UNIVERSIDAD POLITÉCNICA DE VALENCIA
Departamento de Tecnología de Alimentos

DESARROLLO DE RECUBRIMIENTOS COMESTIBLES CON ACTIVIDAD ANTIFÚNGICA EN FRUTOS CÍTRICOS

DEVELOPMENT OF EDIBLE COMPOSITE COATINGS WITH ANTIFUNGAL ACTIVITY ON CITRUS FRUIT

TESIS DOCTORAL

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CERTIFICAN: Que la memoria titulada "Desarrollo de Recubrimientos Comestibles con Actividad Antifúngica en Frutos Cítricos", que, para aspirar al grado de Doctor en Ciencia y Tecnología de los Alimentos presenta Dña. Silvia Azucena Valencia Chamorro, realizada bajo nuestra dirección en el Centro de Tecnología Poscosecha del Instituto Valenciano de Investigaciones Agrarias, cumple las condiciones adecuadas para su aceptación como Tesis Doctoral, por lo que

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"El mundo está en las manos de aquellos
que tienen el coraje de soñar....

.... Y correr el riesgo de vivir sus sueños"

Paulo Coelho.

Dedicado a mis padres Leopoldina y Gabriel, a quienes debo todo lo que soy, por su infinito amor y cuidados, por apoyarme incondicionalmente para convertir mis sueños en realidad.

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En la industria citrícola, las pérdidas económicas más importantes en poscosecha se deben a las podredumbres verde y azul, causadas por los hongos *Penicillium digitatum* (Pers.:Fr.) Sacc. y *Penicillium italicum* Wehmer. Durante muchos años, se utilizaron ampliamente los fungicidas químicos para el control de estas enfermedades. Sin embargo, la preocupación de los consumidores por el uso excesivo y prolongado de estos productos para el control de las podredumbres ha orientado a los investigadores a buscar métodos alternativos no contaminantes que no depositen residuos peligrosos ni contaminen el ambiente.

El uso de películas y recubrimientos comestibles es un método respetuoso con el ambiente que incrementa la vida útil de muchos alimentos incluidas las frutas y verduras. Sin embargo, muy poca investigación se ha enfocado al desarrollo de recubrimientos comestibles compuestos con la adición de compuestos antifúngicos como un nuevo método para controlar las enfermedades poscosecha en frutos cítricos frescos.

El objetivo general de esta tesis doctoral fue desarrollar nuevos recubrimientos comestibles compuestos con la adición de aditivos alimentarios antifúngicos para el control de las podredumbres verde y azul en cultivares de cítricos comercialmente importantes. Primero, se desarrollaron las nuevas películas comestibles compuestas en base a hidroxipropil metilcelulosa (HPMC)-lípido con la adición de aditivos alimentarios o compuestos generalmente reconocidos como seguros (GRAS, por sus siglas en inglés), y se seleccionaron de acuerdo a su capacidad de formar emulsiones estables. Las películas se evaluaron por su actividad *in vitro* contra *P. digitatum* y *P. italicum* y sus propiedades mecánicas y de barrera (Capítulo 1). Luego, las emulsiones seleccionadas se usaron para pruebas *in vivo* en especies y cultivares de cítricos comercialmente importantes y se determinó su actividad antifúngica curativa (fruta recubierta después de la inoculación) y preventiva (fruta recubierta antes de la inoculación fungíca) contra las podredumbres verde y azul (Capítulo 2). Después, se evaluó el efecto de la aplicación de los recubrimientos antifúngicos seleccionados en el desarrollo de las podredumbres y en la calidad fisico-química y sensorial de naranjas 'Valencia Late', y mandarinas 'Ortanique' y 'Clemenules' durante el almacenamiento en frío prolongado (Capítulos 3, 4 y 5). Finalmente, se determinó la efectividad de recubrimientos comestibles de quitosano en

el control de las podredumbres verde y azul en naranjas 'Valencia Late' y mandarinas 'Oronules' (Capítulo 6).

De las más de 470 emulsiones preparadas de HPMC-lípido con la adición de aditivos alimentarios (principalmente sales minerales, sales de ácidos orgánicos, y sales de parabenos), solo alrededor de 5% fueron estables. Las películas comestibles y los recubrimientos que contenían parabenos, sorbato de potasio (PS), benzoato de sodio (SB), y algunas mezclas fueron las más efectivas para inhibir el crecimiento *in vitro* de *P. digitatum* y *P. italicum*, y controlar el desarrollo *in vivo* de las podredumbres verde y azul en naranjas 'Valencia Late' y mandarinas 'Ortanique' y 'Clemenules' (acción curativa). Se observaron diferencias importantes en la actividad antifúngica entre los ensayos *in vitro* e *in vivo*. En general, la actividad curativa de los recubrimientos después de la incubación a 20 ºC durante 7 días fue mayor en naranjas que en mandarinas. La actividad fue más fungistática que fungicida. Los recubrimientos ensayados no presentaron ninguna actividad preventiva contra los dos hongos. La composición del lípido y las propiedades de los preservativos alimentarios adicionados influyeron de forma importante en las propiedades mecánicas y de barrera de las películas seleccionadas.

Durante un almacenamiento a 5 ºC de hasta 2 meses, los recubrimientos que contenían PS+SP y SB fueron los más efectivos inhibiendo los dos hongos en naranjas 'Valencia Late' y mandarinas 'Ortanique', siendo mayor la actividad antifúngica en naranjas que en mandarinas. Aunque los recubrimientos no redujeron la pérdida de peso en naranjas 'Valencia Late', no afectaron negativamente la calidad físico-química de la fruta. Los recubrimientos redujeron significativamente la pérdida de peso y mantuvieron la firmeza de las mandarinas 'Ortanique' y 'Clemenules'. En todos los casos, la calidad sensorial fue evaluada como aceptable.

Puesto que los recubrimientos comestibles de HPMC-lípido seleccionados redujeron de forma efectiva el desarrollo de los hongos y preservaron la calidad de la fruta durante el almacenamiento en frío, se presentan como una alternativa comercial no contaminante a las ceras convencionales fungicidas usadas en cítricos. Las futuras investigaciones deberían enfocarse en mejorar su brillo y aspecto visual, y en la combinación con otros métodos alternativos de control para encontrar

sinergias o actividad complementaria dentro de un programa de control integrado de las enfermedades.

En general la efectividad de los recubrimientos de quitosano dependió de la densidad de inóculo, del cultivar, y de las condiciones de almacenamiento, pero no fue suficiente para el control efectivo de la pudrición a nivel comercial.

En la indústria citrícola, les pèrdues econòmiques més importants en postcollita són degudesa les podridures verda i blava causades pels fongs *Penicillium digitatum* (Pers.:Fr.) Sacc. i *Penicillium italicum* Wehmer. Durant molts anys, es van utilitzar contínuament els fungicides químics per al control d'estes malalties. No obstant això, la preocupació dels consumidors per l'ús excessiu i prolongat d'estos productes per al control de les podridures ha orientat els investigadors a buscar mètodes alternatius no contaminants que no depositen residus perillosos ni contaminen l'ambient.

L'ús de pel·lícules i recobriments comestibles és un mètode respectuós amb l'ambient que incrementa la vida útil de molts aliments, inclosos les fruites i verdures. No obstant això, molt poca investigació s'ha enfocat al desenrotllament de nous recobriments comestibles compostos amb l'addició de compostos antifúngics com un nou mètode per controlar les malalties postcollita en fruits cítrics frescos.

L'objectiu general d'esta tesi doctoral va ser desenrotllar nous recobriments comestibles compostos amb l'addició d'additius alimentaris antifúngics per al control de les podridures verda i blava en cultivars de cítrics comercialment importants. Primer, es van desenrotllar les noves pel·lícules comestibles compostes basades en hidroxipropil metilcelulosa (HPMC)-lípid amb l'addició d'additius alimentaris o compostos generalment reconeguts com a segurs (GRAS, per les seues sigles en anglès), i es van seleccionar d'acord amb la seua capacitat de formar emulsions estables. Les pel·lícules es van avaluar per la seua activitat *in vitro* contra *P. digitatum* i *P. italicum*, i per seues propietats mecàniques i de barrera (Capítol 1). Després, les emulsions seleccionades es van usar per a les proves *in vivo* en espècies de cítrics i cultivars comercialment importants i es va determinar la seua activitat antifúngica curativa (fruita recoberta després de la inoculació fúngica) i preventiva (fruita recoberta abans de la inoculació) contra les podridures verda i blava (Capítol 2). Posteriorment, es va avaluar l'efecte de l'aplicació dels recobriments antifúngics seleccionats en el desenrotllament les podridures i en la qualitat fisicoquímica i sensorial de taronges 'Valencia Late', i mandarines 'Ortanique' i 'Clemenules' durant l'emmagatzemament prolongat (Capítols 3, 4 i 5). Finalment, es va determinar l'efectivitat de recobriments comestibles de quitosan en el control de les podridures

verda i blava en taronges 'Valencia Late' i mandarines 'Oronules' (Capítol 6).

De les més de 470 emulsions preparades de HPMC-lípid amb l'addició d'additius alimentaris (principalment sals minerals, sals d'àcids orgànics, i sals de parabens), només al voltant del 5% van ser estables. Les pel·lícules comestibles i els recobriments que contenien parabens, sorbat de potassi (PS), benzoat de sodi (SB), i algunes mescles van ser els més efectiu per inhibir el creixement *in vitro* de *P. digitatum* i *P. italicum* i controlar el desenrotllament *in vivo* de les podridures verda i blava en taronges 'Valencia Late' i mandarines 'Ortanique' i 'Clemenules' (acció curativa). Es van observar diferències importants en l'activitat antifúngica entre els assajos *in vitro* i *in vivo*. En general, l'activitat curativa dels recobriments després de la incubació a 20 ºC durant 7 dies va ser major en taronges que en mandarines. L'activitat va ser mes fungistática que fungicida. Els recobriments assajats no van presentar activitat preventiva contra els dos fongs. La composició del lípid i les propietats dels preservatius alimentaris afegits van influir de manera important en les propietats mecàniques i de barrera de les pel·lícules seleccionades.

Durant un emmagatzemament a 5 ºC de fins a 2 mesos, els recobriments que contenien PS+SP i SB van ser els més efectius en la inhibició dos fongs en taronges 'Valencia Late' i mandarines 'Ortanique', sent major l'activitat antifúngica en taronges que en mandarines.

Encara que els recobriments no van reduir la pèrdua de pes en taronges 'Valencia Late', tampoc van afectar negativament la qualitat fisicoquímica de la fruita. Els recobriments van reduir significativament la pèrdua de pes i van mantindre la fermesa de les mandarines 'Ortanique' i 'Clemenules'. En tots els casos, la qualitat sensorial va ser avaluada com acceptable.

Ja que els recobriments comestibles HPMC-lípid seleccionats van reduir efectivament el desenrotllament dels fongs i van preservar la qualitat de la fruita durant l'emmagatzemament en fred, es presenten con una alternativa comercial no contaminant a les ceres convencionals fungicides usades en cítrics. Futures investigacions haurien d'enfocar-se a millorar la seua lluentor i aspecte visual, i també a la combinació amb altres mètodes alternatius de control per trobar sinergies o activitat complementàra dintre d´un programa de control integrat de les malalties.

En general l'efectivitat dels recobriments de quitosan va dependre de la densitat d´inocul, del cultivar, i de les condicion d'emmagatzemament, però no va ser suficient per a un control efectiva de les podridures a nivell comercial.

In the citrus industry, very important economical losses are primarily caused by postharvest green and blue molds, caused by the pathogens *Penicillium digitatum* (Pers.:Fr.) Sacc. and *Penicillium italicum* Wehmer. For many years, chemical fungicides have been widely applied to control these diseases. However, consumer concerns about prolonged and extensive use of chemical fungicides to control citrus postharvest decay lead researchers to look for alternative nonpolluting methods that do not deposit harmful residues in/on fruit and contaminate the environment.

The use of edible films and coatings is an environmentally-friendly alternative method to increase the shelf-life of many food products including fruits and vegetables. However, very little research has been focused on the development of edible composite coatings with the addition of antifungal compounds as a new method to control major fungal postharvest diseases of fresh citrus fruit.

The general objective of the present doctoral thesis was to develop new edible composite coatings with the addition of antifungal food additives for the control of posharvest green and blue molds on commercially important citrus cultivars. Firstly, new hydroxypropyl methylcellulose (HPMC)-lipid edible composite films containing food additives or generally recognized as safe (GRAS) compounds with antifungal properties were developed and selected according to their capability of forming stable emulsions. Films from stable emulsions were evaluated for their *in vitro* activity against *P. digitatum* and *P. italicum* and their mechanical and barrier properties (Chapter 1). Then, selected emulsions were used in *in vivo* tests to coat commercially important citrus species and cultivars and determine their curative (fruit coated after fungal inoculation) and preventive (fruit coated before fungal inoculation) antifungal activity against green and blue molds (Chapter 2). Next, the effect of the application of selected antifungal coatings on the development of penicillium molds and the physico-chemical and sensory quality of 'Valencia' oranges, and 'Ortanique' and 'Clemenules' mandarins during long-term cold storage was assessed (Chapters 3, 4, and 5). Finally, the effectiveness of chitosan edible coatings to control green and blue molds of 'Valencia' oranges and 'Oronules' mandarins was determined (Chapter 6).

Only around 5% of about 470 emulsion formulations prepared by incorporating food preservatives (mostly mineral salts, salts of organic acids, salts of parabens) to HPMC-lipid were stable. Edible films and coatings containing parabens, potassium sorbate (PS), sodium benzoate (SB), and some mixtures were the most effective to inhibit the *in vitro* growth of *P. digitatum* and *P. italicum*, and control *in vivo* the development of green and blue molds on previously inoculated 'Valencia' oranges, and 'Ortanique' and 'Clemenules' mandarins (curative action). Important differences in antifungal activity were observed between *in vitro* and *in vivo* tests. In general, the curative activity of the coatings after incubation at 20 ºC for 7 d was higher on oranges than on mandarins. The activity was fungistatic rather than fungicidal. The tested coatings did not provide any preventive activity against both molds. The lipid composition and the properties of added food preservatives greatly influenced the barrier and mechanical properties of selected films.

During cold storage at 5 ºC for up to 2 months, the PS+SP- and SB-based coatings were the most effective to inhibit both molds on 'Valencia' oranges and 'Ortanique' mandarins, being the antifungal activity higher on oranges than on mandarins. Although the coatings did not reduce weight loss of 'Valencia' oranges, they did not adversely affect the fruit physico-chemical quality. The coatings significantly reduced weight loss and maintained the firmness of coated 'Ortanique' and 'Clemenules' mandarins. Sensory quality was evaluated as acceptable in all cases.

Since selected HPMC-lipid edible coatings effectively reduced mold development and preserved fruit quality during cold storage, they showed promise as nonpolluting commercial alternatives to conventional fungicide-amended citrus waxes. Further research should focus on the modification of some physical characteristics of the coatings to improve their gloss and visual aspect, and their combination with other alternative control methods to find synergistic or complementary activities in an integrated disease management approach.

In general, the effectiveness of chitosan-based coatings was highly dependent on inoculum density, fruit cultivar, and storage conditions, but it was not consistently high enough for commercial decay control.

ABBREVIATIONS

BM	blue mold
BW	beeswax
CFU	colony forming units
db	dry basis
EA	egg albumen
%E	elongation at break
GM	green mold
GRAS	generally recognized as safe
GSE	grape seed extracts
HPMC	hydroxypropyl methylcellulose
IU	international units
MC	methylcellulose
OP	oxygen permeability
PD	*Penicillium digitatum*
PI	*Penicillium italicum*
PS	potassium sorbate
RH	relative humidity
SB	sodium benzoate
SC	solid concentration
SEP	sodium ethyl paraben
SMP	sodium methyl paraben
SP	sodium propionate
SPI	soy protein isolated
SPP	sodium propyl paraben
TS	tensile strength
wb	wet basis
WG	wheat gluten
WPI	whey protein isolated
WVP	water vapor permeability
YM	Young's modulus

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INTRODUCTION

ANTIMICROBIAL EDIBLE FILMS AND COATINGS FOR FRUITS AND VEGETABLES: A REVIEW

New edible films and coatings from natural products have been developed for fresh and processed food products. Edible films and coatings are an environmentally friendly technology that, to some extent, may replace plastic packaging by natural and biodegradable substances. Their use could lead to an important reduction on the overall packaging requirements and, therefore, waste disposal problems.

Edible films and coatings may enhance food quality, safety, stability, and the mechanical–handling properties by providing a semi-permeable barrier to water vapor, oxygen, and carbon dioxide between the food and the surrounding atmosphere (Greener and Fennema, 1994). Edible films and coatings can also be used as carriers of antioxidants, flavoring agents, coloring agents, growth regulators and antimicrobials, that will improve food quality and safety (Vojdani and Torres, 1990; Cuppet, 1994; Yaman and Baymdirh, 2001; Coma et al., 2002). In fresh fruits and vegetables, the creation of a moisture and gas barrier may lead to weight loss and respiration rate reductions with a consequent general delay of produce senescence (Hagenmaier and Baker, 1993; Debeaufort et al., 1998; Pérez-Gago et al., 2002). Furthermore, the application of coatings may improve the visual quality by providing gloss to the coated commodities (Trezza and Krochta, 2000).

In the last decade, many works have focused on the development of films and coatings based on proteins and polysaccharides with food additives from chemical or natural sources to control microbial growth on fresh and processed food. Edible films and coatings containing antimicrobials, such as organic acids and their salts, parabens, chitosan, essential oils, or natural plant extracts have been effective in delaying the growth of contaminating microorganisms and maintaining the quality during storage and distribution of fresh and minimally processed horticultural products.

This paper reviews existing literature on: (1) edible films prepared with antimicrobial food additives ingredients and their effect on the control of target microorganisms; (2) the effect of antimicrobials agents on mechanical and barrier properties of stand-alone edible films; and (3)

the effect of antimicrobial edible coatings on the quality of fruits and vegetables.

1. Edible films and coatings: definition, composition and functional properties

1.1. Films versus coatings

Films are usually defined as a stand-alone thin layer of materials that can be used as covers, wraps or separation layers. However, the main use of stand-alone films is as testing structures for determination of barrier, mechanical, solubility, and other properties provided by a certain film material. Coatings involve the formation of films directly on the surface of the product they are intended to protect or enhance (Krochta, 2002). Therefore, edible coatings are considered part of the final food product and, therefore, should confer acceptable color, odor, taste, flavor and texture to the coated product.

1.2. Edible film and coating materials

According to their components, edible films and coatings can be divided into three categories: hydrocolloids, lipids, and composites. Hydrocolloids include proteins and polysaccharides. Lipids include waxes, acylglycerols, and fatty acids. Composites contain both hydrocolloid components and lipids (Greener and Fennema, 1994; Nisperos-Carriedo, 1994; Baldwin, 1999). Several other compounds such as plasticizers and emulsifiers may be added to edible films and coatings to improve their mechanical properties and to form stable emulsions when lipids and hydrocolloids are combined. In addition, edible coatings and films can also act as carriers of food additives, including antioxidants, colorants, flavoring agents, and antimicrobial compounds (Cuppet, 1994; Baldwin, 1999; Franssen and Krochta, 2000; Cha and Chinnan, 2004; Han and Gennadios, 2005).

Highly polar polymers containing hydroxyl groups, such as proteins and polysaccharides, generally present a good barrier to oxygen at low

relative humidity (RH) due to their tightly packed, ordered hydrogen-bonded network structure and low solubility (McHugh and Krochta, 1994). However, they form a poor moisture barrier due to their hydrophilic character. Polysaccharides film-forming materials include starch and starch derivatives, cellulose derivatives, alginate, carrageenan, pectin, pullulan, chitosan, and various gums (Han and Gennadios, 2005).

Proteins that have received great attention to form edible films and coatings include corn zein, wheat gluten (WG), soy protein, whey protein, casein, collagen/gelatin, pea protein, rice bran protein, cottonseed protein, peanut protein, and keratin (Baldwin and Baker, 2002; Han and Gennadios, 2005). However, some considerations respect to food intolerances such as wheat gluten intolerance (celiac disease), or milk protein intolerance, allergies, or religious believes/banning should be taken into account when protein-based films and coatings are used**.**

Lipids and resins, due to their hydrophobic nature, are used in edible films and coatings to provide a barrier to moisture. In addition, they are often used to provide gloss to food surfaces (Greener and Fennema, 1994). However, because lipids and resins are not polymers, they form films and coatings with poor mechanical properties. Lipids and resins used for the preparation of lipid-based edible films and coatings include neutral lipids, fatty acids, waxes (beeswax (BW), candelilla wax, carnauba wax, rice bran wax), and resins (shellac, wood rosin) (Rhim and Shellhammer, 2005).

Composite films and coatings comprise hydrocolloid components and lipids, thus enhancing the advantages and lessening the disadvantages of each. A composite film can be produced as either a bi-layer or a stable emulsion. In bi-layer composite films, the lipid forms a second layer over the polysaccharide or protein layer. In emulsion composite films, the lipid is dispersed and entrapped in the supporting matrix of protein or polysaccharide (Shellhammer and Krochta, 1997; Pérez-Gago and Krochta, 2005). In composite edible films and coatings, the efficiency of the lipid materials depends on the lipid structure, its chemical arrangement, hydrophobicity, physical state, and its interaction with other components of the film (Rhim and Shellhammer, 2005).

Plasticizers are low molecular weight agents that are incorporated into film-forming materials to decrease the intermolecular forces

between polymer chains, which results in greater film flexibility, elongation and toughness. However, they can also increase film permeability (Han and Gennadios, 2005). Plasticizers used for edible films and coatings include sucrose, glycerol, sorbitol, propylene glycol, polyethylene glycol, fatty acids, and monoglycerides. Water also acts as a plasticizer for polysaccharide and protein edible films (Krochta, 1997). Thus, the film moisture content, as affected by relative humidity (RH), has a large effect on film properties.

Emulsifiers or surfactants are surface-active agents of amphiphilic nature, which interact at the water-lipid interface and reduce surface tension between the dispersed and continuous phases to improve the stability of the emulsion (Han and Gennadios, 2005). They are also used to ensure good surface wetting, spreading, and adhesion of the coating to the food surface. Common emulsifiers used on films and coatings are fatty acids, ethylene glycol monostearate, glycerol monostearate, esters of fatty acids, lecithin, sucrose ester, sobitan monostereate or polysorbates (Tweens).

1.3. Functions and properties of edible films and coatings

The main function of edible films and coatings is to offer a protective barrier to moisture, oxygen, flavor, aroma and/or oil between the food and the environment. In addition, edible films and coatings may also maintain food integrity by providing some mechanical protection. The protective function of edible films and coatings may be enhanced with the addition of antioxidants, antimicrobials, flavors, nutrients, etc.

Given that the main interest in edible films and coatings is generally based on their barrier and protective functions, more of the studies are focused on determining these properties on stand-alone edible films. Barrier properties commonly studied to determine the ability to protect foods from the environment and from adjacent ingredients are water vapor permeability (WVP) and oxygen permeability (OP). Aroma and oil permeability are also very important for many foods but have generally received less attention.

The ability of edible films and coatings to protect food against mechanical damage is usually assessed by determining film tensile

properties: (1) Young's Modulus (YM), which determines film stiffness as determined by ratio of pulling force/area to degree-of-film-stretch, (2) tensile strength (TS), which indicates the pulling force per film cross-sectional area required to break the film, and (3) elongation, which gives the degree to which the film can stretch before breaking and it is expressed as percentage of elongation (%E) (Krochta, 2002). Other film properties that have been investigated include film water solubility, gloss, and color.

Because of the possibility of adding antimicrobials to edible films and coatings, experiments have been performed to examine the antimicrobial properties of stand-alone films. Different methods are being used to determine the effectiveness of antimicrobial films against target microorganisms. Antimicrobial assays include: (1) 'agar diffusion test', in which an antimicrobial film is placed over a lawn of the microorganism growing on a agar media plate, and the radius or diameter of the zone of inhibition of microbial growth around the antimicrobial film is measured. This method is relatively simple and easy to perform, but the quantitative zone measurements from different studies are difficult to compare because of the many specific conditions of the experiments; (2) the colony diameter test that presents a slight variation from the agar diffusion test. The antimicrobial film is placed on a plate covered with inoculum, the plate is incubated and the diameters of the colony that grow are measured. This test is also easy to perform; (3) the 'cell count assay' or 'log reduction assay', involves placing the film in a microorganism growing broth solution and removing samples from the solution over time. The solution sample is then plated on agar media and colonies are counted. The results of these experimental approach are not directly applicable to a coated food product because of different experimental conditions.

There are other tests intended determine the ability of the film to release the antimicrobial. These test may provide information about (1) the antimicrobial release rate or the amount of antimicrobial that is released over time; (2) the antimicrobial diffusion coefficient that gives a quantitative measurement of the rate at which the diffusion process occurs; and (3) the antimicrobial permeability coefficient (Franssen and Krochta, 2000; Nychas and Skandamis, 2000).

2. Antimicrobial food additives

Additives used to prevent biological deterioration are termed antimicrobials or preservatives. As they allowed for food contact applications, this category include natural or synthetic compounds with known and minimal toxicological effects on mammals and the environment. Antimicrobial compounds include organic acids and their salts, carbonates and bicarbonates, parabens, chitosan, enzymes, bacteriocins, polypeptides, natural extracts, or essential oils.

A wide variety of antimicrobials have been added to edible films and coatings to control microbiological growth and extend produce shelf life. Antimicrobials used for the formulation of edible films and coatings must be classified as food-grade additives or compounds generally recognized as safe (GRAS) by the appropriate regulations. International regulatory agencies are in charge of approving antimicrobials for the use on foods. In the European Union those compounds are regulated by the the European Union (EU, 1989) and in the United Sates by the U.S. Food and Drug Administration (FDA, 2008). Table 1 shows the common antimicrobial agents used on edible films and coatings and their number codes for food additives approved by the European Union (E-code) or the FDA (FDA-code).

Table 1. Antimicrobial compounds used on edible films and coatings

Food preservative			
Chemical compounds	E- Code[a]	Natural compounds	E- Code/ REGNUM[b]
Organic acids		***Polypeptides***	
Acetic	E-260	Lysozyme	E-1105
Benzoic	E-210	Peroxidase	-
Citric	E-330	Lactoperoxidase	-
Lactic	E-270	Lactoferrin	-
Malic	E-296	Nisin	E-234
Propionic	E-280	Natamycin	E-235
Sorbic	E-200		
Tartaric	E-334		
Organic acid salts		***Plant extracts, essential oils, spices***	
Sodium acetate	E-262-(i)	Cinnamon	182.10
Sodium diacetate	E-262-(II)	Capsicum	182.10
Sodium benzoate	E-211	Lemongrass	182.20
Sodium citrate	E-331(I)	Oregano	182.10
Sodium formate	E-237	Rosemary	182.20
Calcium formate	E-238	Garlic	184.1317
Sodium L-lactate	E-325	Vanilla	182.10
Sodium propionate	E-281	Carvacrol	172.515
Calcium propionate	E-282	Citral	182.60
Potassium sorbate	E-202	Cinnamaldehyde	182.60
Sodium L-tartrate	E-335 (I)	Vanillin	182.60
		Grape seed extracts	-
Parabens			
Methyl paraben	E-218		
Ethyl paraben	E-214		
Propyl paraben	E-216		
Sodium salt of methyl paraben	E-219		
Sodium salt of ethyl paraben	E-215		
Sodium salt of propyl paraben	E-217		
Mmineral salts			
Sodium bicarbonate	E-500(I)		
Ammonium bicarbonate	E-237		
Sodium carbonate	E-500(II)		
Others			
EDTA-$CaNa_2$[c]	E-385		

[a] E-code = number codes for food additives approved by the European Union [b] REGNUM = Regulation numbers in Title 21 of the U.S. Code of Federal Regulations where the chemical appears. [c] EDTA-$CaNa_2$ = disodium calcium ethylenediamine-tetraacetate.

2.1. Antimicrobial synthetic chemical agents

Organic acids are the most common synthetic chemical antimicrobial agents and include acetic, benzoic, citric, fumaric, lactic, malic, propionic, sorbic, succinic, and tartaric acid, among others. These acids inhibit the outgrowth of bacterial and fungal cells. Potassium sorbate (PS) and sodium benzoate (SB) are the two organic acid salts more widely used as antimicrobial food additives. Benzoic acid is also called phenylformic acid or benzene-carboxylic acid. The antimicrobial activity of benzoic acid and SB is related to pH, and the most effective are the undissociated forms. Therefore, the use of these preservative has been limited to those products that are acid in nature (Chipley, 2005). Sorbic acid is a straight-chain unsaturated fatty acid. The carboxyl group of sorbic acid is highly reactive with calcium, sodium or potassium, and results in formation of various salts and esters (Stopforth et al., 2005b). PS, the most soluble form of sorbate is well known for its potent antifungal activity. The antimicrobial action of sorbate is also pH dependent. In general, PS activity is greater at low pH values, although sorbates may be effective at pH values as high as 7. In contrast, other common organic acid-based antimicrobials, such as propionates or benzoates, only show considerable antimicrobial activity at low pH values such as 5-5.5 and 4-4.5, respectively (Stopforth et al., 2005b). Bacterial species and mold species inhibited by sorbates belong to the genera *Alternaria, Penicillium*, and others. Several studies have also indicated increased antimicrobial effects when sorbate was combined with various phosphates. Combinations of sorbate with benzoate or propionate may be used to expand the range of inhibited microorganisms with reduced concentrations of each preservative (Stopforth et al., 2005b).

Propionic acid is a naturally-occurring monocarboxylic acid. Salts of the acid have a slight cheeslike flavor likewise, the antimicrobial activity of propionate salts is pH dependent, being also more effective in their undissociated form at low pH. Propionic acid is primarily inhibitory to molds, however, some yeast and bacteria have been also inhibited (Doores, 2005).

Parabens are the alkyl esters of *para*-hydroxybenzoic acid. The alkyl chain length of parabens determines their water solubility. The lower alkyl chain length, the higher the water solubility of parabens. Parabens are inhibitory to either several gram-positive and gram-negative bacteria or molds, but fungi are generally more susceptible to parabens than bacteria (Davidson, 2005). For both bacteria and fungi, the inhibitory activity generally increases as the alkyl chain length of parabens increases. The optimum pH for effective antimicrobial activity of parabens is in the range 3.0-8.0.

2.2. Natural antimicrobial agents

Natural antimicrobial agents include chitosan, polypetides, and plant oils, spices and extracts.

Chitosan is a polysaccharide prepared by deacetylation of chitin. It is composed of β-1,4 linked glucosamine units and N-acetyl glucosamine residues. It is obtained by the alkaline deacetylation of chitin, the most abundant component of the shells of crustaceans (Coma et al., 2002; No et al., 2007). Chitosan antimicrobial activity comes from its positive charges that would interfere with the negatively charged residues of macromolecules on the cell surface, rendering membrane leakage (Sebti et al., 2007). Functional properties and antimicrobial effects of chitosan are related to its deacetylation degree and molecular weight. Chitosan inhibits the growth of a wide variety of fungi, yeasts and bacteria. Due to its film forming property, chitosan is used to prepare films and coatings (No et al., 2007).

Nisin, a hydrophobic protein, is a low-molecular-weight polypeptide produced by the bacterial dairy starter *Lactococcus lactis* subspecies *lactis*. Nisin has a broad spectrum of activity against gram-positive bacteria, but do not significantly inhibit gram-negative bacteria, yeasts or molds (Thomas and Delves-Broughton, 2005). Nisin was proved to be non-toxic and GRAS by the U.S. FDA in 1969. Since then, it has been widely used in the food industry as a safe and natural preservative (Sebti et al., 2007).

Natamycin is a tetraene polyene macrolide. It is a natural antifungal agent produced by *Streptomyces natelensis*. Natamycin has no effect on

bacteria, but it is active against nearly all molds and yeasts. Natamycin is usually applied as a surface treatment for hard cheese and dry or ripened sausages (Türe et al., 2009b).

Lactoperoxidase is a hemoprotein present in milk, tears, and salive. The lactoperoxidase system consists of three components: lactoperoxidase, thiocyanate, and hydrogen peroxide. The last compound serves as a substrate for lactoperoxidase in oxidazing thiocyanate and iodide ions, resulting in the generation of highly reactive oxidazing agents (Naidu, 2003). The laxtoperoxidase system has the ability to inhibit bacteria, fungi, parasites, and viruses, and for that reason it is considered a broad-spectrum natural antimicrobial (Stopforth et al., 2005a).

Lactoferrin is an iron-binding, bioactive glycoprotein of the transferrin family that contributes to the control of iron in biological fluids. Lactoferrin inhibits microorganisms by binding iron and making it unavailable for the development of the microorganisms (Stopforth et al., 2005a).

Lysozyme is an enzyme comprising 129 amino acids cross-linked by disulfide bonds (Cagri et al., 2004). Lysozyme exhibits antimicrobial activity against vegetative cells of a wide variety of organism, including numerous foodborne pathogens and spoilage organism. Gram-negative bacteria are generally less sensitive than Gram-positive bacteria to lysozyme mainly as a result of protection of the cell wall by the outer membrane (Johnson and Larson, 2005).

Plants, herbs, and spices as well as their derived essential oils and compounds isolated from extracts contain a large number of substances that are known to inhibit various metabolic activities of bacteria, yeasts, and molds (López-Malo et al., 2005). Essential oils of angelica, anise, carrot, cardamom, cinnamon, cloves, coriander, dill weed, fennel, garlic, nutmeg, oregano, parsley, rosemary, sage or thymol are inhibitory to various spoilage or pathogenic bacteria, molds, and yeasts (Cagri et al., 2004).

3. Antimicrobial edible films: antimicrobial, barrier and mechanical properties

The behavior of an antimicrobial edible film can be predicted by combining studies of antimicrobial, barrier and mechanical properties (McHugh and Krochta, 1994; Cagri et al., 2001). Film properties of these films are strongly influenced by the type and concentration of the antimicrobial compound, and the nature of the film matrix.

3.1. Antimicrobial activity of edible films

Tables 2 and 3 show the antimicrobial activity of different edible films containing antimicrobials against target pathogens. Results reported in different studies are difficult to compare mainly due to differences in experimental conditions such as film composition, antimicrobial agent and concentration, strain and concentration of the target microorganism, and analytical method used to determine the film antimicrobial activity. For this reason, the antimicrobial activity of the films is reported as inhibition (+) or no inhibition (-) of the target pathogenic microorganism with no dependence on the magnitude of the inhibition as concluded by the authors of the different studies, according with their experimental conditions.

Many research works report the addition of antimicrobial agents to different film matrix and their effect on the antimicrobial activity against target pathogens. Polysaccharide-based films containing antimicrobial agents that have been evaluated include those prepared with starch, cellulose derivates, chitosan, alginate, and fruit-puree (Table 2). Regarding protein-based films, there are available studies on those prepared with whey protein isolated (WPI), soy protein and soy protein isolated (SPI), egg albumen (EA), WG and sodium caseinate (Table 3). From the broad variety of microbes, human pathogens of the genus *Listeria*, *Escherichia,* and *Salmonella* have been the most widely studied.

Cellulose-based edible films

Some studies (Table 2) report the antimicrobial effect of nisin in hydroxypropyl methylcellulose (HPMC) films against *Listeria monocytogenes* ATCC 15313*, Staphylococcus aureus* IP 58156*, Aspergillus niger, and Kocuria rhizaphila* ATCC 934. The addition of 15% stearic acid to HPMC films decreased film inhibitory activity by 70 and 40% for *L. monocytogenes* and *S. aureus,* respectively. This phenomenon was explained by electrostatic interactions between the cationic nisin and the anionic fatty acid, which decreased nisin desorption from the film (Sebti et al., 2002). Similarly, a 3-fold reduction of film antimicrobial activity against *K. rhizaphila* was observed when 18% milkfat was added to the HPMC film (Sebti et al., 2007). Incorporation of chitosan to HPMC films at concentrations as low as 0.1% (w/v) showed a complete inhibition of *A. niger* (Sebti et al., 2007). On the other hand, when nisin was added to cross-linked HPMC film (98% cross-linking level with citric acid), no antimicrobial activity against *Micrococcus luteus* 270 was observed (Sebti et al., 2003). The authors concluded that HPMC could potentially graft nisin via ester bonds from the nisin C-terminal carboxylic acid group and cellulosic hydroxyl group. In addition, the primary amine group from the N-terminal position and from the lysine residues could react on the carboxylic function available on citric acid to form amine bonds. In both cases, nisin desorption could be strongly reduced, limiting film antimicrobial activity.

There are few studies on methylcellulose (MC) films containing antimicrobial agents. MC films containing 2% organic acid salts, PS or SB, were very active against *Rodotorula rubra and Penicillium notatum* providing clear inhibitory zones around film disks plated in agar media (Chen et al., 1996). The addition of natamycin and rosemary extract, alone or in combination, to MC films showed different antimicrobial effects against *A. niger* and *P. roquefortii*. The minimum inhibitory concentration (MIC) values of natamycin were 2 and 1 mg/10 g film solution against *A. niger* and *P. roquefortii*, respectively. Rosemary extract did not show any inhibitory antifungal activity alone, however, it acted synergistically with natamycin to prevent the growth of *A. niger*. This

way, although concentrations of natamycin of 1.5 mg per 10 g film solution were not effective against *A. niger*, the combination of this concentration with rosemary extract satisfactorily inhibited the growth of this mold (Türe et al., 2009b).

Chitosan-based edible films

Chitosan ability to form edible films and its antimicrobial activity against a broad spectrum of microbes makes it one of the most studied biopolymers. Results show that the antimicrobial activity of chitosan films depends primarily on the strain of microorganism and the conditions of the assay (Table 2). Coma et al. (2002) reported no inhibition from chitosan films deposited on agar medium inoculated with *L. monocytogenes* after 24 hours of incubation. However, chitosan films showed 100% inhibition of *L. monocytogenes* for at least 8 d, when the bactericidal activity was measured by epifluorescence assays. The authors stated that under the conditions tested, chitosan was incapable of diffusing through the adjacent agar medium, indicating the importance of the test method. These authors also observed a decrease in the antibacterial effect of chitosan with time, which was attributed to a decrease in the availability of amino-groups of chitosan. Möller et al. (2004) reported that a minimum of 1% chitosan content was required in chitosan films to maintain a significant anti-listerial activity using an agar plate method, whereas the kind of solvent (water, aqueous acetic acid and ethanol) used to prepare the film did not influence the anti-listerial activity of the chitosan films.

Chitosan films containing 2% of PS or SB did not clear inhibit the growth of *R. rubra and P. notatum* in agar diffusion test (Chen et al., 1996). These authors concluded that the interaction between chitosan and the preservatives inhibited their release. An increase in the preservatives concentration to 5% resulted in clear inhibitory zones in the agar with chitosan films, indicating that the binding sites for additives were presumably saturated at this high concentration (Chen et al., 1996).

Pranoto et al. (2005a) improved the antimicrobial activity of chitosan films against pathogenic bacteria through the addition of antimicrobial agents, such as garlic oil, PS, and nisin. The addition of garlic oil up to levels of at least 100 μL/g of chitosan, PS at 100 mg/100 g or nisin at 51,000 IU/g revealed an important antimicrobial effect against *S. aureus*,

L. monocytogenes and *Bacillus cereus*. However, these films did not show inhibitory activity against *Escherichia coli* and *Salmonella typhimurium*. They suggested that this behavior was due to the higher sensitivity of gram-positive bacteria to the antimicrobial agents.

Chitosan films have also been effective for the control of *Fusarium moniliforme*, *F. proliferatum*, and *A. ochraceus*. A combination of chitosan and polylactic acid presented antifungal activity (Sébastien et al., 2006).

Several workers have modified chitosan activity by means of blending with other polymers to improve the film physical properties. The similarity of cellulose and chitosan in primary structures has facilitated the formation of homogeneous composite films. Möller et al. (2004) reported that 1% of chitosan in chitosan-HPMC composite films was effective against *L. monocytogenes*. The incorporation of stearic acid into the film-forming solution did not influence the anti-listerial activity of the film. However, when films were cross-linked with citric acid the anti-listerial activity of the film was lost, probably due to a chemical reaction of the amino group, which is eventually responsible for the anti-listerial activity. In another study, chitosan-MC films containing 4% PS or SB induced clear inhibitory zones in the agar medium around the film disk when tested against *R. rubra and P. notatum* (Chen et al., 1996).

Starch-based edible films

Different types of starch-based films containing antimicrobial agents such as PS or natural compounds like lemongrass or grape seed extracts (GSE), have shown antimicrobial activity against different pathogens (Table 2). Sago starch-alginate films containing lemongrass oil (0.1 to 0.4%) exhibited clear inhibition zone around the film disk against *E. coli*. The zone of inhibition increased significantly as lemongrass oil content increased in the presence of glycerol. This result was attributed to the increased solubility of lemongrass oil in the matrix and more uniform dispersion of the oil in the film (Maizura et al., 2007).

Pea starch films containing GSE greatly inhibited the growth of all gram-positive bacteria tested, whereas the films were not effective against the gram-negative bacteria *S. typhimurium* and *E. coli* (Corrales et al., 2009). Bacterial growth inhibition was probably due to the presence

of secondary metabolites such as polyphenols in the seed extracts. These researchers assumed that these polyphenols could penetrate the semipermeable gram-positive bacterial membrane reacting in the cytoplasm with cellular proteins. In contrast, the lipidic wall of gram-negative bacteria represented an impassable barrier for extracted polyphenols to get into the cytoplasm.

Alginate-based edible films

Sodium alginate films containing lactoperoxidase or garlic oil presented antimicrobial activity against different pathogen bacteria (Table 2) (Pranoto et al., 2005b; Yener et al., 2009). The incorporation of garlic oil to sodium alginate films reduced the antimicrobial effect of garlic oil alone. At 0.1% (v/v), garlic oil in the nutrient broth decreased viable cell counts of *B. cereus, S. aureus, E. coli, and S. typhimurium* 5.61, 4.30, 2.28, and 1.24 log cycles, respectively. When garlic oil was incorporated to sodium alginated films, a concentration of 0.2% was needed in agar diffusion test to observe a clear inhibitory zone against *S. aureus* and *B. cereus*; while even at a concentration of 0.4% garlic oil the gram-negative bacteria *S. typhimurium* and *E. coli* were not effectively inhibited. These results were consistent with an *in vitro* test in nutrient broth, in which *E. coli* and *S. typhimurium* were more resistant to garlic oil than *S. aureus* and *B. cereus* (Pranoto et al., 2005b).

Fruit-based edible films

Different fruit-based films prepared with plant essential oils or their major constituents have been effective to control microbial growth (Table 2). In recent work, edible tomato films containing carvacrol were effective to inhibit the microbial growth of *E. coli*. Antimicrobial assays with tomato films indicated that carvacrol levels were approximately of 0.75% when added to tomato purees before film preparation. HPLC analysis of the films indicated that the carvacrol concentration and bactericidal activity of the films remained unchanged over an storage period of up to 98 d at 5 or 25 ºC (Du et al., 2008).

In other research, the antimicrobial activity of oregano essential oil in apple puree edible films against *E. coli* O157:H7 was significantly greater than that of cinnamon oil or lemongrass oil (Rojas-Graü et al., 2006). Similar results were reported by this research group with alginate-apple puree edible films containing plant essential oils such as oregano oil, cinnamon oil or lemongrass oil, or oil compounds such as carvacrol, cinnamaldehyde, or citral. Among all them, carvacrol and oregano oils exhibited the strongest antimicrobial activity against *E. coli* at a concentration of 0.1%, whereas a concentration as high as 0.5% of the other compounds was required to inhibit the microbial growth on agar plate (Rojas-Graü et al., 2007a).

Tabla 2. Antimicrobial activity of edible polysaccharide-based composite films containing antimicrobial agents.

Film matrix	Antimicrobial agent	Concentration	Target pathogen	Pathogen inoculation	Antimicrobial activity[a]	Reference
HPMC[b]	Chitosan	0.1 % (w/w)[c]	*Aspergillus niger*	10^4 spores per Petri dish	+	(Sebti et al., 2007)
HPMC	Nisin	5 x 10^4 IU[d]/ mL	*Listeria monocytogenes, Staphylococcus aureus*	70 µL/wells	+	(Sebti et al., 2002)
HPMC	Nisin	10^3 - 10^5 IU/ mL	*Micrococcus luteus* 270	0.1% v/v[e]	-	(Sebti et al., 2003)
HPMC	Nisin	250 µg/mL	*A. niger, Kocuria rhizaphila ATCC 9341*	10^3 - 10^4 spores	+	(Sebti et al., 2007)
MC[f]	-	-	*Rhodotorula rubra, Penicillium notatum*	0.1 mL of suspension	-	(Chen et al., 1996)
	Potassium sorbate, Sodium benzoate	2 %	*R. rubra, P. notatum*	0.1 mL of suspension	+	
MC	Natamycin	1.5 mg/10 g film solution	*A. niger*	10^4 spores/mL	-	(Türe et al., 2009b)
		1.0 mg/10 g film solution	*P. roquefortii*	10^6 spores/mL	+	
	Natamycin + rosemary extract	0.5 + 1.5 mg/10 g film solution	*A. niger, P. roquefortii*	10^4 , 10^6 spores/mL	-	
	Natamycin + rosemary extract	1.5 + 1.5 mg/10 g film solution	*A. niger*	10^4 spores/mL	+	
Chitosan-MC	Potassium sorbate, Sodium benzoate	4 %	*R. rubra, P. notatum*	0.1 mL of suspension	+	(Chen et al., 1996)
Chitosan-HPMC	-	-	*L. monocytogenes*	300 cells	+	(Möller et al., 2004)
Chitosan	-	-	*R. rubra, P. notatum*	0.1 mL of suspension	-	(Chen et al., 1996)
	Potassium sorbate, Sodium benzoate	2 %	*R. rubra, P. notatum*	0.1 mL of suspension	-	

Film matrix	Antimicrobial agent	Concentration	Target pathogen	Pathogen inoculation	Antimicrobial activity[a]	Reference
Chitosan	Nisin	250 µg/mL	*A. niger,* *K. rhizaphila ATCC 9341*	10^3 -10^4 spores/mL	+	(Sebti et al., 2007)
Chitosan	-	-	*L. monocytogenes*	37 cell per Petri dish	-	(Coma et al., 2002)
Chitosan	-	-	*L. monocytogenes*	300 cells	+	(Möller et al., 2004)
Chitosan	Garlic oil	100 µL/g	*S. aureus,* *L. monocytogenes,* *Bacillus cereus,*	10^5 - 10^6 CFU^g/mL	+	(Pranoto et al., 2005a)
		400 µL/g	*Escherichia coli,* *Salmonella typhimurium*	10^5 - 10^6 CFU/mL	-	
	Potassium sorbate	100 mg/g	*S. aureus,* *L. monocytogenes,* *B. cereus*	10^5 - 10^6 CFU/mL	+	
		200 mg/g	*E. coli,* *S. typhimurium*	10^5 - 10^6 CFU/mL	-	
	Nisin	51,000 IU/g	*S. aureus,* *L. monocytogenes,* *B. cereus*	10^5 - 10^6 CFU/mL	+	(Pranoto et al., 2005a)
		204,000 IU/g	*E. coli,* *S. typhimurium*	10^5 CFU/mL	-	
Chitosan	-	-	*Fusarium moniliforme,* *F. proliferatum,* *Aspergillus ochraceus,*	10^3 spores	+	(Sébastien et al., 2006)
Chitosan	-	-	*A. niger*	10^2 spores per Petri dish	+	(Sebti et al., 2007)
Tapioca-starch	Potassium sorbate	0.3 % (v/w)	*Zygosaccharomyces bailii*	5 x 10^6 CFU/mL	+	(Flores et al., 2007b)
Sago starch	Lemongrass oil	0.4 % (v/w)	*E. coli* O157:H7	10^5 - 10^6 CFU/mL	+	(Maizura et al., 2007)

Film matrix	Antimicrobial agent	Concentration	Target pathogen	Pathogen inoculation	Antimicrobial activity[a]	Reference
Pea starch	Grape seed extracts	1.0 %	*L. monocytogenes*, *S. aureus*, *Enterococcus faecium*, *E. faecalis*, *Brochothrix thermosphacta*	10^6 CFU/mL	+	(Corrales et al., 2009)
		20 %	*S. typhimurium*, *E. coli*		-	
Sodium alginate	Garlic oil	0.2 % (v/v)	*S. aureus*, *B. cereus*	10^5 - 10^6 CFU/mL	+	(Pranoto et al., 2005b)
Sodium alginate	Lactoperoxidase	2 % (w/v)	*E. coli*, *L. inocua*, *Pseudomonas fluorescences*	3–4 $\log_{10}$ CFU/mL	+	(Yener et al., 2009)
		0.4 % (v/v)	*E. coli*, *S. typhimurium*	10^5 - 10^6 CFU/mL	-	
Alginate-apple puree	Oregano oil/ carvacrol	0.1 % (w/w)	*E. coli* O157:H7	10^5 CFU/mL	+	(Rojas-Graü et al., 2007a)
	Lemongrass oil/ citral	0.5 % (w/w)				
	Cinnamon oil/ cinnamaldehyde	0.5 % (w/w)				
Apple puree	Oregano oil	0.1 % (w/w)	*E. coli* O157:H7	10^5 CFU/mL	+	(Rojas-Graü et al., 2006)
	Lemongrass oil	0.5 % (w/w)				
	Cinnamon oil	0.5 % (w/w)				
Tomato puree	Carvacrol	0.75 % (w/w)	*E. coli* O157:H7	10^5 CFU/mL	+	(Du et al., 2008)

[a] Antimicrobial activity ‘ + ’ = inhibition; ‘ - ’ = no inhibition. [b] HPMC = hydroxypropyl methylcellulose. [c] w/w = weight-in-weight. [d] IU = International Units. [e] v/v = volume-in-volume. [f] MC = methylcellulose. [g] CFU = colony forming units.

Protein-based edible films

Among protein-based films, WPI films have been tested with a great number of antimicrobial agents including organic acids and their salts, polypeptides, essential oils, natural extracts, and others antimicrobial agents (Table 3). WPI films containing sorbic acid or p-aminobenzoic acid were effective against *L. monocytogenes*, *E. coli* and *S. typhimurium* (Cagri et al., 2001). As the concentration of the antimicrobial agents in the films increased (range 0.5-1.5%), the activity of the films in disk diameter test also increased. Since the undissociated form of weak acids at low pH increased the ability to penetrate the cytoplasmatic membrane of the bacteria, the pH adjustment to 5.2 using lactic or acetic acids significantly increased the antimicrobial effect of the films.

Irrespective of the concentration of polypectic antimicrobials like lactoperoxidase or lactoferrin, and the microbial test used, WPI films presented high antimicrobial activity against *P. comune*, *L. monocytogenes*, *S. enterica*, and *E. coli* (Min et al., 2005a,b; Min and Krochta, 2005; Seydim and Sarikus, 2006)

WPI films containing oregano and garlic oils were effective against *E. coli, S. aureus*, *S. enteriditis, L. monocytogenes* and *Lactobacillus plantarum*, while films containing rosemary oil had no activity against the same pathogenic bacteria (Seydim and Sarikus, 2006).

Ko et al. (2001) studied the effect of nisin incorporated to different film protein matrix, WPI, SPI, EA, or WG. Among the tested film matrices, WPI films containing nisin were the most effective to reduce *L. monocytogenes* counts, whereas WG films showed the lowest antimicrobial activity. These results correlated with film surface hydrophobicity, indicating that nisin was more active for inhibition of *L. monocytogenes* in a hydrophobic film such as WPI films than in a less hydrophobic film such as WG films. As nisin concentration increased from 4.0 to 160 IU per film disk, the inhibitory activity of all tested films progressively increased. In addition, edible films containing nisin in an acidic environment exerted a greater inhibitory effect against the pathogen, which is because nisin is more active at acidic conditions (Klaenhammer, 1993).

The incorporation of nisin and organic acids (citric, lactic, malic, or tartaric acid) to SPI films was tested in order to improve the film

antimicrobial activity against *L. monocytogenes, S. gaminara,* and *E. coli* O157:H7 (Eswaranandam et al., 2004). The films antimicrobial activity was expressed in terms of inhibition zone in agar plates and log number of survivors. In SPI films, only *L. monocytogenes* was inhibited by the combined effect of nisin and organic acids at all concentrations tested (range 0.9-2.6% (w/w)) and *S. gaminara* and *E. coli* were only inhibited by the combination of nisin with 1.8 or 2.6 % of citric, malic, or tartaric acids. Lactic acid, however, only slightly inhibited *S. gaminara* and *E. coli.* On the other hand, films with 2.6% organic acids without nisin similarly inhibited *L. monocytogenes* and their anti-salmonella activity was lower than that of the nisin-organic acids combination. In a recent work, nisin, GSE, EDTA and their combinations were added to SPI films and tested against *L. monocytogenes, S. typhimurium,* and *E. coli* O157:H7 (Sivarooban et al., 2008). *L. monocytogenes* was more sensitive to various combinations of antimicrobials than the other two pathogens. The authors pointed out that both phenolics and nisin act upon the cytoplasmic membrane of the bacteria, thus the additive or synergistic effect of combinations of these compounds could enhance the inhibitory activity against *L. monocytogenes.* The lower inhibitory activity of combined GSE and nisin against *S. typhimurium* and *E. coli* O157:H7 might be related with the structure of the cell membrane.

Table 3. Antimicrobial activity of edible protein-based composite films containing antimicrobial agents.

Film matrix	Antimicrobial agent	Concentration	Target pathogen	Pathogen inoculation	Antimicrobial activity	Reference
WPI[a]	Sorbic acid	0.75 %	*Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella typhimurium* DT104	0.1 mL	+	(Cagri et al., 2001)
	p-amimobenzoic acid	0.75 %	*L. monocytogenes*, *E. coli* O157:H7, *S. typhimurium* DT104	0.1 mL	+	
WPI	Lactoferrin	0.1 g/g	*Penicillium comune*	10^5 spores	-	(Min and Krochta, 2005)
	Lactoferrin hydrolysate	0.1 g/g			-	
	Lactoperoxidase	59 mg/g film			+	
WPI	Lactoperoxidase	0.5 % (w/v)[b]	*L. monocytogenes*	10^3 CFU/ mL	+	(Min et al., 2005b)
WPI	Lactoperoxidase	3.0 % (w/w)	*Salmonella enterica*, *E. coli* O157:H7	10^8 CFU	+	(Min et al., 2005a)
WPI	Oregano oil	2.0 % (w/v)	*E. coli* O157:H7, *Staphylococcus aureus*, *Salmonella enteriditis*, *L. monocytogenes*, *Lactobacillus plantarum*	10^8 CFU/ mL	+	(Seydim and Sarikus, 2006)
	Garlic oil	3.0 % (w/v)	*E. coli* O157:H7, *S. aureus*, *S. enteriditis*, *L. monocytogenes*, *L. plantarum*		+	

Film matrix	Antimicrobial agent	Concentration	Target pathogen	Pathogen inoculation	Antimicrobial activity	Reference
	Rosemary oil	4.0 % (w/v)	*E. coli* O157:H7, *S. aureus*, *S. enteriditis*, *L. monocytogenes*, *L. plantarum*		-	
WPI, SPI[c], EA[d], WG[e]	Nisin	4.0 IU[j]/ film disk	*L. monocytogenes*	10^3 CFU/ g	+	(Ko et al., 2001)
SPI	Nisin + citric acid	205 IU/ g protein + 2.6 % (w/w)	*L. monocytogenes*, *Salmonella gaminara*, *E. coli* O157:H7	10^6 CFU	+	(Eswaranandam et al., 2004)
	Nisin + lactic acid					
	Nisin + malic acid					
	Nisin + tartaric acid					
SPI	Grape seed extracts	1.0 %	*L. monocytogenes*	10^6 CFU/mL	+	(Sivarooban et al., 2008)
	Nisin	10,000 IU/g				
	Sodium-EDTA[f]	0.16 %				
	Grape seed extracts	1.0 %	*S. typhimurium*, *E. coli O157:H7*	10^6 CFU/mL	-	
	Grape seed extracts + nisin + sodium-EDTA	1.0 % + 10,000 IU/g + 0.16 %	*L. monocytogenes*, *S. typhimurium*, *E. coli* O157:H7		+	
Sodium caseinate	Sodium lactate	40 %	*L. monocytogenes*	10^2 CFU/cm^2	+	(Kristo et al., 2008)
	Potassium sorbate	25 %				
	Nisin	0.075 %				

[a] WPI = whey protein isolated. [b] w/v = weight-in-volume. [c] SPI = soy protein isolated. [d] EA = egg albumen. [e] WG = wheat gluten.
[f] sodium-EDTA = sodium ethylenediamine-tetraacetate.
Other abbreviations are similar to those presented in Tables 2.

3.2. Barrier and mechanical properties of antimicrobial edible films

The barrier and mechanical properties of films depend basically on intrinsic factors such as film composition, thickness, on preparation techniques, but also on other secondary factors like the test conditions. Therefore, the incorporation of antimicrobial food additives or other ingredients into edible films may cause significant changes on the mechanical and barrier properties that need to be considered when new films are developed (Greener and Fennema, 1994).

Tables 4 and 5 show the mechanical and barrier properties of edible films containing antimicrobial agents. Properties of controls refers to those of films prepared without antimicrobial agents. Considering all the different factors that affect film properties, it is very difficult to compare the performance of different films. However because such comparison is important, it will be made when possible in this review.

In the literature, polysaccharide-edible films containing antimicrobial agents presented a wide range of WVP values (1-160 g mm/m^2 d kPa) depending on film composition and RH gradient. HPMC, chitosan, alginate, or sago starch films presented WVP values lower than 35 g mm/m^2 d kPa at a ΔRH around 50-0% or 0-100%. WVP values were higher than the former when films were prepared with MC, pea starch, or tomato puree (50-90 g mm/m^2 d kPa), and the highest WVP values were those of alginate-apple puree and apple puree films (>120 g mm/m^2 d kPa) (Table 4). The high WVP values for MC films could be related to the high RH during WVP measurements (50-100%), since the WVP of hydrophilic films is highly influenced by RH. However, alginate-apple puree films presented a high WVP, even at ΔRH similar to other polysaccharide films.

Similarly to WVP, the mechanical properties YM, TS and %E clearly depend on the type of film matrix and film composition. In the literature, edible films containing antimicrobial agents had YM, TS and %E values lower than 30 MPa, 60 MPa and 74%, respectively, except for tomato puree- or MC-based films, which had YM values higher than 200 MPa, or pea starch films with TS values higher than 500 MPa.

Cellulose-based edible films

Sebti et al. (2002; 2003) studied the effect of stearic acid and the degree of cross-linking level of HPMC-nisin films on the film mechanical properties and WVP. Citric acid was used as the cross-linking agent to produce films with 0-98% cross-linking level. The addition of 15% (w/w) of stearic acid improved film moisture barrier, but reduced the mechanical resistance with a decrease in film elasticity and extensibility. The negative effect of lipid addition to different polymer matrix has been repeatedly observed in many research works and it is usually attributed to the partial replacement of the polymer by the lipids in the film matrix, which flavors the disruption of the film. Contrary to what was expected, an increase in the cross-linking decreased YM, which might be explained by a higher heterogeneity of the space between cross-links related to the ester bond rate that induced the formation of cracks and the worst mechanical properties of cross-linked films.

The addition of low concentrations of natamycin to MC films slightly increased YM and decreased %E values, while it did not modify TS value. Similarly, TS was not affected by the addition of low concentration of natamycin plus rosemary extract. However, the incorporation of high concentrations of natamycin (10 or 20 mg per 10 g of film solution) resulted in a significant decrease of film TS (Türe et al., 2009a). These changes were attributed to the weakening of some of the chemical bonds in the polymer structure.

In another study, the mechanical properties of MC-chitosan films did not significantly change by the addition of PS or SB (Chen et al., 1996).

Chitosan-based edible films

In chitosan films (Table 4), WVP values increased as the concentration of the antifungal ingredients PS or nisin increased, while an increase of garlic oil (up to 400 μL/g of chitosan) did not affect the film WVP (Pranoto et al., 2005a). The addition of PS and nisin contributed to extend the intermolecular interaction and lose the compactness of the structure, which enhanced moisture diffusion through the film. However, this behavior did not occur when garlic oil was added to the chitosan film, probably due to its hydrophobic

character. In the same tests, the addition of PS or nisin produced higher reduction of TS than that the incorporation of garlic oil. The authors confirmed that the incorporation of additives other than cross-linking agents generally reduced TS values. In contrast, %E values increased with addition of PS or nisin to chitosan films, being such increase higher with nisin than with PS. As with TS, garlic oil did not significantly affect %E value.

Starch-based edible films

Among the research conducted with antimicrobial starch-based edible films (Table 4), very recent work by Corrales et al. (2009) showed that GSE added to pea starch films significantly increased film elongation and decreased TS. Despite the swollen structure of films containing GSE, observed by a considerable increase in film thickness, the WVP did not significantly change compared to the control film. This result was explained by the reduced solubility of water vapor to film surface due to the less polar property of the GSE compounds incorporated to the pea starch film. However, the reduced polarity of these films accelerated the absorption of oxygen to the film surface, which resulted in an increase in OP compared to control films. The incorporation of GSE to the films reduced TS by 50% and increased %E (Corrales et al., 2009). The authors attributed these results to the chemical disposition of flavonoids and phenolic acids from GSE, with amylose chains that lose intermolecular interactions

Flores et al. (2007a) observed no effect on WVP as PS was incorporated to tapioca-starch-glycerol edible films, whereas film YM and %E decreased. However, the gelatinization/drying method used to prepare the films significantly affected the barrier, mechanical, and antimicrobial stability of the films. It was concluded that short gelatinization and drying times were optimal to produce films of better antimicrobial stability. However, these films showed poor mechanical and moisture barrier properties.

Alginate-based edible films

The addition of increasing amounts of garlic oil to alginate-based edible films greatly modified the film mechanical and barrier properties. TS and %E values were reduced by incorporation of garlic oil at 0.3 and 0.4% (v/v), respectively (Table 4). Considering that unpeeled films were dipped in a calcium chloride solution to help to form a network between polymer chains, the presence of garlic oil in the alginate film probably interfered with ionic interactions facilitated by calcium ions, causing an important reduction of TS at the higher garlic oil concentration. WVP values of the films were not affected by garlic oil incorporation at a concentration range of 0.1-0.3%. However, film WVP was significantly higher at a garlic oil concentration of 0.4%. In spite of the hydrophobic character of garlic oil, the increase in film WVP was attributed to an extension of the intermolecular interactions in the structural matrix, which enhanced moisture diffusion through the film (Pranoto et al., 2005b).

In alginate-apple puree films, the presence of plant essential oils or oil compounds did not significantly affect WVP and OP of the films, but modified tensile properties. Only a slight decrease in WVP was reported with the addition of 0.5% (v/v) cinnamaldehyde (Rojas-Graü et al., 2007a). Since water vapor transfer generally occurs through the hydrophilic portion of the film and depends on the hydrophilic-hydrophobic ratio of the film components, these workers suggested that the amount of essential oils or oil compounds were not enough to reduce film WVP. In general, the addition of antimicrobial agents to films significantly reduce TS and increase %E values. In this research, films containing oregano oil and carvacrol presented lower TS values, and films with carvacrol had the highest %E value. YM was reduced in all films containing essential oils or oil compounds, but no significant differences were reported among films.

However, work by this group with apple puree edible films (no alginate), showed that the addition of essential oils decreased WVP and increased OP, but did not significantly alter the tensile properties of the films even though the concentrations were similar to those reported in the previous trials (Rojas-Graü et al., 2006). While the effect of essential oils on WVP was important for oregano oil (at 0.1% (w/w) induced a significant decrease in film WVP), it was not for lemongrass and

cinnamon oils (a concentration of 0.5% (w/w) was required to reduce WVP). The effect of essential oils on OP can be explained by their nonpolar character, which makes them less effective oxygen barriers.

Fruit-based edible films

Du et al. (2008) reported two different film casting methods (batch- and continuous-cast) to develop tomato puree edible films with the addition of carvacrol as antimicrobial agent (Table 4). YM and TS decreased with the addition of carvacrol. Batch-cast films presented lower YM and TS and higher %E values than continuous-cast tomato films. The authors pointed out that the continuous-cast films had a higher density that to explain this behavior in the batch-cast films. WVP was significantly higher for batch-cast films than for continuous-cast films. The addition of carvacrol increased WVP batch-cast films. The differences were attributed to the higher casting temperatures for continuous-cast films that increased the evaporation of both carvacrol and water, reducing the amount of interstitial spaces for molecular diffusion.

Table 4. Mechanical and barrier properties of polysaccharide-based edible composite films containing antimicrobial agents

Film matrix	Antimicrobial agent	Concentration	Mechanical properties			Barrier properties			References
			YM[a] (MPa)	TS[b] (MPa)	E[c] (%)	Water vapor permeability WVP[e] (g mm/m^2 d kPa)	Water vapor permeability Δ RH[f] (%)	OP[d] (cm^3 µm/m^2 d kPa)	
HPMC-stearic acid	Nisin	5 x 10^4 IU/mL	-	-	6.6	7.4	50-0		(Sebti et al., 2002)
HPMC	Control[g]	0.0 %	19.0	34.0	6.6	-	-	-	(Sebti et al., 2003)
	Nisin (0 % cross-linked HPMC-citric acid)	10^4 – 10^5 IU/mL	21.0	32.0	2.8				
	Nisin (98 % cross-linked HPMC-citric acid)	10^4 IU/mL	14.0	27.0	2.7				
HPMC	Control	-	19.0	34.0	6.6	-	-	-	(Möller et al., 2004)
Chitosan-HPMC	-	-	18.0	24.0	3.9				
Chitosan-HPMC-stearic acid	-	-	31.0	30.0	1.8				
MC	Control	0.0 mg/10 g film solution	313.2	36.6	74.0	84.0	50-100	-	(Türe et al., 2009a)
	Natamycin	2.0 mg/10 g film solution	380.7	37.2	60.5	82.3			
	Natamycin + rosemary extract	1.0 + 1.5 mg/10 g film solution	426.8	36.1	62.2	93.1			
Chitosan-MC	Control	0 %	-	2.8	19.6	-	-	-	(Chen et al., 1996)
	Potassium sorbate	4 %	-	3.8	28.5				
	Sodium benzoate		-	3.0	22.5				

Film matrix	Antimicrobial agent	Concentration	Mechanical properties			Barrier properties			References
			YM[a] (MPa)	TS[b] (MPa)	E[c] (%)	Water vapor permeability WVP[e] (g mm/m^2 d kPa)	Water vapor permeability Δ RH[f] (%)	OP[d] (cm^3 µm/m^2 d kPa)	
Chitosan	-	-	11.0	23.0	22.0	0.05	50-0	-	(Sébastien et al., 2006)
Chitosan	Control	0.0	-	37.0	3.5	0.02	-	-	(Pranoto et al., 2005a)
	Garlic oil	100 µL/g		33.4	3.0	0.02			
		400 µL/g		29.0	2.5	0.03			
	Potassium sorbate	100 mg/g		26.4	3.1	0.02			
		200 mg/g		13.5	4.9	0.04			
	Nisin	51,000 IU/g		23.7	14.1	0.02			
		204,000 IU/g		13.6	30.7	0.03			
Pea starch	Control	0.0 %	-	510.3	36.9	62.6	10-100	1.1	(Corrales et al., 2009)
	Grape seed extracts	1.0 %(w/v)	-	249.5	56.1	57.6		3.1	
Tapioca-starch	Control (long gelatinisation time, slow drying rate)	0.0 %(w/w)	29.0	-	2.4	54.4	43-0	-	(Famá et al., 2005; Flores et al., 2007a)
	Potassium sorbate (long gelatinisation time, slow drying rate)	0.3 % (w/w)	7.6		0.7	52.7	44-0		
	Control (long gelatinisation time, fast drying rate)	0.0 % w/w)	13.0	-	2.0	70.0	41-0	-	
	Potassium sorbate (long gelatinisation time, fast drying rate)	0.3 % (w/w)	4.3		0.6	70.0	42-0		

Film matrix	Antimicrobial agent	Concentration	Mechanical properties			Barrier properties			References
			YM[a] (MPa)	TS[b] (MPa)	E[c] (%)	Water vapor permeability WVP[e] (g mm/m^2 d kPa)	Δ RH[f] (%)	OP[d] (cm^3 µm/m^2 d kPa)	
	Control (long gelatinisation time, fast drying rate)	0.0 % w/w)	3.2	-	1.0	124.4	49-0	-	
	Potassium sorbate (long gelatinisation time, fast drying rate)	0.3 % (w/w)	1.3		0.2	139.1	50-0		
Sago starch-alginate	Control		-	16.0	3.7	20.7	-	-	(Maizura et al., 2007)
	Lemongrass oil	0.4 % (v/w)		12.9	13.2	34.5	52-0		
Sodium alginate	Control	0.0 % (w/w)	-	66.1	4.1	20.3	0-100	-	(Pranoto et al., 2005b)
	Garlic oil	0.1 % (w/w)		64.7	4.1	18.7			
		0.2 % (w/w)		55.2	4.4	21.8			
		0.3 % (w/w)		49.1	4.8	23.4			
		0.4 % (w/w)		38.7	2.7	30.9			
Alginate-apple puree	Control	0% (w/w)	7.1	2.9	51.1	118.8	0-65	10.2	(Rojas-Graü et al., 2007a)
	Oregano oil	0.1 % (w/w)	5.8	2.5	57.0	126.0	0-64	11.0	
	Carvacrol		6.0	2.6	58.3	120.5	0-64	10.9	
	Lemongrass oil	0.5 % (w/w)	6.0	2.6	56.0	117.8	0-66	9.4	
	Citral		6.5	2.5	57.4	122.9	0-64	9.9	
	Cinnamon oil		6.9	2.8	57.9	117.6	0-65	10.5	
	Cinnamaldehyde		6.8	2.8	55.5	104.9	0-67	11.0	

Film matrix	Antimicrobial agent	Concentration	Mechanical properties			Barrier properties			References
			YM[a] (MPa)	TS[b] (MPa)	E[c] (%)	Water vapor permeability WVP[e] (g mm/m^2 d kPa)	Water vapor permeability Δ RH[f] (%)	OP[d] (cm^3 µm/m^2 d kPa)	
Apple puree	Control	0.0 %	5.1	0.6	25.4	169.0	0-63	22.6	(Rojas-Graü et al., 2006)
	Oregano oil	0.1 %	4.7	0.6	26.5	148.1	0-63	38.1	
	Lemongrass oil	0.5 %	4.5	0.6	24.8	158.9	0-64	30.3	
	Cinnamon oil	0.5 %	4.0	0.6	22.6	163.7	0-63	32.3	
Tomato puree	Control (batch-cast method)	0.0 %	248.1	11.4	11.2	58.6	0-82	-	(Du et al., 2008)
	Carvacrol (batch-cast method)	1.5 % (w/w)	187.2	8.9	11.6	62.7	0-82		
	Control (continuous- cast method)	0.0 %	316.9	13.7	9.6	52.8	0-85		
	Carvacrol (continuous-cast method)	1.5 % (w/w)	259.1	10.4	8.6	54.7	0-83		

[a] YM = Young's modulus. [b] TS = tensile strength. [c] E = elongation at break (%). [d] OP = oxygen permeability. [e] WVP = water vapor permeability. [f] Δ RH = relative humidy gradient. [g] Control = film without antimicrobial agent.
Other abbreviations are similar to those presented in Tables 2 and 3.

Protein-based edible films

Mechanical and barrier properties of protein-based films based on WPI, SPI, EA, WG with antimicrobial agents have been reported (Table 5). Among them, WPI has been more extensively studied as a structural matrix for antimicrobial edible films. Cagri et al. (2001) reported the effect of incorporating *p*-aminobenzoic acid or sorbic acid on mechanical and barrier properties of WPI films. The addition of *p*-aminobenzoic acid or sorbic acid increased %E and decreased TS. Films containing sorbic acid presented lower TS and higher %E than those containing *p*-aminobenzoic acid. It was suggested that the straight chain of sorbic acid could more easily penetrate into WPI films than *p*-aminobenzoic acid, which has a benzene ring. Consequently, sorbic acid may allow higher mobility between WPI chains resulting in lower YM and TS and greater flexibility of the films. Film WVP increased with the addition of *p*-aminobenzoic acid or sorbic acid, probably due to the hydrophilic character of both antimicrobial agents. Moreover, these compounds weakened chain packing in the film to produce a looser structure, which increased water mobility.

The addition of 59 mg/g of film, dry basis lactoperoxidase system to WPI films, which exhibited most efficient inhibition effects in a microbial test, did not significantly modify film mechanical properties and OP, suggesting that the lactoperoxidase system did not change the structure of WPI films (Min and Krochta, 2005). Nevertheless, an increase of lactoperoxidase system higher than 0.15 g/g film (dry basis) in WPI films worsened the tensile properties and improved the oxygen barrier properties suggesting the formation of protein aggregates in lactoperoxidase system-WPI films due to the presence of gluconolactone (Min et al., 2005a). Ozdemir and Floros, (2008a,b) studied the effect of plasticizer (sorbitol), lipid (BW) and antimicrobial agent (PS) concentrations on the mechanical, barrier, optical, and sensory properties of WPI films. Film WVP decreased as protein and BW concentration increased, but it increased as sorbitol and PS concentration also increased. On the other hand, YM, TS, and %E were influenced by protein, sorbitol, and PS concentrations. As in other edible films, the addition of PS decreased YM and TS and increased %E. The curvilinear increased of %E with increasing PS was considered as an indicator of the

fact that PS significantly interacted with some components in the mixture.

Ko et al. (2001) studied the effect of nisin on mechanical and barrier properties of different protein film matrices (WPI, SPI, EA, or WG). Theoretically, a decrease in the WVP of the protein films was expected due to the hydrophobic character of nisin. However, film WVP was not affected by nisin addition, which might be due to the low concentration of nisin incorporated into the film forming solution. The addition of nisin only affected the TS of WPI films, and no effect on SPI, EA, or WG was observed. The increase in TS of WPI films was attributed to possible rearrangements of disulfide and hydrophobic bonds, more protein-protein interactions, or the electrostatic interaction between molecules of nisin and protein. The lower hydrophobicity of the other protein films compared to that of WPI films may have resulted in lower number of potential hydrophobic bonds between nisin and protein molecules, which may explain the differences in mechanical properties between WPI and the other protein films.

The incorporation of natamycin into WG films did not cause major changes on their mechanical properties. However, the incorporation of a mixture of natamycin and rosemary extract into the films decreased TS and %E, whereas WVP were not affected by the addition of antimicrobial agents alone or in combination (Türe et al., 2009a).

Kristo et al. (2008) studied the effect of the addition of an increasing concentration of sodium lactate, PS, and nisin on the mechanical and barrier properties of sorbitol-plasticized sodium caseinate films. The addition of sodium lactate (0-40% dry basis) and PS (0-25% dry basis) to the films increased film WVP. Films containing PS presented lower WVP than films with sodium lactate. The addition of an increasing concentration of both antimicrobials to sodium caseinate films resulted in a reduction of YM and TS, and an increase of %E, suggesting that both antimicrobials acted as a plasticizer for those films. In contrast, the addition of nisin did not cause significant changes in WVP or the tensile properties of sodium caseinate films, probably due to the low concentration of nisin in the films.

Table 5. Mechanical and barrier properties of protein-based edible composite films containing antimicrobial agents

Film matrix	Antimicrobial agent	Concentration	Mechanical properties			Barrier properties			References
			YM (MPa)	TS (MPa)	E (%)	Water vapor permeability WVP (g mm/m^2 d kPa)	Water vapor permeability Δ RH (%)	OP (cm^3 µm/m^2 d kPa)	
WPI	Control	0.0 %	-	5.9	6.4	27.2	0-85	-	(Cagri et al., 2001)
	Sorbic acid	1.5 %		3.6	73.0	43.8	0-85		
	p-amimobenzoic acid	1.5 %		5.3	35.0	56.1	0-85		
WPI	Control	0.0 mg/mL	-	2.0	-	34.8	55-100	-	(Ko et al., 2001)
	Nisin	0.2 mg/mL		3.5		38.2	55-100		
WPI	-	0 %	84.0	3.3	27.6	9.6	0-50	-	(Ozdemir and Floros, 2008a,b)
	Potassium sorbate	10 %	44.4	3.6	57.2	239.3			
WPI	Control	0.0 mg/mL	-	244.1	-	34.8	55-100	-	(Ko et al., 2001)
	Nisin	0.2 mg/mL	-	244.4	-	38.2			
SPI	Control	0.0 mg/mL	-	8.6	-	41.3			
	Nisin	0.2 mg/mL	-	10.4	-	42.5			
EA	Control	0.0 mg/mL	-	1.8	-	57.8			
	Nisin	0.2 mg/mL	-	1.4	-	52.8			
WG	Control	0.0 mg/mL	-	1.8	-	63.4			
	Nisin	0.2 mg/mL	-	2.0	-	51.6			
WPI	Control	-	21.9	2.3	147.2	-	-	228.8	(Min and Krochta, 2005)
	Lactoperoxidase	59 (mg/g film, dry basis)	23.2	2.3	140.3			231.7	

Film matrix	Antimicrobial agent	Concentration	Mechanical properties			Barrier properties			References
			YM (MPa)	TS (MPa)	E (%)	Water vapor permeability WVP (g mm/m^2 d kPa)	Water vapor permeability Δ RH (%)	OP (cm^3 µm/m^2 d kPa)	
WPI	Control	0.0%	25.8	1.1	119.5	-	-	270.1	(Min et al., 2005a)
	Lactoperoxidase	150 (mg/g film, dry basis)	17.2	2.1	129.5			141.0	
WG	Control	0.0 mg/10 g film solution	28.8	2.1	224.8	164.4	50-100	-	(Türe et al., 2009a)
Sodium caseinate	Control	0 % (w/w)	2400.0	63.0	3.0	1.4	53-79	-	(Kristo et al., 2008))
	Sodium lactate	10 % (w/w film, dry basis)	1400.0	38.0	3.0	2.8	53-72		
	Potassium sorbate	40 % (w/w film, dry basis)	250.0	8.0	20.0	9.4	53-60		
		10 % (w/w film, dry basis)	2350.0	70.0	5.0	2.3	53-75		

Film matrix	Antimicrobial agent	Concentration	Mechanical properties			Barrier properties			References
			YM (MPa)	TS (MPa)	E (%)	Water vapor permeability		OP (cm^3 µm/m^2 d kPa)	
						WVP (g mm/m^2 d kPa)	Δ RH (%)		
Sodium caseinate	Nisin	25 % (w/w film, dry basis)	900.0	28.0	28.0	3.3	53-71	-	(Kristo et al., 2008)
		0.075 % (w/w film, dry basis)	2200.0	63.0	4.0	1.4	53-78		
		0.0075 % (w/w film, dry basis)	-	-	-	1.6	53-76		

Abbreviations are similar to those presented in Table 4.

4. Antimicrobial edible composite coatings applied to fruits and vegetables

Nowadays, many commercial edible coatings for use on fruits and vegetables are available on the market to reduce produce weight loss, physiological disorders, and maintain produce quality. Most of them are assigned to maintain the quality of citrus and apples and, to a lesser extend, mangoes, papayas, pomegranates, avocados, and tomatoes (Olivas et al., 2008). However, no commercial edible coatings are found to inhibit microbial growth on fruits and vegetables. Several studies have been published in the literature showing the potential of edible antimicrobial coatings to control microbial decay of fruits and vegetables. Table 6 shows the most important antimicrobial edible coatings applied to date fresh or minimally processed fruits and vegetables, the target pathogen, and an assessment of their antimicrobial activity. A summary of what has been reported in the literature is given below.

Citrus fruits

Postharvest green and blue molds, caused by *Penicillium digitatum (*Pers.:Fr.) Sacc. and *Penicillium italicum* Wehmer, respectively, are the most economically important postharvest diseases of citrus fruits, in all production areas characterized by low summer rainfall such as Spain, California, or Israel (Eckert and Eaks, 1989). Commercial fungal control on citrus fruit has been performed by the use of conventional synthetic chemical fungicides (Eckert and Eaks, 1989) or, the use of more recently, new reduced-risk fungicides (Smilanick et al., 2006; Kanetis et al., 2007; Palou et al., 2008). However, consumers concerns about human health and environmental contamination, lead researchers worldwide to increase the efforts to find non-contaminant alternatives like for instances, antifungal edible coatings.

In the literature, many works report the effect of edible coatings, on the postharvest quality of citrus fruits (Hagenmaier et al., 2002; Hagenmaier and Shaw, 2002; Pérez-Gago et al., 2002; Hagenmaier, 2004;

Porat et al., 2005; Navarro-Tarazaga and Pérez-Gago, 2006; Navarro-Tarazaga et al., 2007; Navarro-Tarazaga et al., 2008; Rojas-Argudo et al., 2009). However, there are not much information about edible coatings containing antimicrobial food additives to control postharvest diseases fruit contamination by human pathogens and their effects on the quality of coated citrus fruit (Table 6).

Chien et al. (2007) studied the effects of low and high molecular weight chitosan coatings (0.05-0.2%) on the antifungal activity against *P. digitatum* and *P. italicum* and the quality of coated 'Murcott' tangor fruit. Low molecular weight chitosan (0.2%) exhibited effective antifungal activity against both molds and improved fruit water content, firmness, and titratable acidity. Moreover, the performance of low molecular weight chitosan was better than that of the synthetic chemical fungicide thiabendazole. Significant reduction of citrus penicillium decay and fruit senescence retardation during long-term cold storage of different citrus species and cultivars have been observed after the application of certain chitosan formulations (El-Ghaouth et al., 2000; Benhamou, 2004; Chien and Chou, 2006).

Shellac formulations at various pH and concentrations of ethanol with and without parabens were applied to 'Ruby Red' grapefruit and 'Valencia' oranges over a carboxymethyl cellulose layer that facilitated shellac adherence (McGuire and Hagenmaier, 2001). The results showed that a shellac formulation at pH 9.0 with 5.2% ethanol was more toxic to the coliform bacteria *Enterobacter aerogenes* and *E. coli* than a formulation at pH 7.25 with 12% ethanol. Paraben addition to the shellac formulation at pH 9.0 further inhibited coliform growth.

Pome fruits

Rojas- Graü et al. (2007b) incorporated lemongrass and oregano oils and vanillin into apple puree-alginate edible coatings to extent the shelf-life of fresh-cut 'Fuji' apples. It was reported that all antimicrobials significantly inhibited the growth of psychrophilic aerobes, yeasts and molds. Coatings containing lemongrass or oregano oils exhibited the strongest antimicrobial activity against *L. inocua*. In addition, the coatings reduced respiration rate and ethylene production of coated fresh-cut apples. The addition of calcium chloride to the coatings effectively

maintained firmness and color, while coatings containing lemongrass caused severe softening. Coatings containing vanillin were the best in terms of sensory quality.

In another study, whole apples were coated with soy protein coatings containing malic or lactic acid (Eswaranandam et al., 2006). The main objective of this work was to evaluate the effect of the coatings on the sensory quality of fresh-cut fruit, without studying the antimicrobial effect of the coatings. In general, organic acids incorporated to films did not adversely affect the sensory properties of coated apples after cold storage. In a previous work, these authors reported the *in vitro* antimicrobial activity of soy films containing malic or lactic acid (agar diffusion test) (Eswaranandam et al., 2004). However, even though the results from *in vitro* assays are a good approach to evaluate the potential of antimicrobial films, the actual antimicrobial activity on coated produce could considerably differ from that of stand-alone films, due to important factors such as the type of surface of the produce, the diffusion rate of the antimicrobial to the coated produce, or the fruit storage conditions.

Tropical and subtropical fruits

Chitosan-based composite coatings have shown the ability to delay ripening and extend the shelf-life of banana and mango. These coatings significantly retarded color development, reduced weight loss and respiration rate, maintained firmness, and reduced titratable acidity of the coated fruits compared to controls. The application of an additional 1% chitosan to the fascicle region reduced the incidence of molds (Kittur et al., 2001). Chitosan coatings containing natamycin were also effective to control decay of 'Hami' melon caused by *Alternaria alternata* and *Fusarium semitectum* (natural infection). This coating also improved the quality properties of coated fruit (Cong et al., 2007).

In recent research, Sangsuwan et al. (2008) evaluated the inhibitory effect of chitosan/MC stand-alone films, with and without vanillin, against *E. coli* and *Saccharomycis cerevisiae* on wrapped fresh-cut cantaloupe and pineapple. Both films inhibited the growth of *E. coli* and *S. cerevisiae* on fresh-cut cantaloupe, being the film with vanillin more effective.

However, it took a longer time for this film to show the inhibitory effect than for chitosan/MC films without vanillin.

The use of vanillin film showed a different response in cantaloupe and pineapple. In low pH fruit like pineapple, the vanillin film was more effective to inhibit microorganisms than in cantaloupe, which was attributed to a higher release rate of vanillin out of the film. In general, quality attributes of fresh-cut cantaloupe and pineapple were reported as acceptable in this study. However, the application of the antimicrobial film reduced the ascorbic acid content in pineapple, remaining after storage only 10% of its original content.

Berries

Starch-based coatings containing PS reduced the microbial counts of 'Selva' strawberries, extending the storage life of coated fruit to up to 28 d from a period of only 14 d that lasted uncoated fruits. The addition of citric acid to the coating enhanced the antimicrobial action of PS. The coating also reduced fruit weight loss and improved fruit quality (García et al., 1998).

Several studies reported the effect of chitosan coatings on the antimicrobial activity and quality of strawberries (Table 6). Chitosan-based coatings containing calcium or vitamin E were used to extend the shelf life of fresh and frozen strawberries and raspberries (Han et al., 2004). The coatings decreased decay incidence and weight loss, improving the storability and enhancing the nutritional value of fresh and frozen fruits. Park et al. (2005) found that chitosan-based coatings reduced weight loss of strawberries during storage and observed an antifungal activity against *Rhizopus* sp. and *Cladosporium* sp. on inoculated fresh strawberries. Although a significant synergic inhibition activity was reported in *in vitro* tests when PS was incorporated into chitosan, no significant synergies inhibitory effects were reported for inhibition of fungal growth on fresh strawberries.

The addition of oleic acid to chitosan coatings enhanced the antimicrobial activity, improved water resistance, and reduced the respiration rate of cold stored 'Camarosa' strawberries. However, chitosan-oleic acid coatings decreased aroma and flavor of coated

samples. In order to avoid unpleasant changes in sensory attributes, it was recommended to incorporate oleic acid in a chitosan:oleic ratio lower than 4:1 (Vargas et al., 2006). In another study, starch, carrageenan, and chitosan were used to optimize coating compositions. The optimized coatings were applied to fresh strawberries to determine the fruit microbiological and quality properties (Ribeiro et al., 2007). Calcium chloride added to the coatings decreased the microbial growth rate on the treated fruit. The lowest microbial growth rate was found on strawberries coated with chitosan and calcium chloride. This chitosan-calcium chloride coating was also the most effective to reduce weight loss and firmness loss of coated strawberries.

Other fruits and vegetables

Chitosan-based coatings delayed ripening and reduced respiration rate, and ethylene production, and postharvest decay of coated tomatoes (El-Ghaouth et al., 1992). Badawy et al. (2009) reported that chitosan of different molecular weight inhibited the growth of *Botrytis cinerea* in both *in vitro* and *in vivo* assays. In addition to the antifungal activity, chitosan had the potential to elicitation defence markers.

HPMC-based coatings containing sorbic acid (0.4%) enhanced the inactivation of *Salmonella montevideo* on the surface of tomatoes. However, these coatings caused a chalky appearance of the fruit surface, limiting its potential for commercial application (Zhuang et al., 1996). The addition of citric or acetic acid to HPMC film solutions did not enhanced the inactivation of *S. montevideo* on the surface or core tissue of tomatoes. Treating with HPMC coatings delayed changes in color and firmness of tomatoes.

Film forming solution made of sodium caseinate, chitosan, or carboxymethyl cellulose containing 1% of oleoresins (olive, rosemary, onion, capsicum, garlic, oreganum) showed limited antimicrobial activity against *L. monocytogenes* in *in vitro* studies. Similarly, in *in vivo* studies, chitosan coatings enriched with rosemary and olive oleoresin applied to butternut squash did not show a significant antimicrobial effect. Those coatings did not induce deletorious effects on the sensory acceptability of coated squash (Ponce et al., 2008).

Table 6. Antimicrobial edible composite coatings applied to fresh or minimally processed fruits and vegetables

Application Fruit/ vegetable	Coating	Antimicrobial agent	Concentration	Target pathogen	Pathogen inoculation	Antimicrobial activity	Reference
CITRUS							
'Valencia' oranges	Shellac (pH =7.3)	Ethanol	12 %	*Escherichia coli, Enterobacter aerogenes*	10^6 CFU/cm^2	+	(McGuire and Hagenmaier, 2001)
	Shellac (pH = 9.0)	Ethanol	5.2 %	*E. coli, E. aerogenes*	10^6 CFU/cm^2	+	
	Shellac (pH = 9.0)	Paraben	0.1 %	*E. coli, E. aerogenes*	10^6 CFU/cm^2	+	
'Ruby Red' grapefruit 'Murcott' tangor	Shellac Chitosan (LMWC[a], 0.2 %) Chitosan (HMWC[b], 0.2 %)	-	-	*P. digitatum, P. italicum, Botrydiplodia lecanidion, Botrytis cinerea*	10^5 spores/mL	+	(Chien et al., 2007)
POME FRUIT							
'Fuji' apple pieces	Apple puree-alginate	Oregano oil, lemongrass, vanillin	0.5 %	*L. inocua*	10^5 CFU/mL	+	(Rojas-Graü et al., 2007b)
TROPICAL AND SUBTROPICAL FRUIT							
Mango, banana	Chitosan-glycerol	-	-	-	Natural infection	+	(Kittur et al., 2001)

Application Fruit/ vegetable	Coating	Antimicrobial agent	Concentration	Target pathogen	Pathogen inoculation	Antimicrobial activity	Reference
Pineapple	Chitosan-MC	Vanillin	0.9 g	*E. coli, Saccharomices cerevisiae*	10^5 CFU/mL	+	(Sangsuwan et al., 2008)
'Hami' melon	Chitosan	Natamycin	20 mg/L	*Alternaria alternata, Fusarium semitectum*	Natural infection	+	(Cong et al., 2007)
BERRIES							
Strawberry	Starch (mixtures of corn and potato)	Potassium sorbate	0.2 g/L	-	Natural infection	+	(García et al., 1998)
Strawberry	HPMC	Potassium sorbate	0.3 %	*Cladoporium* sp., *Rhizopus* sp.	1.1 x 10^4 spores /mL	+	(Park et al., 2005)
Strawberry	Chitosan	-	-	*Cladoporium* sp., *Rhizopus* sp.	1.1 x 10^4 spores /mL	+	(Park et al., 2005)
		Potassium sorbate	0.3 %	*Cladoporium* sp., *Rhizopus* sp.			
Strawberry	Chitosan	-	-	-	Natural infection	+	(Vargas et al., 2006)
Strawberry, raspberries	Chitosan-vitamin E or Chitosan-calcium lactate and calcium gluconate	-	-	-	Natural infection	+	(Han et al., 2004)

Application Fruit/ vegetable	Coating	Antimicrobial agent	Concentration	Target pathogen	Pathogen inoculation	Antimicrobial activity	Reference
OTHER FRUIT AND VEGETABLES							
Tomatoes	Chitosan	-	-	*B. cinerea*	Natural infection	+	(El-Ghaouth et al., 1992)
	HPMC	Sorbic acid	0.4 %	*Salmonella montevideo*	-	+	(Zhuang et al., 1996)
	Chitosan (pH adjusted with lactic acid)	-	-	*B. cinerea*	10^5 conidia/mL	+	(Badawy and Rabea, 2009)
Squash slices	Chitosan	Oleoresins olive Rosemary Capsicum	1 %	*L. monocytogenes*	Natural infection	+	(Ponce et al., 2008)

[a] LMWC = low molecular weight chitosan. [b] HMWC = high molecular weight chitosan.
Other abbreviations are similar to those presented in Tables 2.

5. Future trends

To date, most of the research has been focussed on the development of antimicrobial edible films and the effect of composition and preparation techniques on film antimicrobial, barrier, and mechanical properties. However, few studies report the application of antimicrobial edible coatings to food products, and their *in vivo* effect on physico-chemical, microbiological and physiological properties. Coatings developed for one species or cultivar of fruit may not be appropriate for another, because of important differences in skin resistance, gas diffusion or respiration rate.

Although aqueous solutions of organic acid salts have been effective to control postharvest citrus diseases (Palou et al., 2002; Montesinos-Herrero et al., 2009), there is very little information on the use of antimicrobial edible films and coatings containing those preservative compounds to inhibit the growth of *P. digitatum* or *P. italicum* growth and their effect on the quality of coated citrus fruit. Therefore, much research is needed to evaluate the real impact of such postharvest treatments from the point of view of both commercial feasibility and consumer acceptance.

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OBJECTIVES

GENERAL OBJECTIVE

To develop new edible composite coatings with the addition of atifungal food additives for the control of posharvest green and blue molds of fresh citrus fruit.

Specific objectives

1. To develop hydroxypropyl methylcellulose (HPMC)-lipid edible composite coatings with antifungal food preservatives based on their capability of forming stable emulsions.
2. To evaluate the *in vitro* activity of selected HPMC-lipid films against *Penicillium digitatum* and *Penicillium italicum*.
3. To determine the mechanical and barrier properties of selected HPMC-lipid films.
4. To evaluate *in vivo* the curative and preventive activity of HPMC-lipid coatings with antifungal food preservatives to control green and blue molds on commercially important citrus species and cultivars.
5. To evaluate the physico-chemical and sensory quality, as well as mold control of coated oranges and mandarins after long-term cold storage.
6. To evaluate the effect of chitosan edible coatings against green and blue molds of oranges and mandarins.

CHAPTER 1

Inhibition of *Penicillium digitatum* and *Penicillium italicum* by hydroxypropyl methylcellulose-lipid edible composite films containing food additives with antifungal properties

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ABSTRACT

New hydroxypropyl methylcellulose (HPMC)-lipid edible composite films containing low-toxicity chemicals with antifungal properties were developed. Tested chemicals were mainly salts of organic acids, salts of parabens, and mineral salts, classified as food additives, or generally recognized as safe (GRAS) compounds. Selected films containing food preservatives were used for *in vitro* evaluation (disk diameter test) of their antifungal activity against *Penicillium digitatum* (PD) and *Penicillium italicum* (PI), the most important postharvest pathogens of fresh citrus fruit. Mechanical properties and oxygen (OP) and water vapor permeabilities (WVP) of selected films were also determined. Film disks containing parabens and their mixtures inhibited PD and PI to higher extent than the other chemicals tested. Among all organic acid salts tested, potassium sorbate (PS), and sodium benzoate (SB) were the most effective salts in controlling both PD and PI. The use of mixtures of parabens or organic acid salts did not provide an additive or synergistic effect for mold inhibition when compared to the use of single chemicals. Barrier and mechanical properties of films were affected by the addition of food preservative. Results showed that HPMC-lipid films containing an appropriate food additive should promise as potential commercial antifungal edible coatings for fresh citrus fruit.

KEYWORDS: hydroxypropyl methylcellulose, edible films, food preservatives, *Penicillium digitatum, Penicillium italicum*, mechanical and barrier properties.

INTRODUCTION

Postharvest diseases of citrus are mainly caused worldwide by the pathogens *Penicillium digitatum* (Pers.:Fr.) Sacc. (PD) and *Penicillium italicum* Wehmer (PI), which cause green and blue molds, respectively (*1*). Chemical fungicides such as imazalil, sodium ortho-phenyl phenate, or thiabendazole have been widely applied in order to control these diseases (*1*). Consumer concerns about prolonged and extensive use of chemical fungicides to control citrus postharvest decay lead researches to look for alternative non-contaminant methods that do not deposit harmful residues and contaminate the environment.

Several alternative methods of different nature have been assayed against both PD and PI including cold storage in conventional controlled hypobaric or ozonated atmospheres, application of heat treatments, use of ionizing radiation, dips in aqueous solutions of food additives, or chemical compounds of low toxicity (*2-4*), or biological control (*5*).

The use of edible coatings and films is an alternative method to preserve the postharvest quality of fruits and vegetables (*6*). In the food industry, they offer opportunities to increase the shelf life of many products. Edible coatings and films form a semi-permeable barrier to exchange oxygen, carbon dioxide, water vapor leading to weight loss reduction, respiratory rate modification, and senescence delay (*7*). Furthermore, the visual quality of treated produce is improved due to gloss provided by edible coatings and films (*8*).

Polysaccharides, proteins, lipids, and resins are mainly used to form edible coatings. Plasticizers and emulsifiers are usually added to increase flexibility and surface tension between aqueous and lipid phases in those formulations that combine lipids and hydrocolloids (*9, 10*). In addition, edible coatings and films are a viable mean for incorporating food additives or other substances to enhance flavor, color and texture, control microbial growth, and improve general coating performance (*11, 12*). Antimicrobials can be added to edible coatings to retard the growth of bacteria, yeasts and molds during storage and distribution of fresh or minimally processed products. Generally, these compounds include organic acids (acetic, benzoic, lactic, propionic, sorbic) and their salts (*13-15*), parabens (*16*), bacteriocins such as nisin (*17*), sucrose esters (*18*), chitosan (*19*), essential oils (*20*), or natural antimicrobials such as natamycin, lactoferrin, lysozyme, or lactoperoxidase (*15, 21*). Several studies reported that the addition of potassium sorbate to edible coatings controlled microbial proliferation on strawberries (*22, 23*), the addition of acetic, citric, and sorbic acids effectively controlled the pathogen *Salmonella montevideo* on tomatoes (*24*), and the addition of parabens reduced coliform bacteria on citrus fruit (*25*). However, little research has been done to develop new edible composite coatings with the addition of antifungal compounds as a new technique to control major fungal postharvest diseases of fresh citrus fruit.

The objectives of this study were to develop new hydroxypropyl methylcelullose-lipid edible composite films containing food additives

with antifungal properties and evaluate the *in vitro* activity of selected films against PD and PI. Barrier and mechanical properties of selected films were also studied.

MATERIALS AND METHODS

Materials. Hydroxypropyl methylcellulose (HPMC) (Methocel E15) was from Dow Chemical Co. (Midland, MI, USA). Beeswax (BW) (grade 1) and resin (shellac) were supplied by Fomesa Fruitech, S.L. (Valencia, Spain). Glycerol and stearic acid were from Panreac Química, S.A (Barcelona, Spain). Ammonia (25%) and silicone antifoam (FG-1510) were from Scharlau (Sentmenat, Spain) and Dow Corning® (Belgium), respectively. Food preservatives were purchased to Sigma (Sigma-Aldrich Chemie, Steinheim, Germany) and included mineral salts, salts of organic acids, sodium salts of parabens, and several other compounds. Molecular formulas and the corresponding E-list code for food additives in the European Union (EU) are shown in Table 1. Most of them are likewise classified as food additives or generally recognized as safe (GRAS) compounds by the United States Food and Drug Administration (US FDA).

Film formation. Films from edible composite emulsions were prepared by combining the hydrophilic phase (HPMC) and the lipid phase (BW and shellac) suspended in water. Glycerol and stearic acid were used as plasticizer and emulsifier, respectively. Ratios of HPMC-glycerol (2:1) dry basis (db) and lipids (BW and shellac)-stearic acid (5:1) (db) were kept constant throughout the study. For emulsion preparation, an aqueous solution of HPMC (5% w/w) was dispersed in hot water at 90 ºC and later hydrated at 20 ºC. Shellac was previously dispersed in water at 40 ºC, and ammonia (15% w/w shellac/ammonia) was added to dissolve the resin. BW, glycerol, stearic acid, and water were added to HPMC solution and heated to 90 ºC. Shellac was heated separately at the same temperature and added to the former compounds. Once the lipids were melted, samples were homogenized with a high-shear probe mixer (Ultra-Turrax Model T25, IKA-Werke, Steufen, Germany) for 4 min at 22,000 rpm. Emulsions were cooled under agitation to a temperature lower than 25 ºC by placing them in an ice water bath. To ensure completed hydration of HPMC, emulsions were further agitated during

25 min.

A preliminary study was performed to prepare emulsions capable of forming homogeneous composite films containing food preservatives with antifungal properties. Edible composite formulations were optimized based on a solid concentration (SC) range of 4-12% and total lipid concentration (BW-shellac) range of 0-60% (db).

The addition to the coating of food preservatives was from 0.05 to 4.5% (wet basis, wb). In each case, the maximum concentration of food preservative that formed stable emulsions was determined. All food preservatives and concentrations tested to prepare HPMC-lipid emulsions are presented in Table 1. Emulsions were degassed and films were cast by pipetting emulsions onto rimmed, smooth plates resting on a leveled wooden slab and allowed to dry at room temperature over 24 h.

Determination of *in vitro* antifungal activity. Antifungal activity of selected edible films was evaluated through the disk diameter test. This method was adapted from that described by Min and Krochta (*21*).

Media. Potato dextrose agar (PDA) supplied by Sigma and Dichloran rose-bengal chloramphenicol agar (DRBC) provided by Merck (Darmstadt, Germany) were used to prepare the media for the disk diameter test.

Fungal inoculum. P. digitatum isolate NAV-7 and *P. italicum* isolate MAV-1, obtained from decayed oranges from Valencia packinghouses, were isolated, identified, and maintained in the IVIA culture collection of postharvest pathogens. These strains were selected for their aggressiveness on the most commercially important mandarin and orange cultivars. Prior to each experiment, the isolates were grown on PDA in petri dishes at 25 ± 1 ºC for 7–10 d. A high-density conidial suspension was prepared in Tween 80 (0.05%, w/v; Panreac Química S.A.) in sterile water, passed through two layers of cheesecloth, measured with a haemacytometer, and diluted with sterile water to achieve the desired inoculum density.

Disk diameter test. Selected films were cast by pipetting the emulsion onto sterilized HDPE plates (14.1 cm internal diameter). Most of the films were very brittle, which made difficult to peel them from the plates. Therefore, the plates had to be covered with wax paper (0.2 mm

thickness) before using them. The wax paper was disinfected with alcohol (97%). For 6 and 8% SC, 48.3 and 46.6 g of emulsion were weighted respectively in order to obtain 0.2-0.3 mm thick films. Emulsions on plates were allowed to dry about 48 h at room temperature under aseptic conditions, inside a previously sterilized laminar flow hood. Dry films were peeled intact from the casting surface and aseptically cut into 16 mm-diameter disks using a sterile cork borer. Films that could not be separated from the wax paper were cut together with it. All films were aseptically stored at 5 ºC until used. For each emulsion 3-5 films were prepared. Ten measurements of film thickness on each film were randomly taken, and the mean value of film thickness was reported. Films from emulsions with the selected percentage of SC and BW-shellac ratio but without added food preservatives were used as controls. Three levels of inoculum were prepared for each fungal species: 10^3, 10^4, and 10^5 spores/mL. For each inoculum level, 100 µL of conidial suspension were spread on the surface of DRBC agar plates (9 cm diameter). Film disks were aseptically transferred to the agar surface previously inoculated with PD or PI. The plates were refrigerated at 4 ºC for 3 h to allow the diffusion of film ingredients and then incubated at 25 ºC for 5 d. For each pathogen, inoculum density, and film, three agar plates (replicates) were prepared. After incubation, the length of the inhibition zone around the film disk (from the perimeter of the film disk until the edge of the inhibited area) was measured with a digital caliper and reported in mm. Four measurements were performed for each petri dish.

Properties of selected emulsions and films. The pH of the emulsions was measured using a digital pH-meter (model C830, CONSORT, Turnhout, Belgium). Emulsion viscosity was measured with a viscometer (Model LVF, Brookfield Engineering Laboratories Inc., Stoughton, MS, USA). Three measurements were performed from replicated emulsion samples. Results were expressed in centipoises (cp). Sample viscosities were measured at 20 ºC.

Emulsion stability was measured according to an adapted method from Taherian et al. (*26*). Sixty mL of prepared emulsion were placed in a 100 mL volumetric flask and left there at 25 ºC for 48 h. The results were expressed as percentage of phase separation respect to total height of the emulsion in the tube. The determinations were done in duplicate.

Water vapor permeability (WVP). WVP of the films was determined using the gravimetric method of the ASTM E96-92 with modifications by McHugh et al. (*27*) Films were casted as described before. Two disk samples (5 cm diameter) were cut from each film and mounted on polymethacrylate cups containing 6 mL of distilled water. To study if phase separation occurred during drying, film surface, which had been exposed to air during drying, was placed either facing the low relative humidity (RH) environment ("facing up") or the high RH environment ("facing down"). The film was sealed to the cup base with a ring using four screws located symmetrically around the cup circumference. The cups were placed on desiccator cabinets containing fans and held around 0% RH using anhydrous silica gel (Scharlau Chemie S. A.). Weights were taken periodically after steady state was achieved and used to calculate the RH at the film underside and the resulting WVP.

Oxygen permeability (OP). An oxygen permeation analyzer (Systech Instruments Mod. 8001, Oxfordshire, UK) was used to measure oxygen transmission rate through films according to the ASTM method D3985 (*28*). OP of the films was measured at 23 ± 2 ºC and 50 ± 5% RH. A square film (3x3 cm) was placed on a stainless steel mask with an open testing area of 5 cm^2. One side of the film was exposed to flowing nitrogen gas, and the other side was exposed to flowing oxygen gas at the same conditions. OP was calculated by dividing the oxygen transmission rate by the oxygen pressure and multiplying by the film thickness (*29*).

Mechanical properties. Films were cast onto rectangular 15x24 cm, rimmed, smooth high density polyethylene (HDPE) plates covered with Teflon® by applying 4 g total solid per plate to minimize thickness variation between treatments. Three replicates of selected emulsions were used to prepare films. Dried films were conditioned at 53% RH in a chamber containing magnesium nitrate hexahydrate (Scharlau Chemie S.A.) saturated solution for more than 2 d. Tensile measurements were tested using an Instron Universal Machine (Mod. 3343; Instron Corp., Canton, MA, USA) according to the ASTM method D882-97 (*30*). Each film was clamped between pneumatic grips and stretched at 5 mm/min with a 0.3 kN load cell. Testing conditions were controlled throughout the measurement and held constant at 50 ± 5% RH and 23 ± 2 ºC. The mechanical properties reported were Young's modulus (YM, in MPa), maximum tensile stress (TS, in MPa) and elongation at break (E, in %).

Fifteen sample strips (50.0x8.0 mm) were measured for each film.

Film thickness. Film thickness was measured with a digital micrometer (Mod. Quickmike Series 293-IP-54; Mitutoyo Corp., Kanawava, Japan) at four random positions in the film. Mechanical properties OP and WVP were calculated using the average thickness.

Statistical analysis. Statistical analyses of data on film properties or fungal inhibition were performed using the software Statgraphics Plus 2.1 (Manugistics, Inc., Rockville, MD, USA). Specific differences between means were determined by Fisher's Protected least significant difference test (LSD, $P < 0.05$) applied after an analysis of variance (ANOVA). For inhibition data, ANOVA were applied to values transformed to the square root of the value plus 0.5.

Table 1. Characteristics and tested concentrations of antifungal food preservatives used to prepare HPMC-lipid emulsion films.

Food preservative	Molecular formula	E- Code[a]	Solid concentration tested (%, wb)	Food preservative concentration tested (%, wb)
Mineral salts				
Sodium bicarbonate	$NaHCO_3$	E-500(I)	6; 8; 10; 12	0.05; 0.5; 1.0; 1.5; 2.0; 2.5; 3.0; 3.5; 4.0; 4.5
Ammonium bicarbonate	NH_4HCO_3	E-237	6; 8; 10; 12	0.05; 0.5; 1.0; 1.5; 2.0; 2.5; 3.0; 3.5; 4.0; 4.5
Sodium carbonate	Na_2CO_3	E-500(II)	6; 8; 10; 12	0.05; 0.5; 1.0; 1.5; 2.0; 2.5; 3.0; 3.5; 4.0; 4.5
Sodium silicate (SiO_2 ; 27%)	$Na_2SiO_3O_7$	-	6; 8	1.0; 2.0; 2.5
Sodium molybdate	$Na_2MoO_4.2H_2O$	-	6; 8; 10; 12	1.0; 1.5; 2.0; 2.5; 3.0; 3.5; 4.0; 4.5
Ammonium molybdate	$(NH_4)_6Mo_7O_{24}.4H_2O$	-	6; 8; 10; 12	1.0; 1.5; 2.0; 2.5; 3.0; 3.5; 4.0; 4.5
Organic acid salts				
Potassium sorbate	$C_6H_7O_2K$	E-202	6; 8; 10; 12	0.05; 0.5; 1.0; 1.5; 2.0; 2.5; 3.0; 3.5; 4.0; 4.5
Sodium benzoate	$C_7H_5O_2Na$	E-211	6; 8; 10; 12	0.05; 0.5; 1.0; 1.5; 2.0; 2.5; 3.0; 3.5; 4.0; 4.5
Sodium acetate	CH_3COONa	E-262-(i)	6; 8	1.0; 2.0; 2.5
Sodium diacetate	$(CH_3COO)_2HNa$	E-262-(II)	6; 8	1.0; 2.0; 2.5
Sodium propionate	CH_3CH_2COONa	E-281	6; 8	1.0; 2.0; 2.5
Calcium propionate	$Ca(CH_3CH_2COO)_2$	E-282	6; 8	1.0; 2.0; 2.5
Sodium formate	$HCOONa$	E-237	6; 8	1.0; 2.0; 2.5
Calcium formate	$Ca(HCOO)_2$	E-238	6; 8	1.0; 2.0; 2.5
Sodium citrate	$C_6H_5Na_3O_7$	E-331(I)	6; 8	1.0; 2.0; 2.5
Sodium L-lactate	$C_3H_5NaO_3$	E-325	6; 8	1.0; 2.0; 2.5
Sodium L-tartrate	$C_4H_4Na_2O_6.2H_2O$	E-335 (I)	6; 8	1.0; 2.0; 2.5
Organic acid salts (mixtures)				
Potassium sorbate + sodium propionate	-	-	6	1.5; 2.0
Sodium benzoate + potassium sorbate	-	-	8	1.5; 2.0; 2.5
Sodium benzoate + sodium propionate	-	-	8	1.5; 2.0; 2.5

Food preservative	Molecular formula	E- Code[a]	Solid concentration tested (%, wb)	Food preservative concentration tested (%, wb)
Parabens				
Sodium salt of methyl paraben	$C_8H_7NaO_3$	E-219	6; 8; 10; 12	1.0; 1.5; 2.0; 2.5; 3.0; 3.5; 4.0; 4.5
Sodium salt of ethyl paraben	$C_9H_9NaO_3$	E-215	6; 8; 10; 12	1.0; 1.5; 2.0; 2.5; 3.0; 3.5; 4.0; 4.5
Sodium salt of propyl paraben	$C_{10}H_{11}NaO_3$	E-217	6; 8; 10; 12	1.0; 1.5; 2.0; 2.5; 3.0; 3.5; 4.0; 4.5
Paraben (mixture)				
Sodium salt of methyl paraben + sodium salt of propyl paraben		-	6, 8, 10, 12	1,0; 1,5; 2,0; 2,5; 3,0; 3,5; 4,0; 4,5
Other compounds				
EDTA	$C_{10}H_{12}N_2O_8CaNa_2$	E-385	6; 8	1.0; 2.0; 2.5
2-deoxy-D-glucose	$C_6H_{12}O_5$	-	6	0.3; 0.5; 1.0

[a] E-Code = Number codes for food additives approved by the European Union.

RESULTS AND DISCUSSION

Selection of stable edible films. Among the large amount of HPMC-lipid edible composite coatings containing antifungal food preservatives that were prepared (470 emulsions, Table 1), only those in Table 2 were selected to be evaluated for antifungal activity. Their preservative concentration, SC, total lipid content (BW-shellac), pH, and viscosity are presented. In order to obtain stable emulsions capable of forming homogeneous coatings, HPMC-lipid edible composite coatings were optimized based on the percentage of SC, total lipid content, and food preservative concentration. The emulsions showed in Table 2 were stable, since they exhibited no phase separation (*26*). It was found that HPMC-lipid stable emulsions suitable to be used as coatings contained from 6 to 8% SC, 50% (db) total lipid content, and a maximum of 2.5% (wb) food preservative (Table 2).

Viscosity is one of the main factors affecting coating thickness (*31*); therefore, in our HPMC-lipid edible composite coatings, the effects of SC and type and concentration of food preservative on viscosity were evaluated. In this study, emulsions were ranked as follows according to their viscosity: "non-viscous" for emulsion viscosity lower than 40 cp,

"viscous" for emulsion viscosity between 40-70 cp, and "very viscous" for emulsion viscosity higher than 70 cp. Viscosity of selected emulsions was on the range of 7-22 cp, corresponding to non-viscous emulsions. Differences in emulsion SC of selected formulations did not translate in important differences on emulsion viscosity (Table 2). Cisneros-Zevallos and Krochta (*31*) found that viscosity of HPMC solutions increased as SC increased. In our emulsions, the formulations not only differed on SC, but also on lipid ratio, and food preservative concentration, which also contribute to the final viscosity value.

Food preservatives added to the emulsions in a concentration lower than 2.5% resulted in non-viscous emulsions. However, food preservatives added at a concentration up to 4.5% resulted on viscous emulsions or gels (data not shown). When a mixture of two food preservatives, such as two salts of organic acids or two sodium salts of parabens, was added to the emulsions, their viscosity did not increase and the resulting emulsion was non-viscous.

Table 2. Composition, pH and viscosity of selected HPMC-lipid edible composite emulsions containing antifungal food preservatives.

HPMC-lipid edible coatings with food preservative	Food preservative concentration tested (%, wb)	Solid concentration tested (%)	BW-shellac (%, db)	pH	Viscosity (cp)
Mineral salts					
Sodium bicarbonate	2.0	6	45-5	8.88	7.0
Ammonium bicarbonate	2.0	6	25-25	9.18	16.5
Organic acid salts					
Potassium sorbate	2.0	6	25-25	7.42	11.6
Sodium benzoate	2.5	8	25-25	7.33	13.3
Sodium acetate	1.0	6	50-0	6.89	10.8
Sodium diacetate	1.0	6	50-0	4.70	14.7
Sodium propionate	2.0	6	25-25	7.39	11.2
Calcium propionate	1.0	6	50-0	5.41	17.8
Sodium formate	1.0	6	50-0	6.41	14.3
Calcium formate	1.0	6	50-0	4.85	18.5
Sodium citrate	1.0	6	50-0	3.77	12.7
Sodium L-lactate	1.0	6	50-0	7.15	15.2
Sodium L-tartrate	1.0	6	50-0	7.04	9.8
Organic acid salts (mixtures)					
Potassium sorbate + sodium propionate	1.5 + 0.5	6	25-25	7.46	11.9
Sodium benzoate + potassium sorbate	2.0 + 0.5	8	25-25	7.43	15.1
Sodium benzoate + sodium propionate	2.0 + 0.5	8	25-25	7.42	16.9
Parabens					
Sodium salt of methyl paraben	1.5	6	50-0	9.06	13.2
Sodium salt of methyl paraben	1.0	6	50-0	8.73	17.5
Sodium salt of ethyl paraben	1.0	6	50-0	9.40	11.1
Sodium salt of propyl paraben	1.0	6	50-0	9.43	14.8
Paraben (mixture)					
Sodium salt of methyl paraben + sodium salt of propyl paraben	1.0 + 0.5	6	50-0	9.21	14.7

HPMC-lipid edible coatings with food preservative	Food preservative concentration tested (%, wb)	Solid concentration tested (%)	BW-shellac (%, db)	pH	Viscosity (cp)
Other compounds					
EDTA	1.5	6	45-5	6.52	18.3
2-deoxy-D-glucose	0.5	6	25-25	7.14	22.2
2-deoxy-D-glucose	0.3	6	25-25	6.94	27.5

Emulsions with food preservatives that contained calcium propionate or calcium formate produced gels when shellac was included in the formulation. Stable and non-viscous emulsions were obtained when shellac was not present in the emulsion.

Since sodium carbonate, sodium molybdate, and ammonium molybdate showed promise for the control of postharvest decay of citrus fruits (*3, 32-34*), these compounds were also evaluated as ingredients of HPMC-lipid emulsions. Unfortunately, the addition of all of them resulted in unstable emulsions or gels. Emulsions containing sodium carbonate formed gels, even when shellac was not included, and sodium carbonate content was as low as 1% (data not shown). Aleuritic acid is one of the most important derivatives of shellac. It is obtained from shellac by saponification (*35*). It seems that saponification of shellac leading to gel formation occurred in our HPMC-lipid emulsions containing sodium bicarbonate, probably due to the alkaline properties of sodium carbonate.

Among all mineral salts tested, only sodium bicarbonate (SBC) and ammonium bicarbonate (ABC) were selected as ingredients of HPMC-lipid emulsions.

Most of the HPMC-lipid emulsions containing shellac and salts of organic acids or sodium salts of parabens precipitated or formed gels. When shellac was removed from most of these emulsions, non-viscous and stable emulsions were obtained (Table 2). Potassium sorbate (PS), sodium benzoate (SB), and sodium propionate (SP) were three of the organic acid salts tested that permitted the inclusion of shellac in their formulations.

Acid or alkaline properties of the emulsions depended on the kind of food preservatives that were added to them. Depending on the food preservative that was added into the emulsions, a wide range of pH was found, from acid emulsions containing sodium citrate (pH 3.77), through neutral emulsions such as those containing sodium tartrate or sodium lactate, to basic emulsions containing parabens (pH 9.40).

***In vitro* antifungal activity.** The determination of film antifungal activity in this study was based on the disk diameter test. In this assay, the inhibition area surrounding the film disks was measured and

compared to control films in which food preservatives were not added and hence there were no inhibition area. Results on the antifungal activity against different inoculum concentrations of both PD and PI are shown in Table 3.

Mineral salts. Among HPMC-lipid films with mineral salts, those with 2% SBC significantly inhibited ($P < 0.05$) the growth of both PD and PI on DRBC agar irrespective of the inoculum density tested (Table 3). Films with SBC controlled better PD than PI. On the contrary, films with ABC produced no significant inhibition of both PD and PI. At all inoculum densities, pH of all these films were above 8.5 (Table 2), pH value at which the germination and growth of PD are inhibited (*36*). However, results from this study suggest that factors other than emulsion pH influence the antifungal activity against PD or PI of these edible films. Smilanick et al. (*36*) observed in an *in vitro* study that sodium salts were superior to ammonium or potassium carbonates, and bicarbonates for the control of green mold, suggesting that the sodium cation played some important role in the control of PD. Furthermore, brief immersions in water solutions of SBC, alone or in a combination with other nonpolluting control methods, have been reported to be effective for the control of PD and PI on citrus fruit (*4, 5, 37*).

Table 3. Antifungal activity of HPMC-lipid edible composite films with food preservatives against *P. digitatum* and *P. italicum* at different inoculum concentrations.

HPMC-lipid films with food preservative[b]	Length of inhibition zone (mm)[a]					
	PD inoculum concentration (spores/mL)			PI inoculum concentration (spores/mL)		
	10^3	10^4	10^5	10^3	10^4	10^5
Mineral salts						
Sodium bicarbonate	8.9 defg	6.2 b	9.0 cd	4.3 cd	5.0 c	3.8 b
Ammonium bicarbonate	1.0 ab	1.1a	0.0 a	0.0 a	0.0 a	0.0 a
Organic acid salts						
Potassium sorbate	16.8 ghi	15.8 ef	17.3 ef	9.2 cde	6.6 e	5.9 de
Sodium benzoate	12.8 fgh	11.3 d	7.2 c	9.8 de	3.9 cd	2.9 bc
Sodium acetate	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Sodium diacetate	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Sodium propionate	6.0 cd	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Calcium propionate	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Sodium formate	6.6 cde	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Calcium formate	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Sodium citrate	1.2 ab	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Sodium L-lactate	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Sodium L-tartrate	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a

HPMC-lipid films with food preservative[b]	Length of inhibition zone (mm)[a]					
	PD inoculum concentration (spores/mL)			PI inoculum concentration (spores/mL)		
	10^3	10^4	10^5	10^3	10^4	10^5
Organic acid salts (mixtures)						
Potassium sorbate + sodium propionate	16.6 ghi	12.0 d	4.3 b	0.0 a	0.0 a	0.0 a
Sodium benzoate + potassium sorbate	13.6 fgh	10.6 cd	9.1 cd	11.0 e	4.5 de	4.3 cd
Sodium benzoate + sodium propionate	10.2 efgh	7.8 bc	0.7 ab	2.8 b	1.1 b	0.0 a
Parabens						
Sodium salt of methyl paraben (1.5%)	22.1 i	24.2 g	21.1 f	18.3 f	18.5 g	19.9 g
Sodium salt of methyl paraben (1.0%)	22.3 i	21.9 fg	17.4 ef	15.8 f	14.8 f	15.8 f
Sodium salt of ethyl paraben	15.3 fghi	15.2 de	12.2 de	15.3 f	14.3 f	13.3 f
Sodium salt of propyl paraben	2.8 bc	4.9 b	5.7 c	7.8 cde	6.1 e	7.0 e
Paraben (mixture)						
Sodium salt of methyl paraben + sodium salt of propyl paraben	21.3 hi	18.9 efg	19.9 f	18.8 f	17.4 fg	19.8 g
Other compounds						
EDTA	7.5 de	4.7 b	0.0 a	6.3 c	0.0 a	0.0 a
2-deoxy-D-glucose (0.5%)	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a

HPMC-lipid films with food preservative[b]	Length of inhibition zone (mm)[a]					
	PD inoculum concentration (spores/mL)			PI inoculum concentration (spores/mL)		
	10^3	10^4	10^5	10^3	10^4	10^5
Controls[c]						
Control (8% SC; 25%BW-25%shellac)	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Control (6% SC; 45%BW-5%shellac)	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Control (6% SC; 25%BW-25%shellac)	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Control (6% SC; 50%BW-0%shellac)	0.6 ab	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a

[a] Values are measurements of length (mm) of inhibitory zones around film disk (from the perimeter of the film disk until the edge of the inhibited area). Values within columns followed by unlike letters are different by Fisher Protected LSD test ($P < 0.05$) applied after an analysis of variance of the square root of the inhibition zone plus 0.5. Non-transformed data are shown. [b] Characteristics of these films are presented in Table 2. [c] HPMC-lipid films without food preservatives at 6 and 8% SC, and different %BW-shellac were used as control films.

Organic acid salts and mixtures. Among all organic acid salts added into HPMC-lipid films, only films containing PS (pH 7.42) or SB (pH 7.33) clearly inhibited ($P < 0.05$) the growth of both PD and PI at 10^3 to 10^5 spores/mL on DRBC agar (Figure 1). Films with PS or SB controlled better PD than PI. Films containing 2.0% PS produced larger inhibition zones for PD (16.8-17.3 mm) and PI (5.9-9.2 mm) than films with 2.5% SB (7.2-12.8 mm for PD and 2.9-9.8 mm for PI) (Table 3). In general, films with other organic acid salts did not inhibit both PD and PI. The only exceptions were SP, sodium formate, and sodium citrate that inhibited PD at 10^3 spores/mL.

PS, the most soluble form of sorbate, is well known for its potent antifungal activity. In food systems, PS is one of the most widely used compounds to prevent the growth of molds and thus extend produce shelf-life (*38*). Sorbates inhibit mold species including *Penicillium*. The antimicrobial activity of PS against PD and PI has been observed in both *in vitro* and *in vivo* studies. For instance, both pathogens were inhibited in PDA agar when PS were added at a concentration of 0.15-0.20 g/L (*39*). Moreover, aqueous solutions of PS and other common food preservatives such as SB applied to citrus fruit controlled to some extent both postharvest green and blue molds (*2, 4, 40*). The antimicrobial action of sorbate is pH dependent. In general, PS activity is greater at low pH values, although sorbates may be effective at pH values as high as 7 (*38*). In our work, pH of HPMC-lipid films containing PS was around 7 (Table 2). In *in vivo* trials with Valencia oranges artificially inoculated with conidia of PD, brief dips in warm water solutions of 2-3% PS at neutral pH significantly inhibited mold development (*40*). In contrast, other common organic acid-based food preservatives, such as propionates or benzoates, only showed considerable antimicrobial activity at low pH values such as 5-5.5 and 4-4.5, respectively (*38*).

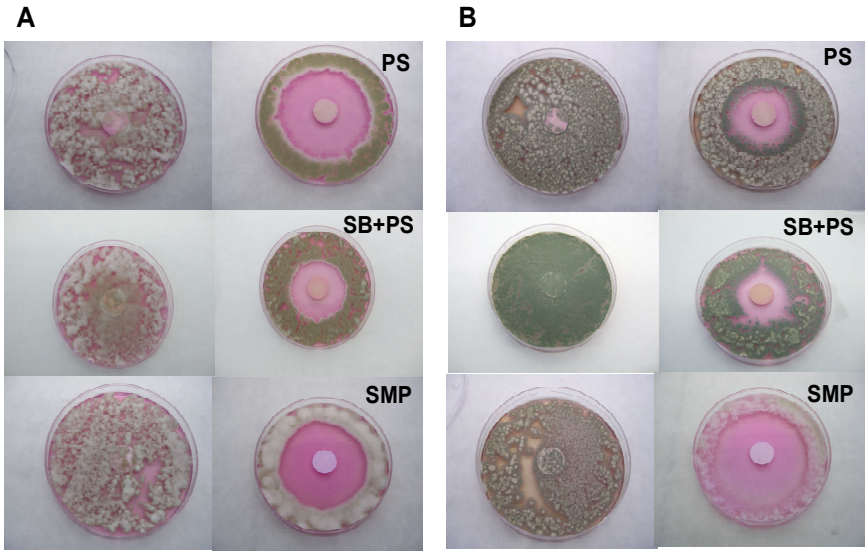

Figure 1. Disk diameter tests on DRBC agar plates for evaluation of the *in vitro* inhibition of *Penicillium digitatum* (A) and *Penicillium italicum* (B) by control HPMC-lipid films (left-side images) and HPMC-lipid films containing potassium sorbate (PS), a mixture of sodium benzoate and potassium sorbate (SB+PS) and sodium salt of methyl paraben (SMP) (right-side images).

In this study, mixtures of the most effective organic acid salts were added to HPMC-lipid films with the goal of achieving an additive or synergistic effect for the inhibition of PD or PI. Films with a mixture of PS+SP significantly inhibited ($P < 0.05$) the growth of PD on DRBC agar at all inoculum concentrations tested. PD at 10^3 spores/mL was more intensely inhibited (16.6 mm) than at 10^4 (12.0 mm) or 10^5 spores/mL (4.3 mm). The inhibition ability was therefore greatly dependent on the pathogenic inoculum density (Table 3). In contrast, PI was not inhibited at all by films formulated with this mixture. Films containing a mixture of SB+PS significantly inhibited ($P < 0.05$) the growth of PD and PI on DRBC agar (Table 3). Stopforth et al. (*38*) observed that the combination of sorbates with either benzoates or propionates may be used to inhibit microorganisms using lower concentrations of each preservative. However, in this work, the use of mixtures of PS+SP, SB+PS, and SB+SP incorporated to HPMC-lipid films resulted in smaller inhibition zones ($P < 0.05$) than the use of films containing PS or SB alone. Thus, there was not an additive effect for the inhibition of PD or PI (Table 3).

Paraben salts and mixtures. Films with sodium salts of parabens and mixtures were the most effective among all food preservatives tested for the inhibition of both PD and PI (Table 3). Large inhibition zones ($P < 0.05$) were observed on DRBC agar at all inoculum densities (Figure 1). Films containing sodium salt of methyl paraben (SMP) inhibited the growth of PD (22.1–24.2 mm) and PI (18.3-19.9 mm) in DRBC agar more than films containing other parabens and mixtures. However, an increase of SMP concentration in the film from 1.0 to 1.5% (wb) did not significantly increase the inhibition zone ($P > 0.05$) for both PD and PI (Table 3). When sodium salt of propyl paraben (SPP) was mixed with SMP (SMP+SPP), the resulting film was not superior to films with SMP alone in reducing the development of PD and PI ($P > 0.05$). Hence, the mixture of parabens did not provide a synergistic effect to control PD or PI.

Effective diffusion of antimicrobials from a film disk depends on the size, shape (linear, branched, or cyclic) and polarity of the diffusing molecule, chemical structure of the film, and the degree of molecular cross-linking (*41*). Cagri et al. (*42*) reported that the application of whey protein isolated (WPI) films containing sorbic acid (linear structure) resulted in higher inhibition of *Listeria monocytogenes* than the application

of films containing p-aminobenzoic acid (cyclic structure), which was related to differences in diffusion rates. It is unknown how these factors affect the antifungal properties of HPMC-lipid films.

Other compounds. Films with disodium-calcium ethylendiaminetetraacetate (EDTA) showed clear zones of inhibition on DRBC agar for PD at 10^3 and 10^4 spores/mL and for PI at 10^3 spores/mL (Table 3). Shelef and Seiter (*43*) reported that EDTA may act as a direct inhibitor of several species of microorganisms and may act synergistically with other antimicrobial agents to promote bacterial destruction. Films containing 0.5% 2-deoxy-D-glucose did not inhibit the growth of PD and PI ($P > 0.05$) (Table 3). *In vivo* studies showed that the combination of low doses of 2-deoxy-D-glucose (0.2% w/w) with *Candida saitona* applied to fruit wounds before artificial inoculation with PD was effective for decay control on oranges and lemons, but this effect was reduced when either 2-deoxy-D-glucose or *C. saitona* were applied separately (*44*).

Properties of selected films. Water vapor permeability. HPMC-lipid films containing PS, SB, and parabens, and their mixtures exhibited higher antifungal activity than other food preservatives tested (Table 3). Therefore, permeability and mechanical properties of these films were determined.

WVP is a measure of the ease with which a material can be penetrated by water vapor (*45*). In general, cellulose-based films present poor moisture barrier due to their hydrophilic character. Incorporation of hydrophobic material improve film resistance to water vapor (*46*). In this study, HPMC-lipid films with 50% (db) total lipid content were used to increase the moisture barrier of the films. Films containing organic acid salts and their mixtures were made with a BW:shellac ratio of 1:1. Films with sodium salt of parabens only contained BW (Table 2).

The effect of food preservatives on WVP of selected HPMC-lipid films is shown in Table 4. Films containing PS alone or the mixture PS+SP exhibited higher WVP than the rest ($P < 0.05$). These values of WVP were around two times higher than those of films containing SB alone or the mixtures SB+PS and SB+SP ($P < 0.05$). These results indicate that PS alone or in combination with SP modified the HPMC-

lipid film structure in a greater extent than films containing SB alone or in combination. Park et al. (*23*) reported that the addition of PS or chitosan to HPMC films increased WVP. This was attributed to a disruption of the crystalline structure of the homogeneous polymer network leading to an increase of WVP.

Table 4. Water vapor and oxygen permeabilities of selected HPMC-lipid edible composite films containing food preservatives.

HPMC-lipid films with food preservative[a]	Permeabilities[b]		
	Water vapor (g mm/kPa h m[c])		Oxygen (cm^3 μm/m^2 d kPa)
	up[z]	*down*[z]	
Potassium sorbate	6.62 c B	5.21 b A	153.11 b
Sodium benzoate	2.85 b B	1.77 a A	82.01 a
Potassium sorbate + sodium propionate	7.38 c A	6.21 b A	293.86 c
Sodium benzoate + potassium sorbate	2.45 b A	2.56 a A	164.58 b
Sodium benzoate + sodium propionate	3.24 b A	2.27 a A	170.44 b
Sodium salt of ethyl paraben	1.11 a A	1.02 a A	713.73 d
Sodium salt of propyl paraben	0.86 a A	1.00 a B	866.27 e

[a] Water vapor and oxygen permeabilities means (n = 3) in columns with different lower-case letters are significantly different according to Fisher Protected LSD test ($P < 0.05$). Water vapor permeability means (n = 3) in rows with different capital letters are significantly different according to Fisher Protected LSD test ($P < 0.05$). [b] Characteristics of the films are shown in Table 2. [c] Film orientation during water vapor permeability test according to drying direction on casting plates

HPMC-lipid films containing parabens had the lowest WVP. This could be due to interactions of parabens with the polymer matrix and/or differences in lipid composition of these films. These films had BW as the only hydrophobic component compared to films with organic acid salts that also had shellac on their formulation. Waxes have been shown to be more effective moisture barriers than resins such as shellac (*47*).

To determine the lipid phase separation in HPMC-lipid composite films, WVP was measured as a function of film orientation during

drying. Phase separation was only observed on films containing PS or SB, with higher WVP when the films were in the "up" position than when the films were in the "down" position, indicating creaming of the lipid phase during drying. This finding contrasted with emulsion stability measurements that showed no phase separation after 48 h at 25 ºC. Surprisingly, when SPP was incorporated into the HPMC-lipid film, the "down" position showed higher WVP than the "up" position ($P < 0.05$), indicating some enrichment on the lipid phase of the film surface in contact with the casting plate during drying. These results show that food additives have an important role in film morphology and final barrier properties.

Oxygen permeability. The OP of HPMC-lipid films containing food preservatives is shown in Table 4. Films containing SB acted as good oxygen barriers and exhibited the lowest OP values. When HPMC-lipid film containing SB was combined with PS or SP, OP values were about two-fold higher than that of the film with SB alone ($P < 0.05$). On the other hand, OP values of films containing PS were lower than that of films with the mixture PS+SP ($P < 0.05$). The mixture of two food preservatives added to the coating formulation might modify the film matrix structure by increasing polymer mobility that permits oxygen migration through the film, thus increasing OP values. The films containing sodium salts of parabens exhibited the highest values of OP among all films tested ($P < 0.05$). These results, as with WVP values, could be due to the presence of the paraben salts and/or the difference in lipid composition of the films. Total amount of lipid in all the films were 50% (db). However, films containing organic acid salts presented a combination of BW and shellac, while films with parabens contained only BW. Hagenmaier and Shaw (*48*) reported that OP was generally lower for coatings with shellac and rosin than for coatings with natural or synthetic waxes.

Mechanical properties. Table 5 shows mechanical properties of HPMC-lipid films containing selected food preservatives. Films containing SB were very brittle and film samples were not possible to obtain for this analysis. The type of food preservative had a significant effect on YM, TS, and E. In general, HPMC-lipid films containing food preservatives presented low TS. HPMC-lipid films containing PS and the mixture PS+SP showed lower YM and TS, and higher E than the rest of assayed

films ($P < 0.05$). Low YM and high E values generally indicate high flexibility of films. The addition of parabens to HPMC-lipid films resulted in an important increase of YM and TS values and a reduction of E values (Table 5), reducing the flexibility of the films and conferring them stiffness and less extensibility. The different behavior of HPMC-lipid films with PS and parabens could be related to the different chemical structure of these food preservatives. Structurally, PS is a straight chain, which can penetrate more easily into film matrix than parabens, which have a benzene ring. Therefore, PS may confer more mobility between HPMC chains resulting in lower YM and TS and greater flexibility of films. Accordingly, similar behavior related to TS and E was reported by Cagri et al. (*42*) in their study of WPI films containing stearic acid (straight chain structure) and p-aminobenzoic acid (benzene ring). HPMC-lipid films containing mixtures of SB+PS and SB+SP showed the highest YM and TS and the smallest E of all the films tested ($P < 0.05$) (Table 5). These samples contained a total concentration of food preservatives higher than the rest (Table 2). In addition, these films contained a benzene ring (SB) that may influence the mobility in the film matrix and thus reduce film flexibility.

Table 5. Mechanical properties of selected HPMC-lipid edible composite films containing food preservatives.

HPMC-lipid films with food preservative[x]	Mechanical properties[a]		
	Young´s modulus (MPa)	Tensile strength (MPa)	Elongation at break (%)
Potassium sorbate	64.18 ab	0.23 a	5.05 c
Sodium benzoate	-	-	-
Potassium sorbate + sodium propionate	32.11 a	0.14 a	7.78 d
Sodium benzoate + potassium sorbate	331.04 d	1.98 c	0.94 a
Sodium benzoate + sodium propionate	329.52 d	1.61 cd	0.92 a
Sodium salt of ethyl paraben	135.53 bc	0.60 ab	2.93 b
Sodium salt of propyl paraben	171.86 c	1.13 bc	4.76 c

[a] Mechanical properties means (n = 3) in columns with different letters are significantly different accordingly to Fisher Protected LSD test ($P < 0.05$).
[b] Characteristics of the films are shown in Table 2.

In conclusion, stable emulsions were obtained from HPMC-lipid containing food preservatives. HPMC-lipid stable emulsion suitable to

use as coatings contained from 6 to 8% SC, 50% (db) total lipid content, and a maximum of 2.5% food preservative. Films containing sodium salts of parabens, PS, SB, and their mixtures exhibited clear inhibition activity against PD and PI. Barrier and mechanical properties of selected HPMC-lipid composite films depended on lipid composition and the properties of the food preservative. Films containing parabens formulated with BW had lower WVP and higher OP than films with organic acid salts formulated with BW and shellac. Further studies should follow to determine the ability of these selected antifungal edible coatings to control PD and PI in *in vivo* tests with fresh citrus fruits, as well as the effect of their application on fruit postharvest quality. On the other hand, these films with antimicrobial properties could also be evaluated as low-toxicity means to reduce the risks associated with the presence of microbes of food safety concern.

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CHAPTER 2

Curative and preventive activity of hydroxypropyl methylcellulose-lipid edible composite coatings containing antifungal food additives to control citrus postharvest green and blue molds

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ABSTRACT

Edible composite coatings based on hydroxypropyl methylcellulose (HPMC), lipid components (beeswax and shellac), and food preservatives with antifungal properties were evaluated *in vivo* on clementine mandarins cv. 'Clemenules', hybrid mandarins cv. 'Ortanique', and oranges cv. 'Valencia'. Their curative and preventive activity against citrus postharvest green (GM) and blue molds (BM), caused by Penicillium *digitatum* (PD) or *Penicillium italicum* (PI), respectively, were determined. Fruit were artificially inoculated before or after the application of the coatings and incubated up to 7 d at 20 ºC. Selected food preservatives included mineral salts, organic acid salts, parabens, and 2-deoxy-D-glucose. Inoculated but uncoated fruit were used as controls. For curative activity, HPMC-lipid edible composite coatings containing sodium benzoate (SB) were most effective to reduce the incidence and severity of GM on clementine mandarins cv. 'Clemenules' (86 and 90%, respectively). On this cultivar, the reduction in GM incidence by the SB-based coating was twice that of potassium sorbate (PS)-based coating. On mandarins cv. 'Ortanique', PS- and SB-based coatings reduced the incidence of GM and BM by more than 40 and 21%, respectively. However, the HPMC-lipid coating containing a mixture of PS and sodium propionate (PS + SP) exhibited a synergistic effect in the reduction of the incidence of GM (78%) and BM (67%). Coatings with parabens modestly reduced disease incidence and severity. On oranges cv. 'Valencia', coatings with food preservatives controlled better BM than GM. Coatings containing SB + PS and SB + SP reduced the incidence and severity of BM by 85% and 95%, respectively. PS- and SB- based coatings controlled GM more effectively than coatings formulated with other food preservatives. In every cultivar, fruit coated before inoculation did not show any incidence or severity reduction of both GM and BM (preventive activity). In every test, the antifungal action of the coatings was fungistatic rather than fungicidal.

Key words: edible coatings, food preservatives, mandarins, oranges, postharvest

INTRODUCTION

Main postharvest diseases of citrus fruit are caused by *Penicillium digitatum* (Pers.:Fr.) Sacc. (PD) and *Penicillium italicum* Wehmer (PI), which causes green (GM) and blue molds (BM), respectively. For many years, these diseases have been traditionally controlled by synthetic fungicides such as thiabendazole, imazalil, or sodium ortho-phenyl phenate (*1*). New synthetic fungicides such as azoxystrobin, fludioxonil, pyrimethanil, or trifloxystrobin, have been largely tested in Europe and in the USA (*2-4*), and some of them are already in use in the USA. These more recent compounds have been classified as "reduced-risk" fungicides by the United Sates Environmental Protection Agency (US EPA). However, extensive and prolonged use of these fungicides has elicited consumer concerns about human health risks and environmental contamination. In general, decay control methods that are alternatives to conventional synthetic fungicides can be classified as physical, chemical, or biological (*5*). Physical control methods include application of heat (curing and hot water) or irradiation (UV-C and ionizing radiation) treatments (*6-8*) and cold storage in controlled environments such as ozonated atmospheres (*9*). Chemical control methods comprise the use of natural or synthetic chemicals such as food additives or low toxicity compounds, classified as generally recognized as safe (GRAS) by the Unites States Food and Drug Administration (U.S. FDA) (*10-12*). Biological control methods use yeasts, bacteria, or filamentous fungi as microbial antagonists against postharvest pathogens (*13, 14*). In an effort to control postharvest diseases of citrus fruit, some studies have combined physical, chemical, and biological methods (*5, 15, 16*).

In the food industry, the use of edible films and coatings is a potential method to increase the shelf-life of many food products including fruits and vegetables (*17, 18*). Consumer interest towards natural products has led researchers to develop new edible films and coatings, which could be recognized as safe. Several advantages have been observed from the use of edible films and coatings. In fresh fruit, the creation of a semi-permeable barrier to gas exchange and water vapor between the fruit and the surrounding atmosphere reduce respiration rate and moisture loss which delay produce senescence (*19, 20*). Moreover, edible films and coatings can add gloss and improve fruit visual quality (*21, 22*). The main components of edible coatings are polysaccharides, proteins, lipids, and natural resins (*23-25*). Several other

compounds such as plasticizers and emulsifiers may be added to edible coatings to improve their mechanical properties and form stable emulsions when lipids and hydrocolloids are combined (*23, 26*). In addition, edible coatings and films can also act as carriers of food additives, including antioxidants, colorants, flavoring agents, and antimicrobial compounds (*25, 27-29*). Edible films and coatings containing antimicrobials, such as organic acids and their salts (*30-32*), parabens (*33*), chitosan (*34, 35*), essential oils, or natural antimicrobials (*36, 37*) have been effective in delaying the growth of contaminating microorganisms during storage or distribution of fresh or minimally processed horticultural products.

In a previous study, hydroxypropyl methylcellulose (HPMC)-lipid stand-alone edible composite films containing food additives with antifungal properties were tested *in vitro* for inhibitory activity against PD and PI on dichloran rose-bengal chloramphenicol agar (DRBC) using a disk diameter test (*38*). Among a wide variety of tested films, those containing either some organic acid salts and their mixtures or parabens and their mixtures resulted in a significant inhibitory activity against PD and PI. It was concluded that selected HPMC-lipid films containing an appropriate food additive may hold promise as (delete potential) commercial antifungal edible coatings for fresh citrus fruit (*38*).

The objective of this work was to evaluate the curative and preventive activity of selected HPMC-lipid edible composite coatings containing food preservatives to control GM and BM on artificially inoculated fruit of commercially important cultivars of mandarin and orange.

MATERIALS AND METHODS

Materials

HPMC (Methocel E15) was purchased to Dow Chemical Co. (Midland, MI, USA). Shellac and beeswax (BW) (grade 1) were supplied by Fomesa Fruitech, S.L. (Valencia, Spain). Stearic acid and glycerol were from Panreac Química, S.A (Barcelona, Spain). Silicone antifoam (FG-1510) and ammonia (25%) were to Dow Corning® (Belgium) and Scharlau (Sentmenat, Spain), respectively. Food preservatives were

purchased to Sigma (Sigma-Aldrich Chemie, Steinheim, Germany) and included mineral salts, salts of organic acids, sodium salts of parabens, and 2-deoxy-D-glucose. Most of these chemicals are classified as food additives or GRAS compounds by the European Union or USA regulations. Table 1 shows the characteristics of the selected food preservatives applied in this study to each citrus cultivar.

Emulsions/Coating preparation

HPMC-lipid edible composite emulsions were prepared combining the hydrophilic phase (HPMC) and the hydrophobic phase (BW and shellac) suspended in water. Glycerol and stearic acid were used as plasticizer and emulsifier, respectively. Ratios of HPMC-glycerol (2:1) (dry basis, db) and lipid components (BW/shellac)-stearic acid (5:1) (db) were kept constant throughout the study. Emulsions were made as described previously by Valencia-Chamorro et al (*38*). Briefly, an aqueous solution of HPMC (5% w/w) was prepared. The corresponding food preservative, BW, glycerol, stearic acid, water, and two drops of antifoam were added to the HPMC solution and heated at 90 ºC to melt the lipids. Shellac was previously dispersed in water at 40 ºC and ammonia (15% w/w shellac/ammonia) was added to dissolve the resin. Shellac solution was heated separately at 90 ºC and added to the HPMC dispersion. Samples were homogenized with a high-shear probe mixer (Ultra-Turrax model T25, IKA-Werke, Steufen, Germany) for 4 min at 22,000 rpm. Emulsions were cooled to less than 25 ºC and further agitated for 25 min. Emulsions were kept 2-3 d at 5 ºC before use. These formulations were stable and no phase separation was observed. Table 1 shows the total solid content, the concentration of the food preservative (% wet basis, wb), the BW-shellac content (%, db), and the pH and viscosity of the formulations.

Fungal inoculum and fruit inoculation

PD isolate NAV-7 and PI isolate MAV-1, obtained from decayed oranges from Valencia packinghouses, were isolated, identified, and maintained in the IVIA culture collection of postharvest pathogens. These strains were selected for their aggressiveness on the most commercially important mandarin and orange cultivars. Prior to each experiment, the isolates were grown on potato dextrose agar (PDA) (Sigma) in petri dishes at 25 ± 1 ºC for 7–10 d. A high-density conidial

suspension was prepared in Tween 80 (0.05%, w/v; Panreac Química S.A.) in sterile water, passed through two layers of cheesecloth, measured with a haemacytometer, and diluted with sterile water to achieve the desired inoculum density. Oranges (*Citrus sinensis* [L] Osbeck) cv. 'Valencia', clementine mandarins (*Citrus reticulata* Blanco) cv. 'Clemenules' and hybrid mandarins (*Citrus reticulata* x [*C. sinensis* x *C. reticulata*]) cv. 'Ortanique' from commercial orchards in the Valencia area (Spain) were selected by hand and used in the experiments before any postharvest treatment were applied. The fruit were stored up to one week at 5 ºC and 90% relative humidity (RH) before use. Before each experiment, the fruit were randomised, washed with fresh water and allow to dry at room temperature. The fruit were artificially inoculated with PD and/or PI (inoculum density of 10^5 spores/mL) by immersing a stainless steel rod with a probe tip 1 mm wide and 2 mm in length into the spore suspension and wounding each fruit on the equator.

Evaluation of the curative and preventive activity of the coatings

In order to determine curative activity, the fruit were inoculated with the pathogens, incubated at 20 ºC for 24 h, coated by immersion (15 s at 20 ºC) with the selected HPMC-lipid edible composite coatings, drained, and allowed to dry at 20 ºC. To test preventive activity, the fruit were coated with the selected HPMC-lipid edible composite coatings (15 s at 20 ºC), drained, allowed to dry, kept at 20 ºC for 24 h, and then inoculated with the pathogens. Inoculated but uncoated fruit were used as controls. In every experiment, each treatment was applied to three replicates of 20 fruit each. All fruit were placed on plastic trays on corrugated cartons and then incubated up to 7 d at 20 ºC and 90% RH to resemble typical shelf-life of citrus fruit. On clementine cv. 'Clemenules' different fruit were used to inoculate each pathogen. On oranges and mandarins cv. 'Ortanique', each fruit was inoculated with both fungus, each one on the opposite side of the equator.

Disease incidence of GM and BM was assessed as the percentage of decayed fruit after 7 d at 20 ºC. For each treatment, the percentage of incidence reduction with respect to control fruit was calculated. Disease severity was determined as the diameter of the lesion (mm) and the results were reported as severity reduction (%) with respect to control fruit.

An additional study to test the performance of related coatings during longer periods of storage was conducted with oranges. Fruit inoculated with both PD and PI were coated by immersion (15 s at 20 ºC) with PS- and SB-based coatings, drained, and allowed to dry at 20 ºC. Inoculated but uncoated fruit were used as controls. Each treatment was applied to three replicates of 20 fruit each. Treated fruit were stored up to 21 d at 20 ºC and 90% RH, and disease incidence and severity were determined after 3, 7, 11, 18, and 21 d of storage.

Statistical analysis

Statistical analyses of data were performed using the software Statgraphics Plus 2.1 (Manugistics, Inc., Rockville, MD, USA). For each disease, mean differences were determined by Fisher´s protected least significant difference test (LSD, $P < 0.05$) applied after an analysis of variance (ANOVA). For incidence and incidence reduction data, the ANOVA was applied to the arcsine of the square root of the percentage of decayed fruit. Non-transformed means are shown.

Table 1. Composition and characterisation of HPMC-lipid edible composite coatings containing antifungal food additives applied to clementine mandarins cv. 'Clemenules', hybrid mandarins cv. 'Ortanique', and oranges cv. 'Valencia'.

Food preservatives added to HPMC-lipid edible composite coatings	Molecular formula	E- Code[a]	Food preservative (%, wb)	SC [b] (%, wb)	BW-shellac[c] (%, db)	Viscosity[d] (cp)	pH[e]
Mandarins cv. 'Clemenules'							
Mineral salts							
Sodium bicarbonate	$NaHCO_3$	E-500(I)	2.0	6	45-5	10.50	9.15
Ammonium bicarbonate	NH_4HCO_3	E-237	2.0	6	25-25	5.33	9.63
Organic acid salts							
Potassium sorbate	$C_6H_7O_2K$	E-202	2.0	6	25-25	12.50	6.83
Sodium benzoate	$C_7H_5O_2Na$	E-211	2.5	8	25-25	20.67	7.44
Parabens							
Sodium salt of methyl paraben	$C_8H_7NaO_3$	E-219	1.5	6	50-0	12.50	8.98
Sodium salt of ethyl paraben	$C_9H_9NaO_3$	E-215	1.0	6	50-0	12.67	9.39
Sodium salt of propyl paraben	$C_{10}H_{11}NaO_3$	E-217	1.0	6	50-0	13.17	9.57
Paraben (mixture)							
Sodium salt of methyl paraben + sodium salt of propyl paraben			1.0 + 0.5	6	50-0	13.17	9.17
Mandarins cv. 'Ortanique'							
Mineral salts							
Sodium bicarbonate			2.0	6	45-5	11.92	8.86

Food preservatives added to HPMC-lipid edible composite coatings	Molecular formula	E- Code[a]	Food preservative (%, wb)	SC [b] (%, wb)	BW-shellac[c] (%, db)	Viscosity[d] (cp)	pH[e]
Organic acid salts							
Potassium sorbate			2.0	6	25-25	23.50	7.29
Sodium benzoate			2.5	8	25-25	17.25	7.39
Sodium acetate	CH_3COONa	E-262-(i)	1.0	6	50-0	11.83	7.09
Sodium diacetate	$(CH_3COO)_2HNa$	E-262-(II)	1.0	6	50-0	11.17	4.61
Sodium propionate	CH_3CH_2COONa	E-281	2.0	6	25-25	19.08	7.09
Sodium formate	$HCOONa$	E-237	1.0	6	50-0	8.57	6.55
Organic acid salts (mixtures)							
Potassium sorbate + sodium propionate			1.5 + 0.5	6	25-25	32.50	6.95
Sodium benzoate + potassium sorbate			2.0 + 0.5	8	25-25	12.08	8.23
Sodium benzoate + sodium propionate			2.0 + 0.5	8	25-25	8.23	8.19
Paraben							
Sodium salt of methyl paraben			1.5	6	50-0	13.92	9.06
Paraben (mixture)							
Sodium salt of methyl paraben + sodium salt of propyl paraben			1.0 + 0.5	6	50-0	36.00	9.36
Other compounds							
EDTA	$C_{10}H_{12}N_2O_8CaNa_2$	E-385	1.5	6	45-5	13.92	6.79
2-deoxy-D-glucose	$C_6H_{12}O_5$	-	0.3	6	25-25	27.50	6.94

Food preservatives added to HPMC-lipid edible composite coatings	Molecular formula	E- Code[a]	Food preservative (%, wb)	SC [b] (%, wb)	BW-shellac[c] (%, db)	Viscosity[d] (cp)	pH[e]
Oranges cv. 'Valencia'							
Organic acid salts							
Potassium sorbate			2.0	6	25-25	14.33	7.48
Sodium benzoate			2.5	8	25-25	21.83	7.47
Calcium propionate	$Ca(CH_3CH_2COO)_2$	E-282	1.0	6	50-0	13.17	5.45
Calcium formate	$Ca(HCOO)_2$	E-238	1.0	6	50-0	15.00	4.93
Organic acid salts (mixtures)							
Potassium sorbate + sodium propionate			1.5 + 0.5	6	25-25	22.33	7.38
Sodium benzoate + potassium sorbate			2.5 + 0.5	8	25-25	12.17	7.09
Sodium benzoate + sodium propionate			2.5 + 0.5	8	25-25	31.67	7.12
Paraben							
Sodium salt of methyl paraben			1.5		50-0	17.25	8.83
Other compounds							
2-deoxy-D-glucose			0.5	6	25-25	24.33	7.57

[a] E-Code = Number codes for food additives approved by the European Union. [b] SC = solid concentration of HPMC-lipid edible composite emulsions with food preservatives. wb = wet basis. [c] BW-shellac = concentration of Beeswax-shellac. db = dry basis. [d] Viscosity (centipoise, cp) of HPMC-lipid edible composite emulsions with food preservatives. [e] pH of HPMC-lipid edible composite emulsions with food preservatives.

RESULTS

Curative activity

Clementines cv. 'Clemenules', mandarins cv. 'Ortanique', and oranges cv. 'Valencia' were coated with 8, 14, and 9 HPMC-lipid composite coatings containing food preservatives, respectively (Table 1). HPMC-based coatings reduced to some extend the incidence and severity of both GM and BM on clementines cv. 'Clemenules' coated 24 h after artificial inoculation with PD or PI showing, therefore, variable curative activity against PD and PI (Figure 1). In general, the coatings controlled GM more effectively than BM. Coatings containing minerals salts such as sodium bicarbonate (SBC) and ammonium bicarbonate (ABC) reduced disease incidence very slightly. In contrast, HPMC-lipid coatings containing sodium benzoate (SB) reduced disease incidence (86%) and severity (90%) greatly. GM was reduced twice as effectively on mandarins by SB-based coating the potassium sorbate (PS)-based coating ($P < 0.05$). The rest of food preservatives tested, including parabens or their mixtures, caused an incidence reduction of both GM and BM lower than 20%. The combination of sodium salt of methyl paraben and sodium salt of propyl paraben (SMP + SPP) did not cause any synergistic effect on the control of GM or BM (Figure 1).

The curative activity of HPMC-based coatings against GM and BM on mandarins cv. 'Ortanique' is shown in Figure 2. In general, the coatings controlled GM more effectively than BM. Coatings with SBC reduced the incidence of BM and GM by approximately 20%. Among the six organic acid salts tested, only coatings with PS or SB reduced the incidence of GM by more than 40%. However, these coatings only reduced the incidence of BM by 21%. When mixtures of two organic acid salts were used, only the coating containing a mixture of PS and sodium propionate (PS + SP) showed a synergistic effect for the incidence reduction of GM (78%) and BM (67%) ($P < 0.05$). Moreover, this coating most effective results overall and reduced GM and BM by 91 and 86%, respectively ($P < 0.05$). When HPMC-lipid coatings containing SB with PS (SB + PS) or SP (SB + SP) were applied to 'Ortanique' mandarins, the incidence reduction of GM and BM were not significantly different than in mandarins coated with HPMC-lipid coatings formulated with SB alone. The rest of the coatings containing parabens, alone or in combination, modestly reduced the incidence of

GM and BM. The incidence reduction of both GM and BM after the application of coatings containing 2-deoxy-D-glucose or EDTA was very low (Figure 2).

HPMC-lipid edible composite coatings containing food preservatives had effective curative activity on 'Valencia' oranges. Both the incidence and severity of both GM and BM were markedly reduced (Figure 3). Coatings with PS, SB, or their mixtures controlled GM more effectively than coatings with the rest of organic acid salts or 2-deoxy-D-glucose ($P < 0.05$). Among all coatings tested, those containing mixtures of SB + PS or SB + SP reduced the incidence and severity of BM by 85 and 95%, respectively ($P < 0.05$). The SMP-based coating reduced the incidence of GM and BM by 40% and 50%, respectively and the severity of these pathogens by 67 and 75%, respectively. These reduction values were similar to those obtained with coatings formulated with organic acid salts. Low incidence reduction of GM and BM was observed on fruit coated with the rest of the food preservatives tested (Figure 3).

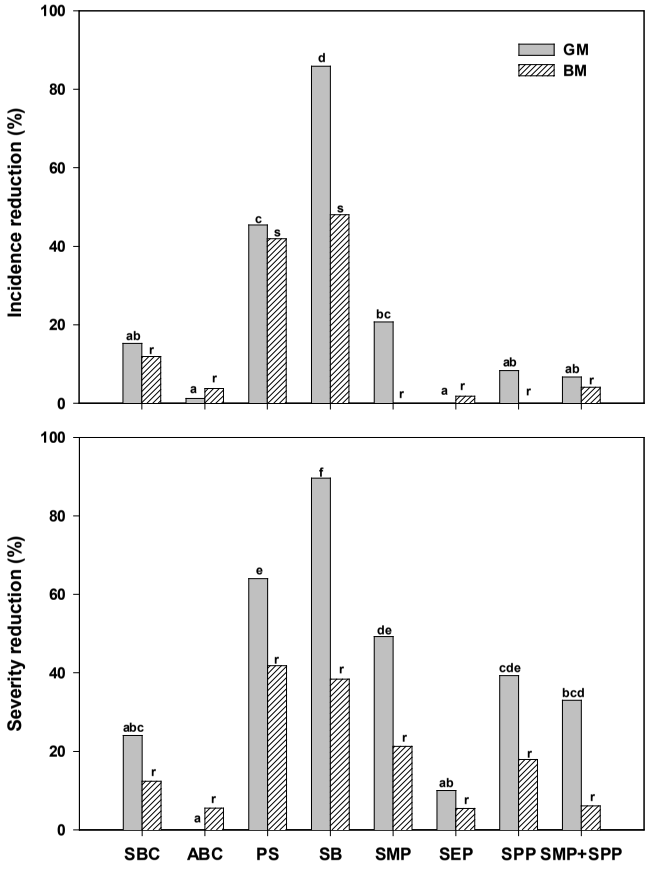

Figure 1. Curative activity of HPMC-lipid edible composite coatings against green (GM) and blue (BM) molds on mandarins cv. 'Clemenules' artificially inoculated with *Penicillium digitatum* and *Penicillium italicum*, coated 24 h later, and incubated for 7 d at 20 ºC and 90% RH. Coatings contained the following preservatives: SBC = sodium bicarbonate, ABC = ammonium bicarbonate, PS = potassium sorbate, SB = sodium benzoate, SMP = sodium salt of methyl paraben, SEP = sodium salt of ethyl paraben, SPP = sodium salt of propyl paraben or SMP + SPP = mixture of sodium salts of methyl and propyl parabens. For each mold, disease incidence and severity reductions were determined with respect to control fruit (inoculated but uncoated fruit). Disease incidence in control treatments was 85-98% and 80-98% for *Penicillium digitatum* and *Penicillium italicum,* respectively. Disease severity in control treatments was 53-68 mm and 24-29 mm for *Penicillium digitatum* and *Penicillium italicum,* respectively. For disease incidence reduction, the ANOVA was applied to the arcsine-transformed values. Non transformed means are shown. For each mold, columns with different letters are significantly different according to Fisher´s protected LSD test ($P < 0.05$) applied after an ANOVA.

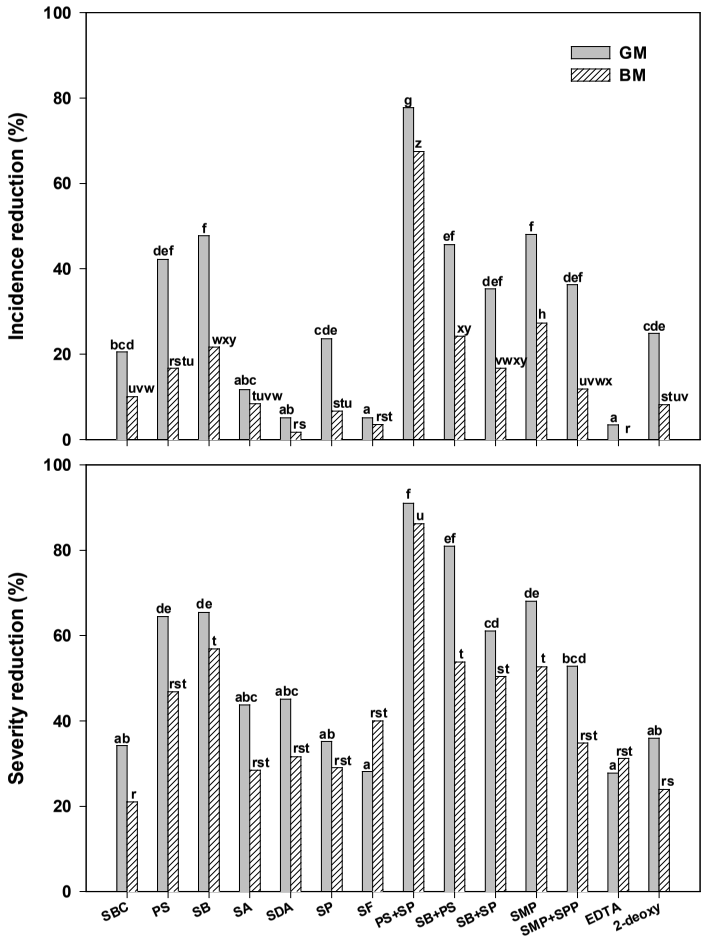

Figure 2. Curative activity of HPMC-lipid edible composite coatings against green (GM) and blue (BM) molds on hybrid mandarins cv. 'Ortanique' artificially inoculated with *Penicillium digitatum* and *Penicillium italicum* coated 24 h later and incubated for 7 d at 20 ºC and 90% RH. Coatings contained the following preservatives: SBC = sodium bicarbonate, PS = potassium sorbate, SB = sodium benzoate, SA = sodium acetate, SDA = sodium diacetate, SP = sodium propionate, SF = sodium formate, PS + SP = the mixture of potassium sorbate and sodium propionate, SB + PS = mixture of sodium benzoate and potassium sorbate, SB + SP = the mixture of sodium benzoate and sodium propionate, SMP = sodium salt of methyl paraben, SMP + SPP = mixture of sodium salts of methyl and propyl parabens, EDTA = Disodium-calcium ethylendiamine-tetraacetate or 2-deoxy = 2-deoxy-D-glucose. For each mold, disease incidence and severity reductions were determined with respect to control fruit (inoculated but uncoated fruit). Disease incidence in control treatments was 95-100% and 85-100% for *Penicillium digitatum* and *Penicillium italicum,* respectively. Disease severity in control treatments was 50-80 mm and 30-40 mm for *Penicillium digitatum* and *Penicillium italicum,* respectively. For disease incidence reduction, the ANOVA was applied to the arcsine-transformed values. Non transformed means are shown. For each mold, columns with different letters are significantly different according to Fisher´s protected LSD test ($P < 0.05$) applied after an ANOVA.

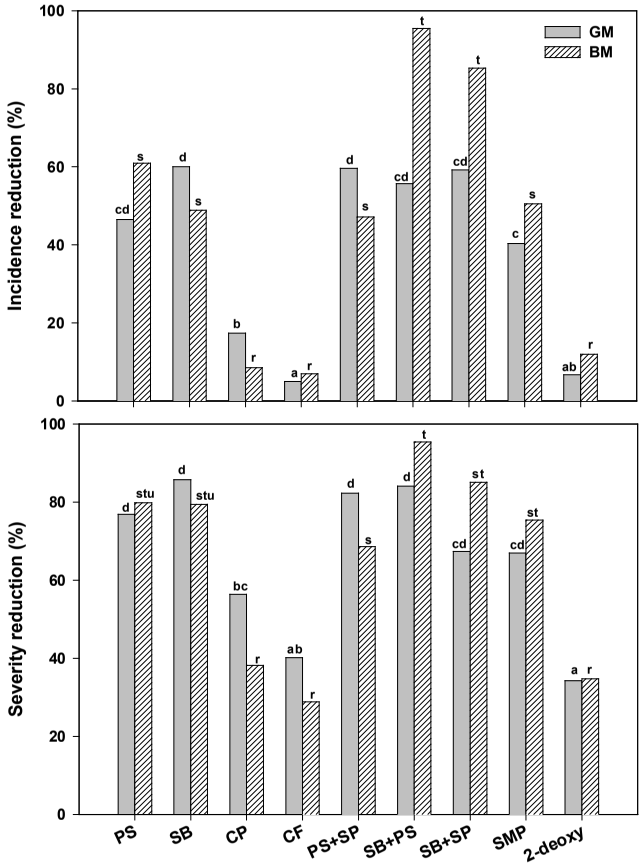

Figure 3. Curative activity of HPMC-lipid edible composite coatings against green (GM) and blue (BM) molds on oranges cv. 'Valencia' artificially inoculated with *Penicillium digitatum* and *Penicillium italicum* coated 24 h later and incubated for 7 d at 20 ºC and 90% RH. Coatings contained the following preservatives: PS = potassium sorbate, SB = sodium benzoate, CP = calcium propionate, CF = calcium formate, PS + SP = the mixture of potassium sorbate and sodium propionate, SB + PS = mixture of sodium benzoate and potassium sorbate, SB + SP = the mixture of sodium benzoate and sodium propionate, SMP = sodium salt of methyl paraben or 2-deoxy = 2-deoxy-D-glucose. For each mold, disease incidence and severity reductions were determined with respect to control fruit (inoculated but uncoated fruit). Disease incidence in control treatments was 65-95% and 45-95% for *Penicillium digitatum* and *Penicillium italicum,* respectively. Disease severity in control treatments was 45-115 mm and 6-45 mm for *Penicillium digitatum* and *Penicillium italicum,* respectively. For disease incidence reduction, the ANOVA was applied to the arcsine-transformed values. Non transformed means are shown. For each mold, columns with different letters are significantly different according to Fisher´s protected LSD test ($P < 0.05$) applied after an ANOVA.

PS- and SB-based coatings effectively controlled GM and BM on 'Valencia' oranges. Thus, an additional assay was conducted to study the performance of these coatings on 'Valencia' oranges stored up to 21 d at 20 ºC. The coatings greatly reduced disease incidence and severity when compared to uncoated fruit (Figure 4). After 7 d, GM was effectively controlled by these coatings (Figure 4). The incidence of GM on oranges coated with PS- and SB-based coatings was around 4 and 7%, respectively, while incidence on control fruit was as high as 78% ($P < 0.05$). In addition, disease severity was approximately 3 and 11 mm, respectively, whereas disease severity on control fruit was 65 mm ($P < 0.05$) (Figure 4). After 14 and 21 d of incubation, GM incidence on oranges treated with PS- and SB-based coatings was less than 40 and 60%, respectively, while it was of 100% on control fruit. GM was better controlled than BM by HPMC-lipid coatings containing PS and SB. After 7 d of incubation, BM incidence was 14 and 17% for PS- and SB-based coatings, respectively. After prolonged a incubation time of 14 and 21 d, the incidence of BM was as high as 74 and 96%, respectively (Figure 4).

Preventive activity

Neither on clementines cv. 'Clemenules', mandarins cv. 'Ortanique', nor oranges cv. 'Valencia', did coating the fruit before inoculation significantly reduce the incidence and severity of either GM and BM (data not shown). Therefore, none of the tested coatings showed any preventive activity against the pathogens on these citrus cultivars.

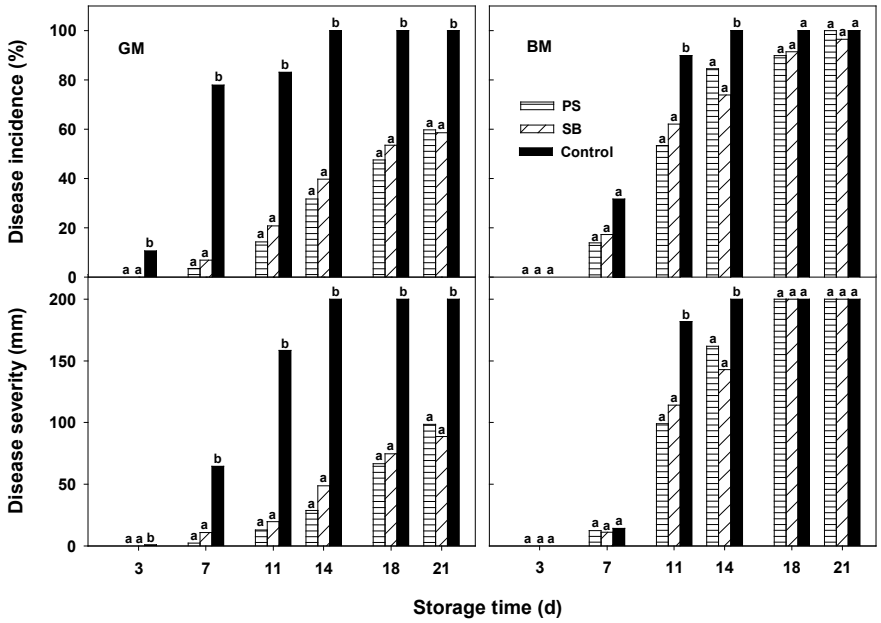

Figure 4. Curative activity of HPMC-lipid edible composite coatings against green (GM) and blue (BM) molds on oranges cv. 'Valencia' artificially inoculated with *Penicillium digitatum* and *Penicillium italicum*, coated 24 h later, and incubated for 21 d at 20 ºC and 90% RH. Treatments were as follows: Control = inoculated but uncoated, PS = potassium sorbate, SB = sodium benzoate. For each mold, disease incidence and severity were analysed. For disease incidence, the ANOVA was applied to the arcsine-transformed values. Non-transformed means are shown. For each mold, columns with different letters are significantly different according to Fisher´s protected LSD test ($P < 0.05$) applied after an ANOVA.

DISCUSSION

In a previous study, stand-alone HPMC-lipid edible composite films containing 23 food preservatives, including mineral salts, organic acid salts and their mixtures, parabens and their mixtures, and other compounds were tested *in vitro* against PD and PI using the disk diameter test (*38*). Among 14 films containing organic acid salts, only those containing PS and SB were consistently effective for the control of both

PD and PI, PS showing the highest inhibitory activity (*38*). In the present *in vivo* study, it was found on 'Ortanique' mandarins and 'Valencia' oranges that HPMC-lipid composite coatings containing PS, SB, and their mixtures were, among all organic acid salts tested, the most effective to reduce the development of GM and BM. PS and SB are generally considered as safe compounds by regulations all over the world and they are therefore widely used as food preservatives with a broad-spectrum activity against a variety of yeasts and molds (*39, 40*). To control molds, these salts have been usually applied as aqueous solutions, but they have also been incorporated into films. Palou et al. (*11*) reported that among more than forty food additives and low-toxicity chemicals, aqueous solutions of PS or SB were the most promising compounds to control postharvest penicillium decay on citrus fruit. In the same study, it was pointed out that GM was better controlled on lemons than on oranges. The inhibitory ability of the chemical was attributed to interactions between salt residues present in the wound infection courts, the fungus, and constituents of the rind (*39, 40*). In other work, PS or SB were added to methylcellulose/chitosan films to study the antimycotic properties of films containing 2, 4, or 5% of these food preservatives (*31*). Clear inhibition zones for *Penicillium notatum* and *Rhodotorula rubra* on PDA were reported for methylcellulose films containing 2% of PS or SB. In contrast, chitosan films with the same concentration of food preservatives did not produce inhibition zones. This behavior was attributed to interactions between chitosan and the food preservatives which prohibited their release from the films. When food preservatives were added at a concentration up to 5% to chitosan, clear inhibitory zones were reported.

Some mixtures of food preservatives were added to the coatings with the aim of obtaining a synergistic effect on antigungal activity against target molds. On 'Ortanique' mandarins, PS + SP-based coating caused an incidence reduction of GM and BM almost two-times and three-four times higher than coatings with PS alone, respectively. SB + PS- and SB + SP-based coatings caused the highest incidence and severity reductions of BM on 'Valencia' oranges. Specifically, the incidence reduction was almost two-times higher than that observed with coatings containing SB or PS alone. These results obtained with coated citrus fruit contrasted with those obtained with stand-alone films in previous work (*38*). Stand-alone films containing the mixtures of the food preservatives PS + SP, SB + PS, and SB + SP had similar or lower antimicrobial activity against

PD and PI than films with PS or SB alone. These dissimilar results are probably due to the complex interactions between host, pathogen and environment that occur during *in vivo* disease development. Likewise, it is probable that notable variations on the growth of PD and PI *in vitro* and *in vivo* resulted from differences on the release rate of food preservatives from films located on agar medium and from coatings located on the rind of citrus fruit. Any of the three steps involved in the release of antimicrobial agents from polymer matrices, diffusion within the polymer matrices, mass transfer across the interface, and dispersion into the bulk food (*41*), can greatly affect the release of antimicrobial agents. Ponce et al. (*42*) reported some differences on the antimicrobial activity of natural extracts against *Listeria monocytogenes* when they were applied alone and when they were incorporated to coatings on squash slices. Film-forming solutions included chitosan, casein, and carboxymethyl cellulose alone as well as films enriched with oleoresins. Lower antibacterial activity were reported when olive and rosemary extracts were applied to chitosan coatings than when the extracts were applied alone. They concluded that those results were probably due to the dispersion effect of the active compounds and the interactions among them.

When coatings containing parabens were applied to citrus fruit, the incidence and severity reduction of GM and BM on mandarins were very low (Figure 1, 2). In addition, the use of SMP + SPP-based coatings did not cause any synergistic effect for the control of GM and BM, resulting in lower incidence reductions than those obtained with coatings containing parabens alone. These results also contrasted with those observed with similar stand-alone films (*38*). Among all additives tested, HPMC-lipid films containing parabens with SMP, sodium salt of ethyl paraben (SEP), and the mixture SMP + SPP exhibited the highest inhibition zone for PD and PI. The poor inhibitory activity of HPMC-lipid edible composite coatings containing parabens and their mixtures when applied *in vivo* to citrus fruit could be likely attributed to a limited chemical release from the coating matrix to the fruit surface. In a study simulating the release of propyl paraben from a polymer coating (styrene-acrylate copolimer), Chung et al. (*33*) found that the release of the chemical from the coating into water and food-simulating solvents depended on the interactions among propyl paraben, the polymer coating, and the solvents. In addition to the release ability of the antimicrobial from the polymer matrix, each type of fruit may

considerably differ in skin resistance to the diffusion of the antimicrobial agent, gas diffusion, and fruit respiration rate, among other attributes. Therefore, coatings developed for one fruit cultivar may not be suitable for another (*43*). These potential differences were confirmed in this work, in which coatings such as those containing SB or PS were generally more effective on oranges than on mandarins.

On 'Valencia' oranges, SB- and PS-based coatings greatly reduced the disease incidence and severity of GM after 7 d of storage at 20 ºC (reductions even higher than 90%). Since these reductions were lower after longer storage periods (Figure 4), it can be concluded that, in general, the effects on citrus fruit of HPMC-lipid edible composite coatings containing food preservatives were fungistatic rather than fungicidal. Similarly, some aqueous solutions containing food preservatives applied to citrus fruit such as organic acid salt aqueous solutions (*11*), hot water, sodium carbonate, or sodium bicarbonate treatments (*44, 45*), have been reported to be primarily fungistatic rather than fungicidal.

The application of HPMC-lipid edible composite coatings containing food preservatives are a simple and environmentally-friendly method to reduce the losses caused by citrus postharvest diseases. Thus, these coatings could be used as an alternative to synthetic chemical fungicides for decay control in citrus packinghouses. The lack of preventive activity could be compensated for with their integration with other postharvest treatments such as the application of biological control antagonists.

Further research with HPMC-lipid coatings containing food preservatives, at a semicommercial or commercial scale, is needed to fully define the impact of these edible composite coatings on citrus fruit quality.

ACKNOWLEDGEMENTS

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CHAPTER 3

Effect of antifungal hydroxypropyl methylcellulose (HPMC)-lipid edible composite coatings on postharvest decay development and quality attributes of cold-stored 'Valencia' oranges

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Abstract

Edible composite coatings based on hydroxypropyl methylcellulose (HPMC), hydrophobic components (beeswax and shellac), and food preservatives with antifungal properties were evaluated on 'Valencia' oranges during long-term cold storage. Selected food preservatives included potassium sorbate (PS), sodium benzoate (SB), sodium propionate (SP), and their mixtures. Intact oranges or oranges artificially inoculated with *Penicillium digitatum* or *Penicillium italicum* were coated and stored up to 60 d at 5 ºC followed by 7 d of shelf life at 20 ºC. Some antifungal HPMC-lipid coatings significantly reduced the incidence and severity of both green (GM) and blue (BM) molds on inoculated and cold-stored oranges and PS+SP-based coating was the most effective. In general, the coatings controlled better GM than BM. Analytical and sensory fruit quality was evaluated on intact oranges. After 30 and 60 d at 5 ºC plus 7 d at 20 ºC, weight loss, rind firmness, internal gas concentration, ethanol and acetaldehyde content of the juice, sensory flavor, off-flavor, and fruit appearance were not adversely affected by the application of the antifungal coatings, which showed promise as potential substitutes for citrus commercial waxes. However, further studies should follow to improve some coating physical characteristics in order to provide better water loss control and higher gloss on coated oranges

KEYWORDS: citrus, food additives, *Penicillium digitatum*, *Penicillium italicum*, green mold, blue mold

1. Introduction

Fruits and vegetables have been traditionally kept under cold storage to maintain their postharvest quality. In many cases, however, benefits from refrigeration are not important enough to preserve produce quality. In the citrus industry, in addition to cold storage, fruit coating is a normal practice to replace the natural waxes that are generally removed during washing. Wax coatings are made of natural or synthetic waxes and fatty acids (beeswax, carnauba, polyethylene, oleic acid), oils, shellac, emulsifier, plasticizers, anti-foam agents, and surfactants (Baldwin, 1994;

Hagenmaier and Baker, 1994; Hagenmaier, 1998). Coatings form a semi-permeable barrier to water vapor and gas exchange, leading to weight loss reduction, respiration rate modification, and senescence delay of coated produce (Hagenmaier and Baker, 1994; Nisperos-Carriedo, 1994; Olivas et al., 2008). Synthetic chemical fungicides are often incorporated into conventional citrus waxes to control postharvest molds. However, the effectiveness to control decay is often lower when the fungicides are dissolved into the coatings than fungicides applied alone. It has been reported that a fungicide concentration increase of two-threefold may be needed to obtain the same level of decay control than with the use of pre-coating fungicidal aqueous solutions (Grant and Burns, 1994). Prolonged and extensive use of postharvest chemical fungicides, either alone or into conventional coatings, may deposit harmful residues on fruits and contaminate the environment.

Edible films and coatings are alternative and non-polluting methods that have been developed to extend produce shelf life (Banker, 1966; Greener and Fennema, 1994; Park, 1999; Rhim and Shellhammer, 2005). Ingredients used in edible films and coatings are proteins, polysaccharides, and lipids, which include natural waxes and resins. In addition, emulsifiers and plasticizers are added to improve coating performance (Nisperos-Carriedo, 1994; Baldwin et al., 1997; Pérez-Gago and Krochta, 2001; Yoshida and Antunes, 2004). Edible films and coatings can also act as carries of food additives, including colorants, flavoring agents, antioxidants, or antimicrobial compounds (Cuppet, 1994; Franssen and Krochta, 2000; Ozdemir and Floros, 2003; Cha and Chinnan, 2004; Valencia-Chamorro et al., 2008). There are much research in the literature that reports the effect of edible films and coatings containing a broad variety of components on the postharvest quality of coated fruits and vegetables (Hagenmaier and Baker, 1995; Mannheim and Soffer, 1996; Pérez-Gago et al., 2002; Porat et al., 2005; Chien et al., 2007; Navarro-Tarazaga et al., 2007).

In the last decade, several works have focused on the development of coatings based on proteins and polysaccharides with natural food preservatives to control microbial growth on fruits. For example, starch-based coatings with potassium sorbate (PS) reduced microbial growth on strawberries (García et al., 1998), and chitosan-based coatings containing PS showed an effective antifungal action against *Rhizopus* sp. and *Cladosporium* sp. on inoculated fresh strawberries (Park et al., 2005). Coatings based on carboxymethyl cellulose-shellac containing parabens

reduced coliform bacteria on coated citrus fruit (McGuire and Hagenmaier, 2001). In recent work, hydroxypropyl methylcellulose (HPMC)-lipid edible composite films containing sodium salts of parabens, PS, sodium benzoate (SB), and their mixtures exhibited clear *in vitro* inhibitory activity against the main postharvest diseases of citrus fruit, green (GM) and blue (BM) molds, caused by *Penicillium digitatum* and *Penicillium italicum*, respectively (Valencia-Chamorro et al., 2008). In subsequent work, *in vivo* selected coatings reduced the incidence and severity of GM and BM on 'Clemenules' clementine mandarins, 'Ortanique' hybrid mandarins, and 'Valencia' oranges stored at 20 ºC (Valencia-Chamorro et al., 2009). However, no information is available on the performance of these edible coatings on cold-stored citrus fruit.

The objective of this work was to determine the effect of selected HPMC-lipid edible composite coatings containing food additives with antifungal properties on the development of penicillium molds and the physico-chemical and sensory quality of 'Valencia' oranges during long-term cold storage

2. Materials and methods

2.1. *Materials*

HPMC (Methocel E15) was purchased from Dow Chemical Co. (Midland, MI, USA). Shellac and beeswax (BW) (grade 1) were supplied by Fomesa Fruitech, S.L. (Beniparrell, València, Spain). Stearic acid and glycerol were from Panreac Química, S.A (Barcelona, Spain). Silicone antifoam (FG-1510) and ammonia (25 %) were from Dow Corning® (Belgium) and Scharlau (Sentmenat, Barcelona, Spain), respectively. Food preservatives were purchased from Sigma (Sigma-Aldrich Chemie, Steinheim, Germany) and included the salts of organic acids, PS, SB, and sodium propionate (SP). These chemicals are all classified as food additives or generally recognized as safe (GRAS) compounds by EU and US regulations.

2.2. *Emulsions preparation*

HPMC-lipid edible composite emulsions were prepared combining the hydrophilic phase (HPMC) and the hydrophobic phase (BW and shellac) suspended in water. Glycerol and stearic acid were used as plasticizer and emulsifier, respectively. Ratios of HPMC-glycerol (2:1) (dry basis, db) and lipid components (BW/shellac)-stearic acid (5:1) (db) were kept constant throughout the study. BW and shellac content was 50 % (db) at a ratio BW:shellac of 1:1. Emulsions were prepared as described previously by Valencia-Chamorro et al. (2008). Briefly, an aqueous solution of HPMC (5 % w/w) was prepared. Food preservative (w/w), BW, glycerol, stearic acid, water, and two drops of antifoam were added to the HPMC solution and heated at 90 ºC to melt the lipids. Shellac was previously dispersed in water at 40 ºC and ammonia (15 % w/w shellac/ammonia) was added to dissolve the resin. Shellac solution was heated separately at 90 ºC and added to the HPMC dispersion. Samples were homogenized with a high-shear probe mixer (Ultra-Turrax model T25, IKA-Werke, Steufen, Germany) for 4 min at 22,000 rpm. Emulsions were cooled to less than 25 ºC and further agitated for 25 min. Emulsions were kept 2-3 d at 5 ºC before use. Table 1 shows the total solid content of the formulations and the concentration of each food preservative (%, wet basis, wb). The formulations were selected from HPMC-lipid edible composite coatings that were effective inhibiting the development of GM and BM on citrus fruit stored at 20 ºC (Valencia-Chamorro et al., 2009). These formulations were stable and no phase separation was observed.

Table 1. Characteristics of hydroxypropyl methylcellulose (HPMC)-lipid edible composite coatings containing antifungal food preservatives and mixtures.

Food preservatives[a]	Food preservative concentration (%, wb)[b]	Solid concentration (%, wb)	Viscosity (cp)[c]	pH
Organic acid salts				
PS	2.0	6	19.20	7.30
SB	2.5	8	18.90	7.30
Mixtures				
PS+SP	1.5 + 0.5	6	12.20	7.20
SB+PS	2.0 + 0.5	8	18.70	7.20
SB+SP	2.0 + 0.5	8	16.10	7.10

[a] PS = potassium sorbate; SB = sodium benzoate; SP = sodium propionate.
[b] wb = wet basis.[c] cp = centipoises.

2.3. *Effect of coatings on disease development*

2.3.1. *Fungal inoculum*

P. digitatum isolate NAV-7 and *P. italicum* isolate MAV-1, obtained from decayed citrus fruit from packinghouses in the València area (Spain), were isolated, identified, and maintained in the IVIA culture collection of postharvest pathogens. These strains were selected for their aggressiveness on the most commercially important mandarin and orange cultivars. Prior to each experiment, the isolates were grown on potato dextrose agar (Sigma) in petri dishes at 25±1 ºC for 7–10 d A high-density conidial suspension was prepared in Tween 80 (0.05 %, w/v; Panreac Química S.A.) in sterile water, passed through two layers of cheesecloth, measured with a haemacytometer and diluted with sterile water to achieve the desired inoculum density.

2.3.2. *Fruit inoculation and coating application*

Oranges (*Citrus sinensis* [L.] Osbeck) cv. 'Valencia' from commercial orchards in València were selected by hand and used in the experiments before any postharvest treatment was applied. The fruit were stored up to one week at 5 ºC and 90 % relative humidity (RH) before use. Before

each experiment, the fruit were randomized, washed with fresh water, and allowed to air-dry at room temperature. Fruit were artificially inoculated (inoculum density of 10^8 conidia L^{-1}) by immersing a stainless steel rod with a probe tip 1 mm wide and 2 mm in length into the spore suspension and wounding each fruit once on the equator. Each fruit was inoculated with both fungus, each one on the opposite side of the equator, incubated 24 h at 20 ºC, coated manually by immersion (about 15 s at 20 ºC) with the HPMC-lipid edible composite coatings (Table 1), drained, and allowed to air-dry at room temperature. Inoculated but uncoated fruit were used as controls. Each treatment was applied to three replicates of 20 fruit each. Fruit were placed on plastic trays on corrugated cartons and stored for 60 d at 5 ºC and 90-95 % RH, plus 7 d at 20 ºC to simulate retail conditions.

2.3.3. *Determination of disease incidence and severity*

Disease incidence of GM and BM was calculated as the percentage of decayed fruit. Disease severity was determined as the diameter of the lesion (mm). Incidence and severity were assessed every 15 d during the storage period at 5 ºC and also after a shelf-life period of 7 d at 20 ºC following cold storage.

2.4. *Effect of coating on fruit quality*

2.4.1. *Fruit coating and storage*

For the quality study, fruit were washed with water containing dodecil benzene sulphonate sodium salt (4 % w/v) applied as a foam cascade in an experimental packingline, rinsed with tap water, and allowed to dry in a tunnel at 45±2 ºC during 130 s. Fruit were divided into six groups of 90 fruit each, which corresponded to the five coating treatments described in Table 1 and one control (uncoated fruit). The oranges were coated as described above, drained of excess coating, dried in the tunnel at 45±2 ºC during 130 s, and stored for up to 60 d at 5 ºC and 90-95 % RH. Physico-chemical and sensory fruit quality was assessed every 30 d at 5 ºC plus a shelf life period of 7 d at 20 ºC.

2.4.2. *Assessment of fruit quality*

2.4.2.1. *Weight loss.* Lots of 30 fruit per treatment were used to measure weight loss. The same marked oranges were weighted at the beginning and at the end of each storage period. The results were expressed as the percentage of initial weight lost.

2.4.2.2. *Fruit firmness.* Firmness of 20 fruit per treatment was determined at the end of each storage period using an Instron Universal testing machine (Model 4301, Instron Corp., Canton, MA, USA). Each fruit was compressed between two flat surfaces closing together at the rate of 5 mm min^{-1}. The machine gave the deformation (mm) after application of a load of 9.8 N to the equatorial region of the fruit. Results were expressed as percentage of deformation, related to initial diameter.

2.4.2.3. *Internal gas concentration.* Gas sampling from the fruit were performed by withdrawing 1 mL internal gas from the orange cavity with a syringe. To avoid external gas contamination samples were submerged under water. The gas sample was injected into a gas chromatograph (GC) (Thermo Trace, Thermo Fisher Scientific, Inc. Waltham, MA, USA) equipped with a thermal conductivity detector (TCD) and fitted with a Poropack QS 80/100 column (1.2 m x 0.32 cm i.d.). Temperatures were 35, 115, and 150 ºC, respectively for the oven, injector, and thermal conductivity detector. Helium was used, as carrier gas at a flow rate of 22 mL min^{-1}. The O_2 and CO_2 concentration was calculated using peak area obtained from standard gas mixtures of 15.0:2.5 % O_2:CO_2. Results were expressed as percentages. Ten fruit per treatment were analyzed.

2.4.2.4. *Ethanol and acetaldehyde contents.* Ethanol and acetaldehyde were analysed from the head-space of juice from samples using a GC (Thermo Trace, Thermo Fisher Scientific) equipped with an auto-sampler (Model

HS 2000), flame ionization detector (FID), and 1.2 m x 0.32 cm (i.d.) Poropack QS 80/100 column. The injector was set at 175 ºC, the column at 150 ºC, the detector at 200 ºC, and the carrier gas at 28 mL min^{-1}. A composite juice of three replicates of ten fruit per treatment was analyzed. Five mL of juice were transferred to 10-mL vials with crimp-top caps and TFE/silicone septum seals. Samples were frozen and stored at –18 ºC until analyses. A 1-mL sample of the headspace was withdrawn from vials previously equilibrated in a water bath at 20 ºC for 1 h, followed by 15 min at 40 ºC, to reach equilibrium in the headspace, and then injected into the GC. Ethanol and acetaldehyde was identified by comparison of retention times with standards. Results were expressed as mg of gas per 1 L of juice.

2.4.2.5. *Sensory evaluation.* Sensory quality of treated samples was evaluate by 8 to 10 trained judges at the end of each storage period. Judges rated flavor on a 9-point scale where 1 = very poor and 9 = optimum. Each judge was given samples from each batch and requested to evaluate off-flavor on a 5-point scale where 0 = absence of off-flavor and 5 = high presence of off-flavor. Four fruit per treatment were pealed and separated into individual segments. Two segments from two different fruit were presented to judges in trays labeled with 3-digit random codes and served to them at room temperature. The judges had to taste several segments of each sample in order to compensate, as far as possible, for biological variation of the material. Spring water was provided for palate rinsing between samples. External aspect of treated fruit (coating cracks, spots, etc.) was also evaluated by the panelists. A 3-point scale was used in which the aspect was classified as 1 = bad, 2 = acceptable, and 3 = good. Panelists were also asked to rank visually the treatments from highest to lowest gloss.

2.5. *Statistical analysis.*

Statistical analysis was performed using Statgraphics 5.1. (Manugistics Inc., Rockville, MD, USA). Specific differences between means were determined by Fisher's protected least significant difference test (LSD, $P < 0.05$) applied after an analysis of variance (ANOVA). For sensory gloss, specific differences were determined by Friedman test, which is

recommended for ranking by the UNE 87023 (AENOR, 1997). For disease incidence data, the ANOVA was applied to the arcsine of the square root of the proportion of decayed fruit.

3. Results and discussion

3.1. *Effect of coatings on disease development*

During cold storage at 5 ºC, all the HPMC-based coatings significantly reduced both GM and BM incidence and severity on artificially inoculated and coated 'Valencia' oranges (Fig. 1). The incidence and severity of both molds increased with storage time, but those of GM were lower than those of BM. No differences were found for disease incidence and severity of GM among coating treatments. After 60 d of storage at 5 ºC, the coatings reduced the incidence and severity of GM by about 2-4 times and 4-9 times, respectively, with respect to those on uncoated controls. In contrast, the incidence reduction of BM on coated fruit was small, except on fruit treated with the PS+SP-based coating. However, the coatings greatly reduced the severity of BM. When coated oranges were transferred to 20 ºC after 60 d at 5 ºC, the development of both GM and BM markedly increased, and the differences in incidence reduction previously observed with some of the coatings were not then significant. Nevertheless, disease severity on coated samples was 2-4 times lower than that on control samples. Among all the coatings, the PS+SP-based coating was the most effective to control both GM and BM after 60 d at 5 ºC plus a shelf life period of 7 d at 20 ºC.

It is known that during storage at temperatures lower than 10 ºC, *P. italicum* grows more rapidly than *P. digitatum* (Eckert and Eaks, 1989), and this reason might account for the lower effectiveness of the coatings to control BM than GM. In an *in vitro* study, Valencia-Chamorro et al. (2008) reported that among 14 HPMC-lipid edible composite films containing organic acid salts, only those containing PS and SB were effective to control GM and BM. Those findings were further confirmed in an *in vivo* study with citrus fruit stored at 20 ºC. Among a wide number of coatings tested, those containing PS, SB, and their mixtures were the most effective to reduce the development of GM and BM on 'Valencia' oranges (Valencia-Chamorro et al., 2009). In the present work, the

effectiveness of HPMC-lipid coatings containing organic acid salts and their mixtures was also confirmed on coated 'Valencia' oranges under cold storage at 5 ºC. Therefore, the HPMC-lipid edible composite coatings containing a mixture of PS+SP at 1.5+0.5 % (wb) could be promising to be used on citrus fruit under cold storage.

In the current commercial context, methods alternative to conventional fungicides are needed worldwide to control citrus penicillium molds (Palou et al., 2008). Differences on the effects of the coatings containing food preservatives on mold incidence and development may be related to either differences in their composition and/or physical properties (Waks et al., 1985) or differences in the release ability of the food preservative from the coating (Chung et al., 2001). The performance of the coatings during cold storage might be also influenced by the type of fruit. According to previous research, their inhibition activity after incubation at 20ºC was higher on 'Valencia' oranges than on 'Ortanique' or 'Clemenules' mandarins (Valencia-Chamorro et al., 2009). These differences are probably related to the different susceptibility of these fruit to infections by *Penicillium* spp. Work is in progress in our laboratory to determine the effectiveness and persistence of selected coatings containing food preservatives during long-term cold storage of mandarins as we expect that they will be lower than in the case of oranges. In previous work with food preservatives applied as aqueous solutions, we also observed a clear influence of the type of citrus fruit and its susceptibility to decay on the efficacy and persistence of the antifungal treatment (Palou et al., 2001, 2002a,b; Montesinos-Herrero et al., 2009).

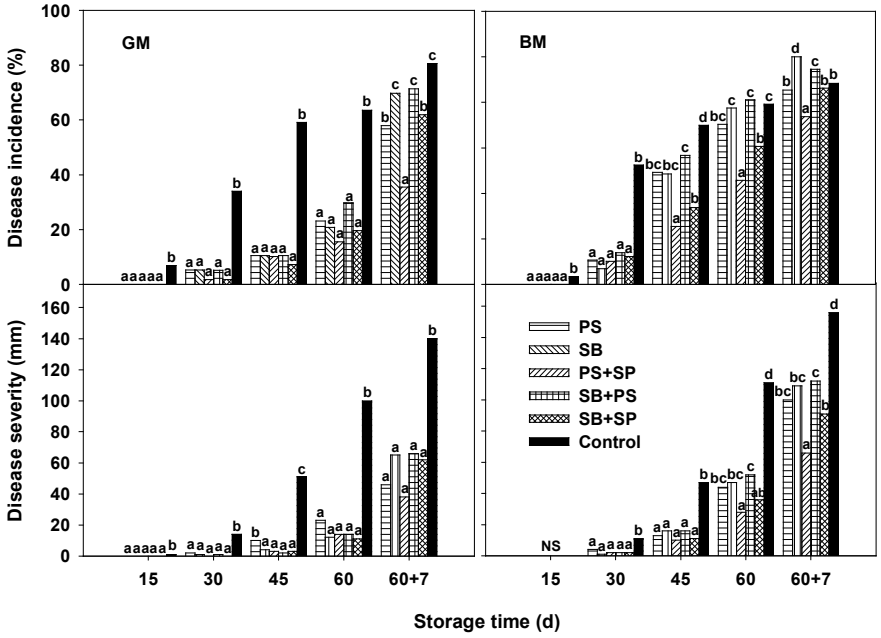

Fig. 1. Disease incidence and severity of green (GM) and blue (BM) molds, caused by *Penicillium digitatum* and *Penicillium italicum*, respectively, on 'Valencia' oranges artificially inoculated, uncoated (Control) or coated 24 h later with hydroxypropyl methylcellulose (HPMC)-lipid edible composite coatings containing potassium sorbate (PS), sodium benzoate (SB), sodium propionate (SP) or their mixtures, and stored up to 60 d at 5 ºC, followed by 7 d at 20 ºC (60+7). For each storage period, columns with different letters are significantly different according to Fisher's protected LSD test ($P < 0.05$) applied after an ANOVA. For disease incidence, the ANOVA was applied to arcsine-transformed values. Non-transformed means are shown.

3.2. *Effect of coating on fruit quality*

3.2.1. *Weight loss*

Fig. 2 shows the weight loss on coated and uncoated samples stored for 30 and 60 d at 5 ºC, followed by 7 d at 20 ºC. The coatings containing SB, alone or in mixtures, significantly reduced weight loss of

coated oranges after the first storage period, which indicates the effectiveness of these coatings as a moisture barrier. At the end of this storage period, weight loss on all samples were around 3.9-4.4 %. In general, weight loss slightly increased with storage time. At the end of the second and final storage period (60 d at 5 ºC plus 7 d at 20 ºC), weight loss of oranges treated with most of the coatings was not significantly different from that of control samples. In contrast, oranges coated with PS+SP- and SB+SP-based coatings suffered higher weight loss than uncoated samples.

Edible coating application has been contradictorily reported both with and without significant effects on weight loss of some fruits. For example, Pérez-Gago et al. (2002) reported that HPMC-lipid composite containing different types of lipids reduced weight loss of coated 'Fortune' mandarins. Coatings containing 0.1 % low molecular weight chitosan effectively retarded weight loss of coated 'Murcott' tangors (Chien et al., 2007). Navarro-Tarazaga et al. (2008b) observed that HPMC-BW coatings containing different types of plasticizers did not reduce weight loss of 'Angeleno' plums as compared with uncoated samples. In another study, HPMC coatings containing soybean oil or carnauba wax had minimal effect on water loss of coated cherries or cucumbers (Baldwin et al., 1997).

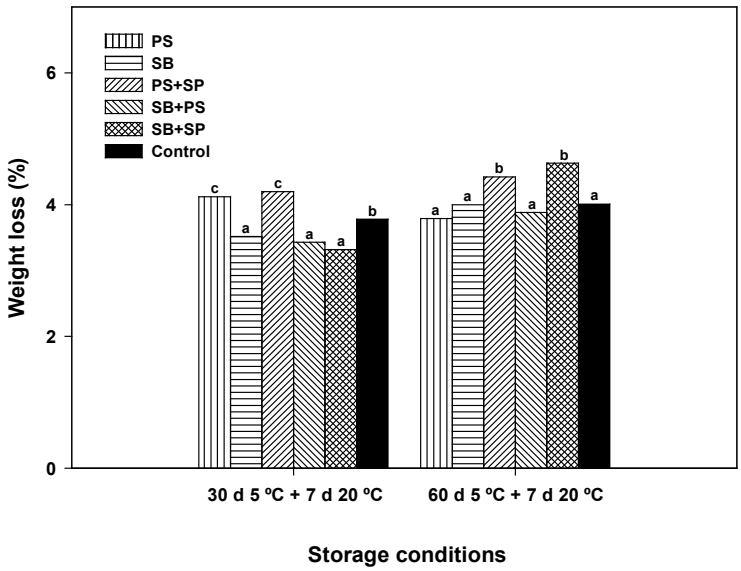

Fig. 2. Weight loss of 'Valencia' oranges uncoated (Control) or coated with hydroxypropyl methylcellulose (HPMC)-lipid edible composite coatings containing potassium sorbate (PS), sodium benzoate (SB), sodium propionate (SP) or their mixtures, stored at 5 ºC followed by 7 d at 20 ºC. For each storage period, columns with different letters are different by Fisher's protected LSD test ($P < 0.05$) applied after an ANOVA.

In previous research work, the water vapor permeability (WVP) of HPMC-lipid films containing food preservatives with the same composition than the coatings tested here was studied (Valencia-Chamorro et al., 2008). Among all films tested, PS- and PS+SP-stand-alone films exhibited the highest WVP. From those results, it was concluded that films containing PS and PS+SP modified the HPMC-lipid film structure in a greater extend than films containing SB alone or in combination. In the present work, the application of PS+SP-based coating to 'Valencia' oranges resulted on high weight loss after both cold storage periods. As on stand-alone films, it seems that water vapor from the fruit could easily diffuse through the coating leading to high weight loss. On the other hand, the mechanical properties of the film could also explain the performance of the SB+SP-based coating after a long storage

period. This film presented high Young's modulus, tensile strength, and low elongation at break, which indicates stiffness and very brittle films (Valencia-Chamorro et al., 2008). It is likely that coatings containing SB+SP could more easily form pits or cracks on the fruit surface that might enhance water loss leading to high weight loss.

3.2.2. *Fruit firmness*

Firmness of 'Valencia' oranges were not modified on coated fruit after both storage periods as compared to uncoated samples (data not shown). Fruit rind deformation after both storage periods was around 4 %. The effect of coatings on the maintenance of fruit firmness is usually related to their control of weight loss. On coated citrus fruit, however, contradictory results have been reported on the correlation between weight loss and firmness. For instance, while a positive correlation was reported by Navarro-Tarazaga et al. (2008a), no correlation was observed in the studies by Hagenmaier (2000) or Pérez-Gago et al. (2002). Apparently, it seems that for the coatings to affect fruit firmness significantly, they should induce a clear negative effect in fruit weight loss.

3.2.3. *Internal gas concentration*

Fig. 3 shows the internal gas concentration of coated and uncoated 'Valencia' oranges. At the end of the 30- and 60-d storage periods, there were an increase of internal CO_2 and a decrease of internal O_2 in coated fruit compared to uncoated fruit, and the concentration of internal CO_2 and O_2 on coated oranges reached values around 3-6 and 12-18 kPa, respectively. In general, these levels of O_2 are not low enough to create anaerobic conditions inside the coated fruit (Baldwin et al., 1997). When 'Valencia' oranges were coated with the HPMC-lipid coatings containing food preservatives, the internal atmosphere created on coated oranges fluctuated with storage time, possibly due to the presence of coating cracks and/or handling of the fruit altering coating permeability. Moreover, it seems that gas permeability was not affected by the small differences in total solids content of the HPMC-lipid coating emulsions (Table 1).

In this study, internal CO_2 values were lower than those reported by Navarro-Tarazaga et al. (2007) for HPMC-BW-shellac-coated 'Valencia' oranges after 15 d of storage at 24 ºC, or those reported by Baldwin et al. (1995) for shellac-coated 'Valencia' oranges after 15 d at 21 ºC. Under cold storage conditions, however, comparable values (around 8 kPa) of internal CO_2 were reported by Peeples et al. (1999) for shellac-based coated 'Valencia' oranges after 70 d of storage at 4 ºC, or by Pérez-Gago et al. (2002) for HPMC:lipid-coated 'Fortune' mandarins after 30 d of storage at 9 ºC.

Valencia-Chamorro et al. (2008) determined the oxygen permeability (OP) of HPMC-lipid-stand-alone films of the same composition than the coatings tested here. OP clearly depended on the food preservatives added to the formulations. In general, stand-alone films containing a mixture of different organic acid salts presented higher OP values than films with one salt alone. Thus, lower internal O_2 concentration was expected on oranges coated with PS- or SB-based coatings than on fruit treated with coatings containing a mixture of these organic acid salts. However, the results in the present work showed that fruit internal O_2 was usually higher on coatings containing only one salt than on mixture-based coatings.

Comparable differences have also been reported between stand-alone films and coatings with hydrocolloids-wax (Chen and Nussinovitch, 2001) or HPMC-BW coatings (Navarro-Tarazaga et al., 2008a) applied to citrus fruit. Even though film OP can be considered as a good predictor of internal gas transfer, other factors like fruit peel morphology or some physical properties of the coating formulation should also be considered when the coatings are applied to the fruit. These factors could influence the coating flexibility or its ability to adapt to the fruit surface. The coatings restrict the air exchange of fruit depending not only on how the coating is distributed over the surface of the fruit to form a continuous layer, but also on the coating ability to plug openings present in the peel (Hagenmaier and Baker, 1993; Mannheim and Soffer, 1996).

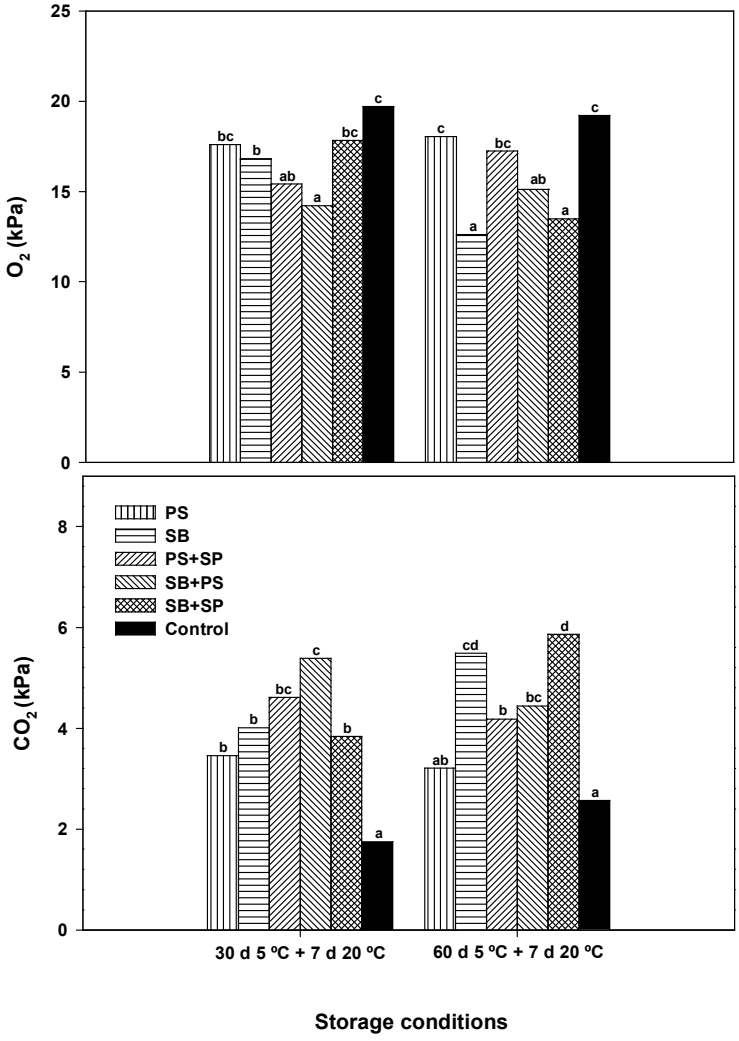

Fig. 3. Concentration of internal O_2 and CO_2 in 'Valencia' oranges uncoated (Control) or coated with hydroxypropyl methylcellulose (HPMC)-lipid edible composite coatings containing potassium sorbate (PS), sodium benzoate (SB), sodium propionate (SP) or their mixtures, stored at 5 ºC followed by 7 d at 20 ºC. For each storage period, columns with different letters are different by Fisher's protected LSD test ($P < 0.05$) applied after an ANOVA.

3.2.4. *Ethanol and acetaldehyde content*

The application of HPMC-lipid coatings increased ethanol content in the juice of coated 'Valencia' oranges ($P < 0.05$; Fig. 4). Thus, the creation of a modified atmosphere within the fruit was further confirmed. After 30 d at 5 ºC plus 7 d at 20 ºC of shelf life, all coated samples had similar ethanol content, which was higher than that of the uncoated control ($P < 0.05$). After the second storage period, 60 d at 5 ºC plus 7 d at 20 ºC, ethanol content in the juice of coated fruit slightly varied and the highest level was found in oranges treated with the PS+SP-based coating ($P < 0.05$).

In general, the concentration of ethanol in the juice of coated oranges after both storage periods was in the range of 1,040-1,240 mg L^{-1}, while it was in the range of 770-866 mg L^{-1} in uncoated samples. Different workers have reported higher amount of ethanol content on coated fruit after prolonged cold storage of citrus fruit. For instance, 'Fortune' mandarins coated with HPMC:lipid (20 % lipid content) reached ethanol values between 3,000 and 4,000 mg L^{-1} after 30 d at 9 ºC plus 7 d at 20 ºC (Pérez-Gago et al., 2002). In another study with 'Ortanique' mandarins coated with HPMC:BW, the ethanol content was higher than 4,000 mg L^{-1} after 45 d at 5 ºC plus 7 d at 20 ºC (Navarro-Tarazaga et al., 2008a). In this work, however, ethanol concentration in coated oranges did not exceed 2,000 mg L^{-1}. On the other hand, amounts of acetaldehyde lower than 6 mg L^{-1} were found on both coated and uncoated fruit, with no significant differences among samples (data not shown).

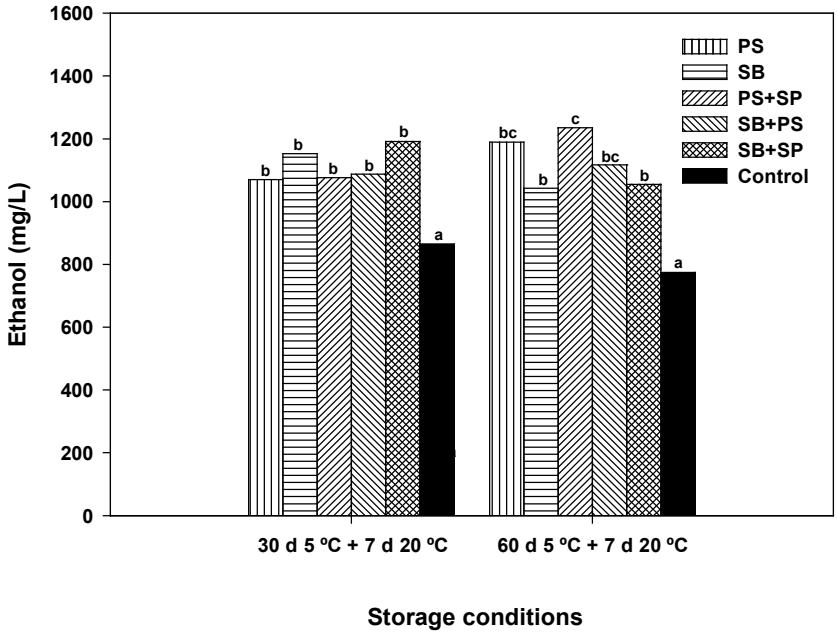

Fig. 4. Ethanol content in the juice of 'Valencia' oranges uncoated (Control) or coated with hydroxypropyl methylcellulose (HPMC)-lipid edible composite coatings containing potassium sorbate (PS), sodium benzoate (SB), sodium propionate (SP) or their mixtures, stored at 5 ºC followed by 7 d at 20 ºC. For each storage period, columns with different letters are different by Fisher's protected LSD test ($P < 0.05$) applied after an ANOVA.

3.2.5. *Sensory evaluation*

HPMC-lipid based coatings containing food preservatives slightly modified the flavor of 'Valencia' oranges compared to uncoated samples, as determined by the semi-trained judges of the sensory panel (Table 2). At the end of the storage period, after 60 d of storage at 5 ºC plus 7 d at 20 ºC of shelf life, flavor scores were around 4.8-5.8 (considered as acceptable) and no differences were detected among coated samples. The flavor of uncoated oranges was scored around 6.2-6.7.

Increases in the ethanol content of the juice beyond a minimal value of 2,000 mg L^{-1} have been associated with off-flavors in citrus fruit (Ke and Kader, 1990). In this study, ethanol values on coated samples were lower than this limit value (Fig. 4), and the panelists detected a very slight off-flavor after 60 d of storage at 5 ºC plus 7 d at 20 ºC, observing no differences between coated and uncoated samples, which indicates that the coatings did not induce off-flavor.

The addition of food preservatives to HPMC-lipid emulsion resulted in stable emulsions, but with a milky appearance due to the formation of a macroemulsion as the hydrophobic components were dispersed in the aqueous phase. Some coated fruit presented small white spots on their surface that reduced the general good appearance of the samples. Among all coated samples, fruit coated with PS-based coatings was evaluated with the highest external appearance value after 30 d at 5 ºC plus 7 d of shelf life at 20 ºC.

Table 2. Sensory evaluation of flavor, off-flavor, and coating appearance of 'Valencia' oranges coated with hydroxypropyl methylcellulose (HPMC)-lipid edible composite coatings containing antifungal food preservatives and mixtures and stored at 5 ºC followed by 7 d of shelf life at 20 ºC.

Food preservatives[v]	30 d 5 ºC + 7 d 20 ºC			60 d 5 ºC + 7 d 20 ºC		
	Flavor[w]	Off- flavor[x]	Coating appearance[y]	Flavor	Off-flavor	Coating appearance
PS	5.4 b[z]	0.8 abc	2.5 d	4.8 a	1.1 a	1.8 b
SB	4.6 ab	1.6 cd	1.4 ab	4.9 ab	0.9 a	1.0 a
PS+SP	4.0 a	2.2 d	2.0 c	4.9 ab	1.3 a	1.8 b
SB+PS	5.5 b	1.3 bc	1.0 a	5.0 ab	1.1 a	1.8 b
SB+SP	5.4 b	0.7 ab	1.6 bc	5.8 bc	0.6 a	1.8 b
Control	6.7 c	0.0 a	3.0 e	6.3 c	0.6 a	3.0 c

[v] PS = potassium sorbate; SB = sodium benzoate; SP = sodium propionate; Control = uncoated. [w] Flavor ranked from 1 (very poor) to 9 (optimum). [x] Off-flavor ranked from 0 (absence) to 5 (presence). [y] Coating appearance ranked 1 (bad) to 3 (good). [z] Means in columns with different letters are significantly different according to Fisher's protected LSD test ($P < 0.05$) applied after an ANOVA.

In general, after 60 d at 5 ºC plus 7 d at 20 ºC, coated samples were evaluated as acceptable except for those treated with SB-based coatings, which appearance was evaluated as bad. After both storage periods, none of the tested coatings provided higher gloss than the uncoated controls and the oranges coated with SB+PS were significantly less glossy than the controls after 60 d at 5 ºC plus 7 d at 20 ºC (Table 3). This behavior could be related to the macroemulsion character of the coating formulations (Hagenmaier and Baker, 1994).

Table 3. Ranked fruit gloss of 'Valencia' oranges coated with hydroxypropyl methylcellulose (HPMC)-lipid edible composite coatings containing antifungal food preservatives and mixtures and stored at 5 ºC followed by 7 d of shelf life at 20 ºC.

Gloss rank	30 d 5 ºC + 7 d 20 ºC		60 d 5 ºC + 7 d 20 ºC	
More glossy	Control[x]	c[y]	Control	b
	SB+PS	bc	PS	ab
	PS+SP	bc	PS+SP	ab
	PS	bc	SB+SP	ab
	SB+SP	ab	SB	ab
Less glossy	SB	a	SB+PS	a

[x] Control = uncoated; SB = sodium benzoate; PS = potassium sorbate; SP = sodium propionate. [y] Treatments in columns with different letters are significantly different according to Friedman test.

4. Conclusion

All coatings effectively reduced GM and BM on artificially inoculated and coated 'Valencia' oranges during cold storage, and the PS+SP-based coating was the most effective at inhibiting both molds during storage at 20 ºC after a cold storage period of 60 d at 5 ºC. Therefore, HPMC-lipid edible composite coatings containing antifungal organic acid salts and mixtures such as PS+SP could be a promising treatment for oranges that should be kept in cold storage. Although the coatings did not reduce weight loss or improve fruit gloss, they did not adversely affect the physico-chemical and sensory quality of 'Valencia' oranges.

Further research should be conducted to improve the physical characteristics of these HPMC-lipid edible composite coatings in order to obtain better water loss control and enhance gloss and visual quality of coated fruit. Moreover, new research should focus on the evaluation of these antifungal coatings on other commercially important citrus species and cultivars and also on their combination with other alternative non-polluting control methods to establish a cost-effective multi-faceted strategy for integrated decay control in citrus packinghouses.

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CHAPTER 4

Effect of antifungal hydroxypropyl methylcellulose (HPMC)-lipid edible composite coatings on penicillium decay development and postharvest quality of cold-stored 'Ortanique' mandarins

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ABSTRACT

Edible composite coatings based on hydroxypropyl methylcellulose (HPMC), hydrophobic components (beeswax and shellac), and food preservatives with antifungal properties were evaluated on 'Ortanique' mandarins during long-term cold storage. Selected food preservatives included potassium sorbate (PS), sodium benzoate (SB), sodium propionate (SP), and their mixtures. Intact mandarins or mandarins artificially inoculated with the pathogens *Penicillium digitatum* and *Penicillium italicum* were coated and stored up to 8 wks at 5 ºC + 1 wk of shelf-life at 20 ºC. HPMC-lipid coatings containing food preservatives controlled better green mold (GM) than blue mold (BM) on 'Ortanique' mandarins. SB- and SB + SP-based coatings reduced the incidence of GM by about 35% after 4 wks at 5 ºC. Among all coatings, only the SB-based coating reduced the incidence of GM (about 16%) after 6 wks at 5 ºC. All coatings significantly reduced disease severity of both GM and BM after 6 wks at 5 ºC. Analytical and sensory fruit quality was evaluated on intact mandarins. All coatings, especially the SB + SP-based coatings, were effective to control weight loss and maintain the firmness of coated mandarins. Internal gas concentration, juice ethanol and acetaldehyde content, sensory flavor, off-flavor, and fruit appearance were not adversely affected by the application of the antifungal coatings. Further studies should focus on the modification of some physical characteristics of the coatings to improve the gloss and visual aspect of treated mandarins.

KEYWORDS: citrus, postharvest quality, diseases, *Penicillium digitatum*, *Penicillium italicum*, green mold, blue mold.

INTRODUCTION

In the fresh citrus industry, fruit washing is a normal procedure during handling in the packinghouses, but the natural waxes of the fruit cuticle are usually removed together with undesirable dust, dirt, or bacterial or mold spores. Thus, clean fruit have lost their protective layer and may become more susceptible to water loss by transpiration.

Moreover, increasing respiration rates and fungal infection levels may reduce shelf-life of these fruit and lead them to general poor fruit quality (*1*). Waxing of citrus, to replace the fruit natural protection, is a usual commercial practice in the packinghouses. The application of waxes provides a barrier to gas and water exchange, improving fruit appearance and marketability. Chemical fungicides are often incorporated into waxes to control the main postharvest citrus diseases, green (GM) and blue (BM) molds, caused by the pathogens *Penicillium digitatum* (Pers.:Fr.) Sacc (PD) and *Penicillium italicum* Wehmer (PI), respectively (*2*). However, prolonged and extensive use of chemical fungicides to control postharvest decay may produce consumer concerns and adverse environmental consequences. Methods alternative to conventional chemical fungicides are currently needed worldwide to control these diseases (*3*).

Edible films and coatings have been developed as environmentally-friendly or non-polluting methods to replace natural waxes and extend produce shelf-life (*4-6*). Components of edible films and coatings include proteins, polysaccharides, lipids, and resins. Typically, functional and mechanical properties of coatings are improved by adding emulsifiers and plasticizers (*4, 7, 8*). In addition, antioxidants, flavors and pigments, vitamins, and antimicrobial agents can be incorporated into edible films and coatings. Films containing proteins and polysaccharides present a good barrier to gases, but a poor moisture barrier. On the contrary, lipid films are used as an adequate barrier to water vapor. Composite films comprise hydrocolloid components and lipids, thus enhancing the advantages and lessening the disadvantages of each.

Several works in the literature report that edible composite coatings based on hydroxypropyl methylcellulose (HPMC) and lipids such as beeswax (BW), carnauba wax, or resin (shellac) preserved the postharvest quality of citrus fruit by reducing weight loss and keeping firmness and sensory quality of coated fruit (*9-11*).

Successful development of films and coatings with added food preservatives or natural plant extracts to control microbial growth *in vitro* or on fresh fruits and vegetables has been reported. The antimicrobial activity of potassium sorbate (PS) supported in tapioca starch edible films against *Zygosaccharonyces bailii* was reported by Flores et al. (*12*). The film containing the preservative was available to prevent external *Z. bailii*

contamination and control yeast growth in an acidified high water activity semisolid product. In an *in vitro* study using the film disc method, it was reported that carvacrol-containing-tomato-based edible film inactivated the virulent pathogen *E. coli* O157:H7 (*13*). Chitosan/methyl cellulose film incorporating vanillin (as natural antimicrobial agent) provided an inhibitory effect against *E. coli* on inoculated fresh-cut cantaloupe stored at 10 ºC (*14*). A chitosan-based coating containing PS showed an antifungal action against *Rhizopus* sp. and *Cladosporium* sp. on inoculated fresh strawberries stored at 5 ºC (*15*). A low molecular chitosan-based coating with PS maintained postharvest fruit quality and showed effective antifungal activity against PD and PI on 'Murcott' tangors stored at 15 ºC (*16*). Natamycin added to a bilayer film formulated with chitosan and polyethylene wax decreased decay severity of *Alternaria alternata* and *Fusarium semitectum* on 'Hami' melons stored at 30 ºC (*17*).

In previous studies, Valencia-Chamorro et al (*18*) developed HPMC-lipid edible composite films and coatings with food additives, such as mineral salts, organic acid salts, salts of parabens, and some mixtures, that presented antifungal properties i*n vitro* against PD and PI. In subsequent work *in vivo,* selected coatings reduced the incidence and severity of GM and BM on 'Clemenules' clementine mandarins, 'Ortanique' hybrid mandarins and 'Valencia' oranges coated and incubated at 20 ºC for 7 d to simulate typical fruit shelf-life conditions (*19*). However, no information is available on the performance of this type of edible coatings on cold-stored citrus fruit.

The objective of this work was to determine the effect of selected new HPMC-lipid edible composite coatings containing food additives with antifungal properties on the development of penicillium molds and the physico-chemical and sensory quality of 'Ortanique' mandarins during long-term cold storage.

MATERIALS AND METHODS

Materials

HPMC (Methocel E15) was purchased from Dow Chemical Co. (Midland, MI, USA). Shellac and BW (grade 1) were supplied by Fomesa

Fruitech, S.L. (Valencia, Spain). Stearic acid and glycerol were from Panreac Química, S.A (Barcelona, Spain). Silicone antifoam (FG-1510) and ammonia (25%) were from Dow Corning Ibérica S.A. (Barcelona, Spain) and Scharlau Chemie S.A. (Sentmenat, Spain), respectively. Food preservatives were purchased from Sigma (Sigma-Aldrich Chemie, Steinheim, Germany) and included the salts of organic acids, PS, sodium benzoate (SB), and sodium propionate (SP). These chemicals are all classified as food additives or generally recognized as safe (GRAS) compounds by the European Union and the United States regulations.

Emulsions preparation

HPMC-lipid edible composite emulsions were prepared by combining the hydrophilic phase (HPMC) and the hydrophobic phase (BW and shellac) suspended in water. Glycerol and stearic acid were used as plasticizer and emulsifier, respectively. Ratios of HPMC-glycerol (2:1) (dry basis, db) and lipid components (BW/shellac)-stearic acid (5:1) (db) were kept constant throughout the study. BW and shellac content was 50% (db) at a ratio BW: shellac of 1:1. Emulsions were prepared as described previously by Valencia-Chamorro et al. (*18*). Briefly, an aqueous solution of HPMC (5% w/w) was prepared. Food preservative (w/w), BW, glycerol, stearic acid, water, and two drops of antifoam were added to the HPMC solution and heated at 90ºC to melt the lipids. Shellac was previously dispersed in water at 40 ºC and ammonia (15% w/w shellac/ammonia) was added to dissolve the resin. Shellac solution was heated separately at 90 ºC and added to the HPMC dispersion. Samples were homogenized with a high-shear probe mixer (Ultra-Turrax model T25, IKA-Werke, Steufen, Germany) for 4 min at 22,000 rpm. Emulsions were cooled to less than 25 ºC and further agitated for 25 min. Emulsions were kept 2-3 d at 5 ºC before use. Table 1 shows the total solid content of the formulations and the concentration of each food preservative (%, wet basis, wb). The formulations were selected from HPMC-lipid edible composite coatings that were effective inhibiting the growth of PD and PI on citrus fruit incubated at 20 ºC (*19*). These formulations were stable and no phase separation was observed.

Table 1. Characteristics of hydroxypropyl methylcellulose (HPMC)-lipid edible composite coatings containing food preservatives and mixtures.

Food preservative[x]	Food preservative concentration (%, wb)	Solid concentration (%, wb)	Viscosity (cp)[c]	pH
Organic acid salts				
PS	2.0	6	9.30	8.03
SB	2.5	8	17.30	8.13
Organic acid salts (mixtures)				
PS + SP	1.5 + 0.5	6	12.20	8.22
SB + PS	2.0 + 0.5	8	11.00	8.24
SB + SP	2.0 + 0.5	8	11.20	8.19

[a] PS = potassium sorbate; SB = sodium benzoate; SP = sodium propionate.
[b] wb = wet basis. [c] cp = centipoises.

Effect of coatings on disease development

Fungal inoculum

P. digitatum isolate NAV-7 and *P. italicum* isolate MAV-1, obtained from decayed oranges from Valencia packinghouses, were isolated, identified, and maintained in the IVIA culture collection of postharvest pathogens. These strains were selected for their aggressiveness on the most commercially important mandarin and orange cultivars. Prior to each experiment, the isolates were grown on potato dextrose agar (Sigma) in petri dishes at 25±1 ºC for 7–10 d. A high-density conidial suspension was prepared in Tween 80 (0.05%, w/v; Panreac Química S.A.) in sterile water, passed through two layers of cheesecloth, measured with a haemacytometer and diluted with sterile water to achieve the desired inoculum density.

Fruit inoculation and coating application

Hybrid mandarins cv. 'Ortanique' (*Citrus reticulata* x [*C sinensis* x *C. reticulata*]) from commercial orchards in Valencia (Spain) were selected by hand and used in the experiment before any postharvest treatment was applied. The fruit were stored up to one week at 5 ºC and 90% relative humidity (RH) before use. Before each experiment, fruit were randomised, washed with fresh water, and allowed to air-dry at room

temperature. Fruit were artificially inoculated (inoculum density of 10^5 spores/mL) by immersing a stainless steel rod with a probe tip 1 mm wide and 2 mm in length into the spore suspension and wounding each fruit once on the equator. Each fruit was inoculated with both fungus, each one on the opposite side of the equator. Inoculated fruit were incubated 24 h at 20 ºC, coated manually by immersion (about 15 s at 20 ºC) with the HPMC-lipid edible composite coatings (Table 1), drained, and dried in a tunnel at 45±2 ºC for 130 s. Inoculated but uncoated fruit were used as controls. Each treatment was applied to three replicates of 20 fruit each. Fruit were placed on plastic trays on corrugated cartons and stored for 8 wks at 5 ºC and 90-95% RH, followed by 1 wk at 20 ºC and 90% RH to simulate retail handling conditions.

Determination of disease incidence and severity

Disease incidence of GM and BM was calculated as the percentage of decayed fruit. Disease severity was determined as the diameter of the decay lesion (mm). Incidence and severity were assessed every 2 wks during a total storage period of 6 wks at 5 ºC.

Effect of coatings on fruit quality

Fruit coating and storage

For the quality study, fruit were washed with water containing dodecil benzene sulphonate sodium salt (4% w/v) applied as a foam cascade, rinsed with tap water and allowed to dry in a tunnel at 45±2 ºC for 130 s. Fruit were divided into six groups of 90 fruits each, which corresponded to five coating treatments and one control (uncoated fruit). The mandarins were coated as described above, drained of excess coating, dried in a tunnel at 45±2 ºC for 130 s, and stored either at 20 ºC for 1 wk or at 5 ºC for 8 wks followed by 1 wk at 20 ºC to simulate retail handling conditions.

Assessment of fruit quality

Weight loss

Lots of 30 fruit per treatment were used to measure weight loss. The same marked fruit were weighted at the beginning and at the end of each storage period. The results were expressed as the percentage loss of initial weight.

Fruit firmness

Firmness of 20 fruit per treatment was determined at the end of each storage period using an Instron Universal testing machine (Model 3343, Instron Corp., Canton, MA, USA). Each fruit was compressed between two flat surfaces closing together at the rate of 5 mm/min. The machine gave the deformation (mm) after application of a load of 10 N to the equatorial region of the fruit. Results were expressed as percentage of deformation, related to initial diameter.

Internal gas concentration

Gas sampling from the fruit were performed by withdrawing 1 mL internal gas from the fruit cavity with a syringe. To avoid external gas contamination samples were submerged under water. The gas sample was injected into a Thermo Trace (Thermo Fisher Scientific, Inc. Waltham, MA, USA) gas chromatograph (GC) equipped with a thermal conductivity detector (TCD) and fitted with a Poropack QS 80/100 column (1.2 m x 0.32 cm i.d.). Temperatures were 35, 115, and 150 ºC, respectively for the oven, injector, and thermal conductivity detector. Helium was used as carrier gas at a flow rate of 22 mL/min. The O_2 and CO_2 concentrations were calculated using peak areas obtained from standard gas mixtures of 15.0:2.5% O_2:CO_2. Results were expressed as percentages. Ten fruits per treatment were analysed.

Ethanol and acetaldehyde concentration

Ethanol and acetaldehyde were analysed from the head-space of juice from samples using a GC Thermo Trace (Thermo Fisher Scientific) equipped with auto-sampler (Model HS 2000), flame ionization detector (FID), and 1.2 m x 0.32 cm (i.d.) Poropack QS 80/100 column. The injector was set at 175 ºC, the column at 150 ºC, the detector at 200 ºC, and the carrier gas at 28 mL/min. A composite juice of three replicates of 10 fruit per treatment was analysed. Five mL of juice were transferred to 10-mL vials with crimp-top caps and TFE/silicone septum seals. Samples were frozen and stored at –18 ºC until analyses. A 1-mL sample of the headspace was withdrawn from vials previously equilibrated in a water bath at 20 ºC for 1 h, followed by 15 min at 40ºC, to reach equilibrium in the headspace, and then injected into the GC. Ethanol and acetaldehyde were identified by comparison of retention times with standards. Results were expressed as mg/100mL juice.

Sensory evaluation

Sensory quality of treated samples was evaluated by 8 to 10 trained judges at the end of each storage period. Judges rated flavor on a 9-point scale, where; 1 = very poor and 9 = optimum. Each judge was given samples from each batch and requested to evaluated off-flavor on a 5-point scale where 0 = absence of off-flavor and 5 = high presence of off-flavor. Four fruit per treatment were pealed and separated into individual segments. Two segments from two different fruit were presented to judges in trays labelled with 3-digit random codes and served to them at room temperature. The judges had to taste several segments of each sample in order to compensate, as far as possible, for biological variation of the material. Spring water was provided for palate rinsing between samples. External aspect of treated fruit (coating cracks, spots, etc.) was also evaluated by the panellists. A 3-point scale was used, where 1 = bad, 2 = acceptable, and 3 = good aspect. Panellists were also asked to rank visually the treatments from highest to lowest gloss.

Statistical analysis

Statistical analysis was performed using Statgraphics® 5.1 (Manugistics Inc., Rockville, MD, USA). Specific differences between means were determined by Fisher's protected least significant difference test (LSD, $P < 0.05$) applied after an analysis of variance (ANOVA). For sensory gloss, specific differences were determined by the Friedman test, which is recommended for ranking by the UNE 87004 (*20*). For disease incidence data, ANOVA was applied to arcsine of the square root of the proportion of decayed fruit.

RESULTS AND DISCUSSION

Effect of coatings on disease development

After 2 wks of cold storage at 5 ºC, all the coatings reduced both GM and BM incidence and severity compared with uncoated samples (Figure 1). Disease incidence of GM and BM were around five to ten and two to seven times lower, respectively, on coated 'Ortanique' mandarins than on control samples ($P < 0.05$). However, a fast incidence increase of both molds was observed after 4 and 6 wks of cold storage at 5 ºC. Among all coatings tested, SB- and SB + SP-based coatings were the most effective to reduce the disease incidence of GM (about 35% reduction) as compared with control samples after 4 wks of cold storage. When storage was prolonged to 6 wks, only the SB-based coating significantly reduced disease incidence as compared with control samples (about 16% reduction). The lower effectiveness of the coatings to control BM than GM during cold storage at 5 ºC may be influenced by the fact that PI grows more rapidly than PD at temperatures lower than 10 ºC (*2*).

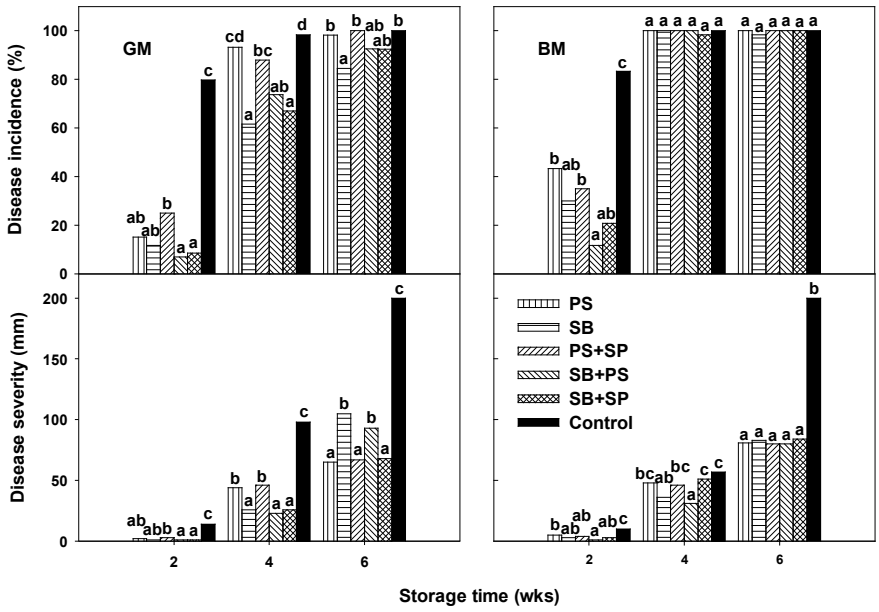

Figure 1. Disease incidence and severity of green (GM) and blue (BM) molds, on 'Ortanique' mandarins artificially inoculated with *Penicillium digitatum* and *Penicillium italicum*, uncoated (Control), or coated 24 h later with hydroxypropyl methylcellulose (HPMC)-lipid edible composite coatings containing potassium sorbate (PS), sodium benzoate (SB), sodium propionate (SP) or their mixtures, and stored up to 6 wks at 5 ºC. For each storage period, columns with different letters are significantly different by Fisher's protected LSD test ($P < 0.05$) applied after an ANOVA. For disease incidence, the ANOVA was applied to arcsine-transformed values. Non-transformed means are shown.

All the coatings effectively reduced disease severity of GM and BM on coated 'Ortanique' mandarins ($P < 0.05$). Although disease severity on coated samples increased during the entire cold storage period, it was 2-3 times lower than on control samples after 6 wks ($P < 0.05$). Moreover, the coatings were very effective in inhibiting the sporulation of GM and BM after 4 wks of cold storage (data not shown).

HPMC-lipid edible composite films and coatings with food additives (organic acid salts, salts of parabens, or their mixtures) presented

antifungal properties i*n vitro* against the pathogens PD and PI (*18*), and *in vivo* against the diseases GM and BM on coated 'Clemenules' mandarins, 'Ortanique' hybrid mandarins and 'Valencia' oranges stored at 20 ºC (*19*). In this study with fruit in cold storage at 5 ºC, it was confirmed that SB and SB + SP were the best antifungal compounds to be added as coating ingredients for reduction of penicillium molds on 'Ortanique' mandarins. These coatings were effective to control disease incidence after 2 wks of storage at 5 ºC and to reduce disease severity even after 6 wks.

From this and previous work (*19*), it can be concluded that the effect of the coatings containing organic acid salts or their mixtures on mold growth and development is dependent on the citrus cultivar. HPMC-lipid edible composite coatings containing these food preservatives were effective to control GM and BM on 'Valencia' oranges stored for 8 wks at 5 ºC. In this study with 'Ortanique' mandarins, the control ability of the coatings was lower and the incidence reduction, especially of BM, was not important after 4 wks of cold storage. Therefore, the treatments were less persistent on mandarins than on oranges. Coating composition and/or physical properties, as well as differences on the release rate of the food preservatives from the coatings, may influence coating effectiveness on fresh produce (*21, 22*). Furthermore, since coating effectiveness was also highly dependent on the type of citrus fruit, it can be assumed that the relative fruit susceptibility to infections by PD or PI may be strongly influence the performance of the coatings. In previous research, Palou et al. (*23, 24*) reported that the effectiveness of brief immersions in aqueous solutions of antifungal food preservatives such as sodium carbonate or bicarbonate to control penicillium molds was lower on mandarins than on oranges, mostly due to the lower susceptibility of the later to decay. In general, comparable differences on performance depending on the fruit species or cultivars have been observed with most of the alternative antifungal treatments which mode of action is rather fungistatic than fungicidal (*3*)

The application of HPMC-lipid edible composite coatings containing food preservatives is a simple and environmentally-friendly method to reduce the losses caused by citrus postharvest diseases. Thus, these coatings could be used as a commercial alternative to synthetic chemical fungicides for decay control in citrus packinghouses, especially in combination with other postharvest treatments that provide complementary activity. Another interesting alternative to chemical

control is the use of antagonistic microorganisms that biologically interfere with and control fungal pathogens. Several coatings that support microorganisms for biological control of postharvest fruit diseases have been developed (*25, 26*). Therefore, the addition of selected biocontrol agents to HPMC-lipid edible composite coatings to control penicillium molds of citrus fruit is a possibility that might be also considered for further research. Moreover, if they are compatible, both biocontrol agents and antifungal food additives might be considered together as ingredients of new edible coatings.

Effect of coatings on fruit quality

Weight loss

The effect of HPMC-lipid edible composite coatings containing food preservatives on weight loss of coated 'Ortanique' mandarins is shown on Figure 2. For all storage periods, coated mandarins presented lower weight loss than the uncoated control ($P < 0.05$), which indicates the effectiveness of these coatings as moisture barriers. However, weight loss increased during the storage period. At the end of the storage period, weight loss on coated samples were around 7.6-9.5%. After 1 wk storage at 20 ºC and at the end of the entire storage period (8 wks at 5 ºC + 1 wk at 20 ºC), the coatings reduced weight loss by about 20-37% and 25-40%, respectively, as compared to uncoated control samples. Similar weight loss on 'Fortune' and 'Ortanique' mandarins coated with HPMC-BW coatings was reported after 5 wks (4 wks at 9 ºC + 1 wk at 20 ºC) and 7 wks (6 wks at 5 ºC + 1 wk at 20 ºC) of cold storage, respectively (*9, 11*). Among all treatments, SB + PS- and SB + SP-based coatings provided the most effective barrier for prolonged cold storage. These results on coated fruit correlated well with the low water vapor permeability (WVP) of stand-alone films containing the same mixtures of antifungal ingredients (*18*). Those films were also stiff and brittle as a result of their low flexibility. Despite the low flexibility, the coatings containing those mixtures showed a good ability to adapt to changes in the fruit surface as the coated fruit lost weight and volume during cold storage.

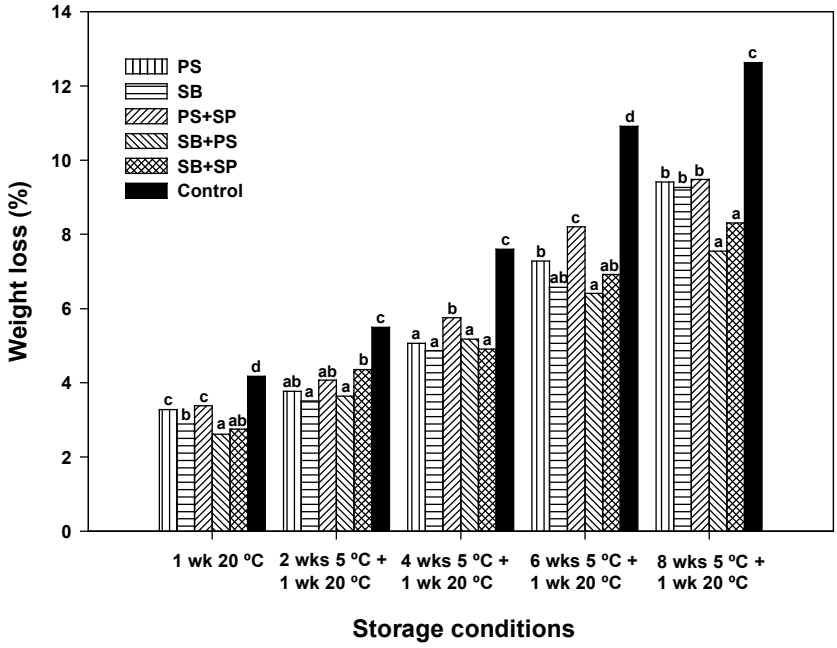

Figure 2. Weight loss of 'Ortanique' mandarins uncoated (Control) or coated with hydroxypropyl methylcellulose (HPMC)-lipid edible composite coatings containing potassium sorbate (PS), sodium benzoate (SB), sodium propionate (SP) or their mixtures, stored at either 20 ºC for 1 wk or 5 ºC for up to 8 wks followed by 1 wk at 20 ºC. For each storage period, columns with different letters are different by Fisher's protected LSD test ($P < 0.05$) applied after an ANOVA

Fruit firmness

Fruit rind deformation of coated mandarins was significantly lower than that of uncoated control samples after all storage periods, except after 4 wks at 5 ºC + 1 wk at 20 ºC (Figure 3). There were no significant differences after this period. The SB + SP-based coating was the most effective to reduce rind deformation and this could be related to the lower weight loss observed in these samples (Figure 2). On 'Ortanique' mandarins coated with HPMC-BW containing oleic acid (OA) at a BW/OA ratio of 1:0.5, a significant correlation between fruit firmness and weight loss was reported (*11*). However, no correlation between fruit

firmness and weight loss was reported on 'Valencia' oranges coated with HPMC-lipid edible composite coatings containing food preservatives (*27*), 'Valencia' oranges coated with polyethylene-candelilla coatings (*28*), or 'Fortune' mandarins coated with HPMC-lipid with various types and amounts of lipids (*9*). It seems, therefore, that fruit firmness on coated samples was affected not only by the coating type and storage conditions but also by the fruit cultivar. It is likely that the coating application may impact on fruit firmness as a result of the high weight loss reduction.

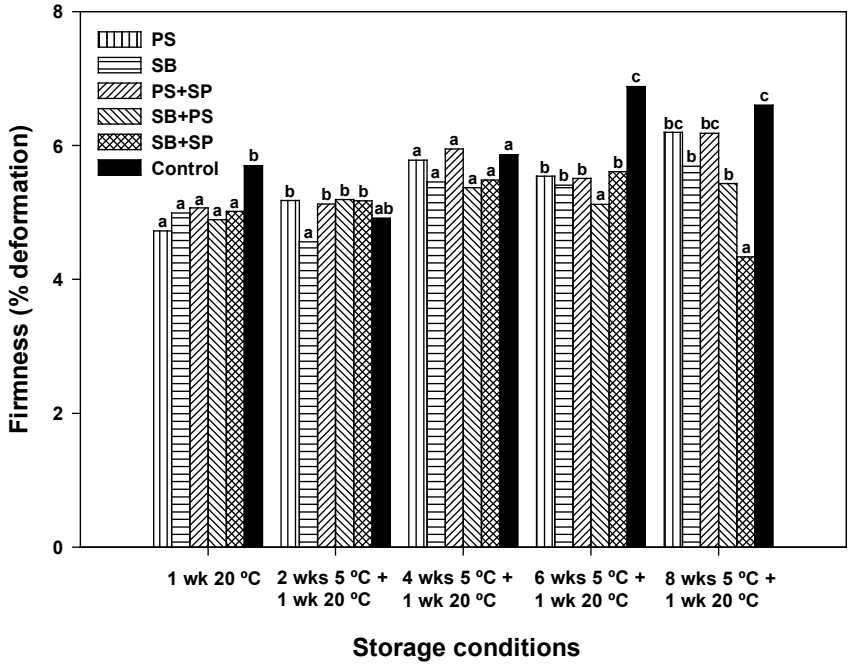

Figure 3. Firmness of 'Ortanique' mandarins uncoated (Control) or coated with hydroxypropyl methylcellulose (HPMC)-lipid edible composite coatings containing potassium sorbate (PS), sodium benzoate (SB), sodium propionate (SP) or their mixtures, stored at either 20 ºC for 1 wk or 5 ºC for up to 8 wks followed by 1 wk at 20 ºC. For each storage period, columns with different letters are different by Fisher's protected LSD test ($P < 0.05$) applied after an ANOVA.

Internal gas concentration

The internal gas concentration of coated and uncoated 'Ortanique' mandarins is shown in Figure 4. In general, there was a decrease of internal O_2 and an increase of internal CO_2 in coated samples, which indicates a significant modification of the internal atmosphere as a result of the application of the coatings. As the storage time increased, the levels of internal CO_2 increased and those of internal O_2 decreased. At the end of the storage period, the concentration of internal CO_2 and O_2 on coated mandarins reached values around 12% and 7%, respectively. In general, these values for O_2 were not low enough to create anaerobic conditions inside the coated fruit (*29*). The addition of food preservatives, either alone or in a mixture, to HPMC-lipid coatings did not result in a clear response with respect to internal gas concentration on coated mandarins. It appears that the total solid content, 6 or 8% of the HPMC-lipid coating emulsion (Table 1) did not affect the gas permeability of coated samples. Lower values (around 6%) for the concentration of internal CO_2 on cold-stored 'Valencia' oranges coated with emulsions containing organic acid salts and their mixtures were reported by Valencia-Chamorro et al. (*27*). Comparable values (around 8%) of internal CO_2 were also reported by Peeples et al. (*30*) on shellac-coated Valencia' oranges after 10 wks of storage at 4 ºC, and by Pérez-Gago et al. (*9*) on HPMC:lipid-coated 'Fortune' mandarins after 4 wks of storage at 9 ºC. However, in this study, internal CO_2 values were lower than those reported by Navarro-Tarazaga et al. (*10*) for HPMC-BW-Shellac-coated 'Valencia' oranges after 2 wks of storage at 24 ºC, and those reported by Baldwin et al. (*31*) for shellac-coated 'Valencia' oranges after 2 wks at 21 ºC.

Oxygen permeability (OP) of HPMC-lipid-stand-alone films containing organic acid salts as antifungal ingredients was determined by Valencia-Chamorro et al. (*18*). OP clearly depended on the kind of food preservative added to the formulations. Stand-alone films containing a mixture of organic acid salts presented higher OP values than films containing only one salt. Thus, higher internal O_2 concentration was expected on fruit coated with mixture-based-coatings than on fruit treated with coatings containing only one salt. However, no significant differences between these two types of coatings were found in the present work. Similar differences have also been reported between stand-alone films and coatings with hydrocolloids-wax (*32*) or HPMC-BW

coatings (*11*) applied to citrus fruit. Such differences were attributed to other factors that may affect the performance of the coatings on the fruit surface. When the coatings are applied to the fruit, it is important to consider that other factors like fruit peel morphology or some physical properties of the coating formulation could influence the coating flexibility or its ability to adapt to the fruit surface. The coatings restrict the air exchange of the fruit depending not only on how the coating is distributed over the surface of the fruit to form a continuous layer but also on the coating ability to plug the openings present in the peel (*33, 34*).

Ethanol and acetaldehyde concentration

Figure 5 shows the ethanol concentration in the juice of coated and uncoated mandarins after the different storage periods. Higher ethanol content was found in coated samples than in the uncoated control irrespective of the storage period tested ($P < 0.05$). Thus, these results confirmed the creation of a modified atmosphere within the fruit. However, this internal atmosphere did not result in a clear response with respect to the ethanol concentration in coated 'Ortanique' mandarins. As the storage time increased, the ethanol concentration increased as well. After 1 wk at 20 ºC and at the end of the whole storage period (8 wks at 5 ºC + 1 wk at 20 ºC) the ethanol content in the juice of coated fruit was about 140-168 and 309-356 mg/100 mL, respectively, while it increased from 110 to 287 mg/100 mL in the uncoated control. After prolonged cold storage of citrus fruit, higher amount of ethanol content on coated fruit has been reported by several authors. For instance, high ethanol concentration (400-500 mg/100 mL) were reported in 'Ortanique' mandarins coated with HPMC-BW coatings after storage of 6 wks at 5 ºC + 1 wk at 20 ºC (*11*), or in 'Fortune' mandarins (300 and 400 mg/100 mL) coated with HPMC:lipid (20% lipid content) after 4 wks at 9 ºC + 1 wk at 20 ºC (*9*). On the other hand, small amounts of acetaldehyde, less than 1.0 mg/100 mL, were found on both coated and uncoated fruit, with no significant differences among samples (data not shown).

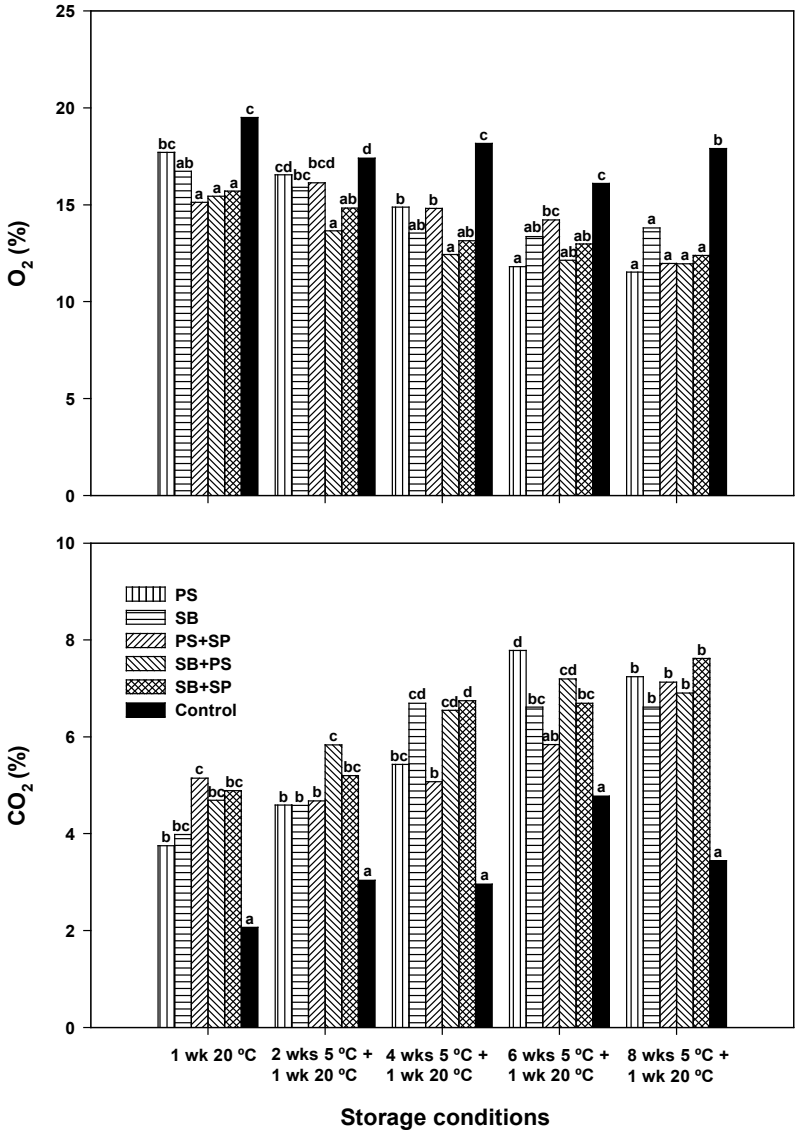

Figure 4. Internal concentration O_2 and CO_2 in 'Ortanique' mandarins uncoated (Control) or coated with hydroxypropyl methylcellulose (HPMC)-lipid edible composite coatings containing potassium sorbate (PS), sodium benzoate (SB), sodium propionate (SP) or their mixtures, stored at either 20 ºC for 1 wk or 5 ºC for up to 8 wks followed by 1 wk at 20 ºC. For each storage period, columns with different letters are different by Fisher's protected LSD test ($P < 0.05$) applied after an ANOVA.

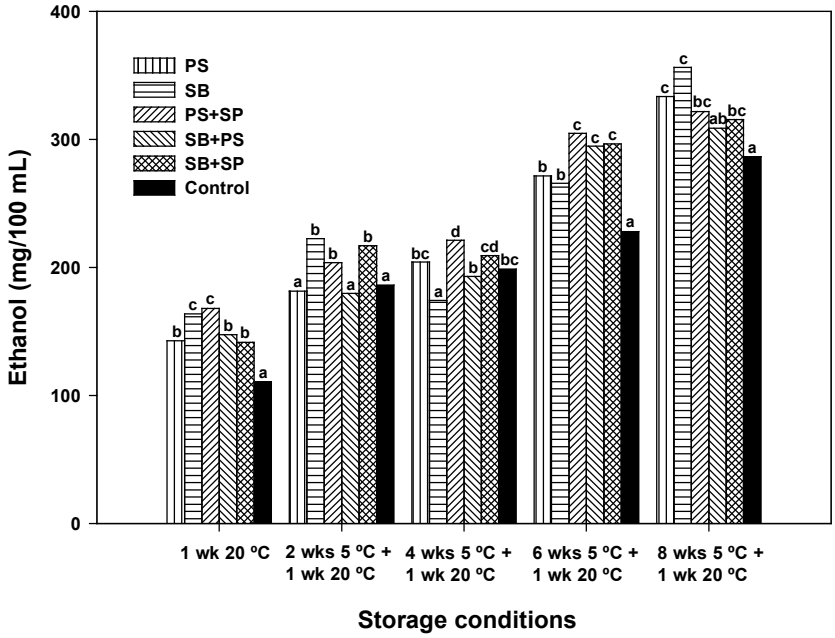

Figure 5. Ethanol concentration in the juice of 'Ortanique' mandarins uncoated (Control) or coated with hydroxypropyl methylcellulose (HPMC)-lipid edible composite coatings containing potassium sorbate (PS), sodium benzoate (SB), sodium propionate (SP) or their mixtures, stored at either 20 ºC for 1 wk or 5 ºC for up to 8 wks followed by 1 wk at 20 ºC. For each storage period, columns with different letters are different by Fisher's protected LSD test ($P < 0.05$) applied after an ANOVA.

Sensory evaluation

HPMC-lipid based coatings containing food preservatives slightly modified the flavor of 'Ortanique' mandarins during the storage time as compared to that of uncoated samples. The panellists considered the flavor as acceptable irrespective of the treatments and the storage time (data not shown).

Ethanol content in excess of about 200 mg/100 mL has been associated with induction of off-flavor in citrus fruit (*35*). In this study,

although ethanol values in cold-stored samples were higher than this limit value (Figure 5) and panellists detected a slight off-flavor after prolonged storage (8 wks of storage at 5 ºC + 1 wk at 20 ºC), no differences between coated and uncoated samples were observed, which indicates that the coatings did not induce off-flavor. The external appearance of all coated samples remained unchanged if compared to that of uncoated fruit and differences were not found among coated and uncoated samples after each storage period (data not shown). Although some coated fruit presented small white spots on their surface that reduced the general good appearance of the samples, in general, the coated samples were evaluated as acceptable after 8 wks of storage at 5 ºC + 1 wk at 20 ºC. The addition of food preservatives to HPMC-lipid emulsion resulted in stable emulsions, but with a milky appearance due to the formation of a macroemulsion as the hydrophobic components were dispersed in the aqueous phase (*36*). Irrespective of the storage period, all tested coatings provided lower fruit gloss than uncoated controls (Table 2). This behavior could be related to the macroemulsion character of the coating formulations (*37*).

Table 2. Ranked fruit gloss of 'Ortanique' mandarins coated with hydroxypropyl methylcellulose (HPMC)-lipid edible composite coatings containing food preservatives[a] and mixtures and stored at either 20 ºC for 1 wk or 5 ºC for 8 wks followed by 1 wk at 20º C.

Gloss rank	1 wk 20 ºC		2 wks 5 ºC + 1 wk 20 ºC		4 wks 5 ºC + 1 wk 20 ºC		6 wks 5 ºC + 1 wk 20 ºC		8 wks 5 ºC + 1 wk 20 ºC	
More glossy	Control[b]	a[c]	Control	b	Control	c	Control	c	Control	c
	PS	a	PS	ab	PS	bc	PS	c	PS	bc
	SB	a	PS + SP	ab	PS + SP	abc	PS + SP	bc	PS + SP	bc
	PS + SP	a	SB	a	SB + PS	ab	SB + SP	ab	SB	ab
	SB + PS	a	SB + PS	a	SB + SP	ab	SB	ab	SB + PS	a
Less glossy	SB + SP	a	SB + SP	a	SB	a	SB + PS	a	SB + SP	a

[a] PS = potassium sorbate; SB = sodium benzoate; SP = sodium propionate.
[b] Control = uncoated. [c] Treatments in columns with different letters are significantly different according to Friedman test.

In conclusion, all coatings were effective to reduce the incidence of GM and BM on artificially inoculated 'Ortanique' mandarins after 2 wks of cold storage at 5º C, and the SB-based coating was effective even after 6 wks of cold storage. Moreover, the coatings significantly reduced the severity of GM and BM after 6 wks of storage at 5 ºC. During cold storage, all the coatings, and especially the SB + SP-based coating, were effective as moisture barriers reducing weight loss and maintaining the firmness of coated mandarins. The coatings did modify the internal gas atmosphere of the fruit, but did not generally induce off-flavor and the sensory quality of coated 'Ortanique' mandarins was evaluated as acceptable. However, the coatings did not improve the gloss of coated samples. Therefore, HPMC-lipid edible composite coatings containing organic acid salts and their mixtures could be used as part of an integrated strategy for non-polluting commercial decay control in citrus packinghouses. Further studies should focus on the modification of some physical characteristics of the coatings to improve their gloss and visual aspect and also on their combination with other control methods alternative to chemical synthetic fungicides in order to find synergistic and /or complementary activities.

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CHAPTER 5

Performance of hydroxypropyl methylcellulose (HPMC)-lipid edible composite coatings containing food additives with antifungal properties during cold storage of 'Clemenules' mandarins

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Abstract

The performance of edible composite coatings containing hydroxypropyl methylcellulose (HPMC), hydrophobic components (beeswax and shellac), and food preservatives as antifungal ingredients was evaluated on 'Clemenues' mandarins. Tested preservatives included potassium sorbate (PS), sodium benzoate (SB), sodium propionate (SP), and their mixtures. Intact fruit or fruit artificially inoculated with *Penicillium digitatum* or *Penicillium italicum* were coated and stored up to 30 d at 5 ºC plus 7 d at 20 ºC to simulate retail conditions. During cold storage, all HPMC-lipid coatings containing food preservatives significantly reduced the development of both green (GM) and blue (BM) molds although the performance was better against GM. When the coated fruit were transferred to 20 ºC, all coatings lost effectiveness. SB+PS-based coating was the most effective in reducing disease severity on coated fruit. All the coatings effectively reduced fruit weight loss and maintained rind firmness on coated samples. The coatings did not adversely affect the ethanol content of the juice, sensory flavor, and fruit appearance of coated 'Clemenules' mandarins. Although the internal gas concentration of the coated fruit was modified, the coatings did not reduce off-flavor.

KEYWORDS: citrus, clementines, food additives, green mold, blue mold, quality, *Penicillium digitatum, P. italicum.*

1. Introduction

Edible films and coatings are an environmentally-friendly alternative method to extend the postharvest life of fresh and minimal processed fruits and vegetables (Baldwin, 1994; Debeaufort, Quezada-Gallo & Violley, 1998; Greener & Fennema, 1994; Pérez-Gago, Serra, Alonso, Mateos & del Río, 2005). They form a semi-permeable barrier to gases and water vapor exchange that reduce respiration and weight loss. The use of edible films and coatings has several advantages such as edibility, biodegradability, and aesthetic appearance. Moreover, coatings may

enhance food quality, safety, and stability of produce conferring good eating and sensory properties (Guilbert, 1986).

According to their components, edible films and coatings can be divided into three categories: hydrocolloids (proteins and polysaccharides), lipids, and composites. Emulsifiers and plasticizers are added to coatings to improve their functional and mechanical properties (Bravin, Peressini & Sensidoni, 2004; Kim, Ko & Park, 2002; McClements, 1999). Hydrocolloid-based films present a good barrier to gases, but a poor moisture barrier. On the contrary, lipid films are used as an adequate barrier to water vapor. Composite films comprise hydrocolloid components and lipids, thus enhancing the advantages and lessening the disadvantages of each (Hagenmaier & Baker, 1994; Nisperos-Carriedo, 1994; Olivas, Dávila-Aviña, Salas-Salazar & Molina, 2008).

Chemical fungicides are often added into commercial waxes to control the main citrus postharvest diseases, green (GM) and blue (BM) molds, caused by the pathogens *Penicillium digitatum* (Pers.:Fr.) Sacc (PD) and *Penicillium italicum* Wehmer (PI), respectively (Eckert & Eaks, 1989). However, prolonged and extensive use of chemical fungicides to control citrus postharvest decay has alerted human and environmental concerns. Therefore, methods alternative to conventional chemical fungicides are currently needed worldwide to control these diseases (Palou, Smilanick & Droby, 2008).

Antioxidants, flavors and pigments, vitamins, and antimicrobial agents can be successfully incorporated into edible coatings. In the literature, several works reported on the efficacy of films and coatings containing antimicrobials to control microbial growth on fruits and vegetables. For instance, starch-based coatings with potassium sorbate (PS) reduced microbial growth on strawberries (García, Martino & Zaritzky, 1998). PS added to tapioca starch-glycerol edible films prevented external contamination by *Zygosaccharonyces bailii* on an acidified high water activity semisolid product (Flores, Haedo, Campos & Gerschenson, 2007). In other study, Maizura, Fazilah, Norziah, & Karim, (2007) reported that sago starch-alginate-based films containing lemongrass oil was effective against *Escherichia coli* O157:H7. Lemongrass and oregano oil added to apple puree-alginate edible coatings inhibited the growth of *Listeria inocua* on inoculated apple pieces (Rojas-Graü, Raybaudi-Massilia, Soliva-Fortuny, Avena-Bustillos, McHugh & Martín-

Belloso, 2007). Low molecular weight chitosan coatings exhibited effective antifungal activity against PD and PI on 'Murcott' tangors (Chien, Sheu & Lin, 2007). In previous studies (Valencia-Chamorro, Palou, del Río & Pérez-Gago, 2008), we reported that a wide variety of food additives such as mineral salts, organic acid salts and their mixtures, and sodium salts of parabens and their mixtures, added to stand-alone hydroxypropyl methylcellulose (HPMC)-lipid edible composite films, exhibited antifungal properties against PD and PI. In a subsequent work, these coatings reduced the incidence and severity of GM and BM on 'Clemenules' mandarins, 'Ortanique' hybrid mandarins, and 'Valencia' oranges incubated at 20 ºC to simulate fruit shelf-life (Valencia-Chamorro, Pérez-Gago, del Río & Palou, 2009a). Further studies confirmed the antimicrobial activity and good physiological performance of HPMC-lipid edible composite coatings containing food preservatives during long-term cold-storage of 'Valencia' oranges (Valencia-Chamorro, Pérez-Gago, del Río & Palou, 2009b).

Nevertheless, there is little information focused on the effect of coatings containing food preservatives on the control of postharvest penicillium molds on different citrus cultivars and the impact of these coatings on the postharvest quality of fresh fruit during cold storage. In general, the activity of these coatings and other low-toxicity or non-contaminant antifungal methods alternative to chemical fungicides is cultivar-dependent. Therefore, the objective of this work was to study the effect of new edible composite coatings prepared with HPMC-lipid containing food additives with antifungal properties on the development of GM and BM and the physico-chemical, and sensory quality of 'Clemenules' mandarins during long-term cold storage.

2. Materials and methods

2.1. *Materials*

HPMC (Methocel E15) was purchased from Dow Chemical Co. (Midland, MI, USA). Shellac and beeswax (BW) (grade 1) were supplied by Fomesa Fruitech, S.L. (Beniparrell, València, Spain). Stearic acid and glycerol were from Panreac Química, S.A (Barcelona, Spain). Silicone antifoam (FG-1510) and ammonia (25 %) were from Dow Corning Ibérica S.A. (Barcelona, Spain) and Scharlau Chemie S. A. (Sentmenat,

Spain), respectively. Food preservatives were purchased from Sigma (Sigma-Aldrich Chemie, Steinheim, Germany) and included the salts of organic acids, PS, SB, and sodium propionate (SP). These chemicals are all classified as food additives or generally recognized as safe (GRAS) compounds by the European Union and the United States regulations.

2.2. *Emulsions preparation*

HPMC-lipid edible composite emulsions were prepared by combining the hydrophilic phase (HPMC) and the hydrophobic phase (BW and shellac) suspended in water. Glycerol and stearic acid were used as plasticizer and emulsifier, respectively. Ratios of HPMC-glycerol (2:1) (dry basis, db) and lipid components (BW/shellac)-stearic acid (5:1) (db) were kept constant throughout the study. BW and shellac content was 50 % (db) at a ratio BW:shellac of 1:1. Emulsions were prepared as described previously by Valencia-Chamorro et al. (2008). Briefly, an aqueous solution of HPMC (5 % w/w) was prepared. Food preservative (w/w), BW, glycerol, stearic acid, water, and two drops of antifoam were added to the HPMC solution and heated at 90 ºC to melt the lipids. Shellac was previously dispersed in water at 40 ºC and ammonia (15 % w/w shellac/ammonia) was added to dissolve the resin. Shellac solution was heated separately at 90 ºC and added to the HPMC dispersion. Samples were homogenized with a high-shear probe mixer (Ultra-Turrax model T25, IKA-Werke, Steufen, Germany) for 4 min at 22,000 rpm. Emulsions were cooled to less than 25 ºC and further agitated for 25 min. Emulsions were kept 2-3 d at 5 ºC before use. Table 1 shows the total solid content of the formulations and the concentration of each food preservative (%, wet basis, wb). The formulations were selected from HPMC-lipid edible composite coatings that were effective inhibiting the development of GM and BM on citrus fruit incubated at 20 ºC (Valencia-Chamorro et al., 2009). These formulations were stable and no phase separation was observed.

Table 1. Characteristics of hydroxypropyl methylcellulose (HPMC)-lipid edible composite coatings containing food preservatives and mixtures.

Food preservative [x]	Food preservative concentration (%, wb)[y]	Solid concentration (%, wb)	Viscosity (cp)[z]	pH
Organic acid salts				
PS	2.0	6	16.0	7.4
SB	2.5	8	13.2	7.6
Mixtures				
PS+SP	1.5 + 0.5	6	13.5	7.5
SB+PS	2.0 + 0.5	8	12.2	7.5
SB+SP	2.0 + 0.5	8	16.3	7.5

[x] PS = potassium sorbate; SB = sodium benzoate; SP = sodium propionate.
[y] wb = wet basis. [z] cp = centipoises.

2.3. *Effect of coatings on disease development*

2.3.1. *Fungal inoculum*

P. digitatum isolate NAV-7 and *P. italicum* isolate MAV-1, obtained from decayed citrus fruit from packinghouses in the València area (Spain), were isolated, identified, and maintained in the IVIA culture collection of postharvest pathogens. These strains were selected for their aggressiveness on the most commercially important mandarin and orange cultivars. Prior to each experiment, the isolates were grown on potato dextrose agar (Sigma) in petri dishes at 25±1 ºC for 7–10 d. A high-density conidial suspension was prepared in Tween 80 (0.05 %, w/v; Panreac Química S.A.) in sterile water, passed through two layers of cheesecloth, measured with a haemacytometer and diluted with sterile water to achieve the desired inoculum density.

2.3.2. *Fruit inoculation and coating application*

Clementine mandarins (*Citrus reticulata* Blanco) cv. 'Clemenules' from commercial orchards in València (Spain) were selected by hand and used in the experiments before any postharvest treatment was applied. The fruit were stored up to one week at 5 ºC and 90 % relative humidity (RH) before use. Before each experiment, the fruit were randomized, washed with fresh water, and allowed to air-dry at room temperature.

Fruit were artificially inoculated (inoculum density of 10^5 spores/mL) by immersing a stainless steel rod with a probe tip 1 mm wide and 2 mm in length into the spore suspension and wounding each fruit once on the equator. Fruit was inoculated with PD or PI, on the equator of each mandarin, incubated 24 h at 20 ºC, coated manually by immersion (about 15 s at 20 ºC) with the HPMC-lipid edible composite coatings (Table 1), drained, and dried in a tunnel at 45±2 ºC for 130 s. Inoculated but uncoated fruit were used as controls. Each treatment was applied to three replicates of 20 fruits each. Fruit were placed on plastic trays on corrugated cartons and stored for 30 d at 5 ºC and 90-95 % RH, followed by 7 d at 20 ºC and 90 % RH to simulate retail handling conditions.

2.3.3. *Determination of disease incidence and severity*

Disease incidence of GM and BM was calculated as the percentage of decayed fruit. Disease severity was determined as the diameter of the lesion (mm). Incidence and severity were assessed every 15 d during the storage period at 5 ºC and also after a shelf-life period of 7 d at 20 ºC following cold storage.

2.4. *Effect of coating on fruit quality*

2.4.1. *Fruit coating and storage*

For the quality study, fruit were washed with water containing dodecil benzene sulphonate sodium salt (4 % w/v) applied as a foam cascade in an experimental packingline, rinsed with tap water, and allowed to dry in a tunnel at 45±2 ºC during 130 s. Fruit were divided into six groups of 90 fruit each, which corresponded to the five coating treatments described in Table 1 and one control (uncoated fruit). The mandarins were coated as described above, drained of excess coating, dried in the tunnel at 45±2 ºC for 130 s, and stored either at 20 ºC for 7 d or at 5 ºC and 90-95 % RH for 30 d followed by 7 d at 20 ºC to simulate retail handling conditions. Physico-chemical and sensory fruit quality was assessed every 7 d at 5 ºC plus a shelf life period of 7 d at 20 ºC.

2.4.2. *Assessment of fruit quality*

2.4.2.1. *Weight loss.* Lots of 30 fruit per treatment were used to measure weight loss. The same marked mandarins were weighted at the beginning and at the end of each storage period. The results were expressed as the percentage of initial weight lost.

2.4.2.2. *Fruit firmness.* Firmness of 20 fruit per treatment was determined at the end of each storage period using an Instron Universal testing machine (Model 3343, Instron Corp., Canton, MA, USA). Each fruit was compressed between two flat surfaces closing together at the rate of 5 mm/min. The machine gave the deformation (mm) after application of a load of 10 N to the equatorial region of the fruit. Results were expressed as percentage of deformation, related to initial diameter.

2.4.2.3. *Internal gas concentration.* Gas sampling from the fruit were performed by withdrawing 1 mL internal gas from the mandarin cavity with a syringe. To avoid external gas contamination samples were submerged under water. The gas sample was injected into a gas chromatograph (GC) (Thermo Trace, Thermo Fisher Scientific, Inc. Waltham, MA, USA) equipped with a thermal conductivity detector (TCD) and fitted with a Poropack QS 80/100 column (1.2 m x 0.32 cm i.d.). Temperatures were 35, 115, and 150 ºC, respectively for the oven, injector, and thermal conductivity detector. Helium was used, as carrier gas at a flow rate of 22 mL/min. The O_2 and CO_2 concentration was calculated using peak area obtained from standard gas mixtures of 15.0:2.5 % O_2:CO_2. Results were expressed as percentages. Ten fruit per treatment were analyzed.

2.4.2.4. *Ethanol and acetaldehyde contents.* Ethanol and acetaldehyde were analysed from the head-space of juice from samples using a GC (Thermo Trace, Thermo Fisher Scientific) equipped with an auto-sampler (Model HS 2000), flame ionization detector (FID), and 1.2 m x 0.32 cm (i.d.)

Poropack QS 80/100 column. The injector was set at 175 ºC, the column at 150 ºC, the detector at 200 ºC, and the carrier gas at 28 mL/min. A composite juice of three replicates of ten fruit per treatment was analyzed. Five mL of juice were transferred to 10-mL vials with crimp-top caps and TFE/silicone septum seals. Samples were frozen and stored at –18 ºC until analyses. A 1-mL sample of the headspace was withdrawn from vials previously equilibrated in a water bath at 20 ºC for 1 h, followed by 15 min at 40 ºC, to reach equilibrium in the headspace, and then injected into the GC. Ethanol and acetaldehyde was identified by comparison of retention times with standards. Results were expressed as mg of gas per 1 L of juice.

2.4.2.5. *Sensory evaluation.* Sensory quality of treated samples was evaluated by 10 to 12 trained judges at the end of each storage period. Judges rated flavor on a 9-point scale where 1 = very poor and 9 = optimum. Each judge was given samples from each batch and requested to evaluated off-flavor on a 5-point scale where 0 = absence of off-flavor and 5 = high presence of off-flavor. Four fruit per treatment were pealed and separated into individual segments. Two segments from two different fruit were presented to judges in trays labeled with 3-digit random codes and served to them at room temperature. The judges had to taste several segments of each sample in order to compensate, as far as possible, for biological variation of the material. Spring water was provided for palate rinsing between samples. External aspect of treated fruit (coating cracks, spots, etc.) was also evaluated by the panelists. A 3-point scale was used in which the aspect was classified as 1 = bad, 2 = acceptable, and 3 = good. Panelists were also asked to rank visually the treatments from highest to lowest gloss.

2.5. *Statistical analysis*

Statistical analysis was performed using Statgraphics 5.1. (Manugistics Inc., Rockville, MD, USA). Specific differences between means were determined by Fisher's protected least significant difference test (LSD, $P < 0.05$) applied after an analysis of variance (ANOVA). For sensory gloss, specific differences were determined by Friedman test, which is recommended for ranking by the UNE 87023 (AENOR, 1997). For

disease incidence data, the ANOVA was applied to the arcsine of the square root of the proportion of decayed fruit.

3. Results and discussion

3.1. *Effect of coatings on disease development*

'Clemenules' mandarins were coated with five HPMC-lipid edible composite coatings containing organic acid salts, alone or in mixtures. After 15 d of cold storage at 5 ºC, all the coatings greatly reduced disease incidence of GM (range 70-94 %) and BM (range 54-85 %) on coated 'Clemenules' mandarins compared to control samples ($P < 0.05$) (Figure 1). However, after 30 d of cold storage a fast incidence increase of both molds was observed and some of the coatings lost effectiveness. Among all coatings, SB- and SB+PS-based coatings were the most effective to reduce the incidence of GM on mandarins. The severity reduction of GM (range 37-66 %) was higher than that of BM (range 26-37 %). Moreover, all the coatings were very effective to inhibit the sporulation of GM and BM after 30 d of cold storage (data not shown). When coated samples were transferred from 5 to 20 ºC after 30 d of storage, disease incidence and severity of GM and BM increased, and the incidence of both molds were similar to than on uncoated controls. However, the severity reduction of BM was significantly lower on all coated samples than on the uncoated control. It is know that during storage at temperatures lower than 10 ºC, *P. italicum* grows more rapidly than *P. digitatum* (Eckert et al., 1989) and this reason might account for the lower effectiveness of the coatings to control BM than GM during cold storage at 5 ºC.

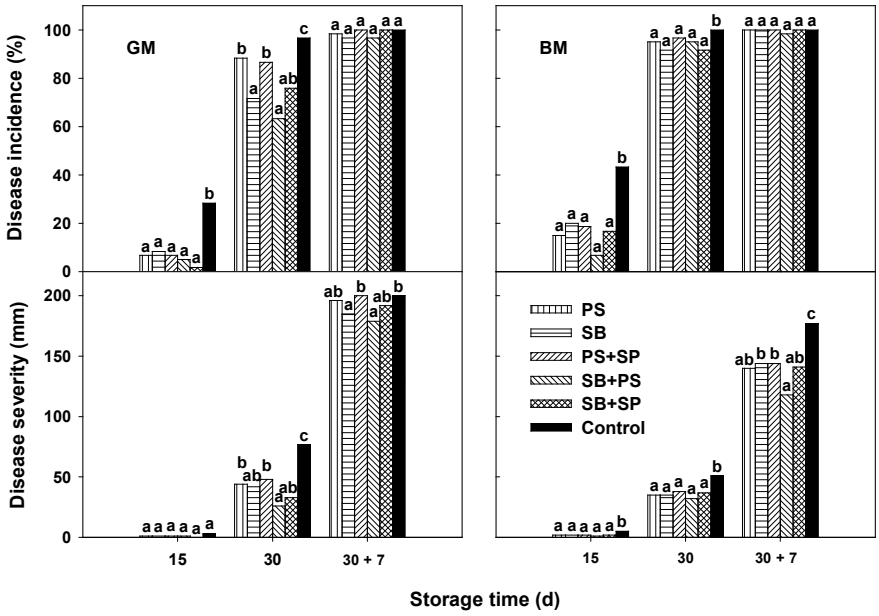

Fig. 1. Disease incidence and severity of green (GM) and blue (BM) molds, on 'Clemenules' mandarins artificially inoculated with *Penicillium digitatum* and *Penicillium italicum*, uncoated (Control), or coated 24 h later with hydroxypropyl methylcellulose (HPMC)-based coatings containing potassium sorbate (PS), sodium benzoate (SB), sodium propionate (SP) or their mixtures, and stored up to 30 d at 5 ºC followed by 7 d at 20 ºC (30+7). For each storage period, columns with different letters are significantly different by Fisher's protected LSD test ($P < 0.05$) applied after an ANOVA. For disease incidence, the ANOVA was applied to arcsine-transformed values. Non-transformed means are shown.

Films containing 23 food preservatives, including mineral salts, organic acid salts and their mixtures, parabens and their mixtures, and other compounds were tested *in vitro* against PD and PI using the disk diameter test (Valencia-Chamorro et al., 2008). Among those films containing organic acid salts, only PS- and SB- based films were effective for the control of both PD and PI. Those findings were further confirmed in an *in vivo* study with artificially inoculated citrus fruit incubated at 20 ºC. Coatings containing PS or SB, and their mixtures

were the most effective to reduce the development of GM and BM on oranges and mandarins (Valencia-Chamorro et al., 2009a). In the present study, it was confirmed that SB and SB+PS were the best antifungal compounds to be added to edible HPMC-lipid coatings for disease reduction on 'Clemenules' mandarins. It has been reported in previous studies that the effect of HPMC-lipid-based coatings containing organic acid salts or mixtures on mold growth and development is dependent on the citrus species and cultivar (Valencia-Chamorro et al., 2009a). Compared to previous studies with 'Valencia' oranges, the antifungal edible coatings were less effective and persistent on 'Clemenules' mandarins than on 'Valencia' oranges.

The effects of the coatings containing food preservatives on mold incidence and development may be related to either differences in their composition and/or physical properties (Waks, Schiffmann-Nadel, Lomaniec & Chalutz, 1985), differences in the release ability of the food preservative from the coating (Chung, Papadakis & Yam, 2001), or differences on the type of citrus fruit (Valencia-Chamorro et al., 2009a). It can be assumed that the relative fruit susceptibility to infections by PD or PI may strongly influence the performance of the coatings. We observed in other research that brief immersions in aqueous solutions of antifungal food preservatives such as sodium carbonate or bicarbonate were more effective to control penicillium molds on oranges than on mandarins, mainly due to the lower susceptibility of the oranges to decay (Palou, Smilanick, Usall & Viñas, 2001; Palou, Usall, Muñoz, Smilanick & Viñas, 2002). Moreover, similar performance differences with different fruit hosts have been reported in studies with most of the alternative antifungal treatments, which mode of action to control GM and BM is rather fungistatic than fungicidal (Palou et al., 2008).

In the current citrus trading commercial context, methods alternative to conventional fungicides are needed worldwide to control citrus penicillium molds (Palou et al., 2008). Therefore, these HPMC-lipid edible composite coating containing organic acid salts and their mixtures could be used as another commercial tool for decay control in citrus packinghouses, especially in combination with other postharvest treatments that provide complementary activity.

3.2. Effect of coating on fruit quality

3.2.1. *Weight loss*

Figure 2 shows the weight loss of coated and uncoated 'Clemenules' mandarins stored at either 20 ºC for 7 d or 5 ºC for 30 d followed by 7 d at 20 ºC. After all storage periods, weight loss on coated samples was significantly lower ($P < 0.05$) than on uncoated controls, which indicates the effectiveness of these coatings as moisture barriers. Nevertheless, weight loss increased as storage time increased. At the end of the storage period, the weight loss reduction on coated samples was up to 28 % as compared to uncoated control samples. Similar results on weight loss during long-term cold storage of citrus fruit were found on 'Fortune' and 'Ortanique' mandarins coated with HPMC-BW coatings after 37 d (30 d at 9 ºC plus 7 d 20 ºC) and 52 d (45 d at 5 ºC plus 7 d at 20 ºC), respectively (Pérez-Gago, Rojas & del Río, 2002; Navarro-Tarazaga, del Río, Krochta & Pérez-Gago, 2008).

Among all treatments, SB+PS- and SB+SP-based coatings were the most effective moisture barriers during prolonged cold storage, reducing weight loss on the range of 25-35 % compared to the control. These results obtained with coated 'Clemenules' mandarins correlated well with the low water vapor permeability (WVP) of stand-alone films containing the same organic acid salts mixtures (Valencia-Chamorro et al., 2008). Even though those films exhibited low flexibility due to their stiffness and brittleness, the coatings containing those mixtures presented a good ability to adapt to fruit surface changes as the coated fruit were losing weight and volume during cold storage.

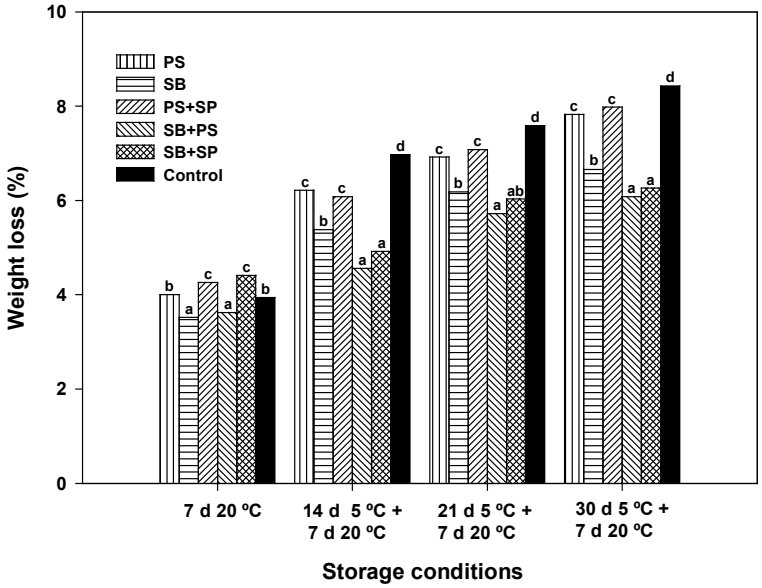

Figure 2. Weight loss of 'Clemenules' mandarins uncoated (Control) or coated with hydroxypropyl methylcellulose (HPMC)-based coatings containing potassium sorbate (PS), sodium benzoate (SB), sodium propionate (SP) or their mixtures, and stored at either 20 ºC for 7 d or 5 ºC for up to 30 d followed by 7 d at 20 ºC. For each storage period, columns with different letters are different by Fisher's protected LSD test ($P < 0.05$) applied after an ANOVA.

3.2.2. *Fruit firmness*

The applied coatings did not influence the fruit firmness after 7 d of storage at 20 ºC ($P < 0.05$) (Figure 3). After 15 and 21 d of cold storage plus 7 d at 20 ºC, all coatings, except the PS+SP-based coating, were effective in reducing the firmness loss of coated 'Clemenules' mandarins. The SB- and SB+SP-based coatings reduced the firmness loss after 30 d at 5 ºC plus 7 d at 20 ºC ($P < 0.05$), which could be related to the lower weight loss observed in these samples (Figure 2).

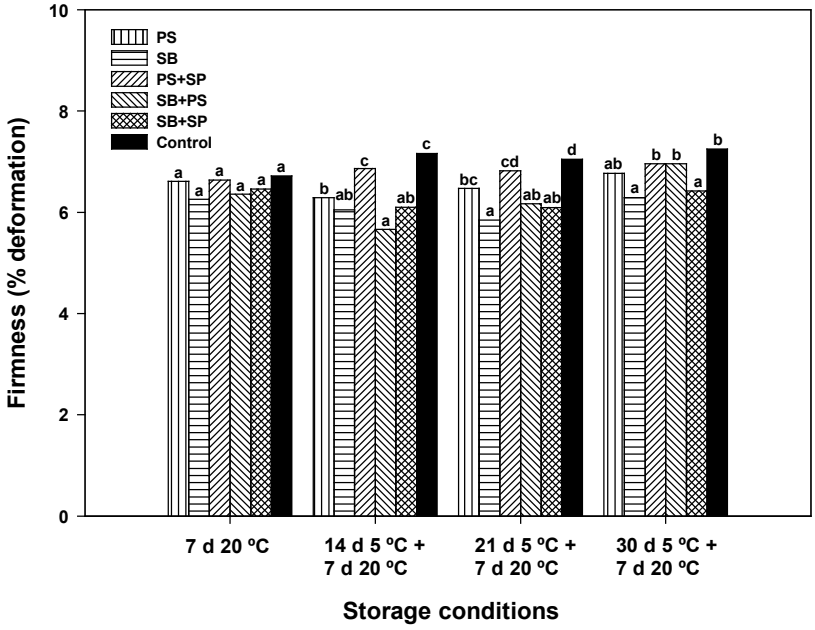

Figure 3. Firmness of 'Clemenules' mandarins uncoated (Control) or coated with hydroxypropyl methylcellulose (HPMC)-based coatings containing potassium sorbate (PS), sodium benzoate (SB), sodium propionate (SP) or their mixtures, and stored at either 20 ºC for 7 d or 5 ºC for up to 30 d followed by 7 d at 20 ºC. For each storage period, columns with different letters are different by Fisher's protected LSD test ($P < 0.05$) applied after an ANOVA.

The effect of the coatings on the maintenance of fruit firmness is usually related to their control of weight loss. Contradictory results have been reported on the correlation between weight loss and firmness on coated citrus fruit. For instance, while a positive correlation was reported by Navarro-Tarazaga, del Río, Krochta & Pérez-Gago, (2008) on 'Ortanique' mandarins coated with HPMC-BW coatings, no correlation was observed in the studies by Hagenmaier (2000) on 'Valencia' oranges coated with polyethylene-candelilla coatings, Pérez-Gago et al. (2002) on 'Fortune' mandarins coated with HPMC-lipid coatings, or Valencia-Chamorro et al. (2009b) on 'Valencia' oranges coated with HPMC-lipid coatings containing food preservatives. In this work, the SB- and SB+SP-based coatings were effective to control both fruit weight (Figure 2) and texture loss (Figure 3). However, after 30 d of storage at 5 ºC plus

7 d at 20 ºC, the weight loss of SB+PS-based coated mandarins did not correlate to firmness loss. Apparently, factors such as coating type, storage condition, or fruit cultivar significantly influence the fruit firmness of coated samples. Moreover, it seems that for the coatings to affect fruit firmness significantly, they should induce a clear effect in fruit weight loss.

3.2.3. *Internal gas concentration*

Figure 4 shows the internal gas concentration of coated and uncoated 'Clemenules' mandarins. HPMC-lipid based coatings containing food preservatives significantly modified the internal gas concentration on coated fruit, which indicates that the coatings provided an additional gas barrier to CO_2 and O_2. In general, internal CO_2 and O_2 concentration on coated mandarins were within the range 3-6 and 13-17 kPa, respectively. These levels of internal O_2 are not low enough to create anaerobic conditions inside the coated fruit (Baldwin, Nisperos, Hagenmaier & Baker, 1997). The total solid content of HPMC-lipid coating emulsions (either 6 or 8 %, Table 1) did not influence the gas permeability of the coated samples.

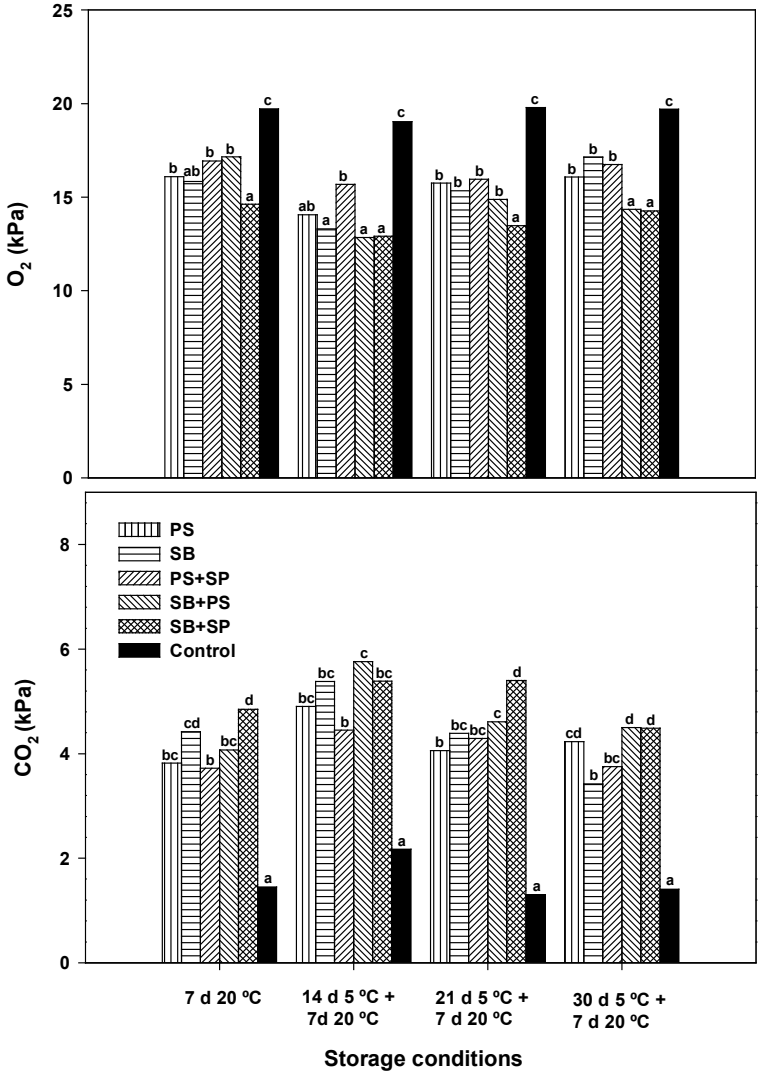

Figure 4. Internal gas concentration of 'Clemenules' uncoated (Control) or coated with hydroxypropyl methylcellulose (HPMC)-based coatings containing potassium sorbate (PS), sodium benzoate (SB), sodium propionate (SP) or their mixtures, and stored at either 20 ºC for 7 d or 5 ºC for up to 30 d followed by 7 d at 20 ºC. For each storage period, columns with different letters are different by Fisher's protected LSD test ($P < 0.05$) applied after an ANOVA.

In spite of differences associated with citrus variety, these values are comparable to those obtained with 'Valencia' oranges coated with the same HPMC-lipid antifungal edible composite coatings and stored for 60 d at 5ºC plus 7 d at 20 ºC (Valencia-Chamorro et al., 2009b). Similar values of internal CO_2 (around 8 kPa) were also reported by Pérez-Gago et al. (2002) on HPMC:lipid-coated 'Fortune' mandarins after 30 d of storage at 9 ºC, Peeples, Albrigo, Pao & Petracek (1999) on shellac-based coated 'Valencia' oranges after 70 d at 4 ºC, and Baldwin, Nisperos-Carriedo, Shaw & Burns (1995) on shellac-coated 'Valencia' oranges after 14 d at 21 ºC. However, higher CO_2 values (about 18 kPa) were reported by Navarro-Tarazaga, Pérez-Gago, Goodner & Plotto (2007) on HPMC-BW-Shellac-coated 'Valencia' oranges after 15 d of storage at 24 ºC.

Oxygen permeability (OP) of HPMC-lipid-stand-alone films containing organic cid salts as antifungal ingredients with the same emulsion composition than the coatings tested in the present work were determined by Valencia-Chamorro et al. (2008). Films containing only one food preservative presented lower OP values than films containing a mixture of them. Thus, lower internal O_2 concentration was expected on fruit coated with PS- or SB-based coatings than on fruit treated with coatings containing a mixture these organic acid salts. In the present work, however, SB+PS- and SB+SP-based coatings produced the highest O_2 barrier on coated 'Clemenules' mandarins. Contradictory results were also reported for stand-alone films and coatings on citrus fruit coated with hydrocolloids-wax (Chen & Nussinovitch, 2001), 'Valencia' oranges coated with HPMC-lipid containing food preservatives (Valencia-Chamorro et al., 2009b) or 'Ortanique' mandarins coated with HPMC-BW coatings (Navarro-Tarazaga et al., 2008). Those differences were attributed to factors such as fruit peel morphology or physical properties of the coating formulation. These factors could influence coating flexibility or its ability to adapt to the fruit surface. The coatings restrict the air exchange of the fruit depending not only on how the coating is distributed over the surface of the fruit to form a continuous layer, but also on the coating ability to plug the openings in the peel (Hagenmaier & Baker, 1993; Mannheim & Soffer, 1996). Therefore, coatings that result appropriate for one cultivar may no to be so suitable for another (Park, 1999; Valencia-Chamorro et al., 2009a).

3.2.4. *Ethanol and acetaldehyde content*

The ethanol concentration in the juice of coated and uncoated 'Clemenules' mandarins after the different storage periods is shown in Figure 5. After 7 d of storage at 20 ºC, no differences were found between coated and uncoated samples. However, during cold storage at 5 ºC, higher ethanol content was found in the juice of coated samples than in the juice of uncoated ones ($P < 0.05$). These results confirmed the creation of a modified atmosphere within the fruit. However, this internal atmosphere did not result in a clear response with respect to the ethanol content in coated mandarins. The highest ethanol concentration, around 700 mg L^{-1}, was found in fruit treated with SB+SP-based coating after 30 d at 5 ºC plus 7 d at 20 ºC, while the content in uncoated samples was 120 mg L^{-1}. In this study, the ethanol content in 'Clemenules' mandarins after 30 d at 5 ºC plus 7 d at 20 ºC was lower than the level of 1240 mg L^{-1} found in 'Valencia' oranges coated with HPMC-lipid containing the same food preservatives (Valencia-Chamorro et al., 2009b). Other authors also reported higher amount of ethanol content in coated mandarins. For instance, Pérez-Gago et al. (2002) reported that the ethanol content was within the range 3000-4000 mg L^{-1} in HPMC-lipid-coated 'Fortune' mandarins after 30 d at 9 ºC plus 7 d at 20 ºC and Navarro-Tarazaga et al. (2008) found that the ethanol content was higher than 4000 mg L^{-1} in HPMC-BW-based coated 'Ortanique' mandarins after 21 d at 5 ºC plus 7 d at 20 ºC. In this study, the ethanol concentration in the juice of coated fruit was lower than 2000 mg L^{-1}, which has been established as the minimum ethanol content associate with off-flavor in citrus (Ke & Kader, 1990). The total solid content of HPMC-lipid coating, either 6 or 8 % (Table 1), did not influence the gas permeability of the coated samples and, therefore, no differences were found in ethanol content. On the other hand, the acetaldehyde content in both coated and uncoated samples was lower than 9 mg L^{-1}, and significant differences were found among coated samples after all storage periods. Coated samples presented higher acetaldehyde values than uncoated control (data not shown).

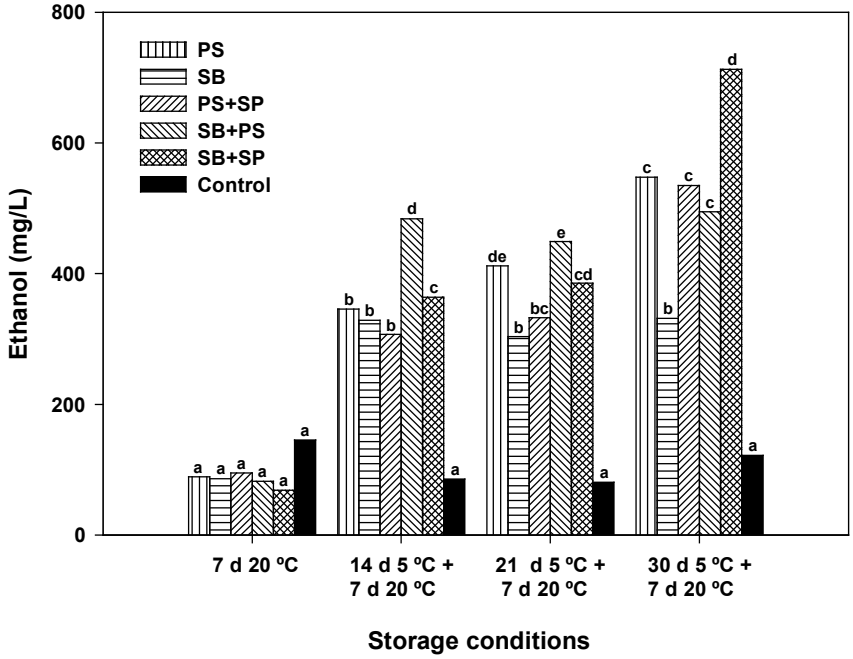

Figure 5. Ethanol content in juice of 'Clemenules' mandarins uncoated (Control) or coated with hydroxypropyl methylcellulose (HPMC)-based coatings containing potassium sorbate (PS), sodium benzoate (SB), sodium propionate (SP) or their mixtures, and stored at either 20 ºC for 7 d or 5 ºC for up to 30 d followed by 7 d at 20 ºC. For each storage period, columns with different letters are different by Fisher's protected LSD test ($P < 0.05$) applied after an ANOVA.

3.2.5. *Sensory evaluation*

HPMC-lipid based coatings containing food preservatives slightly modified the flavor of coated 'Clemenules' mandarins during the storage time (Figure 6). At the end of the storage period, after 30 d at 5 ºC + 7 d at 20 ºC of shelf life, flavor scores were close to 6.0 points, except for those samples coated with SB+SP-based coatings (flavor score of 4.3 points). However, the flavor was considered as acceptable for all treatments. The flavor of uncoated mandarins was scored with 5.8

points. Off-flavor in citrus fruit has been associated with an ethanol concentration in the juice higher than 2000 mg/L (Ke and Kader, 1990). In this study, ethanol values in all cold-stored coated samples were lower than this value. Although the panellists detected a slight off-flavor after 30 d at 5 ºC + 7 d at 20 ºC, they observed no differences between coated and uncoated samples, which indicated that the coatings did not induce off-flavor. At the end of the storage period, off-flavor on coated 'Clemenules' mandarins was lower than that reported by Navarro-Tarazaga (2007) on 'Marisol' tangerines coated with HPMC:BW:shellac composite coating after 21 d at 23 ºC.

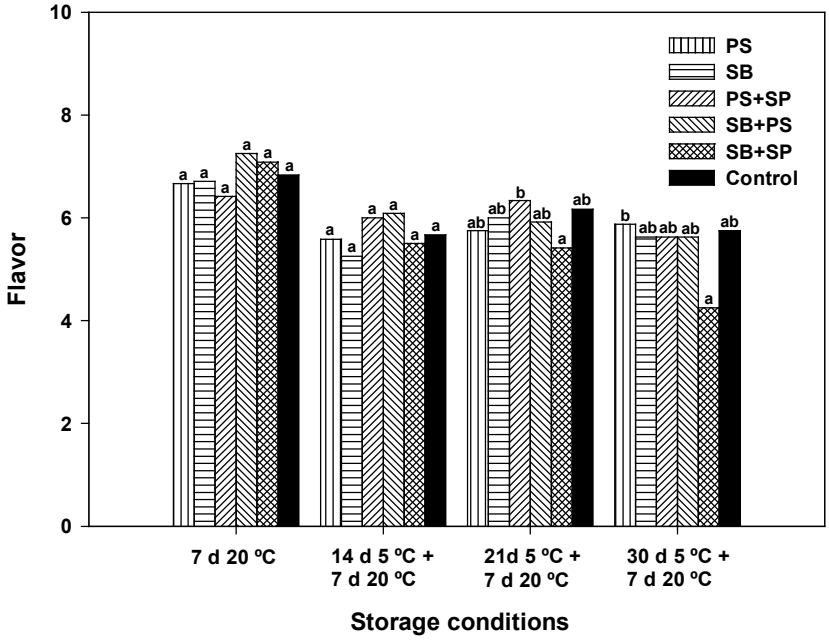

Figure 6. Flavor of 'Clemenules' mandarins uncoated (Control) or coated with hydroxypropyl methylcellulose (HPMC)-based coatings containing potassium sorbate (PS), sodium benzoate (SB), sodium propionate (SP) or their mixtures, and stored at either 20 ºC for 7 d or 5 ºC for up to 30 d followed by 7 d at 20 ºC. For each storage period, columns with different letters are different by Fisher's protected LSD test ($P < 0.05$) applied after an ANOVA.

In general, the coating appearance on coated samples was evaluated as acceptable after all storage periods, except for the SB+SP-based coating, which was assigned the lowest value and was evaluated as showing a bad appearance after 30 d at 5 ºC plus 7 d at 20 ºC (Table 2). The quality of the coating appearance on all coated samples decreased with storage time. Some coated fruit presented small white spots that reduced the general good appearance of the samples. The addition of food preservatives to HPMC-lipid emulsion resulted in stable emulsions, but with a milky appearance due to the formation of a macroemulsion as the lipids were dispersed in the aqueous phase (Hagenmaier, 1998). After either 7 d at 20 ºC or any cold storage period, SB+SP-based coatings were significantly less glossy than the uncoated controls (Table 3). In a similar way as for coatings appearance, this behavior could be related to the milky appearance due to macroemulsion formation of the coatings (Hagenmaier et al., 1994).

Table 2. Sensory evaluation of the coating appearance[x] of 'Clemenules' mandarins coated with hydroxypropyl methylcellulose (HPMC)-lipid edible composite coatings containing antifungal food preservatives and mixtures and stored at either 20 ºC for 7 d or 5 ºC followed by 7 d of shelf life at 20 ºC.

Food preservatives[y]	7 d 20 ºC	14 d 5 ºC + 7 d 20 ºC	21 d 5 ºC + 7 d 20 ºC	30 d 5 ºC + 7 d 20 ºC
PS	2.3 b[z]	2.8 c	2.0 b	2.3 c
SB	2.3 b	1.8 ad	2.0 b	1.5 b
PS+SP	2.2 b	2.0 b	1.8 ab	2.0 c
SB+PS	1.6 a	1.8 ab	1.7 ab	1.9 bc
SB+SP	1.5 a	1.5 a	1.4 a	1.0 a
Control	3.0 c	3.0 c	2.8 c	3.0 d

[x] Coating appearance ranked 1 (bad) to 3 (good). [y] PS = potassium sorbate; SB = sodium benzoate; SP = sodium propionate; Control = uncoated. [z] Means in columns with different letters are significantly different according to Fisher's protected LSD test ($P < 0.05$) applied after an ANOVA.

Table 3. Ranked fruit gloss of 'Clemenules' mandarins coated with hydroxypropyl methylcellulose (HPMC)-lipid edible composite coatings containing food preservatives[x] and mixtures and stored at either 20 ºC for 7 d or 5 ºC followed by 7 d of shelf life at 20 ºC.

Gloss rank	7 d 20 ºC		14 d 5 ºC + 7 d 20 ºC		21 d 5 ºC + 7 d 20 ºC		30 d 5 ºC + 7 d 20 ºC	
More glossy	SB	b	Control[y]	c[z]	Control	c	Control	b
	PS+SP	b	PS	bc	PS	bc	SB	b
	Control	b	SB	ab	PS+SP	abc	PS	ab
	PS	b	SB+SP	ab	SB	ab	PS+SP	ab
	SB+PS	ab	PS+ P	a	SB+PS	ab	SB+PS	a
Less glossy	SB+SP	a	SB+PS	a	SB+SP	a	SB+SP	a

[x] PS = potassium sorbate; SB = sodium benzoate; SP = sodium propionate, [y] Control = uncoated. [z] Treatments within each storage period with different letters are significantly different according to Friedman test.

4. Conclusion

In conclusion, all coatings were effective to reduce the development of GM and BM on artificially inoculated 'Clemenules' mandarins after 15 d of cold storage at 5 ºC. After 30 d of cold storage, the disease incidence of GM and BM on coated samples increased. The disease severity of GM and BM was effectively reduced after 30 d of cold storage. The coatings reduced fruit weight loss, modified the internal fruit atmosphere and did not induce anaerobic conditions that produce off-flavor. Overall, the sensory quality of coated 'Clemenules' mandarins was evaluated as acceptable, but the coatings did not improve fruit gloss. Further research should focus on the modification of physical characteristics of the HPMC-lipid edible composite coatings to improve their gloss and visual aspect. HPMC-lipid edible composite coatings containing organic acid salts and their mixtures could also be applied to other commercially important citrus species and cultivars to evaluate the performance of these antifungal coatings. Moreover, new research should focus on the evaluation of these antifungal coatings in combination with other alternative methods as part of an integrated strategy for non-polluting commercial control of GM and BM in citrus packinghouses.

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CHAPTER 6

Efecto del recubrimiento con quitosano en el control de las podredumbres verde y azul de los cítricos

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RESUMEN

Se evaluó el control de las podredumbres verde y azul, causadas por *Penicillium digitatum* (PD) y *Penicillium italicum* (PI) respectivamente, en naranjas cv. Valencia Late y mandarinas cv. Oronules recubiertas con quitosano comercial (Biorend®) a las concentraciones de 0,66% (C1) y 1,88% (C2). Los frutos recubiertos se mantuvieron a 20ºC durante 1, 3 ó 7 días para naranjas, ó 3 días para mandarinas, se inocularon artificialmente con 10^4 esporas/mL de PD y PI, y se almacenaron a 20ºC ó a 5ºC. La incidencia y severidad (diámetro de la lesión) de las enfermedades se determinaron a los 4 y 7 días de almacenamiento a 20ºC, y a los 15 y 30 días de conservación a 5ºC y después de 7 días de simulación de comercialización a 20ºC. Frutos inoculados sin recubrir se utilizaron como control. En naranjas almacenadas a 20ºC, la incidencia y la severidad de PI fueron menores que lo de PD. En frutos inoculados a los 3 días de aplicar el recubrimiento, el quitosano C2 redujo la incidencia de PI un 75% y 60% a los 4 y 7 días de almacenamiento a 20ºC, respectivamente, y los diámetros de la lesión aunque fueron menores que en el control, no presentaron diferencias significativas. En los frutos inoculados tras 1 y 7 días de la aplicación del recubrimiento no se encontraron diferencias significativas entre los tratamientos con quitosano y el control en la incidencia y severidad de PI en las naranjas almacenadas a 20ºC. Los recubrimientos con quitosano no controlaron significativamente la incidencia y severidad de PD en naranjas durante el almacenamiento a 20ºC, y a los 15 días de conservación a 5ºC. En naranjas, a los 30 días de conservación a 5ºC la incidencia de PD fue superior al 60%, sin existir diferencia significativa entre tratamientos. Al cambiar la fruta de 5ºC a 20ºC, todos los tratamientos presentaron una incidencia de PD y PI mayor del 70% y 90% respectivamente con independencia del tiempo transcurrido entre aplicación del recubrimiento y la inoculación. La incidencia y el diámetro de lesión de PI fueron menores cuanto menor fue el tiempo entre la aplicación del recubrimiento y la inoculación. Cuando este tiempo fue de 3 días, la aplicación del recubrimiento C2 redujo la incidencia de PI en un 19%, mientras que no se encontraron diferencias significativas entre C1 y el control. En mandarinas, tanto a los 15 días como a los 30 días de conservación a 5ºC no existieron diferencias significativas entre tratamientos para la incidencia y la severidad de PD y PI. Los resultados

indican que la efectividad del recubrimiento depende del cultivar, del inóculo y de las condiciones de almacenamiento.

PALABRAS CLAVE: quitosano, recubrimientos, *Penicillium digitatum, Penicillium italicum,* cítricos

INTRODUCCIÓN

Los cítricos son los cultivos de mayor importancia económica en España y unos de los más importantes en el ámbito mundial. En la poscosecha de cítricos, las principales enfermedades son causadas por los hongos *Penicillium digitatum* (PD) y *Penicillium italicum* (PI), los cuales producen las podredumbres verde y azul respectivamente (Tuset, 1987). Los fungicidas químicos de síntesis como el imazalil, ortofenil-fenato de sodio y tiabendazol han sido ampliamente aplicados para controlar estas enfermedades (Eckert y Eacks, 1989). Para el control de estas enfermedades sin el uso de fungicidas químicos se ha ensayado la utilización de diferentes métodos como la conservación frigorífica en atmósferas controladas convencionales, hipobáricas u ozonizadas, la aplicación de calor con aire y agua caliente, el uso de radiaciones ionizantes, la aplicación de soluciones acuosas de aditivos alimentarios y compuestos de baja toxicidad (Plaza et al*.,* 2004; Palou et al*.,* 2008). Sin embargo, la demanda actual de los consumidores exige productos inocuos, más seguros y que respeten el medio ambiente. Por tanto, se hace necesaria la búsqueda de métodos alternativos como los recubrimientos en base a compuestos naturales con propiedades antimicrobianas. El uso de recubrimientos es un método alternativo utilizado para preservar la calidad poscosecha de las frutas. Los recubrimientos proporcionan una barrera semipermeable al intercambio gaseoso de oxígeno, dióxido de carbono y vapor de agua, lo cual reduce la pérdida de peso, modifica la tasa respiratoria y retrasa la senescencia. Además, aportan brillo a las frutas lo que contribuye a mejorar su calidad visual (Trezza y Krochta, 2000).

El quitosano es un producto natural que posee la propiedad de formar películas para usarlo como recubrimiento, además de su actividad biológica como compuesto antimicrobiano (No et al*.,* 2007). El quitosano es un polisacárido catiónico con alto peso molecular, está compuesto por unidades de glucosamina con uniones $\beta(1\rightarrow 4)$, se

obtiene por la deacetilación alcalina de extractos de quitina, el mayor componente del caparazón de los crustáceos (No et al*.,* 2007; Coma et al*.,* 2002). El quitosano producido a nivel comercial presenta diferentes grados de deacetilación y de pesos moleculares, lo cual está directamente relacionado con sus diferentes propiedades funcionales y sus efectos antimicrobianos (No et al*.,* 2007).

En varios estudios realizados se han reportado la capacidad inhibitoria del quitosano y sus derivados en diferentes frutas y hortalizas como fresas, frambuesas, lechuga, tomates, manzanas (Devlieghre et al*.,* 2004; Han et al*.,* 2004, Vargas et al*.,* 2006; El Ghaouth et al*.,* 1992) y en naranjas, mandarinas y limones (El Ghaouth et al*.,* 2000; Benhamou, 2004; Chien y Chou, 2006; Chien et al*.,* 2007; Vargas et al*.,* 2007). El objetivo de este trabajo fue estudiar la capacidad de un recubrimiento de quitosano para inducir resistencia en naranjas cv. Valencia Late y en mandarinas cv. Oronules contra PD y PI a temperatura ambiente y en frigoconservación.

MATERIAL Y MÉTODOS

Preparación del recubrimiento

Los recubrimientos se prepararon a partir de quitosano comercial (peso molecular intermedio) al 1,88% en ácido acético. La formulación es de Biorend® distribuido por Idebio, S.L, Salamanca, España. Para facilitar la adhesión del recubrimiento se añadió 0,1% de Tween 80. Se utilizaron dos concentraciones: 0,66% (C1) y 1,88% (C2). La viscosidad de las emulsiones de quitosano para C1 y C2 fueron 19,7 y 37,3 cp, respectivamente. Estos valores corresponden a emulsiones con viscosidades relativamente bajas, las cuales facilitan la aplicación y secado del recubrimiento en los frutos. Por otro lado, las emulsiones de quitosano fueron emulsiones ácidas, el pH de las emulsiones fue 4,5 y 4,4 para C1 y C2, respectivamente.

Material vegetal

Se utilizaron naranjas cv. Valencia Late y mandarinas cv. Oronules procedentes de plantaciones comerciales de la zona citrícola de Valencia. Los frutos no recibieron ningún tipo de tratamiento previo en

poscosecha. Los frutos se lavaron con agua, se secaron a temperatura ambiente y se almacenaron a 5ºC y 90% humedad relativa (HR), por un tiempo menor a 5 días. Un día antes de la aplicación de los tratamientos, se atemperaron a temperatura ambiente (20ºC).

Preparación de los inóculos de PD y PI

Los inóculos de PD o PI fueron obtenidos a partir de cultivos en PDA de la colección de hongos del Centro de Tecnología Poscosecha del IVIA. Se utilizaron cultivos de 7-14 días de edad incubados a 25ºC. Se preparó una suspensión de conidios en Tween 80 (0,05% p/v) y se diluyó a 10^4 esporas/mL después de contarlas con un hemacitómetro.

Evaluación de la inducción de la resistencia de los frutos a las podredumbres por acción del quitosano

Los frutos se recubrieron, por inmersión, con quitosano a las concentraciones C1 y C2 y se mantuvieron a 20ºC durante 1, 3 ó 7 días para naranjas, ó 3 días para mandarinas. Después de cada uno de esos periodos, los frutos recubiertos se inocularon artificialmente con PD mediante la inmersión de un punzón con una punta de 1 mm de ancho y 2 mm de longitud en la suspensión con 10^4 esporas/mL, haciendo una incisión con el punzón en la zona ecuatorial del fruto. PI, a 10^4 esporas/mL, se inoculó de la misma manera que PD en la cara opuesta del mismo fruto. Frutos inoculados a una concentración de 10^4 esporas/mL y sin recubrir se utilizaron como control. Los frutos se almacenaron a 20 ó a 5ºC. Para cada tratamiento se utilizaron tres repeticiones de 10 frutos cada una.

Incidencia y severidad de las enfermedades

Se determinaron la incidencia (porcentaje de frutos podridos) y la severidad (diámetro de lesión, mm) de las podredumbres verde y azul a los 4 y 7 días de almacenamiento a 20ºC y a los 15 y 30 días a 5ºC más 7 días de simulación de la comercialización a 20ºC.

Análisis estadísticos

El tratamiento estadístico de los resultados se realizó mediante el análisis de varianza (ANOVA). Las diferencias entre las medias se establecieron con la prueba de la mínima diferencia significativa (MDS)

con un nivel de confianza del 95%. Los análisis se realizaron usando el programa estadístico Statgraphics Plus 2.1 (Manugistics, Inc., Rockville, MD, EE UU). Para la incidencia, el ANOVA se aplicó a los valores transformados al arcoseno de la raíz cuadrada de la proporción de frutos podridos.

RESULTADOS Y DISCUSIÓN

Las Figuras 1 y 2 muestran la incidencia de PI y PD, respectivamente, en naranjas cv. Valencia Late inoculadas artificialmente tras 1, 3 y 7 días de la aplicación de quitosano y almacenadas a 20ºC y 90% HR durante 4 y 7 días. En general, en naranjas cv. Valencia Late almacenadas a 20ºC, los recubrimientos con quitosano, C1 y C2, controlaron mejor PI que PD. En frutos inoculados después de 3 días de aplicar el recubrimiento, el quitosano C2 redujo la incidencia de PI en un 75 y 60% (comparados con el control) a los 4 y 7 días de almacenamiento a 20ºC, respectivamente ($P < 0,05$; Figura 1). En un estudio realizado en naranjas cv. Lane Late recubiertas con quitosano reportaron más del 60% de naranjas infectadas con PI después de 7 días a 25ºC (Vargas et al*.,* 2007). De acuerdo a los resultados encontrados en nuestro estudio, en naranjas cv. Valencia Late almacenadas a 20ºC después de 3 días de la aplicación de C2, parece que el quitosano induce algún tipo de resistencia al ataque de PI. En los frutos inoculados tras 1 y 7 días de la aplicación del recubrimiento no se encontraron diferencias significativas entre los tratamientos con quitosano y el control en la incidencia de PI en las naranjas almacenadas a 20ºC.

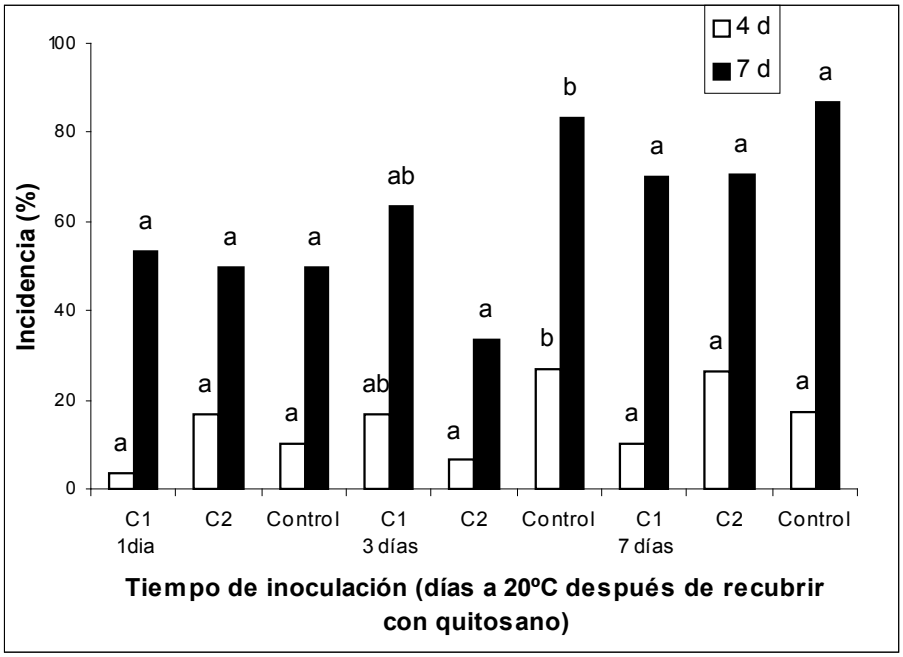

Figura 1. Incidencia de *Penicillium italicum* en naranjas cv. Valencia Late, recubiertas con quitosano a tres concentraciones 0% (Control), 0,66% (C1) y 1,88% (C2), inoculadas artificialmente tras 1, 3 y 7 días de aplicación del recubrimiento, y almacenadas a 20ºC y 90% HR durante 4 y 7 días. Para cada tiempo de inoculación y almacenamiento, columnas con letras diferentes son significativamente diferentes según el análisis MSD ($P < 0,05$). El análisis de varianza se realizó con los datos transformados al arcoseno. Se presentan los datos no transformados

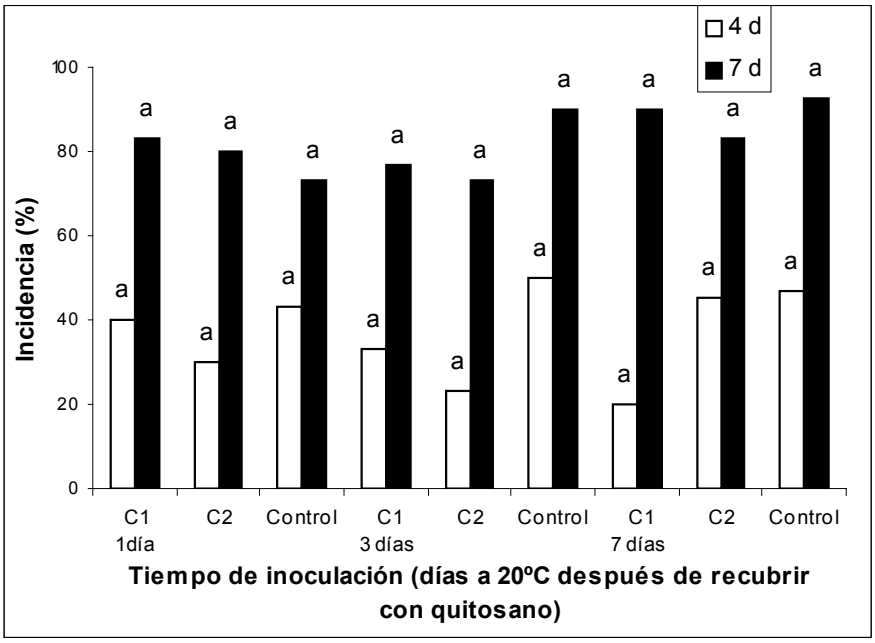

Figura 2. Incidencia de *Penicillium digitatum* en naranjas cv. Valencia Late, recubiertas con quitosano a tres concentraciones 0% (Control), 0,66% (C1) y 1,88% (C2), inoculadas artificialmente tras 1, 3 y 7 días de aplicación del recubrimiento, y almacenadas a 20ºC y 90% HR durante 4 y 7 días. Para cada tiempo de inoculación y almacenamiento, columnas con letras diferentes son significativamente diferentes según el análisis MSD ($P < 0,05$). El análisis de varianza se realizó con los datos transformados al arcoseno. Se presentan los datos no transformados

Se encontró que mientras mayor es el tiempo entre la aplicación del recubrimiento y la inoculación, menor es la incidencia de PD. Así para C1 después de 1, 3 y 7 días de la aplicación de quitosano la incidencia fue 40, 33 y 20%, respectivamente, después de 4 días de almacenamiento a 20ºC. Sin embargo, esa tendencia no se mantuvo después de 7 días de almacenamiento a 20ºC, superando en todos los casos el 76% de incidencia ($P > 0,05$). Para C2, a los 4 y 7 días a 20ºC, la incidencia de

PD fue superior al 20 y 73% para 1, 3 y 7 días ($P > 0,05$) después de la aplicación de quitosano (Figura 2). Una mayor concentración de quitosano, C2, produjo una menor incidencia de PD para 4 y 7 días de almacenamiento a 20ºC, aunque esos resultados no fueron significativos comparados con el control ($P > 0,05$). La aplicación de quitosano C1 y C2 resultó en alta incidencia de PD después de 7 días a 20ºC.

Los diámetros de lesión de PI y PD para C2, después de 7 días de almacenamiento a 20ºC en frutos inoculados tras 3 y 7 días de la aplicación del recubrimiento fueron menores que los del control, sin embargo esas diferencias no fueron significativas ($P > 0,05$). Los diámetros de lesión de PI fueron menores que los de PD para todos los periodos después de la aplicación del recubrimiento con quitosano. En los frutos, tras 3 y 7 días de la aplicación de los recubrimientos C1 y C2, los diámetros de lesión de PI fueron menores que los del control, aunque no se encontraron diferencias significativas ($P > 0,05$) entre los tratamientos y el control en las naranjas almacenadas a 20ºC (datos no mostrados).

En naranjas, a los 30 días de conservación a 5ºC la incidencia de PD fue superior al 60%, sin existir diferencia significativa entre tratamientos ($P > 0,05$). Al cambiar la fruta de 5ºC a 20ºC, independientemente del tiempo transcurrido entre la aplicación del recubrimiento y la inoculación, la incidencia se incrementó hasta valores superiores al 70% y 90% para PD y PI, respectivamente (datos de PD no mostrados). La incidencia y el diámetro de lesión de PI fueron menores cuanto menor fue el tiempo entre la aplicación del recubrimiento y la inoculación. Cuando este tiempo fue de 3 días, la aplicación del recubrimiento C2 redujo la incidencia de PI en un 19%, mientras que no se encontraron diferencias significativas entre C1 y el control (Figura 3).

En naranjas almacenadas a 20ºC, se encontró que a los 3 días después de la aplicación de quitosano indujo algún tipo de resistencia al ataque de PI. A partir de estos resultados se realizó una experimentación en mandarinas almacenadas en frigoconservación. En mandarinas, tanto a los 15 días como a los 30 días de conservación a 5ºC no existieron

diferencias significativas entre tratamientos para la incidencia y la severidad de PD y PI. Para PD y PI, a los 30 días la incidencia fue mayor de 80% y los diámetros de lesión fueron superiores a 40 mm para las concentraciones de quitosano estudiadas (datos no mostrados).

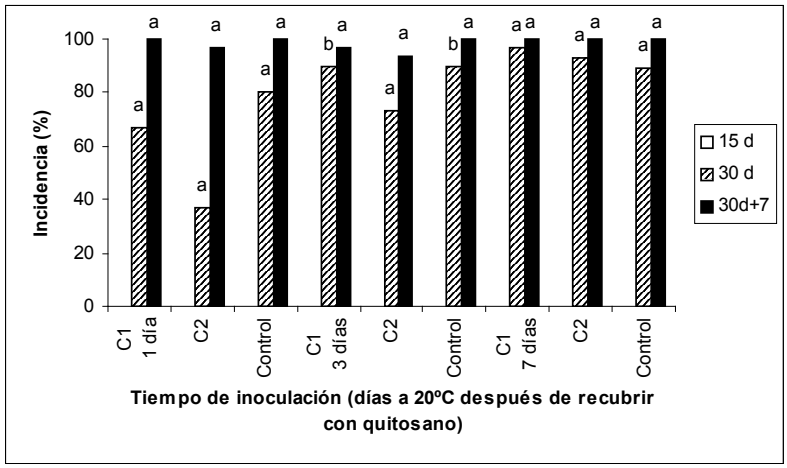

Figura 3. Incidencia de *Penicillium italicum* en naranjas cv. Valencia Late, recubiertas con quitosano a tres concentraciones 0% (Control), 0,66% (C1) y 1,88% (C2), inoculadas artificialmente tras 1, 3 y 7 días de aplicación del recubrimiento y almacenadas a 5ºC y 90% HR durante 15 ó 30 días más 7 días de simulación de comercialización a 20ºC. Para cada tiempo de inoculación y almacenamiento, columnas con letras diferentes son significativamente diferentes según el análisis MSD ($P < 0,05$). El análisis de varianza se realizó con los datos transformados al arcoseno. Se presentan los datos no transformados.

En las condiciones estudiadas tanto para naranjas como para mandarinas no se indujo resistencia al ataque de PD y PI, excepto para PI en naranjas después de 3 días de aplicación de quitosano. En varios estudios realizados en cítricos se reportó que la aplicación de quitosano o derivados de quitosano controlaron el ataque de PD y PI. Así, Chien y Chou (2006) encontraron que la aplicación de quitosano (dependiendo del tipo y concentración) en mandarinas produjo un 25-90% de

inhibición del crecimiento de PI y PD después de 5 días a 24ºC. De manera similar, Chien et al*.,* (2007) reportaron valores menores del 58% de infección de PD y PI en mandarinas tras la aplicación de quitosano. Las diferencias encontradas entre este y otros estudios realizados, podrían explicarse debido a que la actividad antimicrobiana del quitosano depende de su peso molecular, grado de deacetilación, tipo de microorganismo y cultivar utilizado (No *et al.,* 2007).

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GENERAL DISCUSSION

1. Inhibition of *Penicillium digitatum* and *Penicillium italicum* by antifungal hydroxypropyl methylcellulose (HPMC)-lipid edible composite films

The general objective of the present doctoral thesis was to develop new edible composite coatings with the addition of antifungal food additives for the control of postharvest green (GM) and blue (BM) molds on commercially important citrus cultivars, as well as to maintain the quality of coated fruits. Firstly, new edible composite films based on HPMC, beeswax (BW), and shellac, containing food additives or generally recognized as safe (GRAS) compounds (mostly mineral salts, salts of organic acids, salts of parabens, and other compounds) with antifungal properties were developed and selected according to their capability of forming stable emulsions. Edible composite formulations were optimized on the basis of a solid concentration (SC) range of 4-12 % and total lipid concentration (BW-shellac) range of 0-60 % (dry basis, db). The addition to the emulsions coating of food preservatives was from 0.05 to 4.5 % (wet basis, wb). In each case, the maximum concentration of food preservative that formed stable emulsions was determined. Then, stand-alone films obtained from stable emulsions were evaluated for their *in vitro* activity against *Penicillium digitatum* and *P. italicum* and their mechanical and barrier properties.

Among the large amount of HPMC-lipid edible composite coatings containing antifungal food preservatives that were prepared (470 emulsions), only about 5 % of HPMC-lipid emulsions were stable. Selected stable emulsion contained 6 to 8 % SC, 50 % (db) total lipid content, and a maximum of 2.5 % food preservative. The viscosity of film emulsions depended on SC and type and concentration of food preservatives added to the formulation. Food preservatives added to the HPMC-lipid formulations at a concentration up to 4.5 % resulted in viscous emulsions or gels. Emulsions with food preservatives that contained calcium propionate or calcium formate produced gels when shellac was included in the formulation. Stable and nonviscous emulsions were obtained when shellac was not present in the emulsion. It seems that the saponification of shellac leading to gel formation occurred in our HPMC-lipid emulsions containing sodium carbonate, probably because

of the alkaline properties of sodium carbonate. Similarly, most of the HPMC-lipid emulsions containing shellac and salts of organic acids or sodium salt of parabens and their mixtures precipitated or formed gels. Only, three of the organic acid salts tested permitted the inclusion of shellac (25 % db) in their formulations, namely potassium sorbate (PS), sodium benzoate (SB), and sodium propionate (SP). The viscosity of selected stable emulsions was in the range 7-28 cp, corresponding to nonviscous emulsions. Differences in emulsion SC of these formulations did not translate in important differences in emulsion viscosity. Cisneros-Zevallos and Krochta (2003) reported that the viscosity of HPMC solutions increased as SC increased. In this study, the selected HPMC-lipid formulations not only differed in SC but also in lipid ratio and food preservative concentration, which also significantly influenced the final viscosity value.

Similarly to viscosity, the pH of the emulsions depended on the nature of the food preservative added, obtaining either acid, neutral, or basic emulsions. The pH value was in the range from 3.8 to 9.4.

Variability for viscosity and pH of the same selected formulations prepared in different experiments (Chapters 3, 4 and 5) was never higher than 10 cp. and 1 point.

The *in vitro* activity against *P. digitatum* and *P. italicum* of selected HPMC-lipid stable emulsions was determined by means of the disk diameter test. Several works reported the use of different methodologies for the study of the antimicrobial activity of stand-alone films (Chen et al., 1996; Nychas and Skandamis, 2000; Cagri et al., 2001). These assays included measurements of the radius or diameter of the zone of inhibition around the antimicrobial film after diffusion in solid agar media (disk diameter test) or determinations of the survival of target microorganisms in liquid media as log CFU/mL (cell count method/ log reduction assay) (Franssen and Krochta, 2000; Nychas and Skandamis, 2000). The disk diameter test gives quantitative results for microbial inhibition and is relatively simple and easy to perform, so it has been used in many studies (Coma et al., 2002; Min and Krochta, 2005; Rojas-Graü et al., 2006; Maizura et al., 2007). However, this test only proves the antimicrobial character and does not predict the extent of antimicrobial protection on a food product. Nevertheless, it provides a good approach when similar films containing different antifungal food

additives are compared (Franssen and Krochta, 2000; Nychas and Skandamis, 2000). In our assays, the inhibition area surrounding the film disks was measured and compared to that produced by control films, in which the food preservatives were not added and hence the inhibition area was nil.

The film antifungal activity was determined for different inoculum concentrations (10^3 to 10^5 spores/mL) of both *P. digitatum* and *P. italicum*. Among all food preservatives added to the HPMC-lipid films, only films containing sodium bicarbonate, PS, SB, parabens, and their mixtures clearly inhibited *P. digitatum* and *P. italicum* at all inoculum levels. Among these effective films, those containing sodium salts of parabens and mixtures were the most effective irrespective of the inoculum density, films containing PS produced larger inhibition zones for both *P. digitatum* and *P. italicum* than films with SB. Films containing SP, sodium formate and sodium citrate only inhibited *P. digitatum* and *P. italicum*, at the lowest inoculum density of 10^3 spores/mL. The rest of films prepared with other organic acid salts did not inhibit the pathogens at all. PS, the most soluble form of sorbate, is well-known for its potent antifungal activity. The antimicrobial activity of PS against *P. digitatum* and *P. italicum* has been observed in both *in vitro* and *in vivo* studies (Matamoros-León et al., 1999; Palou et al., 2002b, 2008; Smilanick et al., 2008). The antimicrobial action of sorbate is pH-dependent. In general, PS activity is greater at low pH values, although sorbates may be effective at pH values as high as 7 (Stopforth et al., 2005), which was the pH of our HPMC-lipid films containing PS. The antimicrobial activity of films containing food preservatives are strongly influenced by the type of antimicrobial compound (size, shape, and polarity) and its concentration and the nature of the film matrix (Pranoto et al., 2005a,b; Corrales et al., 2009; Türe et al., 2009).

Since HPMC-lipid films containing PS, SB and their mixtures, and parabens and their mixtures exhibited higher antifungal activity than films with the other food preservatives, these films were selected to determine their barrier and mechanical properties. In this study, the lipid composition and the addition of food preservatives to HPMC-lipid films greatly influenced their barrier and mechanical properties. Films containing organic acid salts formulated with BW and shellac had higher water vapor permeability (WVP) and lower oxygen permeability (OP) than films containing parabens formulated with BW as the only

hydrophobic component. Films containing PS alone or in combination with SP exhibited higher WVP than the other films tested, which indicates that these food additives modified the HPMC-lipid film structure to a greater extent than films containing SB, parabens, or their mixtures. The different chemical structure of PS and parabens (i.e. straight chain vs. benzene ring) might also contribute to the differences observed in the mechanical properties of these films. This way, films with PS showed higher flexibility than films with parabens (Greener Donhowe and Fennema, 1994; Krochta, 2002).

2. Curative and preventive activity of antifungal HPMC-lipid coatings to control citrus green and blue molds

A large number of works are found in the literature that focus on the study of the *in vitro* antimicrobial activity of stand-alone films against target microorganisms (Ko et al., 2001; Min et al., 2005; Pranoto et al., 2005a; Sebti et al., 2007). However, there are many less references that compare the *in vitro* antimicrobial activity of stand-alone films with the performance of the same formulations applied *in vivo* as coatings to different fruit products (Cong et al., 2007; Ponce et al., 2008). Although *in vitro* studies can be a good approach for the evaluation and selection of antimicrobial films, the performance of the films on agar medium might not always appropriately predict the performance of the coatings on the surface of fresh produce. The release rate of antimicrobial agents from coatings located on the rind of fruit could be influenced by factors such as interactions of the antimicrobials with the film matrix (Chen et al., 1996), their diffusion rate within the polymer matrix (Limm and Holifield, 1995), the dispersion effect of the antimicrobials (Ponce et al., 2008), or differences in fruit skin resistance to the diffusion of antimicrobials (Park, 1999).

In this work, our objective was to evaluate the curative (fruit coated after 24 h fungal inoculation) and preventive (fruit coated 24 h before fungal inoculation) activity of selected antifungal HPMC-lipid coatings against GM and BM on commercially important citrus cultivars. The results were then compared with the *in vitro* studies previously performed (Section 1).

PS- and SB-based coatings were effective to reduce the development of GM and BM on 'Ortanique' mandarins and 'Valencia' oranges inoculated, treated, and incubated at 20ºC for 7 d. This result was in agreement with those obtained with stand-alone films in *in vitro* assays. However, when mixtures of two organic acid salts were added to HPMC-lipid coatings, the results obtained *in vivo* on coated citrus fruit did not agree with those obtained *in vitro* with stand-alone films. These differences were probably due to complex interactions among fruit host, pathogen, and environment that occur during *in vivo* disease development (Palou et al., 2002b). Coatings containing parabens and their mixtures were not very effective to reduce mold incidence and severity. These results also differed from those obtained in *in vitro* studies. Probably, the poor inhibitory activity of the coatings containing parabens when applied *in vivo* was the result of limited chemical release from the coating matrix to the fruit surface (Chung et al., 2001).

Irrespective of their composition, the inhibition activity of the coatings after 7 d of incubation at 20 ºC was higher on 'Valencia' oranges than on 'Ortanique' or 'Clemenules' mandarins. These differences were probably related to the different susceptibility of the fruit host to infections by *Penicillium* spp. In previous work with food preservatives applied as aqueous solutions, it was also observed a clear influence of the type of citrus fruit and its susceptibility to decay on the efficacy and persistence of the antifungal treatments (Palou et al., 2001, 2002a,b; Montesinos-Herrero et al., 2009). When we further evaluated, the performance of PS- and SB-based coatings on inoculated 'Valencia' oranges after 21 d at 20 ºC we observed that the reduction of disease incidence and severity was considerably lower than after 7 d. Such a low persistence probably indicated that the antifungal effects of these coatings on citrus fruit were fungistatic rather than fungicidal. Similarly, hot water or aqueous solutions of sodium carbonate, sodium bicarbonate, or organic acid salts were reported to be primarily fungistatic against citrus GM and BM (Smilanick et al., 1999; Palou et al., 2001; Palou et al., 2002b).

None of the tested coatings showed any preventive activity against the molds on the assayed citrus cultivars. The lack of preventive activity could be explained by the fact that new wounds were inflicted to the fruit rind for pathogen inoculation 24 h after the application of the coatings. We assume that coating would probably protect from infection

potential old rind wounds inflicted before coating but contaminated with *Penicillium* spores afterwards. In any case, because of lack of preventive activity, the use of coatings may be integrated with other antifungal postharvest treatments such as the application of biological control antagonists.

3. Effect of antifungal HPMC-lipid coatings on postharvest decay development and quality attributes of oranges and mandarins during long-term cold storage

Among usual postharvest techniques applied to fruits and vegetables, refrigeration is a common practice in commercial packinghouses to maintain the postharvest quality of fresh produce. Cold storage conditions influence not only the metabolic rate of the commodity, but also the development of postharvest decay. The results of the previous *in vivo* study showed that HPMC-lipid edible composite coatings containing organic acid salts and their mixtures were effective to control penicillium molds on citrus fruit incubated at 20 ºC. However, the performance of these new antifungal HPMC-lipid edible composite coatings during cold storage was unknown. The knowledge of such performance is fundamental because this work is intended to replace the conventional citrus commercial waxes formulated with synthetic polluting fungicides such as imazalil and thiabendazole. Therefore, another objective of this research was to determine the effect of HPMC-lipid edible composite coatings containing PS, SB, and their mixtures on the development of penicillium molds and the physico-chemical and sensory quality of commercially important oranges and mandarins during long-term cold storage.

3.1. 'Valencia' oranges

All tested coatings effectively reduced GM and BM on 'Valencia' oranges artificially inoculated with 10^5 spores/mL of both *P. digitatum*

and *P. italicum* and stored at 5 º C for up to 60 d, the coatings however, controlled significantly better GM than BM. This behavior could be due to the fact that *P. italicum* grows faster than *P. digitatum* at temperatures lower than 10 ºC (Eckert and Eaks, 1989; Plaza et al., 2003). The incidence and severity of both molds increased with cold storage time, showing again that the persistence of the antifungal coatings was limited. These findings confirmed on coated cold-stored 'Valencia' oranges previous results showing the efficacy of films and coatings containing selected organic acid salts and their mixtures to control penicillium molds *in vitro* and *in vivo* assays. Among all tested HPMC-lipid coatings, PS+SP-based coating was the most effective to inhibit GM and BM after 60 d at 5 ºC plus 7 d at 20 ºC of simulated shelf-life. We suggest that differences in antifungal activity on cold-stored 'Valencia' oranges that were observed between the different coatings containing food preservatives were probably due to differences in their compostition and/or physical properties (Waks et al., 1985), differences in the release ability of the food preservative from the coatings (Chung et al., 2001), differences in skin resistance to the diffusion of antimicrobials (Park, 1999).

In general, the tested coatings did not reduce the weight loss of treated oranges after 60 d at 5 ºC plus 7 d at 20 ºC. In fact, the application of PS+SP- and SB+SP-based coatings even resulted in higher weight loss on coated oranges than on uncoated controls. It is known from our previous studies (Section 1) that films containing PS and PS+SP had higher WVP than the other films tested, which indicated that these food additives modified the HPMC-lipid film structure to a greater extent. Similarly to what occurred with stand-alone films, it seems that water vapor could easily diffuse from the fruit through this type of coatings leading to higher weight loss. In addition, SB+SP-stand alone films were stiff and very brittle as shown by their high Young´s modulus, tensile strenght, and low elongation at break. These particular mechanical properties could be responsible for the the formation of pits or cracks on the SB+SP-based coatings applied to the surface of the fruit, which could lead to an increase of water loss and consequent weight loss.

The coatings modified the internal gas concentration, during storage at 5 ºC, increasing the internal CO_2 in coated fruit to 3-6 kPa and decreasing the internal O_2 to 12-18 kPa. In general, these internal O_2 concentrations were not low enough to produce anaerobic conditions

inside the fruit (Baldwin et al., 1997). Similar values of internal CO_2 were reported by other workers on 'Valencia' oranges (Peeples et al., 1999) and 'Fortune' mandarins (Pérez-Gago et al., 2002) maintained under similar cold storage conditions. However, coated 'Valencia' oranges stored at higher temperature than the used in our study showed higher internal CO_2 values (Baldwin et al., 1995; Navarro-Tarazaga et al., 2007). Typically, film OP has been considered as a good predictor of gas transfer. In our previous work (Section 1), stand-alone films containing a mixture of different organic acid salts presented higher OP values than films with one salt alone. Thus, higher internal O_2 concentration was expected on oranges coated with these films. However, the results did not confirm this assumption and the fruit internal O_2 was usually higher on coatings containing only one salt than on mixture-based coatings. Similar differences between stand-alone films and coatings with hydrocolloids-wax have also been reported, indicating that factors other than OP (physical properties of the coating formulation or peel morphology) should also be considered (Hagenmaier and Baker, 1993; Mannheim and Soffer, 1996).

The sensory quality of 'Valencia' oranges, determinated as flavor, off-flavor, and external appearance, were not adversaly affected by the application of the coatings. The levels of ethanol in the juice of coated oranges never exceeded the limit of 200 mg/100 mL value associated with presence of off-flavors in citrus fruit (Ke and Kader, 1990). However, the coatings did not improve fruit gloss on treated oranges with respect to uncoated control fruit. In general, we observed that small differences in the total solid content of HPMC-lipid coating emulsions did not significantly influence the physico-chemical and sensory quality of coated 'Valencia' oranges.

3.2. 'Ortanique' hybrid mandarins

The incidence of GM and BM on artificially inoculated 'Ortanique' mandarins was effectively reduced by coatings containing PS, SB, and their mixtures as antifungal ingredientes after 15 d of cold storage at 5 ºC. Furthermore, SB-based coating was still effective after 45 d of cold storage. As it was observed on oranges, the coatings controlled better

GM than BM during cold storage because of its worst adaptation to chilling environments (Eckert and Eaks, 1989). All the tested coatings significantly reduced the severity of GM and BM on coated 'Ortanique' mandarins after 45 d at 5 ºC, and effectively inhibited the sporulation of both molds after 30 d of cold storage. When compared to the results obtained with oranges (Section 3.1), the effectiveness and persistence of selected antifungal coatings during cold storage at 5 ºC was lower on 'Ortanique' mandarins. Since this behavior was also observed in *in vivo* tests after inoculation at 20 ºC, it ca be concluded that the effect of coatings containing organic acid salts and their mixtures on mold growth and development is dependent on the citrus cultivar.

On 'Ortanique' mandarins, all the coatings were effective as moisture barrier, significantly reducing the mandarin weight loss. Specifically, SB+PS- and SB+SP-based coatings were the most effective to reduce weight loss of coated mandarins. These results on coated fruit might be satisfactorily explained by the low WVP of stand-alone films containing the same mixtures of antifungal ingredients. Although those stand-alone films were stiff and brittle as a result of their mechanical properties, it seems that the coatings adapted physically better to the surface of 'Ortanique' mandarins than to that of 'Valencia' oranges.

Although the coatings decreased internal O_2 and increased internal CO_2 of the mandarins, the concentration of internal O_2 in coated mandarins reached values around 7 %, which were not low enough to create anaerobic conditions inside the coated fruit (Baldwin et al., 1997). Similarly, at the end of the entire storage period (60 d at 5 ºC plus 7 d at 20 ºC) the ethanol content in the juice of coated samples reached levels of 309-356 mg/100 mL, which, as it has been also reported by other authors (Navarro-Tarazaga et al., 2008) did not induce off-flavors in mandarins. These results were confirmed by the sensory quality analysis, in which both mandarin flavor and external appearance were considered as acceptable irrespective of the treatments and the storage time. However, the coatings did not improve the fruit gloss compared to uncoated samples.

3.3. 'Clemenules' clementine mandarins

On 'Clemenules' clementines, the incidence of GM and BM was significantly reduced by all the tested coatings after 15 d of cold storage at 5 ºC but not after 30 d. The SB+PS-based coating was the most effective to reduce the diseae of both molds. Similarly to what was found with 'Valencia' oranges and 'Ortanique' mandarins, the incidence of BM was higher than that of GM on cold-stored 'Clemenules' mandarins. Although the severity of the molds was significantly lower on all coated samples than on uncoated controls, the effectiveness of the coatings to control disease development was nil when the coated samples were transferred from 5 to 20 ºC after 30 d of storage. Therefore, comparing among citrus cultivars, the effectiveness and persistence of the antifungal coatings were lower than on 'Ortanique' mandarins, and, in general, they were lower on mandarins than on oranges. Probably, the high fruit susceptibility of 'Clemenules' mandarins to infections by GM or BM may strongly influence the performance of these coatings, and therefore the lower effectiveness of the alternative fungistatic treatments (Palou et al., 2001, 2002a,b; Montesinos-Herrero et al., 2009).

As it was also observed on 'Ortanique' mandarins, SB+PS- and SB+SP-based coatings were the most effective to reduce weight loss on coated 'Clemenules' mandarins. These coatings were flexible and adapted very well to the surface of the clementines. They modified the internal gas atmosphere of the fruit, but did not induce off-flavor. In general, the overall sensory quality of coated mandarins was valuated as acceptable, but there was a lack of gloss. The results from the work with all tested cultivars showed a clear need to perform further research to improve the gloss provided by the antifungal coatings. These general lack of gloss compared to that provided by commercial citrus waxes could be related to the formation of a macroemulsion with a milky appearance instead of a microemulsion (Hagenmaier and Baker, 1994).

4. Control of green and blue molds of oranges and mandarins by chitosan edible coatings

The objective of this research work was to evaluate the effect of commercial chitosan edible coatings (intermediate molecular weight, Biorend®) on the development of GM and BM molds on 'Valencia' oranges and 'Oronules' mandarins during both inoculation at 20 ºC and long-term cold storage.

In general, the effectiveness of chitosan-based coatings was highly dependent on the inoculum density, fruit cultivar, and storage conditions, but it was not high enough for commercial decay control of these postharvest diseases. These results contrasted with those reported by Chien et al. (2007) who found that low molecular weight chitosan exhibited greater antifungal resistance than the chemical fungicide thiabendazole against *P. digitatum* and *P. italicum* on coated 'Murcott' tangor fruit. However, different reports on chitosan performance are difficult to compare because, as it has been concluded in many works the functional properties and antimicrobial effects of chitosan are clearly related to its deacetylation degree and molecular weight (No et al., 2007).

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GENERAL CONCLUSIONS AND FUTURE WORK

GENERAL CONCLUSIONS AND FUTURE WORK

1. Only around 5 % of 470 hydroxypropyl methylcellulose (HPMC)-lipid edible composite formulations containing antifungal food additives or generally recognized as safe (GRAS) compounds (mostly mineral salts, salts of organic acids, and salts of parabens) were selected according to their capability of forming stable emulsions.

2. Most of the HPMC-lipid emulsions containing shellac and salts of organic acids or sodium salts of parabens precipitated or formed gels. The addition of shellac into the formulation was possible only in emulsions containing potassium sorbate, sodium benzoate, and sodium propionate.

3. Selected stable HPMC-lipid emulsions contained 6 to 8 % solid content, 50 % (dry basis) total lipid content (beeswax-shellac), and a maximum of 2.5 % (wet basis) food preservative. They were nonviscous emulsions (<40 cp.) and theie pH was in the range from 3.8 to 9.4 depending upon the food preservative added.

4. Films containing organic acid salts formulated with beeswax and shellac had higher water vapor permeability and lower oxygen permeability than those formulated with parabens and beeswax as the only hydrophobic component.

5. Films containing food preservatives with benzene ring molecules, such as sodium benzoate and parabens, were more rigid and less extensible than films containing additives with a straight-chain molecule such as potassium sorbate.

6. Irrespective of the pathogenic inoculum density, stand-alone films containing sodium salts of parabens and their mixtures, and potassium sorbate, sodium benzoate and their mixtures were the most effective to inhibit the *in vitro* growth of *Penicillium digitatum* and *Penicillium italicum*. Their combination did not provide any synergistic effect.

7. Important differences in antifungal activity of HPMC-lipid films and coatings were observed between *in vitro* and *in vivo* tests, probably due to differences on the release of food preservatives from films located on agar medium and from coatings located on the rind of citrus fruit and/or complex interactions among the host, pathogen, and environment that occur during *in vivo* disease development.

8. The curative activity of antifungal coatings after incubation at 20 ºC for 7 d was dependent on the citrus species and cultivars, being in general higher on oranges than on mandarins, probably due to the lower susceptibility of the former to infections by *Penicillium* spp.

9. The *in vivo* curative antifungal activity of the HPMC-lipid coatings applied 24 h after wound pathogen inoculation was fungistatic rather than fungicidal and none of the tested coatings showed preventive activity against the pathogens when they were wound inoculated 24 h after coating.

10. Among all food preservatives tested, coatings containing potassium sorbate, sodium benzoate, and their mixtures were the most effective to reduce the development of postharvest green and blue molds on previously inoculated 'Valencia' oranges and 'Clemenules' and 'Ortanique' mandarins incubated at 20 ºC for 7 d. These coatings were selected for long-term cold storage trials with these citrus cultivars.

11. During long-term cold storage, mold control of antifungal HPMC-lipid coatings was in general higher on oranges than on mandarins.

12. During 60 d of cold storage at 5 ºC and after 7 d of shelf life at 20 ºC, the coating containing the mixture potassium sorbate + sodium propionate was the most effective to inhibit both green and blue molds on artificially inoculated and coated 'Valencia' oranges.

13. During 30 d of cold storage at 5 ºC, sodium benzoate-based coating was the most effective reducing the incidence of green mold and disease severity of both molds on artificially inoculated and coated 'Ortanique' mandarins.

14. During 30 d of cold storage at 5 ºC, sodium benzoate + sodium propionate-based coating was the most effective to inhibit green mold on artificially inoculated and coated 'Clemenules' mandarins.

15. During long-term cold storage, HPMC-lipid coatings containing organic acid salts and their mixtures significantly reduced weight loss and maintained the firmness of coated 'Clemenules' and 'Ortanique' mandarins, but did not reduce weight loss of 'Valencia' oranges. Coatings did not adversely affect the fruit physico-chemical and sensory quality of all tested citrus cultivars.

16. Although HPMC-lipid edible coatings containing food additives significantly modified the internal gas concentration and the volatile content in the juice of citrus fruit, they did not induce anaerobic conditions that lead to the production of off-flavors.

17. Weight loss and internal gas concentration of coated citrus fruit did not always correlate with barrier properties of stand-alone films. The differences may be related to film mechanical properties.

18. Irrespective of the cold storage period, the sensory quality of coated 'Valencia' oranges, and 'Ortanique' and 'Clemenules' mandarins was evaluated as acceptable by a trained sensory panel. The coatings did not improve the gloss and overall external aspect of the tested citrus cultivars.

19. The effectiveness of the commercial chitosan-based coatings to control citrus green and blue molds was highly dependent on inoculum density, fruit cultivar, and storage conditions, but it was not consistently high enough for commercial decay control.

FUTURE WORK

Considering the potential of HPMC-lipid edible coatings containing food additives to control citrus green and blue molds and maintain the physico-chemical and sensory quality of oranges and mandarins, major objectives of future research are:

1. To improve the physical characteristics of antifungal HPMC-lipid edible composite coatings in order to increase the moisture barrier on oranges and enhance the gloss and visual quality of coated fruit.

2. To determine the antifungal effect and general performance of selected HPMC-lipid coatings on other commercially important citrus species and cultivars.

3. To evaluate the use of antifungal natural compounds (plant extracts, essential oils, spices, etc.) as ingredients of new HPMC-lipid edible composite films and coatings to control penicillium molds of citrus fruit.

4. To study the performance of antifungal HPMC-lipid coatings in combination with other non-polluting postharvest disease control methods to establish a cost-effective multi-faceted strategy for integrated decay control in citrus packinghouses.

5. To evaluate selected edible films and coatings with antimicrobial properties to reduce risks associated with the presence of microbes of food safety concern (human pathogens) on fresh and minimally processed horticultural products.

APPENDICES

A 1. PHOTOGRAPHIC APPENDIX

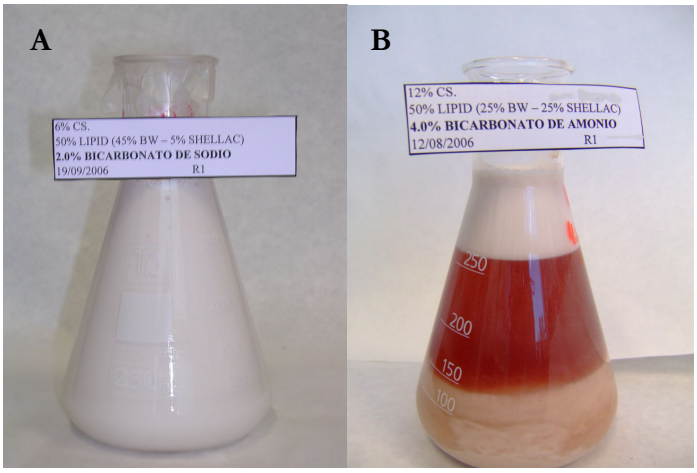


Photo 1. Examples of HPMC-lipid edible composite emulsions: stable (A) and unstable (B).

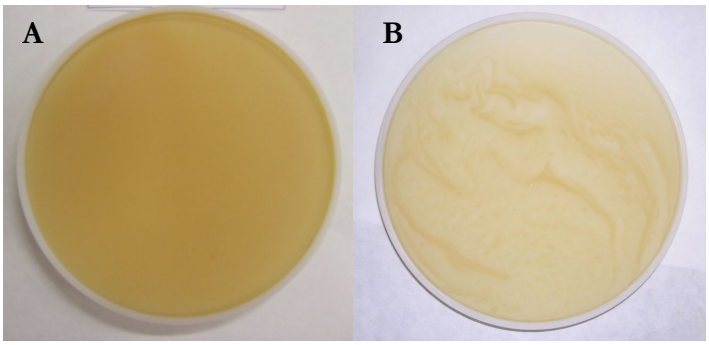


Photo 2. Examples of HPMC-lipid edible composite homogeneous film (**A**) and heterogeneous (brittle) film (B).

Photo 3. Oranges inoculated with *Penicillium digitatum* and *Penicillium italicum*, incubated at 20 ºC for 24 h, coated by inmersion with selected HPMC-lipid edible composite coatings, drained, allowed to dry, and evaluated for disease incidence and severity after up to 21 d of incubation at 20 ºC (Curative activity).

Photo 4. Green mold (left) caused by *Penicillium digitatum* and blue mold (right) caused by *Penicillium italicum* on oranges.

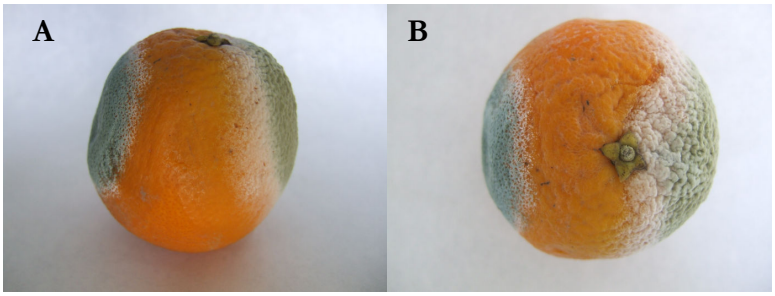


Photo 5. Blue mold (left) and green mold (right) on opposite sides of the same oranges (front view (**A**)**,** upper view (**B**)).

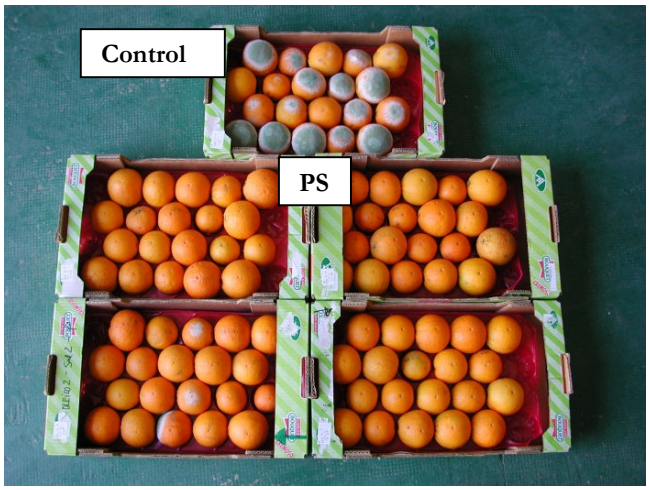


Photo 6. Curative activity against blue mold of HPMC-lipid edible composite coatings containing potassium sorbate (PS) on 'Valencia' oranges artificially inoculated with *Penicillium italicum*, coated, and incubated for 7 d at 20 ºC.

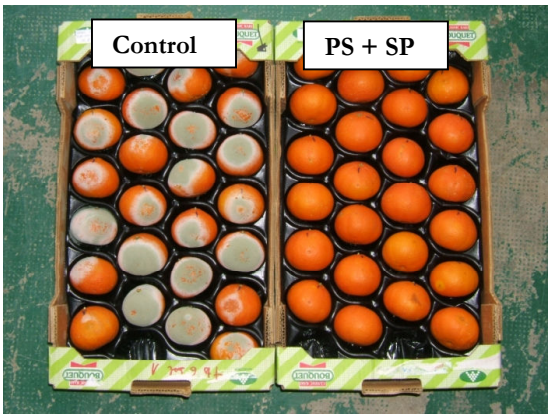


Photo 7. Curative activity against green mold of HPMC-lipid edible composite coatings containing potassium sorbate + sodium propionate (PS + SP) on 'Ortanique' mandarins artificially inoculated with *Penicillium digitatum*, coated and incubated for 7 d at 20 ºC.

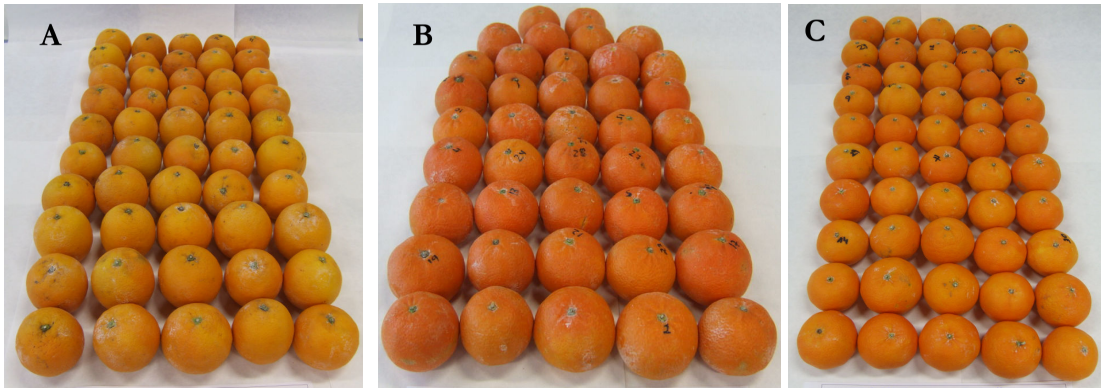
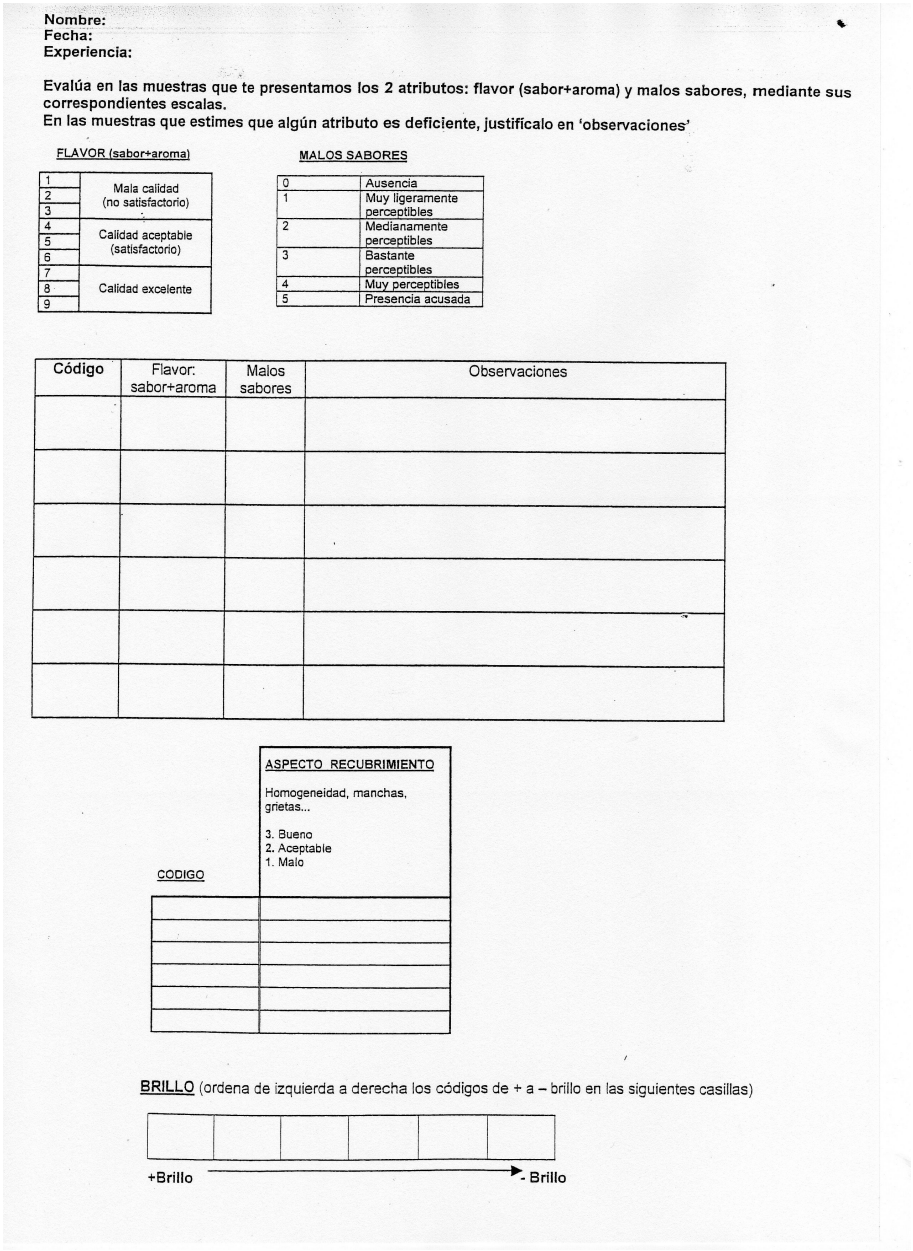


Photo 8. External appearance of 'Valencia' oranges (**A)** and Ortanique' mandarins **(B)** after 60 d at 5 ºC plus 7 d of shelf life a 20 ºC, and 'Clemenules' mandarins (**C)** after 30 d at 5 ºC plus 7 d of shelf life a 20 ºC. Fruits were coated with HPMC-lipid edible composite coatings contained potassium sorbate + sodium propionate.

Photo 9. Samples for sensory analysis for gloss and external apperances of coated mandarins (**A**) and for quality attributes of segments of coated oranges.(**B**).

A 2. QUESTIONNAIRE FOR SENSORY EVALUATION



Nombre:
Fecha:
Experiencia:

Evalúa en las muestras que te presentamos los 2 atributos: flavor (sabor+aroma) y malos sabores, mediante sus correspondientes escalas.
En las muestras que estimes que algún atributo es deficiente, justifícalo en 'observaciones'

FLAVOR (sabor+aroma)

1 2 3	Mala calidad (no satisfactorio)
4 5 6	Calidad aceptable (satisfactorio)
7 8 9	Calidad excelente

MALOS SABORES

0	Ausencia
1	Muy ligeramente perceptibles
2	Medianamente perceptibles
3	Bastante perceptibles
4	Muy perceptibles
5	Presencia acusada

Código	Flavor: sabor+aroma	Malos sabores	Observaciones

CODIGO	ASPECTO RECUBRIMIENTO Homogeneidad, manchas, grietas... 3. Bueno 2. Aceptable 1. Malo

BRILLO (ordena de izquierda a derecha los códigos de + a – brillo en las siguientes casillas)

+Brillo → - Brillo

Figure 1. Questionnaire for sensory evaluation of flavor, off-flavors, external aspect and gloss of coated citrus fruit.