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Influence of Maillard reaction and temperature on functional, structure and bioactive properties of fish gelatin films

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Abstract

This study aims to assess the effect of heating temperatures on structure, physicochemical and antioxidant properties of fish gelatin films crosslinked by the temperature and/or with glucose by the Maillard reaction.Glucose was incorporated at a 0.5 glucose/lysine molar ratio into the fish gelatin film forming solutions. The products of the Maillard reaction were generated by heating the films during 24 h in a temperature range of 90-130 °C. An enhancement of some films properties was obtained after the induction of the Maillard reaction. Indeed, the water solubility and wettability were reduced. On the contrary, a rise of color intensity, UV-barrier property, radical scavenging activities and iron reducing effects was observed. These enhancements occurred also without glucose addition thanks to the heating treatment, but to a lesser extend. In addition, an increase of thermal stability due to structural changes was revealed by means of XRD, TGA and DSC analyses. As evidenced by the different experiments used for the determination of the antioxidant activity (DPPH• and ABTS free radicals scavenging activity, reducing power and β-carotene bleaching inhibition) the antioxidant activity of films containing glucose displayed an optimum after being treated at 90 °C. On the contrary, it decreased or kept constant at higher temperatures, probably due to the degradation or further stages of Maillard reactions. Moreover, the thermal treatment alone (T ≥ 100 °C) can be a useful tool for enhancing the antioxidant activity of gelatin films. Finally, the Maillard reaction has a good potential to induce and generate bioactive compounds in gelatin films for food protection.

Keywords: fish gelatin; thermal treatment; Maillard reaction; crosslinking; physicochemical, thermal and structural properties; antioxidant activity.

1. Introduction

Nowadays, biodegradable films show a high interest in research and industrial fields as an alternative to synthetic packaging based on petrochemical sources, which are considered as a major cause of environmental pollution. Thus, different polymers have been used for film preparation such as proteins and polysaccharides. Amongst biodegradable films, protein-based-films have been widely studied for their physico-chemical and structural properties. Gelatin is a protein derived from collagen by hydrolysis, the primary protein component of animal connective tissues, which includes skin and tendon (Poppe, 1997). Gelatin is widely used by various industries because of its functional and technological properties. Fish gelatins have been equally extensively studied for their good film forming ability leading to produce transparent, colorless, water-soluble, highly stretchable and biodegradable films (Alfaro, Balbinot, Weber, Tonial, & Machado-Lunkes, 2015). However, due to their high hydrophilic amino acid content, films based on gelatin showed high brittleness and moisture sensitivity, which limit their applications (Etxabide, Urdanpilleta, de la Caba, & Guerrero, 2016; Liu et al., 2016). Therefore, the heating process could be a promising alternative to overcome the problems associated with the hydrophilic character of hydrocolloid-based films (Rivero, García& Pinotti, 2012). Heating proteins in the presence of a reducing sugar is a well-known method of crosslinking based on the Maillard reaction.

The Maillard reaction (MR), or non-enzymatic browning reaction, refers to the complex reactions between carbonyl-containing compound (such as reducing sugar) and amino-containing compound (such as amino acid, peptide or protein). It produces a complex array of compounds referred to as Maillard reaction products (MRPs) (Amarowicz, 2009). The rate of Maillard reaction (MR) is influenced by many factors, such as temperature, water activity (a_w), pH, reactant source, concentration, type and ratio of reducing sugar to lysine, and time of exposure (Labuza & Baisier, 1992). The rate of deteriorative reactions and storage stability

71 are particularly linked to water activity of the non enzymatic browning reaction occurs at a
72 maximal rate in the a_w range of 0.5–0.75 according to Kaanane & Labuza (1989) and Labuza
73 & Saltmarch (1981). Among influencing parameters, temperature is considered as the main
74 factor favoring the MR rate and strongly affecting the MRPs properties (Karseno, Yanto,
75 Setyowati, & Haryanti, 2018; Zhang et al., 2018).
76 Furthermore, besides heating temperature, Jiang, Wang, Che, & Tian (2014) indicated that pH
77 is an influencing factor, which modulates the MR rate, as they remarkably influence the
78 biological activities and the characteristics of the MRPs. These include volatile compounds of
79 low molecular mass, non-volatile colored compounds of intermediate molecular mass and
80 brown melanoidins of high molecular weight exhibiting antioxidant properties (Loucif,
81 Chetouani, Bounekhel & Elkolli,2017; Lan et al., 2010). Previous studies demonstrated that
82 MRPs might act as radical scavengers (Vhangani & Van Wyk, 2013), reducing agents
83 (Loucif, Chetouani, Bounekhel & Elkolli, 2017) and metal chelators (Maillard, Billaud,
84 Chow, Ordonaud, & Nicolas, 2007), based on their hydrogen atom and electron transfer
85 capacity.
86 Recently, numerous scientific publications studied the effect of heating temperature on the
87 functional properties of MRPs generated from coconut sap (Karseno, Yanto, Setyowati, &
88 Haryanti, 2018), xylose and chicken peptide (Liu, Liu, He, Song, & Chen, 2015), galactose-
89 bovine casein peptide (Jiang, Wang, Che & Tian, 2014), fructose–lysine and ribose–lysine
90 model systems (Vhangani & Van Wyk, 2013) or silver carp protein hydrolysate–glucose
91 system (You, Luo, Shen, & Song, 2011).
92 Additionally, heat-treatment of protein films and coatings or film forming protein solutions
93 had a noticeable effect on film properties (Caoa, Fua, & Hea, 2007; Kim, Weller, Hanna, &
94 Gennadios, 2002; Gennadios, Ghorpade,Weller, & Hanna, 1996). In this context, several
95 studies showed that heat treatment improved the moisture barrier properties and the

mechanical toughness of cast films. It was observed for films made with soy protein (Gennadios, Ghorpade,Weller, & Hanna,1996; Kim, Weller, Hanna, & Gennadios, 2002; Rhim, Gennadios, Handa, Weller, & Hanna, 2000), whey protein (Miller, Chiang, & Krochta, 1997), collagen (Weadock, Olson, & Silver, 1984), zein, gelatin and fibrin proteins (Julius, 1967). However, there is not enough information about the effect of heat treatment on the antioxidant activity of protein-based films.

In a previous study, we prepared a series of glucose-fish gelatin films and we promoted the MR by heating the films at 90 °C for 24 h. Resulted glucose-gelatin films displayed improved barrier, thermal and mechanical properties. In addition, these films exhibited an important antioxidant activity that was glucose/gelatin concentration and MR time dependent (Kchaou, et al., 2018). The present research aims to evaluate the effect of thermal treatments at different temperatures in the range of 90 to130 °C and the the impact of the Maillard reaction on the structure, and the functional and bioactive properties of gelatin based films with glucose or glucose free.

2. Materials and methods

2.1. Materials

Commercial fish gelatin type A (Rousselot 200 FG, 200 degree bloom, 4 mPa.s viscosity at 45 °C for a concentration of 6.67% in water at pH = 5.4, water content of 11.77 $g_{water}/100g_{dry\ matter}$, ash content 0.10 $g_{ash}/100\ g_{dry\ matter}$) was employed as filmogenic biopolymer for films preparation. Anhydrous glycerol was purchased from Fluka (98% purity, Fluka Chemical, Germany) and was used as plasticizer for the films. D(+) anhydrous-glucose ($C_6H_{12}O_6$; 180 $g.mol^{-1}$) was used as reducing sugar to initiate the Maillard reaction in fish gelatin based films. All other reagents were of analytic grade.

2.2. Film preparation

A mother film-forming solution was prepared by dissolving fish gelatin 4% (w/v) in distilled water at 60 °C and was stirred for 30 min. The solution's pH was set fixed at 5.5 and was controlled during the following steps of film forming. Then, three different films were prepared from a mother film-forming solution. First of all, unplasticized gelatin film named as (G) film was obtained by casting a volume of 25 ml of mother film forming solution in Petri dishes, drying in a ventilated climatic chamber (KBF 240 Binder, ODIL, France) at 25 °C and 50% relative humidity (RH) for 24 h. Then, films were peeled from the surface. A plasticized gelatin film named (GP) film was prepared by adding glycerol to the mother film-forming solution at a concentration of 15% (w/w dry gelatin matter) and was stirred for 30 min. After, the same volume of the plasticized solution was poured and dried in the same conditions stated previously. Finally, plasticized glucose containing film, referred as (GP-glu) film was prepared to favour Maillard reactions development. For this, glucose was added to the plasticized film-forming solution at a 0.5/1.0 glucose/lysine molar ratio according to a previous study (Kchaou, et al., 2018). The same volume of the plasticized solution was poured and dried in same conditions described previously.

All prepared films were then equilibrated at 25 °C and 50% RH before the heating treatment and before all the analyses. Except for FTIR, XRD, TGA and DSC measurements, films were equilibrated at 0% RH.

2.3. Heating treatments of films

After peeling and equilibration, half of all the films (with or without glucose, plasticized and unplasticized) were heated in an oven at 90, 100, 110, 120 and 130 °C for 24 h to induce Maillard reactions. Non heated films will be considered as blank and named as NH-G (non heated, unplastized films), NH-GP (non heated glycerol plasticized films) and NH-GP-glu (non heated glucose-glycerol plasticized films).

As the Maillard reaction is water activity dependent, the water activity of the sample prior heating was 0.5, corresponding to a water content lower than 7%. During the heating treatment, the water activity of films is rapidly equilibrated with the relative humidity in the oven, which ranges from 0.04 down to <0.01 when temperature rises from 90 to 130 °C (calculated from the room relative humidity introduced in the oven and the temperature in oven according the moist air thermodynamics (Mollier's diagram). Thus, the range of aw used is much below that of the maximum rate (Kaanane & Labuza, 1989; Labuza & Saltmarch, 1981).

2.4. Film thickness

Film thickness was measured using a digital thickness gauge (PosiTector 6000, DeFelsko Corporation, USA) with a one µm accuracy. For each film sample, five measurements at different positions were done.

2.5. Determination of the relative degree of crosslinking

The relative degree of crosslinking was adapted from the method of Bubnis & Ofner (1992) and Prasertsung, Mongkolnavin, Kanokpanont & Damrongsakkul (2010). The concept of this method was to react free amino groups of gelatin, which indicate non-crosslinked groups, with 2,4,6-trinitrobenzene sulfonic acid (TNBS). This method only allows to measure the increase or decrease of the NH_2 groups related to an initial (un-heated films) NH_2 content. A negative relative crosslinking degree results from a hydrolysis of the peptide bonds within the protein as there are more terminal NH_2 groups. Approximately 10 mg of each gelatin film were weighed and placed into a test tube. Then, 1 mL of 0.01% TNBS solution and 1 mL of 4% sodium hydrogen carbonate solution ($NaHCO_3$, pH 8.5) were added. The reaction mixture was then heated at 40 °C for 2 h. At this step, the non-crosslinked primary amino groups of

gelatin react with TNBS and form a yellow soluble complex. This solution was further treated with 2 mL of 6 N HCl at 60 °C for 1.5 h in order to hydrolyze and dissolve any insoluble material. The absorbance of the solutions was determined spectrophotometrically at 415 nm. The measured absorbance of a sample was corrected with that of a blank tube prepared in the same manner except the reacted reagent (TNBS). The relative degree of crosslinking was then obtained from the difference between the absorbance values before and after crosslinking using the following equation (1):

$$Relative\ degree\ of\ crosslinking\ (\%) = \left(1 - \frac{\text{absorbance of crosslinked films}}{\text{absorbance of control films non heated}}\right) \text{x } 100 \qquad (1)$$

Where NH-G film at 25 °C is the control film for G heated films, NH-GP film at 25 °C is the control film for GP heated films and NH-GP-glu film at 25 °C is the control one for GP-glu heated films.

2.6. Spectroscopic analysis

FTIR spectra of film samples were recorded with a Perkin-Elmer spectrometer (Spectrum 65, France) equipped with an attenuated total reflectance (ATR) accessory with a ZnSe crystal. 32 scans were collected at a resolution of 2 cm^{-1} in the wavelength range of 600-4000 cm^{-1}. Calibration was performed using background spectrum recorded from the clean and empty cell at 25 °C. The Spectrum Suite ES software (Perkin Elmer) was used for FTIR data treatment.

The films were cut into rectangles (1 cm x 3 cm) and directly placed in the test cell of a UV-Visible spectrophotometer (SAFAS UVmc). Light transmission of the films was determined in the wavelength range from 200 to 800 nm according to the method used by Benbettaïeb, Karbowiak, Bornaz & Debeaufort (2015). An empty test cell was used as a reference.

192 *2.7. Color properties*

193 A CIE colorimeter (CR-200; Minolta, Japan) was used in order to assess the color changes in
194 gelatin films as a function of heating temperature. A white standard color plate (L_0 = 97.5, a_0
195 = -0.1, and b_0 = 2.3) was used as background for the color measurements of the films. Color
196 of films was expressed as L (lightness/brightness), a (redness/greenness) and b
197 (yellowness/blueness) values. The difference in color (ΔE) for GP-glu and GP films as a
198 function of heating temperature was determined as follows:

199 $$\Delta E = \sqrt{(L - L_C)^2 + (a - a_c)^2 + (b - b_c)^2} (2)$$

200 L, a and b are the color parameters of the heated (GP and GP-glu) films; L_c, a_c and b_c are the
201 color parameters of their respective controls (non heated without glucose (NH-GP) and with
202 glucose (NH-GP-glu) films). Three measurements were carried out for each sample and their
203 average was obtained.

204 The obtained CIE Lab values were then used to calculate the browning index (BI) as
205 mentioned in equation (3):

206 $$BI = \frac{100(z - 0.31)}{0.172} \quad with \quad z = \frac{a + 1.75(L)}{5.645\ (L) + \ a - 3.012(b)} \qquad (3)$$

207 *2.8. Thermal properties*

208 A thermogravimetric analysis (TGA) was carried out to determine the thermal stability of the
209 film samples. This technique is based on the continuous weighing of the film as a function of
210 the temperature rise in a controlled atmosphere (nitrogen). A TGA instrument (SDT Q 600)
211 was used in order to assess the thermogravimetric measurements by heating the samples from
212 25 to 600 °C at a heating rate of 5 °C/min under nitrogen atmosphere.

213 Thermal properties of films were investigated using a Differential Scanning Calorimeter
214 (Mettler Instruments). About 5 mg of each film, initially weighed in sealed aluminium pans,
215 were subjected to a double heating cooling cycle from -50 °C to 150 °C at a rate of 10
216 °C/min, under nitrogen flow rate of 25 mL/min. Glass transition temperature (Tg) for each
217 sample was then determined from the mid-point of the second heating cycle using STAR^e
218 SW13.00 software (Mettler-Toledo DSC1).The Cp variation (ΔC_P) was also determined as an
219 indicator of the film crystallinity variation after the heating treatment. Films were previously
220 equilibrated at 25 °C and 0% RH during two weeks before each measurement to obtain the
221 most dehydrated films for both of TGA and DSC analyses. Only two films were triplicated in
222 order to determine the relative error measurement and thus a confidence interval of 10% was
223 chosen for statistical analysis.

224 *2.9. X-ray diffraction (XRD) analysis*

225 X-ray diffraction analysis of the selected films was performed using a diffractometer (D5000,
226 Bruker) equipped with monochromatic Cu-Kα radiation (I = 1.5418 A) operating at a voltage
227 of 40 kV and a current of 40 mA. All samples were analyzed in continuous scan mode with
228 the 2θ ranging from 3° to 50° where θ is the incidence angle of the X-ray beam on the sample.

229 *2.10. Water content (WC) and water solubility (WS)*

230 After being equilibrated at 50% and 25 °C, the water content (g $_{moisture}$/100 g $_{film}$) of gelatin
231 films was determined by measuring the weight loss of samples (100 mg) after drying in an
232 oven at 105 °C until constant weight was reached (dry sample weight) according to the
233 following equation:

234 $$WC = \frac{(m_0 - m'_f)}{m_0} \times 100 \qquad (4)$$

where m_0 is the initial film weight (g) and m_f' is the final film dry weight (g). Three samples of each formulationwere analyzed.

The solubility of film samples in water was determined according to the Gennadios, Handa, Froning, Weller, & Hanna (1998) method. The film samples (2 cm x 5 cm) previously equilibrated were weighed and transferred to centrifuge tube containing 30 mL of distilled water with 0.1% (w/v) sodium azide as antimicrobial agent. The samples were then shaken at a speed of 250 rpm for 24 h at 25 °C. After centrifugation (GYROZEN centrifuge, 1580R) at 8000 rpm for 10 min, samples were dried at 105 °C for 24 h and then were weighed to determine the remaining pieces of films. Water solubility (WS) was calculated as follows:

$$WS(\%) = \frac{\left[\left(m_0\, x\, (100 - WC)\right) - m''_f\right]}{\left(m_0\, x\, (100 - WC)\right)} x\ 100 \qquad (5)$$

where m_0 : film initial weight (g) and $m"_f$: final dry weight of non-solubilised film (g). All tests were carried out in triplicate.

2.11. Water contact angle

The contact angle measurements were carried out using the sessile drop method on a goniometer (Drop Shape Analyzer 30 from KrussGmbH), equipped with an image analysis software (ADVANCE). First, a droplet of water (~2 µL) was deposited on the film surface with a precision syringe. The method is based on image processing and curve fitting for contact angle measurement from a theoretical meridian drop profile, determining contact angle between the baseline of the water drop and the tangent at the drop boundary. Then, the contact angle was measured on both sides of the drop and averaged. Five measurements per

film were carried out. All the tests were conducted in an environmental chamber with a constant environment at a temperature of 25±2 °C and a relative humidity of 50±1%.

2.12. In-vitro antioxidant activity

2.12.1. Free radical-scavenging activity (FRSA)

Two methods for assessing the free radical scavenging were used.

The antioxidant activity of films was first measured using the method of DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging as described by Jridi et al. (2014), adapted to edible films. The films were cut into small pieces (m = 10 mg) and immersed in 500 µL of distilled water, 375 µL of 99.5% ethanol and 125 µL of 0.02% DPPH• in ethanol 99%. The mixtures were then incubated for 24 h at room temperature in the dark. The reduction of DPPH• radical was measured at 517 nm, using a UV–visible spectrophotometer. The DPPH• RSA was calculated as follows (equation 6):

$$DPPH\ radical\ scavenging\ activity(\%) = \frac{A_C - (A_S - A_b)}{A_C}\ x\ 100 \qquad (6)$$

Where A_C is the absorbance of DPPH• solution without addition of the films, A_S is the absorbance of DPPH• solution containing the film samples and $A_b$is the absorbance of blank tubes containing film samples without addition of the DPPH• solution.

A lower absorbance of the reaction mixture, caused by the change of solution color from purple to yellow, indicated a higher radical-scavenging activity. The test was carried out in triplicate.

Antioxidant activity of films was also determined by the ABTS method as described by Re et al. (1999). The $ABTS^+$ radical was generated by mixing of 7 mM $ABTS^+$ stock solution and 2.45 mM potassium persulphate ($K_2S_8O_2$). Then, the mixture was placed under dark

conditions for 12-16 h before use. The $ABTS^+$ solution (stable for 2 days) was diluted with 99.5% ethanol to adjust the absorbance to 0.70 ± 0.05 at 734 nm. The films were cut into small pieces (m = 10 mg) and immersed in 100 µL of distilled water and 900 µL of diluted $ABTS^+$ solution. The solution with samples was then incubated at 37 °C shielded from the light for 10 min, and a solution without samples under the same conditions was recorded as control. The absorbance was determined at 734 nm. The $ABTS^+$ radical scavenging activity could be expressed as follows:

$$ABTS^+ radical\ scavenging\ activity\ (\%) = \frac{A'_C - (A'_S - A'_b)}{A'_C} \times 100 \qquad (7)$$

Where A'_C was the absorbance of the control $ABTS^+$ solution, A'_S was the absorbance of sample with $ABTS^+$ solution and A'_b was the absorbance of blank tubes containing film sample without the addition of $ABTS^+$ solution. The experiments were carried out in triplicate.

2.12.2. Reducing power

The ability of films to reduce iron (III) was determined according to the method of Yıldırım, Mavi & Kara (2001). Films were cut into small pieces (m = 10 mg) and immersed in 0.5 mL of distilled water, 1.25 mL of 0.2 M phosphate buffer (pH 6.6) and 1.25 mL of 1% (w/v) potassium ferricyanide. After incubation for 3 h at 50 °C, a volume of 500 µL of 10% (w/v) trichloroacetic acid was added to the mixture which was centrifuged for 10 min at 10,000 x *g*. Then, 1.25 mL of the supernatant solution of each sample mixture was mixed with 1.25 mL of distilled water and 0.25 mL of 0.1% (w/v) ferric chloride. After a reaction time of 10 min, the absorbance of the resulting solutions was measured at 700 nm. Higher absorbance of the reaction mixture indicated higher reducing power. The values are presented as the means of triplicate analyses.

304 *2.12.3. β-carotene-linoleate bleaching assay*

305 The ability of films to prevent β-carotene bleaching was determined according to the method
306 of Koleva, Van Beek, Linssen, de Groot, & Evstatieva (2002). 0.5 mg β-carotene in 1 mL
307 chloroform was mixed with 25 µL of linoleic acid and 200 µL of Tween-80. The chloroform
308 was completely evaporated under vacuum in a rotator evaporator at 40 °C, then 100 mL of
309 double distilled water were added, and the resulting mixture was vigorously stirred. The
310 emulsion obtained was freshly prepared before each experiment. Aliquots (2.5 mL) of the β-
311 carotene-linoleic acid emulsion were transferred to test tubes containing 10 mg of each film
312 and 0.5 mL of distilled water. The tubes were immediately placed in water bath and incubated
313 at 50 °C for 2 h. Thereafter, the absorbance of each sample was measured at 470 nm. Control
314 tube was prepared in the same conditions without film. The antioxidant activity was evaluated
315 in terms of β-carotene bleaching inhibition using the following formula:

316 $$\beta - carotene\ bleaching\ inhibition\ (\%) = \left(1 - \left(\frac{A^{0}_{sample} - A^{120}_{sample}}{A^{0}_{control} - A^{120}_{control}}\right)\right) x100 \quad (8)$$

317 *where A^0: absorbance at t=0 min, A^{120}: absorbance at t=120 min.*

318 *2.12.4. Inhibition of linoleic acid oxidation*

319 The lipid peroxidation inhibition activity of prepared films was measured in a linoleic acid
320 emulsion system according to the method of Osawa & Namiki (1985). Film samples (10 mg)
321 were added to 2.5 mL of phosphate buffer (50 mM; pH 7.0), 2.5 mL of absolute ethanol and
322 0.0325 mL of linoleic acid. The final volume was then been adjusted to 6.25 mL with distilled
323 water. The reaction mixture was incubated in glass test tubes with aluminum screw caps at 45
324 °C for 10 days in a dark room. A tube without sample addition was used as negative control.
325 The degree of linoleic acid oxidation during time storage was measured by the evaluation of

326 the thiobarbituric acid reactive substances (TBARS) formation, including malondialdehyde
327 (MDA). TBARS were assayed, every three days, by the method described by Yagi (1976).
328 MDA and TBARS were measured by their reactivity with TBA in an acidic condition to
329 generate pink colored chromospheres, which absorb at 530 nm. The capacity of the TBARS
330 formation inhibition in linoleic acid system was determined as follows:

331 $$Lipid\ peroxidation\ inhibition\ capacity\ (\%) = 1 - \left(\frac{A_{Sample}}{A_{Control}}\right) x\ 100 \qquad (9)$$

332 Where A_{sample} is the absorbance value of the film sample and $A_{control}$ is that of the pure linoleic
333 acid used as the standard.

334 *2.13. Activation energy*

335 The Arrhenius model was used to calculate the activation energy (Ea) for ΔE, BI,
336 A_{294nm}, A_{420nm}, water solubility, relative crosslinking degree, free radicals (DPPH• and
337 $ABTS^{+}$) scavenging activity, β-carotene-linoleate bleaching assay as well as reducing power
338 parameters. The Ea was determined from the value at equilibrium, and not from the kinetic
339 rates on each parameter. Ea allows to determine the dependence of the MR on the heating
340 temperature on all the studied parameters. The activation energy was deduced from the
341 following mathematical Arrhenius-equation established by Vant'Hoff in1884, which was well
342 described in the literature (Labuza, 1984):

343 $$Ln\ (x) = f\left(\frac{1}{RT}\right) \qquad (10)$$

344 where Ln (x) corresponds to the natural logarithmic (Ln) of the studied variable (x), R is the
345 universal gas constant (8.314 J/mol K) and T is the absolute temperature (K). Then, Ea was
346 obtained by calculating the slope of the linear part of the plot of the logarithm of (*x) vs* (1/
347 RT). When only a part of the plot displayed a significant linear behavior, the Ea was
348 calculated on a limited range of temperature and was specified. Furthermore, the linear

regressions (R^2) of the corrected experimental fitting points of the Arrhenius plots were determined.

2.14. Statistical analysis

Statistical analyses were performed with SPSS ver. 17.0, professional edition using ANOVA analysis at a (p-value<0.05). Duncan's multiple-range test (p-value<0.05) was used to detect differences among mean values of all the parameters analyzed for the different films. A standard deviation at the 90% confidence level was used to compare the DSC data for the different films.

3. Results and discussion

3.1. Confirmation of the crosslinking in fish gelatin films induced by temperature and/or Maillard reaction

The relative degree of crosslinking, FTIR, color change and UV absorption were techniques selected to assess the efficacy of the thermal treatment and Maillard reaction to induce crosslinking but also to generate new compounds able to have positive effect on the functional properties of films.

3.1.1. The relative crosslinking degree

The relative crosslinking of films heated at different temperatures has been evaluated by measuring the loss of free amino groups using the TNBS method. Even if the TNBS method is an indirect method of assessing the crosslinking, it allowed to display the change in the number of free amine groups, and thus the indirect evaluation of the amine function engaged in crosslinking. Crosslinking degree is a relative value regarding the number of free amino groups at 25 °C, *ie* determined on non-heated films. The negative value of the relative crosslinking degree of the unplasticized glucose-free gelatin (G) films is explained by the

372 hydrolysis of the gelatin chains with high temperature, which is mainly involved for
373 temperatures higher than 100 °C (Fig. 1).

374 For GP-glu films, the relative crosslinking degree increased significantly from 33.6%
375 to 55.5% with increasing the temperature from 90 to 130 °C, respectively. This confirms the
376 consumption of a fraction of the free amino acids during the protein-glucose conjugation.
377 Results showed that the variation of relative crosslinking degree follows a linear trend as a
378 function of heating temperature. Nevertheless, only 55.5% of crosslinking has been revealed
379 at the highest temperature indicating that 44.5% of the amino groups were still in the free
380 form and available for further reactions, linked to the free amino groups available at 25 °C
381 (non-heated films). You, Luo, Shen, & Song (2011) reported that the temperature plays an
382 important role on the ultimate content of free amino acids, which determines the formation
383 rate of MRPs. Loucif, Chetouani, Bounekhel & Elkolli (2017) stated that the crosslinking
384 degree increased to about 45%, 47% and 56% for alginate/gelatin crosslinked systems
385 prepared at pH 11 and heated at 70, 80 to 90 °C, respectively. Jiang, Wang, Che & Tian
386 (2014) displaid a continuous decrease of the free amino groups in galactose-bovine casein
387 peptide systems upon heating ranging from 70 to 120 °C which achieved 51.1% after heat
388 treatment at 120 °C. The increase in relative crosslinking values (%) is correlated with the
389 increase in the browning intensity values (Table 1) in gelatin-glucose films confirming that
390 the heating temperature range used was effective for promoting the MR. For glucose-free
391 gelatin films, the relative crosslinking degree, caused only by the thermal treatment, was
392 13.6%, 17.2%, 25.5%, 26.3% and 28.5% for 90, 100,110, 120 and 130 °C, respectively. Such
393 findings showed that the thermal treatment in absence of glucose facilitate also possible
394 reactions/interactions between amino acid residues of gelatin, including inter-protein and
395 intra-protein crosslinking. Glycerol is present in the glucose-free gelatin films and can
396 promote MR as it plays as a precursor. This was demonstrated by Smarrito-Menozzi,

Matthey-Dorret, Devaud-Goumoens & Viton (2013) and Cerny & Guntz-Dubini (2006). The browning and crosslinking that occurred in glucose-free gelatin films could then be explained by the glycerol effect.

The activation energies of crosslinking degree are respectively 23.3 and 16.1 kJ/mol for GP and GP-glu films. Glucose thus makes the crosslinking easier as it requires less energy. This is logical as crosslinking results of MR were enhanced by the glucose.

3.1.2. FTIR spectroscopy

The ATR-FTIR spectroscopy was used in this study in order to assess the chemical bond modifications following the establishment of interactions in glucose-free gelatin as well as in gelatin-glucose films after heating treatments. Results presented in Fig. 2 showed that similar spectra were obtained for both films. As it can be seen, gelatin films displayed three characteristic peaks located at 1642-1654 cm^{-1}, 1540-1551 cm^{-1} and 1239-1243 cm^{-1} related to C=C and C=O stretching of primary and secondary amine N-H band of amide-I, N-H binding of amide-II and assigned to aromatic primary amine and C-N and N-H stretch of amide-III or vibrations of CH_2 groups of glycine, respectively (Hoque, Benjakul, & Prodpran, 2011). Loucif, Chetouani, Bounekhel & Elkolli (2017) reported a similar spectrum for gelatin films where the amide-I, amide-II and amide-III peaks were located at 1646, 1550 and 1237 cm^{-1}, respectively. Additionally, a peak situated around 1046 cm^{-1} is present in the different films spectra. This peak might be related to the possible interactions arising between the plasticizer (OH group of glycerol) and gelatin (Bergo & Sobral, 2007). Furthermore, small shifts in amide-I, amide-II and amide-III positions have been noted for GP-glu films when heating temperature increased up to 130 °C. In fact, the latter bands shifted from 1648 cm^{-1} to 1646 cm^{-1}, from 1551 cm^{-1} to 1545 cm^{-1} and from 1243 cm^{-1} to 1240 cm^{-1} for amide I, amide II and amide III peaks, respectively for 130 °C heated glucose-gelatin films. Etxabide, Urdanpilleta, Gómez-Arriaran, de la Caba, & Guerrero (2017) reported that the changes in the secondary

422 structure of fish gelatin-lactose films prepared at native pH (5.4) and heated at 105 °C for 24 h
423 were not so noticeable and the chain movement was restricted. In the present study, the 130
424 °C heated glucose-free gelatin films display amide I and amide II bands shifted from 1654
425 cm^{-1} to 1647 cm^{-1} and from 1551 cm^{-1} to 1546 cm^{-1}, respectively. However, no modification
426 in the amide III position has been revealed for control films. In the end, the FTIR only reveals
427 weak changes in the chemical structure of gelatin network, either because no new type of
428 bonds occurred, or new linkage are of similar nature as those already existing in the gelatin, or
429 new linkage are in too low proportion to be clearly displayed.

430 *3.1.3. Color of films*

431 Optical properties are essential to define the ability of films to be applied over a food
432 surface, due to their effect on the appearance of coated foods (Abdelhedi et al., 2018).
433 Changes in color of glucose-free gelatin and gelatin-glucose films as a function of heating
434 temperature were determined and shown in table 1. As can be seen, a considerable decrease in
435 L-values of gelatin-glucose films was observed with increasing temperatures up to 110 °C. As
436 the L-value is related to the lightness of a sample, it reflected the development of dark
437 products after 24 h of heating due to MR. In addition, heating gelatin-glucose films at high
438 temperatures led to increase significantly their dark-yellowish color as illustrated in Fig. 3 and
439 as proved by b-values measurements. Indeed, b-values first increased rapidly when
440 temperature rose from 90 to 110 °C, and then its rate increased slowly in the temperature
441 range between 110 and 130 °C.
442 However, the b-values of GP films showed a slight increase until 120 °C and then increased
443 remarkably at 130 °C to reach a value of 7.8. It reveals the beginning of a yellowish color
444 appearance (Fig. 3). In this context, Leceta, Guerrero, & de la Caba (2013a) reported that
445 color parameters changed notably for chitosan-based films after being heat-treated at 105 °C
446 for 24 h, which could mean that the film structure changed. Furthermore, total color

447 difference (ΔE) was measured in order to assess the observed differences between heated
448 films and their non-heated films. A sharp increase in ΔE-values was noticed as a function of
449 heating temperature for GP-glu films up to 110 °C. Such results of ΔE-values indicated that
450 MR resulted films are dark colored and their barrier ability in the visible region is stronger
451 than that of non heated gelatin films (Fig. 4). ΔE-values for GP films require 5 times more
452 energy when the Ea rises from 25 to 122.1 kJ/mol (table 3, supplementary data). Furthermore,
453 the browning index (BI), was calculated for GP-glu films and displayed a similar trend to ΔE
454 and b-values. The browning index give an overall evaluation of the progress of the Maillard
455 reaction according the development of colored compounds generated. It increased
456 significantly with heating temperatures, from 22.44±1.10 at 90 °C to 85.35±6.93 at 130 °C.

457 *3.1.4.UV-visible spectroscopy*

458 In order to correlate the visual variation of film's color with the elaboration of MRPs,
459 the UV-visible light absorbance of glucose-free gelatin (GP) and gelatin-glucose (GP-glu)
460 films was conducted in the range of 200–800 nm and spectra are presented in Fig. 4. All
461 prepared gelatin films displayed an excellent UV light barrier capacity in the range of 200 nm
462 to300 nm due to the presence of aromatic amino acids namely tyrosine and phenylalanine that
463 absorb UV light (Li, Liu, Gao, & Chen, 2004). For heat-treated glucose-free gelatin films, a
464 slight increase of the UV-absorption (250-350 nm) as a function of the temperature (until 120
465 °C) has been revealed that thermal treatment was not the main factor affecting the UV-light
466 barrier properties of gelatin films. However, the absorbance increases noticeably in the range
467 of 250-400 nm for 130 °C heat-treated films. Regarding GP-glu films, they displayed
468 different spectra ranging from 200 nm to 500 nm. Their absorbances increased markedly of
469 about 6 times until 120 °C to be mainly constant at 130 °C. These results are in line with the
470 color data because these films are also lightly colored. The concomitant rise of absorption

indicated the development of MRPs. Therefore, all the GP-glu heat-treated films and the 130 °C heated GP films effectively prevented UV light. Their potential preventive effects on the retardation of product oxidation induced by UV light is expected (Leceta, Guerrero, & de la Caba, 2013a).

After the first step of MR, which consists on the condensation reaction between the carbonyl group of glucose and the amino group of gelatin, unstable products known as Schiff base products are formed and then transformed via the Amadori rearrangement into protein-bound Amadori products (Etxabide, Urdanpilleta, Gómez-Arriaran, de la Caba, & Guerrero, 2017). These latter are intermediate colorless compounds absorbing in the UV-light (294 nm). Fig. 5.A indicated that the absorbance at 294 nm (A_{294nm}) of GP-glu films was significantly higher than that of GP films for the different heating temperatures, except for non-heated films. As can be seen, glucose-free gelatin films didn't show any variation of A_{294nm} values up to 120 °C. However, an increase in absorbance values has been noted for 130 °C heated gelatin films. For gelatin-glucose (GP-glu) films, A_{294nm}values increased markedly when MR heating temperature increased from 90 to 110 °C and then reached a plateau at110 °C. This finding could be due to the transformation (at temperatures higher than 110 °C) of the intermediate compounds into the final MRP's during the further steps of the Maillard reaction. This was showed by the higher absorbance at 420 nm thanks to MRPs. Indeed, Arrhenius representations (supplementary data) clearly display the change in activation energy below and above 120 °C and the Ea rose respectively from 24.1 to 133.8 kJ/mol for the glucose-free gelatin (GP) films. You, Luo, Shen, & Song (2011) reported that A_{294nm} values obtained for silver carp protein hydrolysate-glucose system at the temperature of 60°C were significantly higher than those obtained at 50 °C.

Further progress of the MR involves the production of high molecular weight compounds, termed melanoidins, with chromophore groups having a characteristic

496 absorbance at 420 nm (Delgado-Andrade, Seiquer, Haro, Castellano, & Navarro, 2010).
497 Indeed, the 420 nm absorbance is directly related to the presence of cycle structures, typical of
498 melanoidins and Amadori rearrangement resulting compounds. These brown nitrogenous
499 compounds have been frequently used to evaluate the extent of this reaction (Nasrollahzadeh,
500 Varidi, Koocheki, & Hadizadeh, 2017). Fig. 5.B illustrated the absorbance at 420 nm of GP
501 and GP-glu films as a function of heating temperature. The absorbance at 420 nm was
502 significatively different between GP and GP-glu films at all temperatures except at 25 °C. GP
503 films didn't show a specific absorbance at 420 nm regardless increasing heating temperature
504 up to 120 °C ($A_{420} \approx 0.06$). Nevertheless, heating at 130 °C increased notably the absorbance
505 ($A_{420} = 0.192$) of glucose-free gelatin (GP) films as previously observed for absorbance at 294
506 nm. For GP-glu films, a high increase in A_{420} values has been noted which was linearly
507 heating temperature-dependent. These findings showed that the brown yellowish color
508 development because of the Maillard reaction was extremely dependent on temperature and
509 thus, the formation of colored melanoidins was faster at higher heating temperatures. This is
510 in accordance with previously obtained BI results. In this context, Lan et al. (2010) reported
511 similar results in terms of A_{420} values for both xylose–soybean peptide MRPs and thermal
512 degradation products, prepared at temperatures ranging from 80°C to 130 °C. The authors
513 indicated that browning development was almost completely due to the MR in the soybean
514 peptides–xylose system and slightly influenced by thermal degradation and sugar
515 caramelization. Jiang, Wang, Che & Tian (2014) reported a high correlation ($R^2 = 0.913$)
516 between the heating temperature (70-120 °C) and the browning intensity (A_{420}) of galactose-
517 bovine casein peptide MRPs. Furthermore, Etxabide, Urdanpilleta, de la Caba, & Guerrero
518 (2016) reported that the increase in the absorption above 420 nm for gelatin-lactose films
519 prepared at native pH (5.4) and heated at 105 °C for 24 h, is due to the fact that the

crosslinking reaction reached the final stage, in which melanoidins, brown and non-soluble compounds, were formed.

Similar trends of variation as a function of heating temperature have been noted for b, ΔE and BI indicating the development of yellow-brown MRPs, caused by the melanoidins color. Furthermore, the Ea was higher in the case of GP films compared to GP-glu films in the case of A_{294nm} and A_{420nm} values (Table 3). All these characterizations confirm that MR occurs in GP-glu films, but probably also in the GP films, either due to glycerol (Smarrito-Menozzi, Matthey-Dorret, Devaud-Goumoens & Viton, 2013) or to sugar impurities in gelatin.

3.2. How temperature and Maillard reactions changed the structure of gelatin-based films

3.2.1. Thermal properties

The thermal stability of gelatin films crosslinked or not with glucose and heated at temperatures ranging from 90 to 130 °C was assessed by means of TGA in a range of temperature between 25 and 600 °C. The weight loss (Δw), temperature of maximum degradation (T_{max}) and final residual mass (Residue %) of the different films are illustrated in table 2. The TGA curves (data not shown) of GP and GP-glu films indicated two steps of transformations corresponding to the main stages of weight loss. The first stage of transformation is related to the loss of free and bound water (below 100 °C). The weight loss in this step was ranged from 4% to 8%. The second stage of weight loss, corresponding to Δw around 69% and 77%, displayed the degradation or the decomposition of gelatin chains at approximately 303-314 °C. Results presented in table 2 showed that MR increases the T_{max} from 310 to 314 °C after 24 h of heating at 90 °C, whereas, there is no differences in T_{max} values when MR temperature increased from 90 to 130 °C. For glucose-free gelatin (GP)

films, the thermal stability increased by heating at T ≥120 °C. Such thermal resistance rise in control films could be due the generation of new bonds or interactions between gelatin strings, which favored their thermal stability. Furthermore, the development of MRPs, which could interact with gelatin chains to stabilize the protein network, may explain the increase in the observed thermal stability for gelatin-glucose films heated at high temperatures (90-130 °C) (Kchaou et al., 2018, González Seligra, Medina Jaramillo, Famá & Goyanes, 2016).

The thermal properties of glucose-free gelatin (GP) and gelatin-glucose (GP-glu) and unplasticized gelatin (G) films were assessed by the DSC analysis in order to determine the glass transition temperature (Tg) from the second heating cycle and the ΔCp (Table 2). As expected, the Tg value of the unplasticized films is much greater than that of GP and GP-glu films. Indeed, it is well known that adding glycerol in the film recipe induces a plasticization revealed by the Tg value decrease by 25 °C. Moreover, the addition of glucose, even at low ratio, also decreased the Tg value but to a smaller extent (non-significant). The plasticizing effect of glucose is less efficient than that of glycerol as demonstrated by Simperler et al. (2006).

All films displayed a clear increase in the Tg values with increasing the temperature of treatment. The increase of Tg values in hydrophilic biopolymer network could be due either to water content decrease, or to crosslinking/reticulation very often simply attributed as an antiplasticization phenomenon. In our case, the water content did not change significantly as all the films were previously equilibrated at 0% relative humidity. The rise of Tg value could thus be attributed to a crosslinking/reticulation. However, not only the Maillard reactions are responsible of the crosslinking as it occurs also in glucose-free films and in unplasticized films. Indeed, the Tg rose from 77 °C to 86 °C for unplasticized G films, from 51 °C to 65 °C for GP films and from 45 °C to 64 °C for GP-glu films when temperature range from 25 to 130 °C, respectively.

569 Furthermore, the higher the ΔCp, the lower the crystallinity, as the ΔCp is only related to
570 the amorphous phase. From table 2, the higher ΔCp is obtained for the unplasticized gelatin
571 (G) films. Adding glycerol or glucose induced plasticization and thus chain mobility, which
572 could make crystallization easier (chain rearrangement in a more ordered configuration). This
573 is confimed by the decrease of the ΔCp values for GP and GP-glu films. By comparing the
574 effect of heat treatment, the ΔCp of the GP-glu films increased because of favoured
575 crosslinking enhanced by the Maillard reaction, which limits chain mobility and thus
576 recrystallization.
577 Moreover, it appears clearly that the improvement of thermal resistance of GP-glu films
578 measured from TGA could be both due to the effects of MR and to a supposed crystallinity
579 increase. Additionally, the increase in Tg values is in accordance with the increase in
580 crosslinking degrees. In this context, Hoque, Benjakul & Prodpran (2010) reported that
581 generally, the increased crystallinity, molecular weight, ionic degree and crosslinking increase
582 Tg.

583 *3.2.2. X-ray diffraction (XRD)*

584 Furthermore, the XRD analysis was carried out in order to assess the structural
585 modifications and the molecular conformation changes caused by the thermal treatment and
586 MR in prepared gelatin films (Fig. 6). The diffractograms of all the films showed a sharp peak
587 at about 8° (2θ), and a broad peak at about 20° (2θ). The other sharp peaks could be attributed
588 to the inorganic impurities (salts, metals) in the gelatin powder, in which the ash content
589 corresponds to around 0,14% of dry matter. The sharp strong peak is due to the triple helix
590 structure, whose peak position corresponds to the diameter of triple helix, and the intensity
591 corresponds to the triple helix content. The broad amorphous peak located at 20° (2θ) is
592 related to the distance between amino acid residues along the helix (Liu et al., 2016). Loucif,

Chetouani, Bounekhel & Elkolli (2017) reported similarly that the spectrum of gelatin film exhibited two peaks ascribed to helical crystalline structure of collagen renatured in gelatin, the first small and narrow peak is at 2θ = 7.75° and the second broad one at 2θ = 20.08°. A slight displacement of the first peak position (2θ = 8°), has been noted for 90 °C GP films compared to NH-GP films. However, this peak disappears for 130 °C applied to GP films indicating a loss of the triple helix structure at 130 °C. For GP-glu films, there were no significant displacements of the sharp peak position as a function of heating temperature, indicating a constant triple helix diameter. For 130 °C, this peak tends to disappear for GP-glu films as previously observed for GP films. Regarding the triple helix content, it decreases remarkably as a function of heating temperature as evidenced by the decrease in the intensity of the sharp peak (8°) for GP films as well as for the GP-glu films. For the broad peak (2θ = 20°), its position increased up to 130°C with increasing heating temperature for GP films . Similar observations have been obtained for GP-glu films up to 90 °C. For 130 °C, this peak was almost absent in GP-glu films confirming the loss of the structure of triple helix. The structural and functional properties of proteins can be influenced by heat treatment leading to the proteins denaturation by the destruction of some forces that stabilize native conformations, such as hydrogen bonds, electrostatic, hydrophobic and disulfide bonds (Pirestani, Nasirpour, Keramat, Desobry -& Jasniewski, 2018). Additionally, Caoa, Fua, & Hea (2007) reported that heating denatures secondary and tertiary structures of proteins and allows possible disulfide interchanges among protein molecules.

3.3. Film functional properties modified by temperature and Maillard reactions

Water resistance properties including water content (WC), water solubility (WS) and water contact angle (WCA) of glucose-free gelatin and gelatin-glucose films were characterized and the results are showed in Fig. 7.

618 *3.3.1. Water content*

619 Water content didn't show a significant modification despite of increasing heating
620 temperature values up to 120 °C for both GP and GP-glu films (Fig. 7.A). However, a
621 significant decrease in WC values has been noted for 130 °C for all films. This finding is
622 explained by the loss of free water from films heated at 130 °C.

623 *3.3.2. Water solubility*

624 For instance, partial water solubility in the saliva could be set for applications of
625 edible films as coating. Water solubility (WS) was calculated based on the percentage of
626 soluble matter to initial dry matter in each film sample (Kim, Weller, Hanna, & Gennadios,
627 2002). WS of all films is shown in Fig. 7.B. Results highlighted that solubility values are kept
628 invariable for G and GP films heated at 90 and 100 °C compared to non-heated films (NH-
629 GP). However, heating at higher temperature (≥110 °C) decreased remarkably the films
630 solubility from 75% to 23%. The relative crosslinking degree values of G and GP films
631 explain the solubility values. Indeed, the higher the crosslinking, the lower the solubility.

632 For GP-glu films, a high reduction in water solubility values has been obtained from
633 24.33% to 13.85% for GP-glu films heated from 90 to 130 °C, respectively when the value
634 was initially 69.26% for NH-GP-glu films. These results displayed that MR and heat
635 treatment are effective methods for reducing the solubility of gelatin-based films. Heating
636 temperature higher than 100 °C did not greatly influence the water solubility of GP-glu
637 crosslinked films. Etxabide, Urdanpilleta, de la Caba, & Guerrero (2016) reported that lactose
638 addition reduced significantly ($p < 0.05$) the solubility of gelatin films prepared at native pH
639 (5.4) and heated at 105 °C as a result of the higher extent of glycation due to the formation of
640 non-soluble compounds, melanoidins. Reduction in water solubility following heat treatments
641 has also been documented by Kim, Weller, Hanna, & Gennadios (2002) for soy protein films.

642 They suggestested that covalent crosslinking, caused by heat denaturation of protein, is
643 responsible for film water insolubility. Furthermore, since total color difference and water
644 solubility followed the same trend of variation as a function of MR heating temperature, it can
645 be concluded that the development of MRPs reduced significantly the water solubility of
646 gelatin films. It has been largely reported in the literature that the early stage of the Maillard
647 reaction involves the formation of conjugates between the carbonyl group of the carbohydrate
648 ends with the amine group of proteins, producing a Schiff base. The Schiff base subsequently
649 cyclizes to produce the Amadori compounds and then colored and insoluble polymeric
650 compounds (referred to as melanoidins) are formed (Yasir, Sutton, Newberry, Antrews, &
651 Gerardard, 2007; Leceta, Guerrero, & de la Caba, 2013a; Leceta, Guerrero, Ibarburu, Duenas,
652 & de la Caba, 2013b; Duconseille, Astruc, Quintana, Meersman & Sante- Lhoutellier, 2015).

653 Additionally, results showed that the water solubility activation energy of GP-glu
654 films was reduced indicating that the glucose addition made the enhancement of water
655 sensitivity of films easier, especially in the range of 90-100 °C (as revealed by the Arrhenius
656 plot given in the supplementary data). Indeed, the Ea decreased from -5.0kJ/mol to -59.8
657 kJ/mol for glucose-free unplasticized gelatin (GP) films and glucose-gelatin (GP-glu) films,
658 respectively.

659

660 *3.3.3. Water contact angle*

661 Furthermore, water contact angle (WCA) is a good indicator of the hydrophilicity
662 degree of films. The final state of a water drop on the film surface is taken as an indication of
663 surface wettability (Leceta, Guerrero, & de la Caba, 2013a). The surface properties of GP and
664 GP-glu films were evaluated by WCA measurements (Fig. 7.C). Results showed that the
665 water contact angle values of GP films didn't vary significantly up to a temperature of 120°C.

However, GP films heated at 130 °C display a significant WCA value decrease from 104° to 77° indicating that heating temperature leads to an increase of gelatin films hydrophilicity. For the GP-glu films, only those heated at T≥110 °C showed a significant decrease in WCA values compared to NH-GP-glu films. Leceta, Guerrero, Ibarburu, Duenas, & de la Caba. (2013b) reported that the heat-treatment caused a slight decrease in contact angle values, attributed to changes in the conformation of molecules and to the exposure of the hydrophilic groups toward the surface.

3.4. Antioxidant activity

The antioxidant activity of MRPs is complex. Thus, it is necessary to employ a number of antioxidant indices to obtain a holistic view of the antioxidant pattern or mechanisms of MRPs (Vhangani & Van Wyk, 2013).The antioxidant potential of GP and GP-glu films was assessed by the free radical scavenging activity (FRSA) using DPPH• and ABTS radicals, the reducing power (RP), the β-carotene bleaching inhibition and the linoleic acid oxidation inhibition assays.

3.4.1. DPPH• free radical scavenging activity (FRSA)

The DPPH• scavenging activity of all the gelatin films (G, GP and GP-glu) heated at 90, 100, 110, 120 and 130 °C is shown in Fig. 8.A. The G and GP (unplasticized and plasticized) films display the same behavior with the temperature changes. For these films, the rise of the FRSA only occurred at temperatures higher than 110 °C. The ability of GP-glu films to scavenge DPPH• free radicals is due to their hydrogen atom transfer capacity, which increased clearly after the MR. Indeed, this increase in FRSA was temperature dependent for GP-glu films up to 100 °C. Thereafter, a sharp decrease in FRSA was found when heating temperature increased from 110 °C to 130 °C which could be attributed to the conformational change and protein denaturation. These findings revealed that heating temperature is a crucial

690 factor for controlling MRPs formation where increasing temperature up to 100 °C was enough
691 to enhance the antioxidant activity of GP-glu films. Also, with the enhanced crosslinking with
692 temperature, the release of the active MRPs is probably limited. Indeed, the farer the Maillard
693 reaction goes with the temperature, the bigger size the MRPs is. The greater the crosslinking
694 and the size of polymerized MRPs is, the lesser the molecular mobility and thus probably
695 lower the release of the active compounds in the DPPH• media is. In this context, Sun & Luo
696 (2011) reported an increase of FRSA by 480% for porcine haemoglobin hydrolysate–sugar
697 model system when MR temperature rose from 40 to 85 °C. Loucif, Chetouani, Bounekhel &
698 Elkolli (2017) reported that there was a positive relationship between the antioxidant capacity
699 and MRPs, particularly compounds crosslinked at higher temperature conditions. For GP
700 films, different results were observed. GP films heated at 90 °C showed the same activity than
701 that of NH-GP films which was equal to 33.88%. The DPPH• activity of NH-GP films, even
702 it was low, could be related to the presence of some antioxidant peptides probably elaborated
703 during the gelatin extraction process (Jridi et al., 2017). Nevertheless, increasing heating
704 temperature from 100°C to 130 °C lead to a significant increase in the radical scavenging
705 activity of GP films that reached around 91% at 130 °C. This could be due to the thermal
706 degradation of gelatin at high temperatures, which generated small active peptides and thus
707 enabling their release. Similarly, Yu et al. (2018) reported a significant increase ($p<0.05$) for
708 DPPH• radical-scavenging activity of different peptide fractions of soybean meal hydrolysate
709 by heating at 120 °C for 2 h. The authors indicated that the thermal degradation might
710 occurred during the heating process and would lead to the changes in antioxidant activity.

711 *3.4.2. $ABTS^+$ radical scavenging activity*

712 Furthermore, the $ABTS^+$ radical scavenging activity was used in order to assess the
713 antioxidant potential of prepared films. As shown in Fig. 8.B, the activity of NH-GP films
714 was similar to the GP films heated at 90 °C (≈38 %). Ge et al. (2018) reported that films

715 exhibited a low $ABTS^+$ radical scavenging activity that is attributed to the electron donation
716 ability of some amino acids of gelatin, which reduces $ABTS^+$ radicals to offer weak
717 antioxidant activity. However, increasing heating temperature ($T \geq 100$ °C) leads to a slight
718 increase of the $ABTS^+$ radical scavenging activity of GP films (44%) which still maintained
719 constant despite of increasing heating temperature. For GP-glu films, an increase of about
720 37% has been noted only for GP-glu films heated at 90 °C. Beyond 90 °C, there is no
721 significant difference in terms of $ABTS^+$ radical scavenging activity between G, GP and GP-
722 glu films. You, Luo, Shen, & Song (2011) reported that the ABTS radical scavenging activity
723 of silver carp protein hydrolysate–glucose system obtained at 60 °C was significantly higher
724 than that those obtained at 50 °C. As observed for the DPPH• FRSA, the decrease of the
725 $ABTS^+$ of GP-glu films heated at temperatures higher than 90 °C could be attributed to the
726 increase of crosslinking degree that limits the molecular mobility and thus their release in the
727 reaction medium.

728 *3.4.3. Reducing power*

729 The reducing power has been used to evaluate the effect of heating temperature on the
730 antioxidant activity of G, GP and GP-glu films (Fig. 8.C). This assay measures particularly
731 the antioxidative activity of MRPs as the hydroxyl groups of MRPs play a role in the reducing
732 activity through their redox potential of transferring electrons (Vhangani & Van Wyk, 2013).
733 For the G and GP, the reducing power, evaluated by measuring the absorbance at 700 nm
734 (OD_{700}), was maintained constant for 90 °C heated GP films compared to non-heated G and
735 GP films. The reducing power of GP films is slightly higher than the one of G films because
736 plasticization enhanced the release of active compounds resulting of gelatin degradation into
737 small peptides at temperatures higher than 110 °C. Regarding GP-glu films, their reducing
738 capacity increased significantly as a function of MR temperature and reached its maximum
739 ($OD_{700} \approx 0.7$) at 100 °C. Loucif, Chetouani, Bounekhel & Elkolli (2017) reported that the

740 reducing power increase of gelatin-alginate system prepared at pH=11 correlated well with
741 increasing MR temperature from 70 to 90 °C.

742 *3.4.4. β-carotene-linoleate bleaching inhibition*

743 The β-carotene-linoleate bleaching inhibition assay was used to assess the antioxidant
744 activity of prepared films. As shown in Fig. 8.D, NH-GP films exhibited the lowest
745 antioxidant power (10%) which increased then to reach only 18% at 130 °C. For GP-glu
746 films, a significant increase of the antioxidant activity has been obtained when heating
747 temperature increases. The generated MRPs could hinder the extent of β-carotene bleaching
748 by neutralizing the linoleate-free radicals formed in the emulsion system, and the activity
749 reached 18%, 22%, 28%, 35% and 43% for 90, 100, 110, 120 and 130 °C heated films,
750 respectively. Additionally, as can be noticed, the antioxidant activity increased with
751 increasing the browning intensity (expressed as A_{420nm}, Fig. 5.B). Thus, the enhancement of
752 the β-carotene bleaching prevention correlate well with the elaboration of the brown colored
753 MRPs in relation with the increase of heating temperature.

754 *3.4.5. Linoleic acid oxidation inhibition*

755 Lastly, the inhibition of the *in-vitro* linoleic acid oxidation of GP and GP-glu films
756 heated at different temperatures was investigated and results are presented in Fig. 8.E. The
757 lipid peroxidation is the oxidative alteration of polyunsaturated fatty acids in cell membranes.
758 It leads to the appearance of several degradation products including malondialdehyde (MDA)
759 which is one of the several low-molecular-weight final products formed via the
760 decomposition of certain primary and secondary lipid peroxidation products. Indeed, the
761 MDA is widely studied as a marker of oxidative stress and index of lipid peroxidation
762 (Janero, 1990). As displayed in Fig. 8.E1 and 8.E2, both of glucose-free gelatin (GP) and
763 gelatin-glucose (GP-glu) films inhibited the MDA formation during linoleic acid storage

under heating conditions. These results prove the ability of films to donate an hydrogen atom to free radicals, leading to decelerate the propagation chain reaction rate occurred during lipid oxidation process (Abdelhedi et al., 2016). As can be seen, the antioxidant activity increased with increasing the incubation time and reached it maximum at the 7^{th} day. The revealed antioxidant activity values extended 69.01%, 70.51%, 76.47%, 76.52% and 75.83% for GP-glu films heated at 90, 100, 110, 120 and 130 °C, respectively. Then, a decrease in the antioxidant activity has been revealed at the 10^{th} day for GP-glu films which reached 31.10%, 49.38%, 54.13%, 51.85% and 55.37% after heat treatments at 90, 100, 110, 120 and 130°C, respectively. Regarding GP films, the decrease in the lipid peroxidation inhibition capacity was more noticeable. Such findings indicated that GP-glu films heated at T ≥100 °C are more effective than GP films. Thus, heated GP-glu films could be used as active packaging in order to retard the oxidative degradation by inhibiting free radicals.

3.5. Activation energy

The activation energy was determined for all the studied parameters. Plots and calculation displayed that the Arrhenius equation did not apply to the antioxidant properties as the R^2 is not significant as displayed in the table 3 and supplementary data. However, we can claim that the Maillard reaction was favored by glucose addition. It always decreases the activation energy, except for the β-carotene bleaching inhibition. Indeed, for the latter, the experiment was conducted at 50°C, which greatly favored the diffusion and thus the release of the MRPs having the greatest bleaching inhibition activity.

The R^2 were significant (≥0.8) for all the studied parameters except those related to antioxidant data. The activation energies of crosslinking degrees are respectively 23.3 kJ/mol and 16.1 kJ/mol for GP and GP-glu films. Glucose thus makes the cross-linking easier as it requires less energy. This is logical as cross-linking results of MR were enhanced by glucose.

This is in line with the color parameters indicating the Maillard reaction degree. Indeed, ΔE-values for GP films require 5 times more energy than the one containing glucose as the Ea varied from 25 to 122.1 kJ/mol (table 3, supplementary data). To confirm color change according temperature, the UV-Visible properties were also considered. Arrhenius representations (supplementary data) of the absorbance at 294 nm which indicates the presence of the intermediate MRPs, clearly display the change in activation energy below and above 120 °C, and the Ea rose respectively from 24.1 to 133.8 kJ/mol for the GP films. Furthermore, the Ea was higher in the case of GP films compared to GP-glu films in the case of A_{294nm} and A_{420nm} values (Table 3).

Additionally, results displayed that the water solubility activation energy of GP-glu films was reduced indicating that glucose addition made the enhancement of water sensitivity of films easier, especially in the range of 90-100 °C (as revealed by Arrhenius plot given in the supplementary data). Indeed, the Ea decreased from -5.0 kJ/mol to -59.8 kJ/mol for GP films and GP-glu films, respectively.

4. Conclusion

A significant decrease of water solubility and an increase of UV-barrier ability were observed for gelatin-glucose films heated for 24 h at different temperatures (90-130 °C). The addition of glycerol and of glucose allowed to preventing more the gelatin hydrolysis during heating treatment. Indeed, an increase of thermal stability and a variation of structural properties have been revealed for gelatin-glucose films by means of XRD, TGA and DSC results. Prepared films showed an interesting antioxidant potential as evidenced by the reducing power, the DPPH• and the $ABTS^{+}$ radicals scavenging assays, the β-carotene-linoleate bleaching inhibition as well as the linoleic acid oxidation inhibition assays. Glucose-gelatin films heated at high temperatures showed antioxidant potential with different modes of actions (electron transfer, hydrogen atom donation). Regarding the heating temperature effect,

it appears clear that 90 °C seems to be enough to improve the antioxidant activity of gelatin films as evidenced by the different *in-vitro* assays. On the other hand, the thermal treatment (T ≥ 100 °C) alone can be a useful tool for enhancing the antioxidant activity of glucose-free gelatin films. Therefore, the Maillard reaction leads to generate bioactive compounds, which confer functional and satisfactory properties to gelatin films, which are suitable for the application as food packaging.

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1016

Figure captions

Figure 1: Degree of crosslinking of G, GP and GP-glu films heated at 90 °C, 100 °C, 110 °C, 120 °C and 130 °C. ^{a,b,c,d}: values with different letters are significantly different at $p<0.05$ in terms of temperature. (■ GP films, ● GP-glu films, ◆ G films).

Figure 2: FTIR spectra of GP and GP-glu films heated at 90 °C, 100 °C, 110 °C, 120 °C and 130 °C.

Figure 3: Images of GP and GP-glu films heated at different temperatures (90 °C, 100 °C, 110 °C, 120 °C and 130 °C).

Figure 4: UV-vis spectra of GP and GP-glu films heated at different temperatures at 90 °C, 100 °C, 110 °C, 120 °C and 130 °C.

Figure 5: A: Absorbance at 294 nm and B: at 420 nm of GP and GP-glu films heated at 90 °C, 100 °C, 110 °C, 120 °C and 130 °C (^{a,b,c,d,e,f} values with different letters are significantly different at $p<0.05$ on the temperature effect) (■ GP films, ● GP-glu films).

Figure 6: XRD patterns of GP and GP-glu films heated at 90 and 130 °C compared to non heated films.

Figure 7: Water content (WC), water solubility and water contact angle (WCA) of GP and GP-glu films (and/or G films) heated at 90 °C, 100 °C, 110 °C, 120 °C and 130 °C (^{a,b,c} significant difference ($p<0.05$) between temperature and $^{\alpha,\beta,\gamma}$ between films samples at a same temperature.) (■ GP films, ● GP-glu films, ◆ G films)

Figure 8: Antioxidant activity of GP and GP-glu films (and/or G films) heated at 90°C, 100°C, 110°C, 120°C and 130°C assessed from the DPPH radical scavenging ability (A), $ABTS^+$ radical scavenging ability (B), reducing power (C), β-carotene bleaching inhibition (D) and lipid peroxidation inhibition capacity (E) tests (^{a,b,c,d,e} values with different letters are significantly different at $p<0.05$ in terms of temperature, $^{\alpha,\beta,\gamma}$ values are significantly different at $p<0.05$ in terms of films sample for each temperature). (■ GP films, ● GP-glu films, ◆ G films)

Supplementary data: Arrhenius plots for all film characteristics measured after heating at temperatures ranging from 90 to 130°C (■ GP films, ● GP-glu films, ◆ G films)

Fig. 1

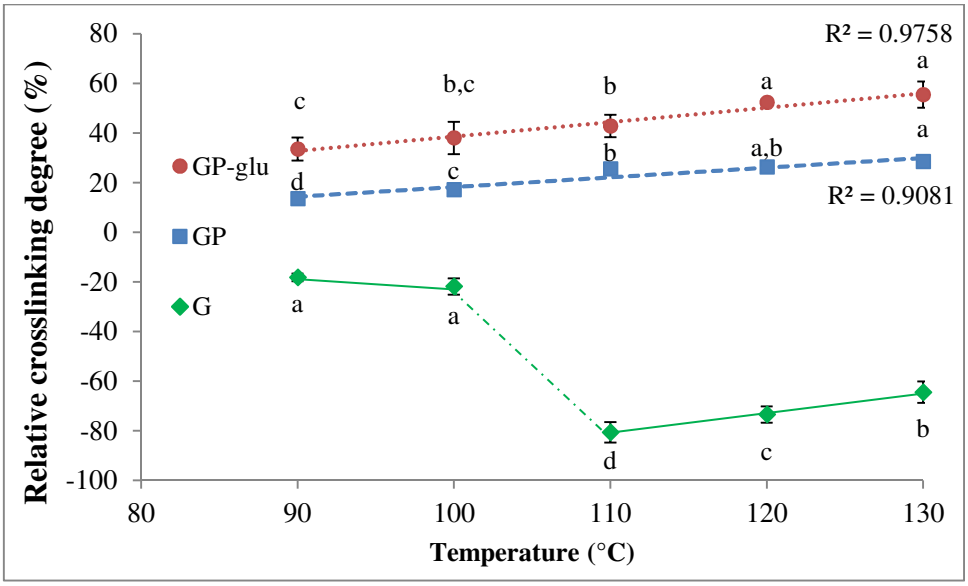
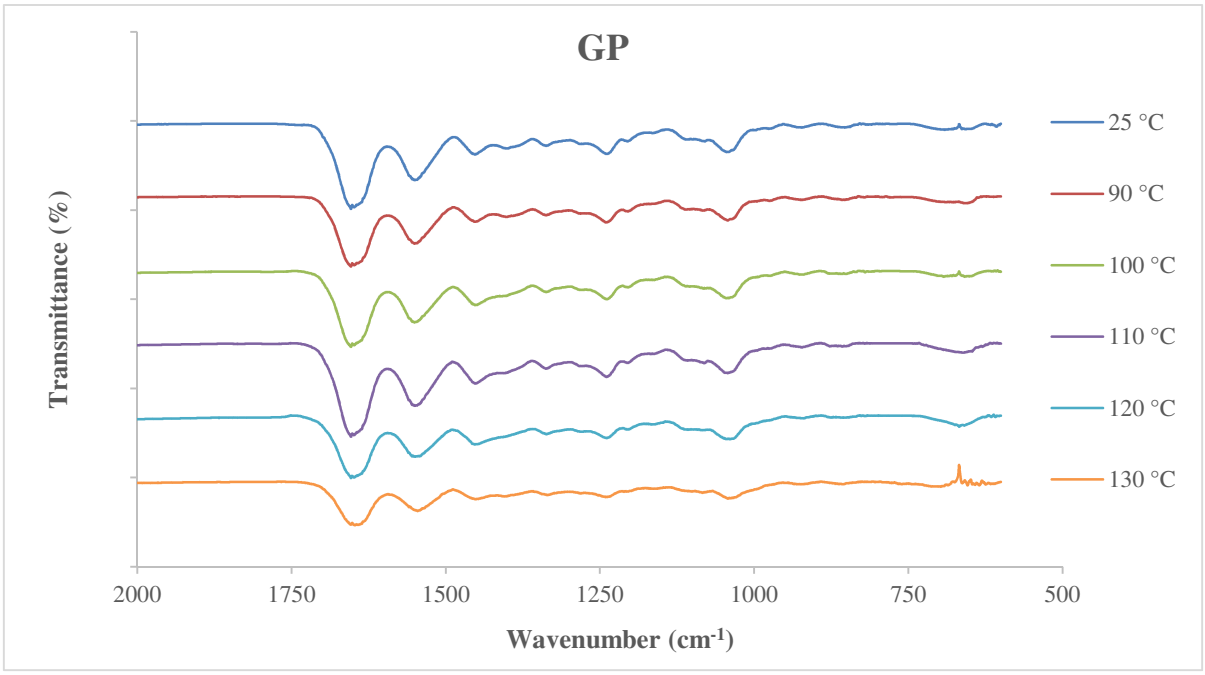

Relative crosslinking degree (%)
80
60
40
20
0
-20
-40
-60
-80
-100
80
90
100
110
120
130
Temperature (°C)
GP-glu
GP
G
R² = 0.9758
R² = 0.9081
c
b,c
b
a
a
d
c
b
a,b
a
a
a
d
c
b

Fig. 2


GP
Transmittance (%)
25 °C
90 °C
100 °C
110 °C
120 °C
130 °C
2000
1750
1500
1250
1000
750
500
Wavenumber (cm-1)

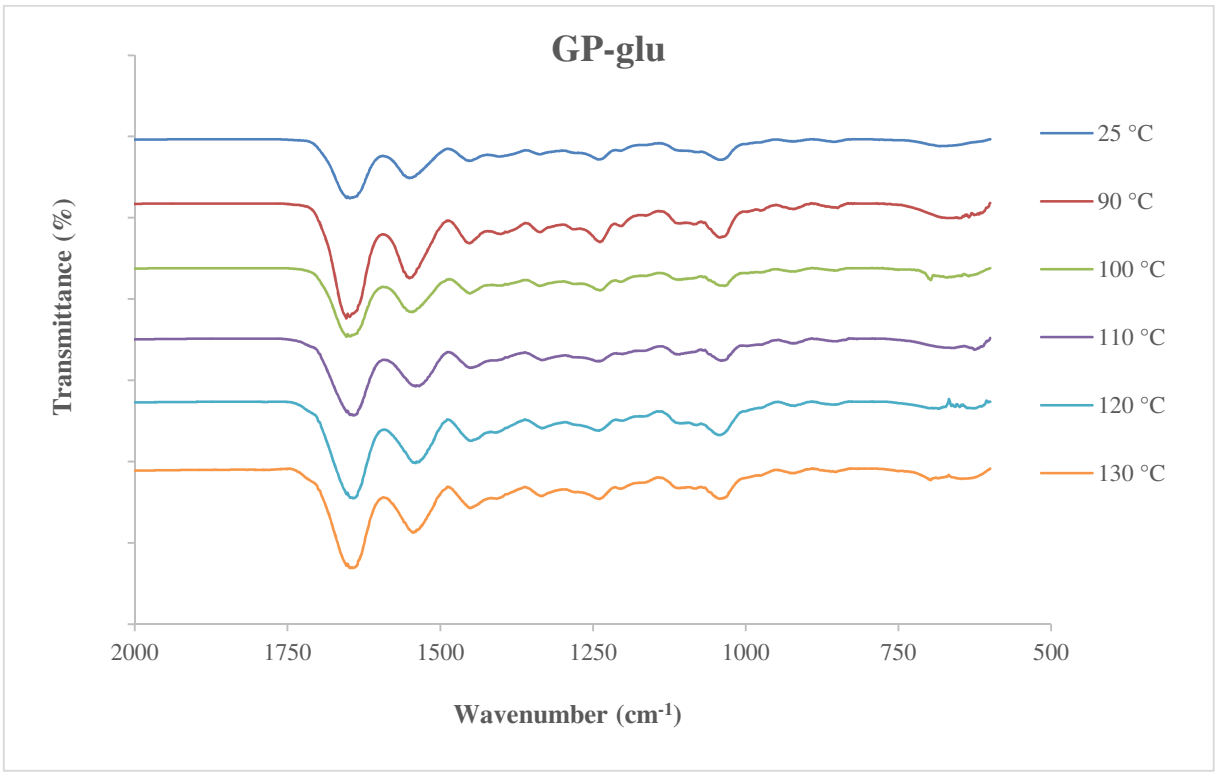

GP-glu
Transmittance (%)
25 °C
90 °C
100 °C
110 °C
120 °C
130 °C
2000
1750
1500
1250
1000
750
500
Wavenumber (cm-1)

Fig. 3

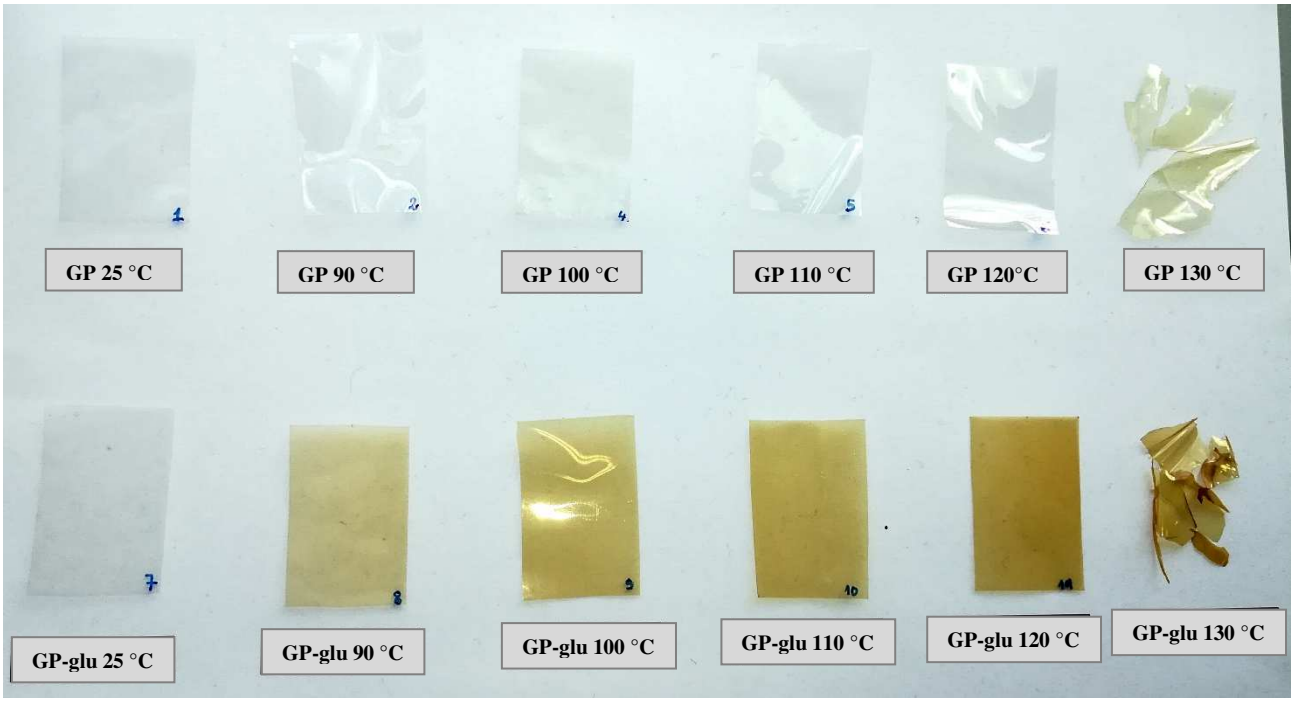
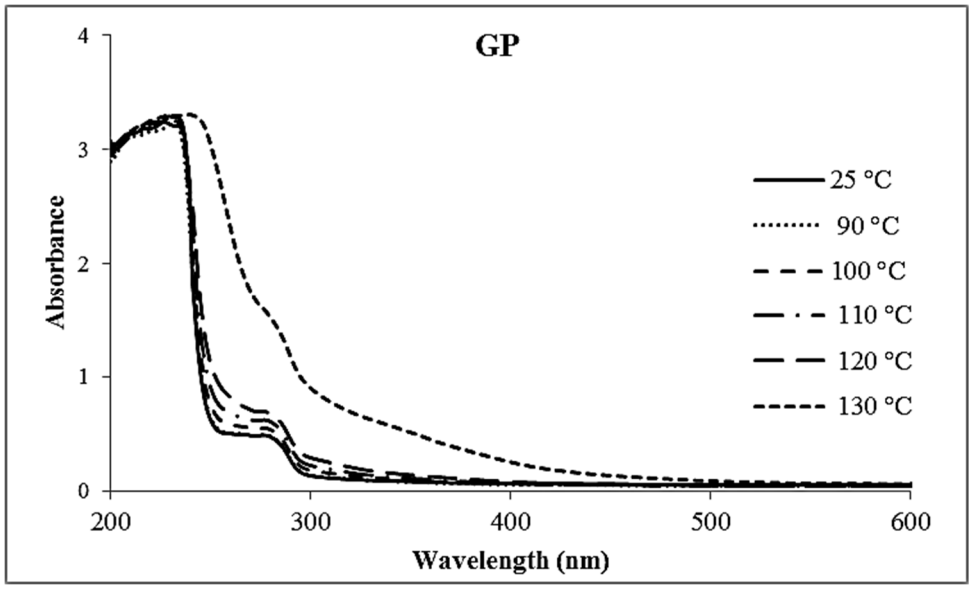

GP 25 °C
GP 90 °C
GP 100 °C
GP 110 °C
GP 120°C
GP 130 °C
GP-glu 25 °C
GP-glu 90 °C
GP-glu 100 °C
GP-glu 110 °C
GP-glu 120 °C
GP-glu 130 °C

Fig. 4


GP
Absorbance
Wavelength (nm)
25 °C
90 °C
100 °C
110 °C
120 °C
130 °C
0
1
2
3
4
200
300
400
500
600

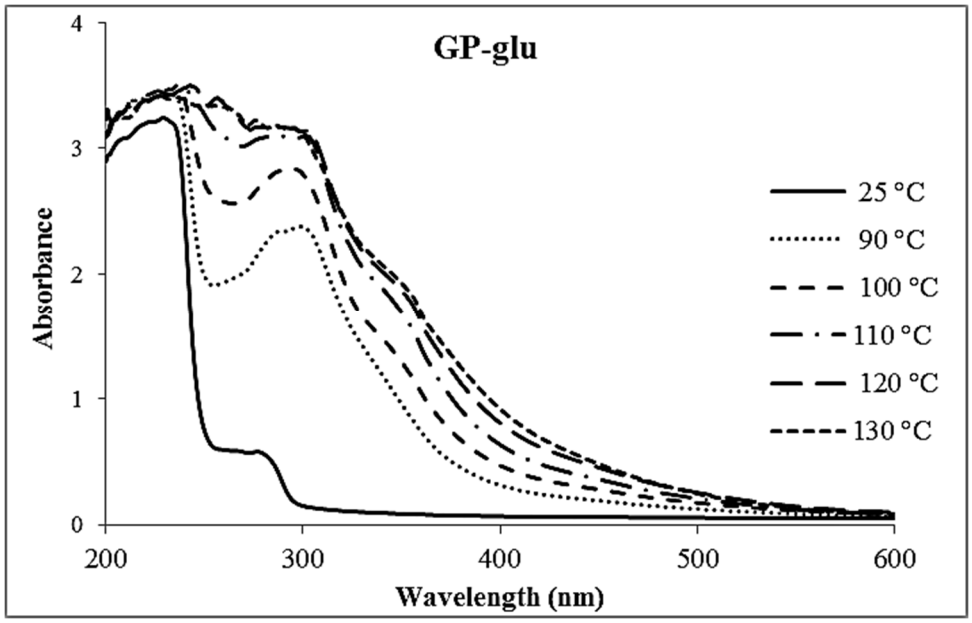
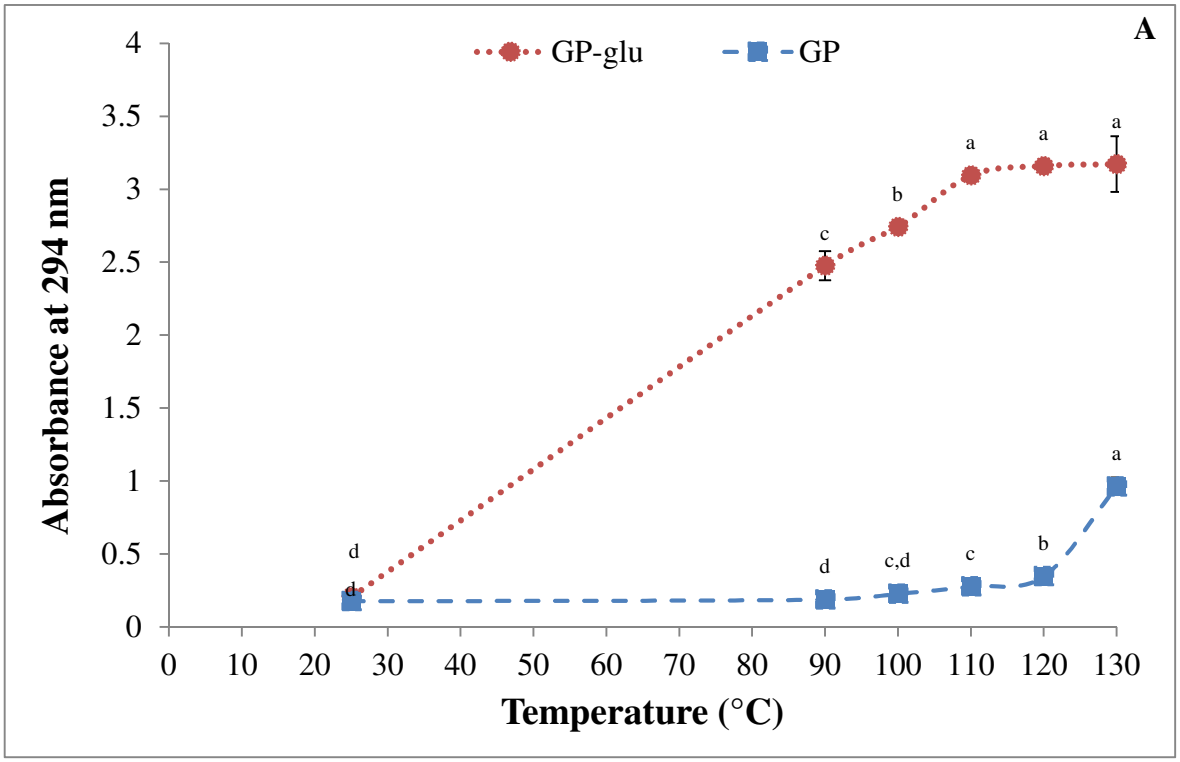

GP-glu
Absorbance
Wavelength (nm)
25 °C
90 °C
100 °C
110 °C
120 °C
130 °C
0
1
2
3
4
200
300
400
500
600

Fig. 5


A
GP-glu
GP
Absorbance at 294 nm
Temperature (°C)
0
0.5
1
1.5
2
2.5
3
3.5
4
0
10
20
30
40
50
60
70
80
90
100
110
120
130
d
d
c
b
a
a
a
d
d
c,d
c
b
a

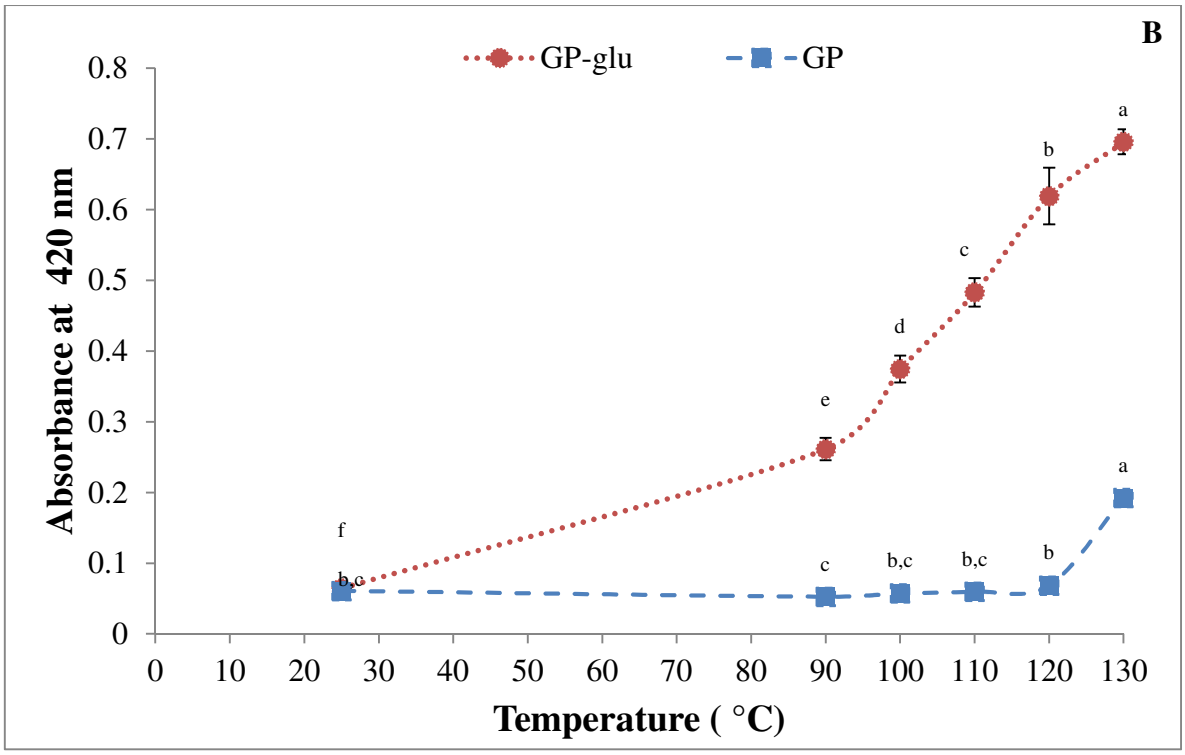
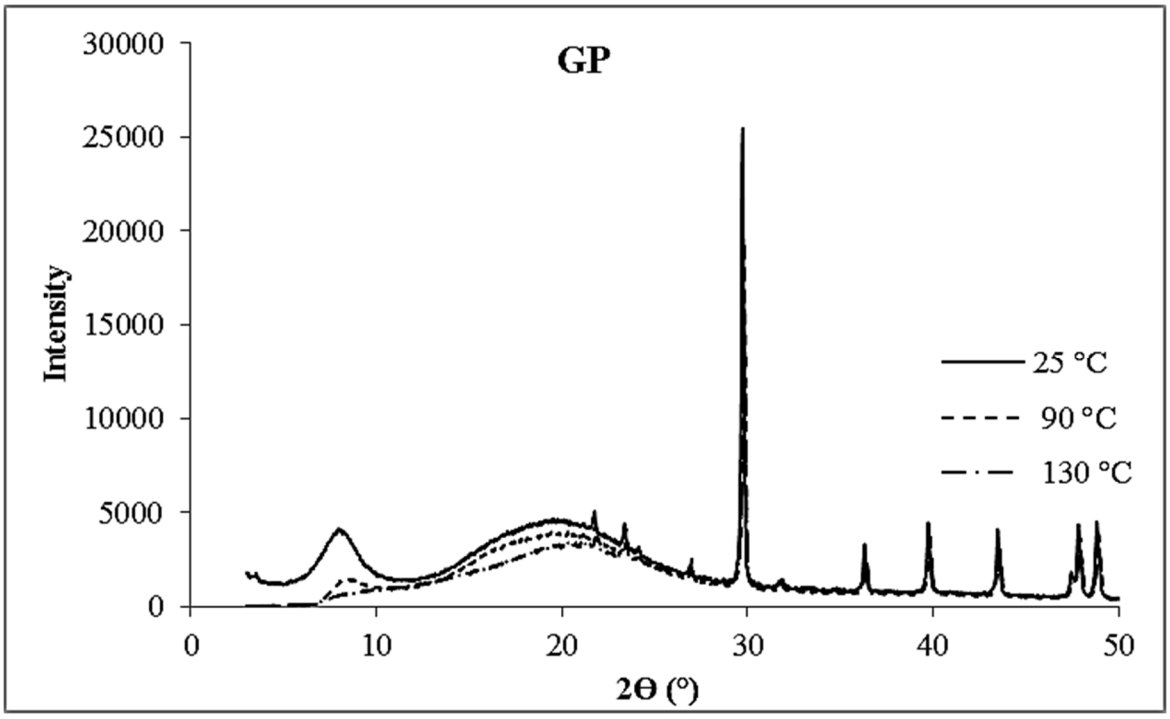

B
GP-glu
GP
Absorbance at 420 nm
Temperature (°C)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
10
20
30
40
50
60
70
80
90
100
110
120
130
f
b,c
e
d
c
b
a
c
b,c
b,c
b
a

Fig. 6


GP
Intensity
30000
25000
20000
15000
10000
5000
0
0
10
20
30
40
50
2Θ (°)
25 °C
90 °C
130 °C

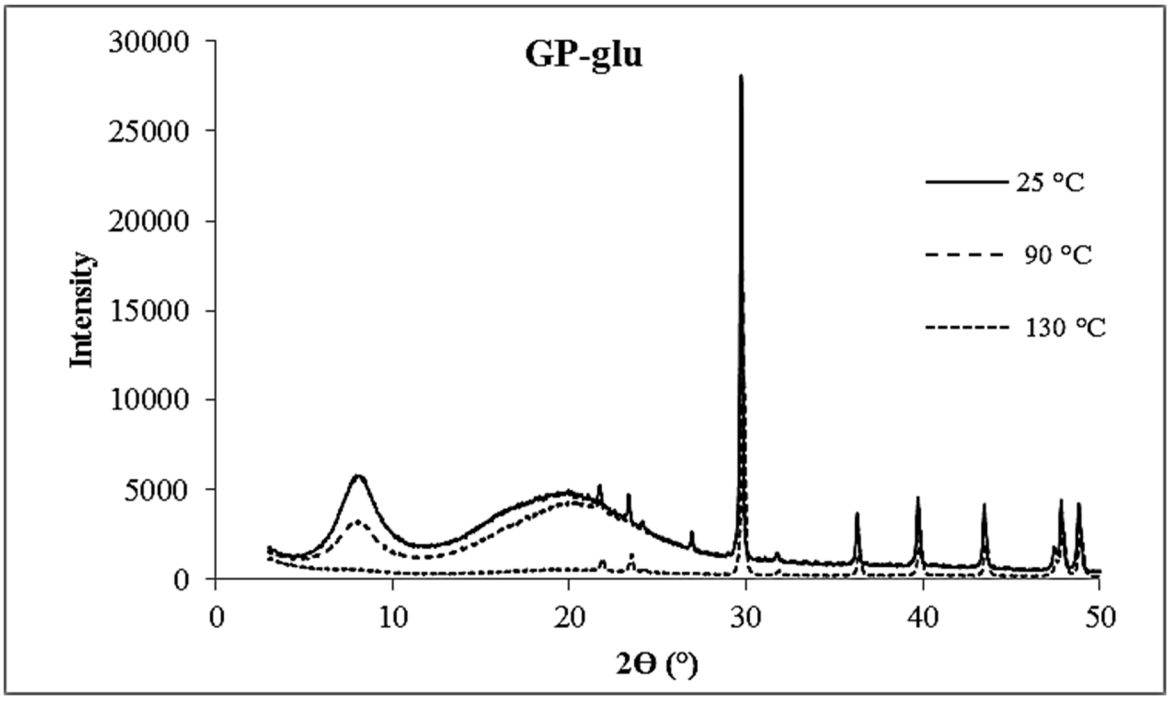
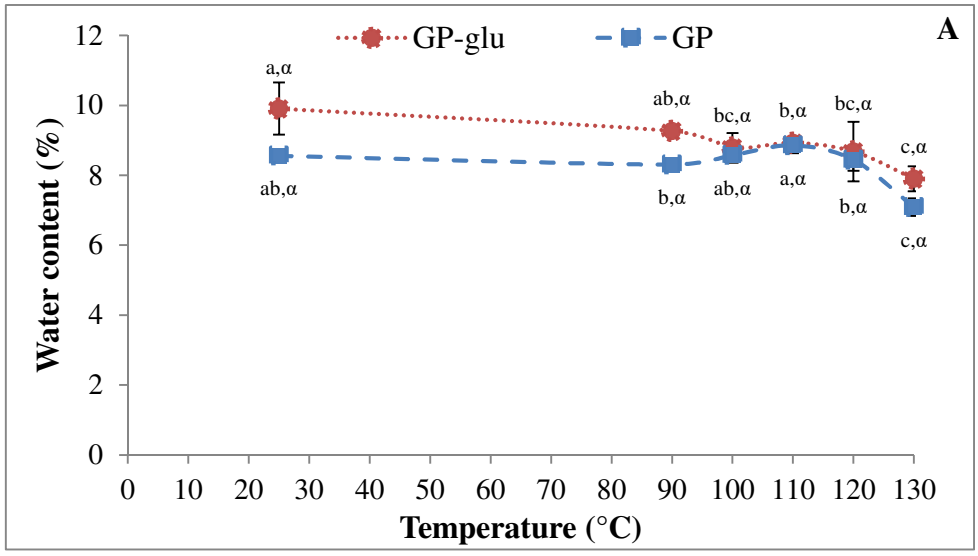
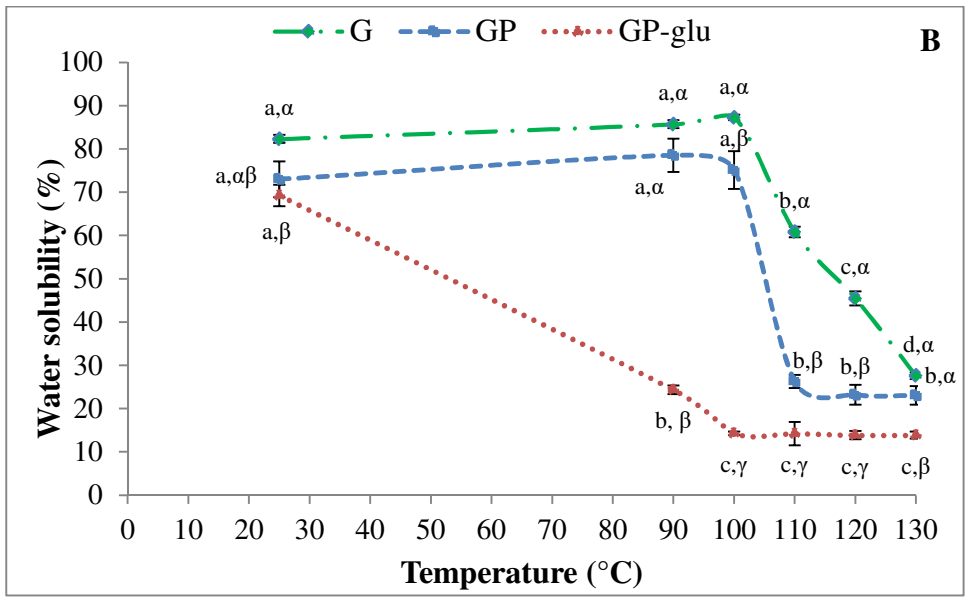

GP-glu
Intensity
30000
25000
20000
15000
10000
5000
0
0
10
20
30
40
50
2Θ (°)
25 °C
90 °C
130 °C

Fig. 7


A
GP-glu
GP
Water content (%)
Temperature (°C)
a,α
ab,α
ab,α
b,α
bc,α
ab,α
b,α
a,α
bc,α
b,α
c,α
c,α


B
G
GP
GP-glu
Water solubility (%)
Temperature (°C)
a,α
a,αβ
a,β
a,α
a,α
a,β
a,α
b,α
c,α
d,α
b,α
b,β
b,β
b, β
c,γ
c,γ
c,γ
c,β

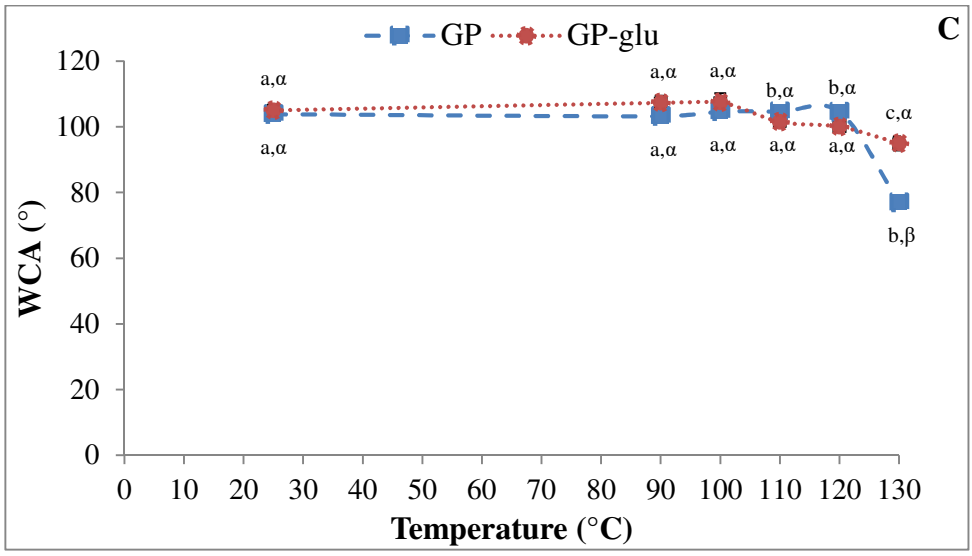
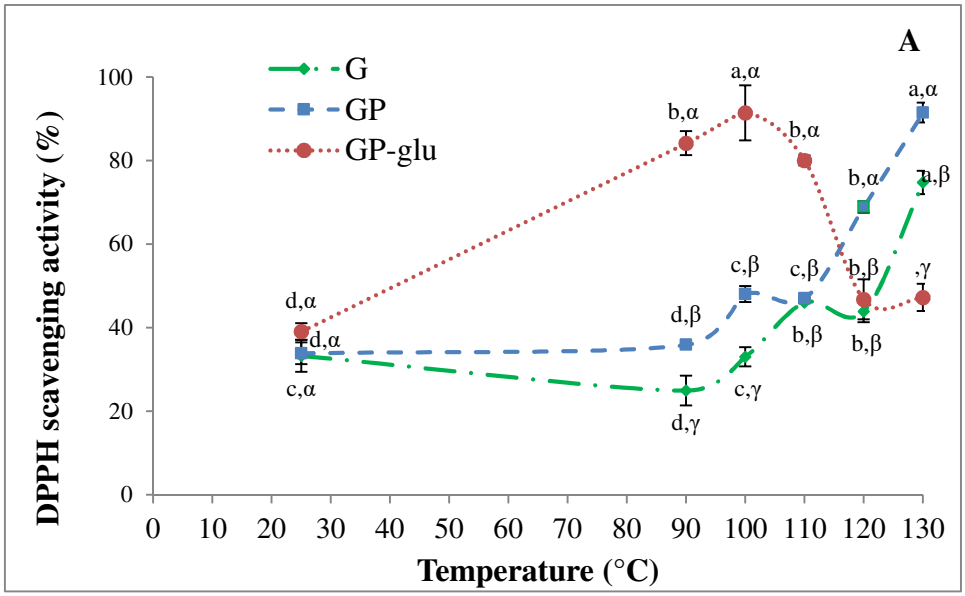
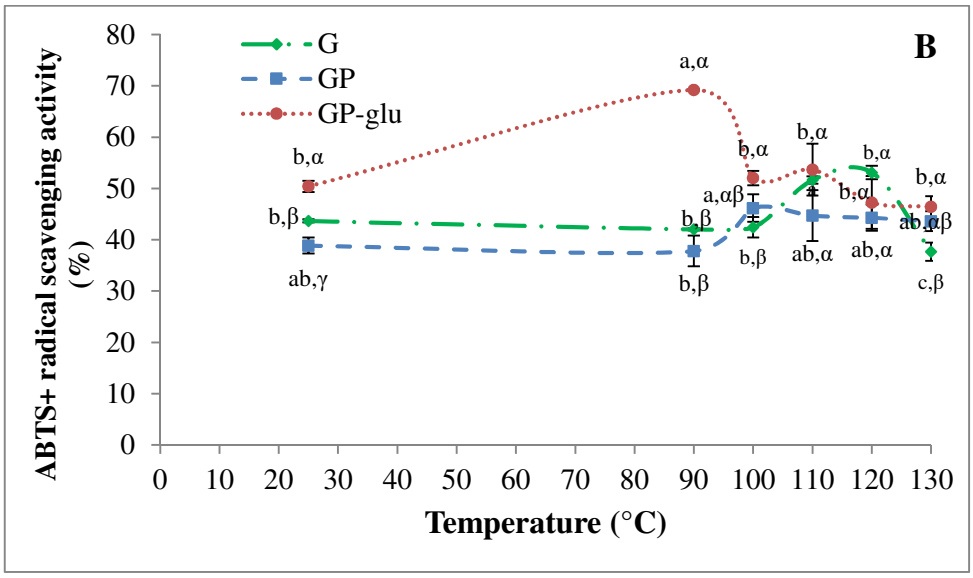
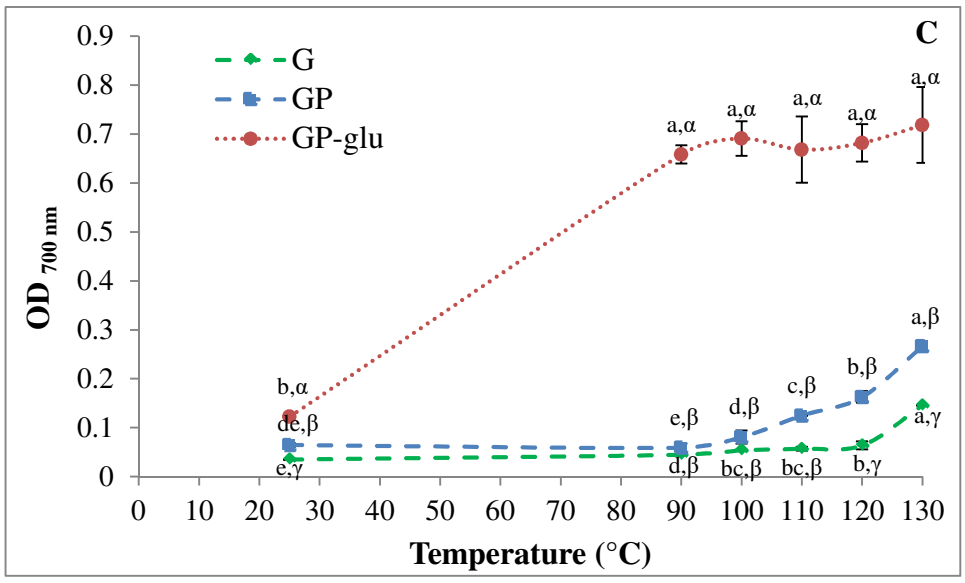

C
GP
GP-glu
WCA (°)
Temperature (°C)
a,α
a,α
a,α
a,α
a,α
a,α
b,α
b,α
a,α
a,α
c,α
b,β

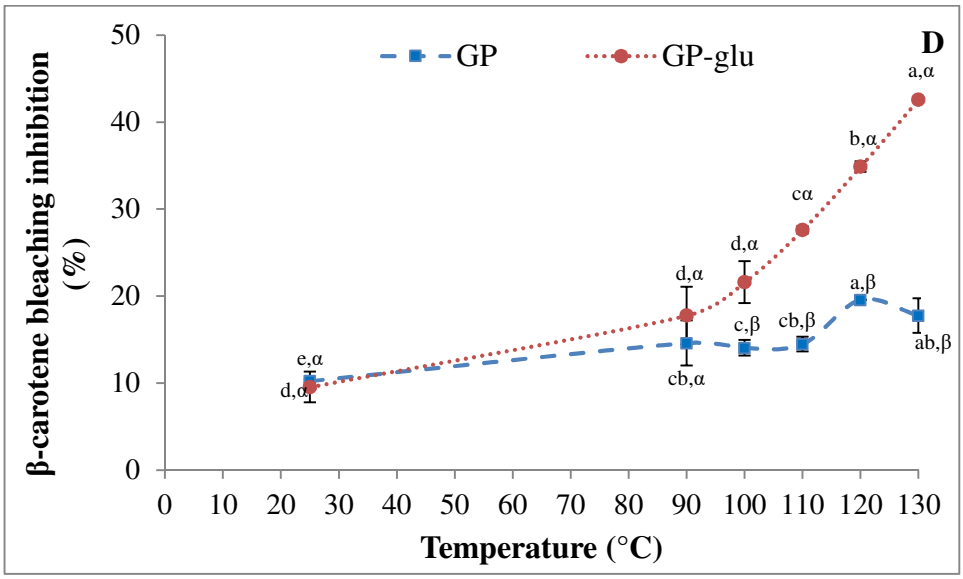
Fig. 8


A
G
GP
GP-glu
DPPH scavenging activity (%)
Temperature (°C)

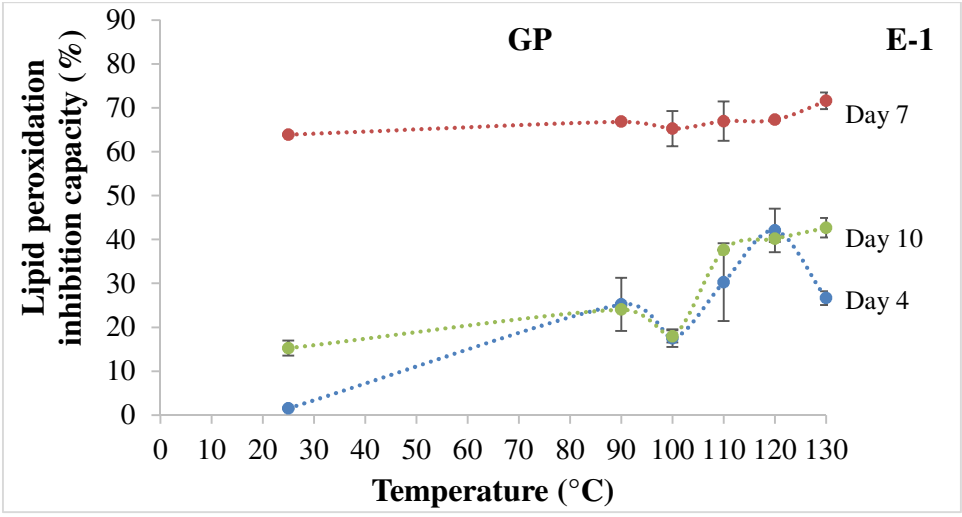

B
G
GP
GP-glu
ABTS+ radical scavenging activity (%)
Temperature (°C)


C
G
GP
GP-glu
OD 700 nm
Temperature (°C)

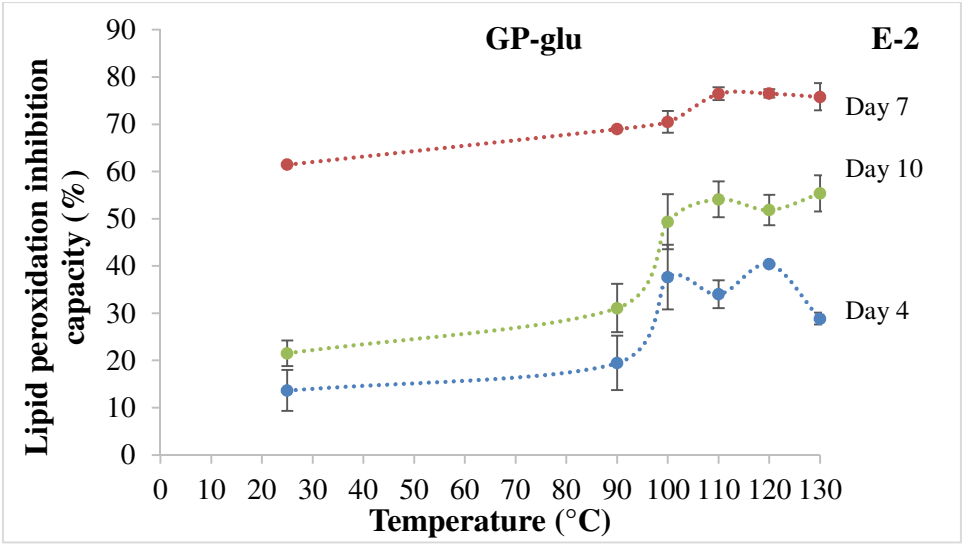
5


D
GP
GP-glu
β-carotene bleaching inhibition (%)
Temperature (°C)
a,α
b,α
cα
d,α
d,α
a,β
c,β
cb,β
ab,β
e,α
d,α
cb,α

6


GP
E-1
Lipid peroxidation inhibition capacity (%)
Temperature (°C)
Day 7
Day 10
Day 4

7


GP-glu
E-2
Lipid peroxidation inhibition capacity (%)
Temperature (°C)
Day 7
Day 10
Day 4

8

Table 1: Color parameters of GP and GP-glu films heated at different temperatures (90°C, 100°C, 110°C, 120°C and 130°C).

Films	T (°C)	L	a	b	ΔE	BI
GP	25	91.47 ± 0.06^{a}	1.47 ± 0.06^{a}	-3.87 ± 0.06^{d}	-	-
	90	91.40 ± 0.10^{a}	1.37 ± 0.06^{a,b}	-3.73 ± 0.06^{d}	0.21 ± 0.03^{d}	-
	100	91.50 ± 0.10^{a}	1.40 ± 0.10^{a,b}	-3.6 ± 0.10^{d}	0.30 ± 0.10^{d}	-
	110	91.53 ± 0.15^{a}	1.20 ± 0.10^{b}	-2.97 ± 0.06^{c}	0.96 ± 0.06^{c}	-
	120	91.23 ± 0.15^{a}	0.83 ± 0.06^{c}	-1.90 ± 0.17^{b}	2.09 ± 0.14^{b}	-
	130	90.60 ± 0.30^{b}	-2.83 ± 0.21^{d}	7.80 ± 0.46^{a}	12.47 ± 0.43^{a}	-
GP-glu	25	91.43 ± 0.06^{a}	1.43 ± 0.06^{a}	-3.83 ± 0.06^{f}	-	-
	90	87.07 ± 0.21^{b}	-1.83 ± 0.12^{d}	19.20 ± 0.72^{e}	5.85 ± 0.18^{d}	22.44 ± 1.10^{e}
	100	84.43 ± 0.15^{c}	-1.33 ± 0.06^{c}	29.70 ± 0.98^{d}	8.46 ± 0.13^{c}	40.42 ± 1.69^{d}
	110	80.53 ± 0.45^{d}	-1.2 ± 0.17^{c}	40.43 ± 1.50^{c}	12.31 ± 0.39^{b}	64.76 ± 3.14^{c}
	120	80.77 ± 0.35^{d}	-0.83 ± 0.12^{b}	44.33 ± 1.45^{b}	12.19 ± 0.36^{b}	73.85 ± 3.74^{b}
	130	79.60 ± 0.62^{e}	-1.33 ± 0.21^{c}	48.43 ± 2.89^{a}	13.47 ± 0.51^{a}	85.35 ± 6.93^{a}

ΔE: total color difference of GP and GP-glu films compared to their non heated films, respectively.

BI: browning index.

Different letters (a-f) in the same column within the same sample (GP or GP-glu) indicate significant difference ($p<0.05$).

Table 2: Glass transition temperature (Tg), Cp variation (**ΔCp**) weight loss (Δ*w*), temperature of maximum degradation (T_{max}) and residue of GP and GP-glu films heated at different temperatures (90°C, 100°C, 110°C, 120°C and 130°C).

Films	T (°C)	Tg (°C)	ΔCp	Δ*w* (%) 1st transformation region	Δ*w* (%) 2nd transformation region	T_{max}(°C)	Residue (%)
G	25	77[a]	0.507[b]	-	-	-	-
	90	83[a]	0.534[ab]	-	-	-	-
	100	83[a]	0.508[b]	-	-	-	-
	110	80[a]	0.61[a]	-	-	-	-
	120	78[a]	0.502[b]	-	-	-	-
	130	86[a]	0.514[b]	-	-	-	-
GP	25	51[b]	0.511[a]	5	77	306	18
	90	56[ab]	0.306[c]	6	70	311	24
	100	50[b]	0.383[b]	8	74	304	18
	110	56[ab]	0.353[bc]	6	70	304	25
	120	56[ab]	0.217[d]	5	72	312	24
	130	65[a]	0.156[e]	5	77	314	18
GP-glu	25	45[c]	0.347[b]	5	70	311	25
	90	52[bc]	0.429[a]	5	74	314	21
	100	55[ab]	0.429[a]	5	75	314	18
	110	59[ab]	0.433[a]	4	73	314	23
	120	59[ab]	0.402[ab]	8	70	314	22
	130	64[a]	0.455[a]	8	69	314	23

The average relative error on data is lower than 5%

[a,b,c,d,e]: values with different letters are significantly different at $p<0.05$ in terms of temperature

Table 3: Activation energy (Ea, kJ/mol) of GP and GP-glu films heated at different temperatures (90 °C, 100 °C, 110 °C, 120 °C and 130 °C), with the linear regression coefficient (R^2).

Parameters	GP		GP-glu	
	Ea	**R^2**	**Ea**	**R^2**
Crosslinking degree	23.3	0.902	16.1	0.981
ΔE	122.1	0.937	25.0	0.869
Browning index	-	-	40.2	0.921
*** Absorbance 294nm**	24.1 (90-120°C)	0.996	7.8	0.873
*** Absorbance 420nm**	9.7 (90-120°C)	0.940	30.1	0.980
*** Water solubility**	-8.5 (110-130°C)	0.799^{NS}	-1.5 (100-130°C)	0.870
DPPH radical scavenging	27	0.922	-22	0.759^{NS}
$ABTS^+$ radical scavenging	2.9	0.235^{NS}	-11	0.798^{NS}
Reducing power	45.1	0.989	1.9	0.566^{NS}
β-carotene bleaching inhibition	8.8	0.672^{NS}	27	0.997

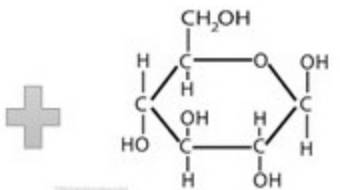
* Arrhenius linear behavior on a limited temperature range (see Arrhenius plots in the supplementary data)

^{NS} : Non significative value of R^2, phenomena cannot be considered as an Arrhenius law's dependence.

Enhancement of gelatin films properties by heat treatments to favour Maillard reactions



Fish gelatin



Glucose

Film making



Dry films

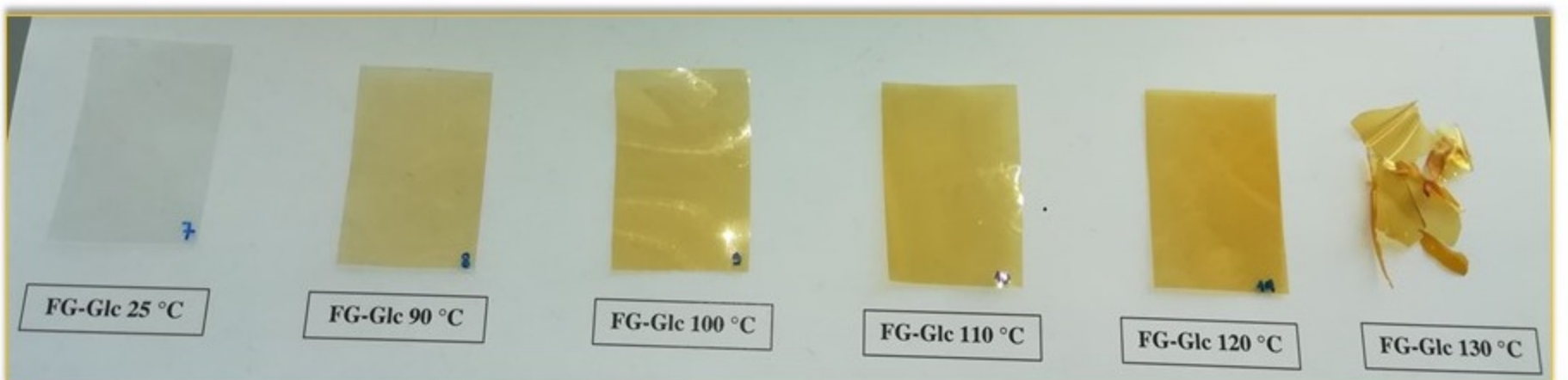
24 h

90 °C
100 °C
110 °C
120 °C
130 °C

Thermal treatment



Maillard reactions improved films



↑ Crosslinking

↓ Solubility

↑ Antioxidant properties