*Research Article*

# Physicochemical and Antioxidant Properties of Chitosan Films Incorporated with Cinnamon Oil

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Chitosan films (CF) with cinnamon bark oil (CO) incorporated at 0% (control), 0.25%, 0.5%, and 1.0% v/v were prepared by an emulsion method. The films were characterized based on their physical properties (solubility, water vapor permeability, optical property, and microstructure) and antioxidant properties (DPPH, ABTS, and its protective effects on human erythrocytes). The results showed that the incorporation of 0.5 and 1.0% of CO into the CF significantly decreased its solubility to 22% of the control ($p < 0.05$). The water vapor permeability of the CF-CO was significantly reduced to 40% with low concentrations of CO (0.25%) incorporated into the CF. In general, the films presented a yellow coloration and an increase in transparency with the incorporation of CO into the CF. It was also observed that the incorporation of CO increased the antioxidant activity between 6.0-fold and 14.5-fold compared to the control, and the protective capacity against erythrocyte hemolysis increased by as much as 80%.

## 1. Introduction

Consumers now demand foods that contain low levels or are free of synthetic chemical preservatives [1]. Some alternatives have aimed to use matrices based on proteins, lipids, and carbohydrates that present film formation properties [2]. Chitosan is a carbohydrate consisting of $\beta$-(1-4)-D-glucosamine and $\beta$-(1-4)-N-acetyl-D-glucosamine, obtained from the thermo-alkaline deacetylation of chitin. This biopolymer is viscous, biodegradable, nontoxic, antigenic, antimicrobial, and biocompatible [3]. These characteristics make it suitable for the development of diverse applications in medicine, agriculture, and the food industry [4]. In the food industry, chitosan has been used as a natural preservative due to its film formation properties. It has been shown that chitosan films can create a semipermeable barrier that can reduce breathing, retard microbial growth, and offer other protective qualities in fruits and vegetables [5]. In addition, its use as

a preservative in food products has been considered safe [6]. However, due to its hydrophilic nature, one of the main disadvantages of the CF is its limited action as a barrier against humidity. Some alternatives that have been used to improve the properties of chitosan have been the incorporation of diverse bioactive compounds such as vitamins, lipids, minerals, drugs, proteins, colorants, and essential oils [7, 8].

Cinnamon bark essential oil (CO) contains a great variety of chemical compounds (mainly cinnamaldehyde, cinnamic acid, coumaric acid, cinnamyl alcohol, and eugenol) that present antioxidant and antimicrobial activities in an individual and collective manner [9]. In the food industry, CO has been used as a flavoring and preservative [10, 11]. However, its direct application to food as a preservative has been limited, as it can be lost during storage due to its high volatility and reactivity with diverse food components [12]. The hydrophobic nature of CO could be exploited to make CF a better barrier against humidity, and in turn the chitosan matrix could conserve the properties of CO, which could improve the physical and antioxidant characteristics of CF. However, the incorporation of CO into CF could also generate undesired changes within the CF. Therefore, the goal of this study was to characterize the physical (solubility, water vapor solubility (WVP), and optical), antioxidant (DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)), and protective effects on human erythrocytes) and microstructural properties of CF surfaces containing CO incorporated by an emulsion method.

## 2. Materials and Methods

### 2.1. Chitosan Preparation.
Chitosan was obtained by the thermo-alkaline deacetylation of shrimp chitin according to the methodology proposed by Hongpattarakere and Riyaphan [13]. For this purpose, 1 g of chitin was homogenized with 15 mL of NaOH (50% w/v) at 95°C for 2 h. The degree of chitosan acetylation obtained was 34%, with an average molecular weight of 128 kDa. The chitosan was characterized in the Polymers Laboratory of the Biotechnology Department at the Metropolitan Autonomous University, Mexico, DF.

### 2.2. FC Preparation and CO Incorporation.
A chitosan solution was prepared by dissolving 0.4 g of chitosan in 20 mL of acetic acid (1% v/v) and homogenizing the solution for 4 min at 15,500 rpm (Ika Ultra-turrax T 18 basic, Germany). Subsequently, glycerol (200 μL) was added as plasticizer and agitated under similar conditions. Before the incorporation of the CO (cinnamon bark oil FCC, Sigma-Aldrich), the chitosan solution was emulsified with tween 80 (1% v/v) as a surfactant. For this study, 0% CO was used as the control, and three levels of CO incorporation were tested: 0.25%, 0.5%, and 1.0% (v/v) CO in chitosan solution, homogenized at 15,500 rpm for 3 min. The resulting emulsion was centrifuged at 2,000 g for 5 min (Hermle Labortechnik, Germany) to eliminate air bubbles. The emulsion was then decanted into glass Petri dishes and left to settle for 24 h at 37°C until the formation of the films.

### 2.3. Film Thickness.
The film thickness was measured with a manual micrometer (Mitutoyo, Japan) with a precision of 0.03 mm. Five measurements were randomly taken for each film. The mean thickness was calculated and used to estimate the WVP and the transparency. The thickness of the films ranged from 0.086 to 0.127 mm.

### 2.4. Scanning Electron Microscopy (SEM).
The surface morphology of the films was examined with a scanning electron microscope (JEOL JSM-5410LV) (Tokyo, Japan) equipped with an INCA system and an EDS (Energy Dispersive X-Ray) microanalysis detector (Oxford Instruments, Buckinghamshire, UK) and operated at 20 kV. For the SEM analysis, the specimens were cut to an adequate size and placed on a cylindrical copper support (1 cm in diameter). The samples were coated with gold to facilitate conduction and prevent the accumulation of charge under electron bombardment. The analysis of the sample was performed under high vacuum conditions.

### 2.5. Solubility.
The solubility determination was performed according to the procedure reported by Casariego et al. [14]. The films were cut into squares ($20 \times 20$ mm) and placed in a vacuum oven (35 kPa for 24 h) to determine the weight of the dry films. Subsequently, the films were immersed in 10 mL of tridistilled water in Petri dishes kept at 25°C for 24 h with occasional agitation. After this time, the films were dried again in the vacuum oven until they reached constant weight to determine the weight of dry matter that was not dissolved in the water. The solubility was calculated based on

$$\text{Solubility} = \left( \frac{\text{Mi} - \text{Mf}}{\text{Mi}} \right) 100, \tag{1}$$

where Mi represents the initial mass and Mf the final mass.

### 2.6. Measurement of the Water Vapor Permeability (WVP).
The WVP was determined gravimetrically based on the ASTM E96-92 method [15]. The films were sealed in the upper part of a 50 mL glass cup, which contained 10 mL of distilled water (100% RH; 3.168 kPa of WVP at 25°C). Subsequently, they were placed in a desiccator with anhydrous calcium sulfate at 25°C and 0% RH (0 kPa of WVP). The cups were weighed at 3 h intervals for 12 h. The ideal state and uniformity of the WVP conditions were assumed, with a constant air circulation outside of the test cup. For that purpose, a miniature fan was used inside the desiccator. The lost weight of the cup was plotted with respect to time to estimate the slope by a linear equation (the correlation coefficient was 0.99). Once the slope was obtained, the water vapor transmission rate (WVTR) was calculated by

$$\text{WVTR} = \frac{\text{Slope}}{\text{Film area}}. \tag{2}$$

The WVP of the films was calculated by multiplying the WVTR by the average thickness of the film ($G$) and dividing by the difference in partial pressures inside (PA1) and outside (PA2) the cup, as expressed in

$$\text{WVP} = \frac{\text{WVTR}}{(\text{PA1} - \text{PA2})} G. \tag{3}$$

*2.7. Optical Properties.* The color and transparency were determined with a UV-Vis spectrophotometer (Cintra 10E, Australia). For the color measurement, three films of each concentration were chosen, from which the average of five samples was taken. The film color was expressed based on the CIE model ($L^*$, $a^*$, and $b^*$). The transparency was determined according to the method established by Han and Floros [16]. For that purpose, the films were cut into rectangles ($9 \times 45$ mm) and placed inside the measuring cell of the spectrophotometer. The transmittance was recorded at 600 nm, and the film transparency was calculated by

$$\text{Transparency} = \log\left(\frac{T600}{G}\right), \tag{4}$$

where $T600$ is the transmittance at 600 nm and $G$ represents the film thickness (mm).

The UV-Vis light (200–800 nm) transmission properties through the films were also determined.

*2.8. Antioxidant Activity.* The CF-CO (50 mg) was homogenized in 5 mL of 80% v/v methanol using a homogenizer (Ika Ultra-turrax T 18 basic, Germany) at 5°C for 5 min at 11,000 rpm. Afterwards, the sample was centrifuged at 16,000 g for 10 min at 5°C; the supernatant was collected and filtered through Whatman number 1 filter paper. These film extracts were used for the assays of DPPH, ABTS, and the protective effect on human erythrocytes.

The DPPH was determined according to the method described by S. Moein and M. R. Moein [17], with certain modifications. A stock solution was prepared by mixing 2.5 mg of the DPPH radical in 100 mL of pure methanol and was adjusted to $0.7 \pm 0.02$ at 490 nm of absorbance. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as the standard and 80% methanol as the blank reactant. The sample (5 μL) was placed on a microplate, and 245 μL of DPPH radical was added. The mixture was kept in the dark for 30 min, and the absorbance was then read in a microplate reader (iMark Bio-Rad, Japan) at a wavelength of 490 nm. The inhibition percentage was calculated for each of the CF-CO films, which indicates the capacity of the antioxidant to reduce the radical absorbance after the final incubation. Finally, the results were expressed in trolox equivalents per gram of film (mmol TE/g).

The ABTS test was determined according to Re et al. [18]. The ABTS$^+$ radical was generated by the interaction of 5 mL of ABTS solution (7 mM) and 88 μL of potassium persulfate solution (0.139 mM). The test was performed by adding 295 μL of the ABTS$^+$ solution and 5 μL of the film extract. Once the components were mixed in the microplate, the absorbance was monitored at 734 nm. The inhibition percentage was calculated and converted to trolox equivalents (TE) per gram of film (mmol TE/g).

*2.9. Evaluation of the Protective Effect on the Human Erythrocyte.* The hemolysis induced by AAPH [2,2'-azobis (2-amidinopropane hydrochloride)] was determined by the method established by Lu et al. [19] with certain modifications. First, the erythrocytes were washed three times with phosphate-buffered saline (PBS, pH = 7.4). Subsequently,

a 5% erythrocyte suspension was prepared with PBS. For the erythrocyte protection test, 50 μL of erythrocytes at 5% was placed with 50 μL of film extract at 400 mmol in a glass tube. A control mixture was prepared in a similar manner but without the film extract (complete hemolysis). The samples were incubated at 37°C with continuous agitation at 30 rpm in the dark for 3 h. After the incubation, the reaction mixture was diluted with 1 mL of PBS and centrifuged at 2000 g for 5 min. The supernatant absorbance was read at 540 nm in a microplate reader (iMark Bio-Rad, Japan). The results were expressed as the hemolysis inhibition percentage in the human erythrocytes, which was calculated using

$$\text{Hemolysis inhibition percentage}$$
$$= \left(\frac{\text{AAPH} - \text{HM}}{\text{AAPH}}\right) \times 100, \tag{5}$$

where AAPH = absorbance with complete hemolysis and HM = absorbance of the supernatant.

*2.10. Statistical Analysis.* Analysis of variance was applied to the obtained data using CF with and without CO incorporation as factors. The transparency, WVP, solubility, antioxidant, and antihemolytic activity were taken as response variables. For the decision-making process, the Duncan comparison test was used ($\alpha = 0.05$). All of the above analysis was conducted using the NCSS statistical package, 2000.

# 3. Results and Discussion

*3.1. Microstructure.* The surface appearance of the film microstructure changed upon the addition of cinnamon oil. Figure 1 shows SEM images of the CF alone and with incorporated CO 1.0%, where a surface without pores and with slight folds can be observed in the CF (control). Recently, CF has been reported to lack apparent pores [14]; however, other authors have documented the presence of pores uniformly distributed on the film surface [20]. It is known that, in general, this characteristic depends on the nature of the chitosan (acetylation degree and molecular weight). In contrast, the CFs with CO presented a rough surface with pores of approximately 2-3 μm in diameter. The presence of these pores is likely due to the flocculation and coalescence of small drops of emulsified cinnamon oil during the drying of the film; similar results have been reported by Peng and Li [21] and López-Mata et al. [22] in films with carvacrol incorporated.

*3.2. Solubility.* The solubility is a very important property of the films that measures their resistance to water. Figure 2 shows the solubility percentages of the CFs with incorporated CO, where it can be observed that the incorporation of CO into the CF significantly decreased ($p < 0.05$) the CF solubility. This decrease was 15% for CF-CO 0.25% and 22% for CF-CO 0.5% and 1.0% compared to the control. It was also observed that increasing the CO concentration from 0.5% to 1.0% did not induce greater insolubility of the film. The solubility percentage of the CF (41%) is consistent with the ranges previously published for CF (16 and 46%) and reported by Casariego et al. [14]. When working with CF, it is complicated
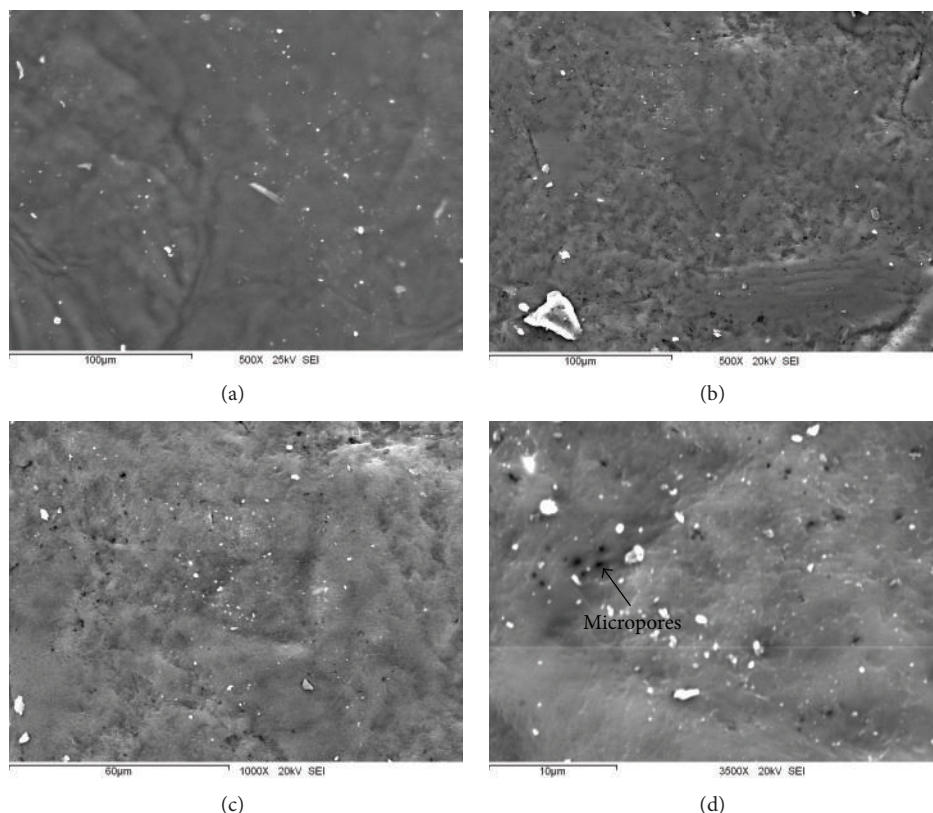
(a)

(b)

(c)

(d)

Figure 1: Scanning Electron Microscopy (SEM) pictures of chitosan film (a) and chitosan film with 1.0% incorporated cinnamon oil (b, c, and d) oriented on the dry side.
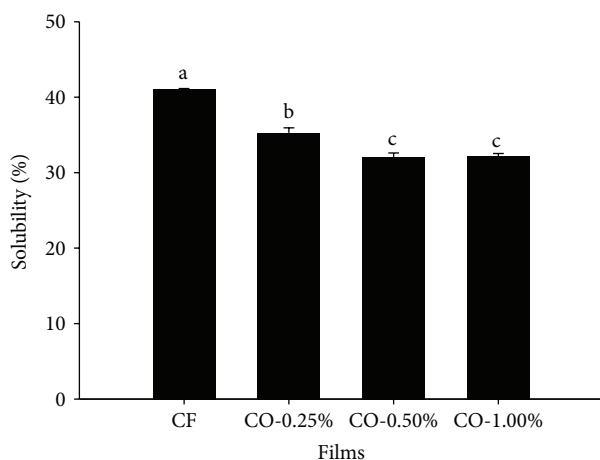


Figure 2: Solubility percentages values of CF and CF-CO films.

to standardize a solubility value, as the chitosan properties can vary depending on the source, acetylation degree, and molecular weight. Therefore, the elevated solubility exhibited by the control film could be influenced by the presence of the plasticizer used during the elaboration of the film and the chitosan functional groups [23]. The solubility reduction of the films incorporated with CO could be due to a decrease in the hydrophobic nature influenced by the loss of its free

functional groups (amino and hydroxyl). The low affinity presented by the films with incorporated CO is important for their application as coatings in food products with high moisture contents.

*3.3. Water Vapor Permeability (WVP).* The WVP is a property that indicates the rate at which water is lost through a film. The WVP behavior of the studied films is shown in **Figure 3**. These results show that the incorporation of 0.25% CO into CF gives a WVP of $0.53 \pm 0.04 \, g \, mm/kPa \, h \, m^2$, a 40% decrease compared to the control ($0.88 \, g \, mm/kPa \, h \, m^2$), which is statistically significant. The incorporation of 0.5 and 1.0% of CO into CF did not show significant difference compared with the control, with values of 0.816 and $1.0178 \, g \, mm/kPa \, h \, m^2$, respectively. Sánchez-González et al. [1] established that increasing concentrations of essential oils in food films do not necessarily translate to a linear reduction of the WVP. The reduction of the WVP at low concentrations of incorporated CO could be due to the action of the diverse compounds that compose the CO, which could, as a whole, form a hydrophobic region in the film [24]. However, incrementally increasing the CO concentration can induce structural changes in the film, as can be observed in Figures 1(a), 1(b), 1(c), and 1(d), where the control film is compared with the CF-CO 1.0%. Here, a surface with a rougher appearance and with the formation of pores is observed in the case of the CF-CO 1.0%. It has been previously documented that the
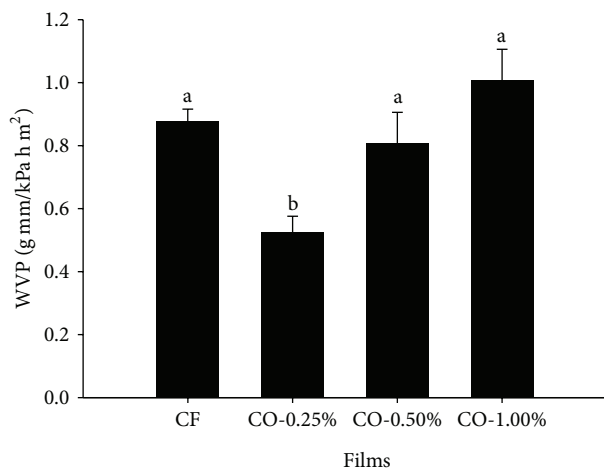
Figure 3: Water Vapor Permeability (WVP) of the CF and CF with the incorporation of CO.

Table 1: Color parameters of CF and CF-CO films.

| Films | $L^*$ | $a^*$ | $b^*$ |
|---|---|---|---|
| CF | 68.15 ± 0.73[a] | −0.12 ± 0.04[a] | 6.25 ± 0.10[a] |
| CO-0.25% | 67.89 ± 1.00[a] | −0.15 ± 0.02[a] | 5.44 ± 0.09[b] |
| CO-0.50% | 65.31 ± 0.99[a] | 0.14 ± 0.01[a] | 5.82 ± 0.08[c] |
| CO-1.00% | 59.53 ± 0.42[b] | 0.13 ± 0.04[a] | 7.77 ± 0.05[d] |

[*] The data represent the mean ± standard deviation of three determinations, and different letters in the same column indicate significant differences ($p < 0.05$).

introduction of cinnamaldehyde (the main component of the CO at ≈80%) into the chitosan structure can react with the amino group of the C2, inducing the formation of a Schiff base [25]. This base can induce cross-linking of the chitosan polymer, and it has been observed that this cross-linking can induce the presence of pores in the films. Also, several authors have shown the properties of cinnamaldehyde as a cross-linking agent with chitosan [26, 27], and these authors reported that the introduction of aldehyde groups in chitosan can induce pore formation in the structure under controlled conditions. The slight increase in WVP of the CF with 1.0% of CO could be due to the formation of pores, which could only occur in superficial parts of the film or in a partial manner; all pores do not necessarily form channels that connect both sides of the film. However, it is also possible that these pores arose from the instability of the emulsion drops, which escaped and left an apparent pore trace. These results are similar to the report by Sun et al. [28], who evaluated chitosan film incorporated with various concentrations of complex of $\beta$-cyclodextrin and essential oils as eugenol, cinnamaldehyde, and carvacrol, and Bonilla et al. [29], who evaluated chitosan film with basil essential oil. These authors showed that when the concentration of oils increased, the WVP of the film increased, and this increase was attributed to excessive oils in the films, which subsequently decreased the intermolecular forces between polymer chains that induce segmental motions and free space, causing a more open matrix.

*3.4. Color Properties.* The measurement of the color parameters is an important property in describing the appearance of films. Table 1 shows the effects of CO incorporation into the CF, where it can be observed that the CF with incorporated CO generally presented a yellow coloration ($b^*$). This coloration was significantly lower when CO was incorporated at concentrations of 0.25% and 0.5%. However, the yellow coloration in the CF was intensified with a higher concentration of incorporated CO (1.0%) ($p < 0.05$). Consequently, this phenomenon could be considered normal

in the control film, as this coloration has been associated with the presence of repeated units of $\beta$-(1-4)-2-amino-2-deoxy-D-glucopyranose [20]. The reduction of the coloration presented in the films with 0.25% and 0.5% incorporated CO could be due to the competition of the diverse CO compounds when interacting with the functional groups of the CF; however, this effect could also be associated with the presence of the surfactant (Tween 80) used during the elaboration of the film, which can also form uniform structures with the chitosan [30]. Therefore, the increase in the yellow coloration of the CF with 1.0% CO could be due to the increased cinnamaldehyde concentration and the possible formation of a Schiff base. However, the values of $L^*$ (luminosity and brightness) significantly decreased in the CF with 1.0% CO. This variation in the luminosity induced by the incorporation of CO could be due to the molecular alteration of chitosan.

The transparency of the film is a desirable property because the consumer needs to clearly see the product covered by the film [31]. The CFs with 0.5% and 1.0% CO incorporated presented greater transparency than the control (Table 2), which indicates that the incorporation of CO can reduce the opacity of the CF. However, even with this reduction, the values indicate that the films are slightly opaque. Another evaluated parameter was the transmission of light through the film, where it could be observed that the films with incorporated CO and the control have the property of being able to block ultraviolet radiation in the region of 280 nm, in a transmission % range of 0.15% to 0.02%. However, the lowest transmission of the films was in the 200 nm region (0.19%–0.01%).

*3.5. Antioxidant Activity.* The antioxidant activity of the films was measured by two methods, DPPH and ABTS. The combination of two methods to measure the antioxidant activity has been recommended for a better understanding of the extract antioxidant properties. Figure 4 shows the antioxidant activity (DPPH) of the extracts of the studied films. A significant increase in the antioxidant capacity of the CF with CO incorporated at 0.5% (6.01 times) and 1.0% (14.5 times) can be observed compared to the control. This activity can be attributed to the presence of the main CO antioxidant, which is eugenol; however, it could also be due to the presence of cinnamaldehyde, which has been reported to have a lower activity than eugenol, or to a synergetic effect among chitosan, eugenol, and cinnamaldehyde [32]. It has been previously demonstrated that chitosan presents antioxidant activity, which has been attributed to the presence of

TABLE 2: Light transmittance (%) and transparency values of CF and CF-CO films.

| Films | Wavelength (nm) | | | | | | | | Transparency value[*] |
| | 200 | 280 | 350 | 400 | 500 | 600 | 700 | 800 | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| CF | 0.19 | 0.15 | 20.36 | 30.46 | 37.25 | 40.20 | 42.09 | 43.47 | $2.66 \pm 0.012^a$ |
| CO-0.25% | 0.08 | 0.17 | 16.82 | 28.51 | 34.37 | 36.64 | 38.05 | 39.07 | $2.69 \pm 0.008^a$ |
| CO-0.50% | 0.01 | 0.02 | 13.75 | 27.19 | 33.42 | 36.01 | 37.58 | 38.71 | $2.56 \pm 0.010^d$ |
| CO-1.00% | 0.00 | 0.02 | 5.25 | 18.40 | 25.56 | 28.47 | 30.17 | 31.25 | $2.34 \pm 0.003^c$ |

[*]The data represent the mean ± standard deviation of three determinations, and different letters in the same column indicate significant differences ($p < 0.05$).
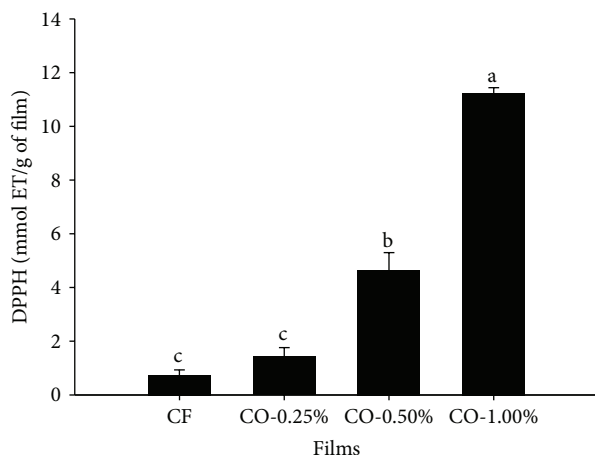


FIGURE 4: Antioxidant capacity of films extract determined by DPPH. The data are the mean values of at least three determinations. The mean values represented by the bars for each type of films extract that are indicated with a different letter are significantly different ($p \leq 0.05$).
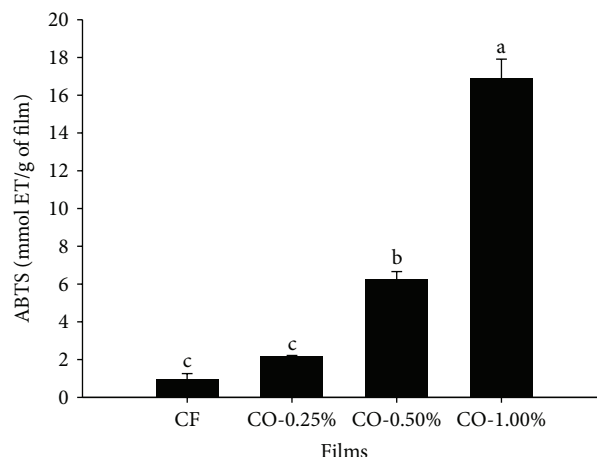


FIGURE 5: Antioxidant capacity of films extract determined by ABTS. The data are the mean values of at least three determinations. The mean values represented by the bars for each type of films extract that are indicated with a different letter are significantly different ($p \leq 0.05$).

nitrogen located at the C2 of the polymeric structure [33]. The antioxidant activity tested by ABTS was similar to the results of the DPPH testing method. Therefore, these results show that the antioxidant capacity is increased between 6.1 and 16.4 times for the CF with CO incorporated at 0.5% and 1.0%, respectively, compared to the control (Figure 5).

*3.6. Evaluation of the Protective Effect on Human Erythrocytes.*
For this evaluation, a radical initializer was used (AAPH), which generates peroxyl/alkoxy radicals in the presence of oxygen at physiological temperature. These secondary radicals are responsible for biological damage to cells. Figure 6 shows the protective effects of the CFs with incorporated CO and the control extracts. In general, all films offered a protective effect to the erythrocytes; this effect provided more than 67% hemolysis inhibition. The extract of the CFs with 1.0% CO presented the greatest protective effect, inhibiting hemolysis by up to 80% ($p < 0.05$). It is important to highlight that the control presented a higher capacity for hemolysis inhibition. Previous studies have shown that chitosan, with its positively charged amino groups, can interact with the negative charges of the erythrocyte membrane and produce a possible chitosan-erythrocyte aggregate that does not seriously damage the erythrocyte membrane [34]. This aggregation most likely forms a net that protect erythrocytes against the
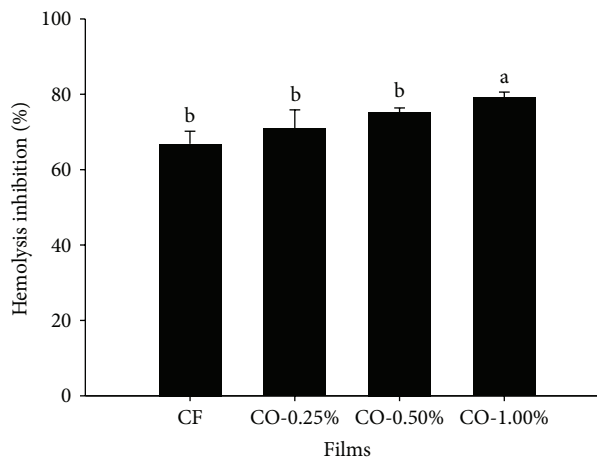


FIGURE 6: Antioxidant capacity of films extract determined by percentage of hemolysis inhibition. The data are the mean values of at least three determinations. The mean values represented by the bars for each type of films extract that are indicated with a different letter are significantly different ($p \leq 0.05$).

attacks of radicals generated by the AAPH. In the case of the CF extract with the highest incorporated CO concentration, its greater hemolysis inhibition effect could be associated

with the probable presence of other biomolecules that might inhibit the radicals, in addition to the possible protective net formed by the chitosan-erythrocyte charges.

## 4. Conclusions

In this study, multiple concentrations of CO were incorporated into CFs, and their physical and antioxidant properties were evaluated. The incorporation of CO was observed to decrease the CF solubility, while WVP was only reduced at low incorporated CO concentrations (0.25%). It was also observed that all films presented a yellow coloration, which was reduced at low incorporated CO concentrations but accentuated at incorporated CO concentrations of 1.0%. Regarding the transparency, it was observed that the incorporation of CO can improve the transparency of the films and also act as a good blocker of ultraviolet radiation. The antioxidant activity of the CF incorporated with CO strengthened the antioxidant activity of the films by 6.0 to 14.5 times compared to the control, and the films with 1.0% CO incorporated could protect erythrocytes against free radical attack by up to 80%.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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