Moreno Marro, O.; Cárdenas Estela, J.; Atarés Huerta, LM.; Chiralt A. (2017). Influence of starch oxidation on the functionality of starch-gelatin based active films. Carbohydrate Polymers. 178:147-158. doi:10.1016/j.carbpol.2017.08.128

Influence of starch oxidation on the functionality of starch-gelatin based active films.

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Abstract

Biodegradable active films were prepared by casting, using corn starch, with differing oxidation degree, bovine gelatin, glycerol, as a plasticizer, and ethyl lauroyl arginate (LAE) as antimicrobial compound. The films were characterised as to their microstructure, physical properties and water affinity. Starch oxidation with sodium periodate (SP) greatly improved polymer compatibility and crosslinking, especially at 1:1 glucose:SP molar ratio, which promoted the films' strength (40-92 %) and barrier capacity (33 and 40 % reduction in water vapour and oxygen permeability, respectively in LAE-free films), while Maillard reactions promoted the film browning. LAE, with amino and carbonyl groups, participated in the condensation reactions, interfering in the network crosslinking. All films with LAE showed bactericidal effect against *Listeria innocua* and *Escherichia coli* and LAE free films with oxidized starch inhibited the growth of both bacteria by 1-2 log CFU, due to the antimicrobial properties of Maillard compounds. Blend films with the most oxidized starch and gelatin containing LAE are promising materials for food packaging applications.

Keywords: Biodegradable active films, oxidized corn starch, bovine gelatin, Ethyl lauroyl arginate (LAE), crosslinking.

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1. Introduction

Nowadays, most of the food packaging systems are based on petroleum-derived synthetic plastics, whose production has increased exponentially over the past two decades (Tharanathan, 2003). Nevertheless, the utilization of these materials involves a serious environmental problem and high recycling costs (Moreno et al., 2014). In order to deal with this issue, current research focuses on the development of biodegradable polymers from renewable sources (Byun and Kim, 2014; Cazón, 2016).

Of the natural polymers available, starch is one of the most promising polysaccharides, since it is an abundant, low cost edible material (Barnett, 2011; Cazón, 2016, Moreno et al., 2015) and capable of being processed by the usual treatments applied in synthetic materials (Moreno et al., 2016). Native starch becomes thermoplastic (TPS) when thermally treated with plasticizers, which reduces the melting temperature below that of decomposition, thus allowing it to be moulded into a determined shape (Jiménez et al., 2012). However, TPS films also show some drawbacks when compared to oil-derived synthetic polymers, such as its highly hydrophilic nature, giving rise to water sensitivity and a poor water vapour barrier capacity. Starch retrogradation is also an inconvenience due to the fact that its mechanical properties worsen throughout storage (Ortega-Toro et al., 2014).

Properties of TPS films have been improved by blending with other biopolymers. More specifically, blends with gelatin have been previously reported to yield films with improved resistance and elongation capacity (Acosta et al., 2015; Fakhoury et al., 2012). Moreno et al. (2016) obtained starch-gelatin based films by thermo-processing and found no improvement in the water sensitivity, which suggests the need for developing new strategies to produce more hydrophobic starch matrices, or crosslinking processes which enhance the functionality of the starch material. In this sense, the oxidation of starch, giving rise to dialdehyde formation (DAS), can be used to promote the crosslinking capacity of the chains, while increasing the hydrophobicity of the matrix. During the oxidizing process, an oxidative cleavage of C-2 and C-3 bond of the anhydroglucose units of the native starch is produced (Yu et al., 2010, Du et al., 2008), yielding a reduction in hydroxyl groups to form carboxyl and carbonyl groups. The condensation reaction between carbonyl and amino groups also enables DAS to act as crosslinking agent with proteins (Rhim et al., 1998; Wang et al., 2015). In this context, it may be hypothesized that starch oxidation may be an adequate strategy to improve functional properties of biodegradable films based on starch and gelatin, on the basis of the crosslinking of polymers, thus reinforcing the inner matrix, and improving the

mechanical behaviour and water resistance of the films. In fact, Rhim et al. (1998) observed improved rigidity, resistance to fracture and extensibility, as well as reduced water solubility, when DAS was added to soy protein films (5:100 mass ratio). On the other hand, the reaction between carbonyls of reducing sugars and protein amino groups led to the formation of Maillard compounds, thus resulting in film browning. The increase in the number of carbonyl groups reduced the thermal stability of DAS (Soliman et al., 1997; Zhang et al., 2007), which can compromise the thermoprocessing of DAS-gelatin blends. Heating DAS-gelatin blends could, hence, extend the reaction between both polymers, resulting in an unsuitable material.

On the other hand, the inner reinforcement of the matrix induced by the crosslinking effect could enhance the retention of bioactive compounds, leading to a more controlled release when applied to foodstuffs. The incorporation of ethyl lauroyl arginate (N-α-lauroyl-l-arginine ethyl ester monohydrochloride, LAE) into DAS-gelatin films, can provide them with antimicrobial properties, while introducing more amino and carbonyl groups in the system, which could also participate in the crosslinking process, affecting the physical properties of the films. LAE is a potent food antimicrobial agent, derived from lauric acid, L-arginine, and ethanol with a wide spectrum of antimicrobial activity, even at low concentrations (Muriel-Galet et al., 2015). LAE has been reported to cause cell growth inhibition or death by increasing the permeability of the cell membrane, as a consequence of the membrane protein denaturation (Rodriguez et al., 2004). Thus, LAE is a promising antimicrobial additive considered as GRAS (Generally recognized as safe) by the FDA, and accepted as food additive E243 in Europe (Hawkins et al., 2009; Higueras et al., 2013). Its antimicrobial properties have been widely studied in different food systems, either directly incorporated into the food formulations or as component of packaging materials. LAE has been effective at controlling the growth of different bacteria in food products such as roasted turkey (Jiang et al., 2011), cold-smoked salmon (Kang et al., 2014), frankfurters (Martín et al., 2010), cheese (Soni et al., 2012) or chicken breast (Nair et al., 2014). Its antimicrobial effectiveness has also been proved when incorporated into different packaging films of polymers, such as chitosan (Higueras et al., 2013; Ma et al., 2016), zein (Kashiri et al., 2016), EVOH (Muriel-Galet et al., 2015) or pullulan (Pattanayaiying et al., 2015).

The present study aims to evaluate the effect of different degrees of starch oxidation and LAE incorporation on the functional properties of starch-gelatin blend films, obtained by casting method. The crosslinking effect in oxidized starch-gelatin matrices was assessed in comparison with non-oxidized starch. Moreover, the tensile and optical

properties and barrier capacity to water vapour after 5 weeks of storage were analysed in order to evaluate the development of the crosslinking effect throughout time.

2. Material and Methods

2.1. Materials

The following materials were employed to obtain the films: Corn starch (CS) (Roquette Laisa España, S.A., Valencia, Spain); Bovine gelatin type A (BG) (Sancho de Borja, S.L., Zaragoza, Spain); Sodium periodate (SP) (Fluka Analytical, Sigma–Aldrich Chemie GmbH, Steinheim, Germany); Ethyl lauroyl arginate (LAE) at 10% w/v in ethanol (Vedeqsa, Lamirsa, Terrassa, Spain) and glycerol as plasticizer (Panreac Química S.A., Castellar de Vallès, Barcelona, Spain).

Stock cultures, *Escherichia coli* (CECT 101) and *Listeria innocua* (CECT 910), employed for the antimicrobial activity analysis, were supplied by the Spanish Type Culture Collection (CECT, Burjassot, Spain). Tryptone Soy Broth, Agar bacteriological and Buffered peptone water were provided by Scharlab, (Barcelona, Spain).

The reagents used for film conditioning (magnesium nitrate or phosphorus pentoxide) and overall migration test (ethanol, acetic acid and isooctane) were all supplied by Panreac Química S.A., Castellar de Vallès, Barcelona, Spain.

2.2. Oxidation of Starch

The oxidation of CS was carried out by using SP as oxidizing reagent following a modification of the method described by Yu et al. (2010). CS was dispersed and gently stirred (10% w/v) in distilled water, then, SP was added obtaining two different SP:Glucose unit molar ratios (1 or 0.5), to obtain two different oxidation levels in the starch samples. The reaction was kept in the dark for four hours at 35 ºC. The solution reached a value of pH 3.5 once the components were mixed. Both oxidized starch (OS) samples were washed with distilled water three times, while being homogenised with a rotor stator homogenizer, Ultraturrax (T25, Janke and Kunkel, Germany) at 8,000 rpm for 30 seconds, and subsequently vacuum filtered (MZ 2C NT, Vacuubrand GMBH + CO KG, Wertheim, Germany). The final solids obtained were used for film preparation, taking into account the water content, which was determined by desiccating both samples in a vacuum oven (60ºC-24h), and then stored in desiccators with P_2O_5 until constant weight was reached.

To verify the different degree of starch oxidation by using two molar ratio of SP (1 or 0.5 SP:Glucose molar ratios), the aldehyde content of each sample was determined, as reported by Yu et al. (2010), through the conversion of aldehydes into oximes at 50ºC for 2 h, using hydroxylamine hydrochloride. To this end, the OS samples (0.2 g) were added in hydroxylamine hydrochloride (25 mL of a 0.25 M solution) and pH was adjusted to 5, using the adequate volume of 0.1 M NaOH solution throughout the reaction. This reaction was also carried out with native starch, as a control, determining the required volume of the NaOH solution to maintain pH=5. The aldehyde content was quantified from the NaOH volume difference with respect to the control, as moles of aldehyde per g of OS sample.

2.3. Film preparation

Six film formulations were prepared using glycerol as plasticizer: three control blend formulations based on starch or DAS and gelatin (mass ratio 1:1) (named OR_0, OR_0.5, OR_1, according to the molar ratio of oxidant used) and three active formulations based on the same blends with LAE (OR_0LAE, OR_0.5LAE, OR_1LAE).

The preparation of the films was carried out using the casting method, by blending two separate dispersions of both starch and gelatin in distilled water (2% wt.). All of the starch dispersions were homogenized in a rotor stator homogenizer, Ultraturrax, for 1 minute at 8,000 rpm, to ensure a complete dispersion of the compound. Then, to induce starch gelatinization, starch dispersions were immersed in a thermostatic bath at 100ºC, for 30, 45 or 60 min, respectively for native and oxidized starch with 0.5 or 1.0 molar ratio of SP, and cooled down to room temperature. Additionally, BG dispersion was prepared with magnetic stirring (30 min at 40ºC). Both starch and BG dispersions were mixed (1:1 mass ratio) to obtain the control films (OR_0, OR_0.5, OR_1), with the incorporation of glycerol in a polymer:glycerol mass ratio of 1:0.25. Moreover, in order to obtain the active films (OR_0, OR_0.5LAE, OR_1LAE) LAE was added in a polymer:LAE mass ratio of 1:0.1. All of the film-forming formulations were kept under magnetic stirring for 30 min, and then, the right amount was poured into Teflon plates (150 mm diameter) to assess 1.5 g of dry solids on each plate, corresponding to 84 g dry solids/dm^2. After drying for 48 h at 45%RH and 25 ◦C, the films were separated from the plates and stored for one and five weeks at 25ºC and 53% RH (in desiccators with saturated solutions of magnesium nitrate) prior to analyses.

2.4. Microstructural and physical characterization of films.

2.4.1. Film microstructure.

The film samples were conditioned in desiccators containing P_2O_5 for 48 h in order to eliminate the water content; then, the films were immersed in liquid nitrogen to obtain cryfractured cross sections. All of the samples were mounted on cupper stubs and, after platinum coating, images of the films' cross section were obtained by Field Emission Scanning Electron Microscopy (FESEM), using a ZEISS®, model ULTRA 55 (Germany) Microscope. An accelerating voltage of 2 kV was used for the observation.

2.4.2. Tensile properties and thickness

The tensile behaviour of the films was determined following the standard method ASTM D882 (2001). Prior to every test, the films were conditioned at 53% RH for one and five weeks to evaluate the crosslinking effect throughout time. A texture analyzer (TA-XTplus, Stable Micro Systems, Surrey, United Kingdom) was employed to obtain the stress–strain curves and the following parameters were determined: Elastic modulus (EM, MPa), tensile strength (TS, MPa) and elongation at break (% E). Firstly, the thickness of all of the film formulations was measured at four different points by using a hand-held digital micrometer (Electronic Digital Micrometer, Comecta S.A., Barcelona, Spain). Then, film stripes (25mm wide, 100 mm long) were mounted in the film extension grips and stretched at 50 mm min^{-1} until breaking. 12 replicates per formulation were run.

2.4.3. Water content and barrier properties of the films.

The moisture of the film samples, conditioned at 53 % RH, was determined by applying a two-step gravimetric method. Firstly, the samples were desiccated in a vacuum oven (60ºC-24h) and, secondly, stored in desiccators with P_2O_5 until constant weight was reached. The final results were expressed as g of water per 100 g of dry film. Six replicates per formulation were considered.

The water vapour permeability (WVP) of the films was determined according to Mc Hugh et al. (1993), following a modification of the ASTM E96-95 gravimetric method (1995). Prior to the test, the thickness of all the samples was measured at six points, as described above. Round film samples (35mm diameter) of each formulation were

195 secured on Payne permeability cups (Elcometer SPRL, Hermelle/s Argenteau, Belgium),
196 with the outwards-facing side in contact with the air during drying . Before securing the
197 films, 5 mL distilled water was placed (100 % RH) inside each cup, and then the cups
198 were placed inside desiccators containing an oversaturated magnesium-nitrate solution
199 in order to generate 53 % RH. Each pre-equilibrated cabinet was fitted with a fan to
200 provide a strong driving force across the film for water vapour diffusion. The cabinets
201 were stored at 25 ºC, weighing the cups periodically (±0.00001, ME36S Sartorius,
202 Germany) every 1.5 h, until steady state had been reached (24 h). Four replicates per
203 formulation were considered.

204 The oxygen permeability (OP) of the films was determined following the standard
205 method ASTM D3985-95 (2005). Measurements were taken using an OX-TRAN (Model
206 2/21 ML Mocon Lippke,Neuwied, Germany), at 53% RH and 25ºC and 50 cm^2 exposed
207 area. The films were exposed to pure nitrogen flow on one side and pure oxygen flow on
208 the other side. The OP was calculated by dividing the oxygen transmission rate by the
209 difference in oxygen partial pressure between the two sides of the film, and multiplying
210 by the average film thickness, which was measured at six points before the test. Two
211 replicates per formulation were made.

212

213 2.4.4. Optical properties.

214 The infinite reflectance spectra (R_∞) of the film samples (400 to 700nm) was obtained
215 with a spectrocolorimeter MINOLTA, model CM-3600d (Minolta CO, Tokyo, Japan),
216 measuring on the side of the films facing the air during drying. To this end, the
217 measurements were taken on both black (R0) and white (R) backgrounds. Additionally,
218 the spectrum of the white background was measured (Rg). Once all the spectra were
219 obtained, the internal transmittance of the films (Ti), an indicator of the transparency, was
220 calculated, using the Kubelka–Munk theory (Hutchings, 1999) for multiple scattering. The
221 same theory allowed R_∞, (reflectance for an infinite film thickness) to be obtained, which
222 was used to calculate the CIE-L*a*b* parameters (CIE, 1986), using illuminant D65 and
223 observer 10º. The colour coordinates L* (lightness), C_{ab}* (chrome) and h_{ab}* (hue) were
224 calculated according to Atarés et al. (2010). Six samples per formulation were measured.

225

226 2.4.5. Film solubility and swelling in water.

A modification of the method described by Balaguer et al., (2011) was applied to determine the solubility and swelling (water uptake capacity) of the films. Film samples were cut into 3 x 3 cm squares and dried for 2 weeks in desiccators over P_2O_5 in order to reduce the water content to practically zero. Firstly, the initial dry weight (W_d^i) of the samples was taken, and then the dry films were immersed in 10mL of distilled water in glass containers, gently stirred and kept in contact for 24h at 25ºC. Directly afterwards, the solvent was poured in a filter, retaining the film sample, the remaining surface water was removed and the final wet weight (W_w^f) was measured. These wet samples were dried till constant weight to evaluate the mas of residual solids in the film after soaking (W_d^f). The solubility of the films was expressed as the weight loss (WL %, g/g dry film) of the samples (Equation 1), while the water update capacity (ΔW %, g water/g dry film) was calculated by Equation 2. Four replicates per formulation were considered.

$$WL\% = \frac{w_d^i - w_d^f}{w_d^i} \cdot 100 \qquad \text{(Equation 1)}$$

$$\Delta W\% = \frac{w_w^f - w_d^f}{w_d^f} \cdot 100 \qquad \text{(Equation 2)}$$

2.4.6. Overall migration

The overall migration of the films conditioned for 1 week at 53% RH was determined according to the current legislation (Commission Regulation (EU) No 10/2011). Film samples were immersed in 50 mL of simulant, keeping a contact ratio of 6 dm^2 of sample/kg of simulant. Three food simulants were used: Simulant A (ethanol 10% v/v, simulating hydrophilic foods), simulant B (acetic acid 3% w/v, simulating hydrophilic acid foods) and simulant D2 (isooctane, simulating lipophilic foods, with free fats at the surface). All the samples were kept in contact with the simulants for 10 days at 20ºC (test number OM1). Directly after the contact time, the film samples were removed from the simulants, which were evaporated until dry. Finally, the obtained residue was weighed and the results were expressed as milligrams of the total constituents released per dm^2 of contact surface (mg/dm^2). All of the tests were run in duplicate.

2.5. Thermogravimetric analysis (TGA)

The films were characterized as to their thermal stability using a thermogravimetric analyzer (TGA/SDTA 851e, Mettler Toledo, Schwerzenbach, Switzerland). Each sample film (about 3 mg) was heated from room temperature to 600 ºC, under nitrogen flow (50 mL/min), at 10ºC/min. Two replicates per formulation were obtained.

2.6. ATR FTIR Spectroscopy analysis

The attenuated total reflectance (ATR) FTIR spectra of the different films were obtained in a Thermo Nicolet 5700 Fourier transform infrared spectrometer (FTIR). The average spectra were collected from 64 accumulations with a resolution of 4 cm^{-1} in the 4000-400 cm^{-1} range, from five different locations of the same specimen.

2.7. In vitro antimicrobial properties of the films.

The antimicrobial activity of the films was analyzed *in vitro* against *Escherichia coli* (CECT 101) and *Listeria innocua* (CECT 910). Both strains were initially frozen in TSB with 30% glycerol, and regenerated by inoculation in 10 mL TSB. After incubation (24 h at 37ºC), 10μl were transferred into 10 ml TSB, which was incubated for 24 h at the same temperature to obtain a work culture in exponential phase of growth.

Film samples were UV treated prior to the microbial assay. TSB tubes of 10 mL were inoculated with 10^4 CFU/mL and a film portion of 5.3 cm of diameter was introduced in each tube. Inoculated tubes without film were used as inoculum control. All tubes were incubated for 24 h at 37 ºC, then, they were ten-fold diluted in order to obtain bacteria enumeration in TSA medium. All the tests were run in duplicate

2.8. Statistical analysis

The statistical analysis of the data was performed through an analysis of variance (ANOVA) using Statgraphics Centurion XVI s for Windows 5.1 (Manugistics Corp., Rockville, Md.). Fisher's least significant difference (LSD) at the 95 % confidence level was used to compare treatments.

3. Results and discussion

3.1. Microstructure and crosslinking analysis in the films.

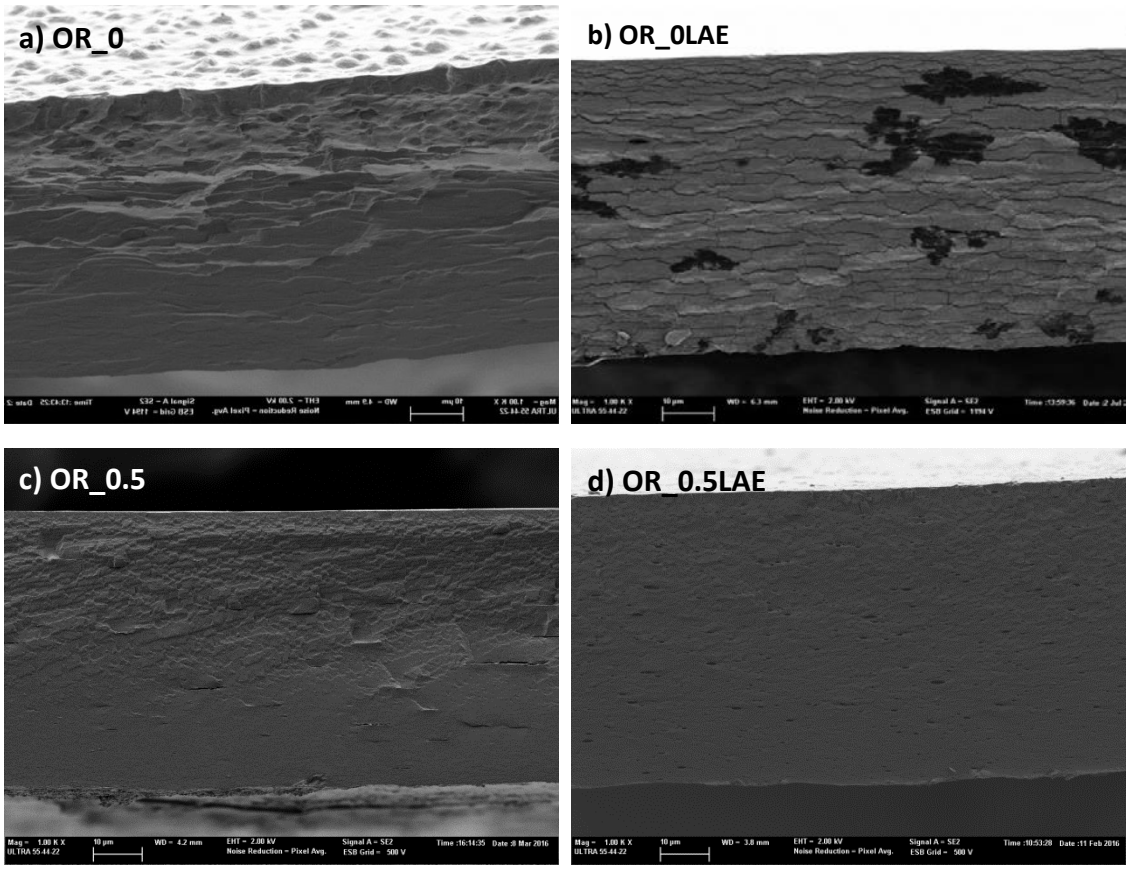
The degree of starch oxidation in starch samples treated with 0.5 or 1 SP:Glucose molar ratio was 0.467 and 0.898, expressed as moles/g sample, which prove the different extent of dialdehyde formation, according to the molar ratio of the oxidant. The effect of starch oxidation on the blend film's structure and the crosslinking process has been assessed through the analysis of the film's microstructure, FTIR spectra and thermal behaviour (thermogravimetric analysis: TGA). Pure starch films obtained with different molar ratios of the oxidant (0, 0.5 and 1.0) were included in the FTIR and TGA analysis to better understand interactions in starch-gelatin films.

FESEM analysis

FESEM cross section images obtained for starch-gelatin blend films, with differing degrees of oxidation, with and without LAE, are shown in Figure 1. In films with a non-oxidised starch (OR_0), polymer separation can be observed during drying, the film exhibiting a gelatin-rich layer at the top and a starch-rich layer at the bottom, depending on their differing densities, as previously reported by Moreno et al. (2016). An oxidation treatment of the starch seems to improve the polymer compatibility by enhancing interactions between starch and gelatin chains; no clearly separated layers were observed in the films with differing degrees of starch oxidation (OR_0.5 and OR_1), showing a greater integration between both polymers. The enhanced cross linking ability provided by OS also led to a smother surface fracture in the cross section images of the films, especially for the OR_1 sample, with the highest degree of oxidation.

The incorporation of LAE favoured the polymer compatibility in non-oxidised starch, giving rise to a different film microstructure with small domains of both polymers due to the LAE surfactant action (sample OR_0LAE); thus avoiding polymer stratification during the film drying step, as reported by Moreno et al. (2016). With oxidized starch, films containing LAE (OR_0.5LAE and OR_1LAE) showed a very smooth fracture surface, thus indicating the good compatibility achieved by all the components. Only small voids could be appreciated in the matrix, which can be attributed to the presence of very small air bubbles incorporated during the homogenization step of the film-forming dispersion.

The surfactant effect of LAE promotes air incorporation and the high degree of viscosity of the film-forming dispersion makes its elimination difficult. Microscopic observations revealed a much more homogenous film structure when oxidised starch was used in the starch-gelatin film formulation, which was clearly enhanced by LAE addition. In this case, separate polymer domains were not observed and a cohesive structure without discontinuities could be appreciated. This suggests LAE participation in the polymer network, through its interaction/reaction with the new side groups of starch chains. The LAE molecule has two primary amino groups that are highly reactive with the carbonyls of oxidised starch and also two carbonyls which are potentially reactive with the amino groups of protein. Therefore, the participation of LAE in polymer bonding could be expected on the basis of its molecular structure.



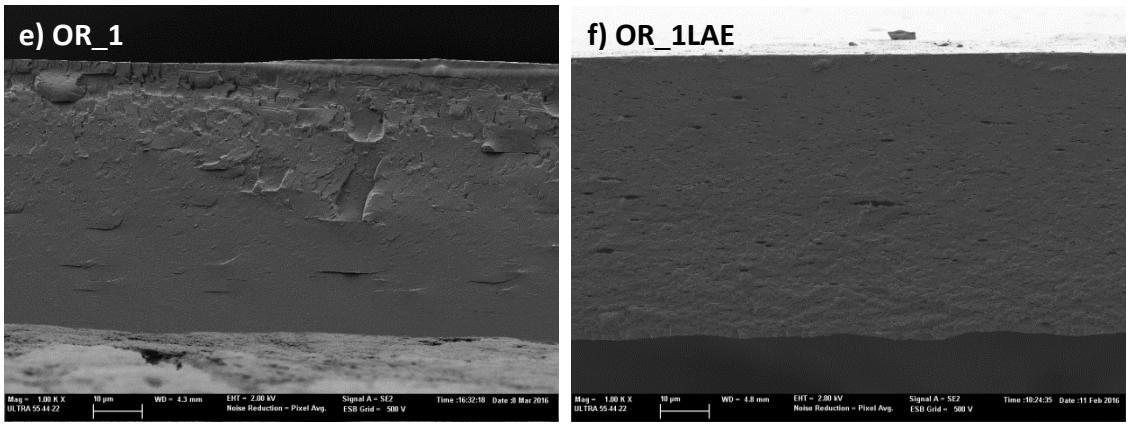
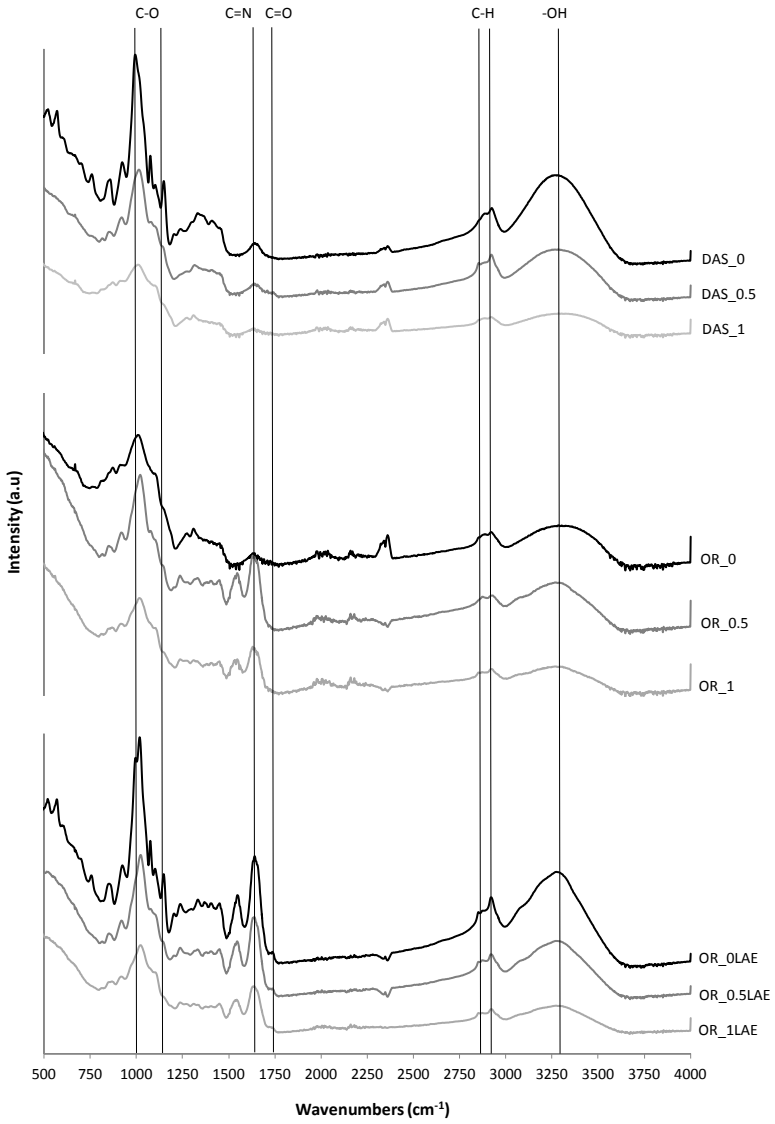


Figure 1. Micrographs obtained by FESEM of the cross-section of the films obtained from starch gelatin blends, with differing degrees of starch oxidation, and with or without LAE.

ATR FTIR analysis

Figure 2 shows the FTIR spectra of the different films, including pure starch films with differing degrees of oxidation. Non-oxidized starch-based films (DAS_0) showed characteristics peaks between 1000 and 1200 cm^{-1}, attributed to the C–O bond stretching of starch (Me et al., 2007 de Yu et al.); the peaks around 2850 and 2926 cm^{-1}, 1450 and 1370 cm^{-1} are related to the C–H stretching and bending modes of the methylene (Sheng et al., 2011); the absorption around 1640 cm^{-1} is a typical band residing in the spectra of starch and its derivative, related to the tightly bound water (Seligra et al., 2016; Yu et al., 2010; Zhang et al., 2012), while the peak observed around 3300 cm^{-1} is associated with the stretching of OH groups from glucose, glycerol and water (Zhang et al., 2014). As the degree of oxidation increased, the absorbance of the bands associated with –OH groups exhibited a lower intensity, which could be related with the reduction of the –OH number in the starch chains and their lower water affinity. In fact, oxidation with periodate leads to the breakage of the C-2 and C-3 bonds of the anhydroglucose units, reducing the content of hydroxyl groups and forming aldehyde groups. Nevertheless, the characteristic absorption peak around 1740 cm^{-1}, corresponding to carbonyl (C=O) stretching vibration, presented a very weak intensity due to the formation of hemiacetal linkage between oxidised and non-oxidised starch residues (Zhang et al., 2007; Zhang et al., 2014). It is remarkable that the absorption peaks in samples with oxidised starch decreased in intensity at a constant wavenumber, as previously described by other authors (Rivero et al., 2010) for crosslinked polymer structures, probably associated with the major restrictions for bond vibration.



355

356 **Figure 2.** FTIR spectra of films obtained from starch films and starch gelatin blend
357 films, with differing degrees of starch oxidation and with or without LAE.

358 Starch-gelatin blend films also exhibited a lower degree of intensity in the absorbance
359 of the different bands, particularly those associated with the vibration of OH groups,
360 which could be related with the strong interaction between both polymers forming a tight
361 network where new bonds are formed. Particularly, dialdehyde polysaccharides are able
362 to crosslink with amino groups of proteins through the formation of Schiff bases, imine
363 group (C=N), which is associated with a stretching vibration around 1630 cm^{-1} (Azeredo
364 and Waldron, 2016; Yin et al., 2008), as observed for both formulations with oxidised
365 starch (OR_0.5, OR_1).

366 The incorporation of LAE introduced new C=O and NH_2 groups, with vibration bands
367 at 1730 and 3400 cm^{-1}, respectively (Kamoun, 2015), which is reflected in a higher

relative intensity of these bands in samples containing this compound. Likewise, LAE could also promote the formation of C=N groups, by reacting with both starch' carbonyls or gelatin amino groups, as revealed by the relative intensity of the peak at 1630 cm^{-1}. A higher intensity of the peaks, associated with the vibration of these groups, was noticed when compared to the corresponding formulations without LAE; this could be related with a greater ability of the network to permit bond vibration and to a greater concentration of C=N groups. Thus, the carbonyl–amino reaction to form C=N groups was enhanced by the presence of LAE. FTIR signal attenuation was also observed in line with the degree of starch oxidation, which could be attributed to a more crosslinked structure, as previously commented on.

From FTIR analyses, the crosslinking between starch and gelatin was evidenced, as well as the participation of LAE in condensation reactions between amino groups and carbonyls. Starch oxidation greatly promotes the inter-chain bonding between polysaccharides and proteins, although the reaction probably involves, to a greater extent, molecules of low molecular weight and higher molecular mobility and reactivity, such as LAE, and short chain macromolecules resulting from the partial de-polymerization of starch during oxidation, as described by Fiedorowicz and Para, (2006). Edge-chain carbonyls of non-oxidised starch can also participate in the crosslinking process. Likewise, cross-linked chains, by means of the formation of acetal bonds between carbonyls and hydroxyls, can also contribute to the polymer network formed in oxidised starch (Du et al., 2008), especially when no proteins were present (samples DAS_0.5 and DAS_1.0).

Thermogravimetric analysis

Figure 3 shows the thermograms obtained for the starch-gelatin films (a), with or without LAE, and for pure starch (b), with differing degrees of oxidation. The evaluation of the normalized mass loss curve with temperature for all of the formulations reflects a slight initial loss, attributed to the water retained in the polymeric network, followed by a pronounced mass loss associated with the polymer degradation. The oxidation of starch greatly affected the thermo-stability of the polymer, decreasing the temperature of both the onset and maximum degradation rates, as previously reported by other authors (Soliman et al., 1997; Zhan et al., 2012), in line with a partial disruption of starch chains (Fiedorowicz and Para, 2006). Likewise, two steps appeared in the thermo-degradation process of oxidized starch, which were different for the two oxidized samples with

different molar ratio of SP. The first step can be attributed to the earlier degradation of the more oxidized chains (more thermosensitive), which is more marked for sample obtained with the highest ratio of oxidant, according to the greater progression of the chain oxidations. The second step, extended to higher temperatures, can be attributed to the thermal changes of crosslinked chains due to the formation of acetalic inter-chain unions through the carbonyl side groups, as reported by Du et al., (2008). This crosslinked structure in oxidized starch films was also deduced from FTIR spectra (as commented on above) and its degradation at higher temperature can be expected because of the higher molecular weight. Thus, an increase in the temperature range of the overall degradation process occurred in oxidized starch samples. Therefore, the different thermo-degradation behavior of starch treated with 0.5 and 1.0 molar ratio of SP was coherent with the different oxidation level of both samples.

For starch-gelatin blend films, two main steps could be observed in polymer degradation. The first one, which starts near 100 ºC, must be attributed to the promotion of the starch-protein reaction induced by the temperature increase. The rate of this step was greatly increased by starch oxidation and LAE addition, on the basis of the significantly lower ($p<0.05$) onset temperature (T_0). This phenomenon points to the prevailing reaction of the starch carbonyl groups with the protein amino groups, or to the fact that LAE is bifunctional and reacts with both carbonyl or/and amino groups in this first step. This behaviour indicates that starch-protein blend films cannot be obtained by thermoplastic processing, especially if oxidized starch is used. The reaction of non-oxidized starch and gelatin or LAE during the thermo-processing of blend films was previously reported (Moreno et al. 2016), but this was greatly enhanced in OS due to the extensive formation of carbonyl groups, which are highly reactive with protein amino groups.

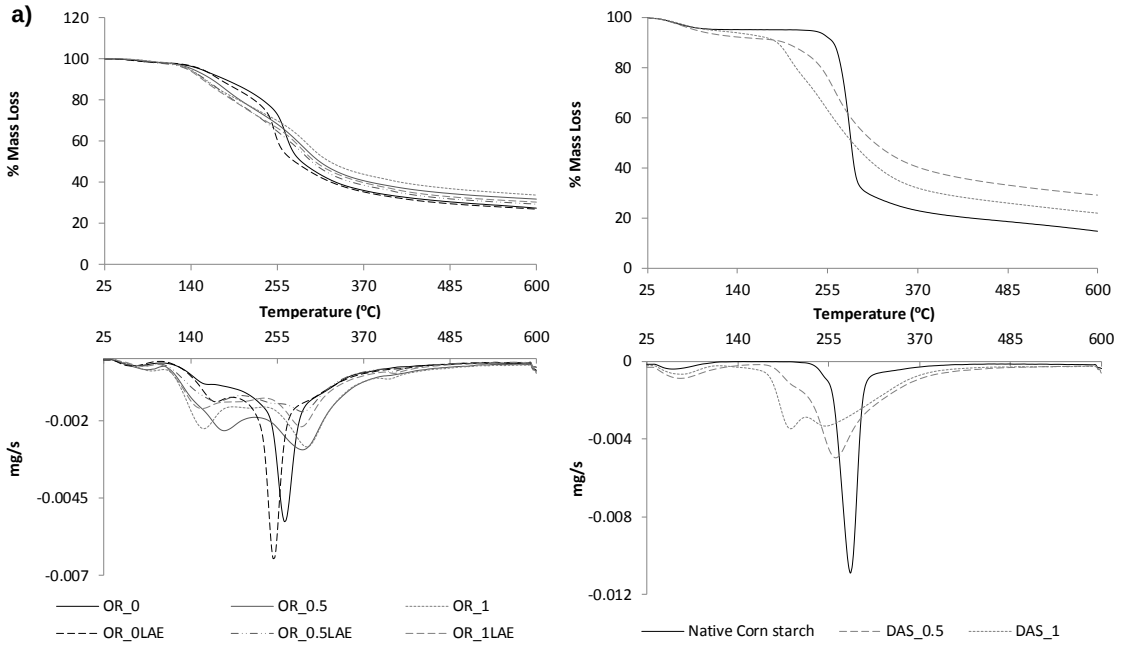


Figure 3. Thermogravimetric curves and the first derivatives of the TGA curves for film samples obtained from (a) starch-gelatin blends with differing degrees of starch oxidation, with or without LAE (b) starch with differing degrees of oxidation.

The second, and main, degradation step of the starch-gelatin films, characterized by the temperature of the fast degradation rate (T_{max}), reflected a significant increase ($p<0.05$) in the T_{max} for films with OS, according to the previous formation of a crosslinked matrix during the first step. In this sense, it is remarkable that films containing LAE exhibited lower values of T_{max}, which suggests that the participation of LAE in the crosslinking reaction did not imply the formation of such a tight network, whereas a more continuous degradation/reaction process (less marked steps) was observed for films containing this compound and oxidized starch.

Thermal analysis reveals that starch-gelatin blend films are very sensitive, when the temperature rises above 100ºC, which is greatly enhanced when the starch was oxidized, or other compounds containing amino or carbonyl groups, such as LAE, are present in the blend. These reactions promote uncontrolled crosslinking in the polymer network, which slowly degrades at higher temperatures. Therefore, the thermal processing of starch-gelatin blend films is limited by the occurrence of these uncontrolled reactions at high temperatures, also provoking the extensive formation of coloured compounds.

On the basis of the film's microstructure and crosslinking analysis, a high level of crosslinking between oxidised starch and gelatin chains can be expected in blend films,

especially when the highest ratio of oxidant was used. The addition of LAE implied its participation in the bonding reactions, given that its molecule has carbonyl and amino groups, which can compete with the macromolecules' functional groups, thus limiting the inter-chain bonds. This occurred mainly when starch was less oxidised and there was lower carbonyl concentration in its chains.

3.2 Tensile, barrier and optical properties of the films

Tensile properties

The values of the tensile parameters (elastic modulus (EM, MPa), tensile strength (TS, MPa) and elongation at break (% E)), and the film thickness are shown in Table 1. The differing degrees of starch oxidation clearly affected the tensile behaviour of the films. The lower starch oxidation (OR_0.5 sample) led to a decrease in the stiffness while increasing the stretchability of the films, in comparison with the non-oxidized formulation (OR_0 sample). However, when the starch oxidation was higher (OR_1 sample), films became more rigid and resistant, with lower extensibility. The crosslinking process associated with starch oxidation involved an increase in the stiffness as a consequence of the reinforcement of the matrix, but this trend was not observed for sample OR_0.5. The poorer progress of chain bonding, together with the possible starch de-polymerization during oxidation could yield a less cohesive polymeric network, thus exhibiting lower strength in tensile behaviour.

The effect of LAE incorporation on tensile behaviour was dependent on the degree of starch oxidation. When the oxidation was higher, LAE did not significantly affect the tensile behaviour of the films. However, the extensibility of films with less oxidized starch decreased when LAE was added, whereas it increased when LAE was added to non-oxidized films. In both cases, LAE addition caused a decrease in stiffness and resistance to break. These different effects, depending on the degree of oxidation of starch, could be related with the different participation of this molecule in bonding reactions with carbonyl groups of starch chains or amino groups of protein, as previously commented on. At higher oxidation ratios, the carbonyls of the starch chains compete with those of LAE with the highest concentration and their reaction with the protein chains could promote inter-chain bonding. However, at lower oxidation ratios, there could be a greater contribution of LAE reactions, which are less effective at promoting the network cohesion, thus reducing the strength of the polymer matrix, but decreasing its stretchability. In fact, due to its lower molecular weight, LAE would be more reactive with

carbonyl groups than gelatin and, consequently, it could be linked to the oxidized chains of starch, decreasing the degree of crosslinking of the polymer matrix. In the case of non-oxidised starch, the interfacial action of LAE between the polymer domains led to films with increased plastic deformability and lower strength, as reported by Moreno et al. (2016).

Table 1. Film thickness (μm) and moisture content; Tensile parameters (Elastic modulus (EM, MPa), Tensile Strength (TS, MPa) and deformation at break (% E)); Barrier properties (Water Vapor Permeability (WVP) and Oxygen Permeability (OP)); Colour coordinates (L*, C_{ab}*, h_{ab}*) and internal transmittance (T_i) at 430 nm of the starch-gelatin blend films, with differing degrees of starch oxidation, with or without LAE, conditioned for 1 week at 53 % RH and 25 ºC.

	OR_0		OR_0.5		OR_1	
	No LAE	LAE	No LAE	LAE	No LAE	LAE
Thickness (μm)	$65\pm6^{b,y}$	$61\pm3^{a,x}$	$60\pm2^{a,x}$	$64\pm2^{b,y}$	$65\pm2^{b,x}$	$65\pm9^{b,x}$
Xw (d.b. g water/ g dry film)	$12,0\pm3,0^{b,x}$	$10,3\pm0,5^{b,x}$	$8,1\pm0,5^{a,x}$	$10,6\pm0,5^{b,y}$	$8,9\pm0,5^{a,y}$	$7,7\pm1,0^{a,x}$
EM (MPa)	$1020\pm190^{b,y}$	$510\pm90^{b,x}$	$470\pm70^{a,y}$	$360\pm70^{a,x}$	$1290\pm80^{c,x}$	$1300\pm200^{c,x}$
TS (MPa)	$33\pm6^{a,y}$	$24\pm7^{a,x}$	$21\pm6^{a,y}$	$35\pm6^{a,x}$	$46\pm8^{b,x}$	$46\pm8^{b,x}$
% E	$14\pm5^{b\,x}$	$35\pm2^{c,y}$	$27\pm5^{c,y}$	$18\pm10^{b,x}$	$6,2\pm1,5^{a,x}$	$7\pm2^{a,x}$
WVP x10^7 (g·mm/kPa·h·m²)	$6,9\pm0,2^{c,y}$	$5,0\pm0,6^{a,x}$	$5,0\pm0,3^{b,x}$	$5,0\pm0,9^{a,x}$	$4,6\pm0,1^{a,x}$	$5,2\pm0,6^{a,y}$
OP x10^{13} (cm^3/m s Pa)	$1,26\pm0,03^{b,x}$	$2,08\pm0,07^{b,y}$	$1,00\pm0,15^{ab,x}$	$2,50\pm0,50^{b,x}$	$0,75\pm0,09^{a,x}$	$0,95\pm0,11^{a,x}$
L_{ab}*	$80,6\pm0,5^{cy}$	$73,8\pm0,6^{a,x}$	$76,8\pm1,1^{a,y}$	$73,4\pm1,0^{a,x}$	$78\pm0,8^{b,y}$	$73,8\pm0,4^{b,x}$
C_{ab}*	$7,27\pm0,17^{c,y}$	$4,41\pm0,08^{a,x}$	$28\pm2^{b,x}$	$28,3\pm0,7^{b,x}$	$33,3\pm1,0^{a,y}$	$29,2\pm0,7^{c,x}$
h_{ab}*	$98\pm0,3^{c,x}$	$104\pm0,9^{b,y}$	$81\pm2^{a,x}$	$79,2\pm0,5^{a,x}$	$91,2\pm0,7^{b,y}$	$79,1\pm0,6^{a,x}$
T_i (430 nm)	$0,833\pm0,004^{c,y}$	$0,826\pm0,005^{c,x}$	$0,74\pm0,02^{b,x}$	$0,73\pm0,014^{b,x}$	$0,689\pm0,016^{a,x}$	$0,711\pm0,004^{a,y}$

Different letters (a, b, c) in the same row indicate statistical differences (p<0.05) between the formulations, either with or without LAE, with differing degrees of starch oxidation.

Different letters (x, y) in the same row indicate statistical differences (p<0.05) between samples with and without LAE, for each degree of starch oxidation.

The tensile behaviour of the films indicated that the degree of crosslinking of the starch-gelatin matrix, caused by the reaction between carbonyl and amino groups, did not progress to the same extent in the case of partial oxidation as a result of the heterogeneity of the chains (containing hydroxyl and carbonyl groups), which probably produces greater steric hindrances to the creation of new bonds. However, the

505 crosslinking effect for OR_1 films was much more noticeable, as reflected by the great
506 reinforcement of the polymer matrix. In the presence of LAE, the lower oxidation of starch
507 offers more opportunities for the reaction of LAE through its carbonyl groups, which did
508 not contribute to a reinforcement of the polymer matrix to the same extent as protein
509 linkages.

510

511 *Barrier properties*

512 Table 1 shows the values of the equilibrium moisture content of the films and their
513 barrier properties to water vapour and oxygen (WVP and OP). Starch oxidation led to a
514 significant ($p<0.05$) decrease in the moisture content and WVP for both oxidation levels,
515 coherently with the greater hydrophobic nature of the matrix, resulting from the
516 transformation of –OH into –C=O groups in the starch chains (Zhang et al., 2007), and
517 the crosslinking effect, which created more resistance to mass transfer in the matrix. LAE
518 addition affected the moisture content of the films, reducing the value for non-oxidised
519 and most oxidised starch, but increasing the value at lower oxidation level. This also
520 points to differences in the network crosslinking when LAE is present, which affects the
521 active points for water adsorption, depending on the oxidation level. Oxygen permeability
522 was also significantly reduced ($p<0.05$) by starch oxidation, which can also be related to
523 the tighter matrix caused by crosslinking and the milder water plasticising effect
524 associated with the lower water content. Only films containing LAE with a lower oxidation
525 level exhibited non-reduced oxygen permeability, which is coherent with the poorer
526 progress of the inter-chain bonds due to the LAE competence in the carbonyl-amino
527 reactions, previously commented on. Thus, starch oxidation allows less polar and tighter
528 polymer matrices to be obtained, with lower water affinity and enhanced barrier
529 properties, thus improving both water vapour and oxygen permeability. This is explained
530 by the weaker water plasticizing effect, since the films' moisture content falls, and also
531 by the crosslinking in the polymer network, which slows down the transport mechanisms
532 by promoting the tortuosity factor of the polymeric network.

533

534 *Optical properties*

535 Table 1 shows the values of the colour coordinates (L_{ab}*, lightness; C_{ab}*, chrome;
536 h_{ab}*, hue) of the different films. Starch oxidation significantly reduced ($p<0.05$) the
537 lightness and hue of films and gave rise to a significant ($p<0.05$) increase in the chrome;

this is coherent with the formation of brown compounds resulting from the carbonyl-amino reactions to form Maillard compounds, greatly promoted at the low moisture content of the films. Specifically, the yellow-brown coloration associated with protein-aldehyde reactions may be attributed to the formation of conjugated Schiff's bases, which are intermediate products of the Maillard reaction (Cheftel et al., 1985; Martucci and Ruseckaite,, 2009). In addition, when the LAE was incorporated, all of the oxidized formulations exhibited a decrease in lightness and hue, obtaining browner films, which is coherent with the involvement of LAE in the browning reactions, due to the presence of amino and carbonyl groups in its molecular structure. Both the oxidized formulations containing LAE and the film with lower degree of oxidation without LAE had very similar colour coordinate values, whereas films with more oxidized starch without LAE were slightly lighter and less red, thus indicating that browning progressed to a lesser extent in this case, where a more effective polymer crosslinking was observed. On the other hand, the incorporation of LAE into films with non-oxidised starch provoked a decrease in the films' lightness and colour saturation, while also promoting the film yellowish, which also points to the formation of coloured compounds to a certain extent.

The internal transmittance (T_i) of the films also decreased in line with starch oxidation, mainly at lower wavelengths, where brown compounds absorb light Table 1 also shows the values of T_i at 430 nm, which reflect that the higher the degree of starch oxidation, the lower the T_i values. This behaviour was coherent with the selective absorption of brown products (Maillard compounds) mainly coming from the reaction of low molecular weight compounds, such as the reducing sugars resulting from the partial hydrolysis of starch. Condensation reactions were encouraged by the greater availability of carbonyl groups obtained during the oxidation process. The addition of LAE did not imply any significant changes in the transmittance of the films, except for OD_1 films, for which its addition led to an increase in internal transmittance. Crosslinking in the matrix can also contribute to a reduction in internal transmittance due to the promotion of the film's opacity (Martucci and Ruseckaite,, 2009).

3.3. Water solubility, water uptake capacity and overall migration of the films.

To analyse the effect that the crosslinking promoted by starch oxidation has on the films' hydrophobicity, their water solubility, water uptake capacity and overall migration in different food simulants were analysed.

Table 2 shows the water solubility values of the films, expressed as weight loss %

572 (WL %) in water, and the water uptake capacity (ΔW %) expressed as the mass of water
573 gained by the dry film. Starch oxidation led to a highly significant decrease ($p<0.05$) in
574 the water uptake capacity of the films, coherently with the polarity reduction in the
575 polymeric matrix, in line with the fall in the number of –OH groups, and by the
576 crosslinking of the polymer chains which restrict the water absorption by steric hindrance.
577 Nonetheless, the solubility in water (WL %) of the films was significantly greater ($p<0.05$)
578 in the case of oxidized samples. The periodate oxidation of starch led to the formation of
579 dialdehyde starch, but also induced a disruption of the starch polysaccharide chains.
580 This fragmentation could result in both changes in the organization of starch chains and
581 solubility (Fiedorowicz and Para, 2006).

582 On the other hand, the addition of LAE involved a significant increase ($p<0.05$) in
583 solubility, while significantly reducing the water uptake values for both oxidized
584 formulations, contrary to what occurred for non-oxidized films. The higher solubility
585 values of oxidized films with LAE could be explained by its tighter attachment to the
586 carbonyl groups present in the low molecular weight molecules resulting from the partial
587 hydrolysis of starch, which were easily released into the water. Likewise, the reduction
588 in the water uptake capacity produced by LAE is coherent with the involvement of this
589 molecule in the crosslinking of the polymers, thus cooperating in the steric limitation of
590 water absorption in the network.

591 Table 2 summarizes the mean values of the overall migration of the films into food
592 stimulants with differing polarity: simulant A (ethanol 10% v/v, simulating food of
593 hydrophilic nature), simulant B (acetic acid 3% w/v, simulating food of hydrophilic nature
594 and pH below 4.5) and simulant D2 (isooctane, simulating food of lipophilic nature and
595 with free surface fats). All of the film formulations exceeded the threshold level of 10
596 mg/dm^2 established by the current law (Commission Regulation (EU) No 10/2011) for
597 every simulant, the highest values being obtained in the acid medium. However, the
598 oxidized formulation in 3 % acetic acid had significantly ($p<0.05$) lower migration values
599 than non-oxidized formulations. This suggests that crosslinking was effective at reducing
600 the disintegration action of the acid medium, thus limiting the release of film compounds.
601 On the contrary, the oxidized films showed a significantly ($p<0.05$) higher degree of
602 migration in ethanol 10 % (v/v) than the non-oxidised. This could be attributed to the
603 formation of low molecular compounds during the oxidation process, which is more easily
604 released into the aqueous media. No significant differences were noticed for the different
605 films' migration in isooctane. The addition of LAE only affected the migration values in
606 the films with non-oxidised starch, promoting their release in aqueous media at neutral

and acid pH. This agrees with the partial attachment of LAE to the polymer network in oxidised matrices, as previously commented on, thus restricting its migration observed in non-oxidised matrices.

Table 2. Overall migration values (mg dm^{-2}) of the different films into different food simulants (A: ethanol (10% v/v), B: acetic acid (3% w/v), D2: isooctane) and weight loss (WL%, g per100 g dried film) and water uptake (ΔW%, g water per 100 g dried film) of the films after 24 h of immersion in distilled water at 25 ºC. Mean values and standard deviation.

	Overall migration values			WL %	ΔW%
	Etanol 10% (v/v)	Acetic Acid 3% (w/v)	Isooctane		
OR_0	108 ±15$^{a, y}$	362 ±36$^{a, z}$	47 ±24$^{a, x}$	35 ±3$^{m, 1}$	1544 ±25$^{n, 1}$
OR_0LAE	348 ±52$^{c, y}$	622 ±148$^{b, z}$	126 ±99$^{a, x}$	37 ±4$^{m, 1}$	2015 ±40$^{n, 2}$
OR_0.5	243 ±30^{b,y}	236 ±21$^{a, y}$	51 ±65$^{a, x}$	40.4 ±0.4$^{n, 1}$	374 ±115$^{m, 2}$
OR_0.5LAE	260 ±18$^{b, y}$	277 ±16$^{a, y}$	29 ±5$^{a, x}$	46.6 ±0.8$^{n, 2}$	182 ±28$^{m, 1}$
OR_1	218 ±40$^{b, y}$	247 ±20$^{a, y}$	25 ±32$^{a, x}$	40.1 ±0.3$^{n, 1}$	367 ±78$^{m, 2}$
OR_1LAE	237 ±3$^{b, y}$	280 ±8$^{a, z}$	30,1 ±0,5$^{a, x}$	46.7 ±0.7$^{n, 2}$	133 ±20$^{m, 1}$

Different letters in the same column indicate statistical differences (p<0.05) between the formulations for the same simulant (a, b, c) or between samples with the differing degree of starch oxidation (m, n).

Different letters (x, y, z)in the same column indicate statistical differences (p<0.05) between simulants for each formulation.

Different numbers (1, 2) in the same column indicate significant differences (p<0.05) between samples, with and without LAE

Starch oxidation and the subsequent crosslinking to gelatin greatly limit the water uptake capacity of the films, although they did not reduce either the film's water solubility or the migration values into neutral aqueous food simulants. Nevertheless, crosslinked films were more resistant to the acid systems, coherent with the formation of a tight network. The partial disruption of starch chains by oxidation led to an increase in the soluble fraction of the films. In the same sense, the addition of a low molecular weight compound (LAE) promoted the film's solubility in water and its migration into aqueous food simulants.

3.4 Effect of starch oxidation on the stability of the film properties.

For the purposes of evaluating the potential progress of both Maillard and crosslinking reactions in the film network throughout time, the colour parameters, tensile properties and water vapour barrier capacity of the films were analysed after 5 storage weeks at 53 % RH and 25 ºC.

Figure 4 compares the values of the tensile properties of the films (EM, TS and % E) and WVP obtained after 1 and 5 weeks of storage. The results obtained pointed to a progression of the crosslinking process throughout storage time in oxidised formulations, since the two films with oxidised starch, both the one with and the one without LAE, exhibited a remarkable increase ($p<0.05$) in EM and a decrease in the film extensibility. Likewise, a slight increase in WVP was observed for these samples when there was no LAE in the formulation.

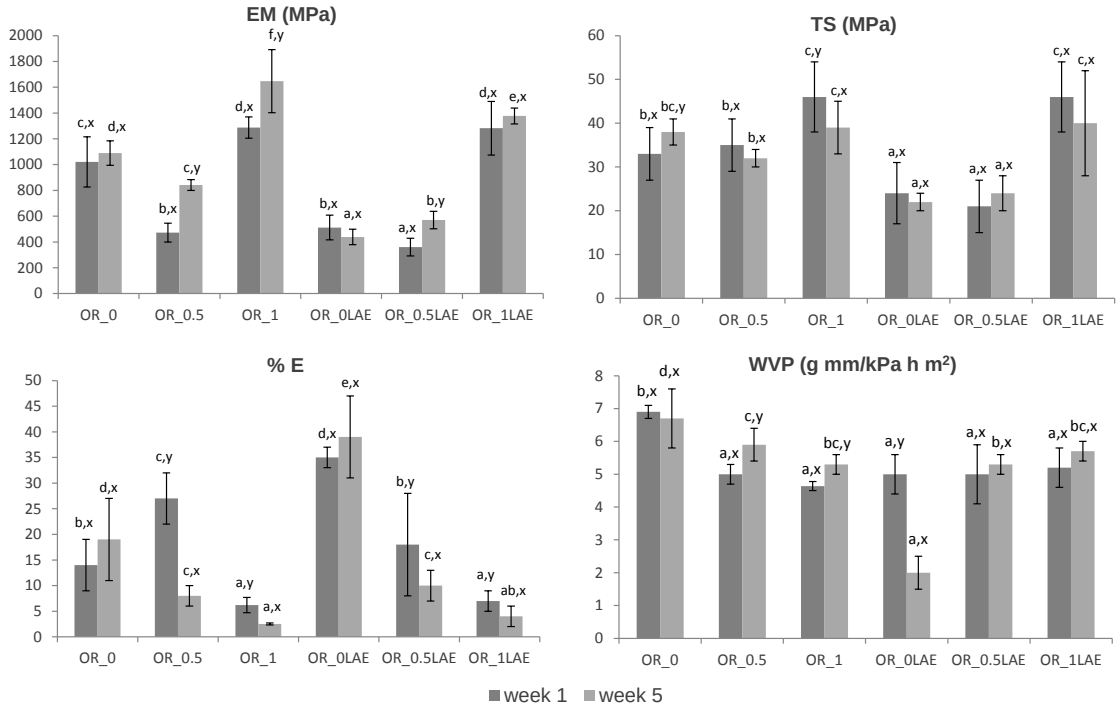
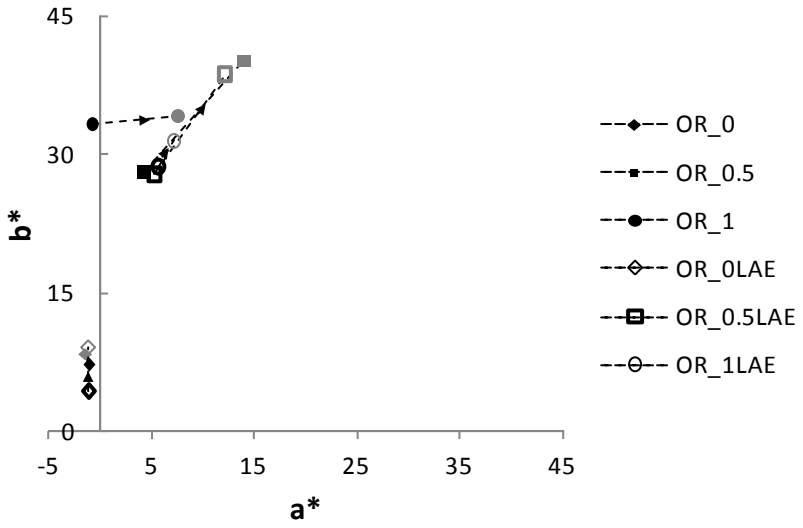


Figure 4. Effect of storage time (1 and 5 weeks) on the tensile parameters (EM, TS and % E) and Water Vapour Permeability (WVP) of the starch-gelatin blend films, with differing degrees of starch oxidation, with or without LAE films, conditioned at 53 % RH. Mean values and standard deviation. Different letters (a, b, c, d, e, f) indicate significant differences ($p<0.05$) between the different film formulations for the same conditioning time. Different letters (x, y) indicate significant differences ($p<0.05$) between both conditioning times for the same formulation.

On the other hand, the colour of the films also changed during storage. Figure 5 shows

655 the *locust* of the different films in the a*b*chromatic diagram after 1 and 5 weeks of
656 storage; here, although no notable changes can be appreciated for the films with non-
657 oxidised starch, either with or without LAE, those films containing oxidized starch evolved
658 towards a redder hue with higher colour saturation. These chromatic changes were
659 associated with a 16% reduction in lightness for the films with lower starch oxidation,
660 both with and without LAE, a 9 % reduction for the LAE-free sample with the most
661 oxidised starch and a 10% increase in the case of non-oxidized starch with LAE. These
662 colour changes also indicate the progression of the formation of conjugated Schiff's
663 bases (intermediate products of the Maillard reaction) causing an increase in colour
664 intensity (Martucci and Ruseckaite, 2009) throughout storage. Browning reactions
665 progressed to a greater extent in the films with oxidized starch, thus indicating the
666 progressive reaction between carbonyls and amino groups. The films with lower
667 oxidation level exhibited the greatest changes, both with and without LAE, whereas in
668 the case of those with the highest oxidation ratio, the browning of the films with LAE was
669 more limited. However, the colour of the LAE-free films with the highest oxidation degree,
670 which exhibited less initial browning, changed until they reached similar browning levels
671 to the OR_1LAE sample.



672

673 **Figure 5**. Colour development in the a*b* chromatic diagram of the starch-gelatin films
674 conditioned at 53 % RH and 25ºC. (1 week: black marks, and 5 weeks: grey marks)

675

676

677 The browning reactions progressed in oxidized starch films, mainly in those with lower

oxidation degree, giving rise to more coloured films, which can limit their application. Nevertheless, these Maillard compounds have antimicrobial activity (Hauser et al., 2014; Wu et al., 2014), which could improve the films' functionality, imparting active properties.

3.5 Antimicrobial activity of the films

Table 3 summarizes the results of the *in vitro* antimicrobial test carried out for the different films, with and without LAE. The bacterial counts after 24 h of incubation, as well as the growth inhibition with respect to the inoculum control of each bacteria are shown. A total growth inhibition was observed for all films containing LAE, thus indicating the high antimicrobial effectiveness of this compound released from the films. Likewise, LAE-free films with oxidized starch also significantly ($p<0.05$) inhibited the growth of both bacteria, although to a lesser extent than the films containing LAE. This mild antimicrobial activity can be attributed to the formed brown compounds (Maillard reaction) and their release from the films. The antimicrobial activity of Maillard compounds has been associated with the generation of hydrogen peroxide (Hauser et al., 2014). OS:BG_1 films (with highest starch oxidation degree) provoked the highest ($p<0.05$) growth inhibition of both bacteria, in agreement with the higher production of Maillard compounds, as deduced from colour parameters (Table 1). The Gram positive bacterium (*L. innocua*) was significantly more ($p<0.05$) sensitive to the antimicrobial action of brown compounds present in the films.

Table 3. Effect of the films on the growth and survival of *Listeria innocua* (CECT 910) and *Escherichia coli* (CECT 101) after 24 h at 37 ºC. Average values and standard deviation.

	Bacterial growth (log CFU/mL)		Growth inhibition (log CFU/mL)	
	E.coli	***L.innocua***	***E.coli***	***L.innocua***
Control	8.9 ±0.08$^{c, 1}$	9.21 ±0.05$^{c, 2}$	-	-
OS:BG_0	9.13 ±0.06$^{d, 1}$	9.15 ±0.14$^{c, 1}$	-0.21 ±0.06$^{a, 1}$	0.05 ±0.14$^{a, 2}$
OS:BG_0LAE	NCD*	NCD*	TOTAL	TOTAL
OS:BG_0.5	8.34 ±0.14$^{b, 1}$	8.0 ±0.5$^{b, 1}$	0.58 ±0.14$^{b, 1}$	1.2 ±0.5$^{b, 2}$
OS:BG_0.5LAE	NCD*	NCD*	TOTAL	TOTAL
OS:BG_1	8.07 ±0.013$^{a, 2}$	7.36 ±0.08$^{a, 1}$	0.841 ±0.013$^{c, 1}$	1.85 ±0.08$^{b, 2}$
OS:BG_1LAE	NCD*	NCD*	TOTAL	TOTAL

**No Cells Detected*

Different letters (a, b) in the same column indicate statistical differences (p<0.05) between formulations for each bacterium.

Different numbers (1, 2) in the same row indicate statistical differences (p<0.05) between bacteria for each formulation.

Conclusion

Starch oxidation with sodium periodate greatly promoted crosslinking in starch-gelatin (1:1) films, enhancing the films' strength and oxygen and water vapour barrier capacity, while reducing their water uptake capacity and migration to acid media. A glucose:periodate molar ratio of 1:1 was more effective than 1:0.5, due to the greater progress of di-aldehyde formation, which offers more reactive groups for the amino-carbonyl reaction and the formation of inter-chain bonds between polysaccharides and proteins. Incorporating LAE as an antimicrobial compound interferes with the polymer crosslinking reactions due to its bifunctional nature (it contains carbonyl and amino groups which also react with the polymer chains), especially when there was a lower degree of starch oxidation because of the lower ratio of oxidant. This affected the tensile behaviour of the films, which were less stiff and stretchable. Nevertheless, an amino-carbonyl condensation reaction also provoked browning in the films, in line with the formation of Maillard compounds. This browning, as well as crosslinking, progressed in the film network throughout storage, especially in the less oxidized starch films, which could compromise their application for certain uses. The formation of Maillard compounds conferred antibacterial activity to the films, while the incorporation of LAE produced a total bactericidal effect. Based on the lower browning progression, reduced water sensitivity, better mechanical performance and the higher antimicrobial activity, blend films with the most oxidized starch and gelatin containing LAE are promising materials for food packaging applications. In order to further improve the film performance, future studies should address film application on real food systems, aiming to quantify the real effect of both Maillard compounds and LAE on the product microbial quality and shelf life.

Acknowledgements

The authors acknowledge the financial support provided by Ministerio de Economía y Competitividad (Projects AGL2013-42989-R and AGL2016-76699-R). The authors also thank Oscar Gil and Amparo Ribes from the Institute of Materials Technology (UPV) for the support and assistance in the use of the infrared spectrometer and the services rendered by the Electron Microscopy Service of the UPV. Olga Moreno Marro also thanks the Ministerio de Educación, Cultura y Deporte for the FPU 2012-1121 grant.

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