UNIVERSITAT POLITÈCNICA DE VALÈNCIA

INFLUENCIA DEL GRADO DE DESACETILACIÓN Y DEL GRADO DE POLIMERIZACIÓN DEL QUITOSANO EN LA REDUCCIÓN DEL CONTENIDO EN ACRILAMIDA EN SISTEMAS MODELO

MÁSTER EN GESTIÓN DE LA CALIDAD Y SEGURIDAD ALIMENTARIA

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RESUMEN: En junio de 2015, la EFSA (Agencia Europea de Seguridad Alimentaria) confirmó las evaluaciones anteriores acerca de la relación existente entre el compuesto acrilamida, generado en aquellos alimentos sometidos a temperaturas superiores a 120 ºC durante su procesado, y el riesgo de padecer determinados tipos de cáncer. Esto, conjuntamente al hecho de que los consumidores demandan cada vez más productos más saludables, obliga a la industria a tomar medidas para satisfacer estas necesidades. Por ello, el objetivo principal de este trabajo ha sido evaluar la influencia del grado de desacetilación (DD) y el peso molecular (Mw) del quitosano sobre la generación de acrilamida, con el tiempo, en un sistema modelo compuesto por asparagina y glucosa/fructosa a 180 ºC. Adicionalmente, se evaluó la actividad antioxidante de los quitosanos con diferentes DD y Mw. Los resultados mostraron que los quitosanos con mayor DD (86.5 y 92.8% alcanzaron reducciones de acrilamida de entre 44 y 81% dependiendo del tiempo de reacción, con respecto al control (ausencia de quitosano en el sistema modelo). Por otro lado, existió una relación inversamente dependiente entre el Mw del quitosano y la formación de acrilamida, de forma que cuanto mayor Mw, menor generación de este compuesto. Concretamente, el quitosano de menor peso molecular (571KDa), sólo supuso reducciones de entre 7 y 17% respecto al control, durante 5 y 10 minutos de procesado. En cuanto a la actividad antioxidante del quitosano se refiere, ésta aumentó conforme lo hicieron su concentración y el DD, y disminuyó a medida que lo hizo su Mw.

PALABRAS CLAVE: Acrilamida, quitosano, desacetilación, polimerización, sistemas modelo, actividad antioxidante.

ABSTRACT: In June 2015, EFSA (European Food Safety Agency) confirmed previous assessments of the relationship between acrylamide compound, generated in those foods subjected to temperatures above 120 ° C during processing, and the risk of suffering certain types of cancer. This, together with the fact that consumers are increasingly demanding healthier products, force food industry to take to meet these needs. Therefore, the aim of this study was to evaluate the influence of the degree of deacetylation (DD) and molecular weight (Mw) of chitosan on acrylamide generation, with respect to time, in a model system consisting of asparagine and glucose / fructose at 180 °C. Additionally, the antioxidant activity of chitosans with different Mw and DD was evaluated. The results showed that those chitosans with higher deacetylation

degree (86.5 and 92.8%) achieved a decrease of acrylamide between 44 and 81%, depending on reaction time, compared to the control (absence of chitosan in the model system). Furthermore, there was an inverse relationship between the Mw of chitosan and acrylamide formation even if in this study the acrylamide reduction was only of 7-17% for the lowest-Mw chitosan (571KDa) with the respect to the control after 5 and 10 minutes reaction time. With regards to the effect of DD and Mw on the antioxidant activity of chitosan, it increased as did their concentration and DD, and decreased as did his Mw.

KEY WORDS: Acrylamide, chitosan, deacetylation, polymerization, model systems, antioxidant activity.

RESUM: Al juny de 2015, l'EFSA (Agència Europea de Seguretat Alimentària) va confirmar les avaluacions anteriors sobre la relació entre el compost acrilamida, que es genera als aliments sotmesos a temperatures superiors a 120 ° C durant el seu pocessat, i el risc de patir certs tipus de càncer. Això, conjuntament amb el fet que els consumidors exigeixen cada vegada productes més saludables, força a la indústria d'aliments a satisfer aquestes necessitats. Per tant, l'objectiu d'aquest estudi va ser avaluar la influència del grau de desacetilación (DD) i el pes molecular (Mw) de quitosà en la generació d'acrilamida, amb el temps, en un sistema model que consisteix en asparagina i glucosa / fructosa a 180°C. A més, es va avaluar l'activitat antioxidant dels quitosans amb diferent MW i DD. Els resultats van mostrar que els quitosans amb major grau de desacetilació (86.5 i 92.8%) van reduir el contingut d'acrilamida entre 44 i 75%, depenent del temps de processat, en comparació amb el control (absència de quitosà en el sistema model). A més, pareix haver una relació inversa entre el Mw de quitosà i la formació d'acrilamida, de fet, la reducció d'acrilamida amb el quitosà de més baix pes molecular (571KDa) va ser de entre 7 i 17% respecte al control després de 5 i 10 minuts de reacció. Pel que fa a l'efecte de DD i Mw a l'activitat antioxidant de quitosà, va augmentar com ho va fer la seva concentració i DD, i al disminuir el seu Mw.

PARAULES CLAU: Acrilamida, quitosà, desacetilació, polimerització, sistemes model, activitat antioxidant.

INTRODUCTION

Consumers are becoming more health-conscious and demand high quality food products, binding the food industry to take measures to provide these needs. An example is to reduce the levels of acrylamide in foods. Acrylamide is an organic amide compound that can be formed during cooking or processing food at high temperatures especially in starchy foods such as potatoes or cereals). Acrylamide is mainly formed in food by the reaction of asparagine (an amino acid) with reducing sugars (particularly glucose and fructose) in the context of the Maillard reactions, during reactions involving 3-aminopropionamida at high temperature (usually above 120° C) and low moisture (AECOSAN, 2014).

In 1994, acrylamide was classified by the IARC (International Agency of Research on Cancer) as “probable carcinogenic for humans” (Group 2A). In February 2005, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) met to evaluate health risks of acrylamide, and concluded that adverse effects including nervous system damage, reproductive and developmental problems were unlikely at average intakes, but nerve structural changes could not be excluded at high intakes. JECFA used the Margin of Exposure (MOE) approach to evaluate risks of acrylamide to humans and recommend reducing its presence in food and collect data concentration of acrylamide in foods ready for consumption (AECOSAN, 2014).

On 4 June 2015, EFSA published its first full risk assessment of acrylamide in food. Experts from EFSA’s Panel on Contaminants in the Food Chain reconfirmed previous evaluations that acrylamide in food potentially increases the risk of developing cancer for consumers in all age groups. Evidence from animal studies shows that acrylamide and its metabolite glycidamide are genotoxic and carcinogenic: they damage DNA and cause cancer. Evidence from human studies that dietary exposure to acrylamide causes cancer is currently limited and inconclusive (EFSA, 2015).

In the last decade some strategies to mitigate acrylamide in food have been developed, based on interfering with the chemical reaction that triggers acrylamide generation. Those strategies can be resumed in two major groups, one related to removing acrylamide precursors and the other based on interfering with Maillard reactions. Blanching or immersions in asparagine before frying promote the lixiviation of reducing sugars and asparagine (Pedreschi *et al*., 2011). Citric acid or natural extracts resulted interferences in acrylamide formation (Pedreschi *et al*., 2007; Morales e*t al.*, 2014) and modifying processing conditions, for example, vacuum frying or roasting, irradiation or air-frying are also useful strategies for this goal (Anese *et al.*, 2014, Tripathi *et al*., 2015, Sansano *et al*., 2015). Certain hydrocolloids (pectin and alginic acid) have also demonstrated good potential as additives to mitigate up to 50% of acrylamide content in foods (Zeng *et al.* 2010). Recently, chitosan (a derivative of chitin) has been tested as a new strategy in reducing acrylamide formation in model and batter systems. The mechanism might be due to free amino groups present in chitosan which compete with asparagine in binding to the reducing sugars, which implies a reduction in acrylamide formation. Gimeno *et al.* (2014) evaluated the potential of chitosan in model systems, achieving reductions up to 70 and 86%, compared to the control using 0.5 an 1% of chitosan, respectively. On the other hand, Aguilera et al. (2014) tested the incorporation of chitosan to batters to corroborate its efficiency in real systems. The results showed an average reduction of acrylamide of 32 ± 1.6 (%) and 57 ± 8 (%) when 0.27 and 0.54% chitosan was added at 7 minutes frying.

Chitosan is obtained by deacetylation of chitin, an abundant polysaccharide in nature (Figure 1) (López, 2012). Chitosan is insoluble in water, on account of its rigid crystalline structure, but soluble in acid dilutes such as acetic acid, formic acid, etc. and its nitrogen content is mostly in the form of primary

aliphatic amino groups (Kurita, 2006). Furthermore, chitosan has been found to be non-toxic, biodegradable, biofunctional, biocompatible and has strong antimicrobial and antifungal activities (Aider, 2010).

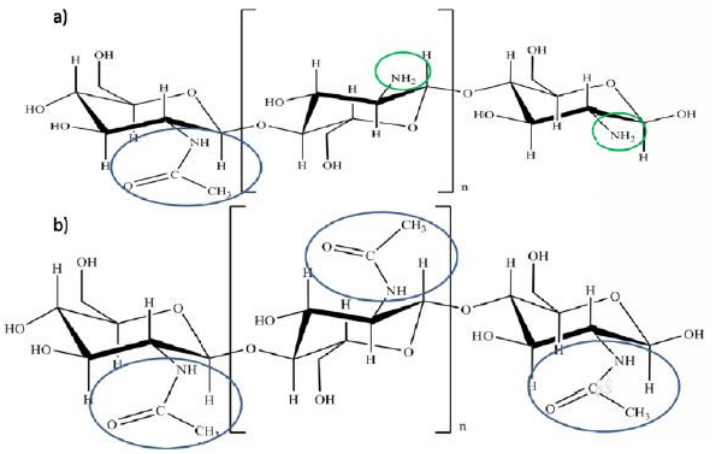

FIGURE 1. Structure of chitosan (a) and chitin (b) (López, M. Á., 2012).

Some of the current applications of chitosan are based on its antioxidant character (Darmadji & Izumimoto, 1994), antitumor (Tsukada *et a*l., 1990), anti-cholesterolemic, anti-ulcer and its antiuricemic properties. This set of properties stems from the capacity to bind specifically fatty acids, bile acids, phospholipids, uric acid and toxic gladin fraction (Muzzarelli *et al*., 2000). It also presents technological applications such as fruit juice clarification and acidification (Imeri & Knorr, 1988) or water purification (Fabris *et al.*, 2010).

The biological properties of chitosan (antimicrobial, antioxidant and anti-cholesterolemic) mainly depend on its deacetylation and polymerization degrees (Aranaz *et al*., 2009). Degree of deacetylation of chitosan can be modified by heterogeneous or homogeneous deacetylation, depending mainly on alkali concentration used, reaction time and temperature (Sannan *et al*., 1975). Heterogeneous deacetylation involved preferential reaction in the amorphous regions of the polymer, leaving almost intact the intractable crystalline native regions in the parent chitin (Kurita *et al.,* 1977). Regarding the degree of polymerization, it can be modified by chemical (acid) or enzymatic hydrolysis, the latter being easier to control. Oligosaccharides obtained are denominated COS (chito-oligosaccharides), which are linear co-oligomers formed by units of 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) and 2-amino-2-deoxy-β- D-glucopyranose (GlcN) linked by β- (1 → 4). Most biological properties are related to the cationic behavior of chitosan and the factor that has a higher effect is degree of deacetylation. However, in some cases, the molecular weight has a predominant role. In addition, other properties such as chain conformation, solubility or degree of substitution also affect to the biological properties (Aranaz *et al*., 2009).

The scientific evidence of chitosan effect on acrylamide mitigation, however needs a deeper understanding of the role of some properties of this polysaccharide on acrylamide formation. Therefore, the objective of this study was to evaluate the influence of deacetylation and molecular weight of

chitosan on the acrylamide generation in model systems (5 µmol of asparagine and 5 µmol of a mixture of glucose-fructose 1:1, with 100 µL of solution of 1% chitosan in 0.5% lactic acid).at 180 ºC during the reaction time (5, 10 and 15 minutes). In addition, the effect of deacetylation and polymerization processes on the antioxidant activity of the different chitosans obtained was also studied.

MATERIALS & METHODS

Reagents

Reducing sugars (glucose and fructose), asparagine, chitosan (Poly (D-glucosamine) *Deacelyled chitin, high molecular weight), acetic anhydride, DPPH (2,2-Diphenyl-1-Picrylhydrazyl) and BHT (Butylated hydroxytoluene) were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Acetic acid, sodium hydroxide, lactic acid, methanol and HPLC grade were from Panreac (Barcelona, Spain). The standard of acrylamide (≥ 99%) was from Merck (Darmstadt, Germany). The bidistilled water was obtained by a purification process of water (Milli-Q, Millipore Corp., Bedford, MA).

Acetylation and deacetylation of commercial chitosan

Chitosans with different degrees of deacetylation (DD) were prepared by acetylation and deacetylation mechanism from commercial chitosan. Both processes are performed according to the protocol described by Zhou *et al*. (2008), with slight modifications. Acetylation was performed dissolving 15 g chitosan in a solution of 2% acetic acid (300 mL), distilled water (400 mL) and methanol (800 mL), and stirred for a further 20 minutes. Then, 2 mL of acetic anhydride were added into the solution and the mixture was stirred for 12 hours. At the end of the reaction, 1M NaOH was added to the solution in order to precipitate the chitosan. The precipitated was washed several times with distilled water and vacuum dried at 60°C. The deacetylation, it was performed by basic hydrolysis of commercial chitosan. For this purpose, chitosan (10 g) was mixed with aqueous NaOH in a ration of 1:10 (w/v) for 30 minutes at 100ºC, washed repeatedly with distilled water and dried 60ºC for 3 days. This process was carried twice in order to obtain the chitosan with the highest DD.

Depolymerization of commercial chitosan by acid hydrolysis

Hydrolysis of chitosan with different molecular weights (Mw) was preformed according to the method described by Zhou *et al*. (2006) with minor modifications. Commercial chitosan was dissolved in 2% acetic acid to obtain the reaction mixture with a final concentration of 2g chitosan/100 g solution. The reaction solution was heated at 70ºC with stirring for 2, 4 and 8 hours to obtain chitosan with different polymerization degrees. The resulting mixture was neutralized with diluted NaOH (0.1M). Absolute ethanol was added to the neutralized solution until the final ethanol concentration reached 70 mL ethanol in 1L solution to completely precipitate the chitosan. The chitosan samples with different molecular weights were collected by filtration, washed with pure water, and dried at 60ºC during three days.

Determination of the degree of deacetylation (DD)

Deacetylation degree was determined by titration method described by Jia & Li (2001). According to this, 0.2 g of each chitosan were dissolved in 20 mL of 0.1 M HCl under stirring for 4 h. Measurements were performed with a solution of 0.1 M NaOH by using a pH meter (Titrando – Metrohm). Potential concentrations were given as pH – log[H^+].

The DD of chitosans was calculated as follows (equation 1):

$$DD = \frac{\Delta V \text{ x } C_{NaOH} \text{x } 10^{-3} \text{x } 16}{M \text{ x } 0.0994} \qquad (1)$$

where ΔV is the volume of NaOH between two inflexion points (the first was the excess HCl and the second was the inflexion point due to the amino group of chitosan), C_{NaOH} is the concentration of the NaOH solution, M was the weight of chitosan sample, and 16 and 0.0994 are the related molecular weight and theoretical amount of amino group (Figure 2).

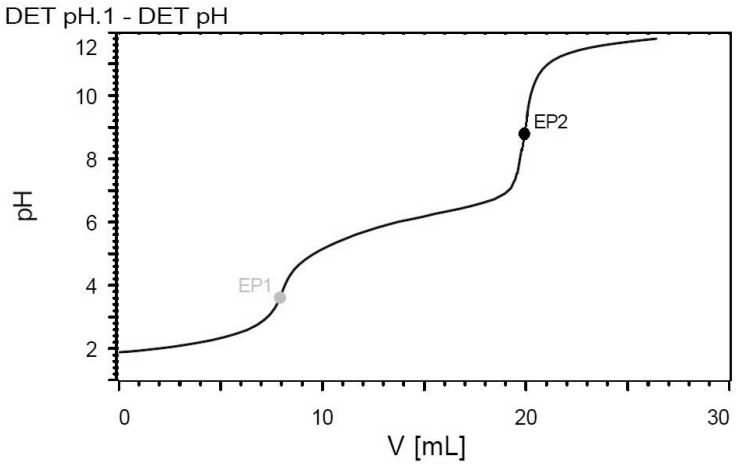

FIGURE 2. Potentiometric titration of chitosan. EP1 and EP2 correspond to the equivalence points of the titration of excessive HCl and the titration of protonated chitosan, respectively.

Determination of the molecular weight of chitosan

There are several techniques that can be used in determining the molecular weight of chitosans, but in this case viscometry has been used (Wang *et al*., 1991) (Argüelles *et al*., 2004). This technique determined the viscosity average molecular weight (Mv), which depends largely on the solvent used to dissolve chitosan and the temperature at which it is determined.

The measurements was performed using a Ubbelohde capillary viscometer No. 2121R, (Ø= 0.4 mm) equipped with a thermostat bath at 25.0° C ± 0.01ºC. Different samples of chitosan were dissolved in 0.1M acetic acid/0.2M NaCl (Roberts solvent). Solutions were prepared at different concentrations $5.0 \cdot 10^{-4}$, $6.5 \cdot 10^{-4}$ y 10^{-3} g/mL) and were filtered before the viscosity determinations. Draining time of a fixed volume of the diluted solutions of chitosan (t) and solvent (t_0) content between two consecutive marks of the capillary was

measured. From these, relative viscosity (η_r) and specific viscosity (η_{sp}) were calculated using the following equations 2 and 3:

$$(\eta_r) = \frac{\eta}{\eta_0} = \frac{t}{t_0} \qquad (2)$$

$$(\eta_{sp}) = \eta_r - 1 \qquad (3)$$

where η is chitosan solution viscosity and η_0 is viscosity of the pure solvent.

The relationship between the viscosity and the concentration was denominated reduced viscosity (η_{red}) and it is calculated by the equation 4:

$$(\eta_{red}) = \eta_{sp}/C \qquad (4)$$

where *C* is concentration of chitosan solution.

The intrinsic viscosity [η] was determined graphically, extrapolating values of reduced viscosity (η_{sp}/C) at zero concentration. The intrinsic viscosity was used to determine the viscosity average molecular weight (Mv) from the classical Mark-Houwink-Sakurada equation (equation 5):

$$[\eta] = Km \cdot Mv^a \qquad (5)$$

where *Km* and *a* are two constant depend on the particular polymer-solvent system. The values of "K" and "a" for chitosan in the solvent used were $1.81 \cdot 10^{-3}$ and 0.93, respectively (de la Paz *et al*., 2013).

Preparation of model systems and methodology for acrylamide determinations

A model system consisted of 5 µmol of asparagine and 5 µmol of a mixture of glucose-fructose 1:1 (2.5 µmol of each). The composition of the model system was based on the results previously obtained by Gimeno *et al*., (2014). This study evaluated the effect of commercial chitosan as a new tool in the mitigation of the generation acrylamide during frying processes in model systems (asparagine + glucose and/or fructose) at different pH and temperatures of reaction. Solution of 1% chitosan in 0.5% lactic acid was added. The pH value of the reaction mixture was adjusted to 4.0. Total reaction volume was 100 µL. Experiments were performed in a 25 mL test tube (Pyrex closed system), which were subjected to 180ºC in an oil bath (Solac Ideal 2000) during 5, 10 and 15 minutes, in triplicate. During frying processes only the bottom of the tubes was covered with oil. After time of reaction, the tubes were immediately placed on ice 5 min and, later, they were allowed at ambient temperature 5 min more.

Then, 2 mL of bidistilled water (Milli-Q) was added and the aqueous extract was obtained by vortexing for 1 min. The mixture was filtered with Nylon filter (0.45 µm) and it was transferred into vials for chromatographic analysis of acrylamide. In the same way a control without chitosan was performed.

The chromatography method used to determine acrylamide content in model systems was validated in a previous work Sansano *et al*., (2015). The method has a linearity >0.998 and a recovery of 82-109%, according to the European Directive 96/23/EC. The chromatographic analysis was performed with an Agilent 1200 Series HPLC system coupled to an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies Inc., CA, USA) with an electrospray type ionization source in positive ion mode. The column used in this study was a Zorbax Eclipse XDB C-18 (2.1 mm x 50 mm^2, 1.8 µm). The elution gradient was as follows: initially the composition was 100% of phase A (formic acid solution in 0.1% water, 2.5% of methanol) and 0% of phase B (methanol), up to 3 minutes. From the minute 3.10 30% of phase B until 3.5 minutes and in the minute 3.6 is set to the initial value: 0% phase B for one minute to equilibrate the column. The column oven temperature was set at 30°C. The flow used was 0.4 mL/minute and the injection volume was 5 µL. The conditions used in the ionization source were: 350 °C at 12 L/min for the drying gas (N_2), a nebulizer pressure of 40 psi and a capillary voltage of 4000 V. The ion monitored for acrylamide was m/z 72, and its product ions were 55.2 (quantitative) and 26.7 (qualitative). The acrylamide content of the samples was determined in triplicate.

Evaluation of the antioxidant activity

The free radical scavenging effect of chitosan was estimated according to the method of Fu *et al*. (2002) with some modifications. According to this method, the intensity of the violet color of the solution free radical 2,2-difenill-1-picrylhydrazyl (DPPH) decreases when there are antioxidants presents, and this change in absorbance recorded spectrophotometrically at 517 nm. Each chitosan solutions were prepared in 0.5% acetic acid at different concentrations (1.0 – 2.0 mg/mL). Reaction mixtures consisted of 1 mL of a chitosan solution, 1 mL etanol and 1 mL of 0.1mM DPPH ethanol solution, were raised to a final volumen of 4mL by 0.5% acetic acid solution. This was stirred with vortexing and then kept at 25 ° C in darkness for 30 minutes. The absorbance of the mixtures was measured at 517 nm against a blank without DPPH using a V-630, Jasco Inc. Spectrophotometer (Tokyo, Japan).

The DPPH radical scavenging activity was calculated using the equation 6:

$$\text{DPPH radical scavenging activity } (\%) = \frac{\mathrm{A_{control} - A_{sample}}}{\mathrm{A_{control}}} \cdot 100 \qquad (6)$$

where $A_{control}$ and A_{sample} are the absorbance of a control mixture without antioxidant and a mixture containing the sample, respectively.

Simultaneously, the antioxidant activity of a compound of reference, BHT (Butylated hydroxytoluene) was studied.

Fourier transformed infrared spectroscopy (FTIR)

The infrared spectra of different chitosan samples were determined using a NICOLET NEXUX FT-IR by the transmission method. Samples were ground with spectroscopic grade potassium bromide (KBr) powder and were

measured in the wavenumber range of 4000 and 400 cm^{-1} using 128 scans. Spectra were analyzed with the informatics program OMNIC™ (Thermo Scientific™ OMNIC™ Series Software).

Statistical analysis.

The influence of degree of deacetylation and the molecular weight of chitosan on acrylamide generation and the antioxidant activity was evaluated using Statgraphics Centurion XVI. Analysis of variance was carried out with a multifactorial ANOVA, obtaining a significance level of 95%.

RESULTS AND DISCUSSION

Deacetylation degree of chitosan

Apart from commercial chitosan (CCH), three other chitosan were obtained with different degree of deacetylation (DD). One of them was obtained by acetylation of commercial chitosan (ACH) and the other two by deacetylation once and twice (1DD and 2DD, respectively). The DDs of the resultant chitosan samples are detailed in Table 1.

TABLE 1. Deacetylation degree (%) (mean value and standard deviation) of commercial chitosan and reacetylated and deacetylated obtained ones**.**

	Deacetylation Degree (%)
Acetylated Chitosan (ACH)	49.4±0.3
Commercial Chitosan (CCH)	64.8±0.8
Deacetylated Chitosan x 1 (1 DD)	86.5±0.6
Deacetylated Chitosan x 2 (2 DD)	92.80±0.12

Viscosity average molecular weight

Different molecular weights (Mw) were obtained by the acid hydrolysis from commercial Chitosan (CCH). Table II shows the preliminary results needed to calculate intrinsic viscosit2

TABLE 2. Fall times and reduced viscosity for each concentration and hydrolysis times tested.

	C (mg/mL)	**CH_0**	**CH_2**	**CH_4**	**CH_8**
FALL TIME (s)	**0.0005**	130.5±0.2	129.6±0.8	130.3±1.0	112.1±1.0
	0.00065	148.72±0.12	143.9±0.5	142.81±1.08	121.4±0.9
	0.001	185.9±0.6	179.6±1.0	199±2	154.0±0.8
	solvent		87.3±0.7		
REDUCED VISCOSITY	**0.0005**	990±5	969±17	974±19	567±22
	0.00065	1082±2	997±9	978±19	601±17
	0.001	1129±7	1057±11	1278±26	764±9
	R^2	0.9144	0.9495	0.9751	0.9797

Reduced viscosity versus concentration was plotted in order to obtain the intrinsic viscosity for each hydrolyzed chitosan, corresponding to the intercepts in y-axis (Figure 3). All samples showed linearity regression, and reduced viscosity was positively correlated with the concentration as expected.

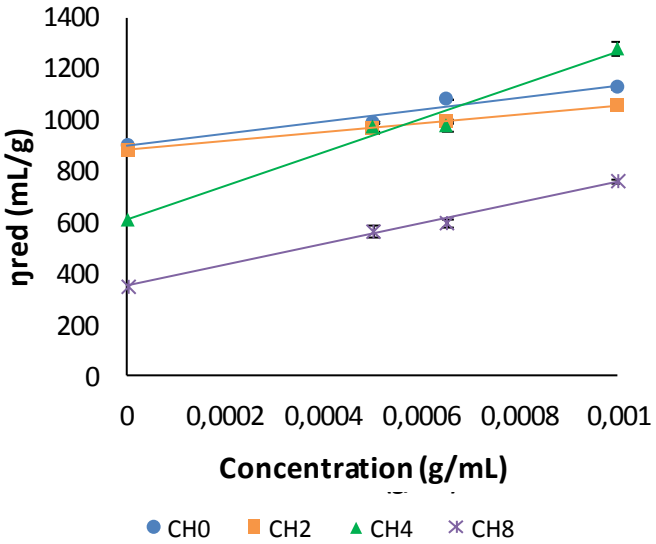

FIGURE 3. Reduced viscosity (mL/g) versus polymer concentration (g/mL) corresponding to commercial chitosan and hydrolyzed samples (during 2, 4 and 8 hours).

The Mark-Houwink-Sakurada model (equation 5), was applied in order to calculate the viscosity average molecular weight of chitosans (Table 3).

$$\text{Equation 5: } [\eta] = \text{Km} \cdot \text{Mv}^{a}$$

TABLE 3. Intrinsic viscosity ([ŋ]) and viscosity average molecular weight (Mw_v) corresponding to hydrolyzed chitosan samples (during 0, 2, 4 and 8 hours).

Time of Hydrolysis(h)	[ŋ] intrinsic viscosity (mL/g)	Mw_v (KDa)
CH_0	902±3	1460±5
CH_2	883±10	1430±16
CH_4	611±20	989±47
CH_8	353±13	571± 21

Viscosity average molecular weights values (Mw_v) of the chitosans indicate that different polymerization degrees were achieved. After 2 hours of hydrolysis, no-significance reduction of Mw_v was achieved with respect to the control (CH_0). Therefore, it was necessary to perform the hydrolysis process at least during 4h in order to obtain chitosans with a significant lower viscosity average molecular weight.

Fourier transformed infrared spectroscopy (FT-IR)

Figure 4 shows the FT-IR spectra corresponding to chitosan with different degree of deacetylation; while Figure 5 shows the FT-IR spectra of chitosan with different average molecular weight.

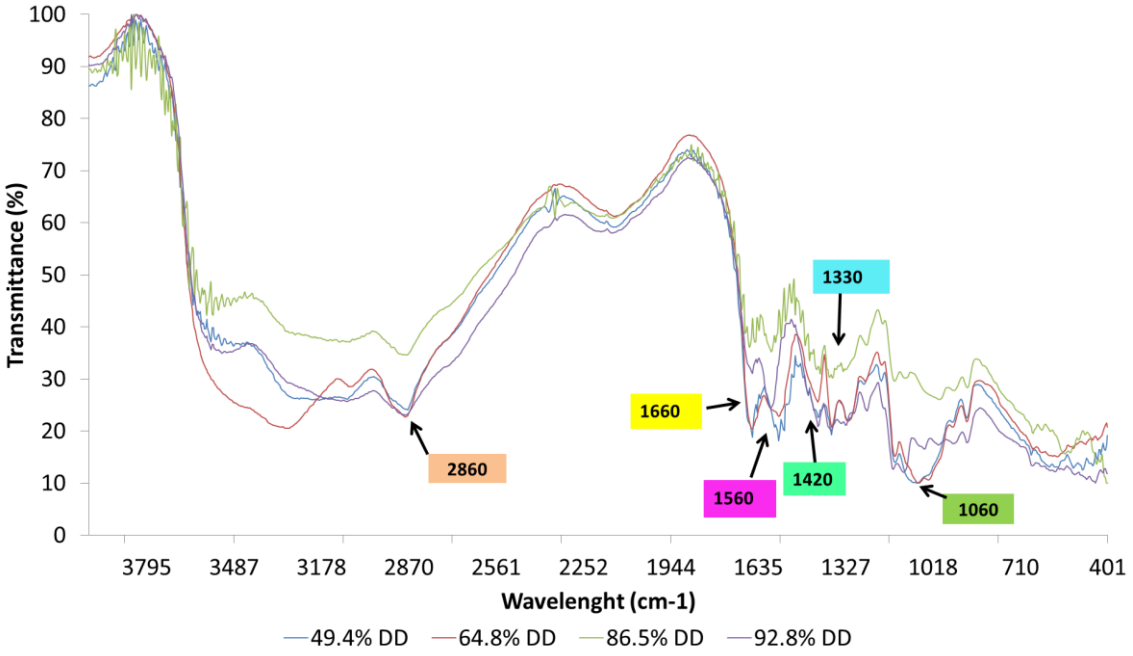

FIGURE 4. FT-IR spectra of chitosans with different degree of deacetylation (DD).

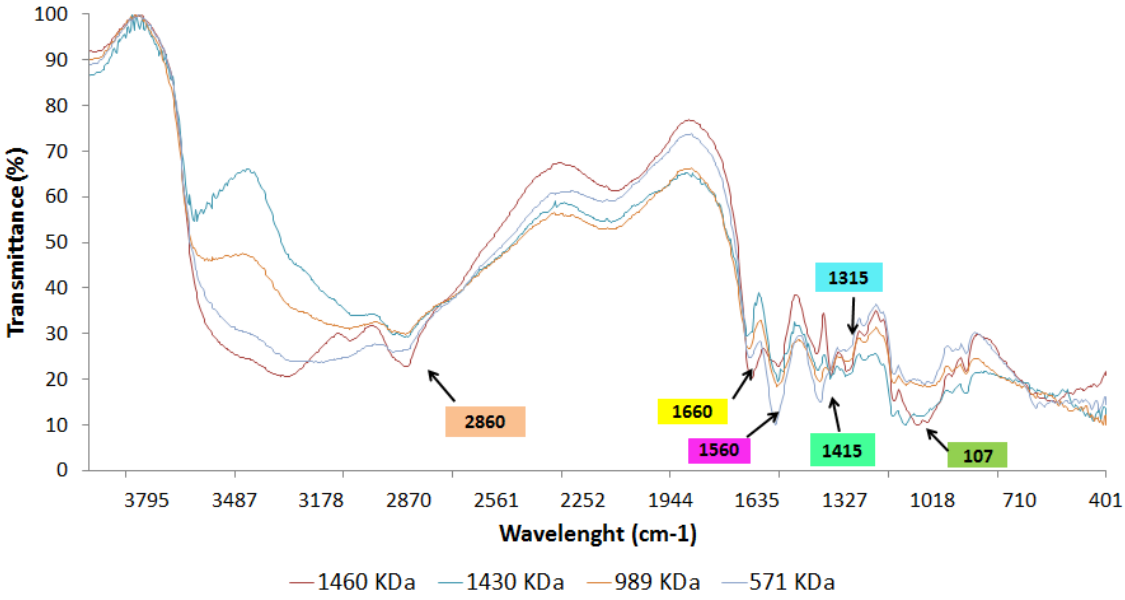

FIGURE 5. FT-IR spectra of chitosans with different average molecular weigth (Mw).

The characteristic absorptions at 3294 and 3497 cm^{-1} corresponded to the hydroxyl groups (OH) and NH groups, while CH groups' bands appear at 2860 cm^{-1} for all chitosan samples. The OH and NH bonds are characteristic of alcohols, amines and amides present in the structure of chitosan. The bands of

the amide groups were visible at 1660 cm^{-1} for all samples tested, and the bands of amino groups (NH_2) were observed at 1560 cm^{-1} in both Figures. The transmittance at 1060 cm^{-1} (Figure 4) and 1070 cm^{-1} (Figure 5) were related to the presence of pyranose group of chitosan (Garcia *et al*., 2015).

The vibration of the methylene and methyl groups' bands are also visible at 1330 cm^{-1} and 1420 cm^{-1} in Figure 4, respectively (1315 and 1415 cm^{-1} in Figure 5) in concordance with the results reported by Mano *et al*., (2003).

It is important to highlight those bands corresponding to amide II (1655, 1560) and amide III (1315). In all bands the representative peaks decrease as deacetylation degree increases, being acetylated and commercial chitosan those with a higher band intensity compared to samples submitted to deacetylation. However, chitosan with different DD and Mw exhibit similar spectrum for all tested samples.

Influence of deacetylation degree and molecular weight on acrylamide formation in model systems

The inhibiting effect of chitosan with different Degree of Deacetylation (DD) on acrylamide formation can be appreciated in Figure 6.

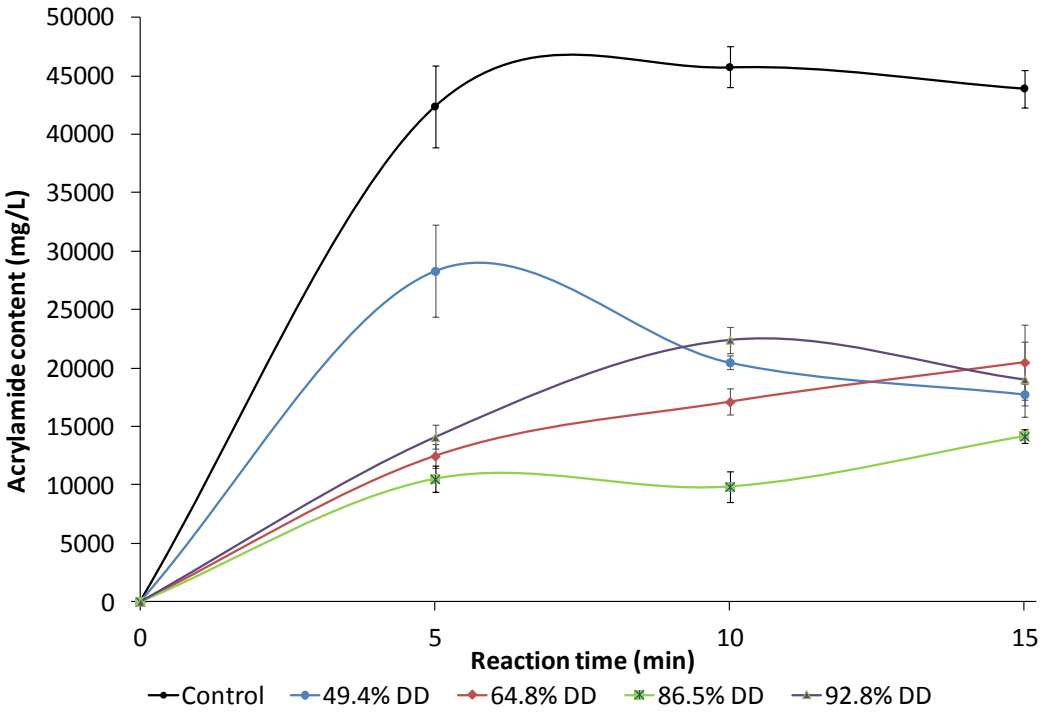

FIGURE 6. Acrylamide generation (mg/L) with respect to the reaction time in the model system consisting of asparagine (5µmol), reducing sugars (5µmol glucose+fructose) and chitosan (1%) of different deacetylation degrees (DD). The reaction was performed at 180 ºC and a control (without chitosan) was also done.

The obtained results neither clearly explain the hypothesis that the higher the number of amino groups in the reaction, the higher the acrylamide inhibition. The low capacity of 92.8%DD chitosan to inhibit acrylamide

generation might be due to the low chemical stability of this chitosan because it was deacetylated twice. A high level of deacetylation tends to form aggregates in aqueous solutions which contribute to form intermolecular interactions that might reduce available sites on the chitosan molecule (Tsai *et al*., 2002).

Results from statistical analysis (ANOVA multifactor) showed that the reaction time, the deacetylation degree (DD) and its interaction were statistically significant, being the reaction time the factor affecting the most the acrylamide generation as expected (Table IV). At short times of reaction (5 minutes), the lowest DD (49.4%) involved the least protective effect, but the rest of tested conditions (reaction times and different DD) implied similar acrylamide content. These results confirm the functionality of chitosan in reducing acrylamide formation (reductions between 44-74%, respected the control). Nevertheless, there was not a clear relationship between DD and acrylamide formation. In this sense, lower chitosan contents (lower than 1%) could be tested in order to clarify the role of the DD in the acrylamide formation.

TABLE 4. F-ratio and p-value parameters resulting from the multifactor variance analysis (ANOVA multifactor) applied to the acrylamide content considering as factors: the reaction time, the deactyilation degree (DD) and the Molecular Weight (Mw_v).

FACTORS	**F-Ratio**	**INTERACTIONS**	**F-Ratio**
Reaction time (min)	122.68**	**Reaction time – DD**	7.77**
Deacetylation degree (DD)	11.41**		
Reaction time (min)	268.29**	**Reaction time – Mw_v**	9.23**
Molecular weight (Mw_v)	53.99**		

** Statistical significance ≥99% (p-value ≤ 0.01);

The molecular weight (Mw_v) of chitosan was also a statistically significant variable that affects acrylamide formation (Table 4).The lowest molecular weight chitosan (571 KDa) resulted no effective in reducing acrylamide formation with respect to the control. High molecular weight chitosan seems to be positively related with the protective effect in acrylamide formation, except for 1430 KDa, that is a value over the expected for this tendency. Probably, chitosans with the same degree of deacetylation but different molecular weight and consequently viscosity, can differently affect to acrylamide formation. The higher the viscosity, the lower the moisture losses during frying, condition that protect the product against an acrylamide formation (Matthaus *et al*. 2004).

Furthermore, the hydrolysis can promote changes in the chain conformation of chitosan and new intermolecular bonds that may hinder the joints of the amino groups with reducing sugars.

It is noteworthy that the acrylamide generation was higher in the model system with chitosan submitted to 2-hour of acid hydrolysis compared with the commercial chitosan (1460 kDa) even if both of them presented the same Mw.

This fact could be due to the undesirable structural changes induced by the acidic hydrolysis even if the Mw is not reduced. In fact, Kumar (2000) recommended the enzymatic hydrolysis because of the better control of the process and the absence of chemical modifications of the structure.

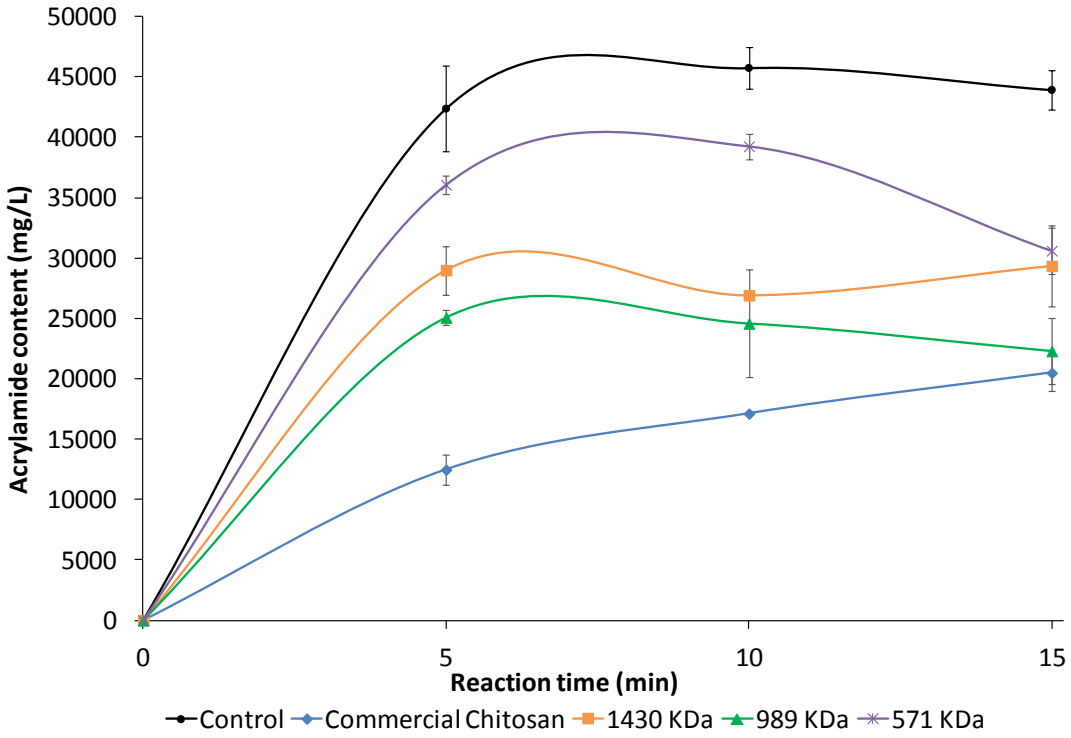

FIGURE 7. Acrylamide generation (mg/L) with respect to the reaction time in the model system consisting of asparagine (5µmol), reducing sugars (5µmol glucose+fructose) and chitosan (1%) with different molecular weight (Mw). The reaction was performed at 180 ºC and a control (without chitosan) was also done.

Influence of deacetylation degree and molecular weight on the antioxidant activity of the obtained chitosans

Chitosan is a biopolymer which presents antioxidant activity. This property could be interesting due to its presence in the final fried foods. As it can be observed in Figure 8, chitosan with 49.4, 64.8 and 86.5% DD exhibited a DPPH radical scavenging activity inferior of 10% at all tested concentrations. Chitosan with higher DD (92.8 %) presented a DPPH radical scavenging activity ranging from 8.7% for 1 mg/mL-concentration to 18.5% for 2 mg/mL-concentration. These results agree with those reported by Park *et al*. (2004) who concluded that the greater DD, the higher the scavenging activity. If this antioxidant activity is compared with the antioxidant compound used as reference this presented the antioxidant BHT a much higher scavenging effect for much lower concentrations.

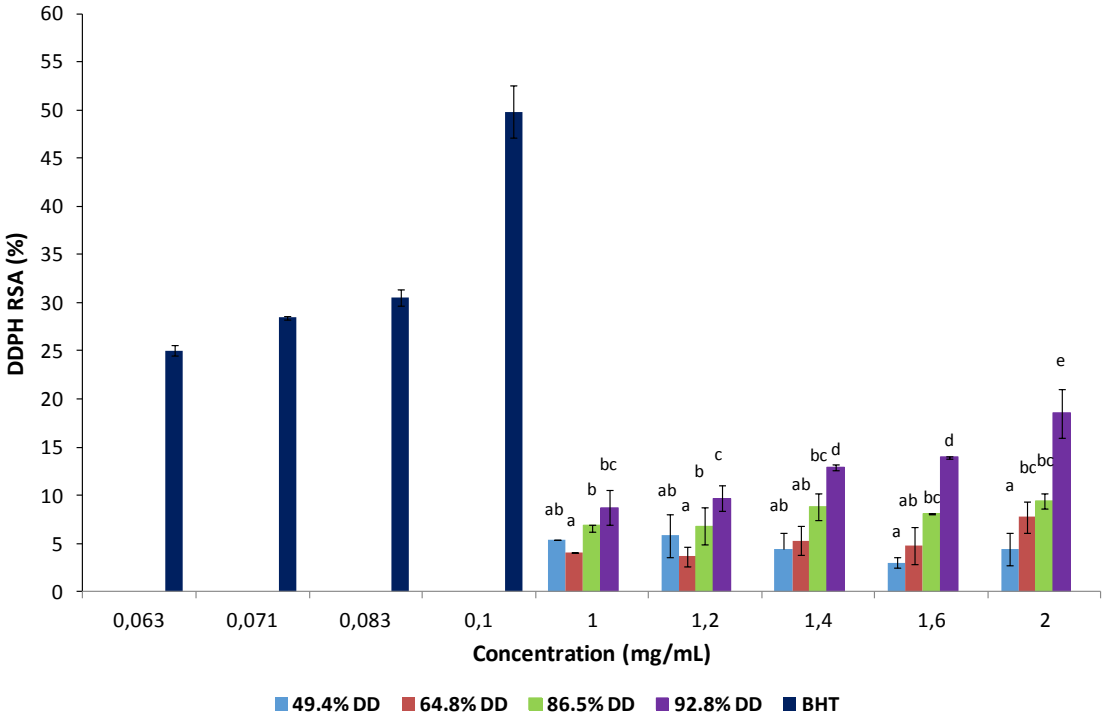

FIGURE 8. DPPH-radical-scavenging activity of BHT and chitosans with different DD.

With regards to the Mw of chitosans on antioxidant activity, chitosan submitted to acid hydrolysis during 2 and 4 hours, with Mw of 1430 KDa and 989 KDa, respectively, showed a DPPH radical scavenging activity less than 5% for all tested concentrations (Figure 9). The 8-hour hydrolyzed chitosan, with a Mw of 571KDa, presented the greatest scavenging activity. These results are similar to those those obtained by other authors (Xing *et al*., 2015), who reported that Mw is inversely related to the antioxidant capacity of chitosan.These values are also very low compared to the antioxidant of reference (BHT).

Kim and Thomas (2007) determined the antioxidant activity of chitosan with much lower Mw than those obtained in this study (30, 90 and 120 kDa) through DPPH assay and reported higher activity for the lower Mw chitosans, with radical scavenging percentages ranging from 40 to 100% when the concentration was increased from 0.2 to 1% (w/v). Chitosan of 90 and 120 kDa showed the least antioxidant capacity, 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), reported that the antioxidant capacity of chitosan increases by increasing the concentration and decreasing the Mw.

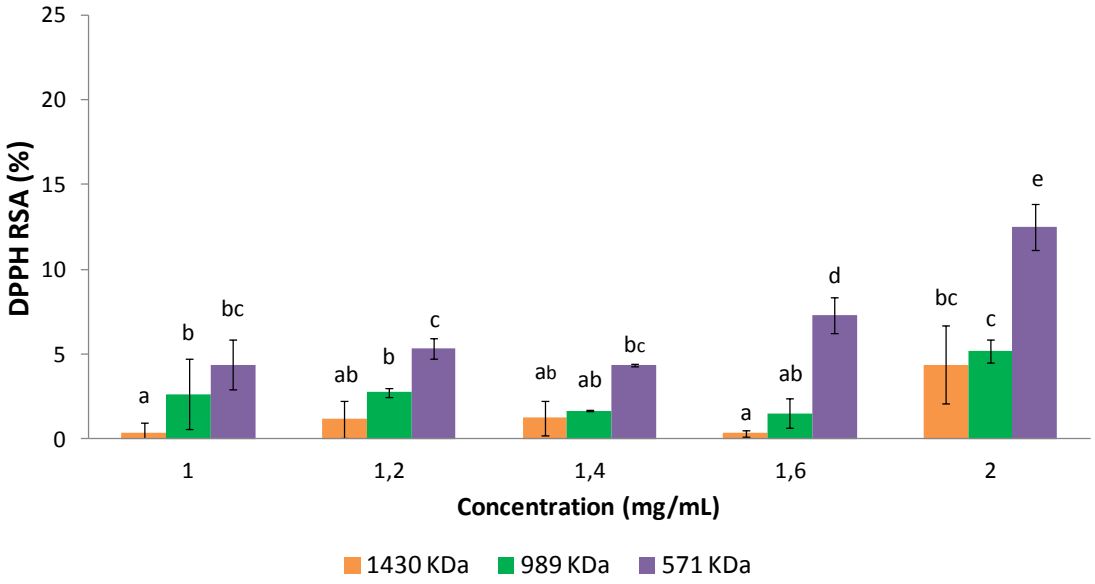

FIGURE 9. DPPH-radical-scavenging activity of BHT and chitosans with different Mw.

One of the mechanisms that explain the antioxidant activity of chitosan is the activity of capturing free radicals. Park *et al*. (2004) suggested that chitosan can eliminate several free radicals by the action of nitrogen in the C-2 position of the chitosan. Meanwhile, Xia *et al*., (2011) explain that the amino nitrogen has a lone pair of electrons; which can be attached to a released proton of the acid solution to form ammonium (NH^{3+}). The free radicals can react with the hydrogen ion from the ammonium ions to form a stable molecule.

CONCLUSIONS

The results showed that the higher deacetylated chitosans tested (86.5 and 92.8%), evidenced a clear reduction (44-81%) compared to the control samples (without chitosan), depending on reaction time.

The molecular weight of chitosan effect on acrylamide formation may be considered. The lower molecular weight chitosan sample (571KDa) did not result in a significant reduction of acrylamide (represented 7-17 % reductions relative to the control at 5 and 10 minutes of reaction), but in general, higher molecular weight samples show a greater protective effect against the formation of acrylamide which could be related to its higher viscosity values and implications of this parameter in the molecular mobility of the precursors of the reaction.

As for the antioxidant activity of chitosan, possibly due to the amino groups present in the molecule, the results suggest that it increases as does the concentration, the deacetylation degree and molecular weight decreases.

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