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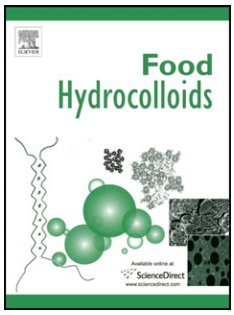

Physical and antioxidant properties of chitosan and methylcellulose based films containing resveratrol

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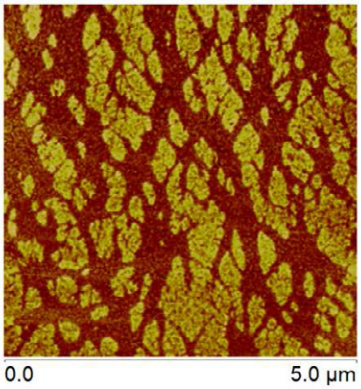

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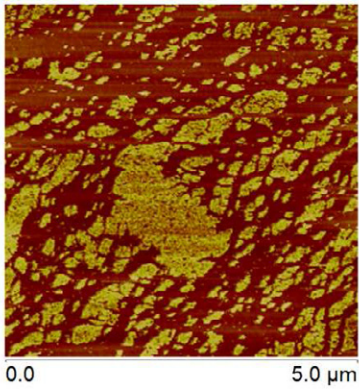

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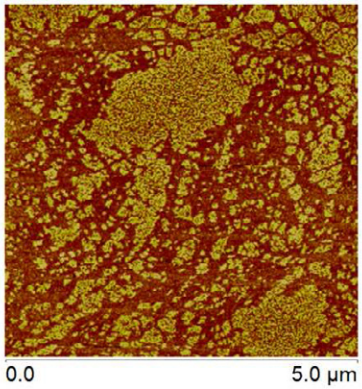

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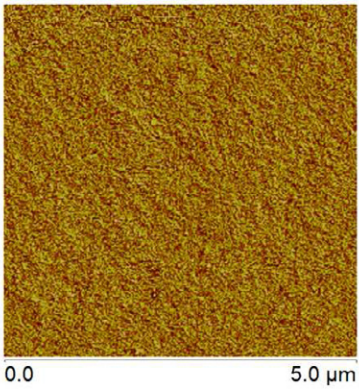

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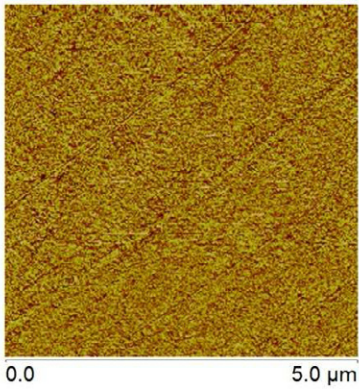

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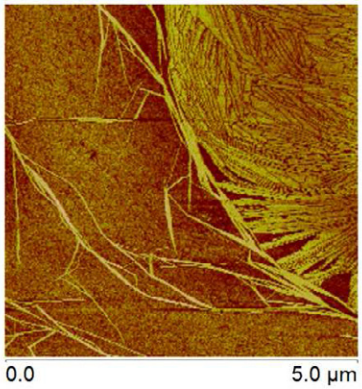

MC-R100

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Physical and antioxidant properties of chitosan and methylcellulose based films containing resveratrol

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Abstract

New trends in edible films focus on the improvement of their functionality through the incorporation of active compounds, such as antimicrobial or antioxidant agents. Resveratrol is a natural antioxidant found in a variety of plant species, such as grapes, and could be used for minimizing or preventing lipid oxidation in food products, retarding the formation of oxidation products, maintaining nutritional quality and prolonging the food shelf life. The aim of this work was to develop and characterize two different polymeric composite films (made with chitosan (CH) and methylcellulose (MC)) containing different amounts of resveratrol. This compound could be incorporated efficiently into both films, but provoke structural changes in the matrices, which became less stretchable (65-70% reduction of deformation at break at the greatest resveratrol content) and resistant to fracture (26 and 54% reduction of tensile at break for MC and CH, respectively, at the greatest resveratrol content) more opaque (significant reduction of the internal transmittance) and less glossy (about 60-65% reduction of gloss at the greatest resveratrol content). Film barrier properties were hardly improved by the presence of resveratrol; water vapour and oxygen permeability tend to slightly decrease when resveratrol was incorporated into both polymers.

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Composite films showed antioxidant activity, which was proportional to the resveratrol concentration in the film. None of the films showed antimicrobial activity against *Penicillium italicum* and *Botrytis cinerea.* Thus, these films could be applied to food products which are sensitive to oxidative processes to prolong their shelf life.

Keywords: microstructure, water vapour permeability, oxygen permeability, mechanical and optical properties, antioxidant activity

1. Introduction

Bioactive edible films may be considered as a natural and biodegradable alternative to chemical preservatives in order to extend food shelf life. Besides acting as protective barriers, these films can be used as carriers of bioactive compounds, such as antimicrobials and antioxidants. Among the biopolymers used to formulate edible films, cellulose derivatives such as methylcellulose (MC), are interesting film forming compounds, as they are odourless, tasteless and biodegradable (Krochta and Mulder-Johnston, 1997). Another biopolymer with excellent film forming ability is chitosan (Li et al. 1992). This non-toxic compound, obtained by deacetylation of chitin, a structural component present in the shell of some crustaceans, presents antimicrobial properties. Edible films should be designed to fulfil a number of requirements, such as having proper mechanical properties, good appearance (adequate gloss and transparency) and water and gas barrier properties.

Resveratrol (3,5,4'-trihydroxystilbene) is a natural polyphenol found in a variety of plant species such as grapes, mulberries and peanuts. This molecule possesses interesting antioxidant and antifungal properties. Antioxidant capacity of resveratrol has largely been studied in vitro by applying physico-chemical methods. Murcia and

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Martínez-Tomé (2001) characterized the antioxidant activity of this stilbene and compared it with those obtained for other antioxidants (alfa-tocopherol, vanillin, butylated hydroxytoluene (BHT), butylated hydroxyacetone (BHA), phenol, propyl gallate, sodium tripolyphosphate). Among the tested molecules, only BHA showed a greater antioxidant activity than resveratrol to inhibit lipid peroxidation. Soto-Valdez et al. (2010) reported that resveratrol had a higher radical-scavenging capacity than propyl gallate, ascorbic acid, and α-tocopherol. Gulçin (2010) demonstrated that 30 μg/mL of resveratrol inhibited 89.1% of the lipid peroxidation of a linoleic acid emulsion.

As regards the antifungal activity, Hoos and Blaich (1990) and Adrain et al. (1997) described inhibitory effects of resveratrol on *B. cinerea* conidia in solid and liquid culture medium. Filip et al. (2003) reported the effectiveness of resveratrol against filamentous fungi *Penicillium expansum* and *Aspergillus niger* and yeast. *A.niger* remains the most sensitive strain out of the three tested genera.

Little information about the incorporation of resveratrol into films is available at the moment. Only some works on resveratrol incorporation into polylactic acid (PLA) based films, obtained by a blow-extrusion process, have been found (Soto-Valdez et al. 2011). The effect of resveratrol on PLA film properties was studied, and the kinetics of the diffusion of resveratrol from the PLA matrix into ethanol was analysed. These authors recommended these films be used as antioxidant release membranes for a variety of pharmaceutical, medical, and food applications.

The aim of this work was to characterize films based on different polysaccharides (chitosan or methylcellulose) containing resveratrol in different polymer:resveratrol ratios (1:0.01 and 1:0.1) by analysing their microstructure, water vapour and oxygen barrier, mechanical and optical properties and to study the effect of resveratrol incorporation on their antioxidant and antifungal properties.

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2. Materials and methods

2.1. Raw materials

Food Grade Methylcellulose (MC, CAS number 9004-67-5), medium molecular weight chitosan (CH, CAS number 9012-76-4) with a deacetylation degree of 75-85% and resveratrol (R, CAS number 501-36-0), supplied by Sigma-Aldrich Química (Madrid, Spain), were used to prepare the film-forming dispersions.

2.2. Preparation of film forming dispersions

To obtain film-forming dispersions (FFDs), 2% (wt) methylcellulose was dispersed in distilled water. Chitosan (1% wt) was dispersed in an aqueous solution of glacial acetic acid (0.25% v/v) and stirred overnight at room temperature. The corresponding amount of resveratrol (R) was dissolved in 96% ethanol and added to the polymer solutions to reach a polymer:ethanol:resveratrol ratio of 1:1:0.01 and 1:1:0.1 in the FFDs. Afterwards, these were homogenized in a rotor-stator ultraturrax DI25 at 13.500 rpm for 4 min. Resveratrol based FFDs were properly protected from light using amber glass flasks during handling.

Pure methylcellulose and chitosan FFDs (resveratrol free) were characterized as controls. In these cases, the same ratio of ethanol as in the films containing R was incorporated into the FFD to ensure the polymer had the same aqueous media solvent properties in all cases.

The mixtures were degasified for 10 min at room temperature by means of a vacuum pump (Diaphragm vacuum pump, Wertheim, Germany).

2.3. Film preparation

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Films were obtained by casting. FFDs were poured onto a framed and levelled polytetrafluorethylene (PTFE) plate (ϕ = 15 cm) and were dried for 48 h, under natural convection, at 25ºC and 60% relative humidity (RH). Film thickness was controlled by pouring onto the PTFE plate the amount of FFD that will provide a surface density of solids in the dry films of 56 g/m^2 in all formulations. The drying process was carried out in darkness to protect the FFDs from the light. Dry films were peeled off the casting surface and preconditioned prior to testing in desiccators at 25ºC and 75% RH (by using an oversaturated NaCl solution). A digital micrometer (Electronic Digital Micrometer, Comecta S.A., Barcelona, Spain) was used to measure the film thickness in at least five random positions around the film.

2.4. Film characterization

2.4.1. Scanning electron microscopy (SEM)

Microstructural analysis of the films was carried out by SEM using a scanning electron microscope (JSM-6300, JEOL Ltd., Tokyo, Japan). Film samples were maintained in a desiccator with P_2O_5 for two weeks to ensure that no water was present in the sample. Then, films were frozen in liquid N_2 and cryofractured to observe the cross-section of the samples. Films were fixed on copper stubs, gold coated, and observed using an accelerating voltage of 15 kV.

2.4.2. Atomic force microscopy (AFM)

The surface morphology of dried film samples (equilibrated in a desiccator with P_2O_5) was analysed using an atomic force microscope (Multimode 8, Bruker AXS, Santa Barbara, USA) with a NanoScope® V controller electronics. The resulting data were transformed into a 3D image. Measurements were taken from several areas of the film

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surface (50x50 and 5x5 μm) using the tapping mode. According to method ASME B46.1 (ASME, 1995), the following statistical parameters related with sample roughness were calculated: average roughness (Ra: average of the absolute value of the height deviations from a mean surface), root-mean-square roughness (Rq: root-mean-square average of height deviations taken from the mean data plane). Phase Imaging mode derived from Tapping Mode, that goes beyond topographical data to detect variations in composition, adhesion, friction, viscoelasticity, and other properties, including electric and magnetic, was also applied. Three replicates were considered to obtain these parameters.

2.4.3. Film thickness

A hand-held digital micrometer (Electronic Digital Micrometer, Comecta S.A., Barcelona, Spain) was used to measure film thickness in six different points of the same film.

2.4.4. Moisture content

Film samples were dried in triplicate at 60ºC for 24 h in a natural convection oven and for 24 h more in a vacuum oven in order to determine their moisture content.

2.4.5. Water vapour permeability

The water vapour permeability (WVP) of films is commonly measured by using a modification of the ASTM E96-95 (ASTM, 1995) gravimetric method using Payne permeability cups (Elcometer SPRL, Hermelle/s Argenteau, Belgium) of 3.5 cm diameter. For each type of film, measurements were replicated seven times and WVP was calculated following the methodology described by Gennadios et al. (1994), at 25ºC

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and a 75-100% relative humidity gradient, which was generated by using an oversaturated NaCl solution and pure water, respectively. To determine WVP, the cups were weighed periodically (each 2 h, for 10 h) after the steady state was reached using an analytical balance (±0.0001 g). Then the slope obtained from the regression analysis (5 points) of weight loss data as a function of time was used to calculate WVP, according to ATSM (1995).

2.4.6. Oxygen permeability

The oxygen permeability of the films (OP) was measured in triplicate by using an oxygen permeation measurement system (OX-TRAN 1/50, Mocon, Minneapolis, USA) at 25ºC and 75% RH (ASTM, 2005). A sample of the film (50 cm^2) was placed in a test cell and pneumatically clamped in place. Films were exposed to pure nitrogen flow on one side and pure oxygen flow on the other side. An oxygen sensor read permeation through the barrier material and the rate of permeation or oxygen transmission rate was calculated taking into account the amount of oxygen and the area of the sample. Oxygen permeability was calculated by dividing the oxygen transmission rate by the difference in oxygen partial pressure between the two sides of the film, and multiplying by the average film thickness.

2.4.7. Mechanical properties

A texture analyser (TA-XTplus, Stable Micro Systems, Surrey, United Kingdom) was used to measure the mechanical properties of films equilibrated at 75% HR and 25ºC. Strips of films (25.4 mm wide and 100 mm long) were mounted in the tensile grips (A/TG model) and stretched at a rate of 50 mm/min until breaking. The elastic modulus (EM) and tensile strength (TS) and percentage of elongation (%E) at break were

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176 determined from stress-strain curves, obtained from force-deformation data. The experiments were carried out at 25ºC on twelve replicates from each film.

178

2.4.8. Optical properties

180 The optical measurements were taken in films previously equilibrated at 25ºC and 75% RH. CIE-L*a*b* coordinates chrome (C^*_{ab}) and hue (h^*_{ab}) of the films were obtained 182 through the surface reflectance spectra determined by means of a spectrocolorimeter (CM-3600d, Minolta Co., Tokyo, Japan) with a 10 mm diameter window, using D65 184 illuminant/10º observer. Measurements were taken on black and white backgrounds and the reflectance infinite (R∞) was determined.

186 The whiteness index (WI) was calculated by applying equation 1:

$$WI = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}} \qquad \text{Eq. (1)}$$

188

The internal transmittance (Ti) of the films was determined by applying the Kubelka–190 Munk theory (Hutchings, 1999) for multiple scattering to the reflection spectra, following the methodology described by Pastor et al. (2010).

192 The gloss of the films was measured at a 60º incidence angle according to the ASTM standard D-523 (ASTM, 1999), using a flat surface gloss meter (Multi-Gloss 268, 194 Minolta Co., Tokyo, Japan). All results are expressed as gloss units (GU), relative to a highly polished surface of black glass standard with a value near to 100.

196 All measurements were taken in quintuplicate for each film at room temperature.

198 *2.4.9. Antioxidant activity*

The potential antioxidant power of films was measured via the in vitro determination of 200 the free radical scavenging effect on 2,2-Diphenyl-1-picrylhydrazyl (DPPH·) radical,

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following the methodology described by Brand-Willians et al. (1995). This method is based on the reduction of DPPH· in an alcoholic solution in the presence of a hydrogen-donating antioxidant, due to the formation of the non-radical form of DPPH in the reaction. In the radical form, this molecule shows absorbance at 517 nm, which disappears after accepting an electron or hydrogen radical from an antioxidant compound thus becoming a stable diamagnetic molecule (Matthäus, 2002).

To this end, dry films (0.12 and 0.012 g, respectively for the film with a lower and higher concentration of resveratrol) were previously dissolved in 15 (for CH films) or 5 (for MC) mL deionised water and maintained under magnetic stirring for 12 h at 25ºC.

In all cases 0.5 mL of the different appropriately diluted samples were added to 3.5 mL of methanol solution of DPPH· (0.030 g l^{-1}).

The decrease in absorbance at 25ºC was determined by using a spectrophotometer (Helios Zeta UV-Vis, Thermo Fisher Scientific, United Kingdom) at 515 nm. Measurements were taken every 15 min until the reaction reached a plateau. The DPPH· concentration (mM) in the reaction medium was calculated from the calibration curve (Eq. 2) determined by linear regression ($R^2 = 0.999$):

$$A_{515nm} = 11.36x[DPPH\cdot] - 0.037 \quad \text{Eq. (2)}$$

The percentage of remaining DPPH· (%$DPPH_{REM}$) was calculated according to equation 3:

$$\%DPPH_{REM} = {[DPPH\cdot]_T}/{[DPPH\cdot]_{t=0}} \quad \text{Eq. (3)}$$

where,

$(DPPH)_T$ is the concentration of DPPH· at the steady state.

$(DPPH)_{T=0}$ is the concentration of DPPH· at the initial reaction time.

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The percentage of remaining DPPH· was plotted *versus* the molar ratio of antioxidant to DPPH· (moles of resveratrol/mol DPPH·) to obtain the amount of antioxidant necessary to decrease the initial DPPH· concentration by 50% (EC_{50}). This parameter was used to measure the antiradical activity of the films. EC_{50} values were expressed in terms of moles of resveratrol per mole of DPPH· and also in terms of kg film per mole DPPH·. The antioxidant activity of 8.98 M resveratrol ethanol solution was also determined, using the same methodology.

2.4.10. Microbiological analysis

To determine the possible antimicrobial activity of the films, *P. italicum* (CECT 2294) and *B. cinerea* (CECT 2100) (CECT 2574) supplied by Colección Española de Cultivos Tipos (CECT, Burjassot, Spain) were used. Both were kept frozen (-25ºC) in Potato Dextrose Broth (PDB) (Scharlab, Barcelona, Spain) supplemented with 30% glycerol (Panreac, Barcelona, Spain). The fungi were inoculated on Potato Dextrose Agar (PDA) and incubated at 25ºC until sporulation. Then the cells were re-suspended in physiological water with 0.1% Tween 80. The cells were counted in a haemocytometer and diluted to a concentration of 10^5 spores per mL. Aliquots of PDA (20 g) were poured into Petri dishes. After the culture medium solidified, the diluted spore solution was inoculated on the plate surface and films of the same diameter as the Petri dishes were placed on the inoculated surface (adapted from Kristo et al. 2008). Inoculated and uncoated PDA Petri dishes were used as control. Plates were then covered with parafilm to avoid dehydration and stored at 20ºC for 5 days. All tests were run in duplicate.

2.5. Statistical analysis

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A statistical analysis of data was performed through a one-way analysis of variance using Statgraphics® Plus for Windows 5.1. Homogeneous sample groups were obtained by using LSD test (95% significance level).

3. Results and discussion

3.1. Film microstructure

Figure 1 shows the SEM micrographs of the cross section of the obtained films with CH or MC with and different amounts of resveratrol. Pure CH film showed a smooth appearance in agreement with an ordered packaging of polymer chains whereas when it contains resveratrol a coarse aspect can be appreciated, more accused when the resveratrol concentration increases. This suggests that the presence of stilbene difficult the chain entanglements giving rise to more disordered network. In the case of MC, SEM micrographs does not reveal appreciable irregularities in the polymer matrix when resveratrol was incorporated at any concentration, but an increase in the film thickness was promoted, the greater the resveratrol concentration, the thickest the film. This was also observed for the CH films as can be seen en Table 1, where the values of the different film thicknesses are shown. These features reveal that resveratrol molecules affect the chain rearrangement in the films for both, MC and CH, modifying the film microstructure. The changes are more intense for CH films where the enhancement of film thickness is near 30% (only 14% for MC) and the cross section of the film shows more pronounced irregularities.

Figure 2 shows the AFM images of the surface of the films where the changes in the surface topography induced by resveratrol incorporation can be seen. These are especially relevant when the highest concentration was used, where a notable increase in the surface roughness can be appreciated for the CH films. For MC films, a different

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surface aspect due to the resveratrol incorporation can be observed, but statistical roughness parameters (Ra, Rq) did not reveals an actual increase in roughness.

A Phase Imaging analysis was also obtained from the Tapping Mode AFM data, which allows us to detect variations in composition, adhesion, friction, viscoelasticity and other properties in the material surface, providing material property contrast. Figure 3 shows the phase images of the obtained films, where very clear differences can be observed between samples at the nano-scale level. Surface of CH films shows two different phases which could correspond to a more crystalline (less hydrated, harder) zones, dispersed in an amorphous (more hydrated, softer) zone. Zhang et al. (2006) described the development of crystallinity in CH matrices due to the formation of hydrogen bonds between flexible chains showing a characteristic X-ray diffraction pattern with two pecks; the strongest one at 2θ of about 20º and a weak peak at 10º (crystal forms II and I, respectively). The more dominate polymorph corresponds to hydrated crystals where water molecules are incorporated in to the crystal lattice which is normally detected by a broad crystalline peak in the corresponding X-ray pattern (Wan et al. 2006). It is remarkable that the presence of resveratrol modify the size and distribution of the “crystalline” zones, as much as its concentration increases in the film. Isolated, wider “crystalline” zones together with other very small can be observed. Polidispersity of the crystalline zone size distribution increases when resveratrol concentration increases. This can be attributed to the fact the resveratrol molecules (more or less heterogeneously distributed) between the CH chains modify the possibility of the hydrogen bond formation between chains, limiting the growth of crystalline zones where the stilbene molecules are more present by creating steric hindrances for chain bonding.

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In the case of MC a more homogenous surface can be observed from the phase analysis. Different authors (Donhowe and Fennema, 1993a and b) describe extensive association of MC by hydrogen bonding and hydrophobic association which induces crystallization likely reinforces the film matrix. The very homogeneous, ordered structure, which is revealed by phase image of MC films, is coherent with the formation of chain association in crystalline lattices. Nevertheless, in samples with the highest concentration of resveratrol, crystal of this compound could be observed, showing the typical dentritic shapes of crystal growth of pure molecular compounds. This could not be observed for MC samples with the lowest resveratrol content, but was present in all the observations with the highest content. This means that resveratrol separates in the MC film forming dispersion during the film drying step when its saturation level is reached, in the form of crystals, as described by Caruso et al. (2004). The resveratrol concentration used in the FFD is well above its critical micelle concentration (CMC around 12.5-37 μM, depending on pH), thus leading to a non-molecularly dispersed compound which forms molecular aggregates. These could be crystals with a planar structure which establishes a network through hydrogen bonds, (Caruso et al. 2004). This phenomenon was not observed for CH films probably due to the highest viscosity of CH in the film forming dispersions (Sánchez-González et al. 2001a) which inhibit the resveratrol crystal growth when it reach the saturation level during the film drying step. At the lowest concentration, probably the resveratrol saturation level was reached when the MC solution has enough viscosity to inhibit crystal formation and it remains more or less homogenously distributed in the matrix, at molecular level, between the MC chains. The presence of resveratrol can cause a large increase in the d101 spacing of the crystal lattice as occurs when plasticizers with low molecular weights are incorporated in the MC films (Donhowe and Fennema, 1993a). This will affect the films thickness,

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increasing its value as can be seen in Table 1: the greater the amount of resveratrol in the film, the thicker the film.

3.2. Barrier properties

Table 1 shows the mean values and standard deviation of WVP and OP of all films equilibrated at 25ºC and 75% RH, together with the values of the film moisture content. The WVP values of resveratrol-free films were in the range of those reported by Sánchez-González et al. (2011a) and Vargas et al. (2011b) for chitosan and methylcellulose, respectively. Furthermore, the OP values of pure MC films agree with that reported by Donhowe and Fenema (1993a).

Significant differences in water vapour and oxygen barrier properties were found due to both the nature of the polysaccharide and the concentration of resveratrol in the films. Due to the more hydrophilic nature of chitosan and the presence of greater amount of water molecules in the matrix (reflected in the higher values of the equilibrium moisture content), pure chitosan based films showed higher water vapour permeability and lower oxygen permeability than pure methylcellulose, in agreement with previously reported values (Vargas et al. 2011b) and with others works (Miller and Krochta, 1997). The chemical affinity of permeant and film greatly affect permeability values. In this sense, the low water solubility of oxygen could be responsible for the low OP values in the more hydrated CH matrix.

The incorporation of resveratrol tended to reduce the water vapour permeability of both kinds of films. The lipophilic nature of this stilbene (López-Nicolás and García-Carmona, 2010) explains the observed effect on the vapour water barrier properties, which were only slightly enhanced, in all likelihood due to the low concentration of the active compound incorporated in the film. This effect was only significant when using

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methylcellulose, with very small induced differences. It seems to indicate that the structural changes provoked by the resveratrol addition did not imply notable changes for mass transfer rate of water molecules.

Oxygen permeability was also slightly affected by the incorporation of resveratrol, this being only significantly reduced ($p<0.05$) in CH matrices. Although resveratrol seems to induce the formation of more open MC lattices, as commented on above, this did not significantly affect the oxygen mass transfer rate, contrary to that observed by other authors (Donhowe and Fennema, 1993a) for low molecular weight plasticizers. In CH films, OP decreased significantly ($p<0.05$), which could be related with the more tortuous pathway for the pass of oxygen molecules through the amorphous zones in the matrix as can be observed in Figure 3 from AFM phase image.

3.3. Mechanical properties

The typical tensile strength *versus* Hencky deformation curves obtained during the mechanical test carried out on the films are shown in Figure 1. As can be deduced from this figure, CH films were mechanically more resistant to fracture (greater TS and EM values) than MC films. The values of chitosan films coincide with those reported by Vargas et al. (2011a) and Sánchez-González et al. (2011a), whereas those of MC films were slightly higher than those reported by Vargas et al. (2011b). The incorporation of resveratrol to the films made them shorter and led to them breaking at a lower deformation degree. The greater the resveratrol concentration in the film, the more brittle the film.

Table 2 shows the mechanical properties of films, in terms of tensile strength (TS) and percentage of elongation at break (E%) and elastic modulus (EM). The mechanical response of the films from both polymers showed similar trends when the resveratrol

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was incorporated into the matrix, in terms of E% and TS. The addition of resveratrol led to a decrease in the tensile strength and deformation at break, in turn leading to films which were less stretchable and resistant to break. This behavior is typical of composite films, where the incorporation of non-miscible compounds provokes structural discontinuities in the polymer network and a reduction in the overall cohesion forces of the matrix (Sánchez-González et al. 2010 and 2011a).

The effect of resveratrol incorporation on the elastic modulus (EM) parameter was dependent on the polymer matrix: it decreased in chitosan composite films, whereas it increased in MC composite films. The structural changes induced by resveratrol in the polymer matrices are responsible for this behavior. In CH films, the dominate formation of smaller crystalline zones will imply the reduction of the stress-strain relationships and so the elastic modulus of the films, whereas the modification of crystalline arrangement of MC by stilbene seems to increase the cohesion forces of the lattices probably by the action of cooperative forces with the resveratrol molecules. The resveratrol crystal formation in MC films could also contribute to an increase in the film rigidity. The decrease in the film stretchability, and the subsequent reduction of the tensile stress at break, can be also justified by the structural changes promoted by resveratrol. CH matrix with smaller and more heterogeneous crystalline zones will be less resistant to deformation, breaking at low extension degree, whereas the presence of resveratrol crystals in the MC films supposes discontinuities in the matrix which favors film rupture at lower deformation levels.

3.4. Optical properties

Film transparency was evaluated through the internal transmittance of the samples: the greater the transmittance value, the more transparent the film. In Figure 2, the spectral

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distribution of transmittance (Ti) of films equilibrated at 25ºC and 75% RH is shown. As can be observed, pure MC and CH films were highly transparent and, in both cases, the incorporation of resveratrol provoked a decrease in the Ti values, thus increasing the film's opacity. This effect was more pronounced at the highest resveratrol concentration. Composite films turned more opaque due to the loss of homogeneity in the polymer matrix, which is caused by the presence of structural heterogeneities in the films with a different refractive index, which promotes light scattering phenomena. The presence of smaller crystalline zones in the CH films when resveratrol was incorporated will promote light scattering, thus increasing the film opacity. In the case of MC, the more open crystalline lattices containing resveratrol aggregates will also reduce the specular light transmission through the films, increasing their opacity.

Table 3 shows the values of the colour coordinates, lightness (L*), hue (h^*_{ab}) and chrome (C^*_{ab}), together with the whiteness index (WI) and the gloss of the different films. In MC composite films, the luminosity, hue and whiteness index significantly decreased when the resveratrol content rose, while color saturation (C^*_{ab}) increased. These effects were barely appreciated in the case of chitosan films probably due to the fact that no crystals of resveratrol are formed, but only changes in their semi-crystalline structure with lower impact in light reflection.

Both composite films became less glossy ($p<0.05$) when the concentration of resveratrol increased in the films, this effect being more marked in MC composite films. This can be attributed to the presence of resveratrol crystals on the film surface, which contributes to an increased surface heterogeneity and so, a reduction in the gloss. This effect has been previously observed by several authors working on composite films (Pastor et al. 2010; Sánchez-González et al. 2010). The above described structural

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changes induced by the resveratrol in the films are responsible for the observed changes in optical properties.

3.5. Antioxidant activity

The antioxidant activity of the films was evaluated by means of the dissolution of the film in a controlled amount of distilled water. Once dissolved, the method described by Brand-Willians et al. (1995) based on the DPPH free radical method was applied. At the pH of the dispersions (6.5 and 4.5 respectively for MC and CH), resveratrol is in protonate form, since the pH<PKa_1~8.8 (López-Nicolás and García-Carmona, 2008). This protonate form needs to be able to exhibit several biological activities, such as antioxidant power. From these experiments, the EC_{50} values were obtained from the plot of the % of remaining DPPH· at the steady state *versus* moles of resveratrol/moles DPPH· (Fig. 3). This parameter indicates the amount of antioxidant needed to reduce the initial DPPH concentration to 50%, once the steady-state of the reaction was reached. Thus, the lower the EC_{50} values, the greater the antioxidant activity of the tested sample. Table 4 shows the EC_{50} values for pure resveratrol and the different films, expressed in terms of moles of resveratrol per DPPH· moles and in kg films per DPPH· mol, by taking into account the amount of resveratrol in each film sample. Resveratrol was found to react slowly with the DPPH· (slow kinetic behavior) coinciding with that reported by Villaño et al. (2007), taking around 100-120 min to reach the steady state.

The EC_{50} value found for the pure resveratrol was greater than those found by Villaño et al. (2007) and Sánchez-Moreno et al. (1998); around 0.50-0.58 moles resveratrol/mole DPPH·. The different solvent medium (water:ethanol and methanol, respectively) used by these authors could explain the observed differences.

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The obtained values for EC_{50}, expressed in terms of moles of resveratrol per mole DPPH· reflect that no losses of antioxidant activity occurred for resveratrol in the CH films, since no significant differences were found either in the EC_{50} values of pure resveratrol or of that encapsulated in the CH films. On the contrary, EC_{50} values for MC films were slightly higher, which indicates that some loss of antioxidant activity occurs in these films. This could be due to the lower extractability of resveratrol from this matrix which reduces the compound reaction capacity in the solvent medium, but also to some degradation of encapsulated resveratrol due to the higher OP of MC films, as compared with CH films.

When referring EC_{50} values in terms of kg film per mole DPPH·, the effect of the antioxidant concentration in the film is clearly shown and, as can be observed, the antiradical efficiency increased in line with the resveratrol content in the film.

The main conclusion of this test is that the antioxidant efficiency of resveratrol did not notable change during film formation, drying and conditioning, which indicates the great antioxidant potential of these films encapsulating resveratrol.

3.6. Microbiological analysis

Microbial analysis showed that none of the composite films show antifungal activity against either *P. italicum* or *B. cinerea*. Filip et al. (2003) found that resveratrol presented antifungal activity against *P. expansum* but no information has been found about *P. italicum*. Regarding *B. cinerea*, the resveratrol concentrations used were higher than the minimal inhibitory concentration (MIC) found for this fungus by Adrian et al. (1997). Taking into account that the release of an active agent into the medium involves several factors, such as solvent and migrant polarities and solubility (Sánchez-González

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et al. 2011b), the low chemical affinity and solubility of resveratrol with the aqueous solvent could explain the observed effect.

4. Conclusions

Resveratrol was efficiently incorporated into chitosan and methylcellulose films. These composite films showed some changes in their microstructural and physicochemical properties, especially when the highest concentration of resveratrol was used: their water vapour and oxygen barrier properties were hardly affected by the induced structural changes, which implied changes in the semi-crystalline arrangement of CH and MC and the appearance of resveratrol crystals in the more concentrated MC films. Nevertheless, films became less stretchable and resistant to fracture, more opaque and less glossy due to structural changes provoked by resveratrol in the matrix, although from a practical point of view, these changes did not negatively affect to the handle, manipulation or appearance of the films.

Composite films also exhibited antioxidant activity, which was proportional to the resveratrol concentration used and no notable losses of this activity during film formation and conditioning were observed. None of the films showed antimicrobial activity against *P. italicum* and *B. cinerea.*

The obtained results points out that resveratrol based films are suitable for coating purposes. The coating of food products with these films could minimize or prevent oxidation processes, maintaining nutritional quality, and prolonging the food shelf life. Thus, future studies will focus on the applications of these films to food products which are sensitive to oxidative processes.

5. Acknowledgements

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Highlights

- Resveratrol was efficiently incorporated into both chitosan and methylcellulose films.

- Composite films exhibited antioxidant properties but no antifungal activity against *P.italicum* and *B. cinerea.*

- Barrier properties, films´ mechanical resistance, gloss and transparency decreased in composite films, especially when using the highest resveratrol concentration.

- Resveratrol incorporation induced changes in the semi-crystalline arrangement of CH and MC, and the appearance of resveratrol crystals in the more concentrated MC films.

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Table 1.- Thickness, moisture content, water vapour permeability (WVP) and oxygen permeability (OP) of films equilibrated at 25ºC and 75% RH. Mean values and standard deviation.

Film	**Thickness (μm)**	**Moisture content (g H_20/ g ss)**	**WVP (g/Pa s m)x10^{10}**	**OP (cm^3μm/m^2 d kPa)**
MC	51 (4)^{a}	7.0 (0.7)^{b}	8.7 (0.9)^{b}	127 (20)^{a}
MC+R10	51 (3)^{a}	6.73(0.13)^{ab}	7.7 (0.7)^{c}	125 (33)^{a}
MC+R100	58 (9)^{b}	5.9 (0.3)^{a}	6.0 (0.6)^{d}	121 (25)^{a}
CH	58 (6)^{b}	14.9 (0.7)^{c}	12 (1)^{a}	14.4 (0.5)^{b}
CH+R10	61 (7)^{b}	14.53 (0.13)^{c}	12 (2)^{a}	16 (1)^{b}
CH+R100	74 (5)^{c}	19.0 (0.5)^{d}	11.9 (0.5)^{a}	11.2 (0.4)^{b}

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

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Table 2.- Elastic modulus (EM) and tensile strength (TS), and percentage of elongation (E) at break of films equilibrated at 25ºC and 75% RH. Mean values and standard deviation.

Film	TS (MPa)	EM (MPa)	E (%)
MC	66 (6)[b]	1604 (92)[a]	15 (2)[c]
MC+R10	65 (7)[b]	1871 (132)[b]	10 (3)[b]
MC+R100	49 (5)[a]	1903 (55)[b]	4 (1)[a]
CH	94 (15)[d]	2739 (93)[d]	14 (7)[c]
CH+R10	78 (2)[c]	2585 (151)[c]	6 (2)[a]
CH+R100	43 (5)[a]	1550 (195)[a]	5.1 (0.4)[a]

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

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Table 3.- Lightness (L*), chrome (C^*_{ab}), hue (h^*_{ab}), whiteness index (WI) and gloss at 60º of films equilibrated at 25ºC and 75% RH. Mean values and standard deviation.

Film	**L***	**C^*_{ab}**	**h^*_{ab}**	**WI**	**Gloss 60º (GU)**
MC	83.8 (0.8)^{d}	11.3 (0.8)^{a}	85.7 (0.8)^{c}	80 (1)^{d}	52 (13)^{c}
MC+R10	64 (1)^{a}	20 (1)^{d}	77 (1)^{b}	59 (1)^{a}	33 (13)^{b}
MC+R100	63 (1)^{a}	19.7 (0.6)^{d}	72 (1)^{a}	58 (1)^{a}	18 (6)^{a}
CH	80.7 (0.4)^{c}	15.5 (0.6)^{b}	89.3 (0.6)^{e}	75 (1)^{c}	47 (13)^{c}
CH+R10	79 (1)^{b}	18 (2)^{c}	88 (1)^{d}	72 (2)^{b}	36 (14)^{b}
CH+R100	81 (2)^{c}	17 (3)^{c}	90 (2)^{e}	75 (3)^{c}	20 (6)^{a}

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

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Table 4.- Efficient concentration (EC_{50}) (amount of antioxidant needed to reduce the initial DPPH· concentration to 50%, once the steady-state of the reaction was reached) of pure resveratrol (R) and the different films.

Film	**EC_{50} (moles R/mole DPPH·)**	**EC_{50} (kg film/mole DPPH·)**
R	0.70 (0.07)[b]	-
MC+R10	0.95 (0.12)[a]	24 (3)
MC+R100	0.87 (0.05)[a]	2.16 (0.13)
CH+R10	0.71 (0.04)[b]	19 (1)
CH+R100	0.73 (0.07)[b]	2.0 (0.2)

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

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Figure captions

Figure 1.- SEM micrographs of the cross-sections of the dried films equilibrated with P_2O_5 at 25ºC.

Figure 2.- AFM micrographs of the surface of the dried films equilibrated with P_2O_5 at 25ºC. Mean values and standard deviation of Rq (nm) and Ra (nm) roughness parameters.

Figure 3.- Phase images of the dried films samples equilibrated with P_2O_5 at 25ºC.

Figure 4.- Typical curves of tensile strength *versus* Hencky deformation of films equilibrated at 25ºC and 75% RH.

Figure 5.- Spectral distribution of internal transmittance (Ti) of films equilibrated at 25ºC and 75% RH.

Figure 6.- Decrease of DPPH· as a function of the number of moles of resveratrol per mole of DPPH·.

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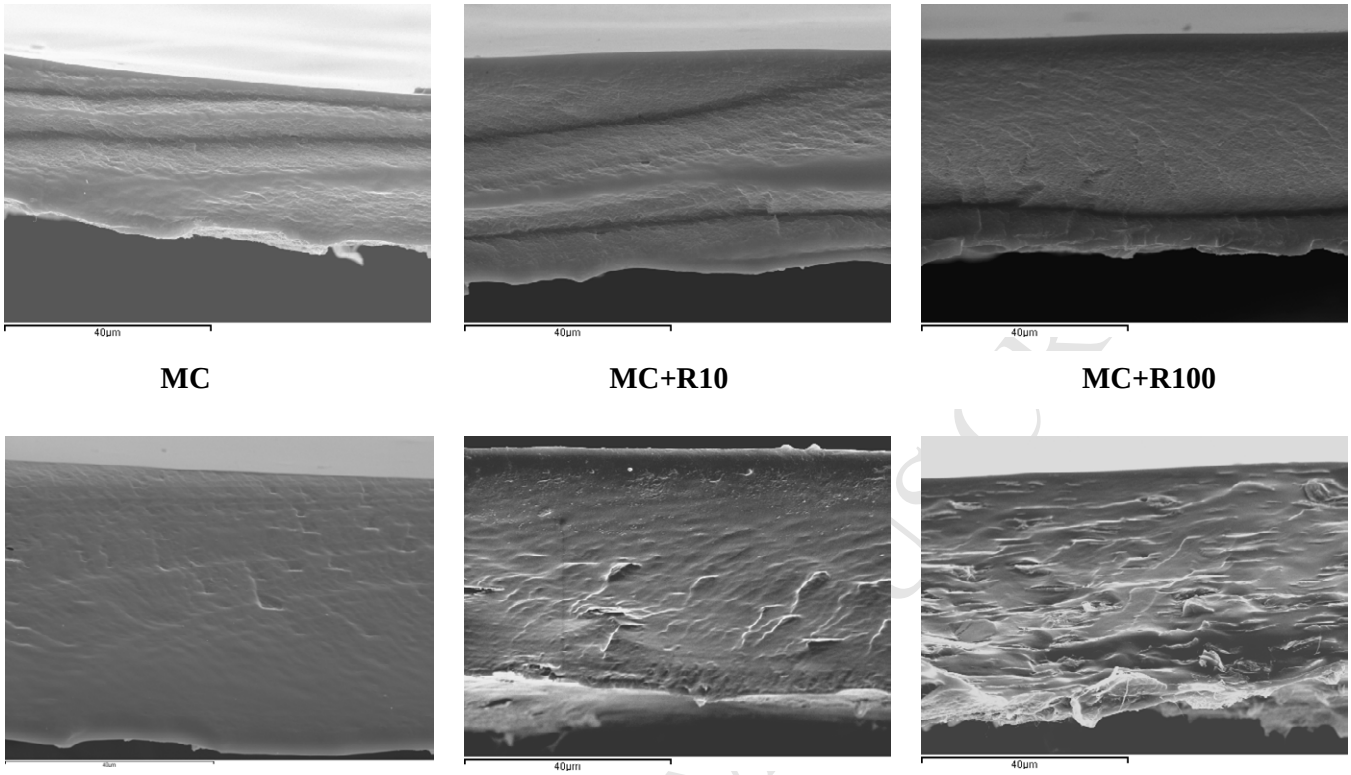

MC **MC+R10** **MC+R100**

CH **CH+R10** **CH+R100**

Figure 1

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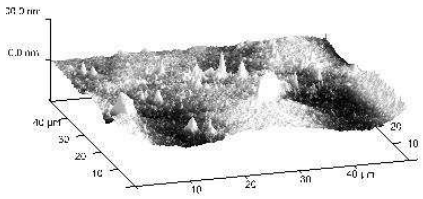
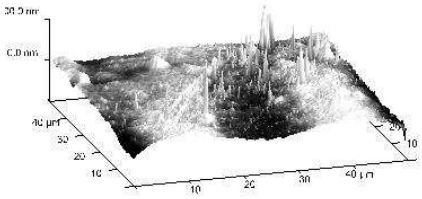
Rq: 19 (1) Ra: 13 (1)



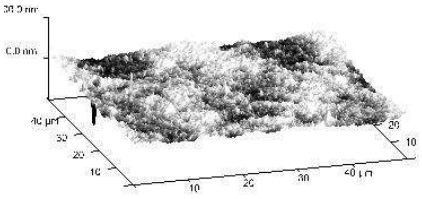
MC

Rq: 15 (1) Ra: 11.5 (0.6)



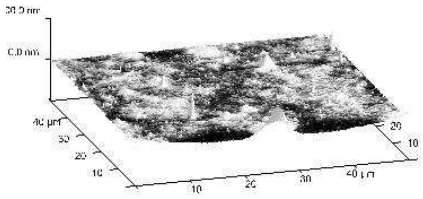
MC+R10

Rq: 11.0 (0.2) Ra: 8.2 (0.3)



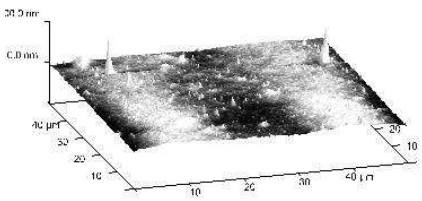
MC+R100

Rq: 9 (3) Ra: 4 (2)



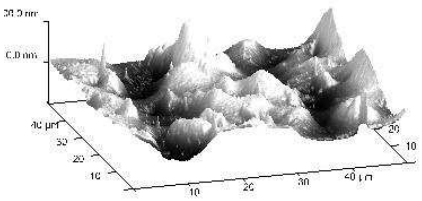
CH

Rq: 6 (1) Ra: 4.4 (0.9)



CH+R10

Rq: 41 (4) Ra: 32 (2)



CH+R100

Figure 2

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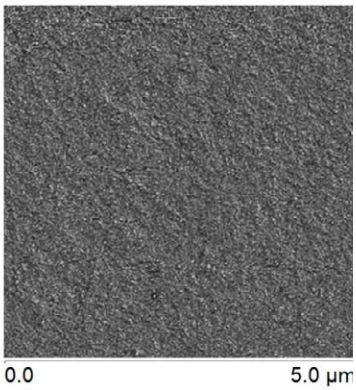
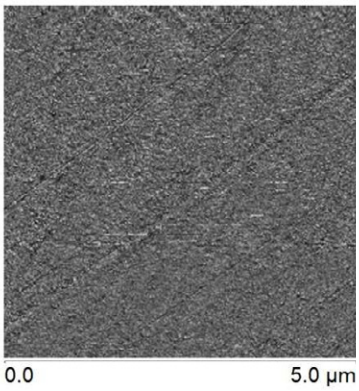
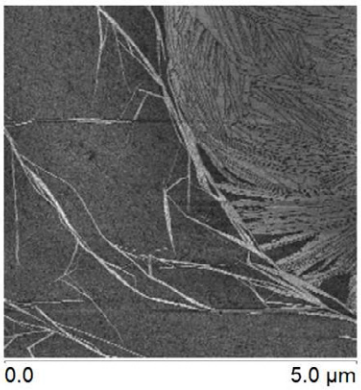


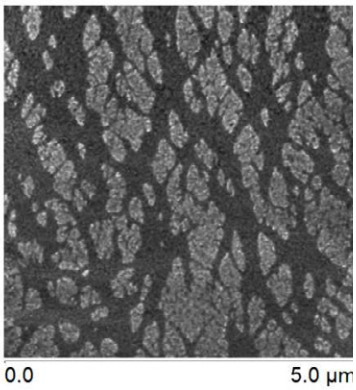
MC



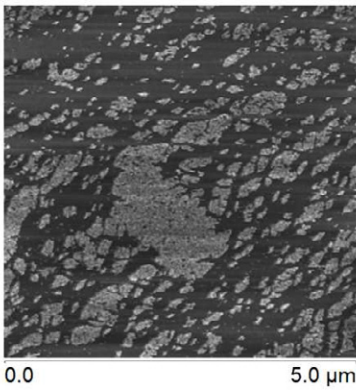
MC+R10



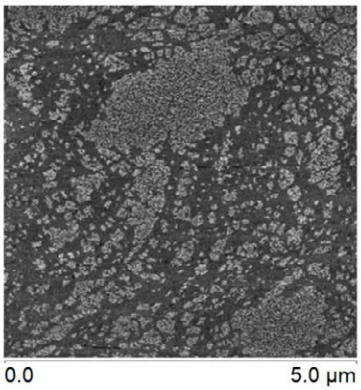
MC+R100



CH



CH+R10



CH+R100

Figure 3

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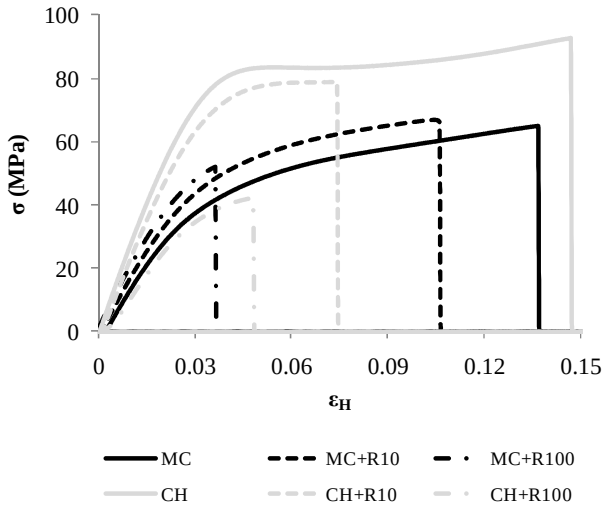

Figure 4

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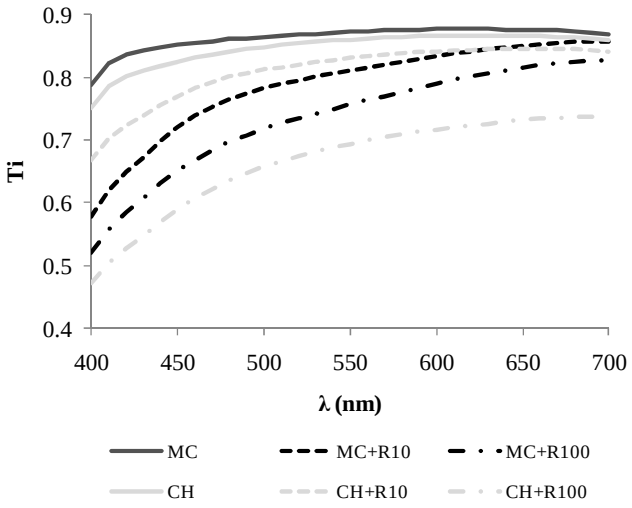

Figure 5

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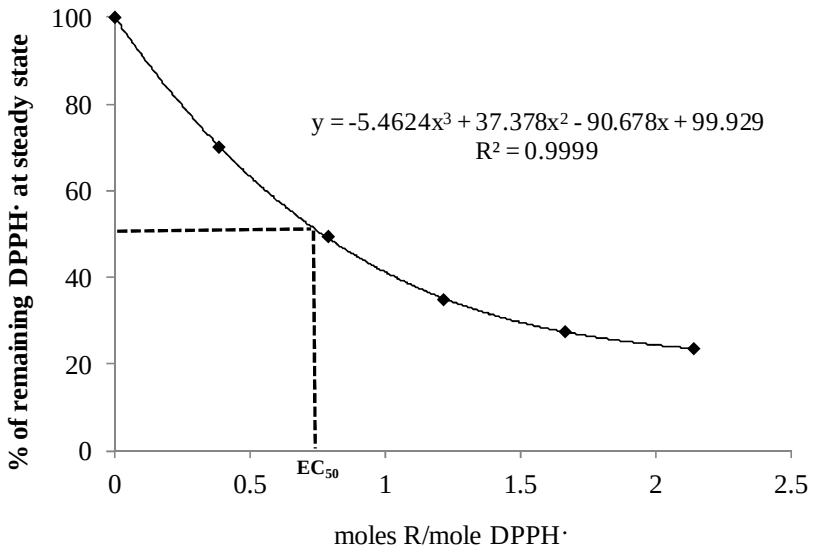

Figure 6

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