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## Using tannins as active compounds to develop antioxidant and antimicrobial chitosan and cellulose based films

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### ABSTRACT

Three tannin extracts from different sources (white peel grape (W), red peel grape (R) and oak bark (O)) have been used to obtain active films based on different polysaccharides: chitosan (CH), methylcellulose (MC) and hydroxypropylmethylcellulose (HPMC). Tannin-rich films were obtained by casting as monolayers (by blending all the components) and as bilayers (one polysaccharide layer plus one tannin layer applied as a coating). Microstructural and physicochemical properties (water solubility, water vapor permeability, mechanical and optical properties), as well as the antioxidant and antimicrobial activities of the films, were analysed. Tannins, regardless of their origin, acted as crosslinking agents when incorporated into CH or MC films due to the establishment of specific associations, mainly hydrogen bonds and hydrophobic interactions. Thus, these tannin-rich films turned less water soluble, mechanically stiffer and less stretchable. In contrast, tannins provoke precipitation in the HPMC film-forming solutions and the application of tannins onto the HPMC film surface gave rise to a weakened network due to the local precipitation of HPMC-tannin complexes. The greatest antioxidant capacity was found in both mono and bilayer tannin-rich MC based films in different food simulants, in line with a tannin release from the polymer matrix. The MC bilayer films also exhibited a bacteriostatic activity against both *E.Coli* and *L. innocua*. So, MC films with tannins could represent a good alternative as active coatings or packaging material so as to prevent the occurrence of oxidative reactions in sensitive food products or to control bacterial growth.

### 1. Introduction

Nowadays, active biodegradable packaging materials based on bio-based compounds are increasingly gaining in importance as a way of diminishing crude oil consumption, thus reducing the packaging's environmental impact and food waste (Shihabudeen, Priscilla & Thirumurugan, 2010; Shirmohammadi, Efhamsisi & Pizzi, 2018; Utama, Willis, Ben-Yehoshua & Kuek, 2002). These materials are usually better alternatives for food contact applications, with fewer problems related to the migration of toxic compounds. On the other hand, the current consumer demand for more natural products free of synthetic chemical additives for food preservation purposes boosts the search for active agents of natural origin able to lengthen the food shelf-life. In this sense, phenolic compounds coming from plant extracts, such as tannins, are currently considered as potential compounds that may be incorporated into polymeric matrices for the development of bioactive films for food packaging purposes (Cano, Andres, Chiralt & Gonzalez-Martinez, 2020).

Tannin compounds are water-soluble polyphenols mainly found in fruits (Ping, Pizzi, Guo & Brosse, 2012), seeds (Teng, Hayasaka, Smith & Bindon, 2019), flowers (Braga et al., 2018) and barks (Sartori, Mota, Miranda, Mori & Pereira, 2018). Their molecular weight ranges between 500 and 3000 Daltons (Porrás-Loaiza & López-Malo, 2009) and their polyphenolic structure is composed of 12–16 phenolic groups and 5–7 aromatic rings per 1000 units of weight. These active agents have the advantages of being renewable, environmentally-friendly and safe to use, while present interesting antioxidant and antimicrobial capacity. A great number of papers have reported the antimicrobial properties of tannins against a wide variety of fungi (*i.e.*, *Candida parapsilosis*) and bacteria, such as *Salmonella*, *Staphylococcus aureus*, *Helicobacter* and *Escherichia coli*, among others, (Arora & Mahajan, 2019; Cano et al., 2020; Khameneh, Iranshahy, Soheili & Bazzaz, 2019; Teanpaisan, Kawsud, Pahununto & Puripattanavong, 2017).

Some polysaccharides, such as chitosan, hydroxypropylmethyl cellulose or methyl cellulose have been extensively studied in order to

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develop biodegradable films/edible coatings that can act as carriers of active agents of natural origin, such as polyphenols. For example, essential oils, green tea, olive leaf or pomegranate seed extracts or resveratrol, among others, have been incorporated into chitosan and/or cellulose-derivative based films to enhance their antioxidant and antimicrobial properties (Sánchez-González, Vargas, González-Martínez, Chiralt & Cháfer, 2009; Siripatrawan & Harte, 2010; Ayana & Turhan, 2009; Pastor, Sánchez-González, Chiralt, Cháfer & González-Martínez, 2013; Nemazifard, Kavooosi, Marzban & Ezedi, 2017), obtaining promising packaging materials to be used for food preservation by using natural, biodegradable and/or edible active compounds safe both for the health of the consumer and the environment.

Polyphenols have been shown to bind with polysaccharides mainly by means of hydrogen bonds and hydrophobic interactions, where electrostatic forces are not usually playing an important role. Hydrophobic interactions may occur between methyl groups of the polysaccharide fraction and the heterocyclic C-ring of polyphenols. Likewise, hydrogen bonds can be established between hydroxyl groups of polysaccharides and phenols, which are promoted by conformational changes of compounds in solution (Renard, Watrelot & Le Bourvellec-Samour, 2017; Yu, Tsai, Lin & Mi., 2015). These interactions could modify some of the film's physical properties, such as water solubility, barrier and mechanical properties.

Few studies into polysaccharide based films using tannins as polyphenols have been reported. Yu et al. (2015), working with tea catechins, showed that the addition of polyphenols to methylcellulose films promoted a crosslinking effect, leading to 64% and 82% reduction in the water vapor permeability and water solubility, respectively and to 37% increase in the mechanical resistance of the composite films. Talon et al. (2017) also reported that the incorporation of tannic acid into chitosan films promoted a reinforcement effect but without enhancing the water barrier or solubility properties of the chitosan films. Zhou et al. (2019) used tannins and nanocellulose to develop polymer structures with antioxidant properties for different applications (food packaging, pharmaceutical wound dressing...). Missio et al. (2018) developed biodegradable nanocellulose-tannin films with a strong hydrophobic structure, resistance to heat and proper antioxidant releasing capacity.

Interactions between polyphenols and polysaccharides in film polymer matrices have been scarcely described, most of the studies focusing on bioaccessibility and bioavailability of polyphenols in the presence of pectin, cellulose or dietary fibers (Jakobek, 2015). These studies pointed out to the fact that different polysaccharide-tannin combinations may lead to different changes in the structural and functional properties of both compounds. The importance of the nature and structure of the phenols (*i.e.*, structural flexibility and number of OH groups) and the nature and structure of polysaccharides (both defining the polyphenols' affinity and bonding to polysaccharides) has also been highlighted by some authors (Dobson et al., 2019; Jakobek, 2015). These phenol-polysaccharide interactions could also contribute to improve the functionality of polysaccharide based films, but further studies are needed to elucidate their response.

The novelty of this work relies on the procedure followed to develop the films, which allows us to compare mono and bilayer films, where components differ in their internal arrangement. Thus, two methods of incorporating tannins into different polysaccharide films are used in order to evaluate if the different extent of the tannin-polysaccharide interactions affect the functionality of the developed films. One method consists of introducing the tannins as dry powders into the film forming solutions to obtain monolayer films. In the other method, the monolayer polysaccharide films are laminated with a tannin aqueous solution, which remains at the top as a second layer.

The aim of the present study was to analyze how the incorporation of tannins from three different sources into hydroxypropylmethyl cellulose, methyl cellulose and chitosan -based films affected their functional and physicochemical properties, when incorporated into the bulk film or on the film surface. To this end, the films' microstructure, optical,

**Table 1**  
Nomenclature and composition of the different formulations.

Matrix	Tannin	Nomenclature	Preparation
Chitosan	–	CH	–
	White (W)	CH <sub>W</sub> <sup>m</sup>	Monolayers
	Oak bark (O)	CH <sub>O</sub> <sup>m</sup>	
	Red (R)	CH <sub>R</sub> <sup>m</sup>	
	White (W)	CH <sub>W</sub> <sup>b</sup>	Bilayers
	Oak bark (O)	CH <sub>O</sub> <sup>b</sup>	
Red (R)	CH <sub>R</sub> <sup>b</sup>		
Methylcellulose	–	M	–
	White (W)	MC <sub>W</sub> <sup>m</sup>	Monolayers
	Oak bark (O)	MC <sub>O</sub> <sup>m</sup>	
	Red (R)	MC <sub>R</sub> <sup>m</sup>	
	White (W)	MC <sub>W</sub> <sup>b</sup>	Bilayers
	Oak bark (O)	MC <sub>O</sub> <sup>b</sup>	
Red (R)	MC <sub>R</sub> <sup>b</sup>		
Hydroxy propyl-methyl cellulose	–	HPMC	–
	White (W)	HPMC <sub>W</sub> <sup>b</sup>	Bilayers
	Oak bark (O)	HPMC <sub>O</sub> <sup>b</sup>	
	Red (R)	HPMC <sub>R</sub> <sup>b</sup>	

barrier and mechanical properties and water solubility were analyzed, as well as their antioxidant and antimicrobial capacity.

## 2. Materials and methods

### 2.1. Materials

High molecular weight chitosan (CH) with a deacetylation degree of 95.8% (Batch MKBH5816V), hydroxypropyl methylcellulose (HPMC), both from Sigma Aldrich Química S.L., Madrid, Spain, and methylcellulose (MC from VWR International Eurolab, S.L., Barcelona, Spain) were used to obtain the films. White (W) and red (R) peel grapes and oak bark (O) commercial tannins, with a total phenolic content of around 82, 55 and 47 mg GAE /g, respectively, were supplied by Dolmar Productos Enológicos S.L. (Haro, La Rioja, Spain). 98% glacial acetic acid, purchased from Panreac Química S.A. (Barcelona, Spain), as well as magnesium nitrate-6-hydrate (Mg(NO<sub>3</sub>)<sub>2</sub>) and di-phosphorous pentoxide (P<sub>2</sub>O<sub>5</sub>), were used to control the relative humidity. The organic chemical compound, 2,2-diphenyl-1-picrylhydrazyl (from Sigma Aldrich Química S.L., Madrid, Spain), was used for antioxidant activity analysis.

### 2.2. Preparation of films

Films were obtained by the casting technique as monolayers by dispersing 2% (wt) MC and 5% (wt) HPMC in distilled water. CH (2% wt) was dispersed into an aqueous solution of 0.5% (v/w) glacial acetic acid (pH~3). Tannins were incorporated in a carbohydrate: tannin ratio of 3:1. To obtain the monolayers, tannins were dispersed into the polysaccharide film forming dispersions using continuous stirring until complete dissolution.

The bilayers were obtained by casting a 10% (w/w) tannin aqueous solution onto the 24 h partially dried carbohydrate films. The tannin aqueous solution provided the same amount of tannins as the ones used in the monolayers. These tannin solutions exhibited a pH of around 5.2, except the pure W tannin solution, that showed the lowest one, of around 2.5. In both procedures, the film-forming dispersions were stirred until completely dissolved and poured into teflon dishes (150 mm), in a proper amount in order to provide a surface density of solids of 113 g m<sup>-2</sup>. The films were dried at 45±2% relative humidity (RH) and 25±1 °C for approximately 48 h. The obtained films were peeled off and stored at 25±1 °C and 53% RH (by using Mg(NO<sub>3</sub>)<sub>2</sub> saturated salt solution) for two weeks until further analysis.

The film nomenclature was selected according to the film composition and preparation as can be observed in Table 1. Seven formulations based on each polymer used: one control with the neat polymer (CH, MC, HPMC), 3 tannin rich (W, O, R) monolayers (m) and 3 tannin rich

(W, O, R) bilayers (b). When using HPMC, only 4 formulations were developed as bilayers. Non-monolayer HPMC films were obtained as a consequence of the extensive precipitation of tannins in the presence of HPMC. Thus, only 6 monolayer (m) and a total of 9 bilayer (b) films were obtained from the different polysaccharides and tannins used.

The film thickness was measured at several random positions with an electronic digital micrometer.

### 2.3. Morphological observation

A morphological analysis of film cross section was carried out using a field emission scanning electron microscope (FESEM) (Supra™ 25-Zeiss, Germany). Sample preparation included cryo-fracturing by using liquid nitrogen, placement on copper stubs, carbon coating and a voltage selection of 1 kV. Two replicates per formulation were prepared.

### 2.4. Physical characterization of films

#### 2.4.1. Mechanical properties

The mechanical behavior was measured with a texture analyzer (TA.XT plus, Stable Micro Systems, Haslemere, England) in accordance with the ASTM D882 standard method for the tensile properties of thin plastic sheeting (ASTM, 2001). A tension test of equilibrated rectangular samples (2.5 × 10 cm) was performed at 50 mm•min<sup>-1</sup> using A/TG tensile grips (separation set at 50 mm) at room temperature. Tensile strength (TS in MPa), elongation (E in%) and elastic modulus (EM in MPa) were obtained from stress vs. strain (Hencky) curves. Eight replicates were considered for each formulation.

#### 2.4.2. Moisture content, water solubility and water vapor permeability

For the water content measurement, the samples were partially dried in a vacuum oven (J.P. Selecta, S.A., Barcelona, Spain) at 60 °C for 24 h. After that, they were placed in desiccators containing P<sub>2</sub>O<sub>5</sub> for total water removal until reaching constant weight. Five replicates were considered for each formulation. For the solubility analysis, five replicates were considered for each formulation. The samples were kept in distilled water in a film: water ratio (w/w) of 1:80 (in order to ensure the complete film immersion) for 24 h at room temperature. Subsequently, the samples were transferred to a convection oven (J.P. Selecta, S.A., Barcelona, Spain) at 60 °C for 24 h to remove the free water, and then the total water elimination was completed in a desiccator with P<sub>2</sub>O<sub>5</sub> at room temperature until constant weight. The water vapor permeability (WVP) was determined in quadruplicate at 25 °C for the water vapor transmission of materials in accordance with ASTM E96-95 (ASTM, 1995) using Payne permeability cups (Payne, Elcometer SPRL, Hermelle/sd Argenteau, Belgium) of 3.5 cm in diameter. De-ionised water was used inside the testing cup to achieve 100% RH on one side of the film, while an oversaturated Mg(NO<sub>3</sub>)<sub>2</sub> salt solution was used to control the RH on the other side of the film (53%). A fan placed on the top of the cup was used to reduce RH resistance to water vapor transport. An analytical balance was used for the periodical weight control of cups. The slopes in the steady state period of the weight loss vs. time curves were determined by linear regression in order to estimate the water vapor transmission rate.

#### 2.4.3. Internal transmittance and gloss

Internal transmittance (Ti) were calculated applying the Kubelka-Munk theory for multiple scattering to the reflection spectra obtained using a spectrophotometer (CM.36000d, Minolta Co. Tokyo, Japan), D65/10° and a 30 mm measurement/illumination area. Measurements were taken on the side of film which was in contact with air during drying. Each formulation was analyzed in triplicate.

The gloss was measured according to the ASTM standard D523 (ASTM, 1999) using a flat surface gloss-meter (Multi-Gloss 268, Minolta Co, Langenhagen, Germany). The measurements were taken over a black matte standard plate. The results quantified at 60° were expressed as

gloss units, relative to a highly polished surface of standard black glass with a value close to 100. Nine replicates were considered for each formulation.

### 2.5. Antioxidant activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH\*) technique was used to determine the potential antioxidant capacity of films in terms of free radical scavenging activity (Brand-Williams, Cuvelier & Berset, 1995; Matthäus, 2002; Pastor et al., 2013). Sample pieces containing 3.125 mg of tannin were cut (specimens of 1.1 cm<sup>2</sup>) and dissolved (under magnetic stirring for 12 h at 25 °C) in 50 mL of three food authorized simulants: ethanol 10% v/v, acetic acid 3% v/v and ethanol 50% v/v. The DPPH\* concentration (mM) in the reaction medium was calculated from the calibration curve  $A_{515\text{ nm}} = 14.546 [\text{DPPH}^*] - 0.005$ , (R<sup>2</sup> 0.999). In every case, different appropriately diluted samples were added to a methanol solution of DPPH\* (0.019 g L<sup>-1</sup>). The scavenging capacity was determined by spectrophotometry (Evolution 201 UV-Vis Spectrophotometer, Thermo Scientific, EEUU) at 515 nm. The measurements were taken after 40 min when the reaction reached a plateau. The remaining DPPH\* (%DPPH\*<sub>REM</sub>) was calculated according to Eq. (1).

$$\% \text{DPPH}^*_{\text{REM}} = [\text{DPPH}^*]_{\text{T}} / [\text{DPPH}^*]_{\text{T}=0} \quad (1)$$

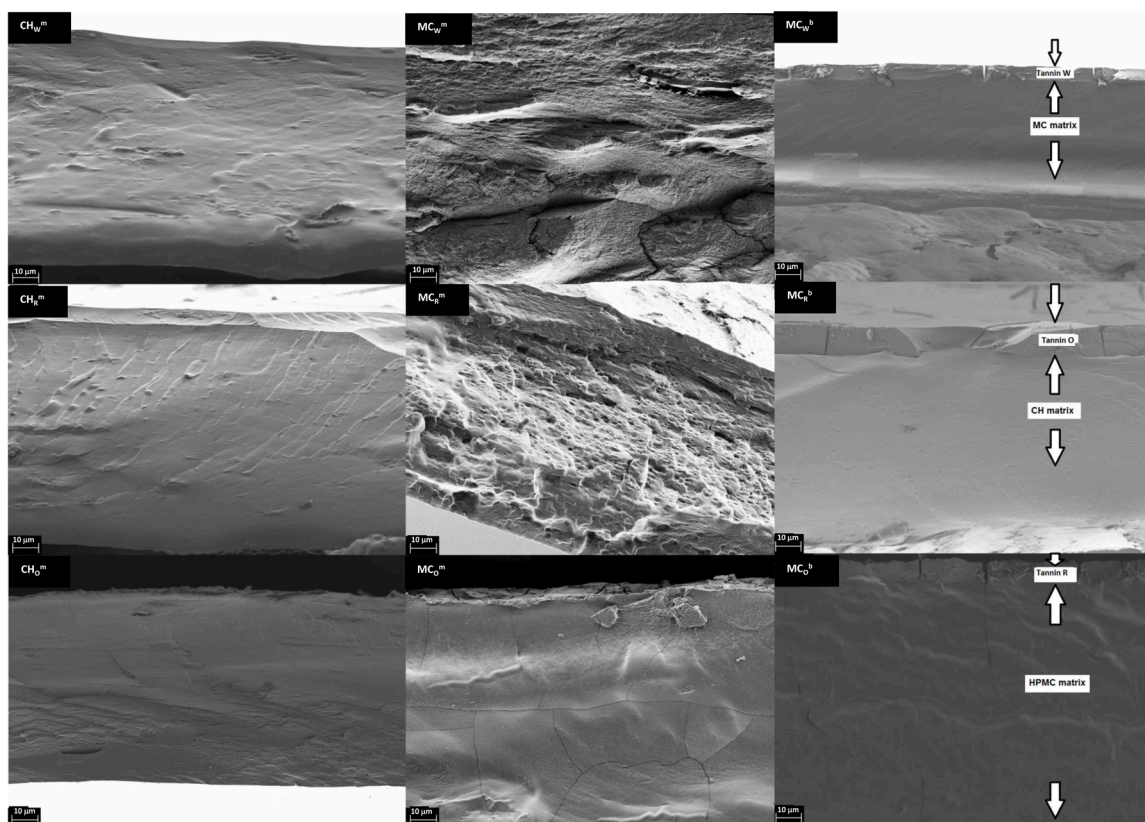
, where [DPPH\*]<sub>T</sub> is the concentration of DPPH\* at the steady state and [DPPH\*]<sub>T=0</sub> is the concentration of DPPH\* at the initial reaction time.

The EC<sub>50</sub> parameter (amount of antioxidant necessary to decrease the initial DPPH\* concentration by 50%) was used to measure the antiradical activity of the films. This value was obtained by plotting the %DPPH\*<sub>REM</sub> vs. molar ratio of antioxidant to DPPH\*, expressed in terms of g of tannin per mole of DPPH\*, taking into account the amount of tannins in each film. Thus, the lower the EC<sub>50</sub> value is, the greater the antioxidant activity of the tested sample will be. In every formulation, three samples were analyzed for each simulant. The antioxidant activity of pure tannin dissolved in the three simulant solutions was also determined in the same way.

### 2.6. Antimicrobial properties

Two bacteria species, one gram-positive (*Listeria innocua*, CECT 910) and one Gram-negative (*E. coli*, CECT 515), which are responsible for most food-related infections, were used to evaluate the antimicrobial effectiveness. The methodology used was previously described by Cano et al. (2020), using a target inoculum of 10<sup>2</sup> CFU/cm<sup>2</sup> of *E. coli* or *L. innocua* supplied by the Spanish Type Culture Collection (CECT, Burjassot, Spain).

Tryptone soy agar with 3% sodium chloride (TSA-NaCl) was used as a model solid food system. Aliquots of TSA-NaCl (10 g) were poured into Petri dishes. After the culture medium solidified, a properly diluted culture was inoculated on the surface. Films of the same diameter as the Petri dishes (containing or not an antimicrobial substance) were placed on the inoculated surfaces, the layer containing the active being in direct contact with the medium. Non-coated inoculated TSA-NaCl Petri dishes were used as controls. Plates were then covered with para-film to prevent dehydration and stored for 10 days at 10 °C. The microbial counts on the TSA-NaCl were examined immediately after the inoculation and periodically throughout the storage period (up to 10 days). To this end, the agar was removed aseptically from Petri dishes, placed in a sterile fitter stomacher bag (Seward, West Sussex, United Kingdom) with 100 ml of tryptone phosphate water and homogenized for 90 s in a Stomacher blender (Bag Mixer 400, Seward, UK). Afterwards, serial dilutions were made and then poured onto microbiological plates, using violet red bilis agar for *E. coli* and palcam agar base supplemented with palcam selective supplement for *L. innocua*. The growth of bacterial colonies was counted after 24–48 h incubation at 37 °C. All the tests were performed in triplicate.



**Fig. 1.** FESEM micrographs of the cross-section of tannin-loaded monolayer chitosan (CH) and methylcellulose (MC) films and some bilayer (b) films containing the different tannins: white peel grape (w), oak bark (O) and red peel grape (R).

## 2.7. Statistical analysis

The results were analyzed by analysis of variance (ANOVA), using the Statgraphics Centurion XV.I (Manugistics Corp., Rockville, MD). Fisher's least significant difference (LSD) was used at the 95% confidence level so as to differentiate samples.

## 3. Results and discussion

### 3.1. Microstructural features

FESEM analyses provide information about the internal arrangement of the films, which depends on the organization and interactions of the different components of the film forming dispersion (FFD) and its evolution during film drying. As the appearance of the films was similar, not all the micrographs have been shown, but a proper selection has been made to show the more remarkable features. Thus, Fig. 1 shows the FESEM micrographs of the cross-section of the composite monolayer films together with the cross-section of some bilayer films incorporating the 3 different tannins. Fig. 2 presents the typical morphology of the surface of mono and bilayer films.

The cross-sections of the pure CH, MC and HPMC polysaccharide films (not shown) were homogeneous and smooth in appearance, similar to what was previously observed by other authors for these films obtained by casting (Pastor et al., 2011; Sánchez-González, Cháfer, Chiralt & González-Martínez, 2010; Pinotti, García, Martino & Zaritzky, 2017). The composite monolayer films (Fig. 1), exhibited a more heterogeneous cross section, revealing a partial miscibility of tannins with the polymer. This effect was very mild for CH-based films and more marked for MC films, especially when using W or R tannins.

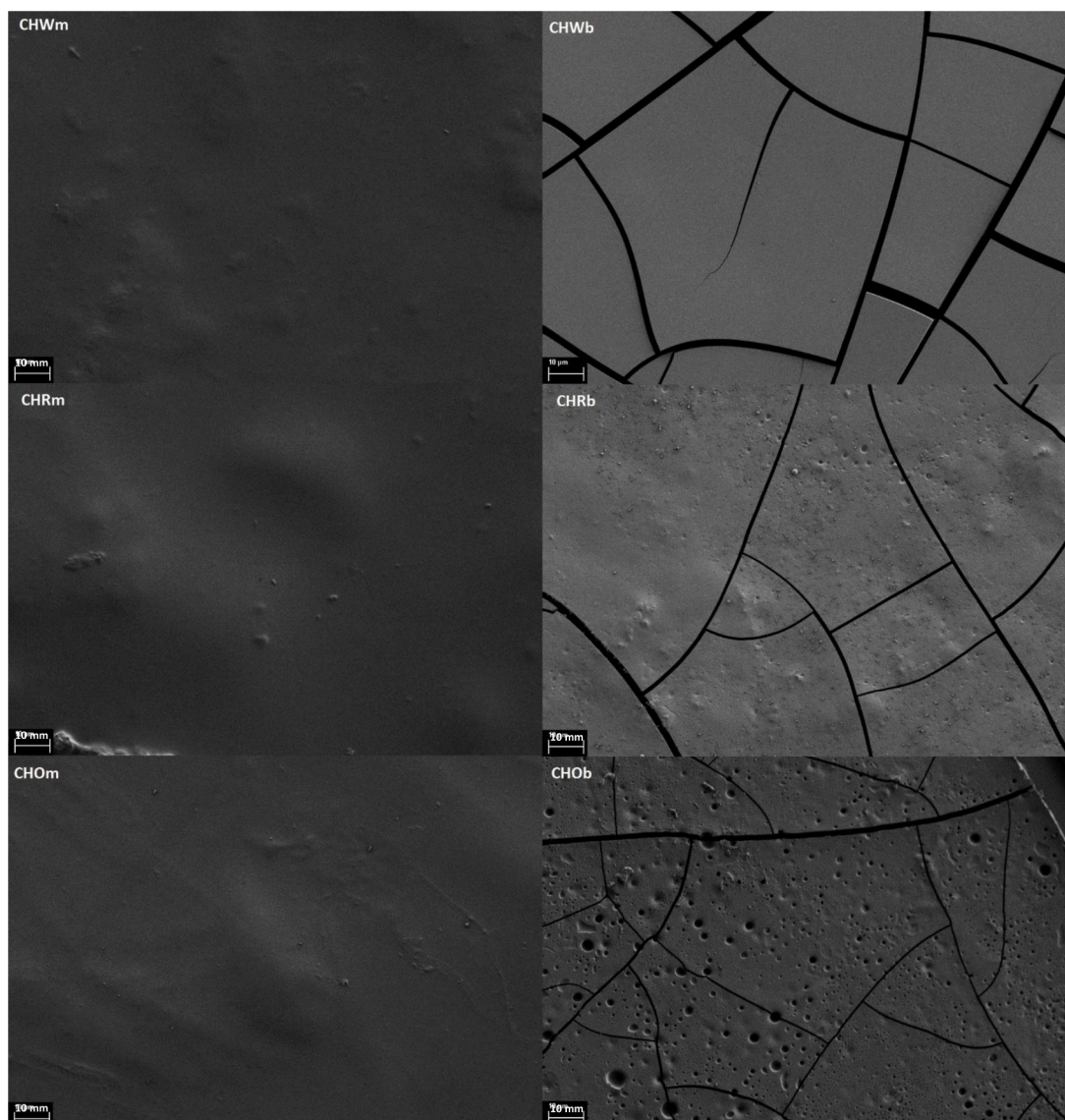
In bilayer films, two different layers were clearly observed: a top tannin-rich layer and a bottom tannin-poor layer (Fig. 1). This points to

the establishment of an abrupt gradient concentration of tannin at the interphase, as expected from the preparation method where tannin solution was cast on the partially dried polymer film. The top tannin layer was compact but showed some cracks due to its fragility in the absence of a high enough polymer ratio. As can be observed in Fig. 2, the surfaces of the top tannin layers exhibited microstructural differences. Thus, the layer containing white tannin (W) was homogeneous and uniform, while the morphology of those containing red (R) or oak (O) tannins showed more discontinuities and pores, especially when using O tannin. Moreover, the surfaces of every bilayer film appeared fractured (Fig. 2) due to the fragility of the tannin layer after drying. Thus, the functionality of these films could be compromised due to the presence of these cracks on the top layer. HPMC films (not shown) exhibited the same behavior as bilayer MC films. However, it is worth noting that the films did not present handling problems.

### 3.2. Mechanical properties

Elastic modulus (EM), tensile strength at break (TS) and percentage of elongation at break (E%) are used to describe the mechanical behavior of films. Table 2 shows the mean values of these mechanical parameters for the films after 2 conditioning weeks at 25 °C and 53% RH. MC control films were less rigid and resistant (lower EM and TS values) but more stretchable (higher E% values) than the pure CH and HPMC films, as previously reported by other authors (Pastor et al., 2013; Sánchez-González et al., 2010; Pinotti et al., 2017; Jiménez, María José Fabra & Chiralt, 2010).

The incorporation of tannins into polysaccharide films led to different types of behavior depending on the polymer matrix. When using CH and MC, tannins acted as reinforcement agents, turning the films more rigid and more resistant to break (around 15–51% and 26–105% greater EM and TS values, respectively). A similar behavior



**Fig. 2.** FESEM features of the surface of mono (m) and bilayer (b) chitosan films containing the different tannins: white peel grape (w), oak bark (O) and red peel grape (R).

has been found by other authors working with the same polymers but using other polyphenolic compounds (Yu et al., 2015; Talon et al., 2017).

In general, this effect was dependent on the effective tannin:polymer ratio. Thus, at a high tannin:polymer ratio (such as occurred at the interphase of the bilayer films), a mechanical reinforcement was promoted (EM values increased around 3–21% and 11–40% in comparison with their respective monolayers for CH and MC films, respectively) than at low ratios, when tannins were diluted in the polysaccharide matrix (monolayers).

This reinforcement effect was the result of the establishment of tannin-CH/MC interactions. As commented on above, polyphenol interactions with polysaccharides are chiefly hydrogen-bonding and hydrophobic interactions (De Freitas, Carvalho & Mateus, 2003; Renard et al., 2017; Yu et al., 2015). These authors suggested that tannins disrupt the hydrogen bonds between chitosan chains by creating new interactions between hydroxyl groups of the tannin molecules and the positively charged amino groups of chitosan, at the pH of the film forming solutions ( $\text{pH} \leq 5.2$ ) (Siripatrawan & Harte, 2010). The possibility that polyphenols may establish also covalent bonding with polar reactive groups of chitosan has been also proposed by several authors

(Zhang & Kosaraju, 2007 and Siripatrawan & Harte, 2010), and verified later on by FTIR techniques. These interactions could have been favoured when using W tannin with the lowest pH (of around 2.5), as these tannin-rich films exhibited the greatest EM and tensile strength values. This is probably due to the higher total phenolic content of W tannin (82 mg GAE/g) as compare to O or R tannins or to the presence of more  $-\text{NH}_3^+$  residues in the polymer chain.

As concerns methylcellulose, specific hydrogen bonds can be formed between phenolic hydroxyl groups on tannins with aliphatic hydroxyl groups on methylcellulose. Moreover, notable hydrophobic interactions can also take place between the methoxyl groups on methylcellulose and the aromatic nuclei of the tannins (Chen, Yu, Tsai, Tang & Mi, 2010; Patel, Seijen-ten-Hoorn & Velikov, 2011; Patel, Seijen-ten-Hoorn & Hazekamp, 2013).

On the other hand, the observed incompatibility between HPMC and tannins, which hindered their solution blend, promoted the formation of layers of low affinity and poor cohesiveness, thus leading to a mechanically weak bilayer material (lower TS values,  $p < 0.05$ ) than HPMC monolayer films, while non-significant changes in the EM values were observed.

In every case, the stretchability of tannin-loaded films was reduced

**Table 2**

Elastic modulus (EM, MPa), tensile strength at break (TS, MPa) and percentage of elongation at break (E,%) of CH, MC, HPMC and composite films conditioned during two weeks. Mean values  $\pm$  standard deviation.

Sample	EM (MPa)	TS (MPa)	E (%)
CH	1850 $\pm$ 40 <sup>bc3</sup>	64 $\pm$ 4 <sup>b2</sup>	11 $\pm$ 4 <sup>b1</sup>
CH <sub>W</sub> <sup>m</sup>	2120 $\pm$ 170 <sup>d1</sup>	86 $\pm$ 13 <sup>d1</sup>	4.7 $\pm$ 0.7 <sup>a1</sup>
CH <sub>R</sub> <sup>m</sup>	1580 $\pm$ 60 <sup>a2</sup>	55 $\pm$ 3 <sup>a2</sup>	4.7 $\pm$ 0.9 <sup>a1</sup>
CH <sub>O</sub> <sup>m</sup>	1850 $\pm$ 30 <sup>b1</sup>	70.6 $\pm$ 1.6 <sup>b1</sup>	5.1 $\pm$ 0.5 <sup>a1</sup>
CH <sub>W</sub> <sup>b</sup>	2190 $\pm$ 70 <sup>d2</sup>	81 $\pm$ 6 <sup>cd2</sup>	5.1 $\pm$ 0.6 <sup>a1</sup>
CH <sub>R</sub> <sup>b</sup>	1920 $\pm$ 50 <sup>c3</sup>	69 $\pm$ 2 <sup>b2</sup>	5.90 $\pm$ 1.13 <sup>a2</sup>
CH <sub>O</sub> <sup>b</sup>	2110 $\pm$ 60 <sup>d2</sup>	79 $\pm$ 2 <sup>c2</sup>	5.8 $\pm$ 0.7 <sup>a2</sup>
MC	1300 $\pm$ 300 <sup>a2</sup>	38 $\pm$ 14 <sup>a1</sup>	21 $\pm$ 5 <sup>c2</sup>
MC <sub>W</sub> <sup>m</sup>	1800 $\pm$ 400 <sup>bc1</sup>	78 $\pm$ 12 <sup>b1</sup>	4.9 $\pm$ 0.6 <sup>a1</sup>
MC <sub>R</sub> <sup>m</sup>	1300 $\pm$ 120 <sup>a1</sup>	42 $\pm$ 4 <sup>a1</sup>	5.01 $\pm$ 1.18 <sup>a1</sup>
MC <sub>O</sub> <sup>m</sup>	1900 $\pm$ 200 <sup>bc1</sup>	72 $\pm$ 8 <sup>b1</sup>	6.46 $\pm$ 1.18 <sup>ab2</sup>
MC <sub>W</sub> <sup>b</sup>	2000 $\pm$ 200 <sup>c2</sup>	65 $\pm$ 21 <sup>b1</sup>	5 $\pm$ 3 <sup>a1</sup>
MC <sub>R</sub> <sup>b</sup>	1800 $\pm$ 200 <sup>b2</sup>	65 $\pm$ 8 <sup>b2</sup>	8 $\pm$ 3 <sup>b3</sup>
MC <sub>O</sub> <sup>b</sup>	1900 $\pm$ 100 <sup>bc1</sup>	65 $\pm$ 11 <sup>b1</sup>	4.5 $\pm$ 0.9 <sup>a1</sup>
HPMC	1695 $\pm$ 40 <sup>b1</sup>	80 $\pm$ 9 <sup>c3</sup>	18 $\pm$ 4 <sup>b2</sup>
HPMC <sub>W</sub> <sup>b</sup>	1662 $\pm$ 87 <sup>b1</sup>	53 $\pm$ 10 <sup>b1</sup>	3.8 $\pm$ 0.9 <sup>a1</sup>
HPMC <sub>R</sub> <sup>b</sup>	1179 $\pm$ 65 <sup>a1</sup>	17 $\pm$ 4 <sup>a1</sup>	1.7 $\pm$ 0.4 <sup>a1</sup>
HPMC <sub>O</sub> <sup>b</sup>	1695 $\pm$ 126 <sup>b1</sup>	61 $\pm$ 4 <sup>b1</sup>	4.6 $\pm$ 0.4 <sup>a1</sup>

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

Different numbers (1,2,3) within a column indicate significant differences among polysaccharide matrices for the same tannin ( $p < 0.05$ ).

**Table 3**

Film's thickness, moisture content, solubility and water vapor permeability (WVP) of CH, MC, HPMC and composite films conditioned during two weeks. Mean values  $\pm$  standard deviation.

Sample	Thickness (mm)	Moisture content (%d.b)	Solubility (% d.b)	WVP (g•mm/kPa•h•m <sup>2</sup> )
CH	0.135 $\pm$ 0.005 <sup>d, 3</sup>	17.99 $\pm$ 0.09 <sup>e3</sup>	35 $\pm$ 7 <sup>cd1</sup>	8.75 $\pm$ 0.19 <sup>cd3</sup>
CH <sub>W</sub> <sup>m</sup>	0.1284 $\pm$ 0.0109 <sup>c,1</sup>	15.30 $\pm$ 0.05 <sup>a2</sup>	31.37 $\pm$ 1.05 <sup>c2</sup>	5.2 $\pm$ 0.2 <sup>a2</sup>
CH <sub>R</sub> <sup>m</sup>	0.147 $\pm$ 0.008 <sup>f,2</sup>	16.58 $\pm$ 0.06 <sup>b2</sup>	19.99 $\pm$ 0.04 <sup>a1</sup>	8.5 $\pm$ 0.5 <sup>c2</sup>
CH <sub>O</sub> <sup>m</sup>	0.139 $\pm$ 0.006 <sup>e,2</sup>	16.5 $\pm$ 0.2 <sup>b1</sup>	20.93 $\pm$ 0.04 <sup>ab1</sup>	9.1 $\pm$ 0.4 <sup>d2</sup>
CH <sub>W</sub> <sup>b</sup>	0.121 $\pm$ 0.006 <sup>b,3</sup>	17.4 $\pm$ 0.4 <sup>d2</sup>	36.76 $\pm$ 1.12 <sup>d2</sup>	5.1 $\pm$ 0.5 <sup>a2</sup>
CH <sub>R</sub> <sup>b</sup>	0.121 $\pm$ 0.004 <sup>b,3</sup>	16.54 $\pm$ 0.17 <sup>b3</sup>	23.0 $\pm$ 0.6 <sup>ab1</sup>	6.8 $\pm$ 0.3 <sup>b3</sup>
CH <sub>O</sub> <sup>b</sup>	0.115 $\pm$ 0.004 <sup>a,3</sup>	17.1 $\pm$ 0.2 <sup>c3</sup>	25.1 $\pm$ 0.8 <sup>b1</sup>	6.3 $\pm$ 0.2 <sup>b3</sup>
MC	0.067 $\pm$ 0.005 <sup>a,1</sup>	3.94 $\pm$ 0.07 <sup>a1</sup>	99.7 $\pm$ 0.5 <sup>c2</sup>	2.9 $\pm$ 0.2 <sup>a1</sup>
MC <sub>W</sub> <sup>m</sup>	0.15 $\pm$ 0.04 <sup>d,2</sup>	8.74 $\pm$ 0.13 <sup>d1</sup>	21.3 $\pm$ 0.3 <sup>a1</sup>	4.2 $\pm$ 0.4 <sup>c1</sup>
MC <sub>R</sub> <sup>m</sup>	0.13 $\pm$ 0.05 <sup>c,1</sup>	5.9 $\pm$ 0.7 <sup>c1</sup>	51.5 $\pm$ 0.6 <sup>c2</sup>	3.4 $\pm$ 0.2 <sup>b1</sup>
MC <sub>O</sub> <sup>m</sup>	0.122 $\pm$ 0.017 <sup>c, 1</sup>	6.01 $\pm$ 0.17 <sup>c2</sup>	58.11 $\pm$ 1.03 <sup>d2</sup>	4.8 $\pm$ 0.4 <sup>d2</sup>
MC <sub>W</sub> <sup>b</sup>	0.093 $\pm$ 0.007 <sup>b,1</sup>	6.3 $\pm$ 0.6 <sup>c1</sup>	34.1 $\pm$ 1.4 <sup>b1</sup>	2.74 $\pm$ 0.12 <sup>a1</sup>
MC <sub>R</sub> <sup>b</sup>	0.102 $\pm$ 0.012 <sup>b,1</sup>	5.83 $\pm$ 0.13 <sup>c1</sup>	53 $\pm$ 7 <sup>c2</sup>	4.3 $\pm$ 0.6 <sup>cd2</sup>
MC <sub>O</sub> <sup>b</sup>	0.094 $\pm$ 0.009 <sup>b,1</sup>	4.5 $\pm$ 0.3 <sup>b1</sup>	54.7 $\pm$ 1.2 <sup>cd2</sup>	3.65 $\pm$ 0.18 <sup>b2</sup>
HPMC	0.092 $\pm$ 0.006 <sup>a,2</sup>	5.07 $\pm$ 0.13 <sup>a2</sup>	99.7 $\pm$ 0.7 <sup>c2</sup>	3.41 $\pm$ 0.09 <sup>d2</sup>
HPMC <sub>W</sub> <sup>b</sup>	0.099 $\pm$ 0.012 <sup>b,2</sup>	5.7 $\pm$ 0.3 <sup>a1</sup>	87.9 $\pm$ 1.8 <sup>a3</sup>	2.35 $\pm$ 0.14 <sup>a1</sup>
HPMC <sub>R</sub> <sup>b</sup>	0.098 $\pm$ 0.02 <sup>c,2</sup>	8.4 $\pm$ 0.9 <sup>b2</sup>	93 $\pm$ 5 <sup>ab3</sup>	3.20 $\pm$ 0.15 <sup>c1</sup>
HPMC <sub>O</sub> <sup>b</sup>	0.0981 $\pm$ 0.0108 <sup>b,2</sup>	5.62 $\pm$ 1.18 <sup>a2</sup>	93.8 $\pm$ 0.9 <sup>b3</sup>	2.92 $\pm$ 0.09 <sup>b1</sup>

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

Different numbers (1,2,3) within a column indicate significant differences among polysaccharide matrices for the same tannin ( $p < 0.05$ ).

(lower E% values) up to 57%, 78% and 90% for CH, MC and HPMC based films, respectively. This was coherent with both the formation of a more resistant tied network where the plastic behavior of the films was limited, and the formation of a cross-linked structure, where tannin links hindered the polymer chain slippage during the tensile test.

All the above-mentioned changes were less noticeable when incorporating R tannin into the films. Several authors pointed out that intrinsic phenolic factors other than the number of peripheral hydroxyl groups of the phenol compounds may also influence the characteristics of the binding events such as the molecular weight, size and number of phenolic groups in each molecule, the native charge of polyphenols, molecular flexibility or the exposure of the polysaccharide binding sites (Dobson et al., 2019; Popa, Aelenei, Popa & Andrei, 2000; Tang, Yu, Ho, Huang & Tsai et al., 2003).

The obtained results suggest that only the polysaccharides favoring hydrophobic/H-bonding collaborative interactions and exhibiting the non-steric hindrance of the binding sites by side chains, such as CH and MC, were prone to binding polyphenols more effectively, thus leading to mechanically enhanced films.

### 3.3. Moisture content, solubility and water vapor barrier properties

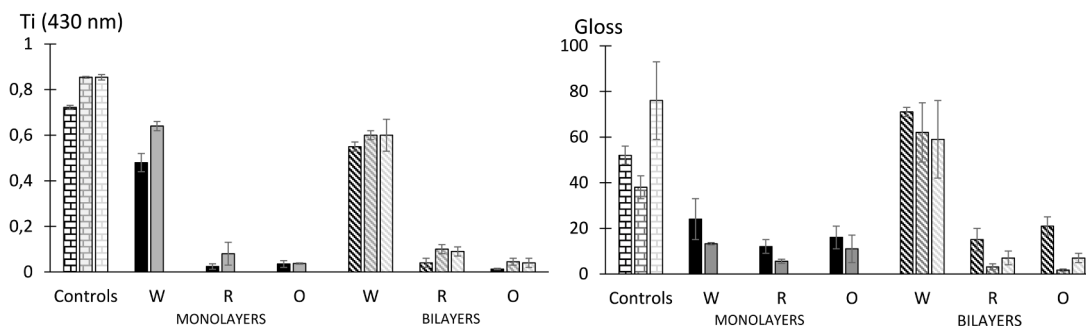
Table 3 shows the thickness, moisture content, solubility and WVP values of the studied films, conditioned for 2 weeks at 25 °C and 53% RH. The WVP and MC values of tannin-free films were in the range of those reported by other authors (Pastor et al., 2013; Jiménez et al., 2010; Sánchez-González et al., 2009) for CH, MC and HPMC, respectively.

The incorporation of tannins affected the thickness, moisture content, solubility and WVP values of the films, depending on the polymer and tannin used. The major changes were detected when using CH and MC as film matrices, whereas almost no changes were detected in HPMC bilayer films. The incompatibility of HPMC with tannins led to non-effective interactions of both components at the interface and HPMC bilayers exhibited similar behavior to the respective monolayer.

In general, the solubility of CH and MC significantly decreased ( $p < 0.05$ ) up to 42% and to 79% when tannins were incorporated in CH and MC films, respectively, regardless of the manufacturing method used (mono or bilayer). This agrees with the formation of non-covalent complexes of both CH and MC where hydrophilic points in the chains are blocked, thus making the film less water soluble (De Freitas et al., 2003). Likewise, the formation of hydrogen bonds between polymer molecules and tannins would imply the re-orientation of the hydrophobic side of the polymer chain, which would reduce the film solubility in water (Bajić, Novak & Ikozar, 2019; Lemes et al., 2018; Peña, de la Cabra, Eceiza, Ruseckaitė & Mondragon, 2010). Yu et al. (2015) also observed a remarkable reduction in the solubility of methylcellulose films incorporating tea catechins. This effect was also observed in bilayer films, thus suggesting tannins were able to partially diffuse to the inner parts of the polysaccharide matrices, thus establishing a tannin concentration gradient inside the films.

The WVP values usually depend on the film composition and the component spatial arrangement inside the matrix, among other factors. As can be observed in Table 3, pure CH films showed greater WVP values ( $p < 0.05$ ) than pure MC or HPMC films, as reported by other authors (Pastor et al., 2013; Jiménez et al., 2010; Sánchez-González et al., 2009) coherently with their more hydrophilic nature, as revealed by their higher equilibrium moisture content.

The addition of tannins significantly enhanced ( $p < 0.05$ ) the water vapor barrier capacity of the films with greater WVP values. This improvement can be related to the development of a more packaged, tighter structure (as shown by the mechanical parameters, Table 2) as well as to the increase in the hydrophobicity of the interchain paths where the permeation of water molecules is more limited. In CH-based films, this improvement was more effective when using the W tannin as the WVP values significantly ( $p < 0.05$ ) decreased by around 40%,



**Fig. 3.** Transmittance (Ti) and gloss values of pure films (brick background), monolayers (full background) and bilayers (raster background) incorporating the different tannins. (CH:black; MC: dark gray; HPMC: soft gray).

**Table 4**

Efficient concentration values ( $EC_{50}$ ) of pure tannins (W, white tannin; R, red tannin and O, oak bark tannin) and composite films in contact with different food simulants. Mean values  $\pm$  standard deviation.

Sample/Simulant	$EC_{50}$ (g tannin/mol DPPH)		
	Ethanol 10%	Acetic acid 3%	Ethanol 50%
W	25.10 $\pm$ 1.04 <sup>a</sup>	31 $\pm$ 4 <sup>a</sup>	27.8 $\pm$ 1.3 <sup>a</sup>
R	40 $\pm$ 2 <sup>b</sup>	56 $\pm$ 6 <sup>b</sup>	50.4 $\pm$ 1.7 <sup>b</sup>
O	47 $\pm$ 8 <sup>b</sup>	49.0 $\pm$ 1.4 <sup>b</sup>	61 $\pm$ 8 <sup>c</sup>
CH <sub>W</sub> <sup>m</sup>	110 $\pm$ 5 <sup>a2</sup>	23.8 $\pm$ 1.2 <sup>a2</sup>	62 $\pm$ 3 <sup>a2</sup>
CH <sub>R</sub> <sup>m</sup>	452 $\pm$ 56 <sup>c2</sup>	139 $\pm$ 12 <sup>c2</sup>	320 $\pm$ 23 <sup>d2</sup>
CH <sub>O</sub> <sup>m</sup>	514 $\pm$ 4 <sup>d2</sup>	110 $\pm$ 2 <sup>b2</sup>	503 $\pm$ 25 <sup>e2</sup>
CH <sub>W</sub> <sup>b</sup>	127 $\pm$ 8 <sup>a2</sup>	28.2 $\pm$ 0.9 <sup>a1</sup>	62 $\pm$ 6 <sup>a2</sup>
CH <sub>R</sub> <sup>b</sup>	174 $\pm$ 5 <sup>b2</sup>	131 $\pm$ 6 <sup>c3</sup>	142 $\pm$ 17 <sup>c3</sup>
CH <sub>O</sub> <sup>b</sup>	130 $\pm$ 7 <sup>a2</sup>	137 $\pm$ 6 <sup>c3</sup>	109 $\pm$ 4 <sup>b2</sup>
MC <sub>W</sub> <sup>m</sup>	24 $\pm$ 2 <sup>a1</sup>	14 $\pm$ 2 <sup>a1</sup>	24.2 $\pm$ 1.6 <sup>a1</sup>
MC <sub>R</sub> <sup>m</sup>	47.8 $\pm$ 1.5 <sup>cd1</sup>	59 $\pm$ 11 <sup>c1</sup>	54 $\pm$ 13 <sup>d1</sup>
MC <sub>O</sub> <sup>m</sup>	53 $\pm$ 4 <sup>d1</sup>	54 $\pm$ 6 <sup>c1</sup>	49 $\pm$ 4 <sup>cd1</sup>
MC <sub>W</sub> <sup>b</sup>	32 $\pm$ 4 <sup>b1</sup>	34 $\pm$ 7 <sup>b1</sup>	26.6 $\pm$ 1.3 <sup>ab1</sup>
MC <sub>R</sub> <sup>b</sup>	41 $\pm$ 5 <sup>c1</sup>	35 $\pm$ 4 <sup>b1</sup>	40 $\pm$ 4 <sup>bc1</sup>
MC <sub>O</sub> <sup>b</sup>	44 $\pm$ 5 <sup>c1</sup>	35.15 $\pm$ 1.19 <sup>b1</sup>	56 $\pm$ 5 <sup>d1</sup>
HPMC <sub>W</sub> <sup>b</sup>	28 $\pm$ 4 <sup>a1</sup>	23 $\pm$ 3 <sup>a1</sup>	19.4 $\pm$ 0.4 <sup>a1</sup>
HPMC <sub>R</sub> <sup>b</sup>	79 $\pm$ 18 <sup>b1</sup>	56 $\pm$ 9 <sup>b2</sup>	36 $\pm$ 3 <sup>b2</sup>
HPMC <sub>O</sub> <sup>b</sup>	48 $\pm$ 8 <sup>a1</sup>	59 $\pm$ 6 <sup>b2</sup>	61 $\pm$ 6 <sup>c1</sup>

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

Different numbers (1,2,3) within a column indicate significant differences among polysaccharide matrices for the same tannin ( $p < 0.05$ ).

surely due to its higher total phenolic content commented on above.

In general, lower WVP values were obtained in bilayer films than in monolayer ones, due to the major resistance to water mass transfer that the tannin-rich layer offers in the bilayer, as has been previously reported by other authors (Kamper & Fennema, 1984; Martin-Polo, Mauguin & Voilley, 1992). Similar results were also obtained by Cano et al. (2020) working with monolayer and bilayer tannin-protein based films.

### 3.4. Optical properties

As regards the optical properties, Fig. 3 shows the gloss and internal transmittance (Ti) at 430 nm, as a measurement of the transparency of the films (low Ti values indicate more opaque films). Control films (CH, MC and HPMC) were highly transparent, colourless and glossier, in agreement with that previously reported by other authors (Jiménez et al., 2010; Pastor et al., 2013).

The optical properties of the tannin-rich films were mainly affected by the type of tannin. The incorporation of tannins provoked a significant reduction ( $p < 0.05$ ) in the transparency (lower Ti values), especially when R and O tannins were used. In fact, films containing R and O tannins were practically opaque, reaching Ti values of under 0.1.

In general, the addition of tannins significantly decreased the gloss of

the films ( $p < 0.05$ ), except in the case of those bilayers containing the W tannin. Thus, b:CH-W and b:MC-W were glossier ( $p < 0.05$ ) than the pure films as a consequence of the presence of a uniform W-tannin top layer with a homogeneous surface with few irregularities, as deduced from the FESEM micrographs (Fig. 2). Nevertheless, this effect was not observed when using the glossiest HPMC polymeric films.

### 3.5. Antioxidant capacity

Table 4 shows the  $EC_{50}$  values for both the pure tannins and the different composite films. Both pure tannins and the composite films were found to react quickly with the DPPH\* (fast kinetic behavior) taking around 40 min to reach the steady state. For all simulants, the  $EC_{50}$  values obtained for the pure W tannin were significantly ( $p < 0.05$ ) lower, thus indicating its greater antioxidant capacity, in agreement with its higher phenolic content (Cano et al., 2020).

As expected, no antioxidant activity (AA) was found in control films (pure CH, HPMC and MC). The antioxidant activity of the composite films depended on the film solubility and the formation of reversible tannin-polymer complexes, which entrap the tannin molecules, inhibiting their redox reaction.

The  $EC_{50}$  values of composite films, expressed in terms of g of tannin per mole DPPH\*, showed that no losses in antioxidant capacity occurred for MC and HPMC tannin-rich films, regardless of the simulant or the manufacture method used, since the  $EC_{50}$  values obtained for these films were similar to those of the pure tannins. These results are coherent with the complete solubilisation of HPMC and the partial solubilisation of MC-based films that released the phenols into the aqueous media, favouring their redox reaction. This also indicates that the formation of tannin-cellulose complexes was reversible, not affecting the antioxidant activity of the compounds that became unbound once the polymer matrix was hydrated (Yu et al., 2015). So, their potential to act as antioxidant films by controlled release is of great interest.

As concerns CH, the  $EC_{50}$  values obtained by the composite chitosan films were greatly affected by the type of tannin and, to a lesser extent, by the simulant. Thus, CH films incorporating R and O tannins in all simulants and the W tannin in non-acidic simulants (ET10% and ET50%) exhibited unexpectedly high  $EC_{50}$  values (low antioxidant capacity). This could be explained by the low solubility of these films and by the formation of irreversible/strong tannin-chitosan complexes with limited release of the antioxidant compound in the reaction medium. Popa et al. (2000) found similar results for chitosan with spruce wood bark (rich in condensed tannins), where polyphenol retention was achieved through strong chemical bonds. On the contrary, when using 3% acetic simulant, the W-rich CH films exhibited similar antioxidant activities to the pure W tannin. So, the obtained results suggested that only the W-tannin was able to form reversible tannin-chitosan complexes, which easily released tannin into the acidic medium once the chitosan matrix was extensively hydrated and solubilized in the diluted aqueous acidic solution (Caiqin, Huiron, Qi, Yi & Juncheng, 2006).

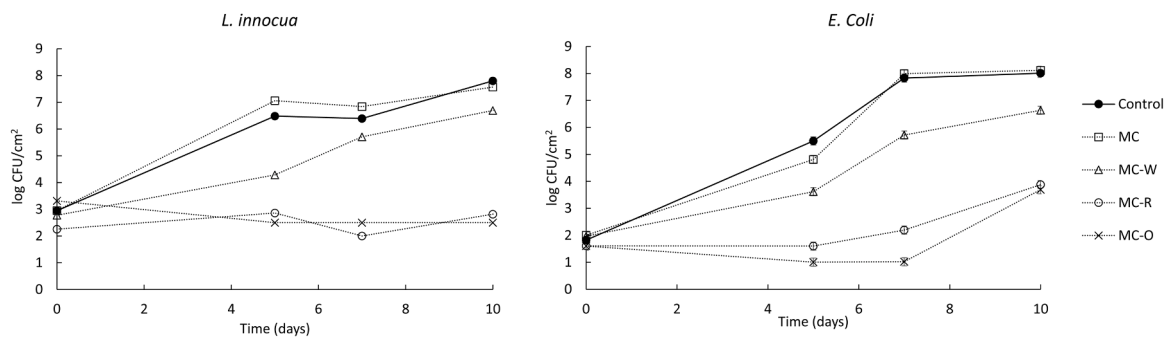


Fig. 4. Effect of methylcellulose (MC) bilayer films incorporating white peel grape (W), oat bark (O) and red peel grape (R) tannins on the growth and survival of *L. innocua* and *E. coli* on TSA-NaCl medium stored at 10 °C.

The functional activity of the active agent incorporated into a film matrix has been shown to depend on several factors, such as its release capacity from the matrix to the simulant.

According to Sanchez-Gonzalez et al. (2010), the release of an active compound from a polymeric network is dependent on the solvent migration to the polymer matrix, leading to network weakening and the diffusion of the active agent through the polymer matrix to the food simulant liquid phase, which in turn, depends on the nature of the interactions established between the active compound and the matrix.

In general, the effect of the manufacturing method on the antioxidant activity was very mild, only being significant ( $p < 0.05$ ) when using chitosan as a film matrix. Thus, CH-bilayers exhibited lower  $EC_{50}$  values than CH monolayers ( $p < 0.05$ ), due to the presence of more tannins unaffected by the polysaccharide interactions, since these were mainly present on the film surface that is poor in chitosan. This favours the preservation of their functional activity and release.

The multifactor ANOVA showed that, in general, the type of simulant did not significantly affect the antioxidant capacity of the tannins (except for CH-W films). Thus, once the tannins were released from the polymeric matrix, its functionality was not significantly affected by the simulant's characteristics, in all likelihood due to the amphiphilic nature of tannins (Mao, Ma, Liao, Zhao & Liao, 2013; Schofield, Mbugua & Pell, 2001).

### 3.6. Antimicrobial activity

The antimicrobial activity was only studied in MC bilayer films incorporating the different tannins, as these films showed the best physical and antioxidant properties when compared with the other matrices and because of their greater antimicrobial potential due to the more ready availability of tannins on the surface of these films, as commented on above.

The population viability of *Listeria innocua* (Gram +) and *E. coli* (Gram -) in control plates (without films) and in plates coated with the different film samples is shown in Fig. 4a and b, respectively. For both facultative anaerobic microorganisms, the population increased from around 2.5–3 to 8 logs CFU/cm<sup>2</sup> at the end of the storage period in the control plates (10 days).

As expected, for tannin-free MC films, no antimicrobial activity was observed against either *L. innocua* or *E. coli* throughout the incubation time at 10 °C. In these films, the *Listeria* and *E. coli* growth was very similar to that of the control plates (without film). The MC bilayer incorporating the different tannins exhibited antimicrobial activity against *Listeria* and *E. coli*, which depended on the tannin used.

The antimicrobial activity of different tannins and polyphenols against both of these bacteria has previously been reported by several authors (Taguri, Tanaka & Kouno, 2004; Xu et al., 2015; Cano et al., 2020). The antimicrobial action of the tannins has been seen to be related to the inhibition of extracellular microbial enzymes, the deprivation of the substrates required for microbial growth (such as metal

ions) or direct action on microbial metabolism through the inhibition of oxidative phosphorylation (Ribeiro, Simões and Simões, 2018).

The minimal inhibitory concentration (MIC) values of the pure tannins used for *L. innocua* and *E. coli* have been reported to range from 6 to 25 mg/ml, the lowest value corresponding to the W tannin (Cano et al., 2020).

MC bilayer composite films exhibited a bacteriostatic effect against both tested bacteria throughout the first 5 days of incubation, probably due to the fact that the total release of tannin concentration into the agar plate would imply around 7 mg/ml, which was below the MIC value. This period lasted longer when using O and R tannins, especially against *Listeria* because of the Gram positive bacteria. After this period, a fast microbial growth was observed, especially in the case of the W-tannin. This could be explained by the lower solubility values of these films (Table 3), which could limit the active release throughout the incubation time.

MC bilayer composite films exhibited a bacteriostatic effect against both tested bacteria throughout the first 5 days of incubation, probably due to the fact that the total release of tannin concentration into the agar plate would imply around 7 mg/ml, which was below the MIC values (Cano et al., 2020). This bacteriostatic period lasted longer when using O and R tannins. After this period, a fast microbial growth was observed, especially in the case of the W-tannin. This could be explained by the lower solubility values of these W- tannin films (Table 3), which could limit the active release throughout the incubation time.

## 4. Conclusions

The incorporation of tannins into the polysaccharide film matrices affected both the microscopic morphology and the functional properties of the films, depending on the ability of polymer chains to interact with tannins. Some polysaccharides, such as MC and CH, were prone to binding polyphenols effectively throughout hydrophobic and H-bonding interactions. In these composite films, tannins acted as cross-linking agents, turning the films less water soluble, more rigid, less stretchable, more opaque and less glossy, regardless of the tannin used. Regarding the manufacture method, bilayers films exhibited better barrier and antioxidant properties, surely due to the more ready availability of tannins on the surface of these films. Likewise, all MC bilayer tannin-rich films showed bacteriostatic activity against *L. innocua* and *E. coli*, especially when incorporating R or O tannins. The results highlight the possibility of using MC bilayer films, incorporating tannins for food coating or packaging purposes, either to extend the shelf life of products sensitive to oxidative processes or to control bacterial growth. Further studies must be conducted in real food systems to verify the obtained results.

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