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Effect of molecular weight reduction by gamma irradiation on the antioxidant capacity of chitosan from lobster shells

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ABSTRACT

This study assessed the effect of molecular weight (MW) reduction by gamma irradiation on the antioxidant capacity of chitosan with potential application in the preservation of foodstuffs. Two batches of chitosan were obtained by heterogeneous chemical N-deacetylation of chitin from common lobster (*Panulirus argus*). Irradiation of chitosan was performed using a ^{60}Co source and applying doses of 5, 10, 20 and 50 kGy with a dose rate of 10 kGy/h. Attenuated Total Reflection Fourier Transform Infrared Spectroscopy was used to identify main chemical features of chitosan. The average viscosimetric MW was determined by the viscosimetric method while the deacetylation degree by a potentiometric method. Thermogravimetric analysis and differential scanning calorimetry were conducted to evaluate the thermal degradation behavior of the chitosan samples, both under nitrogen flow. The antioxidant activity of chitosan solutions at 1% (w/v) in lactic acid at 1% (v/v) and Tween 80 at 0.1% (v/v) was evaluated through the ABTS assay and scavenging of DPPH radical by chitosan. The increase of irradiation dose with ^{60}Co until 50 kGy decreased significantly the MW of chitosan through the scission of glycosidic bonds without affecting its functional groups, while the DD (72–75 %) did not vary ($p > 0.05$). The AC of the chitosan solutions increased with the reduction of MW of chitosan by gamma irradiation.

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1. Introduction

Researches related with effective natural antioxidants for food preservation has increased during the recent years. Consumer behavior has changed from the use of chemical preservatives to the demand for natural additives, especially in ready-to-eat and fresh food products. Consequently, food industry needs to find alternative methods for preservation that covering the same antimicrobial or/and antioxidant properties and compatibility with food than the chemical additives.

Many natural compounds with antioxidant capacity for extending the shelf life of foods have been studied. The use of food additives from natural source involves the isolation, purification, stabilization and its incorporation to food without adversely affecting sensory, nutritional and safety features.

Chitosan, N-deacetylated derivative from chitin, can be included in this category (Hou et al., 2012). It is widely used because of its film-forming properties, good biocompatibility, biodegradability, low cost (Sirinivasa, Ramesh, Kumar, & Tharanathan, 2004), safety (Argullo, Albertengo, Pastor, Rodríguez, & Valenzuela, 2004), and be a renewable resource. The use of chitosan as antioxidant additive had been reported in numerous researches, which had demonstrated the capacity of this polymer for interacting with free radicals through ionic interactions with its amino groups (Mahdy, El-Kalyoubi, Khalaf, & Abd, 2013). Applications as antioxidant include the preservation of strawberries (Wang & Hao, 2013), orange (Martín-Diana, Rico, Barat, & Barry-Ryan, 2009) and apple juices (Chien, Sheu, Huang, & Su, 2007), peanuts, potato chips (Schreiber, 2012), beef hamburger (Georgantelis, Ambrosiadis, Katikou, Blekas, & Georgakis, 2007), fermented dried sausages (Krkic et al., 2013) and mayonnaise (García, Silva, & Casariego, 2014).

Several studies *in vitro* and *in vivo* had demonstrated that de antioxidant activity of chitosan is dependent on its MW (Mahdy et al., 2013). Thus, chitosan with lower MW showed a higher antioxidant activity. Moreover, the low solubility of chitosan is related with its high MW, which affects the applications of this polymer.

Ionizing radiations such us gamma irradiation, constitute one of the most popular tools for modifying the physical and chemical properties of some polymeric materials (Choi, ParK, Ahn, Lee, & Lee, 2002). In that sense, the gamma irradiation can be used to improve its solubility (Mao et al., 2004; Wasikiewicz, Yoshii, Nagasawa, Wach, & Mitomo, 2005) by decreasing the MW (Chmielewski, 2010; Ciechanska et al., 2004) by breaking the polymeric chains and thus enhance its antimicrobial and antioxidant properties (Chmielewski et al., 2007).

Although various studies have reported the application of irradiation in the modification of polymers such us chitosan and it is commercially available, the information about the relationship between the irradiation of chitosan and some of its biological properties is still limited. However, some papers informed about the influence of chitosan MW in its antioxidant (Kim & Thomas, 2007) and antimicrobial (Tikhonov et al., 2006) properties and as biostimulator for plant growing (Gryczka, Gawrońska, Migdał, Gawroński, & Chmielewski, 2008). Accordingly, the present study assessed the effect of MW reduction by gamma irradiation on the antioxidant activity of chitosan derived from lobster chitin by heterogeneous chemical N-deacetylation with potential applications in the preservation of foodstuffs.

2. Materials & methods

2.1. *Irradiation with ^{60}Co*

Two batches of chitosan, one at lab scale (Lot I) and the other at pilot scale (Lot II), were obtained at the Drug Research and Development Center (Havana, Cuba), by heterogeneous chemical N-deacetylation of chitin from common lobster (*Panulirus argus*). Irradiation of chitosan was performed at the Center of Technological Applications and Nuclear Development (Havana, Cuba) using a ^{60}Co source and applying doses of 5, 10, 20 and 50 kGy with a dose rate of 10 kGy/h in an irradiation facility Gamma PX-30. The distribution dose in the installation as well as the calibration of the irradiation process were verified through the Fricke dosimetrical system, while that for controlling the process were used dosimeters Red Perspex (Barrera, Otero, Rodríguez, & González, 2005). Before irradiation, the chitosan was packaged in bags of low-density polyethylene with 50 μm of thickness.

2.2. *Chemical characterization*

2.2.1. *Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (ATR-FT-IR)*

ATR-FT-IR was used to identify main chemical features of chitosan. ATR-FT-IR spectra were recorded on a Nicolet 6700 spectrophotometer in the 4000–400 cm^{-1} range with a diamond ATR crystal. The spectra were recorded with a resolution of 4 cm^{-1} and an accumulation of 64 scans.

2.2.2. *Molecular weight (MW)*

A capillary viscometer Ubbelhode No. 2121R with a constant temperature bath controlled by recirculating water (Haake, Germany) at 25.0 ± 0.01 °C was used to determine the average viscosimetric MW. The chitosan solutions were prepared using the solvent system lactic acid at 0.1 mol/L and sodium chloride at 0.2 mol/L. The initial polymer concentration was 9.6×10^{-3} g/mL in all cases and four dilutions (7.68×10^{-3}, 5.76×10^{-3}, 1.92×10^{-3} and 9.6×10^{-4} g/mL) were prepared. The fall time of each of the polymeric solutions was measured with five replicates for determining the viscometric parameters (Table 1).

Table 1 – Viscosimetric parameters for determining viscosimetric molecular weight.

Parameter	Symbology and equation
Relative viscosity	$\eta_r = \eta/\eta_0 = t/t_0$
Specific viscosity	$\eta_{sp} = \eta_r - 1 = (\eta - \eta_0)/\eta_0 \cong (t - t_0)/t_0$
Reduced viscosity	$\eta_{red} = \eta_{sp}/C$
Inherent viscosity	$\eta_{inh} = (\ln \cdot \eta_r)/C$
Intrinsic viscosity	$[\eta] = (\eta_{sp}/C)_{C=0} = [(\ln \cdot \eta_{red})/C]_{C=0}$

The intrinsic viscosity ([η]) were determined by the graphical method using Huggins's equation (Eq. (1)), which relates the reduced viscosity (η_{red}) vs. concentration (Parada, Crespín, Miranda, & Katime, 2004; Ravi-Kumar, 2000)

$$\eta_{red} = [\eta] + k_H[\eta]^2 C \quad (1)$$

where: k_H, Huggins constant.

After, the intrinsic viscosity was used to determine the average viscosimetric MW (M_v) from the Mark-Houwink's equation (Eq. 2) (Parada et al., 2004; Ravi-Kumar, 2000)

$$[\eta] = K\ M_v^{\alpha} \quad (2)$$

where: K and α, constants that depend on the buffer system. Reported values of K and α for the chitosan in the solvent used were 1.81×10^{-3} and 0.93, respectively (Fernández et al., 2004; Ravi-Kumar, 2000).

The average MW in number was related with the viscosimetric MW trough the Eq. (3) (Rapado et al., 2004):

$$\overline{M}_n = \overline{M}_v \left[(\alpha+1)\sqrt{(\alpha+1)}\right]^{-1/\alpha} \quad (3)$$

where: M_n, average MW in number.

2.2.3. Deacetylation degree (DD)

The DD was determined by the titration (Mettler Toledo, Switzerland) as reported by Parada et al. (2004) and Hernández, Águila, Flores, Viveros, and Ramos (2009). Chitosan sample (0.1 g) was dissolved in 20 mL of a solution of hydrochloric acid at 0.3 mol/L and then was added 20 mL of distilled water. The solution was titrated with sodium hydroxide solution at 0.3 mol/L previously standardized with potassium hydrogen phthalate as primary standard. The titration was achieved by measuring the potential change every 2 mL of added base. The addition was performed slowly and with continuous stirring to homogenize the solution and to avoid possible errors due to precipitation of the biopolymer. The obtained curve showed two inflection points and the difference between them corresponded to the amount of acid required to protonate the amino groups of chitosan. The DD was calculated as follows:

$$NH_2(\%) = \frac{16.1(y-x)}{m} f \quad (4)$$

$$DD(\%) = \frac{203(\%NH_2)}{[16.1 + 42(\%NH_2)]} \cdot 100 \quad (5)$$

where: y, higher inflection point (mL); x, lower inflection point (mL); f, molarity of the NaOH solution (mol/L); m, chitosan weight (g); 16.1, NH_2 milli-equivalent value to 1 mL of HCl at 0.1 mol/L.

2.3. Thermal properties

Thermogravimetric analysis (TGA, Mettler Toledo) was conducted to evaluate the thermal degradation behavior of the chitosan samples. The TGA apparatus was flushed with nitrogen atmosphere and 10 mg of sample was used. Each sample was heated from room temperature to 800 °C at a rate of 10 °C/min.

Differential scanning calorimeter (DSC, Metter Toledo) was used to obtain the thermograms under nitrogen flow. Samples (20–25 mg) were placed in hermetically closed DSC crucibles and heated from 25 to 500 °C and then cooled to 25 °C, both at 10 °C/min. Glass transition temperature (Tg) was measured at the inflection point of the marked increase of temperature.

2.4. Influence of irradiation on the antioxidant capacity of chitosan solutions

2.4.1. Preparation of solutions of chitosan

Chitosans irradiated or not, were dissolved at 1% (w/v) in a solution of lactic acid at 1% (v/v) with magnetic stirring during 2 h. Previously, Tween 80 was added at 0.1% (v/v) in the lactic acid solution.

2.4.2. ABTS assay

Total antioxidant capacity (AC) was evaluated according to the modified methodology proposed by Re et al. (1999) and Chien et al. (2007). The Trolox equivalent antioxidant capacity (TEAC) assay is based on the previous generation of ABTS radical cations through the reaction between potassium persulphate ($K_2S_2O_8$) and ABTS [2,2-azinobis (ethylbenzthiazoline-6-sulfonic acid 3)].

The assay consisted in adding 1 mL of the solution of $ABTS^{\bullet+}$ in 0.01 M phosphate-buffered saline, pH 7.4, with absorbance of 1.00 ± 0.02 AU at 734 nm, to a test tube containing 100 μL of chitosan solution at 1% (w/v) and another test tube with 100 μL of lactic acid at 1% (v/v) as blank. The sample or standard was allowed to react for 10 min at room temperature in the dark. After this period, the remaining $ABTS^{\bullet+}$ was quantified at 734 nm (Van den Berg, Haenen, van den Berg, & Bast, 1999) by using a spectrophotometer (Shimadzu UV-2401PC UV–VIS, Japan). For the calibration curve it was used, as the reference compound, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-carboxylic-2-carboxylic acid) at concentrations between 0 and 7 mM. Due to the addition of chitosan solution reduced $ABTS^{\bullet+}$ to its colorless form, the difference between the absorbance can be used for estimating the AC, expressed as Trolox in mmol/100 mL of chitosan solution at 1% (w/v).

2.4.3. Scavenging of DPPH radical

The ability of chitosan to scavenge free radicals was evaluated by the method of Shimada, Fujikawa, Yahara, and Nakamura (1992) and Chien et al. (2007) with some modifications. The reduction of 2,2-diphenyl-1-picryl hydrazyl radicals (DPPH) by chitosan was quantified spectrophotometrically (Shimadzu UV-2401PC UV–VIS, Japan) at 517 nm against the blank. Ethanolic solution of DPPH at 100 μM was added to a chitosan solution at 1% (w/v), using a proportion DPPH: chitosan of 3: 1. Then, the resulting solution was shaken. The percentage of discoloration of this mixture solution was calculated after 30 min of reaction at 25 °C in the dark, according to the Eq. (6).

$$\text{Scavenging ability } (\%) = \frac{(1 - \text{Absorbance}_{sample})}{\text{Absorbance}_{control}} \cdot 100 \quad (6)$$

2.5. *Statistical analysis*

Two-way ANOVA was performed using Statistics (version 7, 2004, StatSoft. Inc., Tulsa, USA) and the test of Duncan's multiple range to compare differences between samples. The level of significance was $p \leq 0.05$.

3. Results & discussion

3.1. *Chemical characterization*

3.1.1. *ATR-FT-IR*

Fig. 1 shows the ATR-FT-IR spectra corresponding to two lots of chitosan irradiated at different doses. The characteristic absorptions at 3362 and 3354 cm^{-1} corresponded to the hydroxyl groups (OH) for lots I and II, respectively. Bands at 3288 cm^{-1} indicated the existence of NH groups, while CH groups' bands appear at 2916 cm^{-1}. The bands of the amide groups were visible at 1646 and 1642 cm^{-1} for Lots I and II, respectively, and the bands of amino groups (NH_2) were observed at 1560 cm^{-1} for both lots. The transmittance at 1149 (Lot I) and 1059 cm^{-1} (Lot II) were related to the presence of pyranose group of chitosan and the corresponding bands at 1024 (Lot I) and 1028 cm^{-1} (Lot II) to the CO groups of COH, COC and CH_2OH rings.

As already reported, for the case of pure chitosan spectrum, main bands appear due to stretching vibration of the OH groups in the range of 3750 to 3000 cm^{-1}, which coincide with the extension of the vibrations of the N–H groups; and for C–H bonds in $-CH_2$ and $-CH_3$ groups appear at 2920 and 2875 cm^{-1}, respectively. Corresponding to vibration of the methylene and methyl groups' bands are also visible at 1375 cm^{-1} and 1426 cm^{-1}, respectively (Mano, Koniarva, &

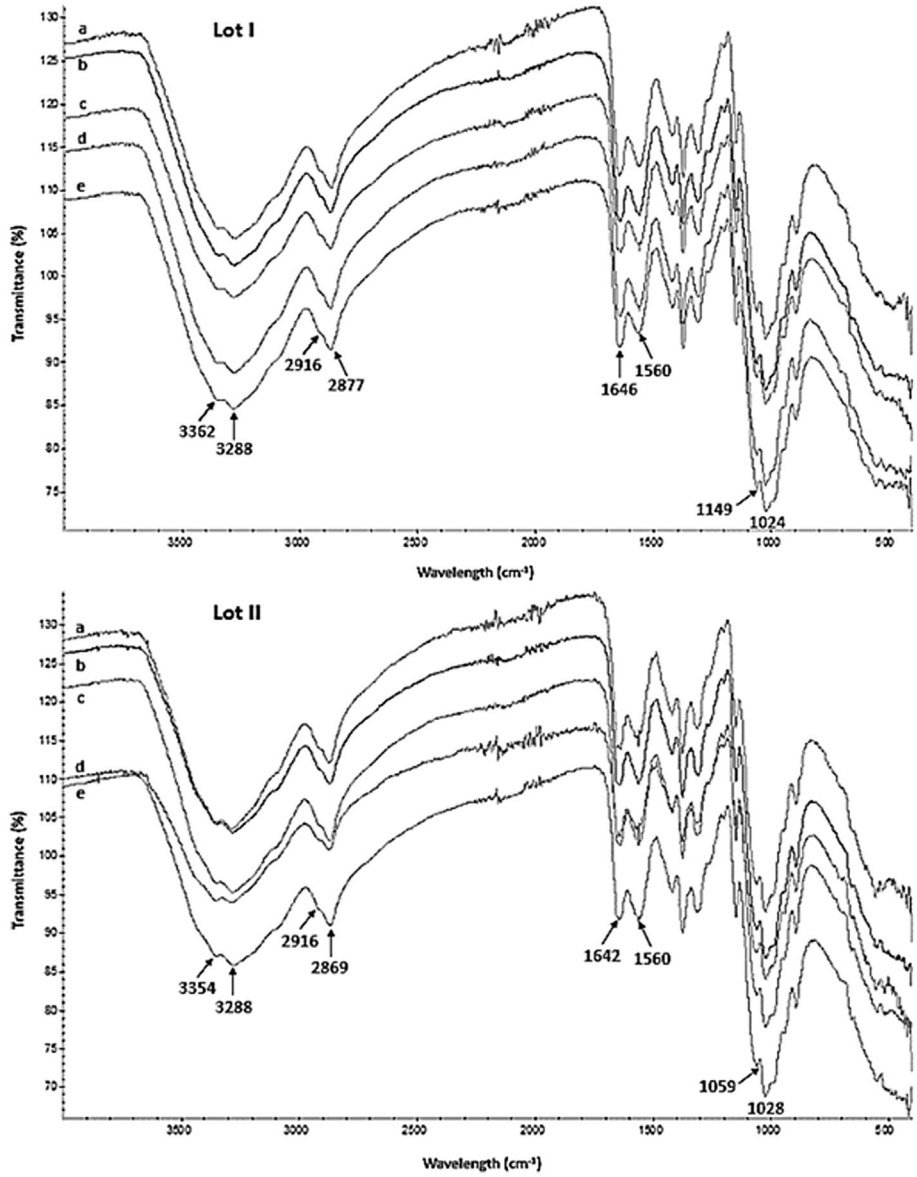

Fig. 1 – ATR-FT-IR spectra of chitosans with different irradiation doses: (a) 0 kGy, (b) 5 kGy, (c) 10 kGy, (d) 20 kGy and (e) 50 kGy.

Reis, 2003). The transmittance in the range of 1680–1480 cm^{-1} is related to the vibrations of the carbonyl bonds (C=O) of the amide groups CONHR (secondary amide: 1645 cm^{-1}) and the vibrations of protonated amino groups show their bands at 1574 cm^{-1} (Marchessault, Ravenelle, & Zhu, 2006).

Values in the range of 1160 to 1000 cm^{-1} are attributed to CO groups (Xu, Kim, Hanna, & Nag, 2004). The bands located near 1150 cm^{-1} are related to the asymmetrical vibration of CO in the bonds with oxygen resulting from the deacetylation of the chitosan. The bands near 1080–1025 cm^{-1} are attributed to CO of the rings COH, COC and CH_2OH. The transmittance values that are around 890 cm^{-1} correspond to the movement of the polysaccharide structure of chitosan (Darder, Colilla, & Ruiz-Hitzky, 2003; Paluszkiewicz et al., 2011; Yuan, Shah, Hein, & Misra, 2010).

The transmittance values shown in Fig. 4 for each functional group of chitosans of both lots are within the ranges reported by others authors, who also obtained that the increase of irradiation dose, did not affect the chemical structure of the polymer related with the detection of new functional groups in the polymer after exposure to γ-ray. Yang, Zhao, Liu, Ding, and Gu (2007) demonstrated that the increase of irradiation dose up to 25 kGy did not affect the chemical structure of the polymer, even if it continues to increase up to 100 kGy. Also Zainol, Md-Akil, and Mastor (2009) obtained similar results.

3.1.2. *Molecular weight*

The reduced viscosity decreased with increasing radiation dose and with decreasing polymer concentration in the solutions, fulfilled in this case the Huggins' expression for the chitosan under the conditions used. Through this equation relating viscosity with the concentration, the intrinsic viscosity of each of the irradiated and non-irradiated chitosans was determined graphically. The graphics of reduced viscosity vs. polymer concentration were obtained and the viscosity value for which the concentration becomes zero was taken as the intrinsic viscosity. This parameter had a tendency to decrease, varying inversely proportional to the absorbed radiation from 207.67 cm^3/g in the non-irradiated sample to 66.806 cm^3/g for the sample irradiated at 50 kGy. For doses of 5, 10 and 20 kGy, the values were 186.755, 167.655 and 99.473 cm^3/g, respectively. Paredes, Altanés, and Rapado (2005) also reported this behavior for chitosan irradiated at 0, 0.5, 1, 5, 10, 15, 20 and 25 kGy for which intrinsic viscosity values were 1590.68, 1504.21, 1146.21, 742.90, 558.18, 459.39, 182.62 and 185.89 cm^3/g, respectively.

The intrinsic viscosity reflects the degree of crosslinking of the chitosan chains in solution and often-higher values indicate higher degree of crosslinking. It has been reported that with the increasing of the irradiation dose, the degree of crosslinking of the chitosan chains and its MW decrease, due to the scission effect induced by the exposure to γ-rays (Shen, Hu, Wang, & Qu, 2011). The rapid decline of the intrinsic viscosity at low irradiation dose could be attributed to the degradation induced by this process. This degradation occurs in the amorphous regions of the polymer at low doses and continues in the crystalline regions at high doses (above 100 kGy), while that the degradation degree is faster in the amorphous that in the crystalline zones (Mitomo, Watanabe, Ishigaki, & Saito, 1994; Nagasawa, Mitomo, Yoshii, & Kume, 2000; Shen et al., 2011). The viscosimetric MW was calculated from the intrinsic viscosity of the chitosan (Table 2).

A direct relationship between the increase of irradiation dose and the decrease of MW was observed, with significant differences among the irradiated samples of the both lots. Apparently, the break of the polymer chains is the predominant process occurring during the exposure of the polymer to the γ-rays, agree with Yoksan, Akashi, and Miyata (2004). According to Zainol et al. (2009), irradiation induces depolymerization reaction that causes the scission of the molecule, leading to smaller chitosan chains through the following scission mechanism:

$$R{-}H \sim R^{\cdot}(C_4{-}C_6) + H^{\cdot}$$

$$R{-}H + H^{\cdot} \rightarrow R^{\cdot}(C_1{-}C_6) + H_2$$

$$R^{\cdot}(C_1,\ C_4) \rightarrow F^{\cdot}{}_1 + F_2(\text{scission})$$

$$R{-}NH_2 + H^{\cdot} \rightarrow R^{\cdot}(C_2) + NH_3$$

where R–H and $R{-}NH_2$ are macromolecules of chitosan; $R^{\cdot}(C_2)$ is a macro-radical of chitosan located in the carbon atom C_n; and $F^{\cdot}{}_1$ and F_2 are chain fragments after the scission.

Table 2 – Viscosimetric molecular weight, average molecular weight in number and deacetylation degree of chitosan with different doses of irradiation with ^{60}Co.

Lot	Irradiation dose (kGy)	Viscosimetric molecular weight (g mol^{-1})	Average molecular weight in number (g mol^{-1})	Deacetylation degree (%)
I	0	274 636 (414) a	95 100 (443) a	75,4 (1,3)
	5	245 361 (758) b	84 963 (700) b	74,4 (0,2)
	10	218 704 (202) c	75 732 (293) c	72,2 (0,1)
	20	125 644 (583) d	43 508 (502) d	71,6 (3,2)
	50	75 971 (772) e	26 307 (789) e	76,0 (2,5)
II	0	275 221 (342) a	95 303 (374) a	74,7 (0,08)
	5	247 847 (568) b	85 824 (508) b	72,6 (2,4)
	10	221 563 (305) c	76 722 (336) c	76,4 (2,9)
	20	126 469 (536) d	43 793 (525) d	73,5 (2,2)
	50	77 063 (673) e	26 685 (667) e	74,4 (0,3)

Mean (standard deviation); n = 3.
Different letters indicate significant differences ($p \leq 0.05$) by the multiple range test of Duncan.

Accordingly, rupture occurs by the glycosidic bonds of the main chain of the chitosan, only site of the molecule involved in the scission reaction. In addition, Zainol et al. (2009) reported a reduction in the intrinsic viscosity and MW with the increase of irradiation dose. Thus, chitosan with 576 kDa was irradiated at 10, 25, 50 and 100 kGy for obtaining MW of 458.35, 242.10, 159.04 and 105.81 kDa for each dose, respectively.

Shen et al. (2011) irradiated chitosan with 497 kDa at 10, 20, 50, 100, 150 and 200 kGy and obtained a reduction in MW corresponding to 399, 371, 228, 106 and 104 kDa for each dose, respectively. Paredes et al. (2005) also described this trend, reporting changes in MW from 2462 kDa for the non-irradiated polymer to 245 kDa for chitosan irradiated at 25 kGy. It should be noted that although previously cited research used chitosans with different MW and irradiation doses, the tendency to decrease the MW with the rise of the absorbed radiation showed the same proportion in all cases.

Fig. 2 depicts the reciprocals of the average MW in number of the chitosan as a function of the irradiation dose. Both lots of chitosan presented a lineal correlation between the reciprocals ($1/M_n - 1/M_{n0}$) and irradiation doses of chitosan, with high correlation coefficients for Lot I and II of 0.9910 and 0.9902, respectively. These results agreed with the reported by Paredes et al. (2005) for the irradiation of chitosan between 0.5 and 25 kGy, and indicates that the chains breaking up of the polymer occurs as the predominant process.

3.1.3. *Deacetylation degree*

Considering the above results, it can be said that the process of chitosan irradiation at reported doses did not affect the DD (Table 2), since no significant differences were found among the samples. DD values ranged between 72 and 75% in all cases. Consequently, the irradiation cleaves the macromolecule producing smaller chain segments with chemical structure identical to the original. It can be said that according to this behavior, the purity of the chitosan evaluated in terms of DD, remains quantitatively in high levels after irradiation at doses used in the present study. Some authors such as Lim, Khor, and Koo (1998) and Zainol et al. (2009) described this trend for irradiated chitosan using different doses. According with them, the scission mechanism of chitosan is based on that only the glycosidic bonds of chitosan molecule are broken by gamma irradiation and the remaining functional groups of the polymer, including the amide groups, are not affected by the attack of free radicals. As in the present research, Zainol et al. (2009) reported no significant changes in the values of DD that ranged between 71 and 74% vs. irradiation doses of 0, 10, 25, 50 and 100 kGy. Meanwhile, Lim et al. (1998), who worked with highly deacetylated chitosan (98.17%) irradiated until 25 kGy, also obtained no significant difference in the DD (98.08–98.93%) before and after irradiation.

Unlike these authors, others suggest that the DD increases with the irradiation dose. In this case, Rashid, Mizanur, Kabir, Shamsuddin, and Khan (2012) determined the DD of chitosans irradiated at 0, 2, 5, 20, 30, 50 and 100 kGy and reported values of 73.8, 74.33, 77.76, 78.32, 78.88, 79.5 and 79.99% for the DD, respectively. This behavior was related with a decrease in transmittance with increasing irradiation dose, according to the pointed out by Baxter, Dillon, Taylor, and Roberts (1992). This fact is associated with the hydrolysis of acetamide to amine, favored by ionizing radiation (Kittur, Prashanth, Udaya, & Tharanathan, 2002).

Contrary to these results, Shen et al. (2011) reported that the DD decreased when the irradiation dose increased from 0 kGy to 10, 20, 50, 100, 150 and 200 kGy, respectively. In this research, chitosans were irradiated at 0, 10, 20, 50, 100, 150 and 200 kGy, with significant differences in the values of DD, especially against 10 and 20 kGy, for which decreased from 88.5 to 84.1%. From 20 to 50 kGy decreased again to 70.8%, without changes compared to higher doses. In this case, the decrease in DD is not the result of an increase in the amide groups, but the decrease of the amino groups (NH_2), due to the effect of chain scission of the polymer by irradiation. It is proposed that, following irradiation some NH_2 groups of chitosan will be removed and converted to gaseous ammonia after joining with the hydrogen radicals. These results did not agree with those informed by Zainol et al. (2009), who concluded that the macromolecule is cleaved only by the glycosidic bonds, and that the remaining functional groups are affected by the irradiation, but these differences may be due to the state of chitosan, namely, a gel (Shen et al., 2011) or powder (Zainol et al., 2009).

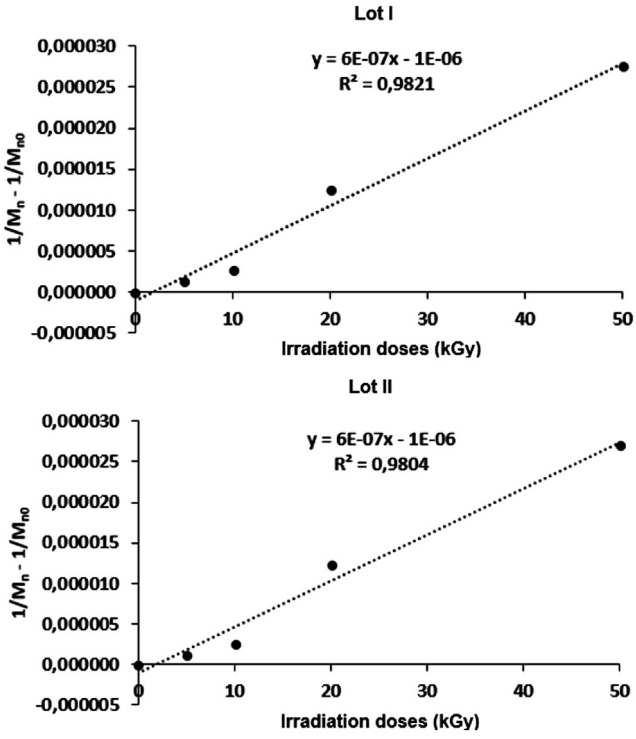

Fig. 2 – Reciprocals of the average molecular weight in number of the chitosan as a function of the irradiation dose.

3.2. *Thermal properties*

The thermogravimetric behavior (Fig. 3) initially displays a mass loss between 40 and 150 °C that is influenced by the moisture content of each sample. As the irradiation dose increases, water loss is lower, indicating that this treatment

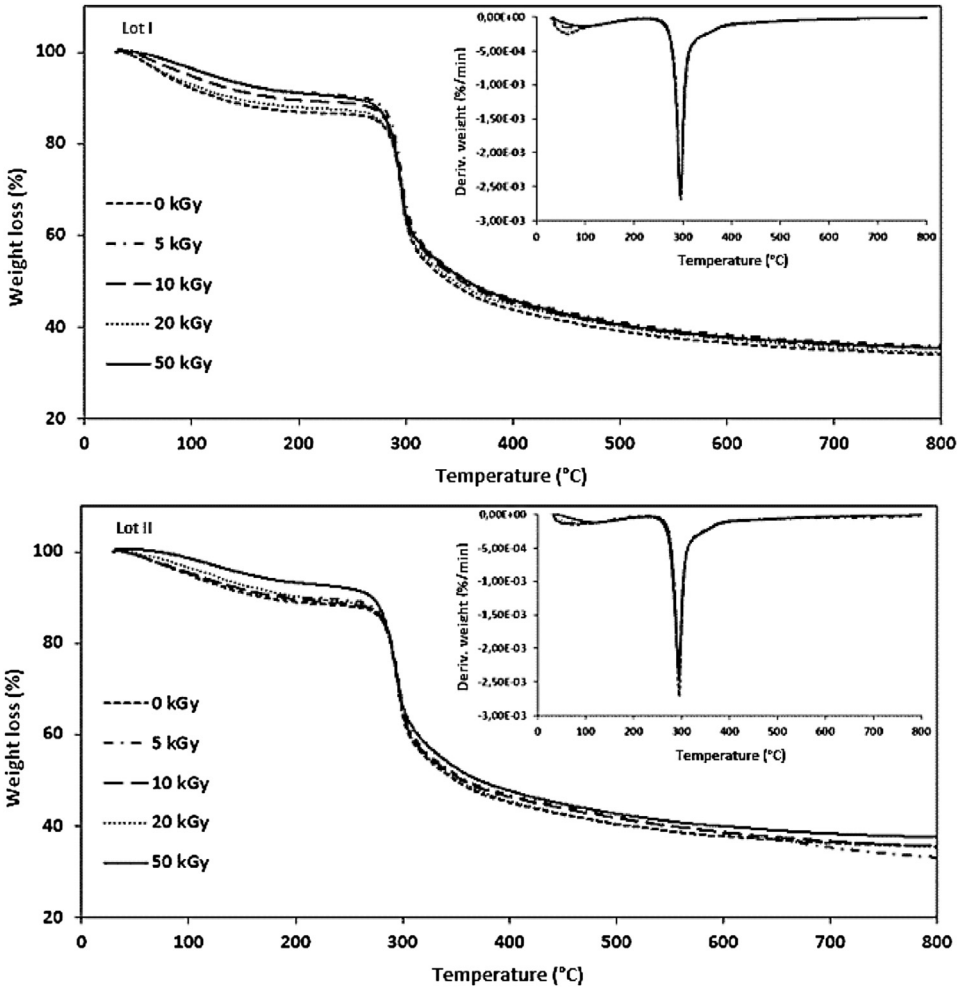

Fig. 3 – Thermal degradation profiles of chitosans with different irradiation doses.

would affect the water retention capacity of chitosan, as also shown in the DSC (Fig. 4).

The second thermal event was due to the chitosan decomposition, including depolymerization phenomena and the degradation of the glucopyranose units and their subsequent oxidation. This decomposition takes place from 280 to 300 °C for all samples, in agreement with Britto and Campana-Filho (2007) and Hong et al. (2007). In the present study, the results did not show changes in the thermal stability of the samples due to the irradiation. The structural configuration of the chitosan is preserved during this treatment.

The DSC thermograms of chitosan samples was obtained to compare the stability of the polymer before and after irradiation. A comparison of the thermograms (Fig. 4) shows the exothermic peaks at the decomposition temperatures in the region between 300 and 310 °C for both lots, in correspondence with the thermogravimetric analysis discussed above and in the temperature range reported by some authors as Rashid et al. (2012) when compared chitosans irradiated at different doses. This trend is also observed for the case of the Tg that is seen in the endothermic peak in the center of the graph, in the region of 270–280 °C for lot I and 265–285 °C for lot II, which, despite having different temperature ranges, had no significant difference between them.

The dewatering temperatures were between 100 and 120 °C, and 90 and 98 °C for lots I and II, respectively. The degradation of chitosan molecules by irradiation may be responsible for certain effects on thermal properties.

Rashid et al. (2012) found that chitosan with the lower MW is degraded at lower temperatures than the higher MW, perhaps due to that in the chitosan with the lower MW, the interactions between molecules are weaker and less energy is required for the thermal movement caused by the temperature, breaks these interactions. The degree of crystallinity of the chitosan also decreases, which contributes to decrease the Tg.

3.3. *Antioxidant capacity*

Considering the potential use of chitosan as antioxidant for food preservation and that it did not find information about the evaluation of AC of Cuban chitosan obtained by N-deacetylation of chitin from common lobster (*P. argus*), the present paper determined this biological activity of solution of irradiated chitosan with different MW through ABTS and DPPH assays.

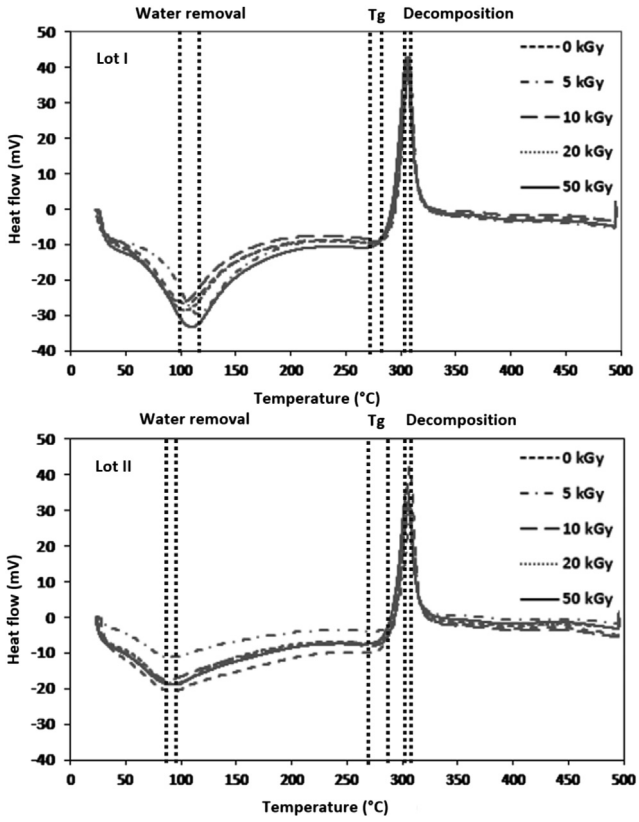

Fig. 4 – DSC thermograms of chitosans with different irradiation doses.

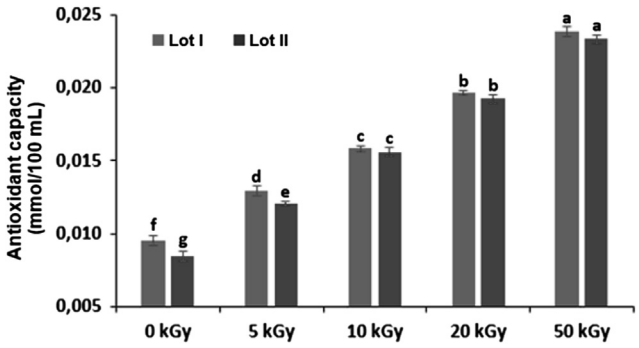

Fig. 5 – Effect of irradiation dose in the antioxidant capacity of a chitosan solution at 1% (w/v). Error bars indicate standard deviation (n = 3). Different letters indicate significant differences ($p \leq 0.05$) by the multiple range test of Duncan.

In both assays of AC it was prepared chitosan solutions at 1% (w/v) due to that in the mostly of papers related with this subject it was used a maximal concentration of 1% (w/v) and the increase of the polymer concentration, increase the antioxidant response (Chien et al., 2007; Mahdy et al., 2013; Yen, Yang, & Mau, 2008). Besides that, the viscosity of solutions with a higher concentration of 1% (w/v) should affect the sample volumes to use in each determination.

3.3.1. *ABTS assay*

Fig. 5 shows that the ^{60}Co exposure increased between 2.5 and 3 times, the Trolox equivalent antioxidant capacity of the chitosan solution at 1% (w/v), with significant higher values for chitosan of both lots irradiated at 50 kGy. This was evidenced by discoloration of the radical solution of $ABTS^{\bullet+}$, because of the reduction by chitosan.

The increase of the AC as function of irradiation dose may be related with the reduction of MW of the chitosan due to the breaking of the acetal bonds (C1 and C4) with the subsequent formation of new active sites, capable of reacting with highly reactive species such as free radicals. Also might occur the rupture of amide bond causing a partial deacetylation of the molecule (Rashid et al., 2012), although this was not evidenced in the present study, because the DD did not present appreciable variations with the increasing of irradiation dose (Table 2).

The AC of the chitosan may be explained by various mechanisms. One of them is the ability for scavenging free radicals, in which the polymer eliminates several of these radicals by the action of the nitrogen in the C2. It has been reported (Xie, Xu, & Liu, 2001) that scavenging capacity is related with the fact that the free radicals can react with the H^+ from the ammonium ions (NH_3^+) of the stable molecules. Various authors have tested the AC of aqueous solutions of chitosan through its ability for scavenging hydroxyl radical (Xie et al., 2001), and chelating metal ions (Xue, Yu, Hirata, Terao, & Lin, 1998).

Chien et al. (2007) evaluated the AC of three chitosans with different MW. They reported an AC of 2.15 μM of Trolox equivalent for chitosan of 12 kDa (lower MW) and 1.46 and 0.89 μM of Trolox equivalent for chitosans of 95 and 318 kDa, respectively. In general, these results are agree with those obtained in the present study, where the chitosan with the lower MW after irradiation, showed the higher AC (2.4 μmol of Trolox equivalent).

Sweetie, Ramesh, and Sharma (2004) compared the antioxidant activity of chitosan irradiated at different doses. The results, expressed as antioxidant activity coefficient, showed that chitosan irradiated at 25 kGy presented a 27 times higher activity than the non-irradiated. However, doses above 40 kGy did not significantly increase its AC.

3.3.2. *Scavenging of DPPH radical*

The values of the scavenging capacity of free radicals by chitosan solutions behaved, in all cases, higher than 52% without significant differences with the increasing of the irradiation dose (Fig. 6) and corroborated the results obtained by the ABTS assay referring to the AC of chitosan against reactive species. This effect can be explained as proposed by Xie et al. (2001) explained in the previous section.

This assay has been widely used by other researchers that, in accordance with the present research, reported AC for chitosan. Sweetie et al. (2004) compared the AC of solutions of 1% (w/v) of non-irradiated and irradiated chitosan at different doses and reported that chitosan irradiated at 20 kGy showed an AC six times higher than non-irradiated chitosan, with a value of 60.8% of DPPH radical scavenging without significant differences compared to higher doses until to 40 kGy.

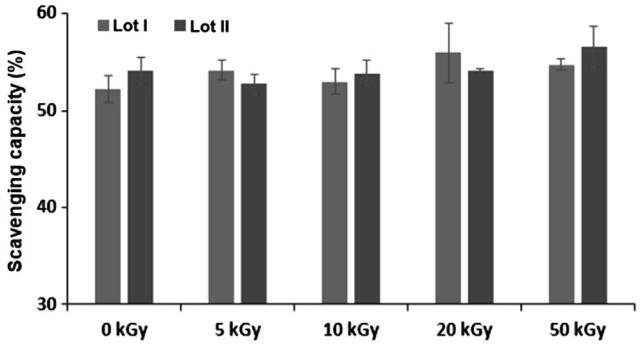

Fig. 6 – Influence of the irradiation dose in the scavenging capacity of DPPH radical by chitosan. Error bars indicate standard deviation (n = 3).

Kim and Thomas (2007) determined the AC of chitosans of 30, 90 and 120 kDa through DPPH assay; reported higher activity for the lower MW, with radical scavenging percentage between 40 and 100% when the concentration was increased from 0.2 to 1% (w/v). Chitosans of 90 and 120 kDa showed less AC, with a scavenging percentage from 9 to 37% for each of the above concentrations. Therefore, it can be suggested that the ability of chitosan to scavenge free radicals depends on the concentrations and MW of the polymer.

Chien et al. (2007), who also used this method, reported that the AC of chitosan increases by the increasing of concentration and decreasing the MW. They used solutions chitosan of 12, 95 and 318 kDa at concentrations of 0.2, 0.4, 0.6, 0.8 and 1% (w/v) each one. The scavenging percentage of chitosan with 12 kDa increased from 25% for solution at 0.2% (w/v) to 53% for the solution at 1% (w/v). Lower MW chitosan exhibited excellent AC, attributable to its strong ability to donate hydronium ions.

It was pointed out that one of the mechanisms through chitosan exerts its scavenging activity is related with that the free radicals can react with residual free $-NH_2$ groups to form stable molecules and the $-NH_2$ groups can form ammonium groups (NH_3^+) by capturing an hydronium ion from the solution (Yen et al., 2008).

Contrary to this, Sweetie, Ramesh, and Arum (2008) suggest that chitosan has poor AC due to the very low scavenging percentage obtained by the DPPH assay. Although the nitrogen atom of the chitosan has a par of unshared electrons that can be potentially donated, in solutions, the $-NH_2$ groups are, mostly, protonated with the impossibility to donate electrons. Moreover, chitosan lacks of an H^+ atom that can be easily donated for acting as a good antioxidant (Schreiber, Bozell, Hayes, & Zivanovic, 2013). By their part, phenolic compounds, classified as primary antioxidants, scavenge free radicals by donating an H^+ atom ($A{-}OH + R \rightarrow A{-}O + RH$) or an electron ($A{-}OH + R \rightarrow AOH^+ + R^-$), and the resulting phenoxyl radicals ($A{-}O$ or $A{-}OH^+$) are stabilized by the delocalization of unshared electrons around of the aromatic ring (Eskin & Przybylski, 2000; Leopoldini, Russo, & Toscano, 2011).

As can be seen, there is usually a tendency of an increase in AC with the increasing of the irradiation dose and decreasing of MW in the above-mentioned researches. In this regard, it is important to note that the determination of AC should take into account various factors that may influence the response variable as the polymer concentrations, irradiation doses, reagent/sample ratio and MW of chitosans, which, as described above, also affect its others properties.

Chien et al. (2007) used a proportion DPPH solution (100 μM): chitosan solution of 1:4, contrary to the ratio employed in the present research (3:1) considering the proposal of Halliwell and Gutteridge (1999), who pointed out that an antioxidant is all substance, that presented in low concentrations respect to an oxidable substrate, retards or prevents significantly the oxidation of this substrate. By other way, Kim and Thomas (2007) used a proportion DPPH solution (0.2 mM): chitosan solution of 1:1, which, besides the differences among chitosans used in each research, limits the comparisons of the results.

According to Frankel and Meyer (2000), various factors influence the effectiveness of antioxidants in complex and heterogeneous systems such as food and biological systems. This includes the properties of the lipid fraction/aqueous phase of the antioxidant, oxidation conditions and physical state of the oxidizable substrate. The influence of all these parameters cannot be evaluated using only a single test method. Consequently, it is noteworthy that in all of cited researches, the AC of chitosan was determined by different methods to compare the behavior of the samples and the results according to each technique, showed in all cases, the protective effects of the chitosan against oxidation reactions.

The different assays for estimating the AC only permits to examine the possibility of that a particular compound should act as antioxidant in one or various forms *in vivo* or in a food matrix. Alternatively, these assays can show that an antioxidant action is viable when the compound shows a protective action *in vitro* at concentration inside of an interval in which it can be present in foods or *in vivo*. However, inclusive an excellent *in vitro* antioxidant, not necessary will function *in vivo* or in a food, due to, for instance, that did not be absorb, or did not reach the correct action place or be rapidly metabolized to inactive products (Halliwell, 2002). That's why, it is necessary to evaluate the effect of chitosan as an additive or coating as active packaging method, in the inhibition of lipid oxidation of food.

4. Conclusions

The increase of irradiation dose with ^{60}Co until 50 kGy decreased significantly the MW of chitosan through the scission of glycosidic bonds without affecting its functional groups, while the DD (72–75 %) did not vary ($p > 0.05$). The AC of the chitosan solutions increased with the reduction of MW of chitosan by gamma irradiation.

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