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Performance of alginate films for retention of L-(+)-ascorbic acid

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ABSTRACT

In view of acting as controlled delivery systems for nutritional supplementation, therapy or antioxidant activity at interfaces, alginate films of different copolymer composition and glycerol plasticizer levels were developed in the presence of Ca^{2+} for achieving higher stability of L-(+)-ascorbic acid (AA). The ability of the alginate network to preserve AA from hydrolysis, tested by storage under vacuum at 25 °C, only decreased with the relative humidity (RH) increase when alginates were mainly constituted by guluronic–guluronic acid blocks (GG), whereas also decreased with the glycerol level increase when mannuronic–mannuronic acid (MM) and/or alternating guluronic–mannuronic (GM + MG) flexible blocks were present in higher proportions. This result could be probably related to the lower capability of the latter alginate block compositions to immobilize water in the network as they are not able to constitute Ca^{2+} mediated junction zones where water molecules are highly retained. Films also studied under air storage showed that even at less favorable conditions of RH and glycerol levels, both GG and GM + MG enriched alginate networks in general preserved AA from oxidation. It also demonstrated that hydrolysis is the principal way by which AA is lost when supported in films.

1. Introduction

Alginate is a biomaterial 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). Alginate hydrogels have been particularly attractive in wound healing, drug delivery, and tissue engineering applications, as these gels retain structural similarity to the extracellular matrices in tissues and can be manipulated to play several critical roles. Alginates are also very useful because of their utility in preparing hydrogels at mild pH and temperature conditions, suitable for sensitive biomolecules (Pawar and Edgar, 2012). Alginic acid, a natural polysaccharide harvested from brown algae, is an unbranched binary copolymer constituted by (1,4)-linked β-D-mannuronic acid (M-block), α-L-guluronic acid (G-block) and sequences of alternating β-D-mannuronic and α-L-guluronic acid (MG-block) (Jothisaraswathi et al., 2006). Physical and mechanical properties as well as biocompatibility of alginate materials are highly dependent on the relative content of L-guluronic to D-mannuronic acids (Klöck et al., 1997; Stabler et al., 2001). Calcium ions can replace in part the hydrogen bonding, zipping guluronate (but not mannuronate) chains together stoichiometrically in an "egg-box" conformation. Guluronate chain pairing through junction zones involves three components: uronate chains, calcium ions and water molecules. The antiparallel arrangement is the macromolecular interaction probably favored in the gel, showing a notable contribution of hydrogen bonds to gel strength. Moreover, the antiparallel association of 2_1 helical chains is the arrangement found in the solid state (Braccini and Pérez, 2001).

Alginates of different monomeric composition can be assayed in their ability to form film matrices for compartmentalization of L-(+)-ascorbic acid (AA), also known as vitamin C. Through a delivery film, AA could provide, for example, nutritional supplementation (Durschlag et al., 2007), selective killing of cancer cells or local treatment of infections where H_2O_2 (formed from AA) may be beneficial (Chen et al., 2005). AA is a water soluble reducing agent and a natural antioxidant which also can be used for pharmaceutical preservation. AA stability is affected by processing and storage conditions because it depends on a large number of factors such as temperature, equilibrium RH, oxygen partial pressure and light (Kitts, 1997). AA reacts with oxygen to produce L-dehydroascorbic acid (DHA) that also has vitamin C activity *in vivo*. Biological activity 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,

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producing 2-keto-L-gulonic acid (Kurata and Sakurai, 1967). On the other hand, non-enzymatic browning 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 browning reaction chain (León and Rojas, 2007). Compartmentalization of AA into a film network could help achieve stabilization because it can preclude the AA interaction with oxygen, with other pharmaceutical preservatives or chemical components of the system where the film is applied, and films can constitute controlled delivery systems and provide localized antioxidant activity at interfaces. In order to evaluate the ability of alginate matrices to stabilize AA, the objective of the present work was to study the effect of alginate composition and level of glycerol (plasticizer) applied to film constitution as well as of the RH (33.3; 57.7, 75.2%) used for film storage (25 °C) on the hydrolytic and oxidative stability of AA in these matrices.

2. Materials and methods

2.1. Chemicals

Manugel DM and Protanal LF240 alginates were a gift from FMC BioPolymer (Billingstad, Norway). Cargill (Mechelen, Belgium) and Sigma–Aldrich (herein called "VR") alginates were also used in this study. All other chemicals were of analytical grade from Merck (Argentina) or Sigma–Aldrich (St. Louis, MO, USA). Deionized water (Milli-Q, USA) was used.

2.2. Analyses of alginates

The diadic frequency composition of alginate (F_{GG}, F_{MM} and F_{GM+MG}) or block-proportions were determined by means of circular dichroism. Spectra of samples containing ≈0.8 mg/mL of alginate in deionized water were recorded on a Jasco J-810 (Japan) spectropolarimeter. Data in the far UV (195–250 nm) region were collected at 25 °C using a 2 mm path length cuvette. A scan speed of 20 nm/min with a time constant of 1 s was used. Each spectrum was measured four times and the data were average to minimize noise. Deconvolution of experimental spectra was done according to the procedure described by Donati et al. (2003). Mollar ellipticity was calculated using a mean residue weight value of 176.14 (the molecular weight of the monomer minus one water molecule). The diadic composition calculations were performed according to Donati et al. (2003). Based on these results the four alginates above mentioned were then selected among others for film development.

Afterwards, these four alginates were submitted to chemical assays to determine the total acid carbohydrate content according to the spectrophotometric method of Edstrom (1969), using 4,5,4′,5′-dibenzo-3,3′-diethyl-9-methylthio-carbocyanine bromide. The protein content was determined according to Lowry et al. (1951). Methanol and acetyl contents respectively derived from methoxyl esterification of carboxylate groups and acetate ether bonding to —OH groups of the acid polysaccharides (alginates), were determined according to Wood and Siddiqui (1971) and Naumenko and Phillipov (1992), respectively. The degrees of methyl esterification (DM) and acetylation (DA) of the acid polysaccharides were then calculated as:

$$DM = 100 \times \frac{\text{moles}_{CH_3OH}}{\text{moles}_{\text{total acid carbohydrates}}}$$

$$DA = 100 \times \frac{\text{moles}_{CH_3COO^-}}{\text{moles}_{\text{total acid carbohydrates}}}$$

Molecular weight profile of alginates was determined through gel filtration using a Fast Protein Liquid Chromatograph (FPLC, Pharmacia, Sweden) with a Superose 12HR 10/30 column (Amersham Biosciences-GE Healthcare, USA). Each alginate sample was dissolved and also eluted by using 0.5 M of imidazole buffer (pH 7.0) (Mort et al., 1991) or deionized water, at 0.5 mL/min. Dextrans of 65,000 and 40,210 molecular weights as well as blue dextran, $CoCl_2$ and sucrose were used as standards for column calibration at both elution conditions. A pectin of known molecular weight was used as reference to control the column performance under both elution conditions. Total carbohydrate content was determined into each collected fraction by the phenol–sulfuric acid spectrophotometric method (Dubois et al., 1956) when samples were collected with 0.5 M imidazole buffer (pH 7.0), and according to the method of Edstrom (1969) when samples were collected with deionized water. The former colorimetric technique underestimated the content of alginates in each fraction.

Iron and copper contents in the alginates were directly determined through inductively coupled plasma atomic emission spectrometry (ICP-AES), using a Thermo Jarrel Ash Atom Scan 25 (Thermo Jarrel, USA), according to Rubio et al. (2009).

2.3. Film formation

For the purpose of this study, each film system was developed from one of the four alginates above mentioned. A 2% (w/w) alginate concentration was used for the film making solution, thus permitting to obtain plasticized films with the adequate handling resistance. The aqueous solution was continuously stirred under controlled high speed (1400-rpm constant) using a vertical stirrer (LH model, Velp Scientifica, Italy) in order to reach homogeneous hydration. While stirring, the obtained viscous, homogeneous and transparent system was then heated up to 85 °C at a constant heating rate (5.3 °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 (P901, Belgium). The following substances were subsequently added: glycerol [26.7, 35.6 or 52.3 g per 100 g of (polymer + glycerol)] for plasticization (Yang and Paulson, 2000), potassium sorbate (0.030%, w/w) as antimicrobial agent and AA (0.100%, w/w). Finally, 1.1×10^{-3} moles of Ca^{2+} (as $CaCl_2 \cdot 2H_2O$) were added for gelling after cooling. The hot solution was placed under vacuum for 20 s to remove air bubbles and then immediately poured onto horizontally leveled polystyrene plates. The solution dispensed into each identified plate was weighted in an analytical scale (0.0001-g precision) in order to have constant thickness as well as a known initial content of AA into the subsequently generated film. The fractionated system was dried for 2.5 h in a forced convection oven at 60 °C. Films were also weighted after drying, peeled from the polystyrene plates and stored in light-protected desiccators over saturated solutions of known water activity (a_W^o), in order to maintain a constant RH for film equilibration:

$$a_W^o = \frac{RH\%}{100}$$

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 °C (Greenspan, 1977). Equilibration was followed by the daily measurement of a_W in the film samples 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 the light-protected desiccators with the 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 = 130 Pa) with controlled RH in order to ensure that AA degradation begins 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 Cargill and Sigma (VR) alginate films made with 35.6% or 52.3% of glycerol were further stored under normal air conditions ($P = 1.013 \times 10^5$ Pa), protected from light, at 25 °C and 57.7% or 75.2% RH, in order to also infer the specific influence of oxygen on the total kinetic of AA destruction.

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

2.4. Water activity

To evaluate film equilibration, the true water activity (a_W^o) 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 of $MgCl_2$, NaBr and NaCl mentioned before.

2.5. Measurement of pH

This was performed on the gel-forming solutions as well as on films equilibrated at the corresponding RH, using a bulb-combined glass electrode or a flat surface electrode (Phoenix, AZ, USA) connected to a pH meter (Consort P901, Belgium). Film pH was determined after a slight surface hydration with 20.0 μL deionized water (Joel et al., 1972). Standard buffer solutions (pH 4.00 and 7.02) were used for calibration.

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

A film sample taken from each of the three batches of films stored at each RH was carefully 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, USA). An aliquot was taken from the supernatant and the AA concentration was determined by using the 2,6-dichloro phenol indophenol (2,6-DPIP) spectrophotometric 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 different aliquots (duplicate) for each film sample.

The initial amount of AA into each identified film sample was known because the solution dispensed into each plate and the corresponding film obtained after drying were both weighted as indicated above. In a previous assay, the AA concentration was spectrophotometrically determined in 10 films of three different batches ($n = 30$) which were processed as described, and it was compared with the expected concentration. The recovery of AA from the films assayed to determine the optimum experimental conditions for extraction ranges from 98.9% to 104.6%. Good interday (relative standard deviation, RSD ≤ 2.84%) and intraday (RSD ≤ 1.98%) precision was achieved.

The procedure retains its accuracy up to 81% of AA degradation kinetics. The calibration curve was constructed with nine AA concentrations ranging between 0 and 34 μg/mL every time the 2,6-DPIP solution was prepared. Regression analysis of Beer's plots showed good correlation in the 0 and 34 μg/mL concentration range, showing the same regression parameters [interception = 0.616 ± 0.001; slope = $-(725 \pm 3) \times 10^{-5}$; residual standard error = 8.7×10^{-6}; $R^2 = 0.9997$]. The limit of detection of the spectrophotometric method is 0.68 μg/mL.

2.7. Color

Measurement of the film color was performed in each sample according to the ASTM E1925 (1995) employing a Minolta colorimeter (Minolta CM-508d) with an aperture of 1.5-cm diameter (León and Rojas, 2007). Film samples for color measurement were taken from each of the three batches of films obtained in order to determine the kinetics of browning (yellowness index, YI%) increase. Also, *L*, *a*, and *b* (HunterLab) color parameters were measured, which ranged from $L = 0$ (black) to $L = 100$ (white or maximum) for lightness (*L*); $-a$ (greenness) to $+a$ (redness), and $-b$ (blueness) to $+b$ (yellowness). Standard values considered were those of the white background.

2.8. Moisture or water content

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 involved approximately 22–30 days. Determinations were performed on six film specimens at each evaluated condition. Moisture or water content was informed on dry basis.

2.9. 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 aluminium 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 usually employed.

2.10. Statistical analyses

The results are reported as the average and standard deviation. Rate constants of AA destruction (k'_{AA} and k_T) were calculated by linear regression according to a first order reaction, where each experimental point corresponded to the ratio between the AA concentration remaining at a given storage time *t* (C_{AA}) and the initial ($t = 0$) concentration of AA (C_{AA}^O):

$$C_{AA}(t) = \frac{\text{Weight}_{AA}(t)}{\text{Weight}_{film}}$$

wherein the "weight" is expressed in grams.

Browning rate constants (k_{YI}) were calculated from the slope of the linear regression of experimental data (YI% *vs* time). Analysis of covariance (ANCOVA) was applied for comparison of slopes, that is, of the rate constants (k'_{AA} and k_T, or k_{YI}), 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 significant difference test. The GraphPad Prism software (version 5.00, 2007, GraphPad Software Inc., USA) was used for all analyses previously detailed.

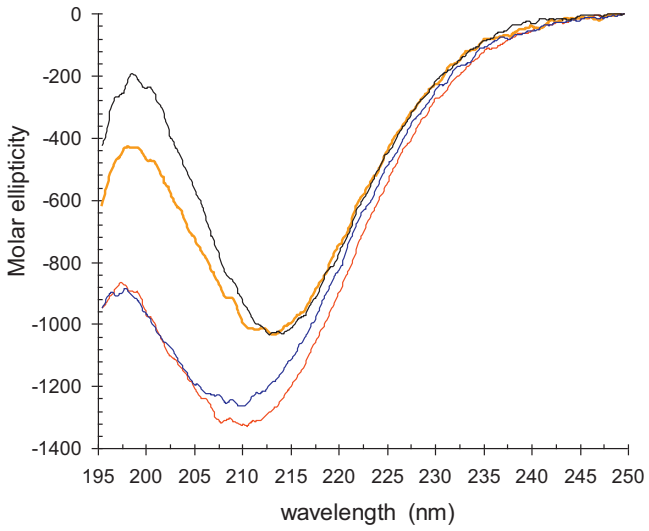

Fig. 1. Circular dichroism spectra recorded for (— black) Protanal, (▬ thick orange line) VR, (▬ blue) Cargill and (▬ red) Manugel alginates.

The effect of two quantitative factors (RH and glycerol) on the calculated rate constants (k'_{AA} and k_{YI}) was analyzed with a complete 3 × 3 experimental design at the three levels described before for both factors, coded as −1, 0, +1. This design was repeated for the four polymers tested. In the first model the polymer type was included as a categorical variable, but subsequently each polymer was analyzed separately. A regression model was applied as a function of the lineal and quadratic values of the quantitative factors and their interactions. This statistical analysis was performed with R (version 2.15: R Core Team, 2012).

3. Results and discussion

3.1. Polymer characterization

The relevant molecular characteristics of the alginate polymers used in this work are listed in Table 1. Proteins were not detectable. Alginates showed an acidic polysaccharide content of ≈95% (Edstrom, 1969) and they were no methoxyl-esterified. As expected from algal alginates, O-acetyl groups were absent (Davidson et al., 1977). Similar and low amounts of iron and copper were observed (Table 1). Molecular weights and their distributions were similar (≈876 kDa). This value corresponds to a high molecular weight alginate which is reported to be related to higher viscosity (Aoyama et al., 2007). Important biophysical properties of alginates are also related to the molecular weight (Kong et al., 2004).

The high selectivity of alginate binding towards calcium ions, which accounts for its capacity to form ionotropic gels, is determined by the polymer composition (Simpson et al., 2004). Furthermore, parameters such as the stability, strength and porosity of the obtained gels are influenced by the diadic frequency composition (F_{GG}, F_{GM+MG} and F_{MM}) of alginate (Donati et al., 2003). In order to study the influence of the macromolecule structure in the development of film networks able to stabilize AA, alginates with different monomeric composition were then used in this work. Alginate composition and block-proportions can be determined by the circular dichroism characteristics of alginate molecules (Morris et al., 1980; Klöck et al., 1997; Donati et al., 2003). Circular dichroism spectra are shown in Fig. 1. All polymers used showed the negative MG and GG diads bands. The circular dichroism spectra of Manugel and Cargill alginates were characterized by the minima at 210 nm (≈−1330 and −1260 molar ellipticity, respectively), whereas VR and Protanal alginates show a shallower spectra with minima at 213 nm (≈−1050 for both alginates). These features can account for the different diadic composition. According to the procedure described by Donati et al. (2003), deconvolution of experimental spectra (Fig. 1) allowed calculating the diadic composition (F_{GG}, F_{MM} and F_{GM+MG}), and results are shown in Table 1. Manugel alginate was mainly constituted by GG-blocks, with lower proportion of MM-blocks. Cargill alginate showed lower proportion of GG- and MM-blocks than Manugel alginate, but Cargill differs mainly in its higher proportion of flexible GM + MG-blocks. On the other hand, Protanal and VR alginates showed similar composition, although Protanal was characterized by a higher proportion of MM-blocks and a lower one of GM + MG-blocks.

Contrary to polymannuronates, a high affinity of polyguluronates to calcium ions was determined by Kohn (1975). By studying the encapsulation of βTC3 cells, Simpson et al. (2004) determined that alginate with high mannuronic acid content was not affected by changes in $CaCl_2$ concentration due to the low percentage of consecutive guluronic acid residues. A cooperative effect in calcium binding is observed for polyguluronic acid at chain lengths above a threshold of ≈20 residues (Braccini and Pérez, 2001; Fang et al., 2008). The alginate fragments with alternating sequence of D-mannuronic and L-guluronic acid units (GM + MG-blocks) exert only a low selectivity in ion exchange reaction, whereas the affinity of the monomers (D-mannuronate, L-guluronate) to calcium ions was found to be virtually the same (Kohn, 1975). GG-blocks are the most inflexible ones in alginate macromolecules, whereas GM + MG-blocks are the most flexible. Chain breakage by oxidants was demonstrated to occur mainly at the most flexible blocks of the alginate macromolecules, whereas the GG-block length largely determines the elastic modulus of calcium cross-linked gels (Kong et al., 2004). In the present work, the amount of Ca^{2+} required for gelling of the film making solutions was then calculated from the proportion of GG-blocks, being it reported in Table 1. For film formulations, 1.1×10^{-3} moles of Ca^{2+} were then used in order to satisfy a minimum requirement for all alginates. This content also permits to obtain films with an adequate handling flexibility, especially at the lowest level of glycerol used for plasticization.

3.2. Film characteristics

Homogeneous and flexible films plasticized by glycerol proportions of 26.7, 35.6 or 52.3% (w/w) were obtained after casting from each alginate solution. Films were transparent, almost colorless or yellowish (b = +6 to +9; YI = 12–18%) and showed high initial lightness (Table 2). The AA concentration initially determined (C^{O}_{AA}) was $\approx 3.02 \times 10^{-2}$ g AA per gram of film, which means that a 100% of AA recovery was achieved after casting. Temperature should be as low as possible to get short periods of drying (≤2.5 h), which avoid AA losses through hydrolysis during this processing. Therefore, films were finally dried at 60 °C. Film samples attained equilibration at 20 h of vacuum storage at each RH, as determined by measurement of the film a^{0}_{W} (0.333, 0.577 and 0.752, respectively) at 25 °C. Thickness measured after equilibration was ≈0.12 mm (Table 2). There was no significant influence of RH and glycerol content on film thickness. The film pH recorded along storage varied as indicated in Table 2. Moisture contents increased with the RH of film equilibration. In general, the increase in the glycerol level produced a significant increase in the moisture content only for films equilibrated at 75.2% RH (Table 3).

At ≈−38 °C and/or 0 °C, MDSC scans did not show any endothermic peak that could correspond to freezable bound and free water, respectively (Hatakeyama and Hatakeyama, 1998). Therefore, water gained from the storage environment was adsorbed

Table 1
Chemical composition of the alginate polymers used for film development.

	Alginate			
	Manugel	Cargill	VR	Protanal
Molecular weight[a] (kDa)	876 ± 180	876 ± 200	876 ± 140	876 ± 180
Protein content[a] (g/100 g)[f]	0.10 ± 0.09	0.59 ± 0.08	0.5 ± 0.3	0.03 ± 0.06
Total acid carbohydrates[a] (g/100 g)[f]	95.9 ± 0.8	93.0 ± 0.6	97.05 ± 0.07	95.6 ± 0.4
DM[b] (%)	0.10	0.10	0.08	0.5
DA[c] (%)	ND	ND	ND	ND
Iron[a] (mg/1000 g)[f]	45 ± 4	39 ± 6	34 ± 6	36 ± 5
Copper[a] (mg/1000 g)[f]	38 ± 7	42 ± 8	24 ± 7	29 ± 5
F_{GG} [d]	0.66	0.57	0.27	0.25
F_{MM} [d]	0.26	0.22	0.32	0.42
F_{GM+MG} [d]	0.08	0.21	0.40	0.33
F_G [d]	0.70	0.67	0.47	0.42
F_M [d]	0.30	0.33	0.53	0.58
Ca^{2+} required[e] (mol/100 g)[f]	2.85×10^{-3}	2.45×10^{-3}	1.18×10^{-3}	1.08×10^{-3}

ND: non detectable.
[a] Mean and standard deviation ($n = 3$) are shown.
[b] Degree of methyl esterification is expressed as 100 × moles of methoxyl group/moles of total acid carbohydrates.
[c] Degree of acetylation is expressed as 100 × moles of acetyl group/moles of total acid carbohydrates.
[d] Diadic frequency composition of GG-, MM- and GM + MG-blocks [guluronic (G); mannuronic (M)] in alginates determined through circular dichroism (Donati et al., 2003). F_G and F_M are the total proportions of G and M monomers, respectively.
[e] Moles of Ca^{2+} required per 100 g of film making solution calculated from the respective F_{GG} value.
[f] Expressed per 100 g or 1000 g of alginate.

or retained by the polymeric network. The T_g values found for all equilibrated films herein studied were lower than the storage temperature (25 °C) (Table 3). Hence, the equilibrated films were amorphous rubber materials at ambient temperature. Into each type of alginate assayed, T_g values in general decreased significantly ($p < 0.05$) with the increase in the glycerol proportion used as well as in the water content (Table 3). Hence, glycerol as well as the water captured during storage plasticized the film networks. At each level of glycerol, Manugel alginate films showed, in general, the highest values of T_g and, hence, the lowest macromolecular mobility. Probably, this result may be associated with its higher proportion of inflexible GG-blocks and/or with a very small proportion of flexible GM + MG-blocks (Table 1). According to Roger et al. (2004), powder samples of alginate exhibited T_g ranging from 95 °C to 136 °C and no significant effect on T_g was observed for different molecular weight samples. However, an increase in T_g values with the G content was observed. This effect was attributed to the presence of residual Ca^{2+} ions in the alginate powder, crosslinking oligomeric G-rich chains.

Alginates are block copolymers and, hence, they can behave as two-phase systems or physical blends. Each phase exhibits its own distinct T_g (Ferry, 1980). Only one T_g was detected in thermograms of alginate films developed in the present work. This could be attributable to a plasticization effect and/or to a probable random alternating distribution of blocks in the alginate macromolecules.

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

The study of AA stability by storage in the absence of air ($P = 130$ Pa) allowed to determine that the ratio between the remaining AA concentration [$C_{AA}(t)$] and the initial one [C^O_{AA}] statistically changed with the storage time (t) according to a pseudo-first order ($p < 0.05$) kinetic law (León and Rojas, 2007). The rate constants of AA hydrolysis (k'_{AA}) were then calculated from the slope obtained after fitting a straight line to the data. On the other hand, browning development was measured as the increment of the YI with time, which statistically fitted ($p < 0.05$) to a pseudo-zero order reaction (Rojas and Gerschenson, 2001). Browning rate constant (k_{YI}) was then calculated from each slope obtained after linear regression fitting to the experimental data. The AA stability to hydrolysis (k'_{AA} values) seemed to be mainly affected by the glycerol level as well as by the RH of film storage at 25 °C, as shown

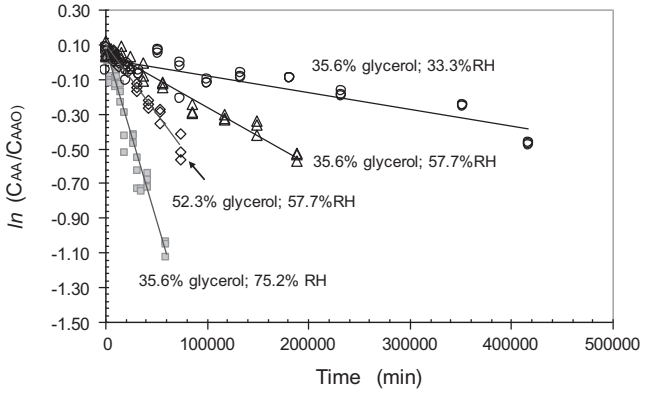

Fig. 2. Kinetics of AA hydrolysis determined in Cargill alginate films are shown for two levels of glycerol and three levels of storage relative humidity (RH).

Table 2
Color parameters[a,b] and thickness[c,d] are reported as well as the pH[a] variation recorded during the complete period of film storage.

Alginate	YI %	L %	+b	Thickness (mm)	pH
Manugel	17 ± 1	82 ± 1	7.9 ± 0.3	0.100 ± 0.040	4.42 ± 0.07
Cargill	12 ± 2	85 ± 1	6.3 ± 0.5	0.100 ± 0.030	4.54 ± 0.08
VR	16 ± 2	80 ± 1	6.1 ± 0.6	0.140 ± 0.070	4.37 ± 0.03
Protanal	18 ± 3	80 ± 3	9 ± 1	0.110 ± 0.030	4.61 ± 0.03

[a] Mean and standard deviation ($n \geq 27$) are shown.
[b] Yellowness index (YI), lightness (L) and b (blue–yellow component) recorded initially.
[c] Mean and standard deviation ($n \geq 11$) are shown.
[d] It was measured after film equilibration at each relative humidity (HR) and 25 °C.

Table 3
Moisture content and glass transition temperature (T_g) determined after film equilibration at each relative humidity (RH) of storage (25 °C).

Alginate	Glycerol (%, w/w)	RH (%)	Moisture content[a] (g water/g *dm*)	T_g [b] (°C)
Manugel	26.7	33.3	14.3 ± 0.9	−40.06
		57.7	22.9 ± 0.1	−44.18
		75.2	27.1 ± 0.1	−66.83
Cargill	26.7	33.3	17 ± 2	−59.05
		57.7	23.42 ± 0.09	−62.57
		75.2	29.8 ± 0.1	−64.37
VR	26.7	33.3	15.3 ± 0.4	−61.70
		57.7	21.4 ± 0.1	−70.57
		75.2	31.3 ± 0.4	−72.66
Protanal	26.7	33.3	17 ± 2	−63.82
		57.7	22.8 ± 0.7	−71.36
		75.2	27.9 ± 0.1	−72.46
Manugel	35.6	33.3	16.3 ± 0.2	−53.14
		57.7	23.9 ± 0.4	−65.72
		75.2	34.1 ± 0.4	−71.4
Cargill	35.6	33.3	16.0 ± 0.4	−58.00
		57.7	23.9 ± 0.4	−66.36
		75.2	28.77 ± 0.07	−72.95
VR	35.6	33.3	17.1 ± 0.5	−63.50
		57.7	23.7 ± 0.3	−71.83
		75.2	37.9 ± 0.4	−75.11
Protanal	35.6	33.3	15.8 ± 0.4	−63.81
		57.7	23.5 ± 0.9	−73.92
		75.2	33.1 ± 0.1	−75.66
Manugel	52.3	33.3	17 ± 1	−63.37
		57.7	25.1 ± 0.3	−75.21
		75.2	35.9 ± 0.6	−84.14
Cargill	52.3	33.3	16.3 ± 0.7	−68.05
		57.7	24.71 ± 0.08	−75.21
		75.2	35.7 ± 0.3	−84.53
VR	52.3	33.3	16.8 ± 0.4	−73.62
		57.7	25.5 ± 0.7	−77.00
		75.2	38.3 ± 0.2	−89.71
Protanal	52.3	33.3	17.1 ± 0.2	−74.19
		57.7	24.2 ± 0.2	−76.25
		75.2	39 ± 3	−88.43

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

in the example depicted in Fig. 2. Similar conclusions were drawn from comparison of k_{YI} values.

Collected k'_{AA} data were analyzed by an experimental design of two quantitative factors (RH and glycerol) at the three levels described before, coded as −1, 0, +1. A regression model was applied to analyze k'_{AA} as a function of the linear and quadratic values of the quantitative terms and their interactions. In a preliminary analysis, the type of polymer was considered as a third quantitative factor which differed in the frequency composition of each alginate (F_{GG}, F_{MM} and F_{GM+MG}) applied to film development. It was observed that AA hydrolysis was only affected by a significant interaction between Protanal ($p < 0.001$) or VR ($p < 0.05$) and the alginate diadic composition and glycerol levels. Hence, only RH and glycerol were considered finally as quantitative factors and separated models were built for each type of polymer.

The statistical results are reported in Table 4. The experimental design of RH and glycerol factors indicated that the rate constant of AA hydrolysis (k'_{AA}) significantly ($p < 0.05$) increased as a consequence of the separated increase in RH or glycerol content, when AA was compartmentalized in Protanal or VR alginate networks. It has been suggested that the previous presence of glycerol permits or facilitates the penetration of water into the polymeric network during storage (Pérez et al., 2009). On the other hand, k'_{AA} only increased significantly ($p < 0.05$) with the RH of film storage when AA was supported either in Manugel ($p < 0.05$) or Cargill ($p < 0.001$) alginate network. The dependence was also significant ($p < 0.05$) for the quadratic term of the RH factor for Cargill alginate films. The proportion of glycerol used for plasticization did not affect the AA stability in Manugel or Cargill alginate film. The highest proportion of GG-block in Manugel followed by Cargill alginate produces ordered templates for polymer chain associations mediated by Ca^{2+} crosslinking between neighboring macromolecules (Braccini and Pérez, 2001). Chandrasekaran et al. (1988) indicated that glycerol can produce disturbance of filament aggregation in the case of gellan polymer, which may also be extended to Manugel and Cargill alginate films. However, zipping of GG-block chains together by calcium ions may overcome the glycerol effect in these films. As previously mentioned, GG-block length determines the elastic modulus of calcium cross-linked alginate gels (Kong et al., 2004).

A somewhat higher hydrolytic stability of AA supported in Manugel or Cargill alginate films is observed by plotting the rate constants of AA hydrolysis (k'_{AA}) *versus* glycerol or RH linear factor (Fig. 3), especially by storage at 33.3% of RH but also at 75.2%. Hence, alginates with a predominant proportion of GG-blocks showed a higher ability to stabilize AA against hydrolysis. This effect could be associated with their higher capability to immobilize water by physical retention, as previously demonstrated for gellan films (León and Rojas, 2007). As mentioned above, guluronate chain pairing junction zones also involve water molecules (Braccini and Pérez, 2001), which correspond to highly adsorbed or non-freezable bound water (Ping et al., 2001).

Water is responsible for hydrolysis and the irreversible opening of the lactone ring of the AA molecule, producing 2-keto-L-gulonic acid (Kurata and Sakurai, 1967). Hence, at constant temperature (25 °C), k'_{AA} depended on the RH factor because k'_{AA} is the product of the true second order rate constant for AA hydrolysis (k) and the concentration of water available for reactions (C_{WATER}) (León and Rojas, 2007). This kind of water is that loosely retained by the solid-like film network. As RH of film equilibration increases, the polymeric network leaves higher proportion of loosely adsorbed water, which is available for chemical reactions. This condition also promotes the parallel development of browning reactions from 2-keto-L-gulonic acid.

The half-life times ($t_{1/2}$) of the AA supported in the alginate films were calculated from the values for k'_{AA}. In the most favorable condition of RH (33.3%), $t_{1/2}$ values ranged between 10 and 16 months for AA supported in Manugel and Cargill alginate films, a result not affected by the glycerol level, and between 3 and 11 months in Protanal and VR alginates, for decreasing proportions of glycerol. At 57.7% RH, the values of $t_{1/2}$ were in general no lower than 2 months. At 75.2% RH, the AA supported in Manugel alginate films showed a $t_{1/2} \approx 27$ days for all glycerol levels, whereas in VR and Protanal films, the $t_{1/2}$ decreased from 32 to 14 and from 32 to 9 days, respectively, as glycerol level increased.

The rate constants of browning development (k_{YI}) were also analyzed through the experimental design applied to AA degradation kinetics, with the polymer type as a categorical variable. The results indicated that k_{YI} significantly ($p < 0.01$) increased in a linear trend with the RH of film storage and glycerol proportion for all polymers assayed (Table 4), excepting for VR alginate films. In the latter system, browning kinetic was only dependent ($p < 0.01$) on the RH of storage. Significant dependence of k_{YI} on the RH in Cargill ($p < 0.01$) and Protanal ($p < 0.05$) alginate films was also observed in a quadratic term. An interaction between RH and glycerol was also detected for Protanal films. Response surfaces were then plotted (Fig. 4). They allowed us to find the best conditions for minimal

Table 4
Results of the statistical analysis are summarized for the rate constants of AA hydrolysis (k'_{AA}) and subsequent browning development (k_{YI}).

	Alginate			
	Manugel	Cargill	VR	Protanal
k'_{AA}				
RH·n	**0.00148**	**0.000516**	**0.00791**	**0.0104**
RH·n^2	0.46283	**0.012284**	0.23020	0.0726
glyc·n	0.13367	0.763096	**0.04400**	**0.0227**
glyc·n^2	0.12316	0.098638	0.63613	0.3497
RH·n:glyc·n[a]	0.28204	0.209220	0.10240	0.1099
Residual standard error	1.655×10^{-6}	1.534×10^{-6}	3.583×10^{-6}	5.72×10^{-6}
Multiple R^2	0.9789	0.9901	0.9520	0.9563
F-test probability	0.0102	0.003335	0.03419	0.02985
k_{YI}				
RH·n	**0.000963**	**0.0013**	**0.00727**	**0.000153**
RH·n^2	0.262219	**0.0071**	0.22641	**0.003147**
glyc·n	**0.016522**	**0.0343**	0.43724	**0.005224**
glyc·n^2	0.511434	0.7844	0.69191	0.474943
RH·n:glyc·n	0.074762	0.0852	0.49912	**0.011385**
Residual standard error	4.49×10^{-5}	3.142×10^{-5}	1.158×10^{-4}	4.195×10^{-5}
Multiple R^2	0.9856	0.9981	0.9396	0.9960
F-test probability	0.005814	0.004774	0.04773	0.000849

Relative humidity (RH) or glycerol (glyc) linear (RH·n; glyc·n) and quadratic (RH·n^2; glyc·n^2) factors. Bold numbers highlight significance ($p < 0.05$).
[a] In no case was the interaction between RH and glycerol level significant ($p < 0.05$) for AA hydrolytic rate constants.

browning, which corresponded to a 41–44% RH for storage and 29% (w/w) of glycerol content for plasticization, whereas the highest values of k_{YI} were observed at the highest RH of storage and glycerol content in films (Fig. 4).

By plotting the rate constants of browning (k_{YI}) *versus* glycerol or RH lineal factor (Fig. 3), no clear tendencies towards slower browning were observed in film systems. In general, lower k_{YI} values were obtained for Manugel alginate films at increasing RH and glycerol levels.

Despite the different kinetic order, k_{YI} correlated significantly (Pearson's correlation coefficient $r = 0.8731$; $p < 0.001$) with the k'_{AA} values.

3.4. Stability of L-(+)-ascorbic acid to chemical hydrolysis and oxygen in films

Films respectively made with Cargill or VR alginate using the two highest glycerol proportions were also studied in their ability to stabilize AA in the presence of oxygen. Storage was performed at 57.7% or 75.2% RH (25 °C) under normal air pressure ($P = 1.013 \times 10^5$ Pa). Hence, the oxygen partial pressure (p_i) was 0.21 atm constant during storage. Under these conditions, a pseudo-first order kinetics could be fitted to the experimental data of AA concentration ($p < 0.05$) in a manner similar to that previously observed in Fig. 2 for AA loss in alginate films stored under vacuum.

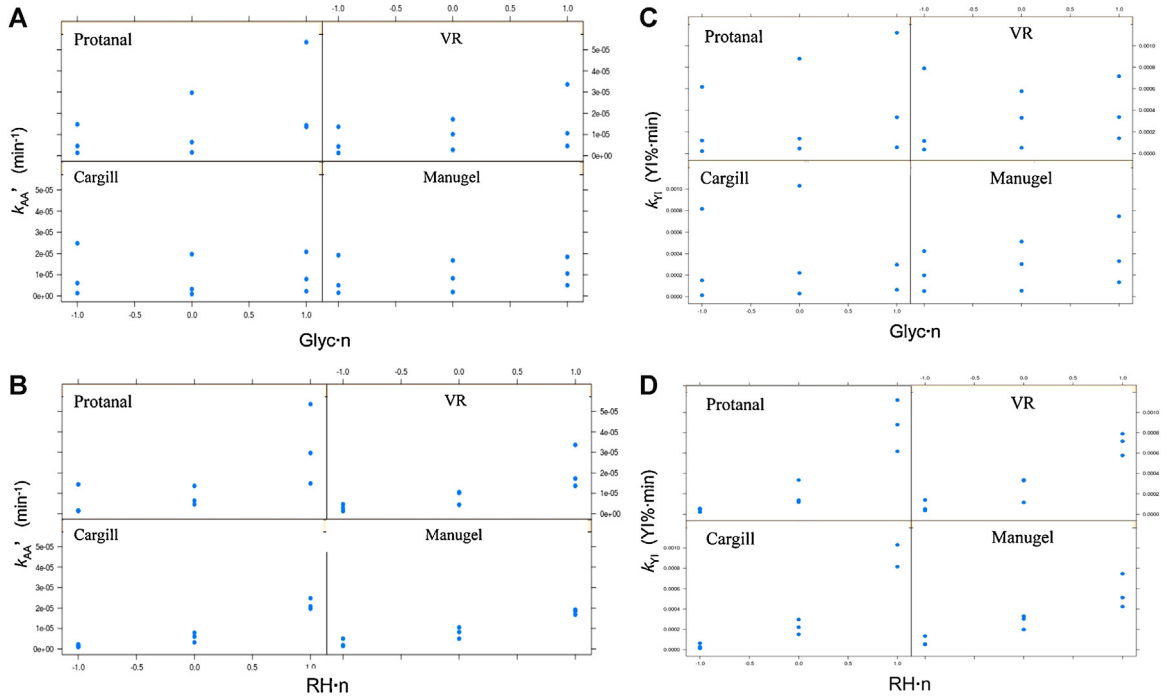

Fig. 3. Rate constants of AA hydrolysis (k'_{AA}) are plotted against glycerol (glyc·n) (**A**) or relative humidity (RH·n) (**B**) linear factor. Idem for the rate constants of browning development (k_{YI}): (glyc·n) (**C**) and (RH·n) (**D**).

Table 5
Rate constants[a] of AA hydrolysis (k'_{AA}) or hydrolysis and oxidation (k_T)[b], as well as of browning development (k_{YI}) at 25 °C, are reported.

Alginate	Glycerol (%, w/w)	Relative humidity (%)	Storage without air		Storage under air	
			$k'_{AA} \times 10^5$ (min^{-1})	$k_{YI} \times 10^4$ (YI% x min^{-1})	$k_T \times 10^5$ (min^{-1})	$k_{YI} \times 10^4$ (YI% x min^{-1})
Cargill	35.6	57.7	0.32 ± 0.01	2.2 ± 0.2	1.07 ± 0.05	3.33 ± 0.09
		75.2	1.97 ± 0.08	10.3 ± 0.5	2.68 ± 0.03	10.7 ± 0.4
VR	35.6	57.7	1.02 ± 0.07	3.3 ± 0.3	0.98 ± 0.08	3.6 ± 0.1
		75.2	2.7 ± 0.1	5.8 ± 0.4	3.1 ± 0.3	11.1 ± 0.9
Cargill	52.3	57.7	0.79 ± 0.04	2.9 ± 0.1	0.78 ± 0.06	3.0 ± 0.2
		75.2	2.8 ± 0.2	6.1 ± 0.5	2.86 ± 0.04	8.0 ± 0.3
VR	52.3	57.7	1.06 ± 0.07	3.4 ± 0.2	1.04 ± 0.08	3.1 ± 0.2
		75.2	3.4 ± 0.1	7.2 ± 0.3	3.7 ± 0.3	10.2 ± 1

[a] Mean and standard deviation ($n > 21$) are shown.
[b] k_T is the total rate constant of AA oxidation (Eq. (2)).

AA destruction in the presence of oxygen occurred simultaneously to the hydrolytic reaction previously studied under vacuum storage of films (Kurata and Sakurai, 1967). It can be then considered that at least two irreversible parallel or competitive reactions proceed: the AA hydrolysis (k'_{AA}) and the AA oxidation (k^{OX}_{AA}), which can be expressed as a differential kinetic equation written for the AA as the reagent, in the form of pseudo-first-order rate reactions:

$$r_{AA} = -\frac{1}{\nu_{AA}}\frac{dC_{AA}}{dt} = k'_{AA} \times C_{AA}(t) + k^{OX}_{AA} \times C_{AA}(t) \quad (1)$$

wherein ν_{AA} is the stoichiometric coefficient for AA hydrolytic reaction, r_{AA} is the AA-reaction rate/unit volume at a constant temperature, $C_{AA}(t)$ is the AA concentration remaining at time t, k'_{AA} is the rate constant of the pseudo first order kinetics for AA hydrolysis, k^{OX}_{AA} is the oxidation rate constant of AA.

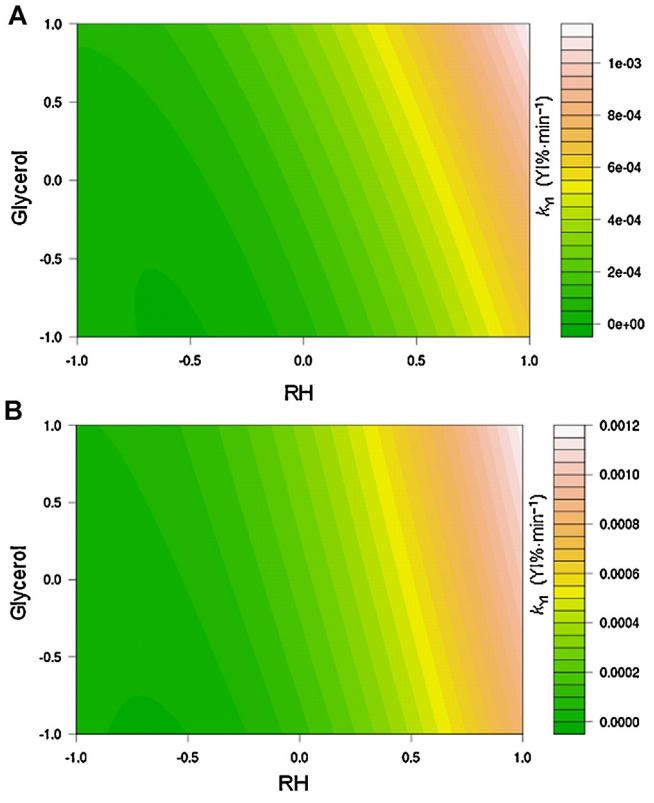

Fig. 4. Relative humidity (RH) and glycerol content influences on the rate constant of browning development (k_{YI}) are plotted as response surfaces for Protanal (**A**) and Cargill (**B**) alginate films.

By integration ($\nu_{AA} = 1$), results:

$$C_{AA} = C^{O}_{AA} \times \exp\left[-\left(k'_{AA} + k^{OX}_{AA}\right) \times t\right]$$

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

$$k_T = k'_{AA} + k^{OX}_{AA} \quad (2)$$

and the oxidation rate constant (k^{OX}_{AA}) can be obtained as the arithmetic difference. For oxygen partial pressures lower than 0.40 atm, the apparent rate constant (k^{OX}_{AA}; Eqs. (1) and (2)) involved the product between the true kinetic rate constant of oxidation (only dependent on temperature) and the oxygen concentration, related to the p_i (Khan and Martell, 1967).

In general, film systems stored under air did not show significant differences between k_T and k'_{AA} values (Table 5). Higher k_T values were only observed for Cargill alginate film formulated with 35.6% glycerol and stored at 57.7% or 75.2% RH. Even in film systems where a non significant difference between k_T and k'_{AA} was observed, browning rate constants (k_{YI}) determined under air storage at 75.2% RH were in general higher than the k_{YI} values found under vacuum (Table 5). It can be concluded that, in general, the alginate film networks seemed to effectively preserve AA from oxidation.

4. Conclusions

Water is the factor responsible for AA hydrolysis, and glycerol may facilitate water penetration from the environment into the polymeric network. In the presence of Ca^{2+}, alginates with higher proportion of GG-blocks ($F_{GG} = 0.66$) and lower one of MM- and, mainly, of GM + MG-flexible blocks, generate film networks that immobilize water sufficiently to reduce the degradation of hydrosensitive biomolecules such as AA. When comparing the hydrolytic with the total rate constant of AA destruction under air, it was observed that even at less favorable conditions of RH and glycerol levels, both GG and GM + MG enriched alginate networks in general preserve AA from oxidation. It also demonstrated that hydrolysis is the principal way by which AA is lost when supported in films and, hence, water immobilization is a key factor to be controlled.

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