Wool keratin film plasticized by citric acid for food packaging

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ABSTRACT

Wool keratin (natural resource) and citric acid (effective preservative) were mixed in water to produce a transparent film for application in active packaging. This film showed excellent biocidal effect, high elongation value (600 %) and little loss of keratin after immersion in water. The capability of citric acid to bind keratin macromolecules by hydrogen bonds is probably responsible for the improvement of film's extensibility. On the other hand, the study of FT-IR spectrum allows understanding that the presence of citric acid in aqueous solution enhances the content of alpha helix structure in the film, with a reduction in the amount of side chain and disordered conformations in the macromolecular structure. Carrot shelf-life was qualitatively improved with this film in comparison with a commercial film for preserving food. Consequently, this film can have possible application for food packaging as a substitute of synthetic polymers replacing them with a natural, environmental friendly and renewable resource.

KEYWORDS: packaging, keratin, citric acid, protein, plasticizer.

1. **Introduction**

Innovative packaging materials that can extend the shelf-life, maintaining consumer safety, reducing losses to the production sector and being environmental friendly, has been the focus of extensive efforts. Particularly, biopolymers such as lipids, proteins, polysaccharides and mixtures thereof, have been investigated to enhance the properties of films as packaging materials (Caro, Medina, Díaz-Dosque, López & Abugoch, 2016). There is a current trend to incorporate active agents into packaging materials, to maintain the quality and to enhance the safety of packaged foods (Fabra, López-Rubio & Lagaron, 2016). In food technology, active packaging with antibacterial property is a concept based on incorporating an antimicrobial compound inside the packaging material or using a packaging material with inherent antimicrobial properties to reduce or inhibit microbial growth (Appendini & Hotchkiss, 2002).

Traditionally, antimicrobial agents are directly mixed into the initial food formulations, but this direct addition may change the taste of the food and result in the inactivation or evaporation of active agents with rapid migration into the bulk of the foods; consequently, antimicrobial activity is rapidly lost. As antimicrobial packaging materials must contact the surface of food, the antimicrobial agents could spread out to its surface, enhancing the shelf-life and safety of packaged food (Long, Joly & Dantigny, 2016).

Keratin is a natural protein extracted from wool or other natural resource (Fraser, MacRae, & Rogers, 1972). In different works it has been used to produce nanofibers (Varesano et al., 2015; Varesano, Vineis, Tonetti, Sanchez Ramirez & Mazzuchetti, 2014; Aluigi, Corbellini, Rombaldoni, Zoccola & Canetti, 2013) because of its special absorption properties of metal ions like Cu (Aluigi, Tonetti, Vineis, Tonin, & Mazzuchetti, 2011), Cr (Aluigi et al., 2012; Aluigi, Vineis, Tonin, Tonetti, Varesano & Mazzuchetti, 2009), dyes like methylene blue (Aluigi,

Rombaldoni, Tonetti & Jannoke, 2014) and volatile organic compounds like formaldehyde (Aluigi, Vineis, Tonin, Tonetti, Varesano & Mazzuchetti, 2009) and because of its possible applications as scaffolds for human body (Tachibana, Furuta, Takeshima, Tanabe & Yamauchi, 2002; Tachibana, Kaneko, Tanabe & Yamauchi, 2005) thanks to its biodegradability (Yamauchi, Maniwa & Mori, 1998). Unfortunately, keratin has not good mechanical properties, so it is necessary to use it in blend with a synthetic and not biodegradable polymer (Aluigi, Tonetti, Vineis, Tonin, & Mazzuchetti, 2011; Aluigi, Vineis, Tonin, Tonetti, Varesano & Mazzuchetti, 2009).

On the other hand, it is possible to find several works where citric acid was employed with natural polymers as compatibilizer and plasticizer to improve the mechanical properties of films. In fact, citric acid has properties to form stronger hydrogen bonds with hydroxyl groups to prevent recrystallization and to enhance the interactions between molecules in polymers as polyvinyl alcohol, starch and polycaprolactone (Ortega-Toro, Collazo-Bigliardi, Talens & Chiralt, 2016; Ghanbarzadeh, Almasi & Entezami, 2011; Reddy & Yang, 2010; Shi et al., 2008; Jiugao, Ning & Xiafoei, 2005). In addition, citric acid has antibacterial properties (In, Kim, Kim & Oh, 2013; Pundir, & Jain, 2011) and it is used in many different products to preserve different types of food (Pundir, & Jain, 2011; Soccol, Vandenberghe, Rodrigues & Pandey, 2006). The aim of this work is to obtain a film produced by casting using citric acid and keratin in aqueous solution, to use it in food industry as an active packaging because of its antimicrobial activity and plasticizer property. The film was analyzed by means of TGA, DSC and FT-IR. Moreover, strength tension, elongation at break, antibacterial efficiency, transparency and loss of weight after immersion in water were measured. Finally, the carrot shelf-life preserved with the keratin-citric acid film was qualitatively evaluated and compared with one of the commercial films for food packaging.

2. Materials and Methods

2.1. Keratin extraction and purification

Keratin was extracted from wool by sulfitolysis with sodium metabisulfite (Aluigi et al., 2007). Preliminarily, the wool fibers were cleaned by Soxhlet extraction with petroleum ether to remove fatty matter and washed with distilled water. An amount of 15 g of cleaned fibers were cut into snippets and treated with 300 ml of a solution containing urea (8M) and sodium metabisulfite (0.5M), adjusted to pH 6.5 with sodium hydroxide (5N), under shaking for 2 h at 65 °C. The mixture was filtered with 30 µm and then 5 µm pore-size filters, and the keratin aqueous solution obtained was dialyzed against distilled water with a cellulose tube (3.500-Da molecular weight cutoff) for 3 days at room temperature, changing the distilled water frequently. The keratin solution was frozen and then lyophilized with a Heto PowerDry PL3000 freeze dryer to obtain soluble and pure keratin.

Citric acid (99 %) was purchased from Sigma Aldrich. Citric acid and keratin were dissolved in distilled water to obtain a final concentration of 15 % wt. of keratin and 10% wt. of citric acid. The solution was shaken until dissolution by means of a magnetic stirrer ensuring its completed homogeneity. After that, 3 ml of solution was poured in a polyethylene bowl and dried at 20 °C and 65 %RH to form a film by casting. The film was separated from the support and cut in a rectangular form of 1 x 2 cm. The same volume of solution and bowl dimension was maintained for each test to standardize the production process.

2.2. UV-VIS (transparency and immersion in water)

In order to measure the keratin-citric acid film transparency, an analysis employing UV-Visible Spectrometer (Lamba 35 PerkinElmer) between 200 and 700 nm was carried out. In addition, sample pictures of film were taken with a camera.

Samples (0.1 g) were immersed in 5 and 20 ml of water up to 170 h in order to verify the water stability. The water after film immersion was qualitative analyzed in UV-Visible spectrometer between 200 and 700 nm. The intensity of peaks were measured at different immersion times. In addition, gravimetric analyses at different times (between 5 min to 2 h) were carried out measuring the loss of weight in the dried samples; the quantification of protein in solution was realized with Bio-Rad Protein Assay (Micro Assay) based on the Bradford method. The Protein Assay Dye Reagent Concentrate with one standard (Bovine Serum Albumin) were purchased from Bio Rad.

The dry weight was measured using the moisture analyzer DBS 60-3 equipped with a Halogen quartz glass heater 400W. The measurements were carried out in Drying mode AUTO (standard drying), where the drying temperature was set at 105°C and the drying process was finished automatically when the present weight loss remained constant for 30 seconds.

In addition, the pH of water after film immersion was measured with a PC 8 Instrumental Bench (pH/mV/COND/TDS/°C) – CARLI Biotec equipped with a polymer electrode type (Basic Pro pH).

2.3. Thermal treatments

Some samples were heated in an oven to study the effect of annealing on the mechanical properties of film. These were subjected to heat treatments in air at different temperatures (80, 100 and 120°C) for one hour. Samples were conditioned for at least 24 h at 20 °C and 65% RH before and after each heat treatment.

2.4. TGA and DSC analysis

The thermogravimetric analysis was done with a Mettler Toledo TGA-DSC. About 2.5 mg of film was put in a 70 ml aluminum oxide crucible for the analysis. The calorimeter cell was flushed with nitrogen at 70 ml min^{-1}. The TGA data were elaborated with a Mettler Toledo STARe system. The run was performed from 30 to 500 °C with a heating rate of 10 °C min^{-1}. Derivative thermogravimetry (DTG) was used to identify the temperature of maximum mass-loss rates.

Differential scanning calorimetry (DSC) was performed with a Mettler Toledo DSC calorimeter calibrated by an indium standard. The calorimeter cell was flushed with 100 ml min^{-1} nitrogen. The run was performed from 30 to 450 °C, at the heating rate of 10 °C min^{-1}. The mass was close to 2.5 mg of sample.

2.5. Mechanical tests and thickness measurement

In order to evaluate tensile strength and elongation at break an Instron (USA) 5500R tensile testing machine, configured with a 10 N load cell with a velocity of 10 mm min^{-1} was used.

Scanning electron microscopy (SEM) instrument was used to make a measurement of thickness in seven different samples on eight different points for each of them. Measurements were carried out with a LEO (Leica Electron Optics) 435 VP SEM. Operative parameters were 15 kV acceleration voltage and 30 mm working distance. Specimens were sputter-coated with gold before SEM analysis in an Emitech K550 Sputter Coater with a current of 20 mA for 5 minutes in rarefied argon at 20 Pa.

2.6. FT-IR analysis

IR spectra were recorded with a Thermo Nicolet Nexus spectrometer by an attenuated total reflection technique with a Smart Endurance accessory (equipped with a diamond crystal ZnSe focusing element) in the range from 4000 to 550 cm^{-1} with 50 scans and 4 cm^{-1} band resolution. Ommic 6.2 software (by Thermo Electron) was used to perform attenuated total reflection baseline

correction. Tests were performed on several parts of each sample to evaluate the citric acid distribution homogeneity.

2.7. Antibacterial test

Antibacterial tests were performed employing the gram positive *Staphylococcus aureus* by using AATCC 100 Test Method. In the procedure was used 1 ml of bacteria standardized inoculum containing 1.5-3x10^5 CFU ml^{-1} on the sample. After one hour 100 ml of sterile buffer were added on the sample, the buffer was then diluted ten times and pleated on Petri dishes with suitable agar. The Petri dish was incubated at least 24 h at 37 °C, then the bacteria colonies were counted. Bacterial reduction is calculated as ((B - A) / B) * 100, where A is the number of colonies of the sample with the film and B is the number of colonies of the sample without film (reference).

2.8. Qualitative assessment of carrot shelf-life

Carrots pieces were stored on the laboratory bench at 20 °C of temperature and 65% of humidity. In order to realize the quality assessment of carrot shelf-life, the different pieces of carrot were packed in a film for preserving food and keratin/citric acid film; then they were compared with a piece unpacked in any film. The film for preserving food was a commercial PET film. Carrot was acquired from a local orchard and was chosen as a sample of food to test the film because its availability and organic origin.

3. Results and Discussions

3.1. UV-VIS (transparency and water immersion)

Figure 1(a) shows the spectrum UV-VIS of the keratin/citric acid film. In the visible range (400 – 700 nm) the film has low absorbance value (close to 0.4), whilst in UV range the film has two strong peaks. The first one at 220 nm related by the carboxylic acid and/or amino groups of peptide

bonds and the second one at 280 nm caused by the aromatic ring portion of amino acids groups (Schmid, 2001; Hegyi et al., 2013; Held, 2016). Figure 1(b) reports a picture of the keratin/citric acid film that shows good property of transparency.

Figure 2 shows the absorbance values of water after the immersion of keratin/citric acid film at 275 nm (Figure 2 (a)) and 214 nm (Figure 2 (b)). On the one hand, in the Figure 2 (a), the signal of aromatic ring of amino acids groups from peptides chains is reported. This figure qualitatively confirms the presence of protein chains in water and shows that the amount of peptide chain increases considerably after 50 h reaching the maximum value at 100 h. At 170 h the absorbance value probably decreased as consequence of the degradation of phenylalanine and tyrosine amino acids groups in the keratin chain. On the other hand, in the Figure 2 (b), the signal of carboxylic acid and/or amino groups of peptide bonds is reported. Unlike in the previous one, the peak can be the result of the contribution of carboxylic groups present in the citric acid and peptide bonds. As a result, the second figure shows a constant trend in time, mainly caused by the citric acid release of film that could hide the signal of peptide bonds.

Table 1 reports the average results of keratin/citric acid film water immersion measured at 5, 10, 15, 30, 1 h and 2 h. After 5 min the parameters of the solutions reached approximatively a constant value with slight changes until 2 h.

As shown in Table 1, the amount of lost keratin decreased and the mean value of lost citric acid increased when the volume was reduced from 20 to 5 ml, but the total loss of weight remained constant at ~55 % wt.. Moreover, considering both the sample volumes, the citric acid contained in the film was almost completely released while the keratin released was 12 % wt. in 20 ml and 6 % wt. in 5 ml. Therefore, the volume of water contacting the film slightly alter the process of keratin release due to the saturation of the solution. It is worth noting that the protein concentration

reached values lower than 0.5 g l^{-1} in both the cases. The pH values slightly change with the volume of water leading a more acid solution using 5 ml, as expected.

3.2. TGA and DSC analyses

In the Figure 3, it is possible to see the thermal behavior differences between pure keratin and film. The pure keratin is thermally more stable than the film (Figure 3(a)): two temperatures with the greatest weight loss are visible before thermal degradation starting at 248 °C. Figure 3 shows the first event with difference in the temperature of water evaporation: 60 °C for pure keratin (Figure 3(b)) and 80 °C for film (Figure 3(c)). It occurs probably because of higher amount of alpha helix conformations in the film that bonded water in the protein chains stronger than pure keratin (Aluigi et al., 2007). The second event at 224 °C for pure keratin (Figure 3(b)) and 195 °C for film (Figure 3(c)), represents the maximum rate of keratin chain decomposition. In the film case, it is overlapped with citric acid degradation, which starts at 155 °C; the maximum decomposition is reached at 230 °C and the melting point at ~157 °C (Barbooti & Al-Sammerrai, 1986). In addition, the onset of this decomposition is 198 °C for pure keratin (Figure 3(b)) and 150 °C for film (Figure 3(c)). The differences are probably due to the citric acid decomposition temperature that is close to 150 °C. In the keratin/citric acid film, the maximum loss of weight is lower (195 °C) than that of pure keratin (224 °C). As a result, the melting points change for citric acid from 157 °C to 153 °C and for the keratin from 227 °C to 198 °C (Figure 3(d) and 3(e)). From this point of view, it is probable that citric acid acts like a catalyzer of thermal degradation (as happens in metabolism process) or produces molecules that allow degrading the keratin. In fact, the heat flow is always lower for film than for pure keratin (less endothermic process).

Thermal analysis also shows that the beginning of thermal denaturation probably occurs between 130 °C and 150 °C (Figure 3(c)); in correspondence of this temperature range, the film loses its

196 mechanical properties. In addition, there is not considerable alteration before 123 °C: before this
197 value, it is possible to avoid any denaturation in the protein chain.

198 Consequently, the temperature range between 80 and 120 °C would be an interval where occurs
199 the plasticization process of protein film. Probably, between these temperatures, the protein chain
200 starts the reticulation process which permits a variation in molecular structures contained in the
201 film; furthermore it changes the moisture contained in it. Obviously, this has consequences in the
202 enhancement of tensile strength and elongation of the film because of annealing-induced
203 phenomena.

204 *3.3. Mechanical test*

205 A mean thickness of 0.067 ± 0.034 mm was obtained from seven different samples. It was used
206 to calculate the tensile strength (Table 2). Figure 4 shows pictures during a tensile stress test of the
207 film without thermal treatment at the beginning of the test and just before its breaking.

208 In the Table 2 are reported mean values of tensile strength and elongation at break corresponding
209 with three different treatment conditions. The data show that the value of elongation at break is
210 very high. Nonetheless, this property could be reduced almost six times from the initial value by
211 using thermal treatment, with a reduction of standard deviation. The value of tension increased
212 five times from the initial value and this growth caused a decrease in the film elongation. The
213 probable cause is that thermal treatment improved tension strength: the movement of molecular
214 chain is not free anymore, due to the presence of the reticulation chain generated by the heating.
215 On the other hand, the values of elongation at break are confrontable to some commercial and
216 semi-commercial biobased materials (such as polylactate, wheat starch and corn starch) or
217 comparable to conventional materials such as LDPE and HDPE (Petersen, Nielsen & Olsen, 2001).

218 *3.4. FT-IR analyses*

In Figure 5 are reported the spectra of the film compared to pure keratin and pure citric acid. In the spectrum of keratin, the absorption bands related to peptide bonds are shown. The amide A band at 3310 cm^{-1} was assigned to the stretching vibrations of N-H bonds. The amide I band at 1650 cm^{-1} and the amide II at 1540 cm^{-1} were related to the stretching vibrations of C=O bonds, and to the in plane bending modes of N-H bonds, with some contributions of C-N stretching vibrations, respectively. The amide III at 1200 cm^{-1} was a complex band assigned to an in-phase combination of N-H in plane bending, C-N stretching vibrations, C-C stretching and C=O bending vibrations (Cardamone, 2008), but it also depended on the nature of the side-chain groups and hydrogen bonding (Jackson & Mantsch, 1995). Finally, the intense peak at 1025 cm^{-1} was attributed to the stretching vibration of the Bunte's salt residues (Erra et al., 1997).

Citric acid has IR band at 3291 cm^{-1} associated with the OH stretching mode. The two strong bands at 1745 and 1698 cm^{-1} are assigned to C=O stretching modes. The bands at 1389, 1242, 1174, 1140 cm^{-1} corresponding to C-O stretching modes (Bichara, Lanús, Ferrer, Gramajo & Brandán, 2011).

The spectrum of the film of keratin with citric acid shows an overlapping of the two previously described spectra. It is possible to notice one additional peak at 1716 cm^{-1} compared to the spectrum of pure keratin due to the presence of citric acid with variation in the wavenumber in the neighboring peaks. In order to study the effect of citric acid in the keratin protein, the peak of amide I was analyzed, because this band is known to be sensitive especially to the secondary structure of proteins (Surewicz, Mantsch & Chapman, 1993). Citric acid is known for its high capacity to generate hydrogen bonds (Ortega-Toro, Collazo-Bigliardi, Talens & Chiralt, 2016; Ghanbarzadeh, Almasi & Entezami, 2011; Reddy & Yang, 2010; Shi et al., 2008; Jiugao, Ning & Xiafoei, 2005) and its presence could change the content of macromolecular structure of keratin.

The peak of amide I was resolved into Gaussian bands, which number was defined by the second order derivative spectrum (Figure 6 (a)). Consequently, at these bands were assigned their correspondent area percentage (calculated respected to the total peak area). Furthermore, the peak of amide II was analyzed (Figure 6 (b)) in order to verify the results of content of macromolecular structure by using the amide I, because amide II peak is well known due to this band also can be sensitive especially to the secondary structures in proteins (Miyazawa & Blout, 1961; Vedantham, Gerald Sparks, Sane, Tzannis & Przybycien, 2000; Adochitei & Drochioiu, 2011).

In Figure 6, it is possible to observe the fitting peaks with Gaussians. Their area percentages were used to determine the amount of molecular structure of alpha helix, beta sheet, side chains, disordered and any additional contribution. In order to make this analysis, it was necessary to obtain the contribution of citric acid and amide I separated. The contribution corresponding only at amide I was compared with results reported in previous works (Aluigi et al., 2007; Aluigi, Corbellini, Rombaldoni, Zoccola & Canetti, 2013), as listed in Table 3. Furthermore, the results for amide II are reported in the Table 4.

Analyzing the information of the Table 3 it is possible to note that reductions in the content of side chain, beta sheet and disordered/turn values are probable. Nevertheless, a growth in the alpha helix structure is present. These results were confirmed when amide II peak was also fitted with Gaussians (Table 4).

On the other hand, it is well known that alpha and beta structures are stabilized by hydrogen bonds between NH and CO groups of the polypeptide chains. In addition, two or more alpha helices can entwine to form a very stable structure. Such as, alpha-helical coiled coils are found in myosin and tropomyosin in muscles, in fibrin in blood clots, and in keratin in hair. The helical cables in these proteins have a mechanical role in forming stiff bundles of fibers (Berg, Tymoczko & Stryer,

2002). From this point of view, changes in the molecular conformation of alpha helix and beta sheet are likely due to the high content of hydrogen bonds originating in citric acid that can replace the hydrogen bonds present between different keratin chains and between keratin chain and water molecules. In this way, the growth of alpha helix and the decrease in all other structure are possible.

In addition, the mechanical response of alpha-keratin has been extensively studied and shows, in some cases, a high elastic deformation (Wang, Yang, McKitrick & Meyers, 2016). As a result, mechanical properties of the keratin/citric acid film improve in comparison to films of pure keratin (Aluigi et al., 2007), which is probably consequence of high content of alpha structure originating in citric acid. In addition, the tensile strength could be increased by thermal treatment because of changes in the moisture content and the amount of reticulation in the protein chains of keratin.

3.5. Antibacterial test

Antimicrobial action of citric acid has been reported in literature (In, Kim, Kim & Oh, 2013; Pundir, & Jain, 2011). In this work, the biocidal effect of citric acid was evaluated on the film following the AATCC 100 Test Method against *S. aureus* (Gram positive). The test confirmed an excellent antibacterial property of the film, before and after immersion in water at 5 and 60 minutes, with a bacterial reduction of 100%.

3.6. Qualitative assessment carrot shelf-life

In the Figure 7 the results of carrot preservation with a film for preserving food and keratin/citric acid film are reported. Firstly, in the control sample, the carrot suffered mainly a drying process that can be observed from the first day and it is kept up eleven subsequent days. Using the film for preserving food, carrot has been conserved for one day, but after eleven days it suffered a drying and oxidation process that is evidenced by the dark color of the surface. Finally, in the case of carrot packed with the keratin/citric acid film, after the first day carrot has been conserved but it

289 released a little amount of water that was evidenced in the support paper underneath it. After eleven
290 days, carrot seemed to be conserved with a change in the physic form that could correspond a
291 drying process. At the end of the test, after 51 days, the observations are confirmed. As a result,
292 the effect of citric acid contented in the film on the carrot surface and on the qualitative assessment
293 carrot shelf-life is noticeable. This film can qualitatively improve the preservation of food and
294 reduce oxidation processes. It demonstrates the possible application of the keratin/citric acid film
295 for food packaging as a substitute of synthetic polymers, replacing them with a renewable source.
296

297 **4. Conclusion**

298 In conclusion, it is possible to produce by casting a plasticized wool keratin/citric acid film with
299 good characteristics of transparency and antibacterial properties. Citric acid promotes the
300 formation of macromolecular arrangements of keratin rich in alpha helix that leads to a molecular
301 structure with improved elongation and flexibility in the film. The resulting material presents high
302 value of elongation at break (higher than 600%) under mechanical tests. Moreover, the film shows
303 an inherently and complete biocidal action. Additionally, the immersion of this film in water
304 showed a small keratin loss (10% wt.) and almost complete citric acid loss. Finally, it was
305 demonstrated that this film can qualitatively improve the shelf-life of carrot. As a result,
306 keratin/citric acid films can be used as substitute of synthetic polymers for packaging, replacing
307 fossil fuels with renewable sources and bio-based products in plastic goods. Therefore, this
308 material could have practical applications in food industry as active packaging.

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REFERENCES

Adochitei, A. & Drochioiu, G. (2011). Rapid Characterization of Peptide Secondary Structure by FT-IR Spectroscopy. *Rev. Roum. Chim.*, *56(8)*, 783-791.

Aluigi, A., Zoccola, M., Vineis, C., Tonin, C., Ferrero, F. & Canetti, M. (2007). Study on the structure and properties of wool keratin regenerated from formic acid. *Int. J. Biol. Macromol.*, *41*, 266-73.

Aluigi, A., Vineis, C., Tonin, C., Tonetti, C., Varesano, A. & Mazzuchetti, G. (2009). Wool keratin-based nanofibres for active filtration of air and water. *J. Biobased Mater. Bioenergy*, *3*, 1-9.

Aluigi, A., Tonetti, C., Vineis, C., Tonin, C. & Mazzuchetti, G. (2011). Adsorption of copper (II) ions by keratin/PA6 blend nanofibres. *Eur. Polym. J. 47*, 1756-1764.

Aluigi, A., Tonetti, C., Vineis, C., Varesano, A., Tonin, C. & Casasola, R. (2012). Study on the adsorption of chromium (VI) by hydrolyzed keratin/polyamide 6 blend nanofibres. *J. Nanosci. Nanotechnol. 12*, 7250-9.

Aluigi, A., Corbellini, A., Rombaldoni, F, Zoccola, M. & Canetti, M. (2013). Morphological and structural investigation of wool-derived keratin nanofibres crosslinked by thermal treatment. *Int. J. Biol. Macromol. 57*, 30-37.

Aluigi, A., Rombaldoni, F., Tonetti, C. & Jannoke, L. (2014). Study of methylene blue adsorption on keratin nanofibrous membranes. *J. Hazard. Mater., 268*, 156-165.

Appendini, P. & Hotchkiss, J.H. (2002). Review of antimicrobial food packaging. *Innovative Food Sci. Emerging Technol. 3* 113–126.

Barbooti, M.M. & Al-Sammerrai, D.A. (1986). Thermal decomposition of citric acid. *Termochimica Acta., 98*, 119-126.

Berg, J.M., Tymoczko, J.L. & Stryer, L. (2002). Secondary Structure: Polypeptide Chains Can Fold Into Regular Structures Such as the Alpha Helix, the Beta Sheet, and Turns and Loops. *In Biochemistry Section 3.3* ; 5th edition; W H Freeman: New York, . http://www.ncbi.nlm.nih.gov/books/NBK22580/ Accessed 03.04.16.

Bichara, L.C., Lanús, H.E., Ferrer, E.G., Gramajo, M.B. & Brandán, S.A. (2011). Vibrational study and force field of the citric acid dimer based on the SQM methodology. *Adv. Phys. Chem., 2011*, 1-10.

Cardamone, J. M. (2008). Keratin transamidation. *Int. J. Biol. Macromol., 42*, 413-9.

Caro, N., Medina, E., Díaz-Dosque, M., López, L. & Abugoch, L. (2016). Novel active packaging based on films of chitosan and chitosan/quinoa protein printed with chitosan-tripolyphosphate-thymol nanoparticles via thermal ink-jet printing. *Food hydrocolloids, 52*, 520-532.

Erra, P., Gomez, N., Dolcet, L. M., Julia, M. R., Lewis, D.M. & Willoughby, J. H. (1997). FTIR analysis to study chemical-changes in wool following a sulfitolysis treatment. *Text. Res. J., 67*, 397-401.

Fabra, M.J., López-Rubio, A. & Lagaron, J.M. (2016). Use of the electrohydrodynamic process to develop active/bioactive bilayer films for food packaging applications. *Food hydrocolloids, 55,* 11-18.

Fraser, R. D. B., MacRae, T. P. & Rogers, G. E. (1972). *Keratins: Their Composition, Structure and Biosynthesis*. (pp. 23). Thomas: Springfield, IL.

Ghanbarzadeh, B., Almasi, H. & Entezami, A.A. (2011). Improving the barrier and mechanical properties of corn starch-based edible films: Effect of citric acid and carboxymethyl cellulose. *Ind. Crops Prod.*, *33*, 229-235.

Hegyi, G., Kardos, J., Kovacs, M., Malnasi-Csizmadia, A., Nyitray, L., Pal, G., Radnai, L., Reményi, A. & Venekei, I. (2013). Absorption spectrum of protein. *In Introduction to Practical Biochemistry- Section 4.6.4* [Online]; Eotvos Lorand University. http://elte.prompt.hu/sites/default/files/tananyagok/IntroductionToPracticalBiochemistry/index.html Accessed 03.04.16.

Held, P. (2016). BioTek. Applications – Application notes: Peptide and amino acid quantification using UV fluorescence in synergy HT multi-mode microplate reader. http://www.biotek.com/resources/articles/peptides-amino-acids-fluorescence.htmln Accessed 03.04.16.

In, Y.W., Kim, J.J., Kim, H.J. & Oh, S.W. (2013). Antimicrobial activities of acetic acid, citric acid and lactic acid against shigella species. *J. Food Saf., 33*, 79-85.

Jackson, M. & Mantsch, H. H. (1995). The use and misuse of FTIR spectroscopy in the determination of protein structure. *Crit. Rev. Biochem. Mol. Biol., 30*, 95-120.

Jiugao, Y., Ning, W. & Xiafoei, M. (2005). The effects of citric acid on the properties of thermoplastic starch plasticized by glycerol. *Starch., 57*, 494-504.

Long, N.N.V. Joly, C. & Dantigny, P. (2016). Active packaging with antifungal activities. *Int. J. Food Microbiol., 220*, 73-90.

Miyazawa, T. & Blout, E.R., (1961). The Infrared Spectra of Polypeptides in Various Conformations: Amide I and II bands. *J. Am. Chem. Soc., 83*, 712-719.

Ortega-Toro, R., Collazo-Bigliardi, S., Talens, P. & Chiralt, A. (2016). Influence of citric acid on the properties and stability of starch-polycaprolactone based films. *J. appl. Polym. Sci.,* 42220.

Petersen, K., Nielsen, P.V. & Olsen, M.B. (2001). Physical and mechanical properties of biobased materials-starch, polylactate and polyhydroxybutyrate. *Starch., 53*, 356-361.

Pundir, R.K. &Jain, P. (2011). Evaluation of five chemical food preservatives for their antibacterial activity against bacterial isolates from bakery products and mango pickles. *J. Chem. Pharm. Res., 3*, 24-31.

Reddy, N. & Yang, Y. (2010). Citric acid cross-linking of starch films. *Food Chem., 118*, 702-711.

Schmid, F.X. (2001). Biological Macromolecules: UV-visible spectrophotometry, *eLs.*.

Shi, R., Bi, J., Zhang, Z., Zhu, A., Chen, D., Zhou, X., Zhang, L. & Tian, W. (2008). The effect of citric acid on the structural properties and cytotoxicity of polyvinyl alcohol/starch films when molding at high temperature. *Carbohydr. Polym., 74*, 763-770.

Soccol, C.R., Vandenberghe, L.P.S., Rodrigues, C. & Pandey, A. (2006). New perspectives for citric acid production and applications. *Food Technol. Biotechnol.*, *44*, 141-149.

Surewicz, W.K., Mantsch, H.H. & Chapman, D. (1993). Determination of protein secondary structure by fourier transform infrared spectroscopy: a critical assessment. *Biochemistry.*, *32*, 389-94.

Tachibana, A., Furuta, Y., Takeshima, H., Tanabe, T. & Yamauchi, K. (2002). Fabrication of wool keratin sponge scaffolds for long-term cell cultivation. *J. Biotechnol.*, *93*, 165-170.

Tachibana, A., Kaneko, S., Tanabe, T. & Yamauchi, K. (2005). Rapid fabrication of keratin-hydroxyapatite hybrid sponges toward osteoblast cultivation and differentiation. *Biomaterials*, *26*, 297-302.

Varesano, A., Vineis, C., Tonetti, C., Sanchez Ramirez, D.O., Mazzuchetti, G. (2014). Chemical and physical modifications of electrospun keratin nanofibers induced by heating treatments. *J. Appl. Polym. Sci.* 40532.

Varesano, A., Vineis, C., Tonetti, C., Sanchez Ramirez, D.O., Mazzuchetti, G., Ortelli, S., Blosi, M. & Costa, A.L. (2015). Multifunctional hybrid nanocomposite nanofibers produced by colloid electrospinning from water solutions. *Curr. Nanosci.*, *11*, 41-48.

Vedantham, G., Gerald Sparks, H., Sane, S.U., Tzannis, S. & Przybycien, T.M. (2000). A Holistic Approach for Protein Secondary Structure Estimation from Infrared Spectra in H_2O Solutions. *Analytical Biochemistry*., *285*, 33-49.

411 Wang, B., Yang, W., McKitrick, J. & Meyers, M.A. (2016). Keratin: structure, mechanical
412 properties, occurrence in biological organisms, and efforts at bioinspiration. *Prog. Mater. Sci., 76,*
413 229-318.

414 Yamauchi, K., Maniwa, M. & Mori, T. (1998). Cultivation of fibroblast cells on keratin-coated
415 substrata. *J. Biomater. Sci. Polym*., Ed. 9, 259.

416

Figure captions

Figure 1. (a) Spectrum UV-VIS of keratin/citric acid film; (b) Picture of the keratin/citric acid film.

Figure 2. Absorbance values in UV-VIS of water after film immersion with dilution 1:10 at different times (a) 275 nm, related to aromatic rings of proteins in water; (b) 214 nm, related to carboxylic groups of both citric acid and peptide bonds.

Figure 3. Thermal graphs for pure keratin and keratin/citric acid film. (a) TGA pure keratin and keratin/citric acid film; (b) and (d) DTG and DSC for pure keratin, respectively; (c) and (e) DTG and DSC for keratin/citric acid film, respectively.

Figure 4. Tensile strength test of keratin/citric acid film. On the right: at the beginning of the test; on the left: before the film breaking.

Figure 5. Spectrum FT-IR of keratin/citric acid film, pure keratin and pure citric acid

Figure 6. Fitting peaks with Gaussians in amide I (a) and amide II (b)

Figure 7. Carrot shelf-life: film for preserving food (Film (a)) and keratin/citric acid film (Film (b)) in comparison with a piece of unpacked carrot (Control)

Tables

Table 1. Results after 2 h water immersion at different volumes.

	20 ml	**5 ml**
Total lost weight (%)	56 ± 5	57 ± 5
Lost citric acid (%)	92 ± 8	97 ± 3
Lost keratin (%)	12 ± 3	6 ± 1
Protein concentration (g l^{-1})	0.28 ± 0.06	0.46 ± 0.11
pH*	2.88 ± 0.03	2.61 ± 0.12

*The initial pH of water was 5.96 (20°C).

Table 2. Values of tensile strength test

Thermal treatment conditions	Tensile strength (MPa)	Elongation at break (%)
As cast (untreated)	0.28 ± 0.19	646 ± 177
80 °C – 1 h	0.37 ± 0.19	366 ± 151
100 °C – 1 h	0.70 ± 0.36	317 ± 24
120 °C – 1 h	1.49 ± 0.80	138 ± 21

Table 3. The result of Fitting peaks with Gaussians in amide I peak

	Keratin-Citric acid 15% wt. (water) [a]		Keratin 5%wt. (water) [b]		Keratin 5%wt. (formic acid) [b]		Keratin 15%wt. (formic acid) [c]	
Band assignment	**Band position (cm^{-1})**	**Content (%)**	**Band position (cm^{-1})**	**Content (%)**	**Band position (cm^{-1})**	**Content (%)**	**Band position (cm^{-1})**	**Content (%)**
Side chains or other	1597	0.1	1596	1	1597 1586	8	1592	8
Beta sheet	1618	29.5	1620	33	1621	42	1621	36
Alpha helix	1651	58.1	1649	47	1650	26	1650	27
Disordered/turns	1674 1682 1691	12.4	1674 1691	19	1675 1697	22	1678	22
Other or COOH					1725	2	1725	7

[a] Present work, [b] Aluigi et al., 2007, [c] Aluigi, Corbellini, Rombaldoni, Zoccola & Canetti, 2013.

Table 4. The result of fitting peaks with Gaussians in amide II peak

	Keratin-Citric acid 15% wt. (water)	
Band assignment	**Band position (cm^{-1})**	**Content (%)**
Other	1502	4.7
Random coil	1535	1.0
Beta sheet	1524	29.3
	1529	
	1556	
Alpha helix	1516	59.1
	1541	
Turns	1549	5.9
	1564	

Figure graphics

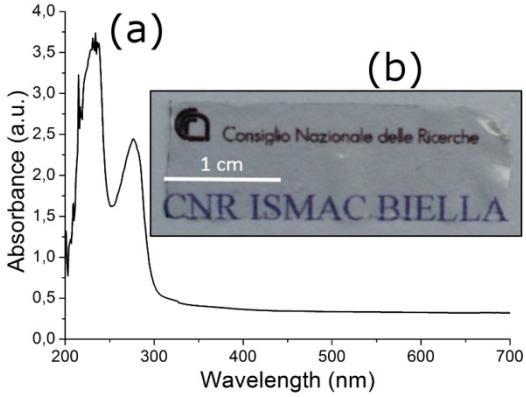

Figure 1

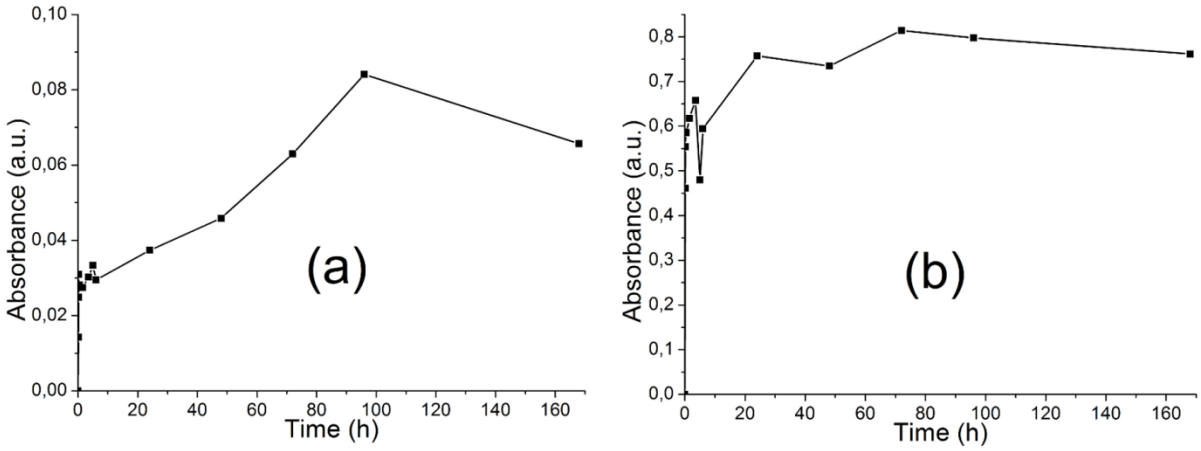

Figure 2

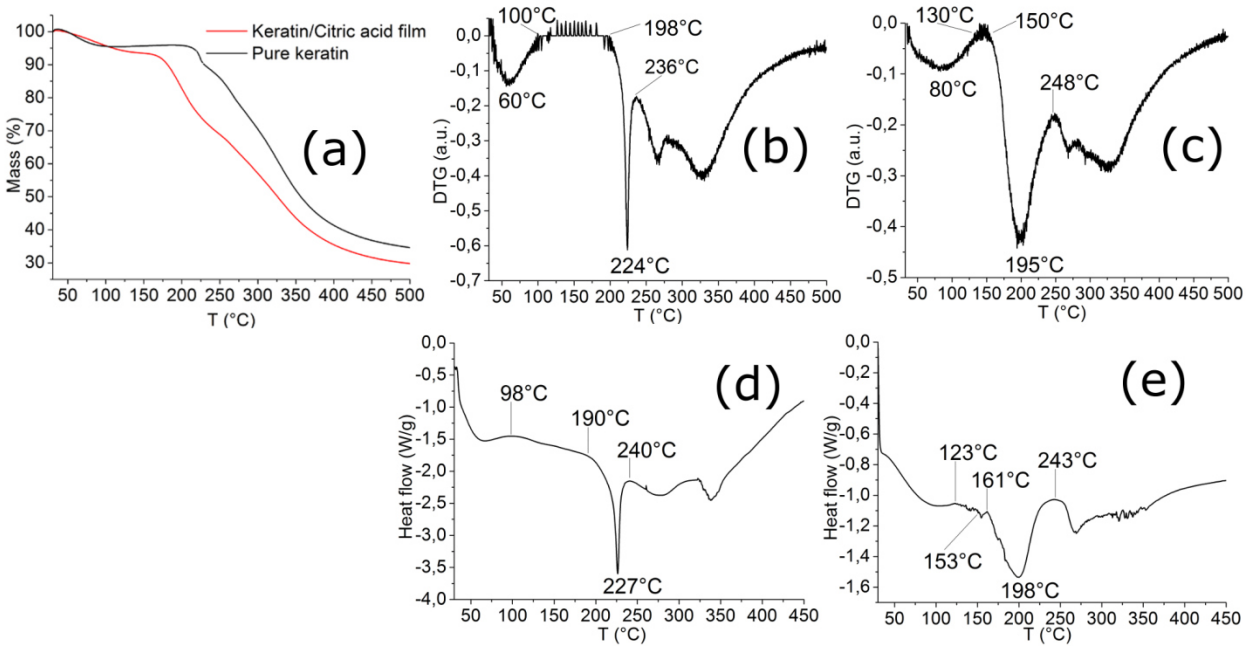
Keratin/Citric acid film
Pure keratin
(a)
Mass (%)
T (°C)
100°C
60°C
198°C
236°C
224°C
(b)
DTG (a.u.)
130°C
80°C
150°C
248°C
195°C
(c)
98°C
190°C
240°C
227°C
(d)
Heat flow (W/g)
123°C
161°C
153°C
243°C
198°C
(e)

Figure 3

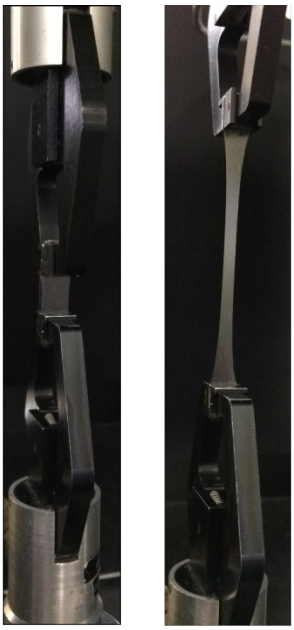

Figure 4

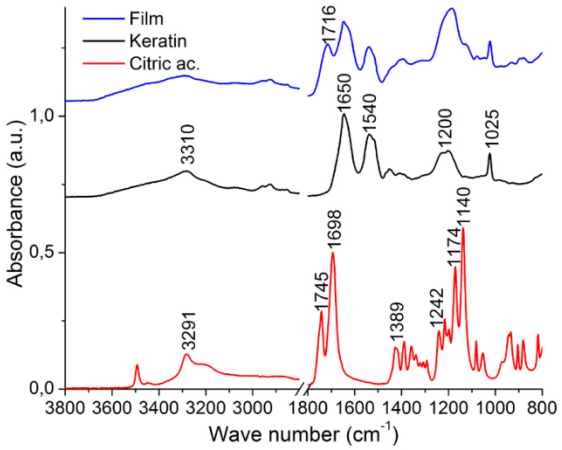
Film
Keratin
Citric ac.
1716
1650
1540
1200
1025
3310
1698
1745
1174
1140
1389
1242
3291
Absorbance (a.u.)
1,0
0,5
0,0
3800 3600 3400 3200 3000 1800 1600 1400 1200 1000 800
Wave number (cm-1)

Figure 5

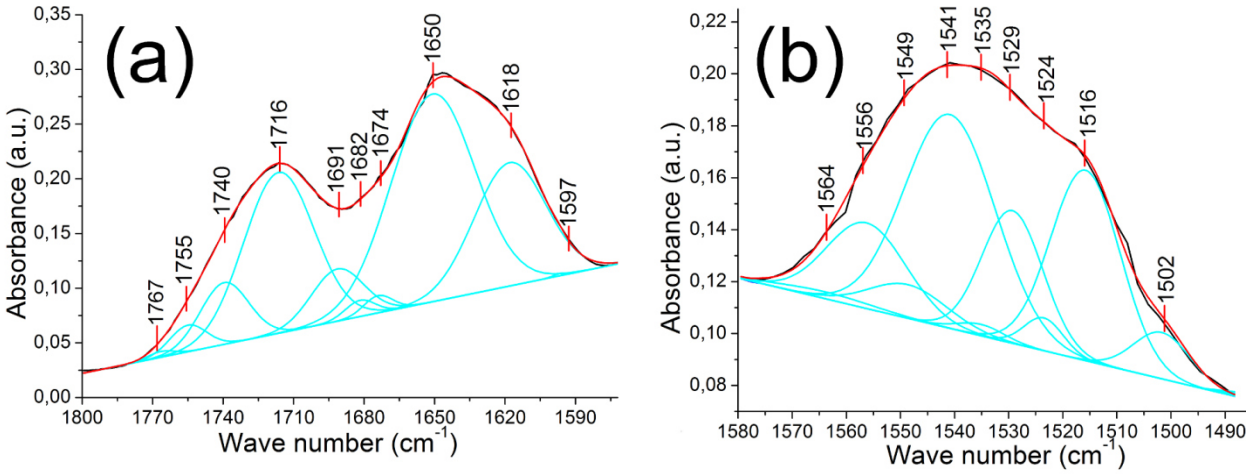
(a)
Absorbance (a.u.)
Wave number (cm-1)
1767
1755
1740
1716
1691
1682
1674
1650
1618
1597
(b)
Absorbance (a.u.)
Wave number (cm-1)
1564
1556
1549
1541
1535
1529
1524
1516
1502

Figure 6

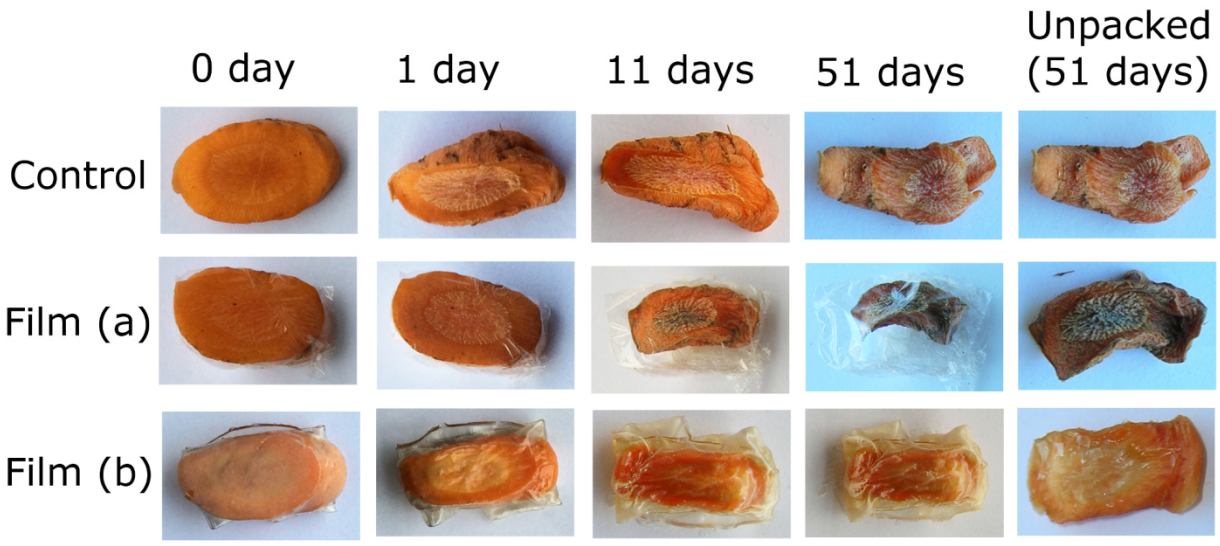

Figure 7

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