron su carácter antimicrobiano. Por tanto, en este caso, la matriz de CS no contribuye a la capacidad antimicrobiana, actuando únicamente como matriz. La actividad antimicrobiana de las películas con nanopartículas de plata es debida a la acción del agente activo incorporado. Se observa que todas las películas probadas presentaron la misma capacidad antimicrobiana, independientemente de la concentración de plata que incorporasen, excepto las de menor concentración en medio de cultivo sin diluir, ya que se probó que las cantidades de plata liberadas por las películas ensayadas al medio de contacto fueron iguales. Probablemente, la plata migrada se encuentre como iones de plata, ya que la difusión de las nanopartículas de plata a través de la matriz es difícil debido a su confinamiento en el polímero. Tal vez, se generen iones de plata en la superficie de las nanopartículas. Esto se vería favorecido por el ambiente acuoso en el que las películas se encuentran inmersas. Además, el hinchamiento de las películas de CS, debido a su capacidad de formar hidrogeles en presencia de agua, favorecería la difusión de estos iones de plata a través del polímero hacia el medio de liberación.

Otro factor que se debe considerar es el medio de cultivo en el que se liberan los iones plata y ejercen su acción antibacteriana. La mayor capacidad antimicrobiana de las películas se dio en medios de cultivo diluidos, donde existe menor presencia de agentes quelantes, como las proteínas y, por tanto, una mayor disposición de iones de plata libre. También se puede pensar que en medios con escasos nutrientes y desfavorables aumenta la susceptibilidad de los microorganismos a los agentes antimicrobianos. Y todo ello debe tenerse en cuenta a la hora de aplicar estas matrices.

Parte de las películas de CS con nanopartículas de plata neutralizadas a 37 ºC se autoclavaron previamente al ensayo de la capacidad antimicrobiana para determinar si este tratamiento hidrotérmico producía más nanopartículas de plata a partir de los iones de plata remanentes en la matriz de CS. No obstante, la capacidad antimicrobiana de las películas mostrada no varió tras el autoclavado. Esto puede deberse bien a que el rendimiento de la reacción durante la neutralización fue muy elevado no quedando iones de plata libre, o bien que el resto de los iones de plata no reaccionados se hubieran eliminado con el lavado con agua destilada tras la neutralización.

Asimismo, se realizaron ensayos de actividad antimicrobiana a lo largo del tiempo. Con ellos, se demuestra que las películas de CS con 0.2% de plata neutralizadas mantuvieron la misma capacidad antimicrobiana, al menos los 15 días que se mantuvieron inmersas en el medio acuoso. Así, la

capacidad antimicrobiana fue independiente del tiempo de inmersión de las películas, aunque la cantidad de plata migrada al medio acuoso se incrementó con este tiempo, cuando se superaron las 10 h de inmersión. Esto puede explicarse ya que la técnica empleada para determinar la liberación de plata (GFAAS) cuantifica globalmente plata iónica y elemental. Así que es posible que la cantidad de plata iónica disponible en el medio sea baja por la reducción de ésta o por su interacción con las proteínas del medio. Por otro lado, se observa que estas películas originan una concentración de plata total migrada durante tiempos cortos (<10 h) que se encuentra alrededor de 0.06 mg/L, valores muy próximos a los límites de migración máxima de plata (0.05 mg/L en agua) y con capacidad antimicrobiana elevada, superior a 3 reducciones logarítmicas. Por lo que, estas películas nanocompuestas podrían ser potencialmente empleadas para el envasado activo de alimentos.

En un segundo estudio, las películas fueron inmersas en un medio acuoso, renovado cada 24 h. Cada día del mes en que se llevó a cabo el ensayo, las películas se extrajeron del medio líquido, transfiriéndose a otro medio para evitar la posible saturación del medio. Se determinó la capacidad antimicrobiana tanto del medio retirado como de esa película tras los 30 días del experimento. Los medios de cultivo presentaron la misma actividad antimicrobiana, 3.5 reducciones logarítmicas, independientemente del día de ensayo. Por otra parte, las películas, presentaron mayor actividad que los medios de cultivos en los que estuvieron inmersas. Por lo que se deduce que las películas actuaron, en parte, mediante la liberación del agente activo y en parte por contacto de las películas con los microorganismos. Y también se prueba que las películas son capaces de liberar iones de plata tras su inmersión en medios acuosos y mantener su efectividad a lo largo del tiempo.

Para verificar esto, en el tercer estudio, se opera de modo similar al anterior con volúmenes de medio fresco elevados, y las películas se probaron a los 30 días. En las películas con baja concentración de plata, la actividad se mantuvo tras los 30 días, mientras que en las de mayor concentración presentaron mayor actividad tras los 30 días. Así se corrobora que, las películas de CS con plata son efectivas a lo largo del tiempo y las películas con mayores cantidades de plata podrían actuar como matrices de liberación sostenida para las nanopartículas de plata siendo grandes reservorios de iones plata.

El tercer mecanismo para incorporar el agente antimicrobiano en la matriz de CS que se empleó fue la inmovilización covalente y reversible del

agente antimicrobiano, de forma que el agente activo es liberado con un determinado estímulo. Esta forma de incorporar el agente activo se llevó a cabo mediante la formación de una base de Schiff entre un aldehído volátil de origen natural con propiedades antimicrobianas, el cinamaldehído, y el CS. Este anclaje covalente se realizó sobre el CS previamente procesado en forma de película. El enlace covalente consistió en la adición nucleofílica del grupo amino del CS, al grupo carbonilo del cinamaldehído. El enlace imino formado puede revertirse para liberar el cinamaldehído en presencia de agua y promovido por la temperatura, pH del medio o presencia de luz. Sin estos estímulos de activación, estas películas fueron estables, evitando la pérdida del volátil durante el almacenamiento de la película hasta su uso. El cinamaldehído permaneció anclado a la matriz de CS, ya que en un ambiente seco la reversibilidad de la base de Schiff es muy baja. A pesar del intenso aroma que caracteriza al cinamaldehído, las películas mantenidas en estas condiciones apenas olían.

Los resultados del análisis elemental y la ATR-FTIR muestran que el rendimiento de reacción del cinamaldehído con las películas de CS fue elevado (>70%), tal como se esperaba según se señala en el estado de la cuestión del **capítulo 3**, cuando la reacción se lleva a cabo entre el aldehído y el CS en forma de película y no como disolución formadora de película.

Las películas de CS tras reaccionar con el cinamaldehído adquirieron un color amarillo, debido a la formación de la base de Schiff insaturada, constituyendo el cromóforo −C=C−C=N−. A continuación, estas películas se sometieron a diferentes combinaciones de temperatura y tiempo características de diferentes tratamientos para la conservación de alimentos. Estos se realizaron en medio acuoso para promover la hidrólisis de la base de Schiff formada. Las películas sometidas a tratamientos más suaves no sufrieron cambios, mientras que a mayores temperaturas, se tornaron naranja rojizo, puede que debido al desarrollo de productos de la reacción de Maillard formados entre los grupos amino del CS y los grupos carbonilo a elevadas temperaturas.

El grado de sustitución, y por tanto el contenido en cinamaldehído en la película, fue disminuyendo conforme aumentó la temperatura de los tratamientos aplicados a las películas, lo que indica una mayor hidrólisis de la base de Schiff. Sin embargo, cabe resaltar que, incluso con temperaturas de esterilización, se mantuvo más del 50% de los enlaces imino en las películas, lo que indica un gran reservorio de cinamaldehído.

Por otra parte, se realizaron estudios de liberación en un medio de cultivo líquido durante el tratamiento de las películas con diferentes combinaciones de temperatura/tiempo y se estudió la actividad

antimicrobiana de la película, tras cada tratamiento. Los resultados mostraron que las películas mantenidas a bajas temperaturas, como las temperaturas habituales de refrigeración, fueron muy estables con una lenta liberación del agente activo y redujeron el crecimiento microbiano aproximadamente 1 log. Las películas bajo condiciones de temperaturas suaves y mantenidas, como la pasteurización suave o el llenado del envase en caliente, presentaron una liberación suficiente y mantenida para reducir el crecimiento microbiano en 5 log., según el microorganismo testado. Las películas activadas a elevadas temperaturas y tiempos cortos mostraron un efecto bactericida.

Se realizaron estudios de migración específica de cinamaldehído en etanol al 50%, simulante de alimentos grasos, tras los distintos tratamientos de temperatura/tiempo, observándose una mayor liberación de cinamaldehído en 50% etanol que en el medio acuoso, debido probablemente a la mayor solubilidad del cinamaldehído en este medio hidroalcohólico. Por lo que, las películas desarrolladas podrían aplicarse en alimentos grasos o emulsiones, como la leche entera, entre otros. Por ello, se comprobó la efectividad antimicrobiana de las películas con cinamaldehído en leche entera pasteurizada refrigerada e inoculada con *Listeria monocytogenes*. En la matriz alimentaria, las películas con cinamaldehído sometidas a temperaturas de refrigeración produjeron una reducción logarítmica de 1.34 a los 3 días, 0.81 a los 6 días y 0.52 a los 12 días. Las películas de CS con cinamaldehído tratadas con temperaturas suaves de 65 ℃ durante 30 minutos presentaron 2.40, 2.16 y 3.46 reducciones logarítmicas, a los mismos tiempos. Mientras que las películas desarrolladas sometidas a 95 ℃ durante 10 minutos mostraron 4.15, 3.41 y 3.87 reducciones logarítmicas, respectivamente. Las películas no se pudieron probar a 121 ℃ durante 5 minutos, ya que la leche coaguló, debido probablemente por la acción coagulante y floculante del CS o bien por la unión del cinamaldehído a las proteínas lácteas. Estas diferencias entre la actividad antimicrobiana en las pruebas *in vitro* y cuando se aplica al alimento, pueden deberse al efecto protector de la matriz alimentaria sobre el microorganismo, la unión del cinamaldehído a las proteínas o la dilución de agente activo en la matriz alimentaria.

Este sistema presenta una gran ventaja y es que la liberación se produciría al aplicar el tratamiento térmico al alimento o durante el llenado en caliente del envase con el alimento. De existir una contaminación en ese momento, ésta coincidiría con la liberación del agente activo antimicrobiano. Así, al inicio del crecimiento del microorganismo en el alimento, el cinamaldehído ya se habría liberado en el medio, en parte, de manera que

podría alargar la fase *lag* de los microorganismos. Posteriormente, la liberación mantenida del cinamaldehído desde las películas coincidiría con la fase exponencial de aquellos microorganismos que superen la fase anterior. Ambas fases del crecimiento microbiano son más susceptibles a los agentes químicos que la fase estacionaria, por lo que este sistema resulta muy conveniente.

Todos estos resultados ponen de manifiesto que las películas desarrolladas pueden aumentar la seguridad de alimentos susceptibles de contaminarse con *Listeria monocytogenes*. Asimismo, es de esperar que las películas con cinamaldehído sean efectivas para otros microorganismos, por lo que se podría aumentar la vida útil de la leche pasteurizada, que se encuentra limitada a 3 días bajo refrigeración.

El inconveniente de emplear aceites esenciales o sus componentes activos como agentes antimicrobianos, es el posible impacto en las propiedades organolépticas de los alimentos en los que se aplican por lo que se llevó a cabo un análisis sensorial de la leche tras el contacto con las películas de CS durante 3, 6 y 12 días de almacenamiento en condiciones de refrigeración. El estudio se realizó con catadores no entrenados que percibieron como olor a canela el cinamaldehído migrado de las películas en las muestras de leche. Los resultados también demostraron una mayor aceptación de las muestras con cinamaldehído que las muestras control sin cinamaldehído.

Por último, se desarrollaron películas de CS introduciendo en su formulación CD y plastificantes con el objetivo de incrementar su capacidad para retener carvacrol. La incorporación de carvacrol se realizó una vez obtenida la película mediante su inmersión en el líquido volátil, evitando de esta forma las pérdidas de agente durante el secado. Seguidamente se estudió la capacidad para liberar el compuesto retenido y el efecto de la RH ambiental en la cinética de liberación. Previamente a estos estudios de sorción y liberación, se estudió el efecto de incorporar CD en las propiedades funcionales de las películas de CS. Este estudio se extendió a otros monoterpenos fenólicos y no fenólicos con el objetivo de estudiar el efecto de la estructura química del compuesto en la capacidad de sorción.

La incorporación de CD en la matriz de CS redujo la elongación a la rotura de las películas mientras que su resistencia a la rotura no se vio afectada. Por otro lado, la permeabilidad al vapor de agua disminuyó y la permeabilidad al oxígeno experimentó un incremento. En el estudio morfológico, mediante SEM, no se observó separación de fases, pero el TGA sí mostró dos temperaturas de degradación correspondientes al CS y a las

CD, ambas temperaturas sufrieron una alteración en varios grados respecto a las correspondientes a los materiales originales revelando cierta interacción entre ambos. Por su parte, como era de esperar, la presencia de G y agua en las películas de CS aumentó los valores de permeabilidad al vapor de agua y al oxígeno, y de elongación; mientras que disminuyó su resistencia a la rotura.

La incorporación de CD incrementó ligeramente la sorción de carvacrol. Sin embargo, cuando la película, además de incorporar CD, se plastificó con G y agua se produjo un elevado incremento en la sorción. Las películas que presentaron una mayor capacidad de sorción fueron las plastificadas con 20 ó 35% de G y acondicionadas a 75% de RH, y aquellas con un 35% G acondicionadas a 50% RH, con valores de 57, 216, 133% (g/100 g de película seca), respectivamente. Mientras que todas las películas de CS sin CD retienen menos del 1% de carvacrol, independientemente del G y del agua que contengan. Teóricamente, el contenido de carvacrol en las películas con CD puede ser entre 5-10%, asumiendo la formación de complejos de inclusión 1:1 o 1:2. Puesto que diversas películas presentaron valores de sorción mayores al 10% de carvacrol, se deduce que se estarían produciendo otros mecanismos diferentes a la formación de complejos de inclusión como la formación de complejos supramoleculares de ciclodextrinas. El agua y el G incrementarían la movilidad de las cadenas poliméricas y, por tanto, el volumen libre de las películas, permitiendo la autoagregación de las ciclodextrinas. Esta disposición de las ciclodextrinas podría aumentar más el volumen libre de la matriz, sin llegar a desestructurar las películas.

Asimismo, la geometría molecular y la cantidad de dextrinas presentes en las películas resultaron fundamentales en la capacidad de sorción de las películas. Por una parte, cuando se incluyeron maltodextrinas en lugar de ciclodextrinas, la sorción de carvacrol por las películas no superó el 1.5%. Por otra, la sorción de carvacrol fue mayor cuanto mayor fue el contenido de CD. Sin embargo, cuando la proporción de CD superó a la de CS, las películas fueron demasiado quebradizas y difíciles de manejar. Por lo que la proporción de CS y CD óptima se estableció en 1:1.

Por otra parte, para determinar el efecto de la estructura química de los agentes naturales volátiles en la capacidad de sorción de las matrices, se seleccionó la película que mostró mayor capacidad de sorción de carvacrol, CS:CD 1:1 (en peso) plastificada con 35% de G y acondicionada a 75% de RH. En estas condiciones, las películas compuestas incorporaron alrededor de un 35% de agua. A continuación, las películas fueron inmersas en otros monoterpenos con actividad antimicrobiana, como ya se hizo con el

carvacrol. Los monoterpenos presentes en AE probados fueron monoterpenos acíclicos, cíclicos y bicíclicos (mirtenol, nerol, carveol, dehidrocarveol e isopulegol), y monoterpenos fenólicos (*m*-cumenol, *o*-cumenol, carvacrol, guayacol, isoeugenol). En general, la sorción de monoterpenos fenólicos (>12%) fue superior a la sorción del resto de monoterpenos (1-4%) por parte de las películas. Esta mayor afinidad de los compuestos fenólicos puede deberse a las interacciones de los dobles enlaces del anillo de benceno con los grupos polares de la matriz de CS, que no se presentan en las estructuras con ciclohexano o acíclicas. Los grupos alquilo en el anillo de fenol del carvacrol e isoeugenol, pudieron aumentar la hidrofobicidad respecto a los cumeoles, por lo que estos últimos tendrían mayor afinidad por la matriz hidrófila de CS y justifica su mayor sorción. En el caso del isoeugenol, la menor capacidad de retención de las películas puede deberse, además, al impedimento estérico producido por el grupo propileno. Por su parte, el guayacol, aunque presente la mayor polaridad, no produjo la mayor capacidad de sorción de los volátiles. Esto probablemente puede atribuirse a la formación de enlaces de hidrógeno intramoleculares en la molécula de guayacol que podrían competir con la formación de los puentes de hidrógeno entre la matriz y el sorbato, disminuyendo la sorción de éste.

De igual forma, se probaron las películas de CS con CD con un 50% de G, que tuvieron que ser acondicionadas al 65% de RH para que el contenido en humedad fuera en torno al 31%. Un contenido similar de agua e igual de CD en las películas, permitió determinar la influencia de la cantidad de G en el sistema. Las películas de CS con CD y 50% de G también fueron capaces de retener elevada cantidad de volátiles naturales, aunque algo menos que las películas que incorporaron el 35% de G. Esto puede ser debido a que una mayor cantidad de G, un componente hidrófilo, en la matriz propicie un entorno más hidrófilo y menos compatible con los volátiles de carácter lipófilo.

Asimismo, se comprobó si el sistema desarrollado era factible con otros hidrogeles hidrofílicos y otros plastificantes. Para ello, se estudió la capacidad de sorción de los diferentes volátiles por parte de hidrogeles, como el CS o PVOH, con CD y con G o PG. Todos los sistemas fueron formulados y acondicionados a una determinada RH para que captaran 30-35% de agua. En este caso, un contenido similar de agua e igual de CD en las películas, permitió observar el efecto del tipo de matriz y plastificante en la capacidad de sorción de componentes presentes en AE.

Se escogió el PVOH por ser, al igual que el CS, un polímero no tóxico, biocompatible, biodegradable y con excelente capacidad filmógena. Además, se trata de un polímero sintético y comercializado, ya que está

aprobado por la FDA como aditivo alimentario indirecto para productos que están en contacto con alimentos. Debido a la semicristalinidad y elevada energía de cohesión del PVOH, fue necesario acondicionar las películas de PVOH con CD y 35% de G a 84% de RH para que incorporen un contenido del 30% de agua. La capacidad de sorción de las películas compuestas de PVOH fue menor que las películas compuestas de CS para todos los monoterpenos, llegando a ser incluso la mitad en el caso de volátiles con estructura fenólica.

Por su parte, el PG se caracteriza por ser algo más apolar que el G. Por ello, hubo que acondicionar las películas de CS con CD que incorporan 35% de PG en lugar de 35% de G, al 90% de RH en lugar de al 75% de RH. La sustitución del G por el PG en la formulación produjo una disminución en la capacidad de retención de casi todos los volátiles por parte las películas de CS con CD, a pesar de la mayor compatibilidad de los volátiles con el PG que la existente con el G. Por lo que parece ser que la plastificación de la matriz es un factor más preponderante que la compatibilidad entre los componentes del sistema. Así, un mayor grado de plastificación implica una mayor retención de volátiles, hasta alcanzar un nivel de plastificación por el plastificante y el agua que provoquen un entorno demasiado hidrófilo, como se veía en el caso de películas compuestas con el 50% de G, o que las películas pierdan su integridad física o maniobrabilidad, como en el caso de películas de CS con CD con 35% de G acondicionadas al 90% de RH.

La capacidad antibacteriana de las películas compuestas con los distintos volátiles dependió de la cantidad de agente activo retenido por las matrices y su posterior liberación, y de la capacidad antimicrobiana y volatilidad de los componentes presentes en el AE. La capacidad antimicrobiana *in vitro* de los monoterpenos probados en fase vapor fue: carvacrol > *o*-cumenol, *m*-cumenol > dehidrocarveol> nerol, carveol > isoeugenol > mirtenol > isopulegol > guayacol. Así, todas las matrices poliméricas con CD que incorporaron cumenoles y carvacrol produjeron una elevada inhibición bacteriana en las pruebas de capacidad antimicrobiana en fase vapor, debido a la mayor cantidad de agente antibacteriano en las películas como consecuencia de su elevada capacidad de sorción. En el caso del guayacol, la inhibición producida fue menor que en los casos anteriores, debido en parte a la menor cantidad retenida de este monoterpeno fenólico que no llega a alcanzar la cantidad mínima inhibitoria en fase vapor. Las películas con isoeugenol y los monoterpenos cíclicos y acíclicos (mirtenol, nerol, carveol y dehidrocarveol) presentaron una actividad antimicrobiana intermedia, dependiendo de la película de la que se trate, y por tanto de su capacidad de retención de los volátiles, y de la capacidad antimicrobiana del

compuesto activo. Mientras que todas las películas compuestas con isopulegol no presentaron capacidad antibacteriana, ya que la cantidad retenida en las matrices compuestas fue menor a la cantidad mínima inhibitoria en fase vapor.

De este modo, es posible adaptar la capacidad de sorción de las matrices en función de la matriz polimérica, de la cantidad y cualidad del oligosacárido, y grado de plastificación por parte del G y el agua. Así como también se puede seleccionar el agente volátil para las distintas aplicaciones. Con este sistema, se consigue una elevada incorporación y liberación de agentes antimicrobianos de carácter lipófilo en una matriz hidrófila.

De los estudios de liberación de carvacrol y de la capacidad antimicrobiana de las películas, se deduce que las películas desarrolladas pueden ser aplicadas en el diseño de envases activos para el control microbiano durante el tiempo de conservación de los alimentos. Las películas podrían formar parte de un sistema de envasado para alimentos con contenido en agua medio o alto. La humedad del espacio de cabeza proveniente del alimento, aseguraría una liberación adecuada del volátil desde la película, que ejercería su acción en fase vapor, por lo que no sería necesario el contacto directo de la película con el alimento.

Con este objetivo, las películas con mayor capacidad de sorción (CS:CD-35G-75RH) de carvacrol se integraron en un sistema de envasado para pechugas de pollo fresco fileteadas. Así, las muestras de pechuga de pollo se colocaron en el fondo de unas barquetas de PP/EVOH/PP, selladas con una tapa de aluminio autoadhesiva, con diferentes tamaños (0.24, 4.8 y 24 cm^2) de películas de CS con un contenido inicial de carvacrol de 230 ± 40 % adheridas en la cara interna de la tapa y conservadas a 4 ºC.

En este caso, las películas de CS no presentaron actividad antimicrobiana, puesto que no mantuvieron contacto directo con el alimento, y la efectividad de las películas de CS con carvacrol dependió de la cantidad de carvacrol aportada por las películas y del tiempo de almacenamiento en refrigeración. Para observar un efecto antimicrobiano significativo, al menos, se precisaron películas de tamaños de 4.8 cm^2 con tiempos de exposición largos. Mientras que las películas de 24 cm^2 produjeron efectos antimicrobianos significativos durante todo el periodo de conservación.

Se observa que los niveles de bacterias aerobias mesófilas se mantuvieron entre $5x10^5$ y $5x10^6$ CFU/g de carne en todos los casos en el día indicado como la fecha de caducidad por el proveedor. Pero únicamente los sistemas de envasado con películas de 4.8 y 24 cm^2 mantuvieron estos niveles hasta 3 días después de dicha fecha. En el caso de *Pseudomonas*, y

bacterias psicrófilas en general, todas las muestras ensayadas se caracterizaron por tener recuentos más bajos de los esperados. Ciertamente, las muestras ensayadas no presentaron, en ningún caso, olores desagradables ni limo como consecuencia del crecimiento microbiano. Debido a las propiedades de alta barrera del envase, el crecimiento de *Pseudomonas* pudo verse desfavorecido, en beneficio de otros microorganismos menos oxígeno-dependiente. Las películas de 4.8 y 24 cm^2 a los 6 y 9 días de exposición presentaron un intervalo de reducciones logarítmicas de bacterias psicrófilas y *Pseudomonas* de 0.62 a 1.16. Respecto a las Enterobacteriaceae, únicamente las películas de 24 cm^2 mantuvieron un recuento microbiano en torno a 3 log. Debido a la mayor resistencia de las bacterias ácido-lácticas a la acción antimicrobiana del carvacrol, la reducción de éstas fue menor en todos los casos. Solamente las películas de 24 cm^2, tras 9 días, presentaron reducciones logarítmicas mayores de 0.5. Por su parte, a causa de la mayor sensibilidad de las levaduras y hongos al carvacrol, las películas de CS con carvacrol de tamaño de 4.8 y 24 cm^2 presentaron 0.78 y 1.77 reducciones logarítmicas tras 9 días de envasado.

Globalmente, se obtuvo una actividad antimicrobiana menor a la esperada, ya que la cantidad de carvacrol presente en todas las películas de CS probadas excedía la cantidad mínima inhibitoria de carvacrol determinada en fase vapor en la pruebas *in vitro*. Una causa puede ser que la mayor parte del carvacrol de las películas acabó siendo sorbido por el pollo, disminuyendo la concentración de carvacrol en el espacio de cabeza por debajo de esta cantidad mínima inhibitoria. De manera que, la cantidad de carvacrol libre y disponible en la superficie del pollo pudo verse disminuida por la interacción del carvacrol con las proteínas del alimento y la difusión del carvacrol hacia el seno del alimento. Además, de la acción protectora que ofrecen las matrices alimentarias a los microorganismos.

Ello hace que se requieran cantidades elevadas de carvacrol y que el producto pueda ser inaceptable sensorialmente. Bitar *et al.* (2008) indicaron que el umbral de olor del carvacrol es de 31 mg/kg en aceite de girasol. Sin embargo, Scramlin *et al.* (2010) apuntaron que el aceite de orégano puede extender la vida útil de los productos a base de carne, pero tiende a desarrollar mal sabor, causando baja aceptabilidad. Mientras que Chi *et al.* (2006) comprobaron que la adición de 45 mg/kg o menos de AE de orégano en los derivados cárnicos, es aceptado por los consumidores. Por su parte, las muestras de pollo envasadas con las películas de CS con carvacrol de 0.24, 4.8 y 24 cm^2 contienen 200, 900 y 4500 mg/kg, respectivamente. Tras el horneado, gran parte del carvacrol fue liberado desde la matriz alimentaria, quedando de 8-12, 36-54 y 180-270 mg/kg, respectivamente. Estas muestras

tras 9 días de conservación, antes y después de su horneado, presentaron un aroma perceptible pero aceptable como "a orégano". No obstante, las muestras de pollo presentaron un gusto amargo, picante y con sensación de quemazón en la lengua, y una textura seca tras el horneado. Otros autores también comprobaron esta merma de la calidad sensorial al emplear AE de orégano en otros productos como en el pan (Passarinho *et al.*, 2014) o la uva (Dos Santos *et al.*, 2012).

A razón de todo ello, una alternativa para no tener que emplear cantidades elevadas de agente activo puede ser aplicar estos sistemas de envasado a otros alimentos con menor contenido en proteínas o en los que habitualmente se condimentan con especias. Gutiérrez *et al.* (2008) indicaron que 300 mg/kg de AE de orégano en zanahorias son organolépticamente aceptables. Muriel-Galet *et al.* (2012) demostraron que la ensalada mínimamente procesada envasada en PP/EVOH con 5% de AE de orégano y atmósfera modificada, además de presentar una mayor vida útil y seguridad, fue más aceptada sensorialmente que la ensalada envasada sin AE. Du *et al.* (2012) describieron que las pechugas de pollo recubiertas con películas de tomate con carvacrol tras su horneado a 204 ºC, 30 minutos fueron aceptadas organolépticamente; mientras que fueron rechazadas en el caso de recubrimientos con películas de manzana con carvacrol.

De manera resumida, la **Tabla 5.2** recoge las propiedades que presenta el CS en los distintos sistemas de envasado desarrollados en la presente Tesis Doctoral. En todos los sistemas, el CS resultó ser una excelente matriz para los agentes antimicrobianos, ya que tiene la capacidad de hincharse en presencia de agua, favoreciendo la difusión de pequeñas moléculas a su través. Por tanto, la humedad propia del alimento puede emplearse como estímulo para la liberación controlada de agentes activos a partir estos sistemas de envasado. Por el contrario, en algunos casos, esta misma propiedad supone un inconveniente. Las películas de CS se muestran muy sensibles a la humedad mermando algunas de sus propiedades, por lo que es necesaria su neutralización. Y la pérdida de sus cargas conlleva la pérdida de sus propiedades antimicrobianas. Por este motivo, en estos casos, se requiere la inclusión de sustancias bioactivas en la matriz biopolimérica para su aplicación en envases antimicrobianos. En la presente Tesis Doctoral, únicamente las películas con LAE no fueron neutralizadas y puestas en contacto con el alimento, por ello son las únicas que presentan capacidad antimicrobiana inherente. En el resto de sistemas desarrollados, los grupos amino del CS han sido neutralizados, por lo que la actividad antimicrobiana es atribuible a las sustancias antimicrobianas incorporadas.

Tabla 5.2. Propiedades del CS en cada uno de los sistemas de envasado antimicrobiano desarrollado.

Sistema	Propiedades del CS
CS/LAE	Soporte Antimicrobiano Matriz compatible, liberación controlada
CS/nanopartículas de plata	Soporte Quelante, estabilizante de nanopartículas Reductor, formador de nanopartículas Reservorio, liberación controlada
CS/cinamaldehído	Soporte Versatilidad, anclaje del volátil al grupo amino Liberación controlada, reversión de la base de Schiff frente a un estímulo
CS/carvacrol	Soporte Liberación controlada

También cabe resaltar otras funciones del CS, como la de reductor en condiciones alcalinas o quelante de iones, actuando como reductor débil y estabilizante en la formación de nanopartículas de plata, respectivamente. O bien, gracias a sus grupos amino reactivos, se pueden anclar moléculas volátiles como el cinamaldehído, estabilizándolas hasta su uso.

Así, este trabajo muestra la versatilidad y "multifuncionalidad" del CS, y la aplicabilidad de los envases activos antimicrobianos, que pueden ser diseñados a medida de las necesidades del alimento que protegen.

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6. CONCLUSIONES

From the results obtained in the development of the present Doctoral Thesis it is possible to highlight the following conclusions:

1. Chitosan films capable of incorporating and releasing various antimicrobial agents using different methodologies were obtained by the solvent-casting technique and developed for active food packaging applications.

2. Chitosan films with LAE were obtained by incorporating the antimicrobial agent into the film-forming solution. Release of LAE from the chitosan matrix in aqueous medium was completed in 10 to 15 h, depending on the temperature. The inherent antimicrobial activity of the chitosan film was significantly increased by the presence of 5% LAE, which satisfactorily reduced the microbial load present in fresh chicken. Therefore these films can be incorporated in the design of an antimicrobial packaging system to extend the shelf life of chicken breasts.

3. Chitosan films with silver nanoparticles were obtained using a novel method that complies with the principles of Green Chemistry. Silver nitrate was incorporated as a precursor to the film-forming solution and nanoparticles were generated *in situ* during the chitosan film neutralization step. The neutralization temperature and the concentration of silver in the film were crucial in determining the shape and size of the nanoparticles. The films showed antimicrobial activity and maintained their effectiveness, releasing silver ions over time. Silver nanoparticles synthesized in chitosan films behaved as nanoreservoirs and systems for sustained release of silver ions in liquid media.

4. The naturally occurring antimicrobial cinnamaldehyde was covalently attached to preformed chitosan films via reversible Schiff base. The reaction yield was quite high. Various temperature/time food preservation treatments in aqueous media successfully produced hydrolysis of the imine bond and cinnamaldehyde release. The films that were developed can be used to improve the microbiological safety of foodstuffs susceptible to contamination with *Listeria monocytogenes*, such as milk. Moreover, these products were well accepted by the sensory panel.

5. HP-β-cyclodextrin was incorporated in chitosan films to modify the capacity for sorption of the naturally occurring antimicrobial carvacrol.

Plasticization by glycerol and water was required to improve and tailor sorption properties of the films immersed in the volatile liquid agent. Loaded films released carvacrol activated by environmental relative humidity. The antimicrobial effectiveness of the carvacrol released by the films was shown in *in vitro* assays employing the microatmosphere test. The films were effective when incorporated in the prototype design of a package for fresh chicken breasts. The volatile exerted its antimicrobial action in vapour phase and without contact between the film and the food. However, because of its high compatibility, large amounts of volatile were retained in the chicken, causing unacceptable sensory deterioration.

6. Tailored amounts of lipophilic antimicrobial compounds were retained in hydrophilic polymer films of chitosan or polyvinyl alcohol, depending on the film formulation and chemical structure of the sorbate. The new films presented higher retention capacity when they were formulated with chitosan instead of PVOH, and employing glycerol as plasticizer rather than propylene glycol. Phenolic monoterpenes were sorbed in greater amounts than cyclohexyl and linear monoterpene alcohols. Films loaded with monoterpenes presented antimicrobial capacity in vapour phase and could be applied in the design of active food packages.

7. The results obtained demonstrate that chitosan can be a versatile, multifunctional matrix applicable to the development of antimicrobial active packaging.

7. ANEXOS

7.1. Anexo A. Ejemplos de materiales biopoliméricos para el envasado antimicrobiano de alimentos

Agente antimicrobiano	Biopolímero	Alimento	Microorganismo	Referencia
alcoholes				
etanol	HPMC	tomate	*S.* Montevideo	Zhuang *et al.* (1996)
etanol	quitosano	uvas sin semilla "Thompson", "Autumn"	*Botrytis cinerea*	Romanazzi *et al.* (2007)
etanol	shellac	naranja "Valencia"	*E. coli, Enterobacter aerogenes*	McGuire y Hagenmaier (2001)
agentes quelantes				
EDTA, nisina, lisozima** EDTA, ED semilla de pomelo	alginato de sodio K-carragenato	medio de cultivo	*Micrococcus luteus, L. innocua,* *S.* Enteritidis, *E. coli, St. aureus*	Cha *et al.* (2002)
EDTA EDTA, sorbato de potasio EDTA, ovotransferrina**	K-carragenato	medio de cultivo pechuga de pollo	*E. coli, S.* Typhimurium, *St. aureus, C. albicans* *E. coli,* ME (TVC)	Seol *et al.* (2009)
EDTA , nisina, lisozima**	gelatina	jamón cocido, mortadela	*Brochothrix thermosphacta, E. coli* O157:H7, *Lb. sakei, Leuconostoc mesenteroides, L. monocytogenes, S.* Typhimurium	Gill y Holley (2000)
EDTA	HPMC	medio de cultivo	*P. digitatum, P. italicum*	Valencia-Chamorro *et al.* (2008)
EDTA, nisina	PLA	medio de cultivo	*E. coli* O157:H7	Liu *et al.* (2010)
EDTA, nisina EDTA, lisozima**	proteína de soja zeína	medio de cultivo	*E. coli*	Padgett *et al.* (1998)
EDTA, ED semilla de uva EDTA, ED semilla de uva, nisina, EDTA, nisina	proteína de soja	medio de cultivo	*L. monocytogenes, E. coli* O157:H7, *S.* Typhimurium	Sivarooban *et al.* (2008)
EDTA, ác, málico, ED semilla de uva, nisina	proteína de suero de leche	salchichas de pavo	*L. monocytogenes, E. coli* O157:H7, *S.* Typhimurium	Gadang *et al.* (2008)
EDTA, lisozima** lactoferrina, lisozima**	quitosano	medio de cultivo	*E. coli* O157:H7, *L. monocytogenes*	Brown *et al.* (2008)
EDTA EDTA, ác. láurico EDTA, nisina	zeína	medio de cultivo	*S.* Enteriditis, *L. monocytogenes*	Hoffman *et al.* (2001)
EDTA, lisozima**	zeína	medio de cultivo	*L. plantarum, B. subtilis, E. coli*	Mecitoğlu *et al.* (2006)
EDTA, lisozima**	zeína, albúmina de garbanzos, albúmina de suero bovino	medio de cultivo	*B. subtilis, E. coli*	Mecitoğlu *et al.* (2007)
carbonato de potasio carbonato de amonio fosfato de amonio hidrogenocarbonato de potasio	HPMC	tomate "Cherry"	*Alternaria alternata, Botrytis cinerea*	Fagundes *et al.* (2013)
hidrogenocarbonato de sodio	HPMC	medio de cultivo	*P. digitatum, P. italicum*	Valencia-Chamorro *et al.* (2008)

ácidos orgánicos y derivados				
ác. acético ác. láctico	alginato de calcio	carne de vacuno magra	*L. monocytogenes, S.* Typhimurium, *E. coli* O157:H7	Siragusa y Dickson (1992, 1993)
ác. acético	agar-agar	dientes de ajo	ME (TVC mesófilos, hongos)	Geraldine *et al.* (2008)
ác. acético ác. acético, ác. láurico ác. acético, cinamaldehído ác. propiónico ác. propiónico, ác. láurico ác. propiónico, cinamaldehído	quitosano	jamón cocido mortadela pastrami	*Lb. sakei, Serratia liquefaciens,* ME (LAB, Enterobacteriaceae)	Ouattara *et al.* (2000)
ác. benzoico	gelatina	pescado "mojarra**"	ME (TVC y anaerobios)	Ou *et al.* (2002)
ác. benzoico	metilcelulosa	ciruela estilo taiwanés	*Zygosaccharomyces rouxii, Zygosaccharomyces mellis*	Chen *et al.* (1999)
ác. cítrico, nisina ác. málico, nisina ác. láctico, nisina	proteínas de suero de leche	medio de cultivo	*L. monocytogenes*	Pintado *et al.* (2009)
ác. cítrico ác. cítrico, nisina ác. láctico ác. láctico, nisina ác. málico ác. málico, nisina ác. tartárico ác. tartárico, nisina	proteínas de soja	medio de cultivo	*L. monocytogenes, E. coli* O157:H7, S. Gaminara	Eswaranandam *et al.* (2004)
ác. láctico, natamicina** ác. láctico, natamicina**, quitooligosacáridos	proteína de suero de leche	queso "Saloio "	ME (TVC mesófilos, *Lactococcus* spp., *Lactobacillus* spp., *Staphylococcus* spp., *Pseudomonas* spp., Enterobacteriaceae, hongos, levaduras)	Ramos, Pereira, *et al.* (2012)
ác. láctico ác. propiónico	proteína de suero de leche	medio de cultivo	*E. coli, St. aureus, Yarrowia lipolytica*	Ramos, Silva, *et al.* (2012)
ác. láctico, quitooligosacáridos	proteína de suero de leche	queso (modelo experimental)	ME (*Staphylococcus* spp., *Pseudomonas* spp., Enterobacteriaceae, hongos, levaduras)	Ramos, Santos, *et al.* (2012)
ác. láurico ác. láurico, nisina	proteína de soja	mortadela de pavo loncheada	*L. monocytogenes*	Dawson *et al.* (2002)
ác. láurico ác. láurico, EDTA ác. láurico, nisina	zeína	medio de cultivo	*S.* Enteriditis, *L. monocytogenes*	Hoffman *et al.* (2001)
ác. láurico ác. láurico, nisina	zeína	medio de cultivo	*Lb. plantarum*	Padgett *et al.* (2000)
ác. málico ác. málico, cinamaldehído ác. málico, citral ác. málico, eugenol	alginato de calcio	manzana "Fuji" troceada	*E. coli* O157:H7, ME (TVC mesófilos y psicrófilos, hongos, levaduras)	Raybaudi-Massilia, Rojas-Graü, *et al.* (2008)
ác. málico ác. málico, citral ác. málico, eugenol ác. málico, geraniol	alginato de calcio	melón "Piel de sapo" pelado	*S.* Enteritidis, ME (TVC mesófilos y psicrófilos, hongos, levaduras)	Raybaudi-Massilia, Mosqueda-Melgar, *et al.* (2008)
ác. málico ác. málico, nisina ác. málico, nisina, natamicina**	proteínas de suero de leche	medio de cultivo	*L. monocytogenes, Penicillium* spp., *Ps. aeruginosa, Yarrowia lipolytica*	Pintado *et al.* (2010)
ác. málico, EDTA, ED	proteína de suero de	salchichas de	*L. monocytogenes, E. coli*	Gadang *et al.*

	semilla de uva, nisina	leche	pavo	O157:H7, *S.* Typhimurium	(2008)
	ác. propiónico	quitosano	masa de hojaldre fresca	ME (hongos, levaduras)	Rivero *et al.* (2013)
	ác. sórbico	gluten de trigo zeína	maíz dulce	*L. monocytogenes*	Carlin *et al.* (2001)
	ác. sórbico PABA	proteína de suero de leche, cera candelilla	medio de cultivo mortadela salchicha	*L. monocytogenes,* *E. coli* O157:H7, *S.* Typhimurium ME (TVC, LAB, hongos, levaduras)	Cagri *et al.* (2003); Cagri *et al.* (2001, 2002)
	benzoato de sodio, nisina benzoato de sodio, sorbato de potasio, nisina diacetato de sodio, nisina lactato de sodio, nisina sorbato de potasio, nisina	alginato almidón κ-carrageenano pectina gelatina HPMC/metilcelulosa	salmón ahumado	*L. monocytogenes,* ME (TVC y anaerobios)	Neetoo y Mahomoodally (2014); Neetoo *et al.* (2010)
	benzoato de sodio sorbato de potasio	celulosa	manzana y patata cortadas	ME (TVC)	Baldwin *et al.* (1996)
	benzoato de sodio propionato de sodio sorbato de potasio	HPMC	medio de cultivo naranjas "Valencia" mandarina híbrida "Ortanique" mandarina clementina "Clemenules"	*P. digitatum, P. italicum*	Valencia-Chamorro *et al.* (2008, 2011); Valencia-Chamorro *et al.* (2009a, b, 2010)
	benzoato de sodio sorbato de potasio	metilcelulosa/quitosano	medio de cultivo	*P. notatum, Rhodotorula rubra*	Chen *et al.* (1996)
	diacetato de sodio diacetato de sodio, nisina	zeína	salchicha de pavo	*L. monocytogenes*	Lungu y Johnson (2005a)
	lactato de sodio	proteínas de suero de leche	carne de vacuno	ME (TVC, LAB, *Pseudomonas* spp.)	Zinoviadou *et al.* (2010)
	lactato de sodio sorbato de potasio	caseinato de sodio	medio de cultivo	*L. monocytogenes*	Kristo *et al.* (2008)
	propionato de sodio	celulosa acetato	pan en rebanadas	ME (hongos)	Soares *et al.* (2002)
	sorbato de potasio	alginato de calcio	patata	ME (TVC)	Mitrakas *et al.* (2008)
	sorbato de potasio	alginato de calcio	queso "mozzarella"	ME (TVC, LAB, lactococos, *Pseudomonas* spp., Enterobacteriaceae)	Mastromatteo *et al.* (2014)
	sorbato de potasio	almidón de boniato	medio de cultivo	*E. coli*	Shen *et al.* (2010)
	sorbato de potasio	almidón de guisante almidón de patata goma guar	manzana pepino tomate	*P. expansum, Cl. herbarum, A. niger* *P. oxalicum, Cl. cucumerinum* *P. expansum, Cl. fulvum*	Mehyar *et al.* (2011)
	sorbato de potasio	almidón de tapioca almidón de tapioca/goma xantana	medio de cultivo	*Zygosaccharomyces bailii*	Arismendi *et al.* (2013); Flores *et al.* (2007); Flores *et al.* (2010)
	sorbato de potasio	almidón de tapioca	calabaza en conserva	ME (TVC mesófilos, LAB, hongos, levaduras)	García *et al.* (2008)
	sorbato de potasio	celulosa y derivados almidón	fresa	ME (TVC mesófilos y psicrófilos, hongos, levaduras)	García *et al.* (1998, 2001)

sorbato de potasio	quitosano	medio de cultivo	*E. coli, St. aureus, S.* Typhimurium, *L. monocytogenes, B. cereus*	Pranoto, Rakshit, *et al.* (2005)
sorbato de potasio	quitosano	bizcocho pan de ajo	ME (hongos)	Sangsuwan *et al.* (2014); Sangsuwan *et al.* (2012)
sorbato de potasio	quitosano quitosano/almidón de tapioca	medio de cultivo salmón en rodajas	*Lactobacillus* spp., *Zygosaccharomyces bailii,* ME (TVC mesófilos y psicrófilos)	Vásconez *et al.* (2009)
sorbato de potasio	zeína	queso	*St. aureus*	Torres (1984)
metales				
óxido de cobre cobre (nano)	celulosa	zumo de piña zumo de melón "Piel de sapo" pelado	ME (hongos, levaduras)	Llorens *et al.* (2012)
cobre cobre (nano)	celulosa (quitosano)	medio de cultivo	*E. coli*	Mary *et al.* (2009)
cobre (nano)	PLA	medio de cultivo	*Ps. fluorescens, Ps. putida*	Longano *et al.* (2012)
cobre (nano)	quitosano	medio de cultivo	*St. aureus, S.* Typhimurium	Cárdenas *et al.* (2009)
cobre (nano)	quitosano	medio de cultivo	*E. coli, S.* Choleraesuis, *S.* Typhimurium, *St. aureus*	Qi *et al.* (2004)
dióxido de titanio (nano)	celulosa	medio de cultivo	*St. aureus*	Daoud *et al.* (2005)
dióxido de titanio (nano)	gelatina bovina	medio de cultivo	*St. aureus, E. coli*	Nassiri y Nafchi (2013)
dióxido de titanio (nano)	proteínas de suero de leche zeína	medio de cultivo	*E. coli*	Kadam *et al.* (2014)
dióxido de titanio (nano)	quitosano	medio de cultivo	*S.* Typhimurium, *E. coli, St. aureus*	Díaz-Visurraga *et al.* (2010)
plata (nano)	alginato de sodio	medio de cultivo	*St. aureus, E. coli*	Fayaz *et al.* (2009)
plata (nano)	celulosa	melón "Piel de sapo" pelado kiwi pechuga de pollo carne de vacuno	ME (TVC mesófilos y psicrófilos, LAB, *Pseudomonas* spp., Enterobacteriaceae, hongos, levaduras)	Fernández, Picouet, *et al.* (2010a, b); Fernández *et al.* (2009); Lloret *et al.* (2012)
plata (nano)	celulosa	medio de cultivo	*B. subtilis, St. aureus, Klebsiella pneumoniae*	Pinto *et al.* (2009)
plata (nano)	celulosa	medio de cultivo	*E. coli*	Tankhiwale y Bajpai (2009)
plata (nano)	celulosa/quitosano	medio de cultivo	*St. aureus, E. coli, B. cereus*	Yoksan y Chirachanchai (2009, 2010)
plata (nano)	celulosa acetato	medio de cultivo	*St. aureus, E. coli*	Chou *et al.* (2005)
plata (nano)	celulosa acetato	medio de cultivo	*St. aureus, E. coli, Klebsiella pneumoniae, Ps. aeruginosa*	Son *et al.* (2004); Son *et al.* (2006)
plata	PLA (zeolitas)	medio de cultivo	*St. aureus, E. coli*	Fernández, Soriano, *et al.* (2010)
plata (nano)	HPMC/PVOH	medio de cultivo	*St. aureus, E. coli*	De Moura *et al.* (2012)
plata (nano)	quitosano lactato	medio de cultivo	*E. coli*	Tankhiwale y Bajpai (2010)
plata plata (nano)	quitosano (zeolitas)	medio de cultivo	*St. aureus, L. monocytogenes, S.* Typhimurium, *E. coli* O157:H7	Rhim *et al.* (2006)

plata (nano)	quitosano	medio de cultivo	*E. coli*	Sanpui *et al.* (2008)
plata (nano)	quitosano	medio de cultivo	*E. coli, Bacillus, Klebsiella pneumoniae*	Vimala *et al.* (2010)
plata (nano), óxido de zinc (nano)	quitosano	medio de cultivo	*B. subtilis, E. coli, St. aureus, Penicillium* spp., *Aspergillus* spp., *Rhizopus* spp., levaduras	Li *et al.* (2010)
plata (nano)	quitosano/dióxido de titanio/celulosa	medio de cultivo	*St. aureus, E. coli*	Xiao *et al.* (2013)
plata plata (nano)	zeína	medio de cultivo	*St. aureus, E. coli*	Zhang *et al.* (2010)
zinc	quitosano	medio de cultivo	*E. coli, Ps. aeruginosa, Proteus mirabilis, S.* Enteritidis, *Enterobacter aerogenes, St. aureus, St. epidermidis, Corynebacterium* spp., *Enterococcus faecalis, C. albicans, C. parapsilosis*	Wang *et al.* (2004)
óxido de zinc (nano)	alginato de calcio	pollo RTE	*S.* Typhimurium, *St. aureus*	Akbar y Anal (2014)
óxido de zinc (nano)	celulosa acetato	agua	*E. coli*	Chaurasia *et al.* (2010)
óxido de zinc (nano) AE albahaca	proteína de pescado/gelatina de piel de pescado	medio de cultivo	*L. monocytogenes, Ps. aeruginosa*	Arfat *et al.* (2014)
péptidos antimicrobianos/bacteriocinas				
dermaseptin S4	almidón de maíz	pepino	ME (TVC, hongos)	Miltz *et al.* (2006)
enterocina	alginato zeína	medio de cultivo jamón cocido loncheado	*L. monocytogenes*	Marcos *et al.* (2007, 2010)
lactocinas	gluten de trigo	medio de cultivo salchicha vienesa	*L. innocua, Lb. plantarum*	Massani, Botana, *et al.* (2014); Massani, Molina, *et al.* (2014)
nisina	agar alginato de calcio	piel de pollo	*S.* Typhimurium	Natrajan y Sheldon (2000)
nisina	albúmina de huevo gluten de trigo proteínas de suero de leche proteínas de soja	medio de cultivo	*L. monocytogenes*	Ko *et al.* (2001)
nisina	alginato de calcio	carne de vacuno	*Brochothrix thermosphacta*	Cutter y Siragusa (1996, 1997)
nisina, lisozima**	alginato de calcio	salmón ahumado	*L. monocytogenes, S.* Anatum	Datta *et al.* (2008)
nisina, EDTA, lisozima**	alginato de sodio K-carragenato	medio de cultivo	*Micrococcus luteus, L. innocua, S.* Enteritidis, *E. coli, St. aureus*	Cha *et al.* (2002)
nisina	almidón de tapioca	medio de cultivo	*L. innocua*	Sanjurjo *et al.* (2006)
nisina, natamicina**	almidón de tapioca	queso "Port Salut"	*Saccharomyces cerevisiae, L. innocua*	Resa *et al.* (2014)
nisina	caseinato de sodio	medio de cultivo	*L. monocytogenes*	Kristo *et al.* (2008)
nisina	caseinato de sodio	queso "Mini Babybel®"	*L. innocua*	Cao-Hoang *et al.* (2010)
nisina	K-carragenano HPMC metilcelulosa quitosano	medio de cultivo	*Micrococcus luteus*	Cha *et al.* (2003)
nisina lacticina 3147	celulosa	jamón cocido, queso "Cheddar"	*L. innocua, St. aureus,* ME (TVC, LAB)	Scannell *et al.* (2000)

		loncheados		
nisina	celulosa bacteriana	salchichas de Frankfurt	*L. monocytogenes,* ME (TVC)	Nguyen *et al.* (2008)
nisina	galactomanano	queso "ricota"	*L. monocytogenes*	Martins *et al.* (2010)
nisina	gelatina	mortadela de pavo	*L. monocytogenes*	Min *et al.* (2010)
nisina, lisozima**, EDTA	gelatina	jamón cocido, mortadela	*Brochothrix thermosphacta, E. coli* O157:H7, *Lb. sakei, Leuconostoc mesenteroides, L. monocytogenes, S.* Typhimurium	Gill y Holley (2000)
nisina	gelatina zeína	medio de cultivo	*L. monocytogenes*	Ku y Bin Song (2007)
nisina	glucomanano de konjac quitosano	medio de cultivo	*E. coli, St. aureus, L. monocytogenes, B. cereus*	Li, Kennedy, *et al.* (2006); Li, Peng, *et al.* (2006)
nisina	gluten de trigo	mortadela de pavo	*L. monocytogenes*	McCormick *et al.* (2005)
nisina	HPMC	medio de cultivo	*L. innocua* *St. aureus*	Coma *et al.* (2001)
nisina	HPMC	medio de cultivo	*L. monocytogenes, L. innocua, St. aureus*	Sebti y Coma (2002); Sebti *et al.* (2002)
nisina	HPMC/metilcelulosa	salchichas de Frankfurt	*L. monocytogenes*	Franklin *et al.* (2004)
nisina nisina, benzoato de sodio nisina, benzoato de sodio, sorbato de potasio nisina, diacetato de sodio nisina, lactato de sodio nisina, sorbato de potasio	HPMC/metilcelulosa	salmón ahumado	*L. monocytogenes,* ME (TVC y anaerobios)	Neetoo y Mahomoodally (2014)
nisina	HPMC/quitosano	leche entera	*A. niger, Kocuria rhizophila*	Sebti *et al.* (2007)
nisina	metilcelulosa/LDPE	salchicha de Frankfurt	*L. monocytogenes*	Cooksey (2005)
nisina	pectina/PLA	medio de cultivo yema de huevo, zumo de naranja	*Lb. plantarum, L. monocytogenes*	Jin *et al.* (2009); Liu *et al.* (2007)
nisina	PLA	medio de cultivo, zumo de naranja, clara de huevo cruda, leche desnatada	*E. coli* O157:H7, *S.* Enteriditis, *L. monocytogenes*	Jin (2010); Jin y Zhang (2008)
nisina, EDTA	PLA	medio de cultivo	*E. coli* O157:H7	Liu *et al.* (2010)
nisina, ED semilla de uva nisina, ED semilla de uva, EDTA nisina, EDTA	proteína de soja	medio de cultivo	*L. monocytogenes, E. coli* O157:H7, *S.* Typhimurium	Sivarooban *et al.* (2008)
nisina, ác. cítrico nisina, ác. láctico nisina, ác. málico nisina, ác. tartárico	proteínas de soja	medio de cultivo	*L. monocytogenes, E. coli* O157:H7, S. Gaminara	Eswaranandam *et al.* (2004)

nisina nisina, EDTA	proteína de soja zeína	medio de cultivo	*Lb. plantarum* *E. coli*	Padgett *et al.* (1998)
nisina nisina, ác. láurico	proteínas de soja	mortadela de pavo loncheado	*L. monocytogenes*	Dawson *et al.* (2002)
nisina nisina, ED semilla de uva nisina, ED té verde	proteína de soja	salchichas de pavo	*L. monocytogenes*	Theivendran *et al.* (2006)
nisina, ác, málico, EDTA, ED semilla de uva	proteína de suero de leche	salchichas de pavo	*L. monocytogenes, E. coli* O157:H7, *S.* Typhimurium	Gadang *et al.* (2008)
nisina, ác. málico nisina, natamicina** nisina, ác. málico, natamicina**	proteínas de suero de leche	medio de cultivo	*L. monocytogenes, Penicillium* spp., *Ps. aeruginosa, Yarrowia lipolytica*	Pintado *et al.* (2009, 2010)
nisina nisina, glucosa oxidasa	proteína de suero de leche	medio de cultivo	*L. innocua, Brochothrix thermosphacta, E. coli, Enterococcus faecalis*	Murillo-Martínez *et al.* (2013)
nisina	quitosano	medio de cultivo	*E. coli, St. aureus, S.* Typhimurium, *L. monocytogenes, B. cereus*	Pranoto, Rakshit, *et al.* (2005)
nisina nisina, diacetato de sodio	zeína	salchicha de pavo	*L. monocytogenes*	Lungu y Johnson (2005a, b)
nisina nisina, EDTA nisina, ác. láurico	zeína	medio de cultivo	*S.* Enteriditis, *L. monocytogenes*	Hoffman *et al.* (2001)
nisina nisina, propionato de calcio	zeína	pollo cocido	*L. monocytogenes*	Janes *et al.* (2002)
nisina, ác. láurico	zeína	medio de cultivo	*Lb. plantarum*	Padgett *et al.* (2000)
pediocina	celulosa	jamón, carne de vacuno, pechuga de pavo	*L. monocytogenes*	Ming *et al.* (1997)
pediocina	celulosa acetato	jamón loncheado	*L. innocua*	Santiago-Silva *et al.* (2009)
sakacina A	pululano	pechuga de pavo RTE	*L. monocytogenes*	Trinetta *et al.* (2010)
antibióticos				
natamicina**	alginato alginato/quitosano	medio de cultivo	*Debaromyces hansenii, P. commune, P. roqueforti*	Da Silva *et al.* (2013)
natamicina**, nisina	almidón de tapioca	queso "Port Salut"	*Saccharomyces cerevisiae, L. innocua*	Resa *et al.* (2013, 2014)
natamicina**	caseína	queso "kashar" fresco	ME (hongos)	Yildirim *et al.* (2006)
natamicina**	celulosa	queso "gorgonzola"	*P. roqueforti*	De Oliveira *et al.* (2007)
natamicina**	celulosa	queso "mozzarella" loncheada	ME (*Staphylococcus* spp., TVC psicrófilos, hongos, levaduras)	Pires *et al.* (2008)
natamicina**	gluten de trigo metilcelulosa	medio de cultivo queso "kashar" fresco	*A. niger, P. roqueforti*	Türe *et al.* (2011); Türe *et al.* (2008)
natamicina**	proteína de suero de leche	medio de cultivo	*E. coli, St. aureus, Yarrowia lipolytica*	Ramos, Silva, *et al.* (2012)
natamicina**, ác. láctico natamicina**, quitooligosacáridos natamicina**, quitooligosacáridos, ác. láctico	proteína de suero de leche	queso "Saloio "	ME (TVC mesófilos, *Lactococcus* spp., *Lactobacillus* spp., *Staphylococcus* spp., *Pseudomonas* spp., Enterobacteriaceae, hongos, levaduras)	Ramos, Pereira, *et al.* (2012)

natamicina**, ác. málico natamicina**, nisina natamicina**, ác. málico nisina	proteínas de suero de leche	medio de cultivo	*L. monocytogenes, Penicillium* spp., *Ps. aeruginosa, Yarrowia lipolytica*	Pintado *et al.* (2010)
natamicina**	quitosano	queso "Saloio "	*A. niger, P. crustosum, P. commune, P. roqueforti*	Fajardo *et al.* (2010)
natamicina**	quitosano	medio de cultivo, melón "Hami"	*Alternaria alternata, Fusarium semitectum*	Cong *et al.* (2007)
enzimas				
glucosa oxidasa glucosa oxidasa, nisina	proteína de suero de leche	medio de cultivo	*L. innocua, Brochothrix thermosphacta, E. coli, Enterococcus faecalis*	Murillo-Martínez *et al.* (2013)
lactoperoxidasa	alginato de calcio	medio de cultivo	*E. coli, L. innocua, Ps. fluorescens*	Yener *et al.* (2009)
lactoperoxidasa	harina de soja desgrasada	medio de cultivo	*S.* Typhimurium	Lee y Min (2013, 2014)
lactoperoxidasa	proteína de suero de leche	medio de cultivo, pavo asado salmón ahumado	*S. enterica, E. coli* O157:H7 *L. monocytogenes, P. commune,* EM (TVC)	Min, Harris, *et al.* (2005a, b); Min *et al.* (2006); Min y Krochta (2005)
lactoperoxidasa	quitosano	medio de cultivo	*Xanthomonas campestris* pv. *mangiferaeindicae, Colletotrichum gloeosporioides, Lasiodiplodia theobromae*	Mohamed *et al.* (2013)
lactoperoxidasa	quitosano	trucha "Arco iris"	ME (TVC mesófilos y psicrófilos, *Shewanella putrefaciens, Ps. fluorescens)*	Jasour *et al.* (2014)
lisozima** lisozima**, nisina	alginato de calcio	salmón ahumado	*L. monocytogenes, S.* Anatum	Datta *et al.* (2008)
lisozima**, nisina, EDTA	alginato de sodio ĸ-carragenato	medio de cultivo	*Micrococcus luteus, L. innocua, S.* Enteritidis, *E. coli, St. aureus*	Cha *et al.* (2002)
lisozima**	almidón de guisante	medio de cultivo	*Brochotrix thermosphacta*	Nam *et al.* (2007)
lisozima**	celulosa triacetato	medio de cultivo	*Micrococcus lysodeikticus*	Appendini y Hotchkiss (1997)
lisozima**	celulosa acetato	medio de cultivo	*E. coli, B. amyloliquefaciens*	Gemili *et al.* (2009)
lisozima**	gelatina de piel de pescado	medio de cultivo	*E. coli, B. subtilis, Streptococcus cremoris*	Bower *et al.* (2006)
lisozima**, nisina, EDTA	gelatina	jamón cocido, mortadela	*Brochothrix thermosphacta, E. coli* O157:H7, *Lb. sakei, Leuconostoc mesenteroides, L. monocytogenes, S.* Typhimurium	Gill y Holley (2000)
lisozima** lisozima**, EDTA	proteína de soja zeína	medio de cultivo	*Lb. plantarum, E. coli*	Padgett *et al.* (1998)
lisozima**	proteína de suero de leche	medio de cultivo salmón ahumado	*L. monocytogenes,* ME (TVC, hongos, levaduras)	Min, Harris, Han, *et al.* (2005)
lisozima**, quitosano	proteína de suero de leche	huevo duro	*S.* Enteriditis, ME (TVC, coliformes, hongos, levaduras)	Kim *et al.* (2008)
lisozima**	proteínas de suero de leche/quitosano	medio de cultivo	*E. coli, St. aureus*	Lian *et al.* (2012)
lisozima**	quitosano	medio de cultivo	*E. coli, Streptococcus faecalis*	Park *et al.* (2004)
lisozima**	quitosano	medio de	*L. monocytogenes, E. coli,*	Duan *et al.* (2008);

		cultivo queso "mozzarella"	*Ps. fluorescens*, hongos, levaduras	Duan *et al.* (2007)
lisozima**, lactoferrina lisozima**, EDTA	quitosano	medio de cultivo	*E. coli* O157:H7, *L. monocytogenes*	Brown *et al.* (2008)
lisozima**, EDTA	zeína	medio de cultivo	*Lb. plantarum, B. subtilis, E. coli*	Mecitoğlu *et al.* (2006)
lisozima**, EDTA	zeína	medio de cultivo hamburguesa de vacuno	*L. monocytogenes, S.* Typhimurium, *E. coli* O157:H7 ME (TVC, coliformes)	Ünalan *et al.* (2011)
lisozima**, EDTA	zeína, albúmina de garbanzos, albúmina de suero bovino	medio de cultivo	*B. subtilis, E. coli*	Mecitoğlu *et al.* (2007)
aceites esenciales, extractos				
AE ajedrea AE canela AE orégano	alginato de calcio	carne de vacuno, mortadela, jamón fileteados	*E. coli* O157:H7, *S.* Typhimurium, *L. monocytogenes*	Oussalah *et al.* (2006, 2007)
AE ajo	alginato de calcio	medio de cultivo	*St. aureus, B. cereus*	Pranoto, Salokhe, *et al.* (2005)
AE ajo AE clavo AE orégano	proteínas de desechos de merluza	medio de cultivo	*Brochothrix thermosphacta, E. coli, L. innocua, L. monocytogenes, Ps. putida, S.* Typhimurium, *Shewanella putrefaciens*	Teixeira *et al.* (2014)
AE ajo AE orégano	proteína de suero de leche	medio de cultivo	*E. coli* O157:H7, *St. aureus, S.* Enteritidis, *L. monocytogenes, Lb. plantarum*	Seydim y Sarikus (2006, 2007)
AE ajo AE orégano AE pimienta de Jamaica	puré de tomate	medio de cultivo	*E. coli* O157:H7, *S. enterica, L. monocytogenes*	Du, Olsen, Avena-Bustillos, McHugh, Levin, Mandrell, *et al.* (2009)
AE ajo	quitosano	medio de cultivo	*E. coli, St. aureus, S.* Typhimurium, *L. monocytogenes, B. cereus*	Pranoto, Rakshit, *et al.* (2005)
AE ajo	quitosano	pan de ajo	ME (hongos)	Sangsuwan *et al.* (2012)
óxido de zinc (nano) AE albahaca	proteína de pescado/gelatina de piel de pescado	medio de cultivo	*L. monocytogenes, Ps. aeruginosa*	Arfat *et al.* (2014)
AE alcaravea**	pululano	zanahoria tierna	*S.* Enteritidis, *St. aureus, Saccharomyces cerevisiae, A. niger*	Gniewosz *et al.* (2013)
AE alga roja *(Gelidium corneum)*	proteína de harina de semilla de girasol	pato ahumado	*L. monocytogenes*	Song *et al.* (2013)
AE árbol del té AE cilantro AE laurel AE romero AE tomillo blanco	proteína de suero de leche	medio de cultivo	*S.* Enteritidis, *Ps. fragi*	Fernández-Pan *et al.* (2012)
AE árbol del té	quitosano	medio de cultivo	*L. monocytogenes, P. italicum*	Sánchez-González, González-Martínez, *et al.* (2010)
AE árbol del té EO propóleos resveratrol	quitosano	brócoli cortado	*E. coli* O157:H7, *L. monocytogenes,* ME (TVC mesófilos y psicrófilos)	Álvarez *et al.* (2013)
AE árbol del té	quitosano	medio de	*E. coli, St. aureus, L.*	Sánchez-

AE bergamota AE limón	HPMC	cultivo	*monocytogenes*	González, Chafer, *et al.* (2011)
AE árbol del té AE bergamota AE tomillo	quitosano	naranja "Navel Powell"	*P. italicum*	Chafer *et al.* (2012)
AE bergamota	quitosano HPMC	medio de cultivo uva "Moscatel"	*P. italicum* ME (TVC mesófilos, hongos, levaduras)	Sánchez-González, Chafer, *et al.* (2010); Sánchez-González, Pastor, *et al.* (2011)
AE bergamota AE hierba limón**	gelatina de pescado (lija, *Aluterus monoceros*)	medio de cultivo lubina	*St. aureus, E. coli, L. monocytogenes, S.* Typhimurium ME (TVC mesófilos y psicrófilos, bacterias sulfuro-productoras, LAB, Enterobacteriaceae)	Ahmad, Benjakul, Prodpran, *et al.* (2012); Ahmad, Benjakul, Sumpavapol, *et al.* (2012)
AE canela, ác. málico AE hierba limón**, ác. málico AE clavo, ác. málico	alginato de calcio	manzana "Fuji" troceada	*E. coli* O157:H7, ME (TVC mesófilos y psicrófilos, hongos, levaduras)	Raybaudi-Massilia, Rojas-Graü, *et al.* (2008)
AE canela AE hierba limón** AE orégano	alginato de calcio/puré de manzana puré de manzana	medio de cultivo	*E. coli* O157:H7	Rojas-Graü *et al.* (2006); Rojas-Graü, Avena-Bustillos, *et al.* (2007)
AE canela, ác. málico AE hierba limón**, ác. málico AE palmarosa, ác. málico	alginato de calcio	melón "Piel de sapo" pelado	*S.* Enteritidis, ME (TVC mesófilos y psicrófilos, hongos, levaduras)	Raybaudi-Massilia, Mosqueda-Melgar, *et al.* (2008)
AE canela AE hierba limón** AE orégano	amaranto almidón de maíz quitosano	medio de cultivo	*A. niger, P. digitatum*	Ávila-Sosa *et al.* (2010); Ávila-Sosa *et al.* (2012)
AE canela	proteínas de suero de leche	medio de cultivo	*Lb. lactis, Ps. putida, Streptococcus agalactiae, E. coli, L. monocytogenes, B. subtilis, C. albicans*	Bahram *et al.* (2014)
AE canela AE clavo AE pimienta de Jamaica	puré de manzana	medio de cultivo	*E. coli* O157:H7, *S. enterica, L. monocytogenes*	Du, Olsen, Avena-Bustillos, McHugh, Levin y Friedman (2009)
AE canela AE clavo AE tomillo	quitosano	medio de cultivo	*L. monocytogenes, St. aureus, S.* Enteriditis, *Ps. aeruginosa*	Hosseini *et al.* (2009)
AE canela	quitosano	medio de cultivo trucha "Arco iris"	*E. coli, Lb. sakei, L. monocytogenes, Lb. plantarum, Ps. fluorescens* ME (TVC mesófilos y psicrófilos)	Ojagh *et al.* (2010a, b)
AE canela	quitosano	azufaifo**	*P. citrinum*	Xing *et al.* (2011)
AE canela	quitosano	medio de cultivo	*E. coli, St. aureus*	Peng y Li (2014)
AE canela	quitosano/ácido oleico	medio de cultivo fresa	*A. niger, Botrytis cinerea, Rhizopus stolonifer* *Rhizopus stolonifer*	Perdones *et al.* (2014)
AE clavo	gelatina	medio de cultivo salmón en rodajas	*Lb. acidophilus, E. coli, Ps. fluorescens, L. innocua* ME (TVC)	Gómez-Estaca *et al.* (2009)
AE clavo	gelatina/quitosano	medio de cultivo	*Ps. fluorescens, Shewanella putrefaciens, Photobacterium phosphoreum, L. innocua,*	Gómez-Estaca *et al.* (2010)

		pescado "dorado"**	*E. coli, Lb. acidophilus* ME (TVC, bacterias sulfuro-productoras, luminiscentes, LAB, *Pseudomonas* spp., Enterobacteriaceae)	
AE clavo	proteínas de girasol	hamburguesa de sardina	ME (TVC, TVC mesófilos, bacterias sulfuro-productoras, luminiscentes, LAB, *Pseudomonas* spp., Enterobacteriaceae)	Salgado *et al.* (2013)
AE clavo AE orégano	proteínas de suero de leche	pechugas de pollo	ME (TVC mesófilos y psicrófilos, LAB, *Pseudomonas* spp., Enterobacteriaceae)	Fernández-Pan *et al.* (2014); Fernández-Pan *et al.* (2013)
AE clavo AE orégano	quitosano	queso "kashar"	*E. coli* O157:H7, *L. monocytogenes, St. aureus*	Torlak y Nizamlıoğlu (2011)
AE cúrcuma	caseinato de calcio	zanahoria	ME (TVC, coliformes, hongos, levaduras)	Jagannath *et al.* (2006)
AE hierba limón**	almidón de sagú/ alginato de sodio	medio de cultivo	*E. coli* O157:H7, *S.* Enteriditis, *St. aureus*	Maizura *et al.* (2008); Maizura *et al.* (2007)
AE hierba limón** AE orégano	alginato de calcio/puré de manzana	manzana "Fuji" troceada	*L. innocua*, ME (TVC psicrófilos, hongos, levaduras)	Rojas-Graü, Raybaudi-Massilia, *et al.* (2007)
AE laurel	gelatina	trucha "Arco iris"	ME (TVC mesófilos y psicrófilos, LAB, Enterobacteriaceae)	Alparslan *et al.* (2014)
AE lima AE tomillo	goma de mezquite	papaya	*Colletotrichum gloeosporioides, Rhizopus stolonifer*	Bosquez-Molina *et al.* (2010)
AE lima AE tomillo	quitosano	tomate	*Rhizopus stolonifer, E. coli*	Ramos-García *et al.* (2012)
AE limón	quitosano	fresa	*Botrytis cinerea*	Perdones *et al.* (2012)
AE pomelo ED semilla de pomelo	alginato de sodio	uva de mesa	*P. digitatum*	Aloui *et al.* (2014)
AE orégano	alginato de calcio	medio de cultivo	*E. coli, S.* Enteritidis, *St. aureus, L. monocytogenes*	Benavides *et al.* (2012)
AE orégano	almidón de boniato	medio de cultivo	*E. coli* O157:H7, *L. monocytogenes, S.* Enteritidis	Ehivet *et al.* (2011)
AE orégano	almidón/quitosano	medio de cultivo	*E. coli, St. aureus, B. cereus, S.* Enteriditis	Pelissari *et al.* (2009)
AE orégano	gelatina de pescado "pez gato"**	pescado "pez gato"**	*S.* Typhimurium, *E. coli* O157:H7	Min y Oh (2009)
AE orégano	gelatina de pescado "carpa plateada"/ quitosano	carpa china	ME (TVC)	Wu *et al.* (2014)
AE orégano AE tomillo ED cítricos	gelatina metilcelulosa gelatina/metilcelulosa	medio de cultivo	*L. innocua, Ps. fluorescens, Aeromonas hydrophila/caviae*	Iturriaga *et al.* (2012)
AE orégano	mucílago de semilla de membrillo	medio de cultivo	*L. monocytogenes, S.* Typhimurium, *B. cereus, Yersinia enterocolitica, Ps. aeruginosa, St. aureus, E. coli, E. coli* O157:H7, *Shewanella putrefaciens, Vibrio cholera, Lb. plantarum*	Jouki, Yazdi, Mortazavi y Koocheki (2014); Jouki, Yazdi, Mortazavi, Koocheki, *et al.* (2014)
		trucha "Arco iris"	ME (TVC mesófilos y psicrófilos, *Pseudomonas* spp., bacterias sulfuro-	

			productoras, LAB, Enterobacteriaceae)	
AE orégano	piel de patata	salmón ahumado	*L. monocytogenes*	Tammineni *et al.* (2013)
AE orégano	proteínas de suero de leche	carne de vacuno	ME (TVC, *Pseudomonas* spp.)	Zinoviadou *et al.* (2009)
AE orégano	proteínas de suero de leche	medio de cultivo	*L. innocua*, *S.* Enteritidis, *St. aureus*	Royo *et al.* (2010)
AE orégano AE pimiento AE orégano, pimiento	proteína de suero leche/caseinato de calcio	carne de vacuno fileteada	*E. coli* O157: H7, *Pseudomonas* spp.	Oussalah *et al.* (2004)
AE orégano AE tomillo AE tomillo, romero	proteínas de soja	hamburguesa de vacuno	ME (TVC, LAB, coliformes, *Staphylococcus* spp., *Pseudomonas* spp.)	Emiroğlu *et al.* (2010)
AE orégano	quitosano	mortadela	*L. monocytogenes, E. coli* O157:H7	Zivanovic *et al.* (2005)
AE orégano	quitosano	pechugas de pollo	ME (TVC, LAB, *Brochothrix thermosphacta,* Enterobacteriaceae, *Pseudomonas* spp., hongos, levaduras)	Petrou *et al.* (2012)
AE orégano	quitosano	uva	*Rhizopus stolonifer, Aspergillus niger*	Dos Santos *et al.* (2012)
AE romero	mucílago de semilla de membrillo	medio de cultivo	*Ps. aeruginosa, St. aureus, E. coli, E. coli* O157:H7, *L. monocytogenes, S.* Typhimurium, *Lb. plantarum, B. cereus, Yersinia enterocolitica, Vibrio cholera, Shewanella putrefaciens*	Jouki, Mortazavi, *et al.* (2014)
AE romero	quitosano	medio de cultivo	*L. monocytogenes, E. coli, Streptococcus agalactiae*	Abdollahi *et al.* (2012)
AE tomillo	quitosano	medio de cultivo	*E. coli, St. aureus, Klebsiella pneumoniae, Ps. aeruginosa*	Altiok *et al.* (2010)
AE tomillo	quitosano	medio de cultivo	*L. innocua, Serratia marcenscens, Aeromonas hydrophila, Achromobacter denitrificans, Alcaligenes faecalis*	Ruiz-Navajas *et al.* (2013)
AE tomillo	quitosano	kebab de pollo	ME (TVC mesófilos, *Pseudomonas* spp., LAB, Enterobacteriaceae, *Brochothrix thermosphacta,* hongos y levaduras)	Giatrakou *et al.* (2010)
AE tomillo	quitosano	hongo "shiitake"	ME (TVC mesófilos y psicrófilos, *Pseudomonas* spp., hongos y levaduras)	Jiang *et al.* (2012)
AE tomillo, cinamaldehído	proteínas de soja/ proteínas de suero de leche/PVOH	gambas cocidas irradiadas a bajas dosis	*Ps. putida*, ME (TVC)	Ouattara *et al.* (2001)
ED canela	quitosano	banana	*Colletotrichum musae, Fusarium* spp., *Lasiodiplodia theobromae*	Win *et al.* (2007)
ED canela ED clavo	almidón de tapioca	pan en rebanadas	hongos, levaduras	Kechichian *et al.* (2010)
ED eucalipto	almidón de tapioca	medio de cultivo	*E. coli*	Rojhan y Nouri (2013)
ED ginseng	alginato de calcio	medio de cultivo	*St. epidermidis, E. coli, B. subtilis, Ps. aeruginosa, L. monocytogenes, S.* Typhimurium	Norajit y Ryu (2011)

ED hoja de olivo	metilcelulosa	queso "kashar"	*St. aureus*	Ayana y Turhan (2009)
ED polen fermentado ED semilla de pomelo ED té verde	alga roja *(Gelidium corneum)*/gelatina	lomo de cerdo	*E coli* O157:H7, *L. monocytogenes*	Hong *et al.* (2009a, b)
ED propóleos	HPMC	uva de mesa	ME (TVC mesófilos, hongos, levaduras)	Pastor *et al.* (2011)
ED semilla de pomelo	alga roja *(Gelidium corneum)*	medio de cultivo	*E coli* O157:H7, *L. monocytogenes*	Lim, Jang, *et al.* (2010)
ED semilla de pomelo	alga roja *(Gelidium corneum)*/ proteínas del suero de leche	pescado triturado	*E. coli* O157:H7, *L. monocytogenes, S.* Typhimurium	Lim *et al.* (2008)
ED semilla de pomelo	alga roja *(Gelidium corneum)*	queso panceta	*E coli* O157:H7, *L. monocytogenes*	Shin *et al.* (2012)
ED semilla de pomelo ED semilla de pomelo, EDTA	alginato de sodio K-carragenato	medio de cultivo	*Micrococcus luteus, L. innocua,* *S.* Enteritidis , *E. coli, St. aureus*	Cha *et al.* (2002)
ED semilla de pomelo	gelatina/proteína de semilla de colza	medio de cultivo fresa	*L. monocytogenes, E. coli* O157:H7 ME (TVC, hongos, levaduras)	Jang *et al.* (2011)
ED semilla de pomelo	gelatina/proteína de salvado de cebada	salmón	*E coli* O157:H7, *L. monocytogenes*	Song *et al.* (2012)
ED semillas de uva	almidón de guisante	medio de cultivo lomo de cerdo	*L. monocytogenes, E. coli, St. aureus, Enterococcus faecalis, Enterococcus faecium, S.* Typhimurium *B. thermosphacta*	Corrales *et al.* (2009)
ED semilla de uva, EDTA ED semilla de uva, nisina ED semilla de uva, EDTA, nisina	proteína de soja	medio de cultivo	*L. monocytogenes, E. coli* O157:H7, *S.* Typhimurium	Sivarooban *et al.* (2008)
ED semilla de uva, nisina ED té verde, nisina	proteína de soja	salchichas de pavo	*L. monocytogenes*	Theivendran *et al.* (2006)
ED semilla de uva, ác. málico, EDTA, nisina	proteína de suero de leche	salchichas de pavo	*L. monocytogenes, E. coli* O157:H7, *S.* Typhimurium	Gadang *et al.* (2008)
ED semilla de uva	quitosano	uva "Redglobe"	*B. cinerea*	Xu *et al.* (2007)
ED té verde	almidón de tapioca/ goma de hoja de hsian-tsao (*Mesona procumbens*)	ensalada con fruta carne de cerdo cortada corazones de lechuga "Romana"	ME (TVC mesófilos y psicrófilos, hongos, levaduras) *B. cereus* *L. monocytogenes, St. aureus*	Chiu y Lai (2010)
ED té verde	proteína de soja	medio de cultivo	*St. aureus, Streptococcus mutans*	Kim *et al.* (2006)
ED té verde	quitosano	salchichas	ME (TVC mesófilos, LAB, hongos, levaduras)	Siripatrawan y Noipha (2012)
componentes de aceites esenciales				
alil-isotiocianato alil-isotiocianato, nisina	PLA	clara de huevo	*Salmonella* spp.	Jin y Gurtler (2011)
alil-isotiocianato	proteínas de soja	brotes de alfalfa, rábano y brócoli	ME (TVC)	Gamage *et al.* (2009)
alil-isotiocianato alil-isotiocianato, nisina	quitosano	melón "Cantalupo"	*Salmonella* spp. ME (TVC, hongos, levaduras)	Chen *et al.* (2012)
benzaldehído 4-dimetilamino-	zeína	medio de cultivo	*B. cereus, L. innocua, L. monocytogenes,*	Soliman *et al.* (2014)

benzaldehído cinamaldehído			*Clostridium sporogenes*, *E. coli*, *S. enterica*, *Yersinia enterocolitica*	
carvacrol	alga roja *(Gelidium corneum)*	jamón	*E coli* O157:H7, *L. monocytogenes*	Lim, Hong, *et al.* (2010)
carvacrol metilcinamato	alginato	fresa	*E. coli* O157:H7, *Botrytis cinerea*	Peretto *et al.* (2014)
carvacrol	caseinato de calcio caseinato de sodio	medio de cultivo	*E. coli*, *St. aureus*	Arrieta *et al.* (2014)
carvacrol	gelatina	medio de cultivo	*Ps. aeruginosa*, *E. coli*, *St. aureus*, *B. subtilis*	Kavoosi *et al.* (2013)
carvacrol	proteínas de soja	medio de cultivo	*Botrytis cinerea*, *E. coli*	Ben Arfa *et al.* (2007); Chalier *et al.* (2007)
carvacrol cinamaldehído	puré de manzana puré de hibisco puré de zanahoria	pechuga de pollo jamón mortadela vegetales de hoja verde	*S.* Enteritidis, *E. coli* O157:H7 *L. monocytogenes* *S.* Newport	Ravishankar *et al.* (2012); Ravishankar *et al.* (2009) Zhu *et al.* (2014)
carvacrol	puré de manzana	medio de cultivo	*E. coli* O157:H7	Du, Olsen, Avena-Bustillos, Mchugh, *et al.* (2008)
carvacrol	puré de manzana	pechuga de pollo	*Campylobacter jejuni*	Mild *et al.* (2011)
carvacrol	puré de tomate	medio de cultivo	*E. coli* O157:H7	Du, Olsen, Avena-Bustillos, McHugh, *et al.* (2008)
carvacrol	quitosano	medio de cultivo	*S.* Typhimurium, *E. coli* O157:H7	López-Mata *et al.* (2013)
cinamaldehído	alginato de calcio/pectina/β-CD	sandía cortada	ME (TVC mesófilos y psicrófilos, coliformes, hongos, levaduras)	Sipahi *et al.* (2013)
cinamaldehído citral carvacrol	alginato de calcio/puré de manzana	medio de cultivo	*E. coli* O157:H7	Rojas-Graü, Avena-Bustillos, *et al.* (2007)
cinamaldehído, ác. málico citral, ác. málico eugenol, ác. málico	alginato de calcio	manzana "Fuji" troceada	*E. coli* O157:H7, ME (TVC mesófilos y psicrófilos, hongos, levaduras)	Raybaudi-Massilia, Rojas-Graü, *et al.* (2008)
cinamaldehído eugenol	celulosa	medio de cultivo	*L. monocytogenes*, *St. aureus*, *E. coli* *S.* Enteritidis, *C. albicans*, *Saccharomyces cerevisiae*	Sanla-Ead *et al.* (2012)
cinamaldehído	gliadina	queso pan	*Penicillium* spp., ME (hongos) *P. expansum*, ME (hongos)	Balaguer *et al.* (2014); Balaguer *et al.* (2013)
cinamaldehído	PLA	medio de cultivo	*E. coli*, *B. cereus*	Makwana *et al.* (2014)
cinamaldehído	proteínas de soja	medio de cultivo	*Botrytis cinerea*, *E. coli*,	Ben Arfa *et al.* (2007)
cinamaldehído	proteínas de soja	brotes de alfalfa, rábano y brócoli	ME (TVC)	Gamage *et al.* (2009)
cinamaldehído, AE tomillo	proteínas de soja/ proteínas de suero de leche/PVOH	gambas cocidas irradiadas a bajas dosis	*Ps. putida*, ME (TVC)	Ouattara *et al.* (2001)
cinamaldehído	puré de manzana	pechuga de pollo	*Campylobacter jejuni*	Mild *et al.* (2011)
ác. acético, cinamaldehído ác. propiónico, cinamaldehído	quitosano	jamón cocido mortadela pastrami	*Lb. sakei*, *Serratia liquefaciens*, ME (LAB, Enterobacteriaceae)	Ouattara *et al.* (2000)
cinamaldehído	quitosano/pectina/β-CD	papaya troceada	ME (TVC mesófilos y psicrófilos, hongos,	Brasil *et al.* (2012); Moreira *et al.*

		melón troceado	levaduras)	(2014)
citral, ác. málico eugenol, ác. málico geraniol, ác. málico	alginato de calcio	melón "Piel de sapo" pelado	*S.* Enteritidis, ME (TVC mesófilos y psicrófilos, hongos, levaduras)	Raybaudi-Massilia, Mosqueda-Melgar, *et al.* (2008)
citral citral, quercetina	kafirina	medio de cultivo	*Ps. fluorescens, Campylobacter jejuni, L. monocytogenes*	Giteru *et al.* (2015)
timol	alga roja *(Gelidium corneum)*	medio de cultivo	*E coli* O157:H7, *L. monocytogenes*	Lim, Jang, *et al.* (2010)
timol	zeína	medio de cultivo	*B. cereus, C. lusitaniae, Pseudomonas* spp.	Del Nobile *et al.* (2008)
vainillina	alginato de calcio/puré de manzana	manzana "Fuji" troceada	ME (TVC psicrófilos, hongos, levaduras) *L. innocua*	Rojas-Graü, Raybaudi-Massilia, *et al.* (2007)
vainillina	metilcelulosa/quitosano	melón "Cantalupo" piña	*E. coli, Saccharomyces cerevisiae*	Sangsuwan *et al.* (2008)
vainillina	quitosano	bizcocho	ME (hongos)	Sangsuwan *et al.* (2014)
otros				
LAE	PLA	jamón cocido loncheado	*L. monocytogenes, S.* Typhimurium	Theinsathid *et al.* (2012)
LAE	quitosano	cáscara de huevo	*S.* Enteritidis	Jin *et al.* (2013)
LAE	quitosano	pavo RTE	*L. monocytogenes, L. innocua, S.* Typhimurium	Guo, Jin, Wang, *et al.* (2014); Guo, Jin y Yang (2014)

*Abreviaturas: ác.= ácido; PABA= ác. *p*-aminobenzoico; *A.*= *Aspergillus*; AE= aceite esencial de; *B.*= *Bacillus;* *C.*= *Candida*; *Cl.*= *Cladosporium*; *E.*= *Escherichia*; EDTA= ácido etildiaminotetraacético; ED= extracto de; HPMC= hidroxipropilmetilcelulosa; (nano) = tamaño 1-100 nm; *L.*= *Listeria;* LAB= bacterias ácido-lácticas; *Lb.*= *Lactobacillus;* LAE = etil-N^{α}-dodecanoil-*L*-arginato; ME= microbiota endógena; *P.*= *Penicillium;* PLA= ácido poliláctico; *Ps.*= *Pseudomonas*; RTE= "*ready to eat*"; *S.*= *Salmonella*; *St.*= *Staphylococcus;* TVC= recuento de microorganismos aerobios totales.

** Sinónimos: alcaravea o comino del prado; azufaifo, chincho o gínjol; hierba limón, citronela o *lemongrass*; dorado o lampuga; lisozima o muramidasa; mojarra o tilapia; natamicina o pimaricina; ovotransferrina o conalbúmina; pez gato, bagre o siluro.

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7.2. Anexo B. Publicaciones

La presente Tesis Doctoral dio lugar a siete publicaciones científicas en revistas internacionales:

• López-Carballo, G., Higueras, L., Gavara, R., Hernández-Muñoz, P., 2013. Silver ions release from antibacterial chitosan films containing *in situ* generated silver nanoparticles. Journal of Agricultural and Food Chemistry 61, 260−267.

• Higueras, L., López-Carballo, G., Hernández-Muñoz, P., Gavara, R., Rollini, M., 2013. Development of a novel antimicrobial film based on chitosan with LAE (ethyl-N^{α}-dodecanoyl-*L*-arginate) and its application to fresh chicken. International Journal of Food Microbiology 165, 339–345.

• Higueras, L., López-Carballo, Cerisuelo, J.P., G., Gavara, R., Hernández-Muñoz, P., 2013. Preparation and characterization of chitosan/HP-β-cyclodextrins composites with high sorption capacity for carvacrol. Carbohydrate Polymers 97, 262– 268.

• Higueras, L., López-Carballo, G., Hernández-Muñoz, P., Catalá, R., Gavara, R., 2014. Antimicrobial packaging of chicken fillets based on the release of carvacrol from chitosan/cyclodextrin films. International Journal of Food Microbiology 188, 53–59.

• Higueras, L., López-Carballo, G., Gavara, R., Hernández-Muñoz, P., 2015. Incorporation of hydroxypropyl-β-cyclodextrins into chitosan films to tailor loading capacity for active aroma compound carvacrol. Food Hydrocolloids 43, 603–611.

• Higueras, L., López-Carballo, G., Gavara, R., Hernández-Muñoz, P., 2015. Reversible covalent immobilization of cinnamaldehyde on chitosan films via schiff base formation and their application in active food packaging. Food and Bioprocess Technology, 1-13.

• Higueras, L., López-Carballo, G., Gavara, R., Hernández-Muñoz, P. Effect of HP-β-cyclodextrins and coadjuvants on the sorption capacity of hydrophilic polymer films for naturally occurring antimicrobial monoterpene alcohols. Submitted to *Carbohydrate Polymers*.

Además de las siguientes contribuciones a congresos:

• Congreso nacional, póster: G. López-Carballo, L. Higueras, A. Fernández, M. J. Galotto, A. Guarda, R. Gavara, P. Hernández-Muñoz. Desarrollo y Propiedades Antimicrobianas de Películas Compuestas de Quitosano/Nanopartículas de Plata. III Congreso del Caribe y I Latinoamericano sobre Higiene y Calidad en la Agricultura y Alimentación. Book of proceedings. Iquique (I Región de Tarapacá), Chile. 4-6 de noviembre de 2009.

• Congreso internacional, póster: L. Higueras, G. López-Carballo, R. Gavara, P. Hernández-Muñoz. Chitosan Matrices as Carriers for the Delivery of Natural Volatile Antimicrobials. BioMicroWorld 2009. III International Conference on Environmental, Industrial and Applied Microbiology. Book of Abstracts, 277. Lisboa, Portugal. 2-4 de diciembre de 2009.

• Congreso internacional, póster: G. López-Carballo, L. Higueras, A. Fernández, R. Gavara, P. Hernández-Muñoz. Antimicrobial Properties of Nanostructured Chitosan-Silver Membranes. BioMicroWorld 2009. III International Conference on Environmental, Industrial and Applied Microbiology. Book of Abstracts, 264. Lisboa, Portugal. 2-4 de diciembre de 2009.

• Congreso internacional, póster: G. López-Carballo, L. Higueras, R. Gavara, P. Hernández-Muñoz. Development of hybrid nanocomposites based on chitosan and silver nanoparticles for active packaging applications. SLIM 2010 (Shelf Life International Meeting). Book of Abstracts, 89. Zaragoza, España. 23-25 de junio de 2010.

• Congreso internacional, póster: L. Higueras, G. López-Carballo, M. P. Balaguer, R. Gavara, P. Hernández-Muñoz. Active packaging technologies based on chitosan/cyclodextrin biocomposites. IFT10, Annual Meeting & Food Expo. Chicago, IL USA.17-20 de Julio de 2010.

• Congreso internacional, póster: G. López-Carballo, L. Higueras, V. Muriel-Galet, R. Gavara, P. Hernández-Muñoz. Long-lasting antimicrobial properties of biological plastics based on silver nanotechnology. 18th IAPRI World Packaging Conference. Book of Abstracts, 408-414. California, USA. 17-21 de junio de 2012.

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Development of a novel antimicrobial film based on chitosan with LAE (ethyl-N$^{\alpha}$-dodecanoyl-L-arginate) and its application to fresh chicken

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ABSTRACT

Chitosan (CS) films incorporating the antimicrobial compound ethyl-N$^{\alpha}$-dodecanoyl-L-arginate (LAE) were developed for food packaging applications. Cast chitosan films were made with 1, 5 or 10% LAE and 20% glycerol in the film forming solution. Optical properties, release of LAE and antimicrobial activity of developed films was determined. The minimum inhibitory concentration (MIC) and the minimum biocide concentration (MBC) of LAE were determined. CS films with LAE were transparent and uniform, without discontinuities or visible particles and no visual differences could be perceived between CS and CS-LAE films. When in contact with an aqueous food simulant, the agent was fully released following a Fickian behavior in a few hours at 4 and 28 °C. Antimicrobial activity of films against mesophiles, psychrophiles, *Pseudomonas* spp., coliforms, lactic acid bacteria, hydrogen sulfide-producing bacteria, yeast and fungi, was evaluated at two, six and eight days for its application on chicken breast fillets. Films were active against bacteria, yeasts and fungi in liquid and solid media. CS films evidenced antimicrobial activity in the range 0.47–2.96 log reductions, while CS-5%LAE film produced 1.78–5.81 log reduction. Results highlighted that LAE incorporation in a chitosan-based packaging structure may provide a relevant antimicrobial activity that could improve the stability of fresh poultry products.

1. Introduction

The incidence of foodborne diseases associated with microbial pathogens is widespread and represents a threat to public health, and a challenge for the food industry (López-Carballo et al., 2008). Considerable efforts are underway to find effective treatments to control recontamination of meat and poultry products to enhance their safety and quality. In this sense, applications of antimicrobial films and coatings to food have received considerable attention in recent years because they can act as protective barriers against microbiological contamination (Suppakul et al., 2003; Cagri et al., 2004). Direct addition of antimicrobial agents into meat formulations may result in partial inactivation of the active substances by interaction with product constituents and is, therefore, expected to have only limited effect on surface microbiota (Torres et al., 1985; Siragusa et al., 1999). Also, direct application of antimicrobial agents onto meat surfaces by dipping or spraying, has been shown to be inefficient, due to the rapid diffusion of the active substances within the bulk of food (Torres et al., 1985; Siragusa et al., 1999; Ouattara et al., 2000a). In contrast, an antimicrobial active film can be developed to deliver a continued and gradual release of an antimicrobial agent during the storage and distribution of food packaging, thus providing an effective prevention of surface contamination of foods (Marcos et al., 2007; Aymerich et al., 2008). In this case it is possible to reduce the amount of active agent required, satisfying the demand of consumers for fewer additives.

One of the most innovative antimicrobial agent is ethyl-N$^{\alpha}$-dodecanoyl-L-arginate hydrochloride (LAE). It is a synthetically derivative of lauric acid, L-arginine and ethanol (Infante et al., 1997, 2004; Ruckman et al., 2004), which is notable for its antimicrobial effectiveness resulting from its chemical structure and surfactant properties (Pinazo et al., 1999; Rodriguez, 2004; Brandt et al., 2010). LAE's antimicrobial properties are due to its action as cationic surfactant on cytoplasmic membrane and the outer membrane of Gram-negative, and cell membrane and cytoplasm of Gram-positive denaturation proteins. These changes produce disturbances in membrane potential, resulting cell growth inhibition and loss of viability (Kanazawa et al., 1995; Infante et al., 1997; Tattawasart et al., 2000; Rodriguez, 2004; Luchansky et al., 2005).

LAE is characterized by a broad spectrum (Infante et al., 1997) and high antimicrobial efficiency against Gram-negatives, Gram-positives, fungi and yeasts, with a low-dose application (Infante et al., 1984; Rodriguez, 2004). In addition, LAE has a low oil–water equilibrium partition coefficient (K_{OW} < 0.1), which means that it tends to concentrate in the aqueous phase, where most bacterial action occurs (Ruckman et al., 2004). On top of that, LAE shows chemical stability and antimicrobial activity in a range of pH 3–7 (Asker et al., 2011).

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Silver Ions Release from Antibacterial Chitosan Films Containing in Situ Generated Silver Nanoparticles

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ABSTRACT: This study aims to develop antimicrobial films consisting of chitosan and silver nanoparticles that are homogeneously distributed throughout the polymer matrix. Nanoparticles were generated in situ during the neutralization of the chitosan acetate film with sodium hydroxide. The temperature of neutralization and the concentration of silver in the film were crucial determinants of the shape and size of the nanoparticles. Neutralized films exhibited antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* in liquid growth media. However, the effectiveness of the films was considerably greater in diluted growth media. Furthermore, no significant differences were found either in the antimicrobial capacities of films incorporating different amounts of silver or in the amount of silver that migrated into the liquid media after 18 h of immersion of the film. Neutralized films maintained their activity after 1 month of immersion in deionized water, which can be attributed to the slow sustained release of silver ions and thus efficacy over time.

KEYWORDS: *synthesis, silver-based nanoparticles, chitosan, antimicrobial activity*

INTRODUCTION

Silver ions have long been recognized as an effective biocide against a broad spectrum of microorganisms, their inhibition mechanism being the subject of considerable research.[1−3] Ionic silver can exert its antimicrobial action in several ways. It has been reported to complex with the thiol groups of enzymes and proteins, altering their structure and function.[4] Silver ions have also been found to bind with DNA and cause structural changes in the cell envelope and cytoplasmic membrane of bacteria.[5,6] Ionic silver has been used as an antimicrobial in the form of salts, and more recently it has been incorporated into inorganic materials such as zeolites, zirconium phosphate, and glass.[7,8] A new approach is the development of silver nanoparticles that can act as nanoreservoirs for the delivery of silver ions, ensuring their availability in the substrate over time. There is a great variety of chemical and physical processes for the synthesis of silver nanoparticles, most of them involving the formation of colloidal nanoparticles or their incorporation in other materials.[9,10] In this regard, increasing attention is being paid to the incorporation of silver nanoparticles in polymer matrices for the development of antimicrobial films and coatings. The antimicrobial capacity of the composites formed will depend on the physical and chemical properties of the nanoparticles and also the silver ion release properties of the carrier matrix. There is also great interest in the use of synthetic and natural hydrophilic polymers and hydrogels as carriers of silver. These materials absorb large amounts of water, owing to the presence in their structure of polar groups (−OH, $-NH_2$, $-CONH_2$, −COOH, ...). Hydrophilic polymers are capable of swelling in a moist environment, thus facilitating the diffusion of the active agent through the polymer matrix and its release to the medium in contact with the polymer.

Chitosan is a biodegradable and biocompatible polymer obtained from biomass and possesses excellent film-forming properties, which have made it of great interest for technological applications in several areas such as pharmacy, medicine, agrochemistry, and packaging. The hydrophilic nature of chitosan has aroused interest in its use as a sustained release carrier when the release of the retained active compound is required in a moist environment. Inclusion of silver-based nanoparticles as an antimicrobial nanofiller in a chitosan matrix could be done directly or by using silver nitrate as a precursor. Chitosan is soluble in aqueous solution and has the ability to bind many metal ions, including silver, via chelation with the amine groups. Chitosan has also been reported to be a mild reducing agent used for reduction of silver ions, and it is frequently employed as an ion capping agent to control the growth of nanoparticles and avoid their aggregation.[11]

The aim of this study, therefore, was to develop films for the slow, sustained release of silver ions, consisting of chitosan polymer as the carrier matrix filled with silver-based nanoparticles synthesized in situ. The resulting films were characterized and the antimicrobial activity was tested in liquid growth media. The ability of the carrier system to exert antimicrobial activity over time was also studied.

MATERIALS AND METHODS

Synthesis of Chitosan/Silver-Based Nanoparticle Films. Low molecular weight chitosan (MW 50−190 kDa, 75−85% deacetylated) from shrimp shells, sodium hydroxide (ACS reagent, ≥97.0%, pellets), and silver nitrate (ACS reagent, ≥99.0%) were obtained from Aldrich Chemical Co., Inc., Milwaukee, WI, USA. A 1.5% (w/w) chitosan solution was prepared in 0.5% (w/w) acetic acid and stirred at 40 °C for 1 h. After the solution had cooled to room temperature, silver nitrate, previously diluted with a small amount of distilled water, was added to the solution and left shaking, protected from light, until complete dissolution. Several chitosan solutions with different silver

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ORIGINAL PAPER

Reversible Covalent Immobilization of Cinnamaldehyde on Chitosan Films via Schiff Base Formation and Their Application in Active Food Packaging

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Abstract In this work, cinnamaldehyde was reversibly anchored to chitosan films via imino-covalent bonding. The Schiff base was synthesized in solid phase employing neutralized chitosan films immersed in acidified 95 % (*v*/*v*) ethanolic solution in which the aldehyde was dissolved. The substitution degree (%) of cinnamaldehyde to the amine group was close to 70 %. Attenuated total reflectance–Fourier transform infrared spectroscopy (ATR-FTIR) analysis revealed the formation of the chitosan-cinnamaldehyde Schiff base. The hydrolysis of the imino bond and subsequent release of cinnamaldehyde were studied after the films had been subjected to different combinations of temperature/time treatments simulating food preservation methods. The amount of aldehyde that remained covalently attached to the films was monitored by ATR-FTIR, and the substitution degree was determined by elemental analysis. Surface contact angle and colour parameters of cinnamaldehyde-imine-chitosan films and these films subjected to different treatments were also evaluated. The antimicrobial properties of chitosan-Schiff base films were tested in vitro against *Staphylococcus aureus* and *Escherichia coli* and in milk inoculated with *Listeria monocytogenes*. The antimicrobial activity varied depending on the treatment applied and consequently the degree of imino bond hydrolysis achieved and cinnamaldehyde released. Films of Schiff base-chitosan derivative subjected to different time/temperature treatments inhibited the growth of *L. monocytogenes* for 12 days under refrigeration conditions, which may extend the microbiological shelf life of such products. Sensory analysis of milk in contact with the films showed that a cinnamon smell does not cause any rejection among potential consumers. These novel films could be used in the design of antimicrobial food packaging and in various other technological areas where sustained-release systems are required.

Keywords Chitosan · Cinnamaldehyde · Schiff base · Hydrolysis · Release · Antimicrobial films

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Introduction

Chitosan is a natural, biocompatible, biodegradable, biorenewable and biofunctional polysaccharide that is finding attractive applications in several industrial areas. Chitosan produces highly transparent grease-resistant films with excellent barrier properties to gases and aroma compounds. These properties make chitosan a good candidate for use in food packaging applications, providing a barrier layer to other polymer films and porous materials such as fibre-based paper (Gallstedt and Hedenqvist 2006). In addition, amino and hydroxyl groups of chitosan can be used to modify the polymer chemically in order to provide it with new properties and widen its technological applications. Moreover, chitosan has been extensively studied as a carrier and system for sustained release of active compounds, and in this regard, it has been applied in a great variety of technological areas, such as agrochemistry, pharmacy, biomedicine, textiles, and active food packaging (Sashiwa and Aiba 2004). The development of antimicrobial materials and their application in the design of active packaging is arousing considerable expectation in the food industry, since food safety is an area of great concern. Regarding chitosan films, many studies in the literature have endeavoured to use the antimicrobial properties of protonated chitosan polymer to form films for active food packaging applications. However, protonated films are water soluble, which limits their applications, whereas neutralized films lack

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Preparation and characterization of chitosan/HP-β-cyclodextrins composites with high sorption capacity for carvacrol

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ABSTRACT

The aim of this work was to design new polymer-based systems exhibiting an adjustable loading capacity of carvacrol depending on the film formulation. For this purpose, biocomposite films were developed employing chitosan (CS) as the polymer matrix and hydroxypropyl-β-cyclodextrins (HP-βCDs) as an adjuvant to improve the sorption of carvacrol in the polymer matrix. The morphology, optical, mechanical and barrier properties of the resulting films were investigated, and the sorption capacity of carvacrol evaluated. Biocomposites resulted highly transparent with higher mechanical resistance and moisture barrier properties. Sorption of carvacrol was greatly affected by the humidity (RH) and glycerol (G) content of the biocomposites. The highest sorption values were achieved for composites incorporating 35% glycerol and conditioned at 75% these composites retained 216% carvacrol (g/100 g dry matter). These results indicate that inclusion of carvacrol in the films could be occurring by mechanisms other than formation of inclusion complexes.

1. Introduction

Chitosan has been widely studied as a polymer for the design of reservoir delivery systems for the slow release of active compounds over an extended period of time. These regulated delivery systems are effective in minimizing the amount of compound used for a specific application and thus improve efficacy and reduce possible side effects associated with the use of large amounts of bioactives (Pedro, Cabral-Albuquerque, Ferreira, & Sarmento, 2009; Sivakumar, Manjubala, & Rao, 2002). Chitosan employed as a delivery system can find applications in a variety of technological areas, such as agrochemistry, pharmacy, biomedicine, textiles and food packaging. The development of antimicrobial materials and their application in the design of active packaging is creating considerable expectation in the food industry, since food safety is an area of great concern. Although there are many studies in the literature that focus on the use of chitosan films as antimicrobials in contact with food, the use of chitosan films for the release of active compounds has received much less attention.

Because of their antimicrobial properties, many plant extracts and essential oils have found applications as natural preservatives. In this regard, carvacrol (5-isopropyl-2-methylphenol) is a constituent of essential oils of oregano and thyme, with known antifungal, insecticidal, antitoxigenic and antiparasitic activities (Burt, 2004; Veldhuizen, Tjeerdsma-Van Bokhoven, Zweijtzer, Burt, & Haagsman, 2006). Carvacrol is categorized as GRAS (Generally Regarded as Safe) by the FDA (Food and Drug Administration) for food. Volatile active compounds can be added to films to achieve a more effective and rational use of them. This step is especially problematic since a large amount of the compounds is lost or inactivated during processing and the remaining amount in the polymer is not enough to exert its effects on the food. In addition, the active compound must be chemically compatible with the polymer matrix to allow good dispersion in the film but not inhibit its release (Chalier, Ben Arfa, Preziosi-Belloy, & Gontard, 2007; Kurek, Descours, Galic, Voilley, & Debeaufort, 2012). Chitosan films have been loaded with active volatile compounds for several purposes (Abdollahi, Rezaei, & Farzi, 2012; Altiok, Altiok, & Tihminlioglu, 2010). However, loading of volatiles in a chitosan matrix presents several difficulties. Its incorporation as an additive into the water-based chitosan film-forming solution is challenging because of general limited aqueous solubility of volatiles and the inevitable partial loss of the compound by evaporation during the casting and drying of the film. In order to overcome these problems, Presence of cyclodextrins in the chitosan matrix could improve compatibility between the polymer matrix and the agent. Moreover, the volatile could be loaded after film casting to avoid agent losses during the film drying step.

Cyclodextrins (CDs) are donut like oligosaccharides with hydrophobic cavities and hydrophilic outer surface. They are widely used as excipients in pharmacy to solubilize lipophilic drugs

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Incorporation of hydroxypropyl-β-cyclodextrins into chitosan films to tailor loading capacity for active aroma compound carvacrol

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ABSTRACT

Chitosan incorporating hydroxypropyl-β-cyclodextrins and glycerol films capable of modulating loading capacity and release of carvacrol have been developed. Films were obtained by casting and conditioned at different relative humidities (RH) prior to immersion in liquid carvacrol. Incorporation of cyclodextrins in the chitosan matrix slightly increased sorption of carvacrol and it was necessary to use glycerol and water as coadjuvants to control loading of the films with the volatile. Good agreement was found between carvacrol retention and plasticization of the film by glycerol and water. The kinetics of carvacrol sorption by the films was evaluated at 25 °C. Diffusion coefficients of sorption varied from 0.011×10^{-14} m^2/s for films incorporating 35% glycerol and conditioned at 0% RH to 1.9×10^{-14} m^2/s for films incorporating 35% glycerol and conditioned at 75% RH. Release of carvacrol from the films was greatly affected by RH. The films showed antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* after 20 days of storage at 25 °C and 43% environmental RH. These films could be useful in the design of systems for delivering active volatiles.

1. Introduction

Delivery systems based on polymers capable of carrying and delivering a continuous supply of biologically active molecules into a specific environment have become of increasing interest in recent years. These systems are able to reduce the amount of active agent required for treatment by maintaining an effective concentration in the system applied over a certain period of time (Ouattara, Simard, Piette, Begin, & Holley, 2000). There is a great need for these devices in diverse technological applications encompassing multidisciplinary areas such as biomedicine, pharmacology, agriculture, packaging, food technology, textiles and the cosmetic industry for the entrapment and delivery of drugs, enzymes, nutraceuticals, agrochemicals, flavours and fragrances, biocides, etc. Currently, most of the recently developed delivery systems consist of natural and synthetic polymers, polymer blends, and composites of organic and inorganic materials that form membranes, capsules or micelles, depending on the application required. Issues concerning biodegradability, biocompatibility and non-toxicity of the materials used for the development of carrier systems need to be considered. Renewable polymers are being widely investigated as delivery vehicles because most of them fulfil the aforementioned requirements.

Chitosan polymer (poly β-(1, 4)N-acetyl-D-glucosamine) has been intensively studied during recent decades (Dutta, Tripathi, & Dutta, 2012; Lopez-Carballo, Higueras, Gavara, & Hernandez-Munoz, 2013; Valencia-Chamorro, Palou, del Rio, & Perez-Gago, 2011). It is a natural cationic linear aminopolysaccharide obtained from partial N-deacetylation of chitin. Chitosan is receiving a great deal of attention in biomedicine and pharmacology for the delivery of drugs (Ramya, Venkatesan, Kim, & Sudha, 2012). Chitosan can also act as a carrier for sustained release and delivery of compounds other than drugs which are of interest in foods, personal care, agriculture, etc. (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Prabaharan & Mano, 2006; Zhang et al., 2009).

Antimicrobial carriers are of great interest in the area of food packaging, and great efforts are being made to develop effective antimicrobial food packaging systems (Appendini & Hotchkiss, 2002; Suppakul, Miltz, Sonneveld, & Bigger, 2003). Antimicrobial food packaging technologies which are based on the incorporation of active volatiles in polymer matrices do not require the film be in contact with the food product to be active. In this case, the volatile is released to the headspace of the package and exerts its activity when contact with the food surface.

There is a wide range of volatile compounds derived from plants or forming part of the aroma profile of fruits presenting

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Antimicrobial packaging of chicken fillets based on the release of carvacrol from chitosan/cyclodextrin films

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ABSTRACT

Chitosan/cyclodextrin films (CS:CD) incorporating carvacrol were obtained by casting, and conditioned at 23 °C and 75% relative humidity prior to being immersed in liquid carvacrol until they reached sorption equilibrium. In a previous work, the in vitro antimicrobial activity of these films was studied. In this work, active films were used to inhibit microbial growth in packaged chicken breast fillets. Samples of CS:CD films loaded with carvacrol, of different sizes and thus with different quantities of antimicrobial agent, were stuck to the aluminium lid used to seal PP/EVOH/PP cups containing 25 g of chicken fillets. These samples were stored for 9 days at 4 °C. The packages were hermetically sealed and it was confirmed that they provided an infinite barrier to carvacrol. The partition of the antimicrobial agent within the food/packaging system was analysed. The antimicrobial devices rapidly released a large percentage of the agent load, amounts that were gained by the adhesive coating of the lid and especially by the chicken fillets. The latter were the main sorbent phase, with average concentrations ranging between 200 and 5000 mg/Kg during the period of storage. The microbiota of the packaged fresh chicken fillets – mesophiles, psychrophiles, *Pseudomonas* spp., enterobacteria, lactic acid bacteria and yeasts and fungi – were analysed and monitored during storage. A general microbial inhibition was observed, increasing with the size of the active device. Inhibition with a 24 cm^2 device ranged from 0.3 log reductions against lactic acid bacteria to 1.8 logs against yeasts and fungi. However, the large amount of antimicrobial that was sorbed or that reacted with the fillet caused an unacceptable sensory deterioration. These high sorption values are probably due to a great chemical compatibility between chicken proteins and carvacrol.

1. Introduction

Antimicrobial packaging systems have received special attention in recent years because they can provide an important obstacle in the so-called "hurdle" technologies that are being implemented to commercialize fresher food products. In these systems, interactions between the food, the surrounding headspace and the package walls or independent devices are responsible for growth inhibition or death of pathogenic and/or spoilage microorganisms (Appendini and Hotchkiss, 2002). Although, exceptionally, this beneficial activity can be carried out by direct contact of the microorganisms with the package surface (Conte et al., 2008; Muriel-Galet et al., 2013; Zi-Xuan et al., 2012), the mechanism of activity in most packaging systems is based on mass transport processes through the packaging/food interface, and especially on the release of antimicrobial substances. The active agent can be incorporated within a suitable polymeric matrix from which it is released following diffusion mechanisms and accumulated into the food package system following thermodynamic principles. Therefore, compatibility between the agent and the various system components and diffusion from the polymeric vehicles are key parameters, and the efficiency and validity of an active packaging has to be tested for the specific product.

Recently, a biopolymeric device consisting of a combination of two biodegradable, renewable materials, chitosan and HP-β-cyclodextrins, and a known natural volatile antimicrobial compound, carvacrol, was developed and characterized. In that report, the film demonstrated a great capacity for sorption of carvacrol. The kinetics of release in ideal conditions were also measured and the in vitro antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* was tested (Higueras et al., 2013; Higueras et al., 2014).

In this work, a package containing this device was designed and used as an active package containing fresh chicken fillets. The partition of carvacrol among the different components of the food/active package/environment system was studied and the effect of the active packaging on the chicken microbiota was analysed.

2. Materials and methods

2.1. Materials

Carvacrol (kosher > 98%), glycerol, acetic acid and low molecular weight chitosan were supplied by Sigma (Barcelona, Spain).

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Quitosano como matriz biopolimérica para el desarrollo de envases activos antimicrobianos de alimentos

En esta Tesis se ha abordado el desarrollo de películas renovables y activas de quitosano con agentes antimicrobianos de diversa naturaleza para su aplicación en el envasado activo de alimentos. Las películas de quitosano se obtuvieron mediante la técnica de extensión y evaporación del solvente, y las sustancias bioactivas fueron incorporadas mediante diferentes metodologías.

En primer lugar, el sólido etil-*N*$^{\alpha}$-dodecanoil-*L*-arginato (LAE) se incorporó previamente a la formación de la película de quitosano. Las películas con LAE liberaron completamente el agente activo en un simulante alimentario acuoso en varias horas, a diferentes temperaturas. Las películas de quitosano, que no fueron neutralizadas, presentaron actividad antimicrobiana frente a microorganismos presentes en pechugas de pollo frescas. Esta actividad antimicrobiana se incrementó al incorporar el LAE a la matriz, reduciendo satisfactoriamente los microorganismos presentes en el alimento para el control del deterioro microbiano de pollo fresco envasado.

En segundo lugar, se incorporó el precursor nitrato de plata a la solución filmógena de quitosano, para generar *in situ* nanopartículas de plata durante la neutralización de las películas de acetato de quitosano. Las películas desarrolladas presentaron una actividad antibacteriana *in vitro* mantenida en el tiempo. Las nanopartículas de plata pudieron actuar como reservorios de iones de plata liberados sostenidamente en un entorno acuoso.

El tercer mecanismo para incorporar el agente antimicrobiano fue el anclaje reversible del volátil cinamaldehído a películas preformadas de quitosano mediante la formación de una base de Schiff. Este enlace covalente fue hidrolizado tras la aplicación de diversos tratamientos de temperatura/tiempo habituales en la conservación de alimentos en un medio acuoso, liberándose el cinamaldehído. Las películas iminoderivadas aumentaron la seguridad microbiológica de la leche. La leche con el cinamaldehído liberado fue aceptada sensorialmente.

El último método empleado se basó en la incorporación de monoterpenos antimicrobianos, principalmente carvacrol, en películas de quitosano con coadyuvantes mediante la inmersión de las películas preformadas en el agente volátil en estado líquido. La presencia conjunta de hidroxipropil-β-ciclodextrinas, glicerol y agua en las películas de quitosano dio lugar a una elevada capacidad de sorción de carvacrol de carácter lipófilo. La liberación del carvacrol presente en las películas compuestas fue activada por la humedad relativa ambiental. Este carvacrol liberado de las películas presentó efectividad antimicrobiana en fase vapor cuando fue incorporado en un sistema de envasado para pechugas de pollo fresco. La capacidad de retención de las películas se pudo modular en función de su formulación y de la estructura química del monoterpeno sorbido. Las películas con dichos monoterpenos presentaron capacidad antimicrobiana en fase vapor *in vitro*, y pueden emplearse en el diseño de envases activos adaptados al alimento aplicado.

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