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Abstract

In the current study, composite films based on blue crab chitosan (Cs) and bluefin tuna collagenous proteins (BTCP) were prepared and characterized. Composite films were assessed for their physicochemical, barrier (water vapor and UV), structural, crystallinity, thermal, mechanical and antioxidant properties. FTIR analysis showed an increase of hydrogen bonding formation between polymers with the increase of BTCP content. Additionally, the addition of BTCP to the Cs solution at a volume ratio of 10:90, consequently forming the Cs90-10BTCP film, improved the water vapor permeability and UV barrier capability of the composite films compared to the chitosan film. Interestingly, the incorporation of BTCP improved significantly the antioxidant activity of composite films, allowing them to be successively used as bioactive packaging materials. The ability of Cs-BTCP film solutions as a coating for shrimp preservation was also investigated. The Cs90-10BTCP film solution showed better preservative effect on shrimps in terms of preventing the lipid peroxidation and delaying the growth of spoilage microorganisms. Hence, these findings suggested that the Cs-BTCP coating preserved the shrimps throughout the refrigerated storage period.

Keywords: Composite films; Shrimp coating; Postharvest shelf life.

1. Introduction

The growth of the world population, leads to a scarcity of biological resources and an increasing degradation of the environment, hence the need for innovation in the food sector

(Kaanane and Mkadem 2020). To minimize food loss and waste in the fish value chains and, at the same time, improve fish waste management strategies, several methods can be used to convert by-products into value added products: animal feed ingredients (fishmeal and fish oil), dietetic products (chitosan), pharmaceuticals (omega-3 oils) and constituents in other industrial processes (Al Khawli et al. 2019; Caruso et al. 2020).

The valorization of marine by-products should be a necessity to guarantee global food security and satisfy of the growing demand for fishery products (Rudovica et al. 2021). Marine by-products (viscera, heads, bones, cartilages, skin, scales, shells, damaged fish), produced by marine processing industries (Hamed et al. 2016), are a good alternative to high-value products (Galiano et al. 2018).

Crustacean products represent a valuable source of proteins, chitin (Hajji et al. 2014), minerals, carotenoids (Hamdi et al. 2019), flavors, nutritive components and enzymes (Affes et al. 2019). Therefore, making use of such waste was due to their biological and economical values (Ozogul et al. 2018). The seafood industry generates about 2,000 tons of chitosan annually, whose main source of extraction is from crustacean exoskeletons (Muñoz et al. 2018; Santos et al. 2020).

In this context, chitosan, a natural polymer obtained from blue crab shell, plays an important role due to its outstanding characteristics such as nontoxicity, low allergenicity, biocompatibility, and biodegradability. It has been evaluated as a biomaterial for various applications (Aranaz et al. 2021). Chitosan, consisting of units of D-glucosamine and N-acetyl-D-glucosamine, linked by β-1,4-glycosidic bonds, is a cationic polysaccharide obtained by deacetylation of chitin (Hamdi et al. 2020). The cationic nature of chitosan allows it to form electrostatic complexes or multilayer structures with other negatively charged natural polymers.

Chitosan is known as an excellent film forming polymer (Wang et al. 2020). The chitosan films have been extensively studied for their good barrier properties against oxygen, water and lights (Kittur et al. 1998; Melro et al. 2020). Despite these advantages, Cs-based films present some drawbacks in food packaging, such as brittleness, poor mechanical resistance, low thermal stability, and sensitivity to moisture (Aider 2010; Yuan et al. 2021).

Therefore, to enhance the performance of the Cs-based film, the blending strategy with other biopolymers would be an alternative approach to improve the film properties (Zhang et al. 2020; Li et al. 2020). Always, films made by mixing polymers usually have enhanced physical and mechanical properties compared to films made of individual components.

Fish protein isolates can be prepared from protein isolation process of fish by-product. The use of protein isolate has already been used not only as food additive but also as film-

forming materials (Valdivia-López et al. 2016; Mihalca et al. 2021). However, fish protein isolates-based films have certainly poor properties. Thus, many studies have been made to show the effect of blending marine protein isolates with chitosan on improving different properties of Cs-based film (Zhang et al. 2019; Azaza et al. 2022).

The composite films, with improved properties, are currently attracting increasing interest in the development of packaging materials for food preservation. The potential of edible coatings is to improve the quality of food products by delaying lipid oxidation, preventing loss of protein functionality and reducing bacterial growth (Barlow and Morgan 2013).

In this study, the head and cartilage of bluefin tuna (*Thunnus thynnus*) were used as a by-product model of fish processing and were valued by the protein isolation process, which is currently the most processed axis for producing biopolymers. This approach involves solubilizing/precipitating of the collagenous protein at low pH separating the soluble proteins. It could be an excellent and viable practice to effectively value this new biomass. As a natural protein, collagenous protein, extracted from by-products of bluefin tuna (*T. thynnus*), was used in polymer-based films due to its functional and antioxidant properties. Cs and BTCP can be used as a potential biological matrix for the development of composite film combined at different ratios, with interesting properties, widely used for various applications. Additionally, this study was carried out to investigate the effect of Cs-BTCP coating on the chemical, microbiological and lipid oxidative changes of shrimp during refrigerated storage was evaluated. This research is of special importance in the sectors of the blue and circular economy and the sustainable use of marine by-products. For this reason, this study aims to valorize these by-products, which can be used as a potential biological matrix for the extraction of bioactive substances with high added value exploitable in several fields.

2. Materials and methods

2.1. Materials

Blue crab samples were obtained under fresh conditions from a fishery market located at Sfax City, Tunisia.

Bluefin tuna by-products were collected from the "The Sultan" tuna fishery in Sfax City, Tunisia. Glycerol and acetic acid were purchased from Sigma-Aldrich. DPPH (2, 2-diphenyl-1-picrylhydrazyl), ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine), ferrous chloride, ferrous ion, α-tocopherol, potassium ferricyanide, trichloroacetic acid (TCA), sodium chloride (NaCl), ammonium thiocyanate, Tris, butylated hydroxytoluene (BHT) and 2/thiobarbituric acid (TBA) were procured from Sigma Chemical Co. (St. Louis, MO, USA).

Hexane, hydrochloric acid (HCl), chloroform and methanol of reagent grade were purchased from Sigma Chemical Co.

2.2. Preparation of blue crab chitosan

Chitosan was extracted from blue crab (*P. segnis)* shells as described by Azaza et al. (2022). Blue crab shells were carefully separated and washed with tap water to remove impurities and cooked for 20 min at 90 °C. Then, the cooked shells were dried at room temperature and powdered in a Moulinex® blender. In the Cs preparation process, there must be at least two distinct steps, namely a demineralization (mineral removal) step, performed chemically in a reactor under agitation and in a solution of HCl (0,55 M) at a ratio of 1/10 (m/v), using three repeated acid baths, for a duration of 30 min each bath and a deproteinization step (protein removal), was carried out using Purafect® for 3 hours with an enzyme/substrate ratio of 5 U/mg protein and under optimal enzyme conditions (pH 10.0 and temperature 50 °C). Finally, the conversion of chitin to chitosan was realized with the treatment of with 12.5 M NaOH at a w/v ratio of 1/10 to 140 °C. After filtration, the residue was washed with distilled water until the pH was neutral, and the crude chitosan was dried at 50 °C for overnight.

2.3. Characteristics of blue crab chitosan

The degree of deacetylation (DD) of Cs was determined by using the direct titration method as described by Hamdi et al. (2018). Briefly, chitosan samples (0.1 g) were dissolved in 25 mL of 0.06 M HCl for 1 h at room temperature. Before being titrated with a 0.1 M NaOH to pH 3.75 under constant stirring, the solutions were diluted to 50 ml with distilled water. The volume of NaOH at pH 3.75 was acquired and recorded. Titration was continued to pH 8 and the total volume of NaOH (0.1 M) was recorded. The degree of deacetylation (DD) was, then, calculated using the following equations:

$$DD\ (\%) = 161.16 \times (V2 - V1) \times N / W1 \qquad \textbf{Eq (1)}$$

where, 161.16 is the mass (g mol^{-1}) of chitosan monomer ; V1 and V2 are the volumes required at pH 3.75 and pH 8.0, respectively, of NaOH solution used (in L); N is the strength of the NaOH solution (0.1 M) and W1 is the mass (in g) of sample after correction for moisture. The degree of deacetylation (DD) of the samples was determined in triplicate.

Steric Exclusion Chromatography (SEC) was performed using multi-detector equipment with a differential refractometer, a multiangle laser light scattering detector and a viscometer from WYATT Technology (DAWN DSP-F). Acetic acid 0.3 M/sodium acetate 0.2 M (pH = 4.5) was adopted as solvent (at 25 ± 2 °C). TSK Gel GMPWXL column type was used and a flow of 0.4 ml/min was adopted. The increment of refractive index dn/dc was 0.190

(Hamdi et al. 2020). Resulted chromatograms were analyzed with ASTRA 6.1.2 (WYATT Technology) software and weight-average molecular weights (Mw; g mol^{-1}), were determined.

The DD of Cs, estimated by potentiometric titration, was found to be significantly high (90.39 ± 1.36 %) as mentioned by Hamdi et al. (2018). Further, the average molecular weight of the obtained Cs was estimated to be 115 000 g mol^{-1}, based on the size exclusion chromatography (SEC) analysis (Hamdi et al. 2020).

2.4. Preparation of bluefin tuna collagenous proteins

The extraction of collagenous proteins was carried out as follows: first of all, the bluefin tuna by-products (equal weight ratio of head and cartilage) were grounded and then mixed with distilled water at a ratio of 1:10 (w/v) using an Ultra-turrax® apparatus (IKA, T18 basic). Afterward, the pH of the mixture was adjusted to 5.0 using 1 M HCl solution, and the mixture was kept under constant stirring at 50 °C overnight. The soluble collagenous proteins were recovered in the supernatant after centrifugation at 6000 rpm for 30 min. Finally, the pH of the supernatant was adjusted to 7.0 using 2 M NaOH solution, and the bluefin tuna collagenous proteins (BTCP) were spray-dried using an atomizer (BUCHI B-290, Arch Spray Drying Services, USA).

The extraction yield of BTCP was calculated as follows,

$$Yield\ (\%) = \frac{W}{W_0} X\ 100 \qquad \textbf{Eq (2)}$$

where W is the weight of dried BTCP (g), and W_0 the wet weight of fresh bluefin tuna by-products (g).

2.5. Chemical analysis of BTCP

The dry matter, moisture, and ash contents of BTCP were determined according to the AOAC standard method (Horwitz 2000). Proteins content of dried BTCP was measured using the Kjeldahl method and the method developed by Lowry et al. (1951). All measurements were performed in triplicate.

2.6. Preparation of Cs-BTCP composite films

Film-forming solutions were prepared as reported in our previous study (Azaza et al. 2022), by dissolving Cs (2%; w/v) in 1% (v/v) aqueous acetic acid and BTCP (2%; w/v) in distilled water, under continuous magnetic stirring at room temperature **(Fig. 1)**. After complete solubilization, both polymer solutions were filtered to remove insoluble impurities, and then glycerol, as a plasticizer, was added to both solutions at a level of 15% (w/w compared to

polymer), respectively. Next, the solutions were blended by mixing under gentle stirring at different Cs-BTCP volume ratios. The film-forming solutions were poured into Petri dishes (25 ml) and allowed to dry under an air flow at 25 °C for 48 h. The dried Cs/BTCP films were peeled off carefully and stored at 25 °C and a relative humidity (RH) of 50%, for further analysis. The obtained composite films were designated as Cs90–10BTCP, Cs80–20BTCP, Cs70–30BTCP, Cs60–40BTCP and Cs50–50BTCP. Pure chitosan (Cs100) films were prepared as control.

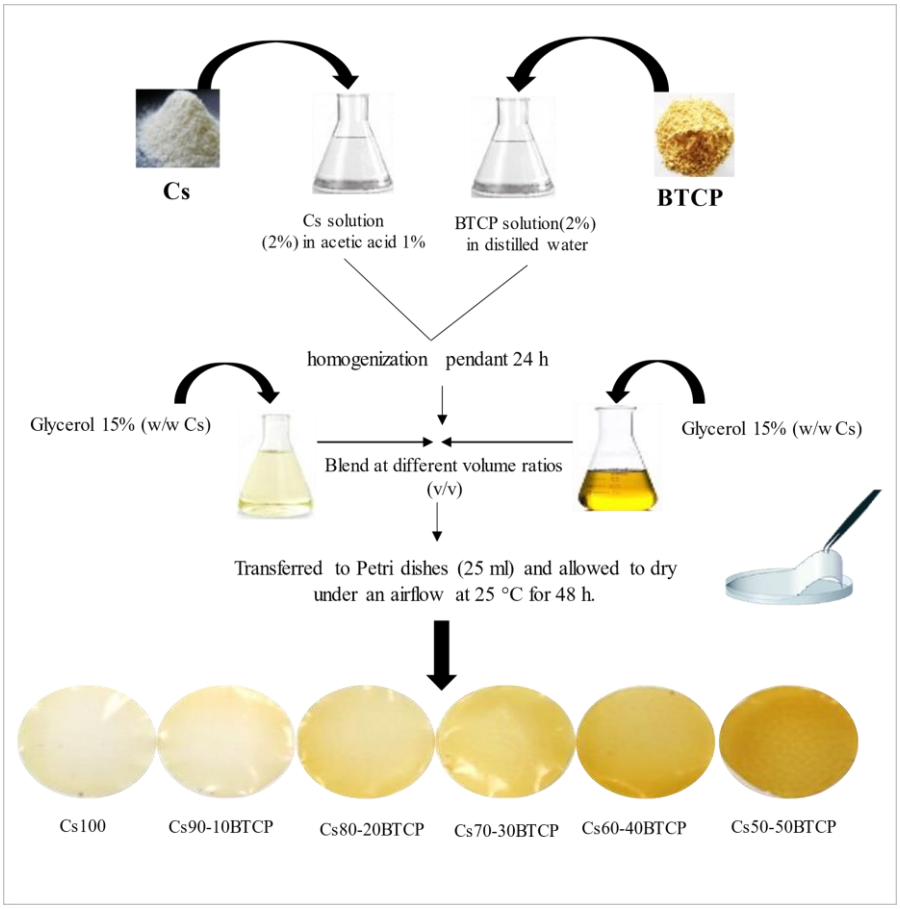


Fig. 1 Preparation scheme of Cs-BTCP composite films

2.7. Characterization of Cs-BTCP composite films

2.7.1. Moisture content and water solubility

Moisture content (MC) was evaluated by drying approximately 100 mg of film samples in an oven at 105 °C up to constant weight. The weights of films before and after drying were measured and the MC of films was calculated as follows:

$$MC\ (\%) = \frac{W0-W}{W0}\ X\ 100 \qquad \textbf{Eq (3)}$$

where W_0 is the initial film weight (g) and W is the final film dry weight (g). Three replicates of each film were carried out.

Water solubility (WS) of Cs-BTCP composite films was assayed as reported by Gennadios et al. (1998) . Briefly, Small pieces of the films (100 mg) were placed in centrifuge tube containing 50 ml distilled water at 25 °C and then shaken for 24 h. After centrifugation (8000 rpm – 15 min), the residual pieces of films were dried at 50 °C for 24 h. The WS was calculated as follows:

$$WS\ (\%) = \frac{[W0\ x\ (100 - MC) - Wf]}{[W0\ x\ (100 - MC)]}\ \text{X}\ 100 \qquad \textbf{Eq (4)}$$

where W_0 is the initial film weight (g), W_f is the final dry weight of film (g), and MC is the moisture content of films. All experiments were performed in triplicates.

2.7.2. Swelling degree

The swelling degree (SD) of Cs-BTCP composite films was determined according to the method reported by Khan and Ranjha, (2014). Film samples (30 mg) were immersed in 10 mL distilled water for 24 h at 25 °C. After incubation, excess water was removed and the films were carefully dried on filter papers and weighed again. Swelling degree was calculated as follows:

$$SD\ (\%) = \frac{\text{Wf}-\text{Wi}}{\text{Wi}}\ \text{X}\ 100 \qquad \textbf{Eq (5)}$$

where W_i is the initial weight of the film (g) and W_f is the final weight of the swollen film (g). The weight measurements were performed in triplicate

2.7.3. Water/vapor permeability (WVP)

Water vapor permeability (WVP) was evaluated gravimetrically according to ASTM E96-95 standards. The film samples (75 × 75 mm) were conditioned at 25 °C and 50% RH for a minimum equilibration time of 48 h and thickness was measured for all samples after equilibration. The WVP cup was placed in a humidity chamber (KBF 240 Binder, ODIL, France) maintained at 25 °C and 50% RH. The change in the weight of the cup was measured at regular intervals of 1 h over 10 h. The WVP (g m^{-1} s^{-1} Pa^{-1}) was calculated by the following equation:

$$\text{WVP} = \frac{\Delta\text{w}\ \text{X}\ \text{e}}{\text{A}\ \text{X}\ \Delta\text{t}\ \text{X}\ \Delta\text{P}} \qquad \textbf{Eq (6)}$$

where Δw is the weight variation of the cup (g); e is the film thickness (m); A is the film area exposed to the transfer (1.39×10^4 m^2); Δt is the time of weight variation (s); ΔP = (p2-p1) is the vapor partial pressure differential across the film (Pa). All films were measured three times.

2.7.4. Light transmission and transparency

The Cs-BTCP composite films light transmittance was determined at wavelengths ranged from 200 to 800 nm using UV/visible spectrometer (T70, UV / Vis spectrometer, PG Instruments Ltd., China) as described by Fang et al. (2002). Briefly, films were cut into rectangular samples (1 x 3 cm) and directly attached on the internal side the spectrophotometer. The transparency was determined at 600 nm and calculated as follows:

$$\text{Transparency} = \frac{\text{LogA600}}{\text{t}} \qquad \textbf{Eq (7)}$$

where A600 is the film absorbance at 600 nm and t is the film thickness (μm). Measurements were carried out in triplicate.

2.7.5. Color properties

Film color properties were measured using a colorimeter Konica Minolta CR/5 (Sensing Europe B.V) to obtain parameters L* (lightness/brightness), a* (redness/greenness), b* (yellowness) and ΔE* (total color difference). The control film (Cs100) was used as a reference for color measurements of the films. The measurements were repeated five times for each sample. The color difference $\triangle E$ was calculated by using the following equation:

$$\triangle E* = [(\triangle L^*)^2 + (\triangle a^*)^2 + (\triangle b^*)^2]^{1/2} \qquad \textbf{Eq (8)}$$

where ΔL*, Δa* and Δb* are the differences between the corresponding color parameter of the sample and that of the control film.

2.7.6. Fourier transform infrared (FTIR) spectroscopy

FTIR spectra were recorded on a spectrometer (Agilent Technologies, Carry 630 series) equipped with horizontal attenuated total reflectance (ATR) crystal (diamond/ZnSe) in the wavenumber range from 650 to 4000 cm^{-1}. A total of 32 scans was made per minute at 4 cm^{-1} resolutions. All spectra were smoothed using the OMNIC Spectra software (ThermoFisher Scientific).

2.7.7. X-ray diffraction (XRD) analysis

The XRD patterns were recorded by a Bruker D5000 ray diffractometer with a radiation source of Cu Kα. Measurements were made from 7 to 40° at a scanning rate of 1°/min, a voltage

of 40 KV and a current of 20 mA. A blank run was done and subtracted subsequently from the sample data.

2.7.8. Thermogravimetric analysis (TGA)

The thermal stability of composite films was conducted by using thermogravimetric analyzer (TGA Q500 High Resolution, TA Instruments). Dynamic scans from 25 to 600 °C were carried out at a constant rate of 20 °C/min under nitrogen flow (40 mL/min). 4 mg of sample were used for each analysis.

2.7.9. Mechanical properties

Tensile strength (TS) and elongation at break (EAB) of film samples were evaluated at 25 °C using a rheometer (Physica MCR. Anton Paar. GmbH. France) equipped with a mechanical property measuring geometry. Rectangular samples (4.5 cm × 1.0 cm) were cut from films using a precision standard cutter. After equilibrating films for one week at 25 °C and 50 % RH, the thicknesses were measured. The samples were placed in the extension grips of the testing machine and stretched uniaxially with a deformation rate of 5 mm/min until breaking. Rheoplus software was used for the estimation of TS (MPa) and EAB (%) corresponding to the maximum load and the final extension at break from the stress/strain curves, respectively. The average values of at least six measurements were listed.

The thickness of Cs-BTCP composite films was measured using micrometer (Digimatic IP65, Mitutoyo, France); with an accuracy of ± 0.001 mm. Ten random measurements at different positions of each sample were used to determine the thickness of the films. Thickness results were taken into account for mechanical properties and water vapor permeability determination.

2.7.10. In vitro antioxidant activity of films

2.7.10.1. DPPH free radical-scavenging assay

The DPPH free radical scavenging activity of prepared films was evaluated according to the method of Bersuder et al. (1998), with some modifications. The films were cut into small pieces (m = 10 mg) and immersed in 375 µl of 99.5 % ethanol and 125 µl of 0.02% DPPH (in 99.5 % ethanol), as free radicals' source. The mixture was homogenized and incubated for 24 h at room temperature (25 °C) in the dark. The reduction of DPPH radicals was measured at 517 nm (T70, UV/Vis spectrometer, PG Instruments Ltd., China). Regarding the solutions of BTCP powder, it was the same process used for all film samples. The butylated hydroxyanisole

(BHA) was used as a positive control and the anti-radical activity was calculated according to the following equation:

$$\text{Radical scavenging activity (\%)} = \frac{[Abs_C + Abs_B - Abs_F]}{Abs_C} \text{ X } 100 \qquad \textbf{Eq (9)}$$

where Abs_C is the absorbance of the control reaction, Abs_B and Abs_F were the absorbance of the blank and the reaction mixture, respectively. The test was carried out in triplicate.

2.7.10.2. Metal chelating activity

The chelating activity of prepared films towards ferrous ion (Fe^{2+}) was studied as reported by Decker and Welch, (1990), with some modifications. Thus, Film samples (10 mg cut into small pieces) were mixed with 450 µl of distilled water. Then, 50 µl of 2 mM $FeCl_2$, 4 H_2O and 200 µl of 5 mM Ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine) were added. The reaction mixtures were vigorously stirred and incubated for 20 minutes at room temperature (25±2 °C). The absorbance of the solutions was measured at 562 nm. Regarding the solutions of BTCP powder, it was the same process used for all film samples. EDTA was used as positive control and the inhibition percentage of ferrozine/Fe^{2+} complex formation was calculated using the following equation:

$$\text{Metal chelating activity (\%)} = \frac{[Abs_C + Abs_B - Abs_F]}{Abs_C} \text{ X } 100 \qquad \textbf{Eq (10)}$$

where Abs_C is the absorbance of the control tube (without sample), Abs_B is the absorbance of the blank tube and Abs_F is the absorbance of the sample in the presence of Ferrozine (reaction).

2.7.10.3. Reducing power assay

The ability to reduce iron (III) was determined using the Yildirim et al. (2001) method, based on following up the ability of an antioxidant molecule to reduce ferric iron from potassium ferricyanide ($K_3Fe(CN)_6$) in ferrous iron (Fe^{2+}). Film samples (10 mg) and BTCP solutions (1-4 mg) were immersed in 1.25 ml of 0.2 M phosphate buffer (pH 6.6) and 1.25 ml of 1% (w/v) potassium ferricyanide, and incubated for 30 min at 50 °C. 1.25 ml of trichloroacetic acid 10% (m/v) was added to the mixture in order to stop the reaction. Finally, the mixture was centrifuged for 10 min at 3 500 g, and the supernatant (1.25 ml) was mixed with 1.25 ml distilled water and 0.25 ml 1% (m/v) ferric chloride. After 10 min reaction, the absorbance of the resulting solution was measured at 700 nm. Regarding the solutions of BTCP powder, it was the same process used for all film samples. The values are presented as the means of duplicate analyses. BHA was used as a standard for BTCP solutions.

2.7.10.4. Total antioxidant activity (TAA)

Total antioxidant activity was assayed using the method of Prieto et al. (1999). Briefly, films were cut into small pieces (10 mg) and immersed in Eppendorf tubes containing 0.1 ml of distilled water and 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and incubated at 90 °C for 90 min. Thereafter, the absorbance was measured at 695 nm. Regarding the solutions of BTCP powder, it was the same process used for all film samples. The total antioxidant activity of the films was expressed as α-tocopherol equivalents using the following formula:

$$C_{toc}\ (\mu\text{mol/mL}) = \frac{[OD_c\ /0.0049\ ]}{0.011} \qquad \textbf{Eq (11)}$$

where C_{toc} is the concentration as α-tocopherol equivalents (µmol/mL) and OD_S is the absorbance of samples at 695 nm.

2.8. Application of Cs-BTCP coating solutions

2.8.1. Preparation and coating treatments on shrimp

Shrimps were purchased from a local fish store in Sfax, Tunisia. Only shrimps with a uniform average weight, healthy appearance, good texture and no visible damage were selected for the experimental work. First, shrimps were washed with running water and then dried in the open air at room temperature. The shrimps were dipped into the respective coating solutions at 4 °C for 4-5 min. Then, the shrimps were placed in an air-tight sterile polyethylene boxes and stored at 4 °C for 9 days. Shrimps were randomly divided into four groups (4 shrimps per group) as follows: (**Sh**) control shrimp (uncoated) without treatment; (**Sh-1**) shrimp soaked in a coating solution of Cs (2%, w/v); (**Sh-2**) shrimp dipped in a Cs90-10BTCP coating solution; (**Sh-3**) shrimp coated with a Cs50-50BTCP coating solution. Finally, the samples from each group were randomly taken out for analysis according to the predetermined time intervals (0, 3, 6 and 9 days of storage), in three replicates.

2.8.2. Chemical evaluation

pH of the shrimp was measured as described by Keller et al. (1974) by a pH-meter using a mixture of 10 g of sample in 50 ml of distilled water.

The dry matter content was determined after evaporating the water contained in 5 g of sample at 105 °C during 24 h until a constant weight was reached (Horwitz 2000).

Color was evaluated using a colorimeter Konica Minolta CR/5 (Sensing Europe B.V) and expressed as L*, a* and b* values, referring to the measuring parameters of lightness, redness/greenness, and yellowness/blueness, respectively. Total color (ΔE) was determined as mentioned above:

$$\Delta E = \frac{[\, a^* + 1.75 \times L^*]}{[\, 5.645 \times L^* + a^* - 3.021 \times b^*]} \quad \textbf{Eq (12)}$$

2.8.3. Determination of thiobarbituric acid reactive substances (TBARS)

The thiobarbituric acid reactive substances (TBARS) test is widely used to evaluate lipid peroxidation. The TBARS values were determined calorimetrically by the modified method of Buege and Aust, (1972). Malondialdehyde (MDA) and other TBARS were evaluated based on their reactivity with 2/thiobarbituric acid (TBA) under acidic conditions allowing a pink colored complex detectable at 530 nm. In brief, a portion (0.5 g) of sample was homogenized with 625 µL of TBS (50 mM Tris containing 150 mM NaCl, pH 7.4) and 375 µl of TCA/BHT (TCA 20%, BHT 1%) in order to precipitate proteins, and then centrifuged (1000 g, 10 min, 4 ºC). Then, 400 µl of the supernatant were mixed with 80 µl of HCl (0.6 M) and 320 µl of Tris/TBA (Tris 26 mM; TBA 120 mM). The homogenate was heated for 10 min at 90 ºC. The absorbance of the resulting solution was measured at 530 nm. TBARS values were expressed as milligram of malonaldehyde equivalents per kilogram of sample.

2.8.4. Determination of the conjugated dienes content

Lipid oxidation was also assessed by the conjugated diene content using the method of Srinivasan et al. (2011). The conjugated dienes were evaluated by increasing absorption at 233 nm.

2.8.5. Determination of peroxide value

The PV test was performed according to the method of Shantha and Decker, (1993) with some modifications. The sample (0.30 g) was mixed with 9.8 ml chloroform–methanol in a glass tube and vortexed for 2-4 s. Ammonium thiocyanate solution (10 mM) (0.05 ml) was added and the sample was vortexed for 2-4 s. Then, 0.05 ml iron (II) solution was added and the sample was vortexed for 2-4 s. Finally, the mixture was incubated for 5 min at room temperature and the absorbance was determined at 500 nm. PV is expressed as milliequivalents of peroxide oxygen combined in a kilogram of fat.

2.8.6. Microbiological analysis

Microbiological analysis of coated shrimps was determined by homogenizing 1 g of shrimp sample in 9 ml of 0.9% NaCl at room temperature. Then, decimal dilutions were prepared from the solution and plated in the appropriate media. The total psychrophilic aerobic bacteria (TPAB) and the total mesophilic aerobic bacteria (TMAB) were estimated after incubation for 48 h at 37 °C and for 7 days at 4 °C, respectively, using Plate Count Agar medium

(PCA). All microbial counts were converted to logarithms of colony/forming units per gram of shrimp sample (log CFU/g).

2.9. Statistical analysis

All the measurements were realized in triplicate, based on the test used. All the statistical computations were performed using SPSS ver. 20.0 professional edition (SPSS, Inc., Chicago, IL, USA) via ANOVA analysis. All data were expressed as mean ± standard deviation. Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Characterization of bluefin tuna collagenous protein

3.1.1. Chemical analysis of BTCP

The collagenous proteins were successfully extracted from the head and cartilage of bluefin tuna via a chemical and thermal treatments with an extraction yield of 8.10 g/100 g of fresh by-products. The proximate composition, including protein, dry matter, moisture and ash contents of BTCP, is presented in **Table 1**. BTCP showed high level of proteins (77.49%) and low amount of fat (1.20%), revealing its high quality. The protein content of BTCP was higher than that of snakehead fish protein concentrate (58.77%) (Romadhoni et al. 2016), but lower than that of sardinella protein isolate (81.3%) (Azaza et al. 2022).

Table 1: Proximate composition of the bluefin tuna by-products and its collagenous protein (BTCP)

	Bluefin tuna by-products	**BTCP**
Dry matter (%)*	36.94 ± 0.87	93.04 ± 0.85
Protein (%)**	72.84 ± 0.31	77.49 ± 0.35
Ash (%)**	17.37 ± 0.48	20.76 ± 0.42
Lipids (%)**	10.09 ± 0.30	1.2 ± 0.04
Yield (%)*	-	8.10

*: g dry matter per 100g wet matter, **: g dry matter per 100g dry matter. All the data are expressed as mean ± SD and are the mean of three replicates.

3.1.2. Antioxidant activities of BTCP

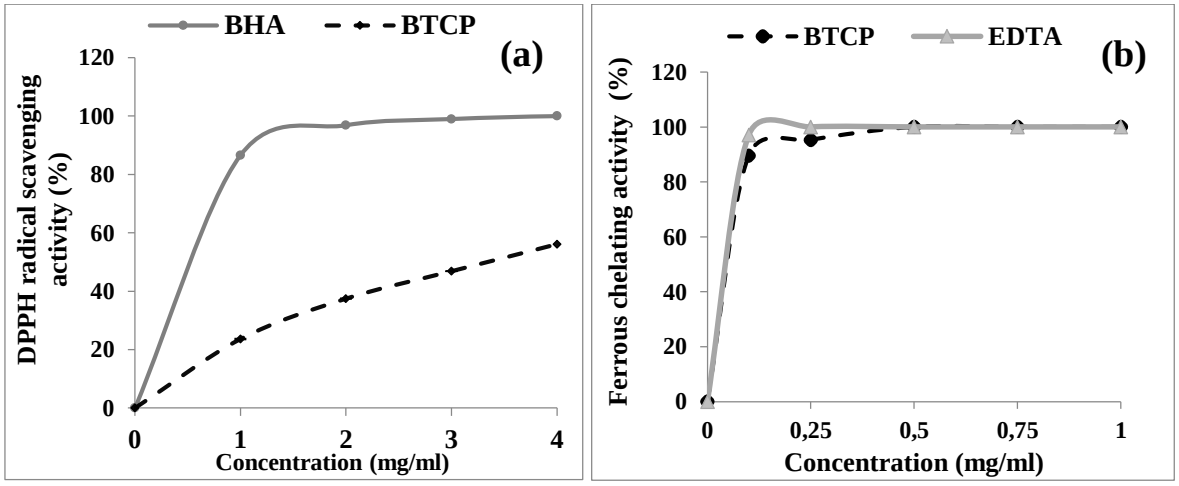
Since antioxidant compounds could act through several chemical mechanisms, the antioxidant activity of BTCP was investigated through multiple assays, including the DPPH radical scavenging, ferrous chelating activity, the reducing power and the total antioxidant capacity (**Fig. 2**).

The DPPH radical scavenging activity of BTPC at various concentrations is shown in **Fig. 2a**. The data revealed that the antioxidant activity of BTCP increased gradually with increasing protein concentration. At a concentration of 4 mg/ml, the BTCP could scavenge 56.12% of the DPPH radicals (**Fig. 2a**). This could be attributed to the ability of BTCP to donate hydrogen and stabilize the radical chain reaction leading to nontoxic species. The values of antiradical scavenging activity of BTCP were significantly higher than that of rice bran protein isolate which displayed 7.3% (Cho 2020).

Furthermore, the ferrous chelating activity is illustrated in **Fig. 2b**. The BTPC showed a chelating effect in a dose-dependent manner. In fact, BTCP displayed significant activity reaching almost the same value as EDTA (100%) at the final concentration (1 mg/ml) (<0.05). Therefore, BTCP could have the potential to improve the shelf life of food products and stop efficiently their oxidative reaction.

However, for the reducing power assay, BTPC exhibited low reducing Fe^{3+} ions (**Fig. 2c**). At 4 mg/ml, the reducing power of BTPC was 0.75 (OD_{700}), which was much lower than that of BHA (control) at the same concentration (OD_{700}=3). It is worthy to note that, the BTCP functions lightly as an electron donor. These results are slightly higher than those of rice bran protein isolate reported by Cho (2020).

Lastly, **Fig. 2d** shows the calculated TAA expressed as α-tocopherol μmol/ml. The obtained results revealed thar the BTPC presented antioxidant activity, which increased significantly with increasing protein concentrations (P<0.05) to reach 180 μmol/ml at 4 mg/ml. These findings suggest that increased values of TAA might have a protective ability against oxidative stress induced by reactive oxygen species (ROS) (Alamdari et al. 2008).



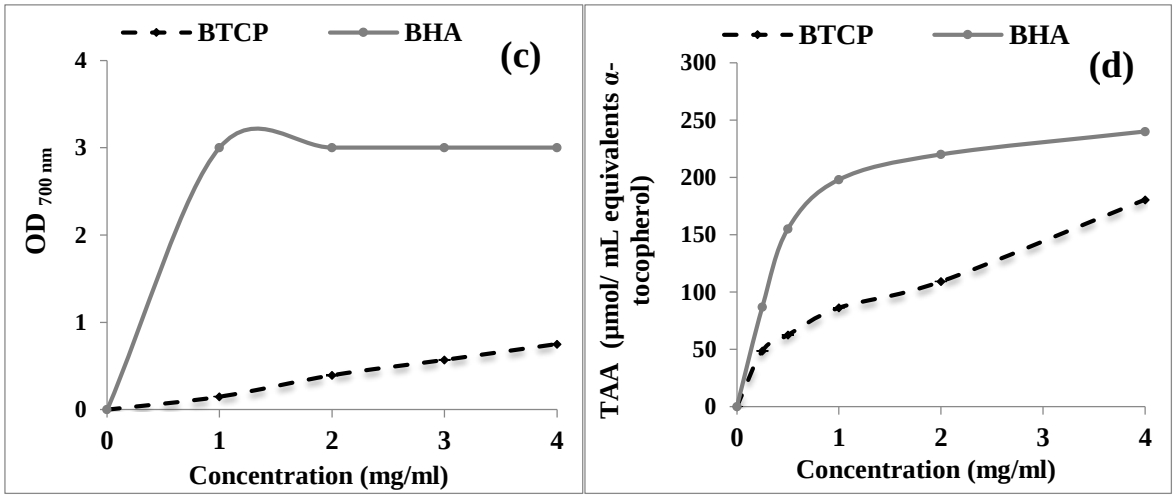


Fig. 2 DPPH radical scavenging activity (**a**), Chelating activity (**b**), Reducing power (**c**) and Total antioxidant activity (**d**) of BTCP

3.2. Characterization of Cs-BTCP composite films

3.2.1. Moisture content, water solubility and swelling degree of the Cs-BTCP composite films

The water-related characteristics of composite films, as well as MC, WS and SD results are presented in **Table 2**. The MC of films is critical for creating a favorable environment for food package films stability.

Regarding MC analysis, all tested films had similar moisture contents (about 20%). The obtained results are comparable to those of collagen-polysaccharide films reported by Ma et al. (2020). The reduction of MC value for composite films could be related to the interaction between chitosan and BTCP, which leads to a decrease in the water content in the film's network (Zhang et al. 2020). So, the low MC value of film permits protecting packaged food for a longer period. (Apriliyani et al. 2020).

The WS of composite films is shown in **Table 2**.Cs control film exhibited high water solubility of more than 57% (**Table 2**), which is slightly higher than WS values reported by Hamdi et al. (2019) and significantly lower than WS values stated by Kaya et al. (2018). Compared with the Cs100, the composite films had relatively lower WS values, reaching a value of 24% for Cs50-50BTCP ($p<0.05$). Lower water solubility could be attributed to the hydrophobic properties of BTCP, which are involved in the weakness of the interactions between polymer-water.

Likewise, the swelling degree of the control chitosan film was 4.4 g/g as shown in **Table 2**, which could be attributed to the hydrophilic property of chitosan (Moalla et al. 2021). As the content of BTCP increased, the SD of Cs-BTCP composite films tended to decrease ($p<0.05$).

This decrease might be related to interactions between chitosan and BTCP, which leads the SD of the composite films to be lower than the control film. According to Di Pierro et al. (2006), the swelling degree of polymer films is closely correlated with the nature and the number of intermolecular chain interactions. These findings were similar to those stated by Zhang et al. (2020), with the addition of plant extracts, the SD of polysaccharide films decreased. Mathew et al. (2006) reported that the water diffusion and the breakdown of hydrogen and ionic bonds strongly depended on the solubility and swelling ability of films.

Table 2: Moisture content (MC), water solubility (WS), swelling degree (SD) and water vapor permeability (WVP) of Cs100 control film and Cs-BTCP composite films

Cs-BTCP ratios (v/v)	MC (%)	WS (%)	SD (g/g)	WVP ($g \cdot s^{-1} \cdot m^{-1} \cdot Pa^{-1} x 10^{-10}$)
0/100	19 ± 0.84^{a}	57.68 ± 0.20^{f}	4.48 ± 0.01^{f}	4.26 ± 0.01^{d}
90/10	20.4 ± 0.28^{a}	50.30 ± 0.28^{e}	3.47 ± 0.04^{e}	4.12 ± 0.17^{d}
80/20	20.2 ± 0.41^{a}	47.38 ± 0.14^{d}	2.11 ± 0.01^{d}	4.02 ± 0.11^{c}
70/30	20.4 ± 1.03^{a}	43.00 ± 0.34^{c}	2.02 ± 0.11^{c}	3.40 ± 0.13^{b}
60/40	20.6 ± 1.13^{a}	33.21 ± 0.53^{b}	1.49 ± 0.01^{b}	3.33 ± 0.04^{b}
50/50	20.8 ± 1.31^{a}	24.06 ± 0.14^{a}	0.64 ± 0.04^{a}	3.10 ± 0.02^{a}

BTCP : Bluefin tuna collagenous protein. **Cs:** Chitosan. Values are given as mean ± standard deviation (SD) of three independent tests. $^{a-f}$ different letters in the same column indicate a significant difference ($p < 0.05$). All films were previously stored at 25 ◦C and 50% RH.

The overall observations showed that the addition of BTCP decreased the hydrophilicity of the Cs-BTCP composite films, thus resulting in the decrease in the water solubility and swelling degree and in the improve in water resistance, implying their potential use as active food packaging materials that protect food, especially in humid environments (Pitak and Rakshit 2011).

3.2.2. Water vapor permeability

WVP is one of the most important physical properties to evaluate the reduction of water vapor transfer between the environment and food. The WVP should be as low as possible to preserve food quality and shelf life. The WVP of Cs-BTCP and Cs films was evaluated with a RH differential of 50% and the results are illustrated in **Table 2**. The Cs100 film showed the highest WVP value (4.26×10^{-10} $g \cdot s^{-1} \cdot m^{-1} \cdot Pa^{-1}$). The WVP of films significantly decreased by adding BTCP to the Cs film matrix, reaching a WVP value of 3.1×10^{-10} $g \cdot s^{-1} \cdot m^{-1} \cdot Pa^{-1}$ for Cs50-50BTCP composite films. Numerous parameters may affect the WVP of film including the hydrophilic character of the polymer, the microstructure, and crosslinking degree in the

structure (Matta et al. 2019). The improvement of water barrier performance of composite films could be attributed to the formation of intermolecular interactions between Cs and collagenous proteins, leading to the increase of crosslinking degree and the decrease of the free volume of the polymeric network and resulting, thereby, in the reduction of water transfer through the composite film. The obtained result suggested that the addition of collagenous protein might reduce the WVP of chitosan film based on its hydrophobic character, in accordance with abovementioned results regarding WS and SD of Cs-BTCP films, inhibiting thereby the diffusion of water vapor through the composite films. Similarly, (Zhang et al. 2019) found that the WVP of the chitosan film was significantly ($p<0.05$) reduced when the zein protein was added and this was based on its hydrophobic character. Hence, Cs-based film incorporated with BTCP had improved barrier water ability for potential application in food packaging.

3.2.3. FTIR spectroscopy analysis

FTIR spectroscopy is one of the most effective techniques for the identification of molecular interactions in the composite films. The FTIR spectrum of Cs100 film shows a broad absorption band at the range of 3400-3000 cm^{-1} assigned to the stretching vibrations of the O-H and N-H groups (**Fig. 3**). The absorption bands detected at 2922 and 2861 cm^{-1} are characteristic of symmetric and asymmetric C-H vibrations (Sun et al. 2017). Additionally, specific bands for chitosan are detected, N-H (1544 cm^{-1}), -CH (1401 cm^{-1}), and glycosidic cycles (1014 cm^{-1}) of chitosan. This trend is in line with previous work on changes in FT-IR spectra occurring for chitosan-based composite film (Liu et al. 2018). As presented in **Fig. 3**, all composite film peaks were roughly similar to those of Cs100 film showing major bands at approximately 3400–3000, 2922–2861, 1632, 1537, 1401 and 1014 cm^{-1}, corresponding to amides A (NH-stretching coupled with hydrogen bonding), amide B (asymmetric stretching vibration of = C–H and $–NH^{3+}$), amide-I (C=O stretching/hydrogen bonding coupled with C═C), amide-II (arising from bending vibration of N-H groups and stretching vibrations of C-N groups), amide-III (vibrations in plane of C-N and N-H groups of bound amide or vibrations of CH_2 groups) and glycosidic cycles, respectively.

When BTCP is added to the films, the wide peak at 3400-3000 cm^{-1} observed for Cs100 has changed to be more pronounced with the increase in BTCP concentration, indicating the decreased stretching of the -NH and/or -OH available groups of chitosan contribute to the hydrogen interactions between the BTCP-Cs bonds (Kaya et al. 2018). Moreover, there is a shift in the region of amide-I (1640 cm^{-1}) and amide II (1544 cm^{-1}) to a lower wavenumber for all composite films (1632 cm^{-1} and 1537 cm^{-1}, respectively). These outcomes indicate that the

formation of polysaccharide (Cs) and protein (BTCP) complexes is mainly due to the interaction between the -COOH group on polysaccharide and groups -NH_3, -COOH on the protein chain which are the sources of hydrogen bond between these two bio-polymers (Bealer et al. 2020).

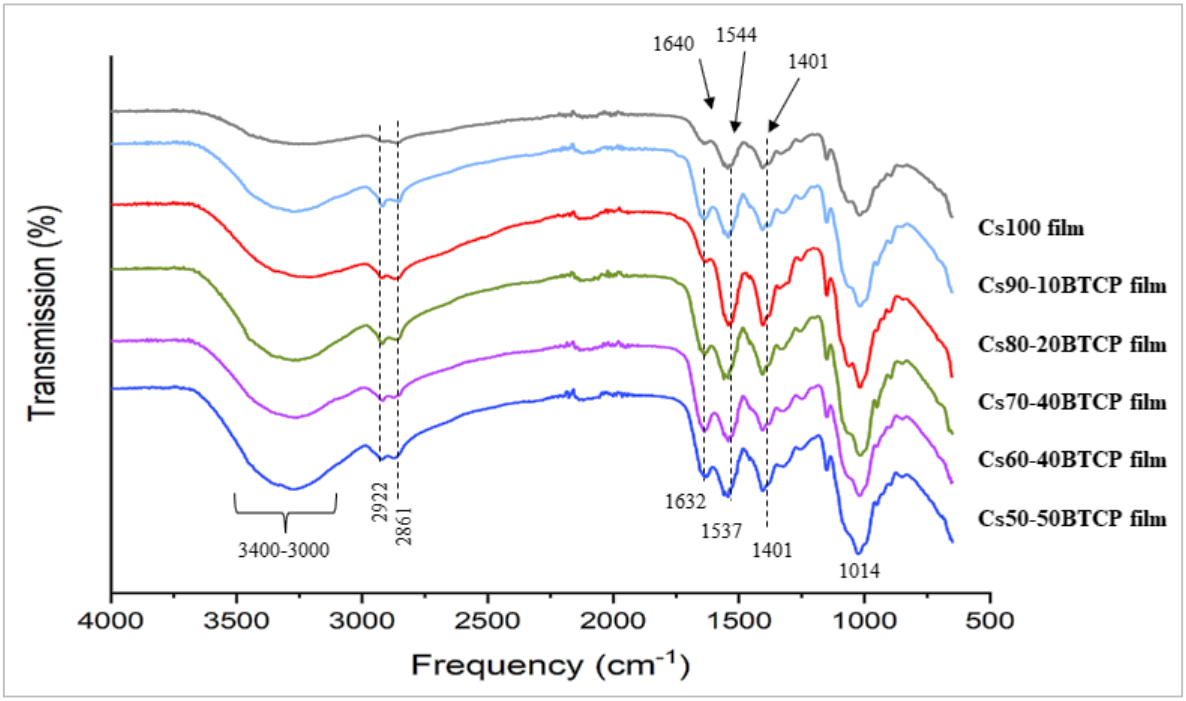


Fig. 3 FTIR spectra of Cs100 film and Cs-BTCP composite films

3.2.4. X-ray diffraction analysis

The X-ray diffraction was conducted to investigate the changes in the morphology of Cs-BTCP composite films. As shown in Fig. 4, Cs100 film diffractogram presents the characteristic diffraction peaks at 2θ of 12° and 18°, attributed to high crystallinity of chitosan (Li et al. 2013). Regarding composite films patterns, the intensity of the crystalline reflection peaks decreased as the BTCP content increased compared to the Cs100 film. This finding could be attributed to the destruction of crystalline structure of the Cs matrix and the formation of an amorphous complex. Therefore, composite films had no strong diffraction peaks owing to amorphous crystalline structures. Similar results were reported by Zhang et al. (2019), who found that with the addition of zein to chitosan film formulation the intensity of peaks decreased, reflecting the good compatibility between zein and chitosan chains.

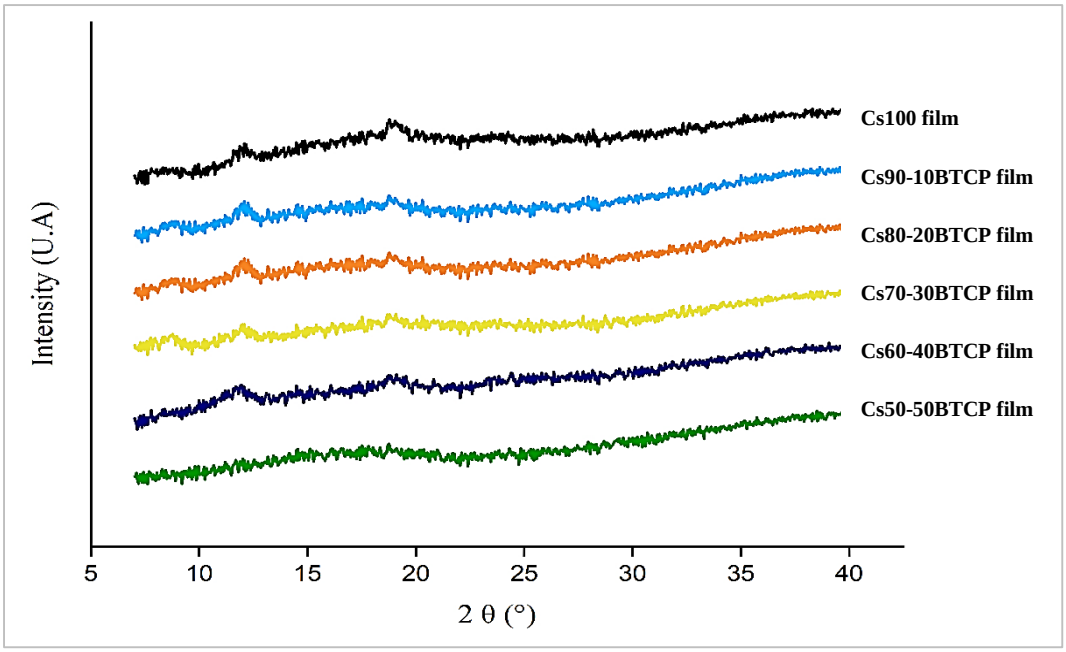


Fig. 4 XRD patterns of Cs100 film and Cs-BTCP composite films

3.2.5. Optical properties of composite films

The light-barrier property of packaging film is an important physical property, which protects the product against oxidation (Jiang et al. 2016). **Table 3** presents the transparency and the light transmittance of composite films in the wavelength range from 200 to 800 nm. Overall, the films incorporated with BTCP showed higher opacity values compared to the chitosan film. In addition, the UV-Vis absorption spectra show that the Cs100 film had poor light barrier property in the UV range, with a high transmission value of 23.7% at 280 nm. It is interesting to note that Cs-BTCP composite films have a low transmission of UV light at 280 nm, ranging from 0.01% to 6.59%. The decreased of UV-light penetration observed in our study is probably attributed to the presence of aromatic amino acids of proteins which absorb UV light below 380 nm (Kalaycıoğlu et al. 2017).

As reported by Hajji et al. (2021), the incorporation of gelatin and shrimp protein hydrolysate improves the chitosan film's barrier property to the UV and visible light of chitosan film. Therefore, Cs-BTCP composite films could be used as UV-screening food packaging materials.

Table 3: Transmission of UV and visible lights and transparency of Cs100 film and Cs-BTCP composite films

Cs-BTPC ratios (v/v)	Light transmittance in different wavelengths (nm)								Transparence
	200	280	350	400	500	600	700	800	

0/100	0.1	23.7	50	65.01	80.72	85.9	86.29	86.29	0.143±0.02[b]
90/10	0.1	6.59	18.87	31.91	45.81	53.57	57.67	60.67	0.047±0.00[a]
80/20	0.1	0.65	4.37	14.62	31.11	39.15	43.85	46.66	0.045±0.04[a]
70/30	0.1	0.39	4.46	11.27	23.06	30.76	36.22	40.83	0.041±0.01[a]
60/40	0.1	0.99	4.84	8.91	15.84	22.43	27.86	32.88	0.032±0.01[a]
50/50	0.1	0.1	1.1	4.32	13.3	19.72	24.83	29.24	0.026±0.01[a]

BTCP : Bluefin tuna collagenous protein ; **Cs:** Chitosan. **[a-b]** different letters in the same column indicate a significant difference ($p < 0.05$).

The color of the film is an interesting optical property that influences the appearance and the marketing value of the packaging. As seen in **Table 4**, there was no significant L* values (lightness) difference among all films ($p > 0.05$). All the films had low L* values that did not exceed L*=32. This property may contribute to preventing oxidative degradation of packaged foods due to exposure to visible and ultraviolet light, which causes nutrient loss, off-flavors, and discoloration. However, the color of composite films changed to a more yellow color with increasing BTCP concentrations (**Table 4**). Similar results has been reported by our previous study (Azaza et al. 2022), when adding sardinella protein isolate to chitosan film matrix.

Table 4: Color parameters (L*. a*. b*) and total color difference (ΔE*) for Cs100 control film and Cs-BTCP composite films

Cs-BTCP ratios (v/v)	L*	a*	b*	ΔE
0/100	29.35±0.18[a]	-0.1±0.01[f]	0.285±0.03[a]	——
90/10	30.51±0.09[b]	-0.52±0.00[d]	0.61±0.00[b]	1.79±0.08[a]
80/20	31.52±0.77[c]	-0.50±0.00[c]	0.60±0.01[c]	2.55±0.58[b]
70/30	31.97±0.28[c]	-0.42±0.06[e]	1.11±0.07[d]	2.72±0.11[b]
60/40	32.04±0.70[c]	-0.63±0.15[ab]	1.29±0.02[e]	3.38±0.50[c]
50/50	31.99±0.72[c]	-0.75±0.04[a]	1.71±0.08[f]	3.62±0.50[c]

BTCP : Bluefin tuna collagenous protein ; Cs : Chitosan. Values are given as mean ± standard deviation (SD) of three independent tests. **[a-f]** different letters in the same column indicate a significant difference ($p < 0.05$).

3.2.6. Thermogravimetric analysis (TGA)

The effect of BTCP addition on the thermal stability of composite films was investigated. The obtained TGA thermograms of films and their derivative (DTG) are shown in **Fig. 5**. The weight loss curve of the Cs100 film presents three distinct phases. The first one (peak I) occurred between 50 and 125 °C, corresponding to the evaporation of water and residual solvent in the film (Altiok et al. 2010). The second weight loss (peak II) detected at 168 °C may be ascribed to degradation of glycerol (Kaya et al. 2018; Kamdem et al. 2019). Finally, the third stage of weight loss (peak III), related to the pyrolytic decomposition of the chitosan matrix, is recorded at 288 °C (de Britto and Campana-Filho 2007).

Notably, for a small amount of BTCP (10%; w/w polymer), a slight shift towards 278 °C was observed, indicating a maintenance of thermal stability similar to chitosan film. Nevertheless, at higher amounts of BTCP (20%, 30%, 40% and 50%; w/w polymer), the weight loss of the decomposition of the chitosan matrix in Cs-BTCP films shifted to lower values, reaching 267 °C, 267 °C, 257 °C and 251 °C, respectively (**Fig. 5**), resulting in reduced thermal stability of composite films. The reduction of the thermal stability could be related to the decrease of the crystallinity of the composite films as reported by the XRD assay. These results are consistent with those reported by Song et al. (2020), which shows that the incorporation of magnolol into the chitosan film results in an decrease in the thermal degradation temperature, thus a low thermal stability of the magnolol-chitosan film.

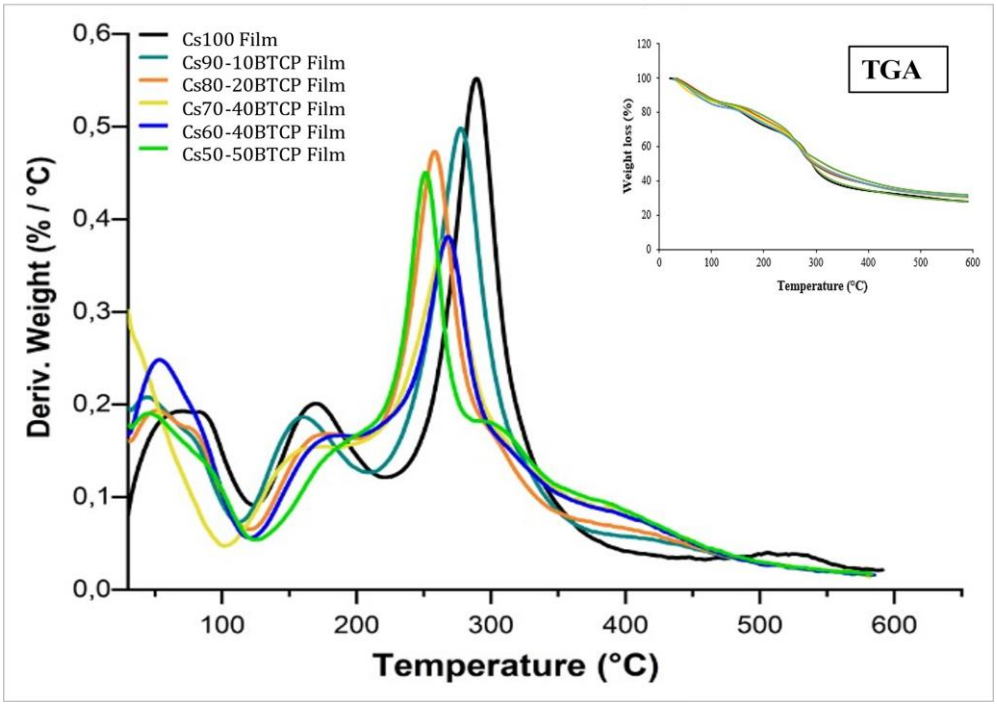


Fig. 5 TGA and DTG thermograms of Cs100 control film and Cs-BTCP composite films

3.2.7. Mechanical properties

The thickness of composite films was measured, and results reveal that Cs-BTCP composite films exhibited higher thicknesses than Cs100 film (**Table 5**). The thicknesses of composite films gradually increased with the increase of BTCP content from 31 µm for Cs90-10BTCP to 52 µm for Cs50-50BTCP ($p<0.05$). Arancibia et al. (2015) also found that the incorporation of protein concentrate increased the film thickness. The increase of composite film thickness can be attributed to the increase of BTCP content on the Cs film matrix, leading to the increase of solids content, and thus a change in the compaction of chitosan, glycerol, and BTCP molecules (Costa et al. 2018; Na et al. 2018).

Tensile strength and elongation at break were usually related to the film network microstructure and the intermolecular force. The TS and EAB values of Cs and Cs-BTCP films were measured and reported in **Table 5**. Results revealed that the Cs100 film showed the highest TS and EAB values of 20.23 MPa and 12.64%, respectively. For Cs10-90BTCP, no significant change was noted for the TS (19.51 MPa) and EAB (11.27%) values ($p>0.05$), suggesting good compatibility and effective crosslinking between BTCP and chitosan. However, at higher BTCP concentration, the TS and EAB values decreased significantly ($p < 0.05$). These findings revealed that the Cs-BTCP films were less resistant and elastic, giving therefore a less deformable film structure, compared to control film. Thus, the higher content of BTCP leads to the formation of agglomerated particles in the composite film, destroying, thereby, the compact structure of Cs films and reducing the molecular mobility and flexibility of the chitosan matrix (Hajji et al. 2021; Azaza et al. 2022).

Table 5: Tensile strength (TS. %), elongation at the break (EAB. MPa) and thickness (µm) of Cs100 control film and Cs-BTCP composite films

Cs-BTCP ratios (v/v)	EAB (%)	TS (MPa)	Thickness (µm)
0/100	12.64 ± 0.26^{d}	20.23 ± 0.18^{e}	13.5 ± 0.02^{a}
90/10	11.27 ± 0.49^{c}	19.51 ± 0.09^{d}	31.5 ± 0.0^{b}
80/20	9.51 ± 0.18^{b}	18.92 ± 0.23^{c}	34 ± 0.00^{c}
70/30	8.93 ± 0.32^{a}	19.09 ± 0.38^{c}	38.5 ± 0.04^{d}
60/40	8.70 ± 0.38^{a}	13.67 ± 0.84^{b}	46.5 ± 0.01^{e}
50/50	8.72 ± 0.39^{a}	4.87 ± 0.06^{a}	52.5 ± 0.0^{f}

BTCP : Bluefin tuna collagenous protein; **Cs:** Chitosan. Values are given as mean ± standard deviation (SD) of three independent tests. $^{a-f}$ different letters in the same column indicate a significant difference ($p < 0.05$).

3.2.8. Antioxidant potential of Cs and BTCP composite films

The effect of incorporating BTCP at different contents on antioxidant activity of chitosan films was studied. The results, illustrated in **Table 6**, show that increasing BTCP content improve the chelating effect of composite films (98.59-99.5%) compared to the Cs100 film (36%). Similarly, the DPPH scavenging activity of composite films (90.17-94.08%) was significant higher that the Cs100 film (74.1%) as the BTPC increase.

As shown in **Table 6**, a higher reducing power was correlated with the addition of BTCP into chitosan matrix, reaching a value of OD_{700}=1.24. The control film presented the lowest ability to reduce ferric ion (OD_{700}=0.165) (Hamdi et al. 2019). This capacity was significantly enhanced for Cs-BTCP composite films.

Lastly, total antioxidant results of composite films are reported in **Table 6**. The control film has a low antioxidant activity (12.51 µmol/mL equivalents α-tocopherol). However, films supplemented with BTCP exhibited a relatively high antioxidant activity, which increased significantly with increasing BTCP content reaching approximately 40.33 µmol/mL equivalents α/tocopherol for Cs50-50BTCP. It is worthy to note that BTCP could convert Mo (VI) to Mo (V) which is more stable.

The significantly high total antioxidant capacity of the composite films could be attributed to the incorporation of BTCP, which showed a distinct antioxidant potential in a dose-dependent manner evaluated in this study by different in vitro antioxidant assays. In addition, it may also be related to the interaction or hydrogen bonds between the functional groups of the added BTCP and those of the chitosan matrix (Kchaou et al. 2017).

Table 6: Antioxidant activities of Cs100 control film and Cs-BTCP composite films

Cs-BTCP ratios (v/v)	Metal chelating effect (%)	Reducing power (OD_{700})	DPPH radical scavenging activity (%)	Total antioxidant capacity (µmol/ mL équivalents α-tocophérol)
0/100	36.00 ± 0.02^{a}	0.165 ± 0.009^{a}	74.1 ± 0.58^{a}	12.51 ± 0.71^{a}
90/10	98.59 ± 0.28^{d}	0.53 ± 0.02^{b}	90.17 ± 0.22^{b}	13.10 ± 0.13^{a}
80/20	96.03 ± 0.22^{b}	0.67 ± 0.005^{c}	90.97 ± 0.07^{b}	21.37 ± 0.51^{b}
70/30	96.32 ± 0.37^{b}	0.77 ± 0.00^{d}	91.26 ± 0.44^{c}	32.92 ± 0.44^{c}
60/40	97.52 ± 0.12^{c}	0.98 ± 0.03^{e}	92.44 ± 0.36^{c}	35.60 ± 0.96^{d}
50/50	99.50 ± 1.57^{e}	1.24 ± 0.08^{f}	94.08 ± 0.80^{c}	40.33 ± 0.71^{e}

BTCP : Bluefin tuna collagenous protein; **Cs :** Chitosan. Values are given as mean ± standard deviation (SD) of three independent tests. $^{a\text{-}f}$ different letters in the same column indicate a significant difference ($p < 0.05$).

3.3. Coating of shrimp

3.3.1. Chemical analysis

The bio-based coatings present a useful and economical technique for shrimp covering, which might preserve and extend food shelf life (Costa et al. 2018b). In this context, the Cs100 bioactive film, used as a control coating, as well as the Cs90-10BTCP and Cs50-50BTCP films, were applied for the preservation of shrimp quality. These different coatings were advantageous with respect to functionality, good stability, mechanical behavior and noticeable antioxidant capacity. To assess the preservative effect of these coatings, moisture content and pH value of coated shrimp samples were measured after 9 days of storage at 4°C and compared to those of uncoated shrimp (**Table 7**).

The moisture of the uncoated pieces of shrimp was assigned a value of 23.23% after 9 days of storage. However, coating with chitosan film/forming solution contributed to the increase in moisture, showing values of 24.27%. The addition of BTCP significantly increased the moisture content of coated shrimp, which was approximately 25.58% and 25.63%, for coated shrimp Sh-2 and Sh-3, respectively.

In addition, the effect of Cs-BTCP coating solution on the pH change of shrimp after 9 days of refrigerated storage is presented in **Table 7**. The results revealed that there were no changes in pH values occurred for shrimp samples after 9 days. The mixture of Cs and BTCP had a significant impact on the shrimp freshness compared to Cs alone. These results are in accordance with those reported by Xiong et al. (2020), suggesting that the incorporation of GSE and/or nisin did not affect the pH value of the coated meat during storage.

Previous studies have shown that there are three levels of quality based on the pH value, as follows: prime quality (<7.7), poor but acceptable quality (7.70-7.95) and unacceptable quality (> 7.95) (Marshall and Wiese-Lehigh 1997). In our study, it was shown that the pH was placed in the first level, suggesting that the coatings of Cs and Cs-BTCP mixtures were effective in preserving the quality of shrimp compared to uncoated shrimp.

Table 7: Changes in MC, pH, color parameters and microbial parameters (TPF: total psychrotrophic flora. TMF: total mesophilic flora) of shrimp samples at 0 and 9 days of storage

Parameters	Days	Sh	Sh-1	Sh-2	Sh-3
MC	0	21.22 ± 0.01^{aA}	22.90 ± 0.02^{aC}	27.53 ± 0.02^{bD}	22.17 ± 0.07^{aB}
	9	23.23 ± 0.07^{aB}	24.27 ± 0.04^{bB}	25.85 ± 0.01^{aD}	25.63 ± 0.12^{bC}
pH	0	7.86 ± 0.01^{bB}	7.22 ± 0.01^{bA}	7.22 ± 0.07^{aA}	7.25 ± 0.01^{bA}
	9	7.76 ± 0.01^{aB}	7.18 ± 0.02^{aA}	7.20 ± 0.07^{aA}	7.22 ± 0.01^{aA}

L*	0	55.67 ± 0.13^{bA}	57.85 ± 0.03^{bC}	57.64 ± 0.13^{bB}	58.74 ± 0.10^{bD}
	9	54.73 ± 0.06^{aA}	56.05 ± 0.31^{aB}	56.27 ± 0.33^{aC}	56.15 ± 0.62^{aBC}
a*	0	2.78 ± 0.01^{aD}	2.57 ± 0.01^{aC}	2.35 ± 0.06^{aB}	2.18 ± 0.03^{bA}
	9	4.71 ± 0.13^{bD}	3.56 ± 0.04^{bC}	2.71 ± 0.40^{aB}	2.31 ± 0.07^{aA}
b*	0	5.07 ± 0.09^{aA}	5.15 ± 0.11^{aA}	7.55 ± 0.23^{aB}	7.98 ± 0.42^{aB}
	9	5.20 ± 0.01^{bA}	8.46 ± 0.04^{bB}	8.84 ± 0.06^{bB}	12.01 ± 0.28^{bC}
ΔE	0	-	2.20 ± 0.11^{aB}	3.20 ± 0.15^{aC}	4.28 ± 0.33^{aD}
	9	-	3.70 ± 0.27^{bB}	4.43 ± 0.39^{bB}	8.23 ± 0.63^{bC}
Browning	0	12.22 ± 0.19^{aA}	12.83 ± 0.20^{aA}	16.57 ± 0.56^{aB}	16.86 ± 0.37^{aB}
Index (%)	9	15.83 ± 0.17^{bA}	20.08 ± 0.78^{bB}	20.50 ± 0.01^{bB}	23.72 ± 0.50^{bC}
TPAB	0	0.38 ± 0.04^{aA}	-	-	-
(log UFC/g)	9	1.97 ± 0.02^{bD}	1.10 ± 0.02^{c}	0.39 ± 0.04^{b}	0.34 ± 0.02^{a}
TMAB	0	0.21 ± 0.02^{aA}	-	-	-
(log UFC/g)	9	4.67 ± 0.04^{bD}	1.13 ± 0.03^{c}	0.60 ± 0.01^{b}	0.46 ± 0.02^{a}

Sh : control shrimp (uncoated) with no treatment; **Sh-1** : shrimp dipped in Cs solution; **Sh-2** : shrimp dipped in Cs90-10BTCP solution; **Sh-3** : shrimp coated with Cs50/50BTCP solution. **(a-d)** Different letters indicate significant differences between different samples on the same storage day ($p < 0.05$). **(A-D)** Different letters in each column mean a significant difference for the same sample on different storage days ($p < 0.05$).

Color is a key factor for consumers to determine the quality of the seafood product. Therefore, the quality of shrimps can be correlated with the skin color browning and/or darkening which results from biochemical reactions with the ripening process during prolonged storage. The changes in color indices (L*, a*, b*, ΔE and BI) of control and coated shrimps during storage at 4 ± 1 °C can be expressed by color indices (**Table 7**). The color of the uncoated shrimp changed after 9 days of storage. The lightness (L*) value of uncoated shrimp decreased, and the redness (a*) value increased. The values were higher than those of coated ones. Chitosan coatings (Sh-1) had a positive effect on the L*, a* and b* values. They increased for chitosan coated shrimps while they decreased for the uncoated one. Therefore, the coating of shrimp by Cs-BTCP coating solutions lead to stability of its lightness (L*), a significant increase of its yellowness (b*) and a clear decrease in the values of the redness (a*). However, it is important to note that the BTCP incorporation positively resulted in lower redness and higher yellowness values due to its initial yellow color. In addition, there was a gradual increase in browning index values (%) suggests a progressively higher rate of enzymatic browning (Kortei et al. 2015). Thus, the Cs-BTPC composite films showed a reduction in color indices of shrimp during storage.

712 Generally, there was a gradual increase in the ΔE values of shrimp for the three groups
713 with the storage time increasing. This was especially apparent in the Sh-3 which has
714 significantly higher ΔE values than the other groups (**Table 7**).

715 *3.3.2. Peroxide value*

716 The most commonly used measure to evaluate autoxidation quality is the peroxide value
717 (PV), which determines the primary lipid oxidation products. The PV values were measured to
718 assess the effectiveness of the Cs-BTCP coating solution in preventing the auto-oxidation of
719 lipids in shrimp (Gray et al. 1996). From the second day until the end of the storage period, PV
720 levels of uncoated shrimp gradually increased ($p < 0.05$). For untreated shrimp, the PV value
721 increased from 0.015 meq O2/kg to 0.03 meq O2/kg of fat on the ninth day of storage (**Fig. 6a**).
722 However, the PV values of coated shrimp were considerably lower ($p < 0.05$) than the uncoated
723 shrimp. On the ninth day of storage, the chitosan coating slightly delayed peroxidation of fat
724 reaching a value of 0.022 meq O2/kg fat. It is interesting to note that the PV values of Sh-2 and
725 Sh-3 coating solutions were 0.019 and 0.017 meq peroxides/kg lipid after 9 days of storage,
726 respectively. Thus, the addition of BTCP improves the antioxidant activity of chitosan coating
727 solution, leading, thereby, to the reduction shrimp peroxidation.

728 Similarly, in a previous study (Azaza et al. 2022), w affirmed that chitosan-sardinella
729 protein isolate packaging films might reduce the production of primary lipid oxidation products
730 in the shrimp under refrigerated storage.

731 *3.3.3. Conjugated dienes (CD)*

732 Lipid oxidation was estimated by the measurement of conjugated dienes (CD) content.
733 The results, presented in **Fig. 6b**, show the increase in CD values over the shelf/life for all
734 samples. In fact, the highest value of CD was noted in uncoated shrimp (Sh) and shrimp coated
735 with a film/forming solution of Cs100 (Sh-1) reaching an OD of 0.47 and 0.31 at day 9,
736 respectively. It is worthy to note that Sh-2 and Sh-3 showed the lowest OD values of 0.27 and
737 0.24 ($p<0.05$), respectively, demonstrating that the addition of the BTCP in the Cs film/forming
738 solution was effective in CD development delay. Therefore, BTCP could be considered as
739 interesting antioxidant additive that can prevent the oxidation of food.

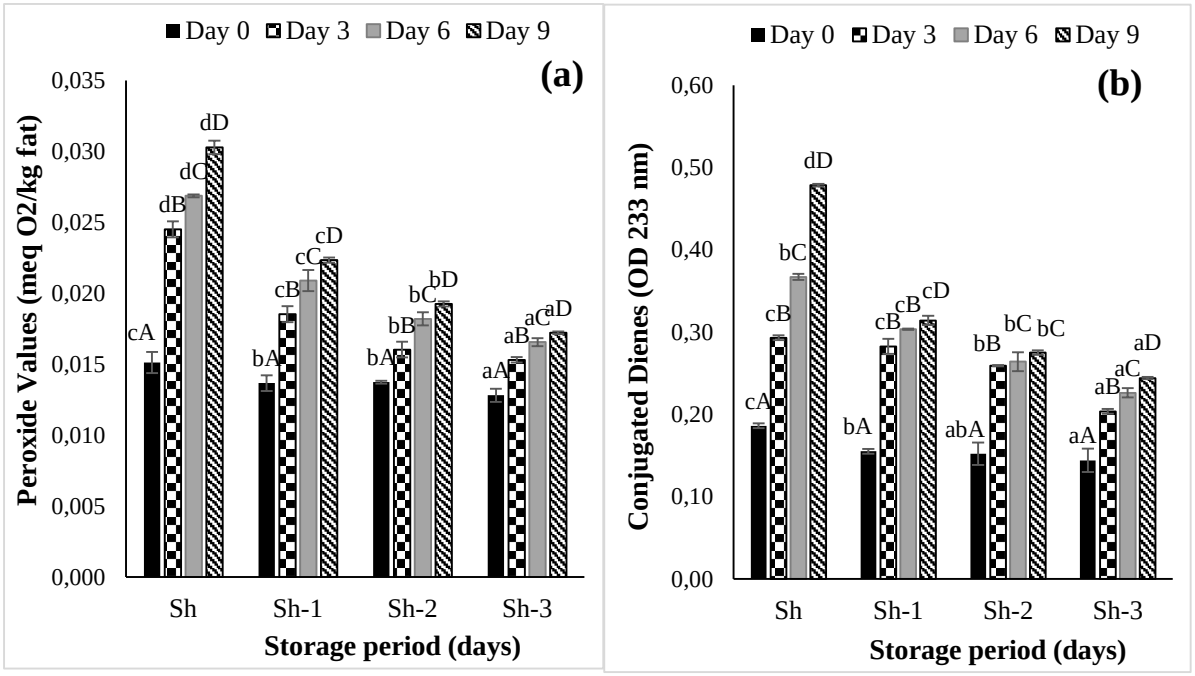
740 *3.3.4. TBARS test*

741 The lipid oxidation of the shrimp sample was also investigated through the measures of
742 the content malondialdehyde (MDA). MDA is defined as a biomarker of oxidative damage to
743 lipids. As shown in **Fig. 6c**, an increase in MDA content was observed for uncoated shrimp

(Sh) samples after 9 days of storage at 4°C. In fact, TBARS value increased from 9.57 mg MDA/kg on day 0 to 27.51 mg MDA/kg on day 9 (**Fig. 6c**).

As compared to the uncoated shrimp (Sh), the extent of secondary oxidation is significantly lower ($p < 0.05$) in the samples coated by Cs film solution enriched with BTCP, reaching 13.81; 11.94 and 9.41 mg MDA/kg on day 9 for (Sh-1), (Sh-2) and (Sh-3), respectively. Hence, the combination of chitosan and BTCP resulted in the reduction of MDA formation.

It is worth mentioning that both antioxidant and oxygen barrier properties of chitosan coating may have contributed to the control of lipid oxidation in the shrimp but also the incorporation of BTCP in Cs coating with different concentrations, had an inhibitory role against lipid peroxidation. Farajzadeh et al. (2016) found that the TBARS values of shrimp samples with chitosan/gelatin coatings had much slower increase during frozen storage.



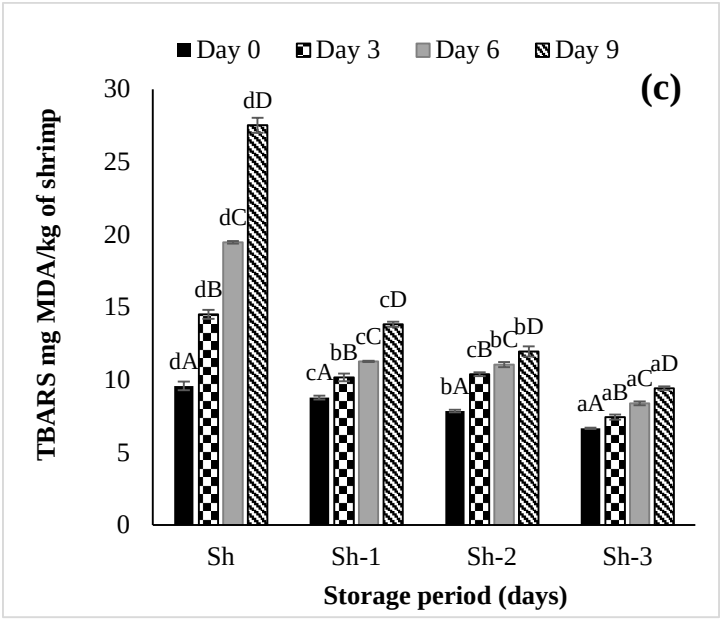


Fig. 6 Effect of BTCP incorporation (10% and 50%, w/w polymer) in the chitosan film matrix on peroxide value (meq O_2/kg fat) (**a**), conjugated dienes (OD 233 nm) (**b**) and thiobarbituric-acid reactive substances values (mg MDA/kg) (**c**) of coated shrimps during refrigerated period. $^{(a-d)}$ Different letters indicate significant differences between different samples on the same storage day ($p < 0.05$). $^{(A-D)}$ Different letters in each column mean a significant difference for the same sample on different storage days ($p < 0.05$)

3.3.5. Microbial analysis

Effects of coatings on the microbiological quality of shrimp stored at 4 °C are shown in **Table 7**. The initial total psychrophilic aerobic bacteria (TPAB) and total mesophilic aerobic bacteria (TMAB) counts for control shrimp were approximately 0.38 and 0.21 (log CFU/g), respectively, signifying a high initial quality of the shrimp. Over the 9 days storage period, the TPAB and TMAB of the control shrimp samples increased quickly to 1.97 and 4.67 log CFU/g, respectively ($p<0.05$). However, coating shrimp with chitosan film/forming solution reduced the growth of micro/organisms, reaching values of about 1.10 and 1.13 log CFU/g for TPAB and TMAB, respectively. Furthermore, Cs50-50 BTCP and Cs90-10BTCP film forming solution were able to reduce significantly the TPAB and TMAB counts, comparing to the Cs100 one. Hence, the incorporation of BTCP to chitosan film forming solution demonstrated an excellent barrier effect around shrimp against bacterial proliferation and oxygen diffusion. Additionally, it is notable that the Cs-BTCP ratio of 90:10 is enough to reduce 2.8/fold and 1.8/fold, respectively, the growth of TPAB and TMAB, in comparison to the Cs100 solution. These findings are in accordance with those reported by Mohebi and Shahbazi, (2017), who confirmed that chitosan and gelatin coatings can be used as an active packaging to delay the growth of spoilage microorganisms and extend the shelf life of the shrimp of at least 11 days.

Conclusion

Composite films based on Cs and BTCP were successfully developed. The properties of the resulted films are dependent on the Cs-BTCP ratio. FTIR analysis are very effective in identifying polymer compositions and their compatibility. The resulting Cs-BTCP composite films showed improved antioxidant activity, reduced WS, SD, light transmittance and WVP properties, as compared to the Cs100 film. Interestingly, the composite films prepared at a Cs-BTCP ratio of 90:10 (v/v) showed interesting mechanical and thermal stability similar to the Cs100 film. The obtained results indicated the incorporation of BTCP improved the chitosan film functionality. Additionally, the Cs-BTCP coating solution was effective in preserving the good quality of shrimp, as it prevented the bacteria growth and decreased the oxidation of coated shrimp.

CRediT authorship contribution statement

Youssra Ben Azaza: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Visualization, Writing – original draft; **Marwa Hamdi**: Investigation and Methodology; **Christophe Charmette**: Investigation and Methodology; **Arie Van der lee**: Investigation and Methodology; **Mourad jridi**: Investigation; **Suming Li**: Funding acquisition, Supervision; **Moncef Nasri**: Supervision, Editing and Validation; **Rim Nasri**: Resources, Supervision, Funding acquisition, Writing - review & editing.

Declaration of competing interest

All cited authors certify that they have sufficiently participated in the work to assume public responsibility for the content. The authors declare that there is no conflict of interest regarding the publication of this article.

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