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High methoxyl pectin–methyl cellulose films with antioxidant activity at a functional food interface

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ABSTRACT

The effect of using increasing proportions of methylcellulose (MC) for the development of glycerol plasticized films based on high methoxyl pectin (HMP) (30:70, 50:50 and 70:30 w/w HMP:MC) and carrying L-(+)-ascorbic acid (AA) was studied with the purpose of achieving higher stability of AA and localized antioxidant activity at food interfaces. MC and 30:70 HMP:MC systems could not be casted. The shelf-life of the other AA-active films was assessed by storage at 25 °C, constant relative humidity (RH: 33.3%, 57.7% and 75.2%) and vacuum conditions. The rate constant for AA hydrolysis increased with the RH and, hence, with water mobility. Browning and AA degradation rates were directly related. When stored at 75.2% RH, both decreased as MC proportion increased. Compared to HMP film, the highest proportion of MC (50:50 HMP:MC) showed the highest AA stabilization under vacuum and greater performance under air atmosphere. They also developed localized antioxidant activity preserving the tocopherol content of walnut oil.

1. Introduction

In the past, foods were primarily recognized for their essential nutrients for normal physiological activity and function. However, during the past two decades, consumers have switched from an emphasis on satisfying hunger to an emphasis on the promising use of foods to promote well-being and help reduce the risk of disease. Nowadays, there is a consensus that eating the "right" foods extends life expectancy and improves quality of life (Agriculture, 2009). The food industry shows increasing interest in product innovation in order to satisfy consumer's demand for high-quality and option variety of healthy products. A preference for foods with natural instead of synthetic additives is also significantly related to health concerns (Devcich et al., 2007). At the same time, preservatives used in healthier food formulations (i.e.: antimicrobial agents, antioxidants) should be used at the lowest level that assures effectiveness. A localized activity of preservatives could be a way to achieve the desired reduction of concentration and edible biopolymer films can be an effective manner of carrying additives while providing localized activity and delivery at food interfaces (De'Nobili, Pérez, Navarro, Stortz, & Rojas, 2011). Known as vitamin C, L-(+)-ascorbic acid (AA) is a natural and water soluble antioxidant also useful in food preservation. AA stability is affected by processing and storage conditions, depending on a large number of factors such as temperature, equilibrium RH, oxygen partial pressure, light, package permeability and package configuration (Kitts, 1997). AA reacts with oxygen to produce L-dehydroascorbic acid (DHA) that also has vitamin C activity in vivo. The latter is irreversibly lost when DHA is hydrolyzed in the subsequent reaction. Furthermore, anaerobic degradation of AA through hydrolysis also occurs simultaneously to AA oxidation when oxygen is present, producing 2-keto-L-gulonic acid (Kurata and Sakurai, 1967a). On the other hand, non-enzymatic browning (NEB) also proceeds with AA concentration decay since the products of the reactions that follow the first step of AA destruction are also part of the NEB reaction chain (Rojas and Gerschenson, 2001; León and Rojas, 2007). Compartmentalization of AA into an edible film network could help achieve stabilization because it can preclude the AA interaction with oxygen, other food preservatives or nutrient components and films can provide a localized antioxidant activity at interfaces.

A high methoxyl pectin (HMP) film formulated to carry AA was previously developed, during which AA degradation and browning kinetics were studied at 25 °C under vacuum conditions (p = 132 Pa) (Pérez, Flores, Marangoni, Gerschenson, & Rojas, 2009) as well as under normal air conditions ($p = 1.013 \times 10^5$ Pa) (Pérez, 2012) in order to determine the films' shelf-life as potentially active interfaces. Methyl cellulose (MC) is a hydrophobically modified cellulose with interesting thermogelation properties (Li, 2002), and most useful in pharmaceutical and food formulation. During film development, MC was applied using polyethylene glycol 400 as plasticizer (Debeaufort and Voilley, 1997; Turhan

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et al., 2001). The hydrophobic/hydrophilic nature of MC films has been recently studied by Innis-Samson and Sakurai (2011), showing it could be able to modify the HMP film network for better immobilization of the water sorbed, necessary to preserve AA from hydrolysis (León and Rojas, 2007). Walnut oil is a valuable functional ingredient, though highly susceptible to oxidation. The aim of the present work was to study the effect of using increasing proportions of MC (compared to those of HMP) in the edible films that were developed, especially focusing on AA stability, browning development and antioxidant activity of the edible films developed. Some aspects related of the polymeric film microstructure were also analyzed.

2. Experimental methods

The kinetics of AA degradation and NEB development in the films were studied under vacuum at constant temperature (25 °C) and at three RH values (33.3%, 57.7% and 75.2%) to specifically determine the hydrolytic stability of AA. The film formulation that could be casted and showed the highest AA stability (50:50 w/w HMP:MC) was then used to evaluate the effect of oxygen on the AA degradation at a RH of 57.7% and 25 °C. It was also used as an antioxidant interface for precluding walnut oil oxidation at the same environmental conditions.

2.1. Chemicals

The MC used was M0512 (molecular weight ≈ 85,000 Da; methoxy substitution ≈ 27.7–31.5%; degree of substitution or average number of substituent groups attached to the ring hydroxyls ≈ 1.5–1.9; viscosity ≈ 4.2 Pa s for a 2% solution in water at 20 °C) from Sigma–Aldrich (St. Louis, MO, USA). Food grade pectin with a high degree of methylation (GENU pectin type B rapid set-Z) was from CP Kelco (Denmark). Its chemical composition was determined and reported by Pérez et al. (2009). All other chemicals were of analytical grade from Merck (Argentina) and Sigma–Aldrich.

In order to evaluate the antioxidant capacity of films containing AA, walnut oil (IL Noce™, Escobar, Buenos Aires province, Argentina) was used. It was the product of the first cold pressing of *Juglans regia* L. edible seeds, whose ripened fruits were harvested in 2010 in the Argentine province of Catamarca.

2.2. Film making procedure

Films constituted only by MC or by 70:30, 50:50 or 30:70 (w/w) HMP:MC ratios were developed for the purpose of this study. Also, HMP film samples were made according to Pérez et al. (2009). For this trial, a 2% (w/w) total polymer concentration was used for film making solution, thus permitting to obtain plasticized films with the adequate handling resistance. This solution was continuously stirred under controlled high speed (1400 rpm-constant) using a vertical stirrer (model LH, Velp Scientifica, Italy) in order to reach homogeneous hydration. While stirring, the obtained viscous, homogeneous and transparent system was then heated up to 75 °C at a constant heating rate (5.0 °C/min) by means of a hot plate (Velp Scientifica, Italy) and with simultaneous recording of the temperature by using a thermocouple connected to a Consort millivoltmeter (P 901, Belgium). Subsequently the following substances were added: Glycerol [36.84 g per 100 g of (polymer + glycerol)] for plasticization (Yang and Paulson, 2000), potassium sorbate (0.03% w/w) as a antimicrobial agent and finally AA (0.100% w/w). The obtained hot solution was placed under vacuum for 20 s to remove air bubbles and then immediately poured onto horizontally leveled polystyrene plates. The fractionated system was dried in a forced convection oven at 60 °C. The films were peeled from the polystyrene plates and stored in light-protected desiccators over saturated solutions of known water activity (a_w°), in order to maintain a constant relative humidity (a_w° = RH%/100) for film equilibration. The salts used were $MgCl_2$ (a_w° = 0.333), NaBr (a_w° = 0.577) and NaCl (a_w° = 0.752) at 25 °C (Greenspan, 1977). Equilibration was followed by the measurement of a_W of the film samples daily until attaining the final equilibrium. Afterwards, the sample thickness was measured at six different locations in each of ten specimens by using a digital micrometer (Mitutoyo, Kawasaki, Japan).

Three batches of films (replicates) were prepared as above described. The film samples obtained from each batch were identified and distributed among light-protected desiccators with different RHs (33.3%, 57.7% or 75.2%) and stored at 25 °C in order to establish the influence of the film making in the following determinations. Storage was first performed under vacuum (p = 132 Pa) with controlled RH in order to ensure that AA degradation was initiated through the irreversible hydrolysis of its lactone ring as the first and limiting reaction step (León and Rojas, 2007). Hence, the specific influence of water in the AA stability could be analyzed. On the other hand, samples of the three batches of the 50:50 HMP:MC film were further stored under air ($p = 1.013 \times 10^5$ Pa) protected from the light, at 25 °C and 57.7% RH, in order to infer the specific influence of oxygen on the total AA kinetics.

The following analyses were performed on each film sample collected from the three batches at each corresponding time and RH of interest.

2.3. Water activity

To evaluate film equilibration, the a_W was determined on the film samples with a Decagon AquaLab (Series 3 Water activity meter, USA) at 25 °C using a calibration curve made with the standard saturated salt solutions above mentioned.

2.4. Measurement of pH

This was performed on the film-forming solutions as well as on the casted films after their equilibration at the corresponding RH, as informed by De'Nobili et al. (2011).

2.5. Determination of L-(+)-ascorbic acid (AA)

A film sample was taken from each of the three batches obtained in order to determine the AA kinetics from the triplicates of film making. The AA concentration was determined by using the 2,6-dichlorophenolindophenol (2,6-DPIP) spectrophotometric method detailed by De'Nobili et al. (2011). The AA concentration was determined from two different aliquots (duplicate) for each film sample.

2.6. Color

Measurement of the film color was performed employing a Minolta colorimeter (Minolta CM-508d) using an aperture of 1.5 cm-diameter, as previously reported by De'Nobili et al. (2011). Film samples for color measurement were taken from each of the three batches of films obtained in order to determine browning (yellowness index, YI%) kinetics. Also, L, a, and b (HunterLab) color parameters were measured, which ranged from L = 0 (black) to L = 100 (white) for lightness; $-a$ (greenness) to $+a$ (redness), and $-b$ (blueness) to $+b$ (yellowness). Standard values considered were those of the white background.

2.7. Moisture content

For determination of moisture content, film samples were evaluated after equilibration with the storage RH and using the same procedure reported by De'Nobili et al. (2011) was used.

2.8. Glass transition temperature (T_g)

Dynamic mechanical analysis (DMTA; Q800, TA Instruments, USA) was used in a tensile mode to determine the T_g. A multifrequency (3, 5, 10, 15 Hz) assay was performed and the storage (E') and loss (E'') moduli as well as the tanδ (E''/E') were isochronically recorded from −140 up to 220 °C at 10 °C/min. Silicone was used to avoid evaporation. The T_g value was read in the middle of the α-relaxation step (E' decay, coincident with the maximum in E''). Assays were carried out in triplicate using films taken from three batches equilibrated at 57.7% RH and at 25 °C.

2.9. Proton nuclear magnetic resonance (NMR) mobility

This study was performed on all the equilibrated film samples using a Bruker Avance II spectrometer (Germany) operating at 300 MHz for ^{1}H. The probe was a Bruker high power CP-MAS and was used under static conditions. The rotor sizes were 18-mm long with a 4-mm outer diameter. All the experiments were conducted on resonance at room temperature. The Carr Purcell Meiboom Gill (CPMG) pulse sequence was used to measure the transverse magnetization decay (T_2) as explained by De'Nobili et al. (2011). The exponential curve fitting was performed at 50 and 10 μs using a Maxwell model:

$$A(t) = \sum_i A_i \exp\left(-\frac{t}{T_{2i}}\right) \quad (1)$$

wherein T_{2i} is the spin–spin characteristic relaxation time (t) of the "i" relaxing element of the relaxation process; $A(t)$ = total peak height at time t of relaxation, being A_i = the peak height of equilibrium magnetization for the "i" relaxing element. An average value of replicates taken from the three batches of films and the corresponding standard deviation (SD) were reported.

2.10. Total elemental analysis

The elemental composition of HMP as well as 50:50 and 70:30 HMP:MC films was determined. A Carlo Erba EA 1108 automatic elemental analyzer (Milano, Italy) was used. The percentages of C, H, N, S and O were informed on a dry basis.

2.11. Surface elemental analysis

The elemental composition of both identified surfaces of each film sample was determined in triplicate through X-ray photoelectron spectroscopy (XPS). A Unispecs spectrometer (SPECS, Berlin, Germany) provided with a dual anode X-ray source (Ag/Al) was used for excitation as well as a hemispheric PHOIBOS 150 analyzer operating in the FAT mode. The electron takeoff angle was 60°. The XPS transmission spectra were acquired by using an analyzer pass energy of 30 eV and a monochromatic Al-anode operated at 200 W. The samples were previously evacuated during 12 h in the load-chamber, with pressure being lower than 2×10^{-5} Pa during the spectral acquisition. To compensate for charge effects, the spectra were acquired by bombardment of samples with low energy electrons (1 eV).

2.12. Water vapor permeability of films

It was determined following the procedure and calculations indicated by Gennadios et al. (1994) for hydrophilic films, by taking into account the resistance of the air column to the vapor transference in the space (10 mm) remaining between the calcium chloride desiccant placed into the cup and the film sample at the top of the beaker; which was in contact with an environmental 73% RH at 25 °C (Ibertest chamber, Spain).

2.13. Antioxidant capacity of films

2.13.1. Sample preparation

An equal weight of walnut oil was dispensed in each of the 25-mm-diameter glass plates (called "samples"). They were then distributed among five groups for dark storage at 25 °C and 57% RH during 50 days. One of them was the "control" group which was stored without film protection. Other two groups were made up of the samples covered by 50:50 HMP:MC and HMP films, respectively, which were prepared as indicated above (see Section 2.2). Finally, the last two groups were constituted by the samples covered by films made without the addition of AA. The latter permitted to determine if the AA supported in the edible films actually contributed to their antioxidant activity beyond the oxygen film barrier effect. For these trials, the films were cut in circles with the same diameter of the glass plates and put on each oil sample to isolate it from the storage environment. Samples were collected in duplicate from the five groups of each film formulation at the following pre-established storage times: 0, 8, 15, 22, 29, 38 and 50 days. The experiment was performed in duplicate. The following analyses were then carried out on each sample replicate.

2.13.2. Fluorescence measurements

The fluorescence spectra were collected employing a fluorescence spectrometer (230 V, PerkinElmer, USA) provided with the front surface device of variable angle. Quartz cuvettes of 10-mm-path length were used for the measurements. On the sample, an incidence angle of 60° was used in order to minimize the light reflection and dispersion as well as the depolarization phenomenon. A xenon lamp source was used for excitation, and the emission and excitation slit widths were 2 nm. The acquisition interval and the integration time were maintained at 1 nm and 0.1 s, respectively. All measurements were performed at room temperature. The three-dimensional spectra were obtained by measuring the emission spectra in the range from 300 to 700 nm repeatedly, at excitation wavelengths from 250 to 500 nm, spaced by 5-nm intervals in the excitation domain. The synchronous fluorescence spectra were collected by simultaneously scanning the excitation and the emission monochromators in the 250–800 nm range, with a constant wavelength difference ($\Delta\lambda$) between them. The fluorescence intensities were plotted in function of the excitation wavelength. Spectra were recorded, in duplicate, from each sample, with $\Delta\lambda$ (*offsets*) of 20, 30, 40 and 50 nm.

2.14. Data analyses

The results are reported as the average and standard deviation (SD) for "n" replicates. Rate constants of AA destruction were calculated by linear regression where AA concentration was in terms of AA (g) per weight (g) of the corresponding film-sample assayed. The analysis of covariance (ANCOVA) was applied for the comparison of slopes, that is, of rate constants, as indicated by Sokal and Rohlf (2000). The statistical analyses of results were performed by applying Analysis of Variance (ANOVA; α: 0.05), followed by

pair wise multiple comparisons evaluated by Tukey's significant difference test (Sokal and Rohlf. 2000). The GraphPad Prism software (version 5.00, 2007, GraphPad Software Inc., USA) was used for all the analyses previously detailed. The OriginPro 7.5 SRO program (Origin Lab Corporation, Northampton, MA, USA) was also used for nonlinear fitting.

3. Results and discussion

3.1. Film characteristics

It was not possible to cast films only constituted by MC or by higher proportions of MC to HMP than 50:50 (w/w). When AA and potassium sorbate were specifically added, non-homogeneous film making solutions were obtained due to segregation of the components and excessive viscosity. After, dehydrated films showed white stains and irregular thickness, and they broke easily during peeling from polystyrene plates. Therefore, homogeneous and flexible films plasticized by glycerol were only obtained from 50:50 and 70:30 (w/w) ratios of HMP to MC for studying. The initial film pH was ≈3.68. These casted films were transparent and colorless (b = +6 ± 1; YI = 11% ± 2) with high initial lightness (L = 89 ± 1%), whereas the HMP films were slightly yellow (b = +8.90; YI = 16 ± 3%) though with similar lightness (L = 86.0 ± 0.2%), as previously reported by Pérez et al. (2009). The initial AA concentration experimentally determined on samples of the casted films was ≈3.01% (w/w), as calculated on film basis. This value meant that a 100% of AA retention was reached after drying. Film samples attained equilibration at the third day of storage at each RH, as determined from the daily measurement of the film a_W (0.333, 0.577 or 0.752). Thickness was then measured (Table 1) and its value increased with the RH for 70:30-films and, also, at 75.2% RH, with the proportion of HMP. This may demonstrate that MC diminished the water adsorption by the film polymeric network. Moisture contents increased with the RH of equilibration as well as with the HMP proportion for 57.7% and 75.2% RH (Table 1). Film pH and lightness (L) changed to a value of 3.83% and 84% with storage. The increase in film pH could be mainly associated with the decrease in AA (pKa$_1$ ≈ 4.17) concentration as well as in 2-keto-L-gulonic (pKa ≈ 2.80) and 2,3-diketo gulonic acid contents subsequently formed during storage, for producing pentuloses involved in the browning reaction chain (Kurata and Sakurai, 1967a,b). These events becoming from hydrolytic instability of AA could be also responsible for some decrease (5–6%) in film lightness with storage.

Table 1
Film thickness and moisture contents determined after equilibration at each condition of relative humidity (RH) and 25 °C are shown.

RH (%)	Thickness[a,b] (mm)		Moisture content[b,c] (g/100 g *dm*)	
	50:50 w/w HMP:MC	70:30 w/w HMP:MC	50:50 w/w HMP:MC	70:30 w/w HMP:MC
33.3	0.095 ± 0.052 [A]	0.081 ± 0.044 [A]	21.3 ± 0.9 [C]	24 ± 1[C]
57.7	0.084 ± 0.051 [A]	0.086 ± 0.038 [A]	27.3 ± 0.6 [D]	33.5 ± 0.7[E]
75.2	0.106 ± 0.060 [A]	0.185 ± 0.080 [B]	46.3 ± 0.9 [F]	54 ± 1[G]

[a] Mean and standard deviation (n = 15) are shown.
[b] The same capital letter means nonsignificant difference (p < 0.05).
[c] The mean and standard deviation (n = 3) are reported.

3.2. Stability of L-(+)-ascorbic acid to chemical hydrolysis in films

The study of AA stability by storage in the absence of air (vacuum, p = 132 Pa) allowed to determine that the ratio between the AA concentration [$C_{AA}(t)$] and the initial one [C_{AAO}] statistically changed with storage time (t) according to a pseudo-first order (p < 0.05) kinetic. On the other hand, the browning development measured as the increment of YI with time, statistically fitted (p < 0.05) to a pseudo-zero order reaction (Rojas and Gerschenson, 2001). There were no significant (p < 0.05) differences between the rate constants of AA hydrolysis (k'_{AA}) determined at 33.3% or 57.7% RH for the films made with 50:50 and 70:30 w/w HMP:MC (Fig. 1a). However, when carrying out the storage at 75.2% RH, the rate of AA decay increased significantly (p < 0.05) with the proportion of HMP present in the film formulation. The maximal increase was observed in HMP films, which were included for comparison from the work of Pérez et al. (2009). At a RH of 57.7%, only the AA compartmentalized in HMP films showed significantly higher k'_{AA}. The same conclusions were obtained by comparison of the rate constants of NEB development (k_{YI}) (Fig. 1b). Hence, AA destruction and NEB depended on RH levels, and the presence of MC in the film network contributed to a better stabilization of AA (Fig. 1).

Water is responsible for hydrolysis and the irreversible opening of the lactone ring of the AA molecule to produce 2-keto-L-gulonic acid through an acid catalyzed reaction (Kurata and Sakurai, 1967a) which was suggested to occur through a bimolecular nucleophilic mechanism (S_N2) by León and Rojas (2007):

$$r_{AA} = -\frac{1}{v_{AA}}\frac{dC_{AA}}{dt} = k \cdot C_{WATER} \cdot C_{AA}(t) = k'_{AA} \cdot C_{AA}(t) \quad (2)$$

wherein v_{AA} is the stoichiometric coefficient for AA hydrolytic reaction (here v_{AA} = 1); r_{AA} is the AA-reaction rate per unit volume at constant temperature; C_{AA} and C_{WATER} are the AA and water concentration, respectively; k is the rate constant of the second order kinetics for AA hydrolytic reaction; k'_{AA} is the rate constant of the pseudo first order kinetics for AA hydrolysis determined in the present work through measurement of the AA concentration remaining at every time (t). This is the only reaction by which AA is lost under anaerobic storage (vacuum, p = 132 Pa). Since the availability of the adsorbed water increased as the *solid-like* networks (films) were stored at increasing RHs, k'_{AA} also increased as this parameter is a pseudo order rate constant which includes $k{\cdot}C_{WATER}$ (Eq. (2)). Water adsorbed in the *solid-like* polymeric systems equilibrated at 33.3–75.2% RH range is a limiting reactive (León and Rojas, 2007) since it is not available as a solvent. This condition also promotes dehydration reactions as well as a high reactivity of nucleophiles (Morrison and Boyd, 1990). Hence, water availability in the polymeric network is a feature for AA stability and subsequent browning development in films (De'Nobili et al., 2011).

The AA hydrolysis was directly related to browning development as observed from the relationship between the calculated half-life times ($t_{1/2}$) of AA degradation and the times involved to reach a 40% value of YI ($t_{40\%=YI}$), as can be observed in Fig. 2. It is important to state that all films showed similar YI initial values. The $t_{1/2}$ and $t_{40\%=YI}$ variation with the storage conditions (Fig. 2) was related to the increase in the rate constants of AA hydrolysis (k'_{AA}) and browning development (k_{YI}) with the RH levels (Fig. 1). From Fig. 2, it can be concluded that the increase in MC proportion determined a higher $t_{40\%=YI}$ for a given value of $t_{1/2}$ of AA, which means a lower NEB degree for the same AA destruction, showing the relationship between both phenomena.

Hydrophobically modified cellulose (MC derivative) may tightly retain water associated to −OH and C−O−C acetal groups through hydrogen bonding and to $-CH_3$ ether groups through hydrophobic association. Furthermore, MC may also hinder the interaction of water with the HMP or with the whole film network, as inferred from the lower AA decay and browning rates observed for HMP:MC films. Velázquez de la Cruz et al. (2001) determined that the moisture sorption isotherms obtained from MC films showed the typical behavior of hydrophilic and moderately hydrophobic

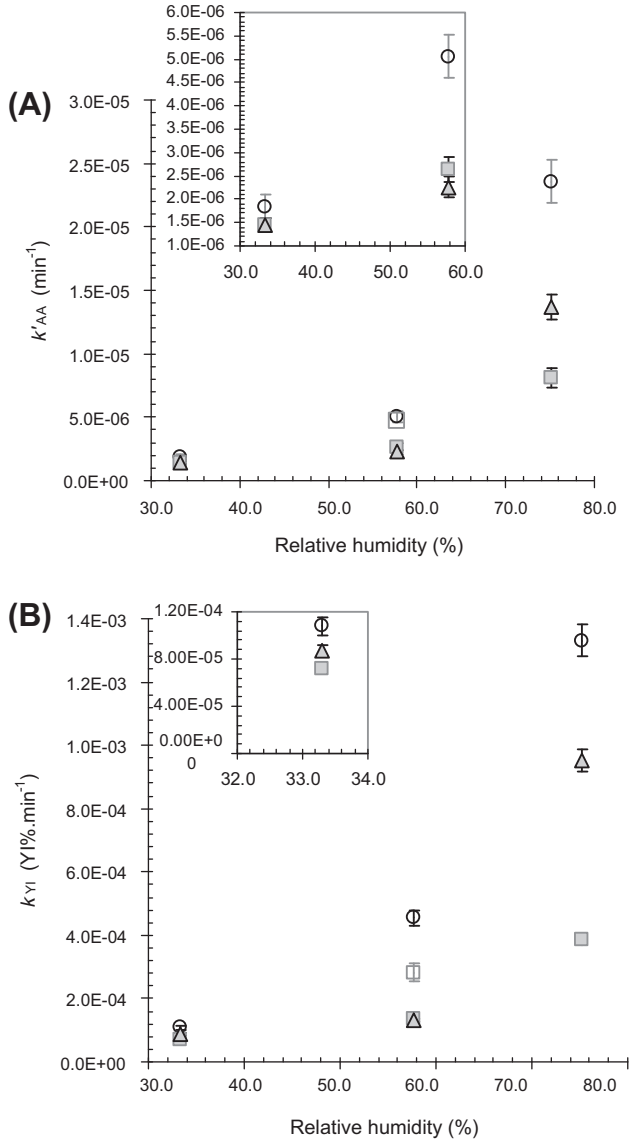

Fig. 1. Rate constants of AA hydrolysis (k'_{AA}) (a) and NEB development (k_{YI}) (b) determined in 50:50 (■), 70:30 (▲) as well as in HMP (○) films, are plotted against RH of film storage under vacuum at 25 °C. Total rate constants of AA decay under air (k_T) for 50:50 HMP:MC film (□) stored at 57.7% RH are also shown (a, b). Error bars (SD, $n = 3$). As a detail, a magnification can be observed for 33.3% and 57.7% RH.

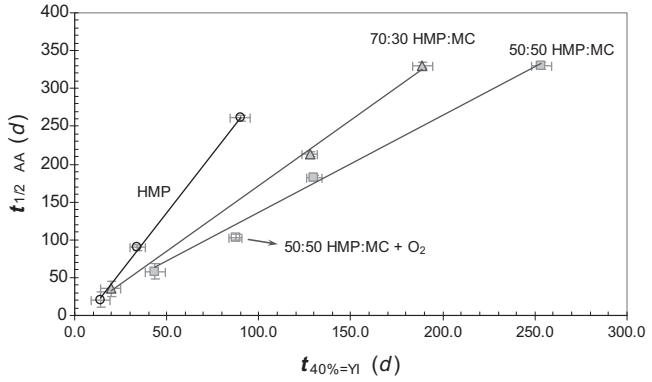

Fig. 2. Half-life time of AA hydrolysis ($t_{1/2}$) plotted against the time needed to reach a yellowness index (YI) value of 40% ($t_{40\%\ =\ YI}$) are reported from 50:50 (■) and 70:30 (▲) HMP:MC as well as HMP (○) films stored under vacuum at 25 °C and 33.3%, 57.7% or 75.2% RH. Also, the kinetic parameters obtained from 50:50 HMP:MC stored under air (□) at 25 °C and 57.7% RH are shown. Error bars (SD, $n = 3$).

materials. Chinnan and Park (1995) found that the moisture content of MC films increased slowly up to an equilibration a_w° of 0.752. The presence of plasticizers like glycerol or other polyols increases the adsorption of water by cellulose derivative (Rachtanapun and Tongdeesoontorn, 2009) or pectin (Maftoonazad et al., 2007) films.

3.2.1. Hydrophilicity and water mobility in films

Hydrophilicity of the polymeric networks is summarized in Table 2 as the elemental oxygen to carbon (O/C) ratio (Shi, Kokini, & Huang, 2009). The total elemental analysis of films showed that the network hydrophilicity (O/C ratio) significantly ($p < 0.05$) increased with the HMP proportion used (Table 2). Very often, the surface hydrophilicity of a film is affected more by its functional groups or elemental composition localized at the surface rather than by the groups distributed in the bulk of materials (Shi et al., 2009). Therefore, the film surface was also specifically characterized in its elemental composition through XPS (Table 3). A higher proportion of HMP (70:30) produced a significant ($p < 0.05$) increase in the surface hydrophilicity although this trend was altered for the 70:30 film on the reverse side (Table 3). Some preferential distribution of hydrophobic ($-CH_3$) groups at the film surface was in general observed for films studied. Hence, water adsorption may be easier in 70:30 than in 50:50 w/w HMP:MC film proportion at each RH of storage, determining higher moisture contents for the former film (Table 1). On the other hand, the water vapor permeability (0%/73% RH) values determined in the present work for 50:50 [$(1.2 \pm 0.1) \times 10^{-9}$ g m^{-1} s^{-1} Pa^{-1}] and 70:30 [$(1.5 \pm 0.4) \times 10^{-9}$ g m^{-1} s^{-1} Pa^{-1}] HMP:MC films were not significantly different ($p < 0.05$).

Water availability in the polymeric network at each RH of film equilibration was also assessed through ^{1}H NMR. Short-range motions or molecular relaxation study (Vittadini and Chinachoti, 2003) gave two spin–spin time constants (T_{2a} and T_{2b}) for the magnetization decay in the *xy*-plane after exponential fitting (Eq. (1)), indicating the existence of multi-relaxation rate behavior. These parameters increased with the RH of film equilibration, thus indicating the main contribution of water. The two spin–spin time constants (T_{2a} and T_{2b}) may be associated with two fractions of water, which have different relaxation rates or mobility degrees in the context of low moisture biopolymer systems with water contents ⩽35% (Chen et al., 1997; Kerr and Wicker, 2000; Kou et al., 2000). The T_{2a} and T_{2b} values were between 1.300×10^{-4} and 80.70×10^{-4} s. Half life time of AA as well as $t_{40\%\ =\ YI}$ in general decreased ($p < 0.05$) with the increment in water mobility as shown through the T_{2a} values (Fig. 3). T_{2b} presented the same trend. These results suggest that more restrictive effects of the plasticized polymers on the surrounding water molecules led to higher hydrolytic stability of AA and lower browning rates, as observed for HMP:MC films. This was more evident at the highest values of T_{2a}, which corresponded to the film samples equilibrated at 75.2% RH

Table 2
Total elemental composition[a] of the films studied.

Film sample	Total elemental composition				
	C (%)	H (%)	N (%)	O (%)	O/C [b]
50:50 HMP-MC	44.5 ± 0.1	6.6 ± 0.2	0.3 ± 0.2	48.5 ± 0.1	1.090^A
70:30 HMP-MC	41.8 ± 0.2	6.5 ± 0.1	0.6 ± 0.2	51.1 ± 0.2	1.222^B
HMP	38.4 ± 0.1	5.9 ± 0.2	0.7 ± 0.4	55.0 ± 0.1	1.431^C

[a] The arithmetic mean and standard deviation ($n = 3$) are reported.
[b] The same capital letter indicates non significant differences ($p < 0.05$).

Table 3
Surface elemental composition[a] of films determined through XPS.

Film sample	Surface elemental composition at one side				Surface elemental composition at the reverse side			
	C (%)	N (%)	O (%)	O/C [b]	C (%)	N (%)	O (%)	O/C [b]
50:50 HMP:MC	62.4 ± 0.1	4.3 ± 0.5	33.3 ± 0.1	0.534^{A}	61.7 ± 0.1	5.4 ± 0.3	32.7 ± 0.1	0.534^{A}
70:30 HMP:MC	60.7 ± 0.1	4.3 ± 0.3	35.0 ± 0.2	0.577^{B}	63.9 ± 0.1	3.2 ± 0.1	32.9 ± 0.2	0.515^{B}
HMP	61.6 ± 0.2	2.7 ± 0.2	35.7 ± 0.1	0.580^{B}	59.5 ± 0.1	4.8 ± 0.2	35.7 ± 0.2	0.600^{C}

[a] The arithmetic mean and standard deviation ($n = 3$) are reported.
[b] The same capital letter as superscripts in a column indicates non significant differences ($p < 0.05$).

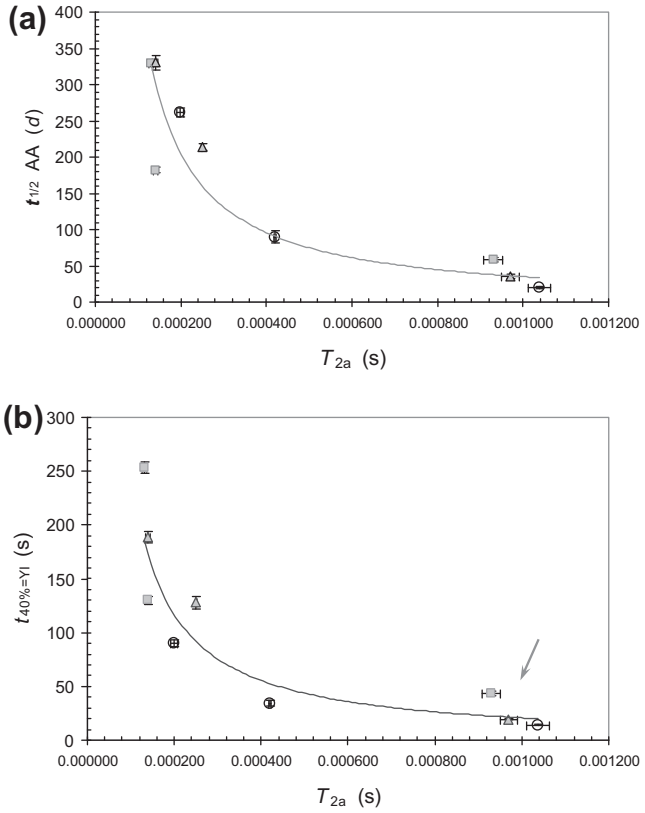

Fig. 3. Half-life time of AA hydrolysis ($t_{1/2}$) (a) and the time needed to reach a YI value of 40% ($t_{40\%=YI}$) (b) are plotted against the spin–spin relaxation time (T_{2a}) for 50:50 (■) and 70:30 (▲) HMP:MC as well as for HMP (○) films stored under vacuum at 25 °C and 33.3%, 57.7% or 75.2% RH. Error bars (SD, $n = 3$).

(Fig. 3; arrows). As can be observed in Fig. 3, T_{2a} decreased with the MC content increase for 75.2% RH.

3.2.2. Macromolecular relaxation

As previously reported for MC by Debeaufort and Voilley (1997), glass transitions were not detectable by DSC scanning of films containing MC developed in the present work. Macromolecular relaxation was then studied through DMTA under tensile mode. A T_g value of −53.78 °C was determined through isochronal scans in the middle of the α-relaxation (E') step observed for 50:50 and 70:30 HMP:MC films. This transition was evident from the maximum in E'' simultaneously recorded during heating at 10 °C/min. The same results were obtained in the DMTA study of HMP film.

On the other hand, a second T_g above 100 °C was expected since T_g values of +185 °C were reported for MC (Gómez-Carracedo et al., 2003). Anyhow, this second α-relaxation was not observed and could have been masked by the melting point (T_m) determined at ≈110 °C for the HMP:MC films. Melting is a first order structural change of the polymeric network, which involves the phenomenon of chain slipping. With few documented exceptions, polymeric mixtures are incompatible, representing a physical blending rather than a solution. Physical blending leaves moderately large regions of homogeneous composition, each type of region experiencing its own glass transition (Ferry, 1980; Liu et al., 2006). Hence, HMP and MC probably constituted physical blends in the films that were studied. Macromolecular relaxation in the HMP:MC film network (T_g = −53.78 °C) corresponded to the amorphous rubber state of the HMP polymeric regions plasticized by glycerol and water, at 25 °C.

3.3. Stability of L-(+)-ascorbic acid to chemical hydrolysis and oxidation

Due to its highest MC content, 50:50 HMP:MC film network was the most suitable to stabilize AA against hydrolysis. Regarding this matter, it was selected to be also evaluated in its ability to stabilize AA under storage at 57.7% RH, 25 °C and normal air conditions ($p = 1.013 \times 10^5$ Pa). Considering its potential antioxidant activity, 50:50 HMP:MC films were subsequently tested on walnut oil at these mentioned environmental conditions.

Khan and Martell (1967) observed that below an oxygen partial pressure of 0.40 atm, the rate constant of AA oxidation (k_{AA}^{OX}) was directly proportional to the oxygen concentration (C_{O_2}) and, hence, to the partial pressure, as observed in the following kinetic equation:

$$r_{AA} = -\frac{1}{v_{AA}}\frac{dC_{AA}}{dt} = k_2 \cdot C_{O_2} \cdot C_{AA}(t) = k_{AA}^{OX} \cdot C_{AA}(t) \quad (3)$$

wherein k_2 is a second order rate constant, which corresponds to the following chemical reaction to be found in AA oxidation:

$$AA + O_2 \rightarrow DHA + H_2O$$

Destruction of AA into pectin films in order to produce DHA under air occurred simultaneously to the hydrolytic reaction previously studied under vacuum condition (Eq. (2)). Therefore, when AA is lost during air storage, it can be considered that at least two irreversible parallel or competitive chemical reactions take place under air (Eq. (4)): AA hydrolysis (Eq. (2)) and AA oxidation (Eq. (3)). Therefore the following differential kinetic equation can be written for AA reactive in the form of the following pseudo-first-order rate reaction:

$$r_{AA} = -\frac{1}{v_{AA}}\frac{dC_{AA}}{dt} = k'_{AA}C_{AA}(t) + k_{AA}^{OX} \cdot C_{AA}(t) \quad (4)$$

As a result of integration ($v_{AA} = 1$) results

$$C_{AA} = C_{AA}^{O} \cdot \exp[-(k'_{AA} + k_{AA}^{OX}) \cdot t]$$

Hence, the slope calculated by fitting of data obtained under air storage gives the total rate constant (k_T):

$$k_T = k'_{AA} + k_{AA}^{OX} \quad (5)$$

in which the oxidation rate constant (k_{AA}^{OX}) can be specifically calculated as the arithmetical difference, which corresponded to

an oxygen partial pressure of 0.21 atm constant under the normal air condition of film storage.

In 50:50 HMP:MC films, the total rate constant of AA decay under air (k_T) increased 76% compared to the rate constant of AA hydrolysis determined under vacuum (k'_{AA}) (Fig. 1a). HMP films were also tested for comparison purposes observing similar values for k_T. At the same time, k_{YI} was doubled under normal air condition (Fig. 1b) though observing a lag or initial period of 15.0 days. Hence, a $t_{40\% = YI}$ of 87.5 days was calculated for 50:50 w/w HMP:MC films under said conditions, whereas, in the case of the AA hydrolysis, a $t_{40\% = YI}$ of 130 days was involved. Anyway, the browning rate was lower in 50:50 HMP:MC film stored under air than in HMP network previously studied under vacuum (Fig. 1b), whereas k_T (Eq. (5)) values of AA decay determined in 50:50 HMP:MC and HMP films were not significantly ($p < 0.05$) different (Fig. 1a).

3.4. Evaluation of the antioxidant activity and oxygen barrier capacity of the developed films

Walnut oil is a valuable functional ingredient due to its high polyunsaturated fatty acid content, but the latter makes it highly susceptible to oxidation. Tocopherols are the natural antioxidants for walnut oil. The shelf-life of tocopherols can determine the start of the early signs of oxidative oil spoilage (lipid peroxides). Walnut oil obtained from the first cold pressing was used as vulnerable substrate of oxidative stress. The 50:50 HMP:MC and HMP films were analyzed as antioxidant interfaces on walnut oil for performance comparison. As above mentioned, the same value of total rate constant of AA destruction (k_T) was observed for both film systems during darkness storage under normal air conditions ($p = 1.013 \times 10^5$ Pa) at 57.7% RH and 25 °C. Also Walnut oil samples were stored under the same environmental conditions. As an example, Fig. 4 shows the recording, during a 50 day storage time, of the synchronous scanning fluorescence spectra of walnut oil covered with HMP film samples carrying AA. An offset between excitation and emission wavelengths ($\Delta\lambda$) of 20 nm was selected since these spectra did not differ from those recorded at higher $\Delta\lambda$ values (30, 40 and 50 nm) from all samples (Sikorska, Khmelinskii, Sikorski, Caponio, Bilancia, Pasqualone et al., 2008). Two important peaks were observed at ≈308 nm and 670 nm for all samples herein assayed. It is to be noted that also while studying the stability of extra virgin olive during storage at different conditions, Sikorska et al. (2008) observed two important peaks at ≈301 nm and ≈666 nm, ascribing this to the main fluorescence compounds identified in this oil, namely tocopherols and chlorophylls, respectively. On the other hand, there was no evidence of formation of new fluorescence groups during the 50 days storage time of walnut oil (Fig. 4). Conversely, Sikorska et al. (2008) found other fluorescence peaks only in the spectra of virgin olive oil stored under light conditions and they were tentatively identified as photodecomposition/photo-oxidation products of chlorophyll or other pigments.

When plotting the values of fluorescence intensity recorded from each sample at 308 nm against time, the effect of the storage conditions was evident (Fig. 5a and b). The tocopherol contents of walnut oil samples covered by HMP film were maintained significantly above ($p < 0.001$) those contents determined in oil samples covered by HMP film developed *without* AA or in uncovered control samples, again during a 50 day storage time (Fig. 5a). This revealed the antioxidant capacity of the HMP films carrying AA. The $t_{1/2}$ calculated for the antioxidant AA compartmentalized in HMP films equaled 83 days under normal air conditions, at 57.7% RH and 25 °C, thus assuring the presence of the antioxidant in the HMP film during the 50-day-trial. When AA was absent in the HMP film, the tocopherol levels of the covered walnut oil samples were significantly higher ($p < 0.05$) than the ones of the uncovered control samples. The trials performed on films *without* AA demonstrated their ability to act as effective barriers to oxygen. On the other hand, 50:50 HMP:MC film system carrying AA maintained the tocopherol content of walnut oil significantly above ($p < 0.05$) the ones shown by the uncovered control samples (Fig. 5b) and films *without* AA, during a 50 day storage time (Fig. 5b). It must be stated that k_T was similar for HMP and 50:50 HMP:MC films, assuring equal concentration of AA while carrying out the assay. Anyhow,

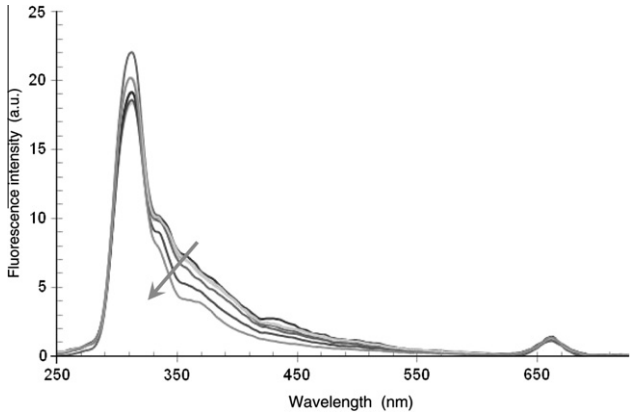

Fig. 4. Synchronous fluorescence spectra recorded from walnut oil covered with HMP film containing AA, during storage in the dark for 50 days at 25 °C and 57.7% RH ($\Delta\lambda$ = 20 nm). Arrow indicates the direction of storage time increase.

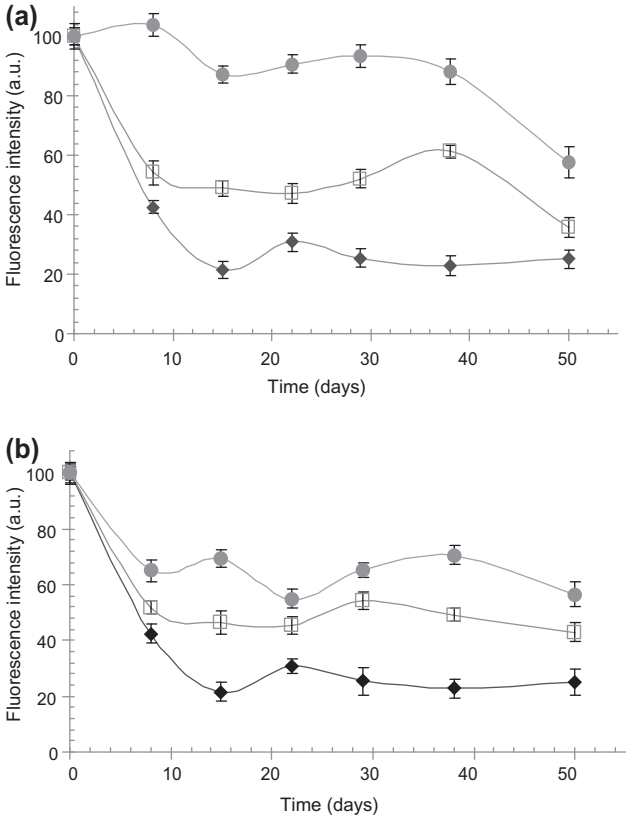

Fig. 5. Fluorescence intensity recorded at 308 nm-peak, attributed to tocopherols, is plotted against the storage time of walnut oil covered with HMP (a) or 50:50 HMP:MC (b) film either with (●) or without (□) AA. Control (uncovered) sample (♦) is also shown in the panels. Fluorescence intensity was normalized with respect to the initial value. Error bars (SD, $n = 4$).

the film containing AA and based on HMP showed a higher antioxidant activity up to 40 days of storage compared to the HMP:MC film with AA. Probably, the peculiar network constituted by the physical blend (HMP:MC) resulted in a lower AA availability to exert its antioxidant effect. Cui (2007) reported that the potential energy states of different phases in pharmaceutical solids are highlighted as the key connection between the physical nature of the materials and their pharmaceutical behavior.

As mentioned above, all fluorescence spectra in this research showed a small emission peak at ≈670 nm (Fig. 4), which corresponded to the presence of some small proportion of chlorophyll pigments in the walnut oil used in these assays. However, the intensity of this band did not change significantly during the 50 days of storage. The dark storage condition could be responsible for the stability of the chlorophyll pigments.

According to the obtained results, the natural tocopherol content of walnut oil was effectively preserved by the use of antioxidant active interfaces such as 50:50 HMP:MC film or HMP film. This demonstrated the utility and activity of films developed as antioxidant active interfaces due to their content of AA.

4. Conclusion

MC and 30:70 HMP:MC systems could not be casted. Hence, the shelf-life of the other AA-active (50:50 and 70:30 HMP:MC) films was assessed. It was observed that water had a profound effect on AA stability and NEB, which are chemically related. The results obtained through this work permitted to determine that MC contributed to increase the AA hydrolytic stability and to reduce the NEB probably due to certain degree of hydrophobicity. HMP and MC may constitute physical blends in the films studied with amorphous rubber state of the HMP polymeric regions plasticized by glycerol and water. The 50:50 HMP:MC films showed an effective performance as antioxidant interfaces for protecting walnut oil. From comparison with HMP film system, it was also observed that the polymeric film microstructure determined a different AA availability for acting as an antioxidant. Hence, the network characteristics seemed to affect not only the stability of the compartmentalized AA but also the performance of the edible films as antioxidant interfaces for food preservation.

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