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Hydrolytic and Oxidative Stability of L-(+)-Ascorbic Acid Supported in Pectin Films: Influence of the Macromolecular Structure and Calcium Presence

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ABSTRACT: The hydrolytic and oxidative stability of L-(+)-ascorbic acid (AA) into plasticized pectin films were separately studied in view of preserving vitamin C activity and/or to achieve localized antioxidant activity at pharmaceutical and food interfaces. Films were made with each one of the enzymatically tailored pectins (50%, 70%, and 80% DM; Cameron et al. *Carbohydr. Polym.* **2008**, *71*, 287−299) or commercial high methoxyl pectin (HMP; 72% DM). Since AA stability was dependent on water availability in the network, pectin nanostructure affected the AA kinetics. Higher AA retention and lower browning rates were achieved in HMP films, and calcium presence in them stabilized AA because of higher water immobilization. Air storage did not change AA decay and browning rates in HMP films, but they significantly increased in Ca-HMP films. It was concluded that the ability of the polymeric network to immobilize water seems to be the main factor to consider in order to succeed in retaining AA into film materials.

KEYWORDS: *pectin nanostructure, edible film, ascorbic acid, browning, water, hydrolysis, oxidation, kinetics*

INTRODUCTION

In many industries, various compounds and ingredients used exhibit significant decrease or degradation in their desired properties due to oxidation and/or hydrolysis. Food, pharmaceutical, nutraceutical, cosmetics, and chemical industries utilize a significant number of substances that are prone to oxidation, the half-life time of these active components being shortened. For example, functional dairy products are usually fortified or enriched with unsaturated lipids of known positive health effects, mainly in prevention of cardiovascular diseases and cancer. The main lipids used are omega-3 long-chain polyunsaturated fatty acids, conjugated linoleic acid, and phytosterols. In order to ensure the integrity of the original lipids and hence guarantee their quality and safety,[2] oxidative stability of functional dairy products also needs functional protection. Additives used in healthier food formulations (antimicrobial agents, antioxidants, etc.) should be as natural as possible. As well additives must be used at the lowest but most effective concentration level. A localized activity of preservatives could be the way to achieve the latter, and edible biopolymer films are effective for carrying additives with localized activity at interfaces. Supporting of active compounds into edible films can overcome negative interactions between preservatives used in a food system, or between a preservative and the nutrients in the case of food formulations.[3] L-(+)-Ascorbic acid (AA) is a reducing agent and also a water soluble vitamin and antioxidant used for pharmaceutical and food preservation and/or supplementation. In recent years, there has been an enormous demand for natural antioxidants mainly because of adverse toxicological reports on many synthetic compounds.[4]

AA is unstable to hydrolysis and oxidation, and its degradation also leads to browning.[5,6] Through a reversible reaction, oxidation of AA under air first produces dehydroascorbic acid (DHA), which also has vitamin C activity. Unlike AA, the DHA undergoes relatively fast spontaneous hydrolysis to 2,3-diketogulonic acid, and the DHA cannot be regenerated. Transition metal catalysts greatly accelerate this AA conversion.[7] In the absence of molecular oxygen, the reactions of AA with certain transition metal ions, like Fe(III), Cu(II), Hg(II), or Cr(VI), result in metal ion reduction and simultaneous oxidation of AA to DHA. However, in the presence of oxygen or other oxidants, such as hydrogen peroxide or lipid peroxides, these metals act predominantly as catalysts in a chain of partially radical reactions.[8] AA carried into edible films may constitute another form of natural antioxidant protection at interfaces and of AA stabilization with controlled delivery. To accomplish this, water should be retained by the polymeric nanostructure in order to be less available for AA hydrolysis.[9] Dietary fiber inclusion in food formulations also confers the functional label to the product. Pectins are soluble dietary fibers mainly present in the primary cell walls and middle lamella of plants, being then renewable as well as biodegradable polymers. Depending on the botanic

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origin, pectin localization, climate, and extractive procedure, the carboxylic groups of the homogalacturonan (HG) regions are either highly or partially esterified with methanol, which determines the DM.[10] Further, the average demethylated block size of HG and the pattern of distribution of demethylated blocks are biologically (or can be in vitro) managed by the blockwise action of pectinmethylesterase, producing pectins with different rheological properties and calcium reactivity, even though they have the same DM.[1,11−14] Enzymatic engineering of the pectin nanostructure for tailoring functionality can expand the role of pectin as a food-formulating agent and the use of in situ modification in prepared foods.[14] Enzymatic engineering can also contribute to produce the pectin nanostructures required for specific demands in film/membrane development. The objective of the present work was to explore the influence of the macromolecular structure on the hydrolytic stability of AA when supported in pectin films. The presence of Ca^{2+} in the film formulation was also analyzed by considering its effect on the hydrolytic and oxidative degradation of AA and the subsequent browning development.

MATERIALS AND METHODS

Chemicals. Three different pectins characterized by a DM of 50%, 70%, and 80%, respectively, and by the nanostructure (average demethylated block size and average number of demethylated blocks of size n per molecule) reported in Table 1 were applied to film development. These pectins were previously obtained through controlled demethylation of a model HG (DM = 94%) by a monocomponent citrus salt-independent pectin methylesterase (PME).[1]

Table 1. Average Length of the Longest Demethylated Block (DP), Average Demethylated Block Size ($\overline{BS}$), and Number of Blocks ($\overline{B}_n$) per Molecule of the Average Block Size for Each Series of Tailored Pectins Used[a]

pectin series (DM[b] %)	oligomer DP	$\overline{BS}$	$\overline{B}_n$
50	52	8.6	3.9
70	42	10.2	2.0
80	14	5.3	1.7

[a]References 1 and 13. [b]Degree of methoxylation.

Food grade pectin with a high degree of methylation (GENU pectin type B rapid set-Z) for manufacturing foodstuffs was provided by CP Kelco (J. M. Huber Corporation, Edison, NJ, USA), called HMP or "commercial" form in this work. Its relevant molecular characteristics were reported by Perez, Flores, Marangoni, Gerschenson, and Rojas.[15] It contains negligible amounts of divalent cations and slightly higher content of sodium and potassium.

All other chemicals were of analytical quality from Merck (Buenos Aires, Argentina) or from Sigma (St. Louis, MO, USA).

Film Making Procedure. The films were made using casting technology. An aliquot of 6.00 g of pectin was slowly poured into deionized water. This solution was continuously stirred under controlled high (1,400 rpm constant) speed shear in a vertical stirrer (model LH, Velp Scientifica, Italy) in order to reach homogeneous hydration of the powder without lumps. During stirring, the obtained viscous, homogeneous, and transparent system was then heated at a constant heating speed (5.0 °C/min) on a hot plate (Velp Scientifica, Italy), with simultaneous recording of the temperature using a thermocouple connected to a Consort millivoltmeter (P 901, CE Belgium). Glycerol was added for plasticization at a constant proportion of 36.80% w/w [glycerol × 100/(pectin + glycerol)],[16] followed by potassium sorbate (0.03% w/w) as antimicrobial agent, AA (0.100% w/w), and $CaCl_2 \cdot 2H_2O$ when needed in the formulation with commercial high methoxyl pectin (HMP). The total weight of the system was completed by adding enough deionized water, followed by stirring for homogenization. The pH of the film forming solution was 3.52. This hot solution was placed under vacuum for 20 s to remove air bubbles and immediately poured onto leveled polystyrene plates. The fractionated system was dried in a convection oven of controlled air speed for 2.5 h at a constant temperature. The films were peeled from the polystyrene plates and stored in desiccators over a saturated solution of known water activity ($a_W°$),[17,18] in order to maintain a constant relative humidity (a_W = RH(%)/100) for film equilibration. The salt used was NaBr ($a_W°$ = 0.577) at 25 °C. Equilibration was assessed by the measurement of the a_W of film samples every day until attaining the same value of RH(%)/100, corresponding to $a_W°$ of the saturated solution used. Afterward, the sample thickness was measured to the nearest 0.001 mm using a digital micrometer (Mitutoyo, Kawasaki, Japan) at six different locations in each of the ten specimens.

Three batches of films (replicates) were prepared as above indicated. The film samples obtained from each batch were identified and distributed among the desiccators for storage at the RH (57.7%) studied, in order to involve the influence of the film making in the following determinations.

Storage was performed under vacuum (P = 132 Pa) at 57.7% constant RH in order to ensure that AA degradation initiated through the irreversible hydrolysis of its lactone ring as the first (and limiting) reaction step. Hence, the specific influence of water in the AA stability can be analyzed. On the other hand, films made with commercial HMP or with HMP and Ca^{2+} (called Ca-HMP system) were alternatively stored at the same RH and temperature under air atmosphere (P = 1.013 × 10^5 Pa), in order to infer the specific influence of oxygen on the AA kinetics in darkness. The following analyses were performed on samples collected from the three batches at each time of interest.

Water Activity. It was determined on the film samples with a Decagon AquaLab (series 3 water activity meter, USA) at 25 °C (constant temperature) using a calibration curve made with the standard saturated salt solutions of $MgCl_2$ ($a_W°$ = 0.333), NaBr ($a_W°$ = 0.577), and NaCl ($a_W°$ = 0.752) at 25 °C. The cell volume of Decagon's Aqualab was half-reduced by inserting a Teflon cylindrical piece, in order to ensure a cell atmosphere conditioned by the RH of the equilibrium vapor pressure coming from the film sample.

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 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 the 2,6-dichlorophenolindophenol (2,6-DPIP) spectrophotometric method but without extraction of the 2,6-DPIP excess with xylene.[19] The AA concentration was determined in two different aliquots (duplicate) for each film sample.

Measurement of pH. pH measurement was performed on the gel-forming solutions as well as on casted films after their equilibration at the corresponding RH. A bulb-combined glass electrode (Phoenix, AZ, USA) connected to a pH meter (Consort P901, ECC) was used for pH measurement of the gel-forming solutions. Alternatively, a flat surface combination electrode (Phoenix, AZ, USA) was used in the same arrangement to determine the film pH after a slight surface hydration with 20.0 μL of deionized water.[20] Calibrations were carried out with standard buffer solutions of pH 4.00 and 7.02.

Film Color. Film color was determined through a Minolta colorimeter (Minolta CM-508d, Tokyo, Japan) using an aperture of 1.5 cm diameter. The exposed area was sufficiently large in relation to the illuminated area to avoid any edge effect. The instrument parameters used were a D-65 (sodium) illuminant and a 2° observer.

Table 2. Rate Constants[a] of L-(+)-Ascorbic Acid (AA) Degradation under Vacuum (k_{AA}') or under Air (k_T) Storage and Nonenzymatic Browning Development[b] (k_{YI}) for Films Stored at 57.7% of Constant Relative Humidity and Temperature (25 °C) in the Indicated Environment Composition; Initial AA Concentration and Yellowness Index (YI) Values as Well as Mean Lightness and pH Recorded during Storage

Vacuum; 57.7% Relative Humidity; 25 °C						
sample	initial AA content (% w/w)[c]	lightness[d] (%)	initial YI (%)	pH[d]	$k_{AA}' \times 10^6$ (min^{-1})	$k_{YI} \times 10^4$ (YI%·min^{-1})
50-pectin film	3.0 ± 0.3	80 ± 3	20.0 ± 0.7	3.8 ± 0.4	8.3 ± 0.4	2.2 ± 0.1
70-pectin film	3.1 ± 0.1	82 ± 2	18.2 ± 0.9	4.0 ± 0.3	8.1 ± 0.3	2.01 ± 0.08
80-pectin film	3.0 ± 0.2	82 ± 2	20 ± 1	4.10 ± 0.4	16.3 ± 0.9	4.0 ± 0.2
HMP film	3.0 ± 0.2	79 ± 3	16 ± 3	3.9 ± 0.4	5.1 ± 0.6	4.6 ± 0.2
Ca-HMP film	3.2 ± 0.1	83 ± 2	17 ± 2	4.0 ± 0.3	0.87 ± 0.08	0.49 ± 0.07
Air Atmosphere; 57.7% Relative Humidity; 25 °C						
sample	initial AA content (% w/w)[c]	lightness[d] (%)	initial YI (%)	pH[d]	k_T[e] $\times 10^6$ (min^{-1})	$k_{YI} \times 10^4$ (YI%·min^{-1})
HMP film	3.2 ± 0.3	83 ± 2	17 ± 2	3.9 ± 0.3	4.9 ± 0.4	lag[f] = 14*d*; 3.7 ± 0.2
Ca-HMP film	3.0 ± 0.2	84 ± 2	17 ± 2	3.6 ± 0.4	2.5 ± 0.4	lag[f] = 15*d*; 1.0 ± 0.5

[a]Standard deviations are shown (n = 3). [b]Measured as yellowness index (YI) increase against time of storage. [c]g of AA/100 g of film. [d]Values reported are the mean and standard deviations corresponding to all values recorded during the total storage period. [e]Total rate constant of AA destructions (eq 5). [f]Lag (d = days) was the delay found for the beginning of the YI increment with the storage time.

The film samples were first rested on a white background standard. L, a, and b (HunterLab) values were averaged from five positions randomly selected across each 55 mm diameter film sample.[21] Color parameters ranged from L = 0 (black) to L = 100 (white); $-a$ (greenness) to $+a$ (redness), and $-b$ (blueness) to $+b$ (yellowness). Standard values considered were those of the white background. White casting surfaces were used because the yellowness is determined as the deviation from whiteness according to the American Society of Testing and Materials.[22] The yellowness index (YI) values of films were calculated to report the NEB developed in film samples, according to the ASTM method D1925.[22] Film samples for color measurement were taken from each of the three batches of films obtained in order to determine the browning (YI) kinetics from the triplicates of film making.

Water Content. Film samples were taken 15 days after equilibration with the RH of storage for moisture determination. Each film was cut into pieces smaller than 1 mm in size, weighing (0.0001 g), placed into small, light glass containers, and dried in a vacuum oven at 70 °C until constant weight (around 30 days). Determinations were performed on six film specimens from each evaluated condition of RH, taking two film samples per batch.

Glass Transition Temperature (T_g). Differential scanning calorimetry (DSC 822[e] Mettler Toledo calorimeter, Schwerzenbach, Switzerland) was used to determine the T_g (onset value[23]) from the second scan performed with the equilibrated film samples (10−15 mg) placed into a hermetically sealed 40 μL aluminum pressure pan. An empty pan served as reference. The first scan performed from −140 to 40 °C (10 °C/min) was followed by a second scan from −140 to 120 °C (10 °C/min), after cooling at 18 °C/min. An average value of replicates taken from the three batches of films, and the corresponding standard deviation, were reported. DSC was calibrated with the melting points of indium (156.6 °C), lead (327.5 °C), and zinc (419.6 °C), in addition to the periodic calibration performed with a sapphire disk, in the whole temperature range where the equipment is usually employed.[24]

Proton Nuclear Magnetic Resonance (NMR) Mobility. All the experiments were performed on the equilibrated film samples using a Bruker Avance II spectrometer 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). The free induction decay (FID) was exported to WIN-NMR (Bruker) software, where it was Fourier transformed and phase and baseline corrected. Overlapping spectra were deconvoluted by analysis. Peak intensity (area) and line width of the deconvoluted peaks were recorded. The exponential curve fitting was performed at 50 and 10 μs using a nonlinear fitting program (OriginPro 7.5 SRO, Origin Lab Corporation, Northampton, MA, USA) and a Maxwell two-component curve was the best fitting model:

$$A_t = A_1 \exp\left(\frac{-t}{T_{2a}}\right) + A_2 \exp\left(\frac{-t}{T_{2b}}\right) \quad (1)$$

wherein T_{2a} and T_{2b} are the spin−spin relaxation times with "a" and "b" subscripts indicating the two components of the relaxation process; A_t = peak height, A_1 and A_2 = equilibrium magnetization. An average value of replicates taken from the three batches of films, and the corresponding standard deviation, were reported

Tensile Test. The tensile strength (N/m) was calculated as the ratio between the tensile force (N) and the corresponding elongation or deformation (m) at film failure, determined from the force-elongation curves recorded at 5 mm/min constant crosshead speed in an Instron Testing Machine (model 3345, Norwood, MA, USA), equipped with a load cell of 100 N and pneumatic grips with flat rubber coated faces. Rectangular specimens of 6 mm width and 60 mm total length were cut and used in the tensile test, in order to be sure that only uniaxial tension was accomplished along testing, as it was determined through previous assays carried out with specimens of different width. A gage length of 20 mm was used along assays, and elongation is reported as relative deformation to this initial gage length. At least ten specimens with nonsignificant difference in thickness ($p < 0.05$) of a given formulation were used, each of them cut from a different film sample.

Statistical Analysis. The results are reported as the average and standard deviation. Rate constants of AA destruction were calculated by linear regression where AA concentration was in terms of g of AA/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.[25] The statistical analyses of results were performed by applying analysis of variance (ANOVA; α: 0.05), followed by pairwise multiple comparisons evaluated by Tukey's significant difference test.[25] The GraphPad Prism software (version 5.00, 2007, GraphPad Software Inc., USA) was used for all the analyses previously detailed.

RESULTS AND DISCUSSION

Stability of L-(+)-Ascorbic Acid to Chemical Hydrolysis in Pectin Films. *1. Effect of the Pectin Structure.* First studied was the influence of the macromolecule structure in the development of film networks able to stabilize the supported

AA molecule to its hydrolysis. In this context, edible films were made with one of the pectins tailored by demethylation of a model HG (94−97% of GalA, DM = 94%; only 3−6% of Gal) through a monocomponent citrus PME at pH 4.5 (DM = 50%) or 7.5 (DM of 80% and 70%). They were characterized by the average demethylated block size ($\overline{BS}$) and the average number of demethylated blocks ($\overline{B}_n$) of size n ($\overline{B}_n$) per molecule[1] indicated in Table 1. The parent HG presented a MW ≈ 43,280 Da, which means a HG stretch with a $GalA_n$ chain of n = 246.

Also, films were made with a commercial HMP (MW ≈ 457,000 Da) with a DM of 72% and a degree of acetylation of ≈14%.[15] The latter can contribute to hinder in some degree the structure at the hydrogen bonding and hydrophobic mediated junction zones in the development of the polymeric film network, modifying or modulating the macromolecular aggregation.[26] The commercial HMP was essentially constituted by GalA (82% per 100 g of pectin) with a 2.3% w/w of Rha, 6.0% w/w of Ara, and 8.6% w/w of Gal. This composition gives a GalA/Rha molar ratio of 32.6 and (Ara + Gal)/Rha and Ara/Gal molar ratios of 6.0 and 0.8, respectively.[15] From these ratios, it can be inferred that the commercial pectin macromolecule of 457,000 Da (molecular weight) could be structurally constituted by ≈49 repeating units of (a) at least 28 molecules of GalA monomers (≈22 of them methylated), followed by (b) other two molecules of free GalA alternating with 2 molecules of L-rhamnose (RG-I), the latter two being substituted by two arabinogalactan I side chains of four galactose and 2−3 arabinose residues.[15] It was then estimated that disorder (hairy) regions of RG-I contributed 18% of the total carbohydrate content of this HMP, while the rest was GalA organized in the HG ("smooth" regions).

Films obtained from pectins tailored by demethylation or from commercial HMP showed similar appearance. Homogeneous and flexible films plasticized by glycerol [glycerol × 100/(pectin + glycerol) = 36.80% w/w] were obtained after casting from each pectin solution. Films were transparent, slightly yellow (b = +9.0; YI = 19% ± 2), and with high initial lightness (L = 83.5% ± 0.8). The AA concentration initially determined was ≈3.00 g of AA/100 g of film (Table 2), which accounted for 100% of AA recovery after casting. Film samples removed from polystyrene plates were stored at constant 57.7% RH and 25 °C under vacuum in order to specifically study the AA degradation through hydrolysis.[3] They rapidly equilibrated during the first 24 h of storage when a a_W° of 0.577 was determined on film samples. Thickness observed after equilibration was 0.134 ± 0.030 mm for film specimens. The film pH recorded during storage varied as shown in Table 2. Moisture contents reported as g of water/100 g of film's dry mass are summarized in Table 3.

Table 3. Glass Transition Temperature (T_g) and Moisture Contents of the Pectin Films Equilibrated at 57.7% of Constant Relative Humidity and 25 °C

film sample	T_g (C)	moisture content[a] (g of water/100 g dry mass)
50%-pectin	−102.43	29.0 ± 0.5
70%-pectin	−99.31	23.4 ± 0.4
80%-pectin	−100.62	23.8 ± 0.2
HMP	−94.20	24.3 ± 0.4
Ca-HMP	−88.56	32.4 ± 0.8

[a]The mean and standard deviation are shown (n = 6).

AA showed different stability to hydrolysis when compartmentalized into each kind of pectin (Figure 1). The decrease of

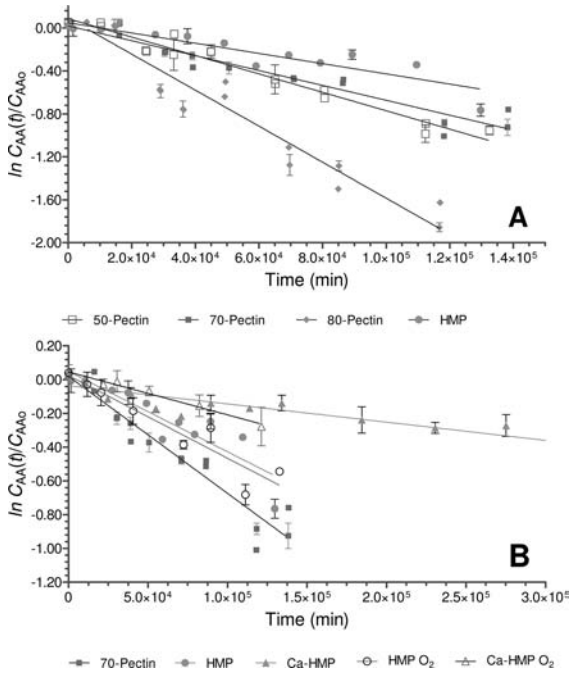

Figure 1. Kinetics of L-(+)-ascorbic acid (AA) destruction into films made with tailored 50%-pectin, 70%-pectin, and 80%-pectin as well as in films made with commercial high methoxyl (72% DM) pectin without (HMP) and with calcium (Ca-HMP) addition. Films were stored at 57.7% RH and 25 °C either under vacuum (A) or under air (B) when indicated (HMP-O_2 and Ca-HMP-O_2). Error bars correspond to standard deviations (n = 3).

the ratio between the AA concentration [$C_{AA}(t)$] and the initial one [C_{AAO}] with time (t) fitted (p < 0.05) to a pseudo first order kinetic from zero storage time of films, as previously observed.[15] The rate constants calculated from the slope obtained by fitting (k_{AA}') are shown in Table 2. As observed in Figure 1, AA was more stable to hydrolysis in the network microstructure developed by commercial HMP (DM = 72%). Between films made with tailored pectins, AA showed the lowest stability in the 80% methylated pectin, with higher retention in 50% and 70% methylated pectin networks (Figure 1). From considering films made with tailored pectins, the DM seemed to affect the stability of AA to hydrolysis. Also, the pectin (and network) nanostructure influenced AA retention as inferred from comparing 70%-methylated tailored pectin film with commercial HMP (DM = 72%) network. Though with similar DM, it has been reported that different distribution patterns of unesterified GalA blocks in pectins produce different gel characteristics[12,14] and calcium sensitivity.[13,27] Figure 2 shows that the pectin films developed from 50%, 70%, or 80% tailored pectins with the block distribution summarized in Table 1 were in general characterized by similar tensile strength at film fracture to that showed by commercial HMP films. However, the former pectin films presented the lowest relative elongation at failure. Although with the same proportion of glycerol and equilibrated at the same water activity, all tailored pectin films were then more brittle in contrast to the films made with commercial HMP, as previously observed by Willats, Knox, and Mikkelsen[12] with respect to gels assayed under compression.

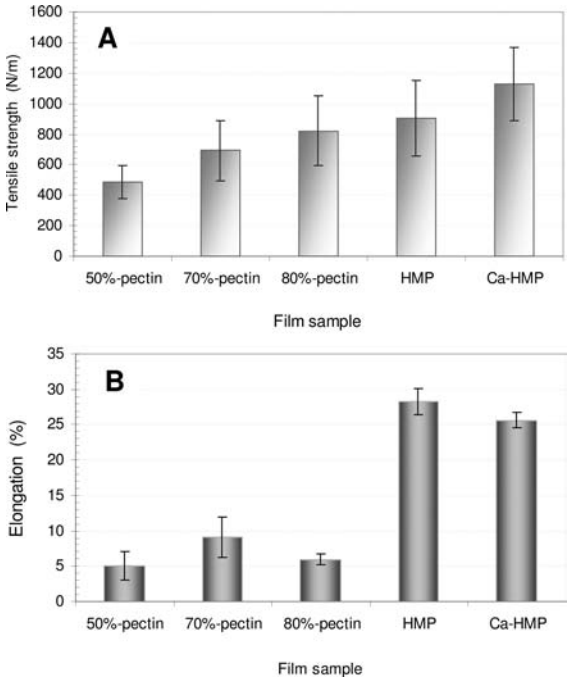

Figure 2. Tensile strength (A) and relative elongation (B) at fracture obtained from the force-elongation curves recorded from films made with the tailored 50%-pectin, 70%-pectin, and 80%-pectin as well as from films made with commercial high methoxyl (72% DM) pectin without (HMP) and with calcium (Ca-HMP) addition. Films were stored under 57.7% RH and 25 °C. Error bars correspond to standard deviation (n = 10).

Since commercial pectins are generally obtained by acidic treatment at high temperature,[28] they are randomly methyl esterified.[12] On the other hand, pectin demethylation by plant PMEs, like that applied to produce the tailored pectins herein used, led to the existence of unesterified GalA blocks of different length as well as to characteristic distribution patterns (Table 1) into the macromolecules. These patterns are also associated with different degrees of water retention.[29] These considerations allowed thinking that the polymeric networks developed by each of these pectins were essentially different with respect to water immobilization, which is the nucleophile responsible for the irreversible opening of the lactone ring of the AA molecule. The latter constitutes the first reaction and limiting step of the AA hydrolysis in the absence of air.[30] León and Rojas[9] found a dependence of the pseudo first order rate constant of AA hydrolysis (k_{AA}') on the RH of film storage (33.3%; 57.7% and 75.2% RH). Hence, it was proposed that a water molecule may be involved through a bimolecular nucleophilic substitution (S_N2) mechanism at the first step of the chemical destruction of AA under anaerobic storage. This reaction leads to hydrolysis of the AA-lactone ring to irreversibly render 2-keto-L-gulonic acid (KGA) through an acid catalyzed reaction.[30] The attack of the AA (lactone) ring by a water molecule (S_N2) was also suggested to be slow enough to determine the total reaction rate. This proposed mechanism explains a second order kinetics for AA hydrolysis and a second order rate constant (k) of AA destruction.[31] Hence, the pseudo first order rate constant (k_{AA}') herein determined from the only measurement of AA concentration against time (Table 2) included the water concentration ($k_{AA}' = k.C_{WATER}$) in the film network, so water is available for reactions:

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

wherein ν_{AA} is the stoichiometric coefficient for AA hydrolytic degradation reaction (here ν_{AA} = 1); r_{AA} is the AA-reaction rate/unit volume at a constant temperature; C_{AA} is the concentration of AA; C_{WATER} is the concentration of water; k is the rate constant of the second order kinetics for AA hydrolytic degradation reaction; k_{AA}' is the rate constant of the pseudo first order kinetics for AA hydrolytic degradation reaction.

The dynamic aspects of water interactions in the film networks were hence studied in order to determine differences in the ability of the networks to retain or immobilize water. For low-moisture biopolymer systems (water content <35%), the slowing of water motion has been reported to be associated with bound water (i.e., immobile water) arising from hydrogen bonding.[32] Short-range motions or molecular (water) relaxations investigated by ^{1}H NMR[33] led to observe that the magnetization decay in the xy-plane showed two spin−spin time constants (T_{2a} and T_{2b}) as observed after exponential fitting (eq 1), indicating the existence of multirelaxation rate behavior. These parameters may be associated with two fractions of water, which have different relaxation rates or mobility degrees.[34,35] One of the fractions showed transverse relaxation values of T_{2a} between 4.00×10^{-4} and 5.60×10^{-4} ($\pm 4 \times 10^{-6}$) s, while the other water population presented characteristic times of transverse relaxation (T_{2b}) ranging from 1.51×10^{-3} to 3×10^{-3} ($\pm 6 \times 10^{-6}$) s. The increment of the pseudo first order rate constants of AA hydrolysis (k_{AA}') with the water mobility (T_{2b} as an example) in the film network can be observed in Figure 3A. It can be suggested that differences in the structure of pectin macromolecules produced different retention of water molecules in the film network by adsorption and, hence, different stability of the compartmentalized AA. Tailored pectins were of similar molar mass. The 80%-methylated pectin produced the lowest water retention. Probably, concentration of methyl esterified carboxyl groups in blocks of the HG chains, as thought from considering the proportions of demethylated block size ($\overline{BS}$) and the average number of demethylated blocks ($\overline{B}_n$) present (Table 1), led to the prevalence of hydrophobic interactions between water and pectins over hydrogen bonds in most regions of the 80%-methylated pectin. Oakenfull and Scott[36] indicated that mainly hydrogen bonds, though necessarily in conjunction with hydrophobic interactions, contribute to the free energy of gelation or formation of junction zones in high methoxyl pectins. Conversely, tailored 70%-methylated pectin whose $\overline{BS}$ and $\overline{B}_n$ values were not very different from those of 50%-methylated pectin (Table 1) stabilized AA similarly to the 50%-methylated pectin film. Their macromolecular structures produced similar water mobility in their respective film networks as observed in Figure 3. The commercial pectin (HMP) has almost the same DM as the tailored 70%-methylated pectin but with higher molar mass, as above indicated. Water presented the lowest mobility in the film network developed by the commercial pectin with respect to tailored pectin films (Figure 3A). This may be ascribed to the fact that it is randomly demethylated.[12] Hence, the HMP network formed by alternating hydrophobic and hydrophilic channels embedded among the pectin helices provides an interesting motif in the formation of junction zones in this kind

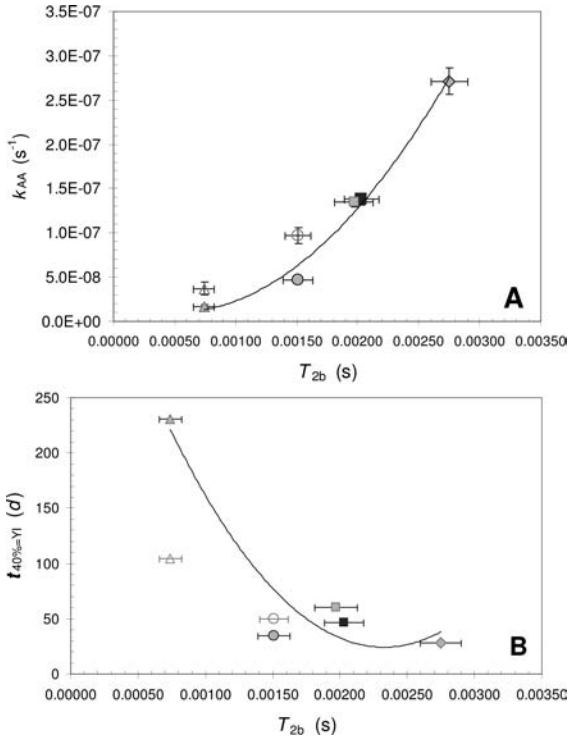

Figure 3. Rate constants for AA loss (k_{AA}') (A) and the time needed to reach a yellowness index (YI) value of 40% ($t_{40\%=YI}$) for browning development (B) are plotted against the spin−spin relaxation time (T_{2b}) for films made with tailored 50%-pectin (■), 70%-pectin (gray ■) and 80%-pectin (gray ◆) as well as in films made with commercial high methoxyl (72% DM) pectin without (HMP; gray ●), and with calcium (Ca-HMP; gray ▲) addition. Films were stored at 57.7% RH and 25 °C either under vacuum (filled symbols) or under air (empty symbols). Error bars correspond to standard deviations (n = 3).

of pectin, the hydrophilic channels being filled with water molecules hydrogen bonded to the surrounding polymer chains.[37,15]

Macromolecular relaxation in the film networks was also considered through determination of the T_g (onset value[23]). As observed in Table 3, their values were well below the storage temperature (25 °C) of films, indicating amorphous-rubbery state as a consequence of their plasticization by glycerol. As observed in previous works,[9,15] water also contributed to plasticization since T_g values changed in ≈10 °C as a function of the RH (33.3; 57.7% and 75.2%) of film storage. Pectin films did not essentially differ from one another in their macromolecular mobility, though some higher plasticization was observed for tailored pectin films since they showed some lower T_g values (Table 3). However, this observation was not accompanied by higher elongation at film failure (Figure 2). It can then be thought that the lowest T_g values shown by the tailored pectin films may be mainly due to higher water adsorption by the macromolecules, though the proportion of glycerol used could not have been sufficient to achieve enough mechanical plasticization as inferred from the lowest percentage of elongation observed. Water can also associate with glycerol producing the shift of T_g. It was previously established that water can only plasticize the polysaccharide networks when glycerol is formerly present in the film network.[15]

Besides, the DSC scans obtained from all film networks studied (36.8% w/w of glycerol content) did not show any other phenomenon beyond the T_g when scans were performed at 10 °C/min between −140 °C and +120 °C. Hence, it can be mentioned that water was sufficiently adsorbed and retained by the polymeric networks developed.

Irreversible hydrolysis of the lactone ring that constitutes the AA molecule leads to KGA through the acid catalyzed reaction,[30] which was suggested to occur through a S_N2 mechanism.[9] Once KGA appears, this reactive molecule (α,β-unsaturated carbonyl, β-hydroxyl carboxyl) suffers successive transformations which involve dehydrations and decarboxylations producing different browning active compounds.[30] These chemical events are favored in solid-like systems like films. Water is a limiting reactive since it is not available as solvent in solid-like systems. Therefore, nucleophiles are highly reactive because they are not solvated as in solutions.[9] Browning development in the pectin films measured through the YI showed pseudo zero order kinetics, and the calculated rate constants are reported in Table 2. The time needed to reach a YI value of 40% ($t_{40\%=YI}$) was plotted versus T_{2b} for the film systems herein analyzed (Figure 3B). It can be observed that their values did not show differences with the lower values of water mobility or with macromolecular relaxation (T_g values; Table 3). The latter is expectable since all films are at the amorphous rubbery condition at room temperature. Browning increased subsequently to AA loss during film storage at 57.7% RH as shown in Figure 4, where the calculated half-life time for AA degradation ($t_{1/2}$) was plotted against $t_{40\%=YI}$.

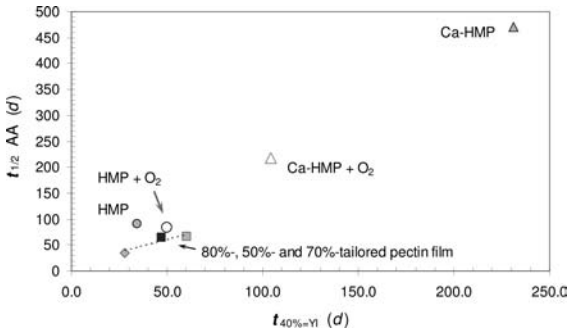

Figure 4. Relationship between the half-life time of L-(+)-ascorbic acid destruction ($t_{1/2}$) and the time needed to reach a yellowness index (YI) value of 40% ($t_{40\%=YI}$) for browning development for films made with tailored 50%-pectin (black ■), 70%-pectin (gray ■), and 80%-pectin (gray ◆) as well as in films made with commercial high methoxyl (72% DM) pectin without (HMP; gray ●) and with calcium (Ca-HMP; gray ▲) addition. Films were stored at 57.7% RH and 25 °C either under vacuum (filled symbols) or under air (empty symbols).

2. Effect of Calcium Presence. A significant hydrolytic stability was previously found by De'Nobili, Perez, Navarro, Stortz, and Rojas[3] for AA when compartmentalized in low methoxyl pectin films where Ca^{2+} was added to cross-link. Also, a low browning development was observed. Hence, the effect of Ca^{2+} addition was also studied in the present work for film constitution from the commercial HMP (DM = 72%) above analyzed. Herein was applied the same level of Ca^{2+} used for the low methoxyl pectin films mentioned. Homogeneous and flexible films plasticized by glycerol [glycerol × 100/(pectin + glycerol) = 36.80% w/w] were also obtained after casting, which were transparent, slightly yellow (b = +8.4; YI = 17% ± 1), and with high initial lightness (L = 84% ± 1). The same AA concentration was initially determined as above indicated (Table 2). Film samples removed from polystyrene plates were

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stored at 25 °C and 57.7% RH under vacuum. They equilibrated at the fourth day of storage. The average thickness was 0.094 ± 0.023 mm. The pH values (Table 2) and moisture content (Table 3) determined after equilibration are reported.

The decrease of the ratio between the AA concentration and the initial one with time also fitted ($p < 0.05$) to a pseudo first order kinetic from zero storage time of films (Figure 1). Higher stabilization to hydrolysis of the compartmentalized AA was observed with respect to the HMP films without Ca^{2+} addition above analyzed. The rate constants (k_{AA}') calculated from the slope obtained by fitting are shown in Table 2. Also, lower browning rate constant of pseudo zero order was determined for Ca-HMP films by measurement of the YI during film storage at 57.7% RH.

Studying the dynamic aspects of water interactions in the complete formulation of edible films through ^{1}H NMR allowed observation that the magnetization decay in the *xy*-plane also presented two spin−spin time constants (T_{2a} and T_{2b}). The lowest pseudo first order rate constant of AA hydrolysis (k_{AA}') was observed joined to the lowest water mobility in the film networks containing Ca^{2+} with respect to the film systems above analyzed (Figure 3A). Also, the lowest browning rate (higher $t_{40\%=YI}$) was observed being coherent with the lowest water mobility determined in Ca-HMP films with respect to those T_{2b} values observed in the other film formulations studied (Figure 3B). Calcium ions may contribute to the junction zones through electrostatic interactions between the remaining unesterified blocks of the HMP chains. This can be inferred from the trend to some higher tensile strength determined for Ca-HMP films than for HMP-ones at fracture as well as for the significantly lower relative elongation (Figure 2). Also, Ca-HMP films showed the highest T_g value (Table 3) which is directly associated with a lower free volume and macromolecular mobility than in the other film networks. The commercial (randomly demethylated) HMP used for film constitution showed some calcium sensitivity. This may demonstrate the presence of some adequate distribution of the remaining unmethylated blocks which also presented a minimal length in order to accomplish some calcium bridges between adjacent chains.[12] In calcium pectate gels, the junction zones involve three components: uronate chains, calcium ions, and water, which corresponds to nonfreezable bound water.[38] Water oxygen atoms may complete the coordination sphere of Ca^{2+} at the pectate junction zone.[39] Adequate distribution patterns of demethylated carboxyl groups are also necessary to constitute Ca junctions.[40] These patterns are also associated with different degrees of water retention.[29] It was reported that blocks of 7−20 unesterified GalA residues are required for association with calcium.[41,28] Hence, additional presence of calcium ions for HMP film constitution involved lower availability of water into the film network, which precluded AA from hydrolysis at a high enough level to sensibly decrease the subsequent browning development.

Stability of L-(+)-Ascorbic Acid to Chemical Hydrolysis and Oxygen in Pectin Films. The films made with commercial HMP (DM = 72%) with or without Ca^{2+} addition above evaluated for hydrolysis of AA were also studied in their ability to stabilize AA in presence of oxygen. Thickness and pH measured after equilibration as well as moisture contents are the same reported in Table 2.

The decrease of the ratio between the AA concentration and the initial one with time also fitted ($p < 0.05$) to a pseudo first order kinetic from zero storage time of films (Figure 1B). Khan and Martell[8] determined the dependence of the AA oxidation rate constant on the concentration of the oxygen dissolved in aqueous solutions with an ionic strength of 0.1000 M. These authors found that, in the range 0.40−1 atm of O_2 pressure, the kinetic equation may be given by the expression

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

They reported that the second order rate constant (k_2), which corresponded to the following chemical reaction for AA oxidation,

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

also depended on the pH (H_3O^+ concentration). Consequently, proton concentration (C_{H+}) also should participate in the kinetic equation (3) included in k_2 value. Hence, k_2 was not the absolute rate constant, which only depends on temperature. When the AA concentration remaining at every time is the only chemical substance measured as in the study herein performed, the rate constant of oxidation determined from experimental data included the oxygen concentration (k_{AA}^{OX}; eq 3), being then a pseudo first order rate constant.

Khan and Martell[8] observed that, below an oxygen partial pressure of 0.40 atm, the rate of oxidation was directly proportional to the C_{O_2} and, hence, to the partial pressure. At these low pressures a very long time was required for the reaction to proceed to any appreciable extent. Destruction of AA into pectin films under air occurred simultaneously with the hydrolytic reaction previously studied under vacuum (eq 2). Therefore, when AA is lost during air storage, it can be considered that at least two irreversible parallel or competitive chemical reactions proceeded under air: the AA hydrolysis (eq 2) and the AA oxidation (eq 3). In this context, the following differential kinetic equation can be written for the AA as the reactive, in the form of pseudo first order rate reactions:

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

$$\nu_{AA} = 1 \Rightarrow \frac{dC_{AA}}{dt} = -(k_{AA}' + k_{AA}^{OX})C_{AA}(t)$$

By integration:

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

Hence, the slope calculated from the experimental data obtained after storage under air (Figure 1B) gives the total rate constant (k_T):

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

and the oxidation rate constant (k_{AA}^{OX}) can be specifically obtained as the difference. The partial pressure of oxygen (p_i) in the air was 0.21 atm constant during storage of pectin films, and a pseudo first order was experimentally observed (Figure 1B). The rate constant calculated (k_{AA}^{OX}; eq 4) involved the oxygen concentration ($C_{O2} = p_i/K_m$ where K_m is the Henry's constant: 756.7 atm/mol·L^{-1} ⇒ $C_{O_2} = 2.775 \times 10^{-4}$ M in pure water at 25 °C as indicated in eq 3). AA supported in HMP films was lost according to nonsignificantly different rate constants in the absence (k_{AA}') or presence of oxygen (k_T) in the storage atmosphere (Table 2). The same was observed for the browning rates. On the other hand, both rate constants

respectively increased at least twice in Ca-HMP films when stored under air, and a k_{AA}^{OX} value of 1.63×10^{-6} min^{-1} was calculated through eq 5 with the rate constants summarized in Table 2. Anyway, AA was more stable in Ca-HMP films at the two storage conditions herein studied than in the HMP network. Probably, higher immobilization of water may contribute not only to lower rate of hydrolysis but also to lower oxidation rate. This may be also associated with a slower irreversible hydrolysis of DHA to 2,3-diketogulonic acid, which is the next step in the reaction chain of AA oxidation.

Half-life times of ≈86 and 195 days were calculated for the AA respectively supported into HMP and Ca-HMP films in aerobic storage ($P = 1.013 \times 10^5$ Pa), whereas $t_{1/2}$ of 90 and 470 days were obtained for the same films stored under vacuum ($P = 132$ Pa). It is known that sorbate used as antimicrobial agent in the films is a short chain fatty acid. Thus, it can suffer oxidation to lipid peroxides. In the presence of oxygen or oxidants such as lipid peroxides, metal ions act predominantly as catalysts in a chain of partially radical reactions, as above-mentioned.[8] As any difference was observed between the rates of AA loss in HMP films stored under vacuum (k_{AA}') or under air (k_T) (Table 2), it can be concluded that the sorbate used as antimicrobial agent did not seem to significantly contribute to AA degradation in the presence of oxygen when compartmentalized in films.

Calcium presence in the HMP network led to higher AA stability and lower browning rates when films were stored under air (Table 2). Browning rate constants led to calculate similar $t_{40\%=YI}$ for HMP-films stored under vacuum or air, whereas the $t_{40\%=YI}$ value determined in Ca-HMP films stored under vacuum doubled that found under air storage in the same film (Figure 4). Higher AA oxidation in Ca-HMP films may be attributed to higher solubility of oxygen in the Ca-HMP film matrix. Permeance through polymeric films includes the product of diffusion coefficient (D) and solubility (S). Cross-linking in general can decrease diffusivity,[42] but oxygen solubility in the Ca-HMP polymeric network could increase probably due to lower water availability (Figure 3) and, hence, higher oxygen adsorption by the solid matrix through the −OH groups of the polymeric network.

Even in the presence of oxygen during storage, AA was better stabilized in the film networks made with the randomly demethylated (commercial) HMP than in the tailored 70% DM pectin films stored under vacuum. It can be suggested that the presence of disorder (amorphous) regions (RG-I, random coils) interspersed with HG ("smooth regions", semiflexible chains)[43] in the pectin macromolecules may produce better immobilization of water by physical adsorption than more rigid networks like those developed from the tailored pectin macromolecules. Calcium presence in the HMP film network also led to lower rates of AA degradation and browning development under air. It can be concluded that the ability of the polymeric network to immobilize water seems to be the main factor to consider in order to succeed in retaining AA into film networks in view of preserving vitamin C under the AA form as well as for potential antioxidant activity localized at pharmaceutical and food interfaces.

SAFETY

This research work was performed in accordance with the Safety Protection Plan of the Facultad de Ciencias Exactas y Naturales of the University of Buenos Aires, where the Laboratories are periodically submitted to the inspections of the Department of Hygiene and Safety.

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Author Contributions

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Notes

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