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Properties of Chitosan-Genipin Films Grafted with Phenolic Compounds from Red Wine

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Abstract

Chitosan has been studied as a renewable biopolymer to form edible films and coatings to improve the shelf life of food products. Chemical modification of chitosan is a strategy to prepare chitosan films with enhanced properties to be used as food preservatives. Wine, particularly red wine, is a rich natural source of phenolic compounds, namely anthocyanins, proanthocyanidins, monomeric catechins, and phenolic acids. Phenolic compounds, in general, present strong antioxidant properties. The aim of this work was to develop chitosan-based films with higher antioxidant activity by grafting wine phenolic compounds. Fractions of red wine phenolic compounds, rich in phenolic acids, anthocyanins, and total phenolic compounds, were grafted to the glucosamine residues of chitosan by a radical mechanism using ammonium cerium (IV) nitrate. In order to increase the stability of the films in aqueous acidic medium, genipin, a natural compound with capacity of cross-linking chitosan, was also used in film preparation. The grafting of all phenolic compounds-rich fractions obtained from wine provided films with antioxidant activity more than 100% than those films prepared only with chitosan-genipin. Moreover, the use of genipin allowed obtaining stable films in a large range of pH. The chitosan-genipin film grafted with anthocyanins and total phenolic compounds lost less than 3% of their mass in acidic media after an exhaustive stirring during 7 days, only due to the diffusion of glycerol used as plasticiser. Therefore, these films are promising active materials to be used to improve food preservation.

Keywords: Chitosan; Antioxidant activity; Phenolic acids; Anthocyanins; Genipin.

Introduction

Chitosan, a functional linear biopolymer constituted mainly by (β,1→4)-2-amino-2-deoxy-D-glucan, is being studied extensively owing to its biocompatibility, biodegradability, absence of toxicity, and ability to form films.[1,2] During the last decade, there was an increasing interest to develop chitosan-based films, characterized by their antioxidant, antimicrobial, and antifungal activities, to be used in food conservation, avoiding the use of non-natural chemical preservatives.[3,4]

Chitosan has shown a significant scavenging capacity against different radical species and the ability to chelate metal ions involved in catalysis of oxidative reactions.[5] These properties allow extending the food shelf life, since oxidation is a problem affecting food quality. Furthermore, chemical modifications of chitosans with other antioxidant compounds of natural origin allow preparing functional derivatives with improved physical and biological properties, enlarging their potential applications, as described in several works for chitosan films grafted with phenolic compounds.[3,6-11]

Wine, particularly red wine, is a rich source of natural phenolic compounds, namely anthocyanins, proanthocyanidins, monomeric catechins, and phenolic acids. Phenolic compounds, in general, present strong antioxidant properties namely superoxide and hydroxyl radical scavenging activities.[12]

The use of chitosan films has been restricted due to their inherent water solubility and relatively low stiffness and strength, especially in moist environments or acidic media, because of protonation of the amine groups.[13,14] Cross-linking can be used to enhance mechanical strength and chemical stability, and to control aqueous permeability and solubility of chitosan-based films.[15,16] Genipin has been successfully used as an effective cross-linking agent for polymers containing amino groups, such as chitosan.[11,17,18] Genipin cross-linked chitosan films exhibit a slower degradation rate in aqueous media and, in addition, these compounds revealed to have 4 orders of magnitude less cytotoxicity than glutaraldehyde, the mostly used cross-linker for chitosan.[19,20]

The purpose of this work was to develop chitosan-based films for acidic foods/drinks, such as wine and juices, with higher antioxidant activity by grafting wine

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phenolic compounds. Three different fractions of phenolic compounds extracted from wine, rich in (i) a mixture of phenolic compounds, (ii) anthocyanins, and (iii) phenolic acids, were studied. In order to decrease the solubility of the films in acidic medium, genipin was used as cross-linker. To determine if the developed film would have potential to be used as an active material for application in foods, antioxidant capacity and solubility of the films in acidic media were evaluated.

Materials and methods

Materials.

Chitosan of medium molecular weight with a degree of deacetylation of 85% (according to the producer) and glycerol (99.5%) were supplied by Sigma-Aldrich (St. Louis, MO, USA). 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS, 99%) was purchased from Fluka (St. Louis, MO, USA). Ammonium cerium (IV) nitrate (CAN) with ≥ 98% purity was obtained from BDH (London, UK). Genipin with ≥ 98% purity was acquired from Challenge Bioproducts Co. (Taiwan, China). All other reagents used were analytical grade.

Wine phenolic compounds fractionation.

The red wine was obtained from Dão Appellation, in Portugal, from grapes of *Vitis vinifera* L. Touriga Nacional (2009 vintage). The wine was heat-evaporated until 75% of its initial volume to obtain the de-alcoholised wine. Then, distilled water was added in the same volume of the alcoholic fraction that was removed.[21]

The C18 sep-pak cartridges (SPE-C18, Supelco-Discovery, 20 g) were used to separate the hydrophilic (sugars-rich fraction) from the hydrophobic (phenolic-rich fraction) material. SPE-C18 column was preconditioned with methanol (20 mL) followed by water (20 mL) and 2% acetic acid (20 mL). The sample was loaded into column at pH 3.4 and eluted with water in order to obtain the sugar-rich fraction that was discarded. The total phenolic compounds (TPhC) from wine were eluted with methanol acidified with HCl (0.1%). The methanol was removed using rotary evaporator and re-dissolved in water.

The fractionation of TPhC extract was carried out using the methodology described by Gonçalves *et al.*[21] The column was pre-conditioned with phosphate buffer (0.1M, 25 mL, pH 7.0) before the fractionation of phenolic compounds. The TPhC extract was neutralized by addition of NaOH (0.1 M) followed by addition of phosphate buffer (0.1 M, pH 7.0) to adjust the pH. This solution was loaded into the column in a flow less than 2 mL/min. Phenolic acids-rich fractions were eluted with phosphate buffer (0.01 M, 50 mL, pH 7.0). The column was washed with distilled water (30 mL) and dried under vacuum. The fraction rich in anthocyanins (Ant) was removed from the column by elution with of methanol (30 mL) acidified with HCl (0.1%). The salts of phenolic acids-rich extract were removed by reloading into another C18 cartridge at pH 2 and the phenolic acids-rich fraction (PhAc) was recovered by elution with methanol. All the fractions were evaporated to remove MeOH and re-dissolved in water.

The phenolic content of the different fractions was determined by Folin-Ciocalteu reagent. Each sample (0.125 mL) was added to deionized water (0.5 mL) and Folin-Ciocalteu reagent (0.125 mL). After 6 min, a solution of sodium carbonate (1.25 mL, 7.5%) and deionized water (1.0 mL) were added. The mixture was left for 90 min at r.t. in the dark and the absorbance at 760 nm was measured. A calibration curve was made with standard solutions of gallic acid (range 50 to 500 mg/L), and the results were expressed in equivalents of gallic acid. All analyses were done in triplicate.

Chromatographic analysis of phenolic acids and anthocyanins by HPLC.

The individual phenolic acids and monomeric anthocyanins were analyzed using a HPLC Dionex Ultimate 3000 Chromatographic System (Sunnyvale, California, USA) equipped with a quaternary pump Model LPG-3400 A, a ACC-3000 auto sampler, having a thermostated column compartment (adjusted to 30 ºC) and a multiple wavelength detector MWD-300. The column (250 x 4.6 mm, particle size 5 µm) was a C_{18} Acclaim® 120 (Dionex, Sunnyvale, California, USA) protected by a guard column of the same material.

Phenolic acids

The PhAc fraction obtained from the C18 sep-pak was used for chromatographic analysis of the phenolic acids. The solvents were, (A) water/formic acid (95:5 v/v) and (B) methanol. A linear gradient was used for a total run time of 80 min, starting from 5% solvent B during 2 min, increase to 80% solvent B over 68 min and then isocratic for 8 min, decreasing to 5% solvent B over 2 min, and finally isocratic for 5 min. The sample volume injected was 40 µL and the flow rate was 1.0 mL/min. The column temperature was maintained at 30 ºC during the run. The chromatographic peaks of all phenolic acids were identified by comparing their retention times with the retention time of individual standards. All analyses were done in triplicate.

Monomeric anthocyanins

The monomeric anthocyanins present in the Ant fraction were analyzed by HPLC. The solvents were (A)

40% formic acid, (B) pure acetonitrile, and (C) bi-distilled water. The initial conditions were 25% A, 10% B, and 65% C, followed by a linear gradient from 10 to 30% B, and 65 to 45% C for 40 min, with a flow rate of 0.7 mL/min. The injection volume was 20 µL. The detection was made at 520 nm and a Chromeleon (version 6.8) software program (Sunnyvale, California, USA) was used. The chromatographic peaks of all anthocyanins were identified by comparing their retention times with the retention time of isolated standard anthocyanins. All analyses were done in triplicate.

Chitosan-based film preparation.

A chitosan solution (1.5%, w/v) was prepared by dissolving the chitosan in aqueous acetic acid solution (5%, v/v) with stirring at r.t. for 18 h. An aqueous solution (50 mL) of ammonium cerium (IV) nitrate (CAN, 60 mM) and wine extract (PhAc, 2.4 g/L; Ant, 2.2 g/L or TPhC, 5.4 g/L) was added to the chitosan solution (50 g). This mixture was kept under nitrogen atmosphere at 40 °C, in the dark, for 3 h, with stirring. At the end of the reaction distilled acetone (600 mL) was added to the mixture in order to precipitate the modified chitosan. The precipitate was obtained by centrifugation at 24,600 *g* for 20 min at 4 °C. The precipitate was washed with methanol (100 mL) during 1 h with stirring, to remove the phenolic compounds not covalently bound to chitosan. After centrifugation, the precipitate was dissolved in acetic acid solution (45 mL, 5% v/v) and, after complete dissolution, glycerol (0.4 g) was added. This mixture was placed in a water bath at 50 °C with stirring for 10 min. After cooling to r.t., genipin (10% w/v, 250 µL) in ethanol was added to the mixture. The mixture was homogenized with constant stirring for 30 min. The solution was filtered under vacuum through a porous glass filter (G2) and degassed. This solution (31 g) was transferred into a plexiglass plate (144 cm^2 with 3 mm depth). After 6 h of genipin addition, the plates were placed in an oven for 16 h at 35 ºC for film formation by solvent casting. Chitosan films (Ch) and chitosan cross-linked with genipin (Ch-Ge) were also prepared using the same methodology, except by the addition of the CAN and phenolic compounds extract.

All the films prepared (Ch, Ch-Ge, Ch-PhAc-Ge, Ch-Ant-Ge, and Ch-TPhC-Ge) were washed with methanol in a Soxhlet extractor for 2 h (12 cycles/h) to extract all the compounds not covalently linked to chitosan. The films were neutralized by immersion in aqueous NaOH solution (1 M) for 1 h, thoroughly washed with distilled water until pH 6. These neutralized films were left to dry at r.t.

Solubility in aqueous acidic medium of the chitosan-based films.

The films solubility was determined in acidic aqueous media (water, pH 3.5 adjusted with HCl) according to the method described by Nunes *et al.*[11] One square (4 cm^2) of film was placed in acidic aqueous media (30 mL) at r.t. with orbital agitation (80 rpm) for 7 days. Then, films were placed in an oven at 105 ºC for 16 h. The films were weighed after cooling down to r.t. The solubility was determined by the percentage of weight loss calculated as follows:

$$\% \text{ weight loss} = 100 \times (m_b - m_a)/m_b$$

where m_b and m_a are the weight of dry film before and after being immersed in water at pH 3.5, respectively. This determination was performed in triplicate.

The films moisture content was determined by measuring their loss of weight, upon drying in an oven at 105 ºC until reaching a constant weight (dry film weight). Samples were analyzed at least in triplicate.

Antioxidant activity of the chitosan-based films.

The antioxidant activity of the films was determined by adaptation of the method of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), ABTS, as described by Nunes *et al.*[11] Briefly, a solution of ABTS (7 mM) was prepared in aqueous potassium persulfate solution (2.45 mM). This solution was left in the dark, at r.t., for 12-16 h for $ABTS^+$ formation. One square (1 cm^2) of film was placed in $ABTS^+$ solution (3 mL, diluted 1:80 in ethanol) and left to react in the dark with orbital stirring at 80 rpm. The absorbance at 734 nm of the solution was measured after 6 h. All measurements were performed in triplicate.

Statistical analyses.

The results of solubility and antioxidant activity were statistically evaluated using *F* and *t*-Student tests of Microsoft Excel 2010 at a 95% significance level.

Results and Discussion

Characterization of Phenolic Compounds Extracts.

The wine extracts were obtained by solid phase extraction (SPE) with a C18 polymer. The schematic representation of the phenolic compounds fractionation is shown in Figure 1. The red wine was distilled at atmospheric pressure to concentrate the phenolic compounds and to remove the ethanol. The de-alcoholised residue was eluted through the C18 column at the pH of wine (about 3.5) (Figure 1A). The non-retained fraction was eluted with distilled water (Figure 1B) and the phenolic compounds were eluted with

methanol containing HCl (0.1%, m/v, Figure 1C). The fraction recovered (TPhC) contained the total phenolic compounds present in the wine with a concentration of 5.4 g/L (gallic equivalents). The pH of TPhC fraction was increased to 7 with aqueous NaOH (1 M) and diluted 2 times with phosphate buffer (0.1 M, pH 7). This fraction was again applied into the C18 column, which was previously washed with phosphate buffer (0.1 M, pH 7). The non-retained fraction eluted with the buffer (pH 7), rich in phenolic acids (PhAc), was collected, with a concentration of 2.4 g/L (gallic acid equivalents). After the elution with buffer (pH 7) it was eluted with ethyl acetate and then with acidic methanol (0.1% HCl). The fraction eluted with acidic methanol was mainly composed by anthocyanins (Ant) with a concentration of 2.2 g/L of phenolic compounds (as gallic acid equivalents).

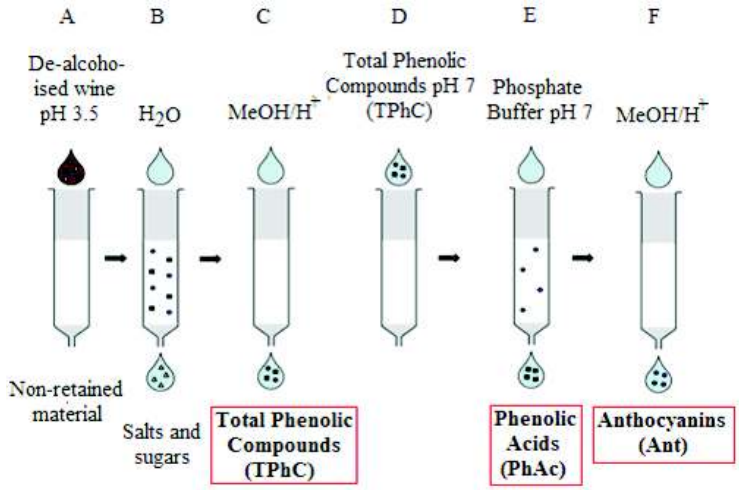

Fig. 1. Schematic representation of the fractionation of wine phenolic compounds.

The composition of phenolic acids and anthocyanin extracts of red wine was determined by HPLC. The representative chromatograms are shown in Figure 2.

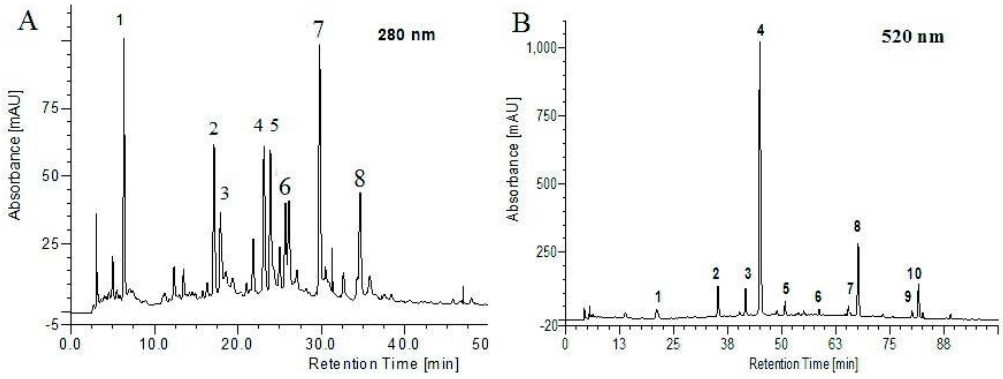

Fig. 2. HPLC chromatograms of A) phenolic acids and B) anthocyanins extracts recorded at 280 and 520 nm, respectively. Identification: A) 1. gallic acid, 2. trans-caftaric acid, 3. *p*-hydroxybenzoic, 4. chlorogenic acid, 5. (+)-catechin, 6. caffeic acid,7. (-)-epicatechin, 8. *p*-coumaric acid; B) 1. Dp3Glc, 2. Pt3Glc, 3. Pn3Glc, 4. Mv3Glc, 5. Dp3AcGlc, 6. Pt3AcGlc, 7. Pn3AcGlc, 8. Mv3AcGlc, 9. Pn3CmGlc, 10. Mv3CmGlc.

The phenolic acids extract was mainly composed by a mixture of gallic, *p*-hydroxybenzoic, trans-caftaric, chlorogenic, caffeic, and coumaric acids (Figure 3A). The anthocyanins extract (Figure 3B) was majorly composed by malvidin 3-glucoside, representing 60% of the anthocyanins quantified. Lower contents of malvidin 3-(6-acetyl)-glucoside, petunidin 3-glucoside, and malvidin 3-(6-coumaroyl)-glucoside were also found.[21]

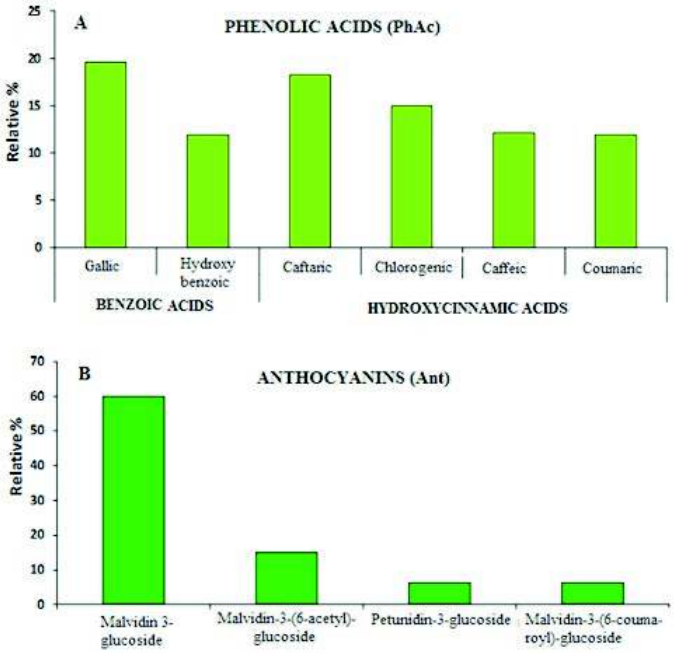

Fig. 3. Composition (expressed as relative percentage) of (A) phenolic acids and (B) anthocyanins extracts.

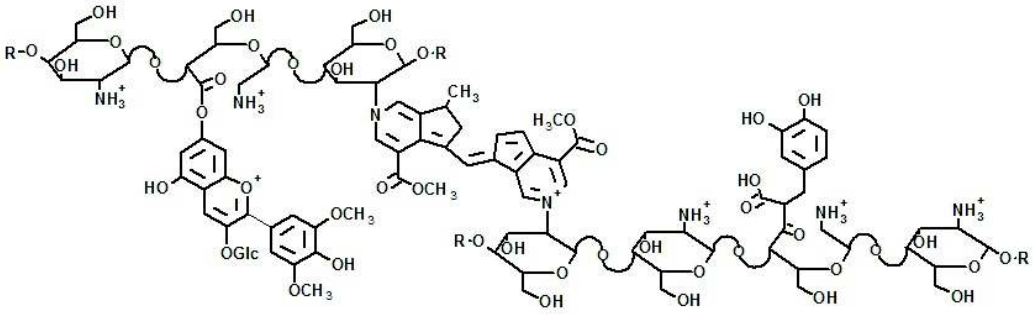

Fig. 4. Proposed structure of chitosan polymer cross-linked with genipin and grafted with caffeic acid and malvidin 3-glucoside (adapted from Nunes *et al.*[11]).

Preparation of chitosan-based films.

In this work, phenolic compounds were covalently linked to the carbon 2 or 3 of glucosamine residues of chitosan, using a procedure described by Nunes *et al.*[11] This grafted polymer was prepared by generating free radicals on the biopolymer backbone and then allowing these radicals to serve as macroinitiators. Ammonium cerium (IV) nitrate (CAN) was used as it is one of the most used reagents for vinyl grafting onto chitin/chitosan.[22]

As the purpose of this work was to develop a chitosan-based film with improved antioxidant activity and insoluble in acidic media, the decrease of the solubility of the films was achieved by adding genipin as a cross-linker to the chitosan grafted with the phenolic compounds extracted from wine. Chitosan-based films were prepared independently with the three fractions of phenolic compounds extracted from wine, namely phenolic acids, forming Ch-PhAc-Ge films, anthocyanins (Ch-Ant-Ge), and total phenolic compounds (Ch-TPhC-Ge). Figure 4 show the proposed structure for the chitosan polymer cross-linked with genipin and grafted with phenolic compounds, caffeic acid and malvidin based on previous studies.[11]

Homogeneous, flexible, and good appearance chitosan based films were obtained (Figure 5). The chitosan films were colourless (Figure 5A) and the cross-linking with genipin gave rise to greenish films (Figure 5B). The films of chitosan cross-linked with genipin and grafted with phenolic compounds from red wine produced yellow (Ch-PhAc-Ge, Figure 5C) and red (Ch-Ant-Ge and Ch-TPhC-Ge, Figure 5D and 5E) films. The films were further characterised and compared with the pristine chitosan (Ch) and the chitosan cross-linked with genipin (Ch-Ge) films in order to evaluate their properties after grafting with the phenolic compounds.

Fig. 5. Films of A) chitosan, B) chitosan cross-linked with genipin, and chitosan cross-linked with genipin and grafted with C) phenolic acids (Ch-PhAc-Ge), D) anthocyanins (Ch-Ant-Ge), and E) total phenolic compounds (Ch-TPhC-Ge) obtained from red wine.

Properties of chitosan-based films.

Solubility in aqueous acidic medium

The films solubility was determined by weight loss after 7 days immersed in acidic medium (pH 3.5) under continuous stirring. Figure 6 shows the weight loss obtained for the chitosan (Ch), chitosan cross-linked with genipin (Ch-Ge), and chitosan with addition of phenolic compounds obtained from wine (Ch-PhAc-Ge, Ch-Ant-Ge, and Ch-TPhC-Ge). The use of genipin allowed obtaining films with low weight loss (< 20%) in acidic media. The solubility of the chitosan-genipin films is attributed to the diffusion of glycerol used as plasticiser. The chitosan-genipin films grafted with phenolic acids (Ch-PhAc-Ge) lost about 10% of their mass, while the chitosan-genipin films grafted with anthocyanins (Ch-Ant-Ge) and with total phenolic compounds (Ch-TPhC-Ge) lost less than 3% of their mass (Figure 6). This low solubility obtained with anthocyanins and total phenolic compounds could be due to the hydrophobic nature of these compounds. This results are in accordance with previous ones that reports the efficiency of genipin to produce chitosan-based films stable in acidic media.[11]

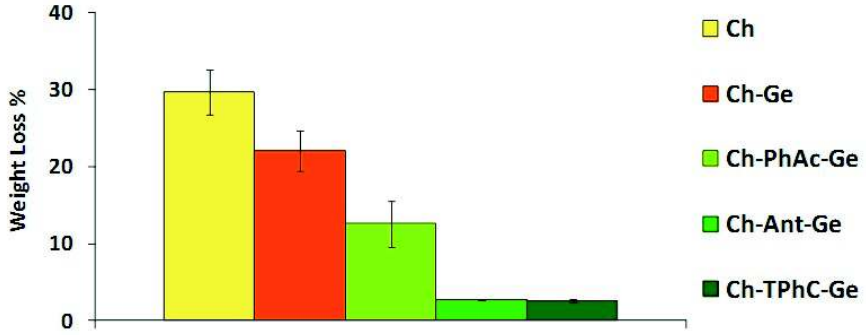

Fig. 6. Solubility (weight loss %) in acidic media (pH 3.5) of chitosan (Ch), chitosan cross-linked with genipin (Ch-Ge), and chitosan cross-linked with genipin and grafted with phenolic acids (Ch-PhAc-Ge), anthocyanins (Ch-Ant-Ge), and total phenolic compounds (Ch-TPhC-Ge) films after 7 days with stirring.

The resistance of these chitosan-based films (solubility of 3%) in acidic media could be important for applications where films with low solubility in water are required, such as packaging of acidic foods with high moisture.

Antioxidant activity

Figure 7 shows the antioxidant activity of all chitosan based films (Ch, Ch-Ge, Ch-PhAc-Ge, Ch-Ant-Ge, and Ch-TPhC-Ge) determined by the ABTS method, which

assays the capacity of scavenging free radicals. The antioxidant activity of the chitosan films represented 40% of inhibition. This activity can be attributed to the capacity of chitosan amino groups to react with free radicals, originating stable macromolecular radicals and protonated ammonium (NH_3^+) groups.[23] Significant differences were not observed between the chitosan and the chitosan cross-linked with genipin films. However, the grafting of the phenolic compounds to chitosan allowed producing films with higher antioxidant activity, around 80% of inhibition of $ABTS^+$ radical, which represents an increase of over than 100%. The Ch-PhAc-Ge and Ch-Ant-Ge films showed slightly higher antioxidant activity (inhibition percentage of 87% after 48 h of reaction with $ABTS^+$ solution) in comparison to Ch-TPhC-Ge films.

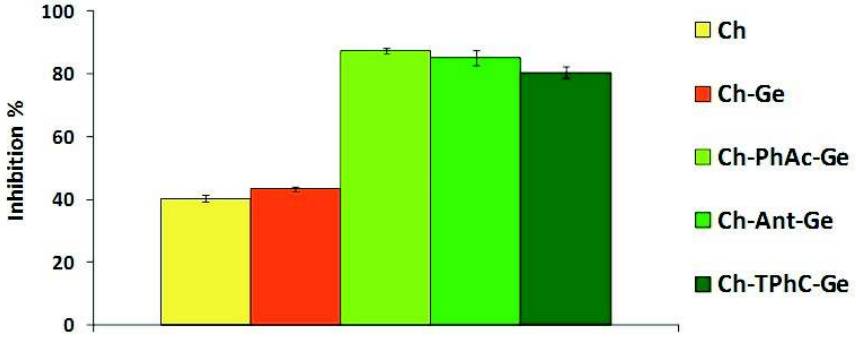

Fig. 7. Antioxidant activity of chitosan (Ch), chitosan cross-linked with genipin (Ch-Ge), and chitosan cross-linked with genipin and grafted with phenolic acids (Ch-PhAc-Ge), anthocyanins (Ch-Ant-Ge), and total phenolic compounds (Ch-TPhC-Ge) films measured by ABTS.

The antioxidant activity of the films prepared with chitosan cross-linked with genipin and grafted with phenolic compounds is considerably higher when compared with the chitosan-based film with caffeic acid and genipin described previous by Nunes *et al.*[11] The $ABTS^+$ inhibition after 48 h was higher (87%, Figure 7) than the inhibition showed by the other films after 72 h (approximately 75%). These results allowed to conclude that the grafting of the phenolic compounds extracted from wine to the chitosan contribute to enhance the scavenge free radical capacity of the chitosan-based films.

Conclusion

This study shows that wine phenolic compounds can be used to prepare chitosan-based films with improved antioxidant activity. These films have also low solubility in a large range of pH, including acidic media. Therefore, they are promising active materials to improve food preservation, mainly acidic foods.

Acknowledgments

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