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Stability of L-(+)-ascorbic acid in alginate edible films loaded with citric acid for antioxidant food preservation

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ABSTRACT

Alginate of known copolymer composition was applied to antioxidant film developed by loading L-(+)-ascorbic (AA) and citric (CA) acids. Relative humidity (RH, 33.3–75.2%) of storage (25.0 °C) and glycerol (plasticizer) level, separately and through their interaction, decreased the AA hydrolytic stability and, hence, the antioxidant half-life of films. Glycerol can facilitate the water penetration from the environment into the polymeric network. The CA content did not vary during film storage. Film browning was then associated to AA degradation. However, CA slowed down the browning development at longer storage times because of the changes in the kinetic order. It may catalyze the formation of unstable color compounds. The present work permitted to determine that AA and CA were 100% recovered after film casting. Also, it allowed finding the adequate film composition and period where the alginate film supporting AA and CA can be used as an antioxidant active interface for food preservation.

1. Introduction

L-(+)-ascorbic acid (AA) or vitamin C is a water soluble weak acid and a natural antioxidant which can be used for food and pharmaceutical preservation, nutritional supplementation and also for therapy (Chen et al., 2005). The concentration of AA decreases during storage depending on environmental conditions such as equilibrium RH, temperature, oxygen content, light (Barba et al., 2012). It reacts with oxygen during food storage to produce L-dehydroascorbic acid (DHA) that also has vitamin C activity in the human body. 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, 1967). Hydrolysis can be even more detrimental on AA than air (De'Nobili et al., 2013). As reported by Hiatt et al. (2011), the moisture present in solid vehicles enhanced the degradation of vitamins A, B_1, and C in pharmaceutical products. It has been determined the detrimental effects of deliquescence on sodium ascorbate stability in powders.

Non enzymatic browning (NEB) proceeds when the AA concentration decays since the products of the reactions that follow 2-keto-L-gulonic acid formation are also part of the browning reaction chain (León and Rojas, 2007). This fact modifies the color and aspect of products containing AA. Compartmentalization into an edible film can not only contribute to stabilize AA but also to overcome negative interactions between preservatives used in a pharmaceutical or food system, or between the preservative and the nutrients in food formulations. Also, antioxidants supported in films could be effective at lower concentrations due to their localized activity and controlled delivery at interfaces for preservation. Edible films are much studied matrices since they can be also applied as a technological hurdle for food preservation because their microstructure can be used to carry food preservatives (antimicrobials, antioxidants).

CA (2-hydroxy-1,2,3-propanetricarboxylic acid) is a weak organic acid naturally found in many fruits and vegetables. It acts as

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a synergist by promoting the activity of proper antioxidants due to its metal chelator activity, binding heavy metals (Pokorný, 2001). Hence, presence of CA together with the AA supported in edible films can be useful for antioxidant effect at interfaces. However, CA and AA can affect each other when both are compartmentalized into the edible film networks and, hence, the half-life time of the antioxidant active interface can be shortened.

Alginate is a biopolymer that has found numerous applications in biomedical science and engineering due to its favorable properties, including biocompatibility and facility for gelation (Lee and Mooney, 2012). Alginic acid is an unbranched binary copolymer extracted from brown algae, constituted by (1,4)-linked β-D-mannuronic acid units (MM-block), α-L-guluronic acid monomers (GG-block) and sequences of alternating β-D-mannuronic and α-L-guluronic acid (MG-block) (Jothisaraswathi et al., 2006). Physical and mechanical properties of alginate materials as well as biocompatibility are highly dependent on the relative content of L-guluronic (G) to D-mannuronic (M) acids (Klöck et al., 1997; Stabler et al., 2001). Different copolymer composition gives rise to different controlled delivery performances of active compounds from alginate networks. Calcium ions can replace in part the hydrogen bonding, zipping guluronate (but not mannuronate) chains together stoichiometrically in an "egg-box" conformation (Braccini and Pérez, 2001). Because alginate films are hydrophilic matrices, the crosslinking process by Ca^{2+} has been used to improve their water barrier properties, mechanical resistance, cohesiveness and rigidity (Benavides et al., 2012). In a previous work, De'Nobili et al. (2013) developed calcium-crosslinked alginate films of determined copolymer compositions and glycerol plasticizer levels with the purpose of achieving higher stability of the compartmentalized AA. The ability of the alginate network to preserve AA from hydrolysis was tested by storage under vacuum at 25 °C and constant RH (33.3–75.2%). It was determined that water was the factor responsible for AA hydrolysis and that the increase in the glycerol level can also affect the AA stability, both depending on the copolymer composition of the alginate used.

The objective of the present work was to develop alginate films supporting AA and CA for antioxidant preservation. The half life time of the alginate edible films as antioxidant interfaces was determined by studying the kinetics of AA and CA destructions as well as of the NEB development during film storage at 33.3, 57.7 and 75.2% RH and 25 °C. Films were made with an alginate of higher proportion of flexible GM + MG-block ($F_{GM+MG} = 0.40$) and lower of the MM–($F_{MM} = 0.32$) and GG–($F_{GG} = 0.27$) blocks, where the total contents of guluronic ($F_G = 0.47$) and mannuronic ($F_M = 0.53$) monomers were similar (De'Nobili et al., 2013), being 1.13 the M/G ratio. Three glycerol plasticizer levels (36.6, 54.8 and 109.6 g/100 g alginate) were also assayed.

2. Materials and methods

2.1. Chemicals

The sodium alginate used in this work was purchased to Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade from Merck (Argentina) or Sigma–Aldrich. Deionized water (Milli-Q, USA) was used. The alginate used for film development was constituted by a total polysaccharide content of 97.0%. It was characterized by a high molecular weight (876 kDa) and a molecular weight distribution of ±320 kDa (De'Nobili et al., 2013).

2.2. Film making procedure

Ca. 300.00 g of total film making solution was elaborated per batch. It was made into a 1 L-glass beaker containing ≈250.00 g of deionized water, where 4.56 g of sodium alginate (1.52 g per 100 g of solution) were slowly poured under continuous controlled high speed (1400 rpm-constant) stirring using a vertical stirrer (LH model, Velp Scientifica, Italy) for homogeneous hydration. A viscous, homogeneous and transparent solution was obtained and then heated up to 85 °C under continuous stirring (1400 rpm-constant) at a constant heating rate (5.2 °C/min) with a hot plate (Velp Scientifica, Italy). The glass beaker was entirely covered as a tent with an aluminum foil, in order to avoid heat dissipation. The temperature was simultaneously recorded by using a thermocouple connected to a Consort millivoltmeter (P 901, Belgium). Glycerol (36.6, 54.8 or 109.6 g per 100 g of sodium alginate) was then added as plasticizer, followed by potassium sorbate (0.030 g/100 g solution) for antimicrobial preservation of films, and L-(+)-ascorbic acid (0.100 g/100 g solution), all of them pre-dispersed or dissolved in ≈7 mL of water. CA (17.50 g/100 g sodium alginate) was also added. Finally, 4.5×10^{-4} mol of Ca^{2+} (as $CaCl_2.2H_2O$) per gram of alginate were added for gelling after cooling, predissolved in ≈7 mL of water, while the temperature of the film making solution was maintained between 85 and 89 °C. Afterwards, the total weight of the film making solution was completed to 300.00 g by addition of enough volume of deionized water while weighing in a scale (Mettler, Germany), followed by stirring for homogenization. Afterwards, the hot solution (70 °C) was placed under vacuum for 20 s to remove air bubbles and immediately poured at 70 °C onto horizontally leveled and identified 55-mm-diameter polystyrene plates of known weight. The hot solution dispensed into each plate (7 g) was weighted with a precision of 0.0001 g in order to have constant thickness as well as a known initial content of AA into each subsequently generated film. Once cooled to room temperature for gelling, drying was performed at 60 °C for 2.5 h under forced convection. Films were also weighted after drying and peeled off from the polystyrene plates.

Three batches of film (a triplicate of the assay) were produced as above indicated. The AA content was then measured at initial time ($t = 0$) in three films (each one taken from each batch) through a spectrofotometric technique (León and Rojas, 2007) and called "$weight_{AA}(t)$" (eq. (1)), knowing the initial weight (in grams) of AA loaded in the identified plate (called "$weight_{AA}(o,loaded)$"). Thus, the AA proportion remaining at any time [$C_{AA}(t)$] (eq. (1)), including $t = 0$, can be calculated as follows:

$$C_{AA}(t) = \frac{weight_{AA}(t)}{weight_{AA,film}(o, loaded)} \quad (1)$$

where,

$$\Rightarrow \quad C_{AA}(t=0) = \frac{weight_{AA}(o)}{weight_{AA,film}(o, loaded)} = 1.00$$

The rest of the films obtained through the three batches of films produced (triplicate of the assay) were distributed among light-protected desiccators (Nalgene, USA) and stored at 25.0 °C, under vacuum (P = 125 Pa), over saturated solutions of known water activity (a_W^o), in order to maintain a constant RH (33.3, 57.7 or 75.2%) for film equilibration:

$$a_W^o = \frac{RH\%}{100} \quad (2)$$

The salts used were $MgCl_2$ ($a_W^o = 0.333$), NaBr ($a_W^o = 0.577$) and NaCl ($a_W^o = 0.752$) at 25.0 °C (Greenspan, 1977). Storage was performed under vacuum 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 can be analyzed. Film equilibration was followed by the daily measurement of the a_W in the film samples, at 25.0 °C, through a Decagon AquaLab (Series 3 Water activity meter, USA), using a calibration curve made with the standard saturated salt solutions above mentioned. After films attained the a_W^o of equilibrium (eq. (2)), the sample thickness was measured at six different locations in each of ten specimens by using a digital micrometer (Mitutoyo, Kawasaki, Japan).

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

2.3. Measurement of pH

This was performed on the film-forming solutions and on the equilibrated films. A bulb-combined glass electrode (Phoenix, AZ, USA) connected to a pH meter (Consort P901, Belgium) was used for pH measurement of the solutions. A flat surface combined electrode (Phoenix, AZ, USA) was used with the same pH meter to determine the film pH after a slight surface hydration with 20.0 μL of deionized water (Joel et al., 1972). Calibrations were performed with standard buffer solutions of 4.00 and 7.02 pH.

2.4. Spectrofotometric determination of L-(+)-ascorbic acid (AA)

Film samples were taken from each of the three batches of films obtained in order to determine the AA kinetics of hydrolysis from the triplicates of film making. Each film sample was first cut into pieces smaller than 1-mm in size, weighed on an analytical scale (0.0001 g), placed into a 25.00 mL-volumetric flask with a 1% (w/v)-oxalic acid solution and submitted to magnetic stirring for 1.5 h at 5 °C to achieve the total extraction of AA from the film sample. During this time, it was also submitted to vortexing (Velp, Italy) for 90 s at 35 Hz, every 15 min. The suspension was finally centrifuged at 10,000 rpm and 6 °C for 30 min (Eppendorf 5810R Refrigerated Centrifuge, USA). An aliquot was taken from the supernatant and the AA concentration was determined by using 2,6-dichloro phenol indophenol (2,6-DPIP) spectrofotometric method (Rojas and Gerschenson, 1991) though xylene was not used for extraction of the remaining 2,6-DPIP. The AA concentration was determined in two aliquots (duplicate) for each film sample.

2.5. Determination of the citric acid content in films

It was analyzed through the high performance liquid chromatography (HPLC) technique reported by Uckoo et al. (2011). The Spectra System HPLC assembled equipment was a Thermo Scientific SCM1000 Quaternary pump, P4000 degasser, AS3000 autosampler and UV2000 Dual λ Absorbance detector. Analysis of chromatograms was carried out using a ChromQuest Chromatography Data System software. Separation of organic acids was achieved with a Spherisorb ODS 2C18 column (Waters, Ireland) (150 mm × 4.6 mm i.d., 5.0 μm). Chromatographic conditions were slightly modified from (Uckoo et al., 2011) as it follows: an injection volume of 10 μL, a column temperature of 25 °C with an isocratic mobile phase prepared with 0.1% w/v-phosphoric acid and a flow rate of 1.0 mL/min. UV-detection was performed at two wavelengths (λ): 210 nm and 254 nm for CA and ascorbic acid, respectively. Chromatographic separation of AA and CA samples were accomplished in less than 10 min.

2.6. Color

Measurement of the film color was performed with a Minolta colorimeter (Minolta CM-508d, Japan) using an aperture of 1.5 cm diameter, as indicated by León and Rojas (2007). The exposed area was sufficiently large in relation to the illuminated area to avoid any edge effect. The instruments parameter used were D-65 sodium illuminant and a 2° observer. Film samples for color measurement were taken from each of the three batches of films obtained in order to determine browning (yellowness index, YI%; ASTM, 1995) kinetics. White casting surfaces were used because the yellowness is determined as the deviation from whiteness according to the American Society of Testing and Materials (ASTM, 1995). Also, the lightness L, a ($-a$ greenness and $+a$ redness) and b ($-b$ blueness and $+b$ yellowness) HunterLab color parameters were measured. Standard values considered were those of the white background.

2.7. Moisture

Films were sampled after equilibration at each RH, cut into pieces smaller than 1-mm size, weighed (0.0001 g) and placed into small, light glass containers. Samples were dehydrated in a vacuum oven at 70 °C until constant weight, which approximately involved approximately 22–30 days. Determinations were performed on six film specimens at each evaluated condition. Moisture (water content) was informed on dry basis.

2.8. Glass transition temperature (T_g)

Modulated differential scanning calorimetry (MDSC, TA Instruments, USA) was used to determine the T_g (midpoint temperature) from the second scan performed on an equilibrated film sample (10–15 mg) placed into an hermetically sealed 40 μL-aluminum medium pressure pan. An empty pan served as reference. Temperature was brought down to −140 °C (20 °C/min) followed by a 5 min-isotherm at −140 °C. A ± 0.5 °C every 40 s modulation was applied. A ramp was then performed up to 40 °C (10 °C/min), followed by a second decrease in temperature to −140 °C (20 °C/min), and a 5 min-isotherm at −140 °C. Afterwards, a second ramp was performed up to 200 °C (10 °C/min), from which the T_g value was determined. MDSC was periodically calibrated with a sapphire disk, in the full temperature range at which the equipment is employed.

2.9. Statistical analyses

The results are reported as the average and standard deviation. Rate constant of AA hydrolysis (k_{AA}') was calculated by linear regression according to a first order reaction, where AA concentration was expressed as g AA/weight (g) of the corresponding film sample assayed. Analysis of covariance (ANCOVA) was applied for comparison of slopes, that is, of the rate constants (k_{AA}'), as indicated by Sokal and Rohlf (2000). The statistical analyses of results were performed by applying ANOVA (α: 0.05), followed by pairwise multiple comparisons evaluated by Tukey's significance difference test. Nonlinear regressions and the analyses previously detailed were performed through the GraphPad Prism software (version 5.00, 2007, GraphPad Software Inc., USA).

The effect of two quantitative factors (RH and glycerol) on the calculated rate constant of AA destruction through hydrolysis (k_{AA}') was analyzed with a complete 3 × 3 experimental design at the three levels described before for both factors, coded as −1, 0 + 1. A regression model was applied as a function of the lineal values of the quantitative factors (RH•n, glyc•n) and their interactions (RH•n:glyc•n). This statistical analysis was performed with R (version 2.15: R Core Team, 2012).

3. Results and discussion

3.1. Alginate characteristics

According to the GG-block proportion previously determined through circular dicroism for the herein used alginate ($F_{GG} = 0.27$), an amount of 7.73×10^{-4} mol of calcium ions per gram of alginate was calculated by us as necessary for complete zipping of the GG-blocks belonging to neighboring alginate macromolecules in films, as previously discussed by De'Nobili et al. (2013). However, an amount of 4.32×10^{-4} mol of calcium ions per gram of alginate was used for film development in the present work, to permit an adequate flexibility, especially at the lowest level of glycerol used for plasticization.

3.2. Film properties

The casted alginate films developed for antioxidant preservation of food were transparent and almost colorless ($a \approx -0.68$; $b = +4$ to $+7$; YI = 12–15%). These homogeneous and flexible films also showed high initial lightness (L%) (Table 1). The AA proportion remaining at initial time [$C_{AA}(t = 0)$] (eq. (1)) after film constitution was ≈1.01, which means that a 100% of AA recovery was achieved after casting. At this proportion [$C_{AA}(t = 0)$], 6893 ± 79 μg of AA per film (14.1 mg AA/g film) were initially entrapped. The temperature used for gel drying (film casting) was as low as possible (60 °C) in order to get short periods of drying (≤2.5 h). The latter avoids AA losses through hydrolysis that can occur when gels are even wet while they are being dried into the forced convection oven. The initial content of CA was ≈17.6 mg per film or 17.5 g per 100 g of alginate, as determined through the HPLC technique.

The AA destruction through hydrolysis was specifically studied by film storage under vacuum (P = 125 Pa) at constant 33.3, 57.7 or 75.2% RH, in the dark. Equilibration of films with the RH of the environment was attained at ≈28 h of storage, which was determined by the daily measurement of the a_W of films (eq. (2)). Film thickness was then measured after film equilibration. Some trend to the thickness increase with the RH of film storage was observed in B and C film systems (Table 1). On the other hand, at a given RH, the thickness values of films in general decreased with the increase in the glycerol content (systems A, B, C, for the two lower values of RH) (Table 1). The grammage of the films obtained was ≈1.80 g/m^2. The pH measured at the surface of films ranged 3.20–4.06 (Table 1). In general, it was not observed any specific influence of the RH and glycerol (plasticizer) level on the film pH. Only in systems A and B, the pH decreased with the increase in the film moisture content (Table 2), due probably to a favored acid dissociation. The water content rose significantly ($p < 0.05$) with the RH of film equilibration into a given film system due to the increase in water adsorption, whereas a non significant or non specific trend between the film moisture and the glycerol content was found (Table 2).

Table 2
Moisture content and glass transition temperature (T_g) recorded after film equilibration at each relative humidity (RH) of storage (25.0 °C).

System	Glycerol (g/100 g alginate)	RH (%)	Moisture content[a] (g water/g *dm*)	T_g[b] (°C)
A	36.6	33.3	14.1 ± 0.2^{A}	−58.27
		57.7	20.8 ± 0.6^{B}	−67.39; −32.73
		75.2	31.0 ± 0.9^{C}	−70.02
B	54.8	33.3	16.5 ± 0.3^{AD}	−57.41
		57.7	21.9 ± 0.3^{B}	−68.64
		75.2	37.0 ± 0.9^{C}	−76.29
C	109.6	33.3	17.0 ± 0.7 ^{AD}	−69.74
		57.7	24.8 ± 0.9 ^{BD}	−76.19
		75.2	36.2 ± 0.8^{C}	−89.31

dm: dry mass.
[a] Mean and standard deviation ($n = 6$) are shown.
[b] Mean is shown. The standard deviation is not reported because it is lower than 1% of the T_g value.

As determined through modulated DSC, films equilibrated at each RH showed T_g values below the storage temperature (25.0 °C) (Table 2). This meant that equilibrated films were at the amorphous-rubber state. At this condition, the macromolecular mobility due to plasticization is important. The T_g values decreased significantly ($p < 0.05$) with the increase in the glycerol proportion as well as in the water content or RH (Table 2). Hence, glycerol as well as the water captured during storage acted as plasticizers of the alginate networks of films. According to Roger et al., 2004), samples of alginate powder exhibited T_g values between 95 °C and 136 °C. Block copolymers like alginates can behave as physical blends rather than true solutions. Physical blending leaves moderately large regions of homogeneous composition, each type of region experiencing its own glass transition (Ferry, 1980; Liu et al., 2006). Each phase exhibits its own distinct T_g. In general, only one T_g was detected in the alginate films developed in the present work (attributable to plasticization and also to random alternating blocks), except at the lowest glycerol (plasticizing) level and 57.7% RH, where a second T_g was detected at −32.73 °C (system A) (Table 2).

At 0 °C, DSC scans of alginate films did not show an endothermic peak corresponding to free water, which meant that water was strongly adsorbed in the film network at every RH assayed (Hatakeyama and Hatakeyama, 1998).

3.3. Stability of antioxidant alginate films

As above mentioned, the hydrolytic stability of the AA

Table 1
Thickness[a,b], pH[a,b] and color parameters[a,c] determined in the alginate films[d].

System	RH (%)	Glycerol (g/100 g alginate)	+*b*	YI (%)	*L* (%)	Thickness (mm)	pH
A	33.3	36.6	5.8 ± 0.6 ^{ABC}	10 ± 1 ^{AB}	83 ± 1 ^{BC}	0.11 ± 0.02 ^{CDE}	3.81 ± 0.03^{FG}
	57.7					0.12 ± 0.05 ^{BCD}	3.89 ± 0.05^{CD}
	75.2					0.13 ± 0.02 ^{ABC}	3.52 ± 0.01^{H}
B	33.3	54.8	5.5 ± 0.9 ^{BC}	11 ± 2 ^{AB}	83 ± 1 ^{BC}	0.07 ± 0.01 ^{FGH}	4.06 ± 0.01^{I}
	57.7					0.08 ± 0.01 ^{EFGH}	3.37 ± 0.01^{MN}
	75.2					0.15 ± 0.03 ^{AB}	3.20 ± 0.06^{O}
C	33.3	109.6	5.4 ± 0.9^{C}	9 ± 2^{B}	84 ± 1^{B}	0.05 ± 0.01^{H}	3.47 ± 0.03^{HKQ}
	57.7					0.06 ± 0.01 ^{GH}	3.59 ± 0.06^{E}
	75.2					0.13 ± 0.03 ^{ABC}	3.99 ± 0.05^{A}

[a] Mean and standard deviation ($n \geq 11$) are shown.
[b] It was measured after film equilibration at each relative humidity (RH) of storage and 25.0 °C.
[c] Lightness (L) and b (blue–yellow component) HunterLab color parameters, as well as the yellowness index (YI) shown were recorded initially, after casting.
[d] The same capital letter as a superscript into a column means that there are non significant differences ($p < 0.05$).

compartmentalized in the alginate films was specifically determined by film storage in the absence of air (vacuum, P = 125 Pa) and at controlled RH. It permitted to determine that, in alginate films, the ratio [$C_{AA}(t)$] between the remaining AA concentration [$weight_{AA}(t)$] in each identified film and the initial one [$weight_{AA}(t = 0)$] loaded in the same film (eq. (1)), statistically changed with the storage time (t) according to the pseudo-first order ($p < 0.05$) kinetic law (eq. (3)) (Fig. 1). Hence, the pseudo first order kinetic rate constant of the AA hydrolysis (k_{AA}') was the slope calculated through the linear regression of the experimental data (Fig. 1) according to a first order reaction whose differential equation is as follows:

$$r_{AA} = -\frac{1}{\nu_{AA}}\frac{dC_{AA}}{dt} = k'_{AA} \cdot C_{AA}(t) \quad (3)$$

wherein ν_{AA} is the stoichiometric coefficient for AA hydrolytic reaction ($\nu_{AA} = 1$), r_{AA} is the AA-reaction rate/unit volume at a constant temperature (25.0 °C), $C_{AA}(t)$ is the AA proportion remaining at any time (t) (eq. (1)). The rate constants of AA hydrolysis (k_{AA}') are reported in Table 3 for the film systems (A, B, C).

As shown in Fig. 1 as an example, at a given RH (e.g. 57.7%), the AA stability to hydrolysis (k_{AA}' -slope- values, because $\nu_{AA} = 1$) was affected by the glycerol level, and also by the RH used for film storage at 25.0 °C. Half-life times of AA in films were also calculated and summarized in Table 3, according to the following equation, derived from equation (3):

$$t_{1/2} = \frac{0.693}{k'_{AA}}$$

The lowest level of glycerol used for plasticization (36.5 g/100 g alginate) together with 33.3% and 57.7% RH of film equilibration produced the longest half-life times of AA in alginate films, which were above 250 and 80 days, respectively (Table 3).

The content of CA was then determined in film samples of the C system stored at 75.2% RH, where the AA showed the lowest stability (the highest k_{AA}' value) (Table 3). It was analyzed through HPLC, together with AA, by applying a methodology used for determination of organic acids, as above indicated. It was found that the initial CA content in films did not change significantly ($p < 0.05$) during storage (Table 4), while the AA content decreased (Fig. 1; Table 4). Consequently, the activity of CA remained in films, but it was paralleled by the loss in the AA reducing activity.

Besides, the browning development was determined as the increment of the YI of films with the storage time. In films

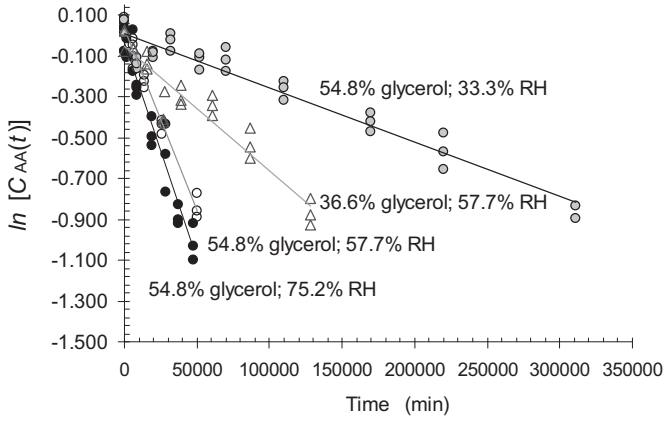

Fig. 1. Kinetics of L-(+)-ascorbic acid hydrolysis in alginate films loaded with citric acid: influence of the glycerol level and relative humidity (RH) of storage (25.0 °C).

developed without CA in previous works (Pérez et al., 2009; De'Nobili et al., 2013), browning data always fitted ($p < 0.05$) to a pseudo-zero order reaction in the AA loaded films (Fig. 2A). Instead, films carrying CA and AA showed that the YI data draw curves (Fig. 2A–C). It was determined that the experimental points fitted according to a zero- and first-order combined kinetic equation (eq. (4)), a two-stage model of browning (continuous lines shown in Fig. 2) postulated by Pérez-Zúñiga et al. (2000):

$$\frac{dA}{dt} = k_o - k_1 A \quad (4)$$

wherein k_0 and k_1 are the zero- and first-order rate constants of browning development, respectively, A corresponds to the YI% and t is the storage time, at a constant temperature of 25.0 °C. This kinetic considers a first faster phase of formation of color compounds, presumably described by the zero-order equation (and k_0 rate constant), followed by a slower phase of dissociation of some color products, yielding reaction compounds with loss of color intensity, presumably described by the first order equation (and k_1 rate constant). Integrating the equation (4) between $A(t)$ and $A_0(t = 0)$ for dA and between t and $t = 0$ for dt, yields:

$$A(t) = \frac{k_o}{k_1} - \left(\frac{k_o}{k_1} - A_o\right).e^{-k_1 t} \quad (5)$$

Rate constants obtained by fitting of experimental data to equation (5) are summarized in Table 3. The k_0 and k_1 browning rate constants increased with the RH of film equilibration for systems A and C. The same was observed in B film system when the RH increased from 33.3% to 57.7% or to 75.2%, but the maximal values of k_0 and k_1 were obtained for films equilibrated at 57.7% RH (Table 3). Hence, the glycerol (plasticizing) level used for film development did not affect the browning rate constants k_0 and k_1 in the same manner as RH. At 33.3% RH, the k_0 browning rate constants were the same in systems A, B and C (Table 3), producing the same initial curves for these three systems (Fig. 2C), whereas the k_1 rate constant (the dissociation rate constant of some color products) decreased as the glycerol level increased (systems A, B, C). It was then observed that at the lowest level of glycerol (system A), the highest value of k_1 (or the highest dissociation rate of some color products) produced the lowest curve of browning development, at 33.3% RH (Fig. 2C). At 57.7% RH, systems A and C showed the same value of k_1 rate constant but k_0 increased with the glycerol level (Table 3), which led to produce the highest curve of browning development for the C system (Fig. 2B). At the intermediate level of glycerol (system B), the highest value of k_0 was found (Table 3), but this seemed to be overcame by the highest value of k_1 (or the highest dissociation rate of color products) obtained, which produced a curve of browning development for B system that was slightly below that shown by the C system, at 57.7% RH (Fig. 2B). At 75.2% RH, the lowest value of k_0 was determined at the intermediate level of glycerol (system B), also accompanied by the lowest value of k_1 (the lowest dissociation rate of some color products). These facts determined the slowest curve of browning development observed for B system (Fig. 2A). At 75.2% RH, the highest values of k_0 obtained for systems A and C (Table 3) determined the highest rates of increment of their browning curves seen in Fig. 2A.

In general, browning was initially faster in the CA loaded films, as shown as an example in Fig. 2A, in comparison to data reported by De'Nobili et al. (2013) for films made without CA, where a pseudo-zero order browning kinetic was observed (straight line shown in Fig. 2A). However, at longer storage times, browning development that proceeded according to a pseudo zero-order kinetic in films made without CA can produce higher YI values

Table 3
Rate constants[a] of L-(+)-ascorbic acid (AA) hydrolysis (k_{AA}') and browning development (k_O[b], k_1[b]) in films stored at constant relative humidity (RH) and 25.0 °C. Half life time ($t_{1/2}$) of AA calculated for films.

System	Glycerol (g/100 g alginate)	RH (%)	$k_{AA}' \times 10^5$ (min^{-1})	$t_{1/2}$ AA (d)	*Browning rate constants*	
					$k_O \times 10^3$ (YI%·min^{-1})	$k_1 \times 10^5$ (min^{-1})
A	36.6	33.3	0.19 ± 0.01	254.4	0.40 ± 0.09	2.4 ± 0.8
		57.7	0.60 ± 0.04	80.6	2.4 ± 0.4	6.4 ± 0.9
		75.2	2.3 ± 0.2	21.0	7.5 ± 0.9	11.0 ± 0.2
B	54.8	33.3	0.27 ± 0.03	179.0	0.31 ± 0.09	1.3 ± 0.4
		57.7	1.77 ± 0.07	27.3	5.1 ± 0.7	10.4 ± 0.2
		75.2	2.1 ± 0.1	23.0	3.3 ± 0.3	4.4 ± 0.6
C	109.6	33.3	0.45 ± 0.03	107.4	0.4 ± 0.1	0.7 ± 0.3
		57.7	1.63 ± 0.09	29.7	3.4 ± 0.4	6.1 ± 0.8
		75.2	4.6 ± 0.1	10.5	5.7 ± 0.4	7.5 ± 0.9

$t_{1/2}$: half-life time of L-(+)-ascorbic acid in films (d: days).
[a] Mean and standard deviation ($n > 11$) are shown.
[b] k_O and k_1 are zero-order and first-order rate constants, respectively, of the combined kinetic equation fitted to the experimental points obtained during browning of films containing citric acid (Pérez-Zúñiga et al., 2000).

Table 4
Citric acid and L-(+)-ascorbic acid (AA) contents determined through HPLC on the "C" film system during storage at 75.2% relative humidity (RH), 25.0 °C.

Glycerol (g/100 g alginate)	RH (%)	Time (d)	Citric acid[a,b] content (mg/film)	AA[a,c] content (mg/film)
109.6	75.2	0	17.2 ± 0.2[A]	6.41 ± 0.09
		11	16.4 ± 0.7[A]	3.24 ± 0.05
		104	17.1 ± 0.6[A]	ND

[a] The mean and standard deviation ($n = 3$) are shown.
[b] The same capital letter as a superscript into a column means that there are non significant differences ($p < 0.05$).
[c] The yield of the AA extraction with water from the film sample for the HPLC method was 93%, whereas a 98% or higher efficiency was reached in the extraction by using oxalic acid as explained in the spectrophotometric method used for the kinetic study of the AA destruction.

(Fig. 2A). Hence, the constant presence of CA in films during storage (Table 4) modified the browning kinetics, which slowed down the browning development, after the initial period of film storage. It was herein found that the CA content of films did not change during storage (Table 4), while browning was developed in films according to a zero- and first-order combined kinetic, subsequently to the AA loss. Hence, as the CA content in films did not change (Table 4) it can then be suggested that CA did not react during the AA degradation and browning development. However, CA modified the kinetic order of the browning reaction, which means that the mechanism of this reaction was changed.

As expectable, the hydrolytic destruction of AA was mainly affected by the RH of film equilibration ($p < 0.001$) (Table 5), which is directly related to the water mobility in the film network (León et al., 2009). It was certainly less dependent on the glycerol level ($p < 0.05$) (Table 5). The experimental design of these two quantitative factors (RH•n and glyc•n) demonstrated that the rate constant of AA hydrolysis (k_{AA}') significantly increased as a consequence of the separate increases in RH or glycerol content. On the other hand, it was also observed a significant ($p < 0.05$) dependence on the RH-glycerol interaction (Table 5). It can then be suggested that the former presence of glycerol as the polymer plasticizer is a necessary condition to permit the following penetration of water into the polymeric network, with additional plasticization during storage (Pérez et al., 2009).

The alginate used in the present work was constituted by 27% of GG-blocks, which are the only ones that can zip alginate macromolecules together through calcium mediated junction zones in an 'egg box' conformation. Guluronate junction zones also involves

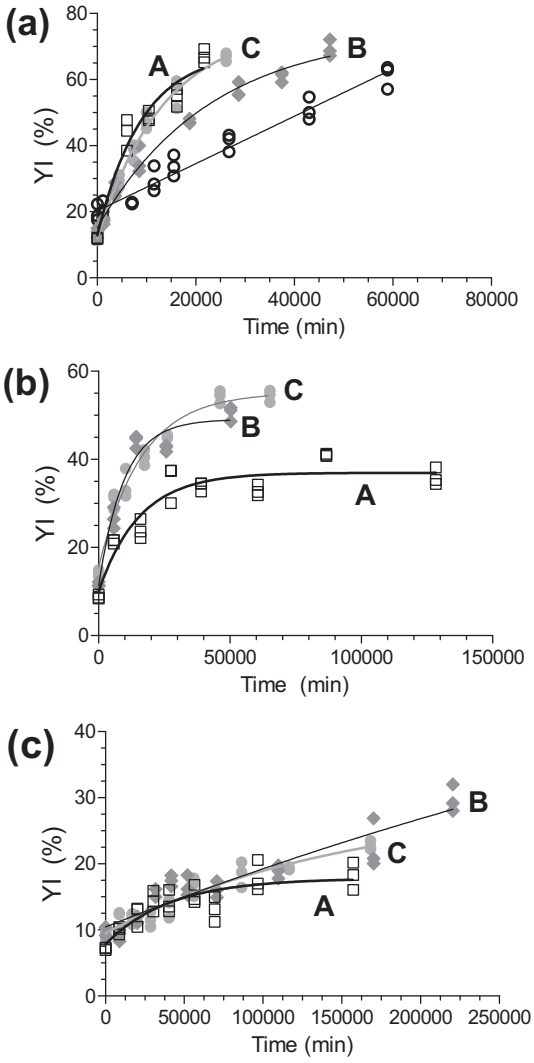

Fig. 2. Kinetics of browning development in alginate films stored at 25.0 °C and 75.2% (a), 57.7% (b) and 33.3% RH (c) [systems A (□), B (◆) and C (●)]. Zero-order kinetic of browning of a film developed by De'Nobili et al. (2013) with L-(+)-ascorbic acid and 109.6% of glycerol, without citric acid (75.2% RH, 25 °C) [(○)] (a).

Table 5
Probability obtained for the rate constants of L-(+)-ascorbic acid (AA) hydrolysis (k_{AA}'): effect of the two quantitative factors (relative humidity and glycerol) analyzed.

	k_{AA}'
RH.n[a]	**1.24×10^{-6}**
glyc.n[a]	**0.0015**
RH.n:glyc.n[a]	**0.0157**
Residual standard error	4.724×10^{-6}
Multiple R^2	0.8839
F-test probability	24.74

Relative humidity (RH) or glycerol (glyc) linear factors (RH•n; glyc•n).
[a] Probability for rate constants. Bold numbers highlight significance ($p < 0.05$).

water molecules (Braccini and Pérez, 2001), which correspond to non-freezable bound water (Ping et al., 2001). This immobilized water is that not involved in chemical reactions responsible for degradation of hydro-sensitive biomolecules such as AA. The rest (73%) of the alginate macromolecule (MM- and flexible GM + MG-blocks) is not able to immobilized water in a non-freezable form. 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, 1967). As RH of film equilibration increases, the polymeric network leaves a higher proportion of water with higher mobility, available for chemical reactions (León and Rojas, 2007).

4. Conclusions

In the presence of CA in films, the RH and glycerol, separately and through their interaction, affected the pseudo first order rate constants of AA hydrolysis in a direct relationship. Water was the factor responsible for AA hydrolysis, and glycerol (plasticizer) may facilitate the water penetration from the environment into the polymeric network. Browning development in films was due only to the AA degradation since CA content did not change during film storage. Additionally, the film browning was slowed down at longer storage times by CA, which changed the kinetic order of the browning reaction and, hence, the reaction mechanism. CA protons may catalyze the formation of unstable color compounds. Hence CA, which improves the activity of proper antioxidants like AA through its metal chelator capacity, can be used together with AA for the development of antioxidant alginate based edible films, using a glycerol (plasticizer) proportion lower than 54.8 g/100 g alginate and RH of storage below 75.2%. In the present work it was possible to determine that the AA and CA were effectively loaded (100% of recovery) after casting. Also, the half life of films as active interfaces to achieve antioxidant protection was determined. Considering these results alginate films can be potentially useful to avoid oxidation in confectionary and nuts.

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