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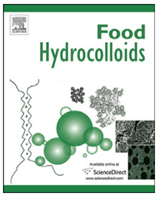

Nanocrystal-reinforced soy protein films and their application as active packaging

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ABSTRACT

Soy protein isolate (SPI) films reinforced with starch nanocrystals (SNC) were developed by simple casting method. The films were transparent and homogeneous. The opacity and degree of crystallinity increased with the amount of nanocrystals. Moisture content, total soluble matter and swelling in water were evaluated, showing a marked effect on SNC additions. As the amount of SNC increased, the films exhibited lower affinity for water. Moreover, mechanical properties were determined showing that SPI-SNC reinforced films became more resistant and less elongable as SNC amount increased. With the incorporation of a considerable amount of reinforcements, a marked variation was observed in these properties. In addition, assays performed demonstrated that β-cyclodextrins (β-CD)-containing SPI-SNC films were able to sequester cholesterol when brought into contact with cholesterol rich food such as milk. This effect was more marked as the amount of β-CD into the films increased. The methodologies developed in this work allowed yielding biodegradable films with optimized physical and mechanical properties which were assayed as active food coating.

1. Introduction

Synthetic polymers, specifically plastics, are the main packaging material on high demand since they offer versatile solutions for several needs variety of purposes. Despite this, conventional packaging polymers are being questioned due to increasing environmental concerns.

Nowadays, a great number of research studies focus on solving the problems produced by plastic waste in order to obtain environmental friendly materials. For this reason, several studies examine the possibility of substituting the traditional petroleum-based plastics by biodegradable and low-cost materials with similar properties (Famá, Goyanes, & Gerschenson, 2007; Jiménez, Fabra, Talens, & Chiralt, 2012a, 2012b). At present, the biopolymer concept is emerging. Biodegradable materials are associated with the use of renewable raw materials such as proteins and polysaccharides extracted from agricultural and animal co-products and by-products, marine or microbial sources. These materials can be environmentally degraded (via exposure to soil optimum moisture, microorganisms and oxygen) into simple substances (water and carbon dioxide) and biomass. In particular, the isolated soy protein (SPI) as raw material has shown advantages over other sources due to its exceptional film-forming properties, low cost (for its extensive production in our country and for being isolated from oil industry waste) and good barrier properties to oxygen, aromas and lipids under intermediate moisture conditions (Gennadios, Weller, & Testin, 1993). This type of proteins also produces softer, more transparent and flexible films compared to those derived from other sources (Guilbert, Gontard, & Cuq, 1995).

As in conventional packaging, bio-based materials must exhibit a number of important properties, including containment and protection of food quality by serving as selective barriers to moisture transfer, oxygen uptake, lipid oxidation and loss of volatile aromas and flavors, maintaining the sensory quality and safety of food (Roy, Saha, Kitano, & Saha, 2012).

On the other hand, materials developed from natural polymers are commonly associated with poor properties, often having less important properties compared to those of commodity polymers. As opposed to most synthetic plastics used as a packaging material, most of the currently available bio-plastics do not fulfill the requirements of food packaging especially in terms of barrier and

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mechanical properties (Hendrix, Morra, Lee, & Min, 2012). Modification techniques are being developed to achieve the required property combinations for specific applications. The chemical structure of biopolymers offers possibilities for chemical reactions. The modification strategies of the starting material should enhance water resistance, the barrier effect against the flow of gases and the mechanical properties (to avoid loss by rupture or break of the material). It should also allow the incorporation of active ingredients, promote adhesion to the surface of food and increase its stability in storage conditions.

Nanocomposites are being used for the development of films with suitable properties as coating material. Improvements in the properties of the final reinforced nanocomposite material, not achieved in the individual component and not conferred by the sum of its properties, involve the synergistic effect produced by the combination of both: the matrix (film precursor) and the reinforcement. This effect arises from the presence of a reinforcement-matrix interface with own physical properties, increased by the high surface/volume ratio showed by the nanomaterials. Their incorporation into polymeric matrices yields composites with excellent properties as compared to those of conventional micro-compounds without impairing density, transparency and film processability (Gao, Dong, Hou, & Zhang, 2011). Various examples of nanomaterials used as reinforcement in natural matrices are reported in the literature, in which improvements in their mechanical properties or a decrease in the total amount of water soluble matter are described. These improvements derive from the strong interactions between the matrices and the nano-reinforcements (Kristo & Biliaderis, 2007). Furthermore, particular important improvements in gas- and water-vapor barrier properties have also been obtained for similar films.

Usually, the reinforcements employed in films of this type are classified according to their dimensions in lamellar, fibrillar and particulates. The most studied nano-reinforcements are the particulates such as nanocrystals formed from semi-crystalline polysaccharides (cellulose, starch and chitin). These nanocrystals are ideal candidates for processing high performance materials, even in low concentrations (D. Chen, Lawton, Thompson, & Liu, 2012; Dufresne, 2010; Lin, Huang, Chang, Anderson, & Yu, 2011; Pires, Neto, Alves, Oliveira, & Pasquini, 2013) since they present excellent mechanical properties by reinforcing ability, abundance, low weight, biodegradability and high surface area. When nanocrystals produced from sources of polar characteristics (such as polysaccharides) are used as a reinforcement of materials based on starch or proteins, the chemical structure of the matrix and filler is similar. These structural similarities promote good miscibility and strong interfacial adhesion between both components (Lin et al., 2011). Reports on the improvements in films reached with starch nanocrystals (SNC) are described (Chen, Cao, Chang, & Huneault, 2008; García, Ribba, Dufresne, Aranguren, & Goyanes, 2011; Kristo & Biliaderis, 2007). These formed materials could be used as packaging. In particular, active food packaging is currently one of the most studied areas, stressing the development of new techniques capable of improving conservation and food quality in terms of their interaction with the packaging. According to Regulation (CE) N 450/2009 of the European Union Commission on active and intelligent materials (EU, 2009, pp. 1–11), an active food packaging comprises the group of materials intended to prolong life or maintain or improve the condition and quality of the packaged food. These containers are designed to incorporate specific components that can absorb or released substances from the packaged food. The absorber substance systems involves oxygen, carbon dioxide, ethylene, water, odors and flavors or other undesirable substances (Restuccia et al., 2010).

On the other hand, a low cholesterol diet is recommended to prevent medical conditions such as atherosclerosis, thrombosis or cardiovascular diseases. The easiest and most efficient way of controlling cholesterol levels is through diet. For this reason, there is a wide range of commercially available food low in cholesterol and fat, such as partial or completely skimmed milk.

In general, cyclodextrins (CD) are cyclic oligosaccharides comprising six (α-CD), seven (β-CD), eight (γ-CD) or more glucopyranose units joined by alpha-(1, 4) linkages. The hydrophobic cavity of β-CD can form inclusion complexes with a wide range of organic guest molecules mainly through weak interactions, such as Van der Waals, dipole–dipole and hydrogen bonding forces, while the hydrophilic surface allows dissolution of the CD in water. A report describes cholesterol/β-CD inclusion complexes in proportions ranging from 1:1 to 1:3 (Yamamoto, Kurihara, Mutoh, Xing, & Unno, 2005). Some reports document cholesterol-retaining systems based on the use of cyclodextrins in food like milk (Alonso, Cuesta, Fontecha, Juarez, & Gilliland, 2009; López-de-dicastillo, Catalá, Gavara, & Hernández-Muñoz, 2011; Tahir et al., 2013), butter (Jung, Kim, Yu, Ahn, & Kwak, 2005; Kim, Jung, Ahn, & Kwak, 2006), cream (Shim, Ahn, & Kwak, 2003), egg (Chiu, Chung, Giridhar, & Wu, 2004) and cheese (Bae, Kim, & Kwak, 2008; Han, Kim, Ahn, & Kwak, 2008).

This article describes the methodologies developed to yield SPI-based biodegradable films reinforced with SNC, which showed optimized physical and mechanical properties. The products were assayed as active food coating with cholesterol sequester properties.

2. Materials and methods

2.1. Materials

The following chemicals were used: soy protein isolate (SPI) SUPRO *E* with 90% protein on fat-free, dry-weight basis (donated by The Solae Company, Argentina), glycerol (Gly) (Taurus, Argentina), sulfuric acid (Cicarelli, Argentina), sodium hydroxide and calcium chloride (Cicarelli, Argentina), unmodified regular corn starch containing approx. 73% amylopectin and 27% amylose (Sigma, USA), cholesterol 99% (Sigma, USA) and β-ciclodextrin (β-CD) (Roquette Freres, France).

2.2. Synthesis of SNC

The SNC synthesis was developed following a method described in the literature which comprises acid hydrolysis of native corn starch (Y. Chen et al., 2008). To this, an aqueous dispersion of 22 g of starch in 150 mL of 3.16 M H_2SO_4 solution was prepared. The reaction proceeded for 5 days at 40 °C and 100 rpm. The nanocrystal purification was performed through five washes with 500 mL of water and successive filtrations to neutral pH. Then, SNC were sonicated in 500 mL of water and stored in the refrigerator. To determine SNC concentration, the dispersion was vigorously shaken and three extractions of 1 mL each were taken, dried in an oven and weighed. The amount of SNC per milliliter of dispersion was calculated as an average of three determinations.

2.3. Preparation of nanocrystal-reinforced films

The starch nanocrystal-reinforced soy protein films (SPI-SNC) were obtained by the casting method. 0.25 g of SPI was dispersed in 30 mL of water and 100 mL of Gly as plasticizer (50% w/w with respect to SPI) was added. This dispersion was stirred for 30 min and the required amount of SNC dispersion was added. Different films were prepared with 0; 2; 5; 10; 20 and 40% of SNC (in mass with respect to SPI). Prior to each addition, SNC dispersion was stirred and sonicated for 30 min. SPI dispersion including SNC was stirred a further 30 min, poured into plastic Petri dishes and dried in an oven at 50 °C for 8 h. The films formed were removed from the capsules and stored at 25 °C and 50% relative humidity (RH). The products were named SPI; SPI-SNC 2%; SPI-SNC 5%; SPI-SNC 10%; SPI-SNC 20% and SPI-SNC 40%.

2.4. Preparation of β-CD-containing films

The β-CD-containing SPI-SNC films (SPI-SNC-β-CD) were prepared using the methodology described in Section 2.3 but adding β-CD in powder to SPI-SNC dispersions (containing 5 or 20% of SNC). The final dispersion was stirred for 1 h and poured into Petri dishes for drying. The β-CD was added in three different proportions (14; 71 and 143% of β-CD in mass with respect to SPI). The products were named SPI-SNC 5%-β-CD 14%; SPI-SNC 5%-β-CD 71%; SPI-SNC 5%-β-CD 143%; SPI-SNC 20%-β-CD 14%; SPI-SNC 20%-β-CD 71% and SPI-SNC 20%-β-CD 143%.

2.5. Characterization of SNC and SPI-SNC films

2.5.1. Particle size distribution

The particle size distribution of SNC was determined by dynamic light scattering (DLS) using a Beckman Coulter Dels Nano C instrument.

2.5.2. Morphology

Morphological properties of SNC and SPI-SNC films were analyzed using optical microscopes (Zenok and Shimadzu), confocal scanning laser microscope (Olimpus Lext 3D) and scanning electron microscopy (SEM) (FE-SEM Σigma).

2.5.3. X-ray diffraction (XRD) analysis

XRD analysis was performed on prepared SNC, native corn starch granules, unreinforced (SPI) and reinforced SPI films (SPI-SNC). Samples were examined with a Philips PW 1710/01 instrument with Cu Kalfa radiation (1.54 A).

2.5.4. Film thickness

Film thickness was determined as the average of ten measurements for each sample with a hand-held micrometer (Schwyz model ESP1-0001PLA, Schwyz, Swiss). The average film thickness was used to assay opacity, water vapor permeability (WVP) and mechanical properties.

2.5.5. Opacity

Each film was cut in a 2.5 × 1 cm rectangle; opacity was determined as the area under the absorbance curve in the visible spectrum ($\lambda = 400–800$ nm) of each sample. The value of the areas was normalized, divided by the thickness of each film.

2.5.6. Swelling ratio (S)

The swelling characteristics of the different specimens were studied in triplicate at different times. All the films were weighed (W_d) and immersed in 30 mL of deionized water for specific time intervals at room temperature. The samples were removed from the swelling medium, wiped with a piece of tissue paper to absorb the excess of water on the surfaces and reweighed (W_s). S was calculated from Eq. (1).

$$S = [(W_s - W_d)/W_d] \times 100 \tag{1}$$

2.5.7. Moisture content (MC) and total soluble matter in water (TSM)

MC was determined according to a method described in the literature (Rhim, Gennadios, Weller, Cezeirat, & Hanna, 1998). Film samples were weighed ($W0$) into glass dishes, dried in an oven at 110 °C for 24 h and weighed again (Wi). MC was determined in quadruplicate for each film by Eq. (2).

$$MC = [(W0 - W\mathrm{i})/W0] \times 100 \tag{2}$$

TSM was determined according to a method described in the literature (Rhim et al., 1998). Dry and soluble matters were measured on different films from each cast film to avoid cross-linking by heating of the samples prior to incubation in water. Four weighed samples of each film were immersed in beakers containing 30 mL of distilled water. The beakers were stored in an environmental chamber at 25 °C for 24 h with occasional stirring. The insoluble matter was then separated and dried in an oven at 110 °C for 24 h (Wf) to determine solubilized dry matter by Eq. (3). The measurements were obtained in quadruplicate for each film. Initial dry matter values needed for TSM calculations were obtained from MC measurements for a film with the same mass (Wi). The reason for using different film specimens to measure initial and soluble dry film matter is that proteins are susceptible to heat-induced cross-linking and this effect would produce errors in the determination of the real TSM values (Gennadios, Ghorpade, Weller, & Hanna, 1996).

$$TSM = [(W\mathrm{i} - W\mathrm{f})/W\mathrm{i}] \times 100 \tag{3}$$

2.5.8. Water vapor permeability (WVP)

WVP was determined in duplicate for each film following the procedure of desiccant, described by a standard method (ASTM Standard (E96-10), 2010). The films were placed in a humidity chamber at 25 °C and 65% RH for a period of 2 days to reach equilibrium. Subsequently, these films were fixed onto aluminum capsules (50 mm diameter, 17 mm depth) containing anhydrous $CaCl_2$ (dried at 180 °C for 24 h), sealed with silicone grease. The desiccant was separated from the atmosphere by the film. These capsules were weighed and placed in a humidity controlled chamber under the same conditions as those in which the films were previously conditioned. The weight variation of the entire system was recorded every 1 h up to 9 measurements. These values were plotted as weight variation versus time, obtaining a linear characteristic graph. Water vapor transmission (WVT) was calculated using Eq. (4) where F and A represent the slope of the linear graph and surface area exposed, respectively. WVP is then calculated according to Eq. (5) where *e* means film thickness, SA is the saturation pressure at 25 °C and (RH1 − RH2) is the difference in RH between the interior and exterior of the capsule.

$$\mathrm{WVT} = \mathrm{F/A} \tag{4}$$

$$\mathrm{WVP} = (\mathrm{WVT} \times e)/[\mathrm{SA} \times (\mathrm{RH1} - \mathrm{RH2}) \times 3600] \tag{5}$$

2.5.9. Mechanical properties

For these analyses, each film was cut into 25 × 100 mm pieces and stress–strain curves were performed. Tensile strength (TS), elongation at break (EB) and Young's modulus (E) were determined according to a standard method (ASTM Standard (D882-02), 2002). An Instron texturometer (model 3342, Norwood, MA, USA) equipped with a 500 N cell was used at a speed of 0.5 mm/s. Five measurements were performed for each sample and averaged.

2.6. Application assays of SPI-SNC-β-CD films

2.6.1. Cholesterol retention effectiveness test

In order to determine the ability of the films to sequester cholesterol from milk samples, each SPI-SNC-β-CD film was contacted with milk (Astray, Gonzalez-Barreiro, Mejuto, Rial-Otero, & Simal-Gándara, 2009; López-de-dicastillo et al., 2011). The films were immersed in distilled water for 6 h to remove the non-incorporated β-CD, dried in an oven at 40 °C for 4 h and sterilized under UV light for 20 min. β-CD-containing films (32 cm^2) were placed in a glass vial and submerged in 50 mL of commercial milk (La Serenisima®). All analyses were performed in duplicate. The vials were sealed and wrapped in aluminum foil and stored in a refrigerator at 10 °C for 10 days.

2.6.2. Determination of cholesterol in milk

The quantification of cholesterol in milk was performed following a procedure described in the literature (Rendón Marín & Pineda Orozco, 2010). For this, a direct saponification, followed by an extraction and subsequent quantification by gas chromatography was conducted. Milk (0.5 g) was weighed into a glass vial. Then, 14 mL of 1 M methanolic KOH was added and stirred for 15 s and heated to 60 °C for 15 min with constant agitation. The extraction was carried out with the addition of 3 mL of water and 14 mL of hexane at room temperature. The organic phase was taken in a vial and injected into the chromatograph equipment. A gas chromatograph Varian 3900 GC with flame ionization detector was used with auto sampler system. The column used was a Varian Factor 4 VF- 5 ms 30 m long by 0.32 mm diameter. Quantification was carried out by external standard method.

2.7. Statistical analysis

Data for each test were statistically analyzed. The analysis of variance (ANOVA) was used to evaluate the significance in the difference between means. Turkey test was used for comparing mean values and differences between means were considered significant when $P \leq 0.05$.

3. Results and discussion

3.1. SNC synthesis and characterization

SNC synthesis was performed from the acid hydrolysis of native corn starch. Various reaction conditions were tested but the best results were yielded using the conditions described in Section 2.2. The presence of nanocrystals was observed as a white suspended particulate that was washed extensively with distilled water to neutral pH and stored in refrigerator.

The morphological and structural characterization of the SNC was developed. DLS of the SNC dispersion was performed in order to determine the size of nanocrystals. High polydispersity with an average size of 35 nm was observed. It is important to note that no proportions of particles smaller than 27 nm was observed probably due to these particles are retained in the filtering process. Fig. 1 shows the proportion of particles of each size.

In order to verify particle size and analyze the morphology of SNC, SEM and confocal scanning laser microscopy was used. Fig. 2 shows the images.

The images show the spherical morphology of the nanocrystals. On the other hand, the average sizes determined by DLS were confirmed.

The crystalline nature of the SNC could be determined by XRD. Native corn starch and SNC were analyzed by this methodology, whose diffractograms are shown in Fig. 3. A typical semi-crystalline A-type polymorphic structure characteristic of the starch from cereals can be identified in the diffractogram of the native corn starch sample (Kristo & Biliaderis, 2007). On the other hand, SNC shows a pattern similar to starch although the presence of more intense crystalline peaks shows a higher crystallinity of SNC compared to the native starch, due to a higher amylopectin proportion.

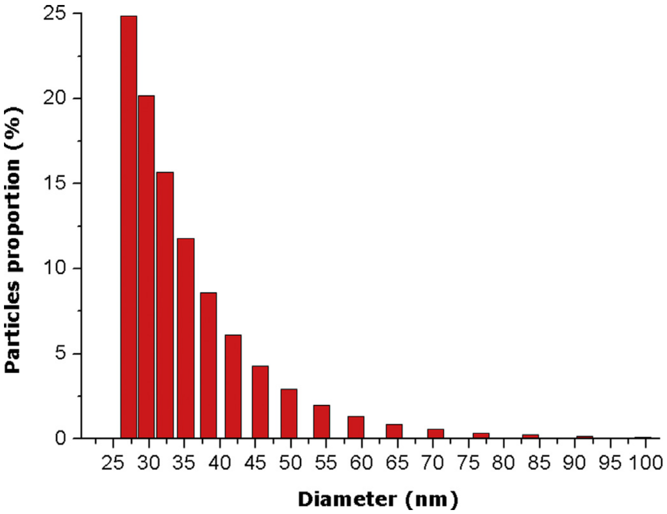

Fig. 1. Size distribution of SNC by DLS.

3.2. Preparation and characterization of SPI-SNC films

The films were obtained by the casting method using Gly as a plasticizer. Those prepared with 0; 2; 5; 10; 20 and 40% w/w (relative to SPI) of SNC showed homogeneous, yellowish and transparent surfaces although the opacity increased as the amount of SNC incorporated. Fig. 4 shows the photographs of the films exhibiting their macroscopic appearance.

From the structural perspective, variation in the amount of SNC incorporated into the different films was reflected in the increase in crystallinity. By the analysis of the XRD of the films, the amorphous characteristics of the proteic film without SNC (determined by the increase of baseline at 20°) could be observed. In addition, an increase was found in the crystallinity of the SPI-SNC films (determined from the appearance of peaks corresponding to crystalline phases) caused by the increase in the amount of SNC. The quantification of the crystalline phases of the films could not be conducted due to the large proportion of amorphous component. Fig. 5 shows the XRD patterns for the films.

In order to determine the structural morphology of the film surfaces, both optical microscope and laser scanning confocal microscope were used. Fig. 6 shows the images. Increases in the amount of solid structures on the surfaces of the samples could be seen, while the quantity of SNC incorporated was higher. Those solid structures show sizes in the order of few microns and, as described above, the average size of the SNC was 35 nm. Hence, it can be concluded that the structures correspond to a small percentage of SNC larger than the average or the presence of aggregates possibly formed in the preparation step by ineffective dispersion of SNC. The smaller nanocrystals cannot be observed probably due to an occlusion effect into the protein matrix.

In order to examine physical characterization, the opacity of the films was determined. For all samples, high transparency was observed, increasing its opacity with the increase in SNC. Accordingly, the most significant change in opacity was observed in the films with 2–5% SNC. Table 1 displays the opacity values measured.

The films were also characterized so as to determine properties in which water plays an important role. They involve swelling ratio (S) as a function of time, MC, TSM and WVP. Fig. 7 shows S values as a function of time. In this work, no comparison with native corn starch-containing films was performed since is described in literature that no positive effects are produced by native starch as reinforcement in biomaterials (Y. Chen et al., 2008).

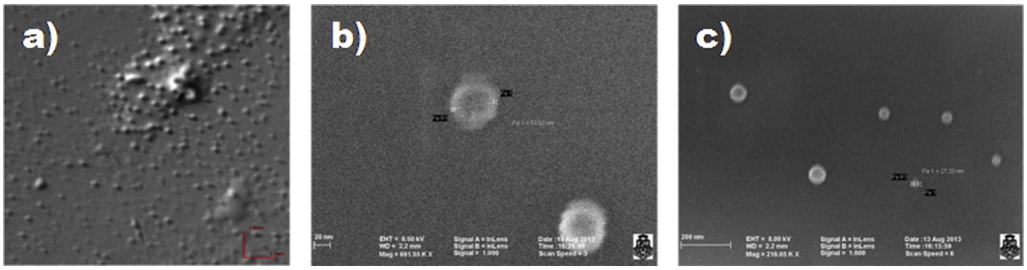

Fig. 2. Images of SNC using: a) confocal scanning laser microscopy; b) and c) scanning electron microscopy.

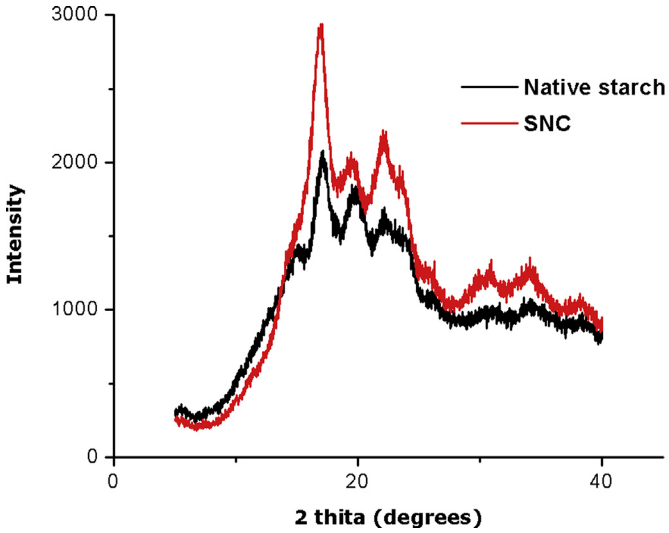

Fig. 3. XRD patterns of native corn starch and SNC.

Fig. 7 shows that all films swelled to the maximum capacity in a relatively short time (approx. 2 min). After reaching this balance, the swelling was generally maintained over time, except for the samples with lower SNC content (0; 2 and 5% SNC) which showed a decrease in swelling after several minutes. This effect was probably caused by the partial solubilization of the samples in water which resulted in a diminution of the mass after immersion in water, reflected in the decrease of S values. By comparing the maximum swelling of the films, it can be observed that S decreased as the amount of reinforcement incorporated increased. This variation represents a diminution by about half for films with higher SNC amount (20 and 40%) compared to the rest. The same behavior was previously described for cross-linked SPI films (González, Strumia, & Alvarez Igarzabal, 2011).

Table 1 shows MC and TSM values which decreased with the increase in the amount of SNC added. For MC, this phenomenon was observed in the films with large additions of SNC (over 10%), while in the case of TSM, a particularly significant decrease (around 43%) was produced with the addition of a low amount of SNC (5%).

WVP was determined using the assay described by ASTM (ASTM Standard (E96-10), 2010). Table 2 shows the results.

It can be noted that this property also decreased with the increase in SNC, especially with large additions (over 20%).

By the analysis of the variation of these physical properties (involving water), it could be concluded that the presence of SNC minimizes the affinity of the films for water. This effect can be accounted in terms of the compatibility in the protein matrix/reinforcement interface. A similar polar nature of both components (hydrophilic materials) probably leads to strong adhesion between them by strong interactions in the interface (bridge hydrogen

Fig. 4. Macroscopic aspect of SPI films with 0; 2; 5; 10; 20 and 40% w/w of SNC.

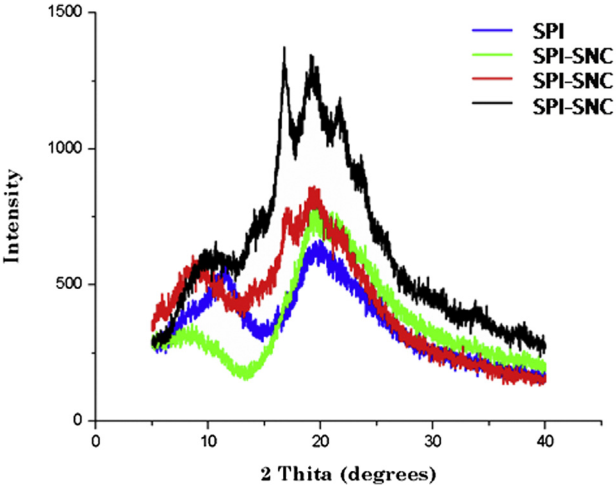

Fig. 5. XRD patterns of SPI films with 0; 10; 20 and 40% of SNC.

bonds mainly), minimizing the interactions of the matrix with water. This effect was more pronounced in materials with greater amount of SNC. In addition, the marked changes observed could be explained by the extremely extensive interface due to the small reinforcement size. The same effect was previously described in the literature for pullulan films (Kristo & Biliaderis, 2007).

The SPI-SNC films were also mechanically studied by tensile tests according to the methodology described by ASTM (ASTM Standard (D882-02), 2002). A universal testing instrument was used to record the stress–strain curves for each specimen. From these, tensile strength (TS), elongation at break (EB) and Young's modulus (*E*) were determined. The results are shown in Table 3. From these values, it can be concluded that, as the amount of nano-reinforcement increased in the film, the material became more rigid since increases in TS and *E* were observed. This effect correlated with that observed and explained for variation in physical properties. The major interactions between the protein matrix and the reinforcement provide high cohesion to the film, requiring more energy to be stretched and increasing TS and E. Likewise, reduction in EB values was achieved yielding less extendible materials.

3.3. Preparation of β-CD-containing films

In order to apply the prepared films as cholesterol sequester active packaging, different amounts of β-CD were added to the filmogenic solutions. The experimental conditions used to prepare CD-containing films, (that require low solubility in water) were the same that were used to prepare both SPI-SNC 5- and −20% but to the incorporation of β-CD into the films were performed. Thus, 0.05; 0.25 and 0.5 g of β-CD (corresponding to 14; 71.4 and 143% in mass relative to SPI, respectively), were added to the filmogenic solutions (as in Section 2.3) after the addition of SNC. It should be noted that high amounts of β-CD (higher than 143%) caused serious problems in the formation of the film. The macroscopic aspects of films with and without β-CD were similar, but their opacity increased as more β-CD was added (results not shown).

Table 1
Opacity, MC and TSM of SPI and SNC-containing SPI films.

Films	Opacity (UA/μm)	MC (%)	TSM (%)
SPI	1.293 ± 0.112^{A}	33.56 ± 0.39^{A}	37.84 ± 2.33^{A}
SPI-SNC 2%	1.021 ± 0.142^{A}	35.24 ± 1.08^{A}	31.35 ± 2.09^{B}
SPI-SNC 5%	2.137 ± 0.003^{B}	33.11 ± 1.69^{A}	21.63 ± 1.24^{C}
SPI-SNC 10%	2.293 ± 0.155^{B}	28.28 ± 1.35^{B}	24.71 ± 3.72^{C}
SPI-SNC 20%	2.377 ± 0.310^{B}	27.27 ± 0.67^{B}	22.84 ± 2.34^{C}
SPI-SNC 40%	2.680 ± 0.125^{B}	24.02 ± 0.20^{C}	20.09 ± 0.54^{C}

Any two means in the same column followed by the same letter are not significantly ($P \geq 0.05$) different according to Turkey test.

3.4. Retention effectiveness of cholesterol in milk using β-CD-containing films

The films were immersed in distilled water in order to remove the non incorporated β-CD and subsequently dried in an oven. Film pieces were immersed in milk in sterilized glass vials. In addition, a milk sample without film was used as a control sample. The vials were sealed, protected from light and stored at 10 °C for 10 days. The number of days was selected on the basis of the minimum and common period of interaction between food and its container. After 10 days, the films were removed from the glass vials and the cholesterol amount was determined in each milk sample. The films remained intact after milk exposure.

The quantification of cholesterol in milk was developed by the external standard method by gas chromatography (Rendón Marín & Pineda Orozco, 2010). By performing a calibration curve, a molar absorptivity coefficient of 719.08 UA/ppm was determined. The peak area of cholesterol in each sample was determined as an average of the corresponding areas of three injections to avoid fluctuations produced by CG detector. Table 4 shows the results.

It can be seen that SPI-SNC -5 and −20% (control films) did not retain cholesterol from milk samples. For films containing β-CD in low proportion (14%), no significant decrease in cholesterol amount

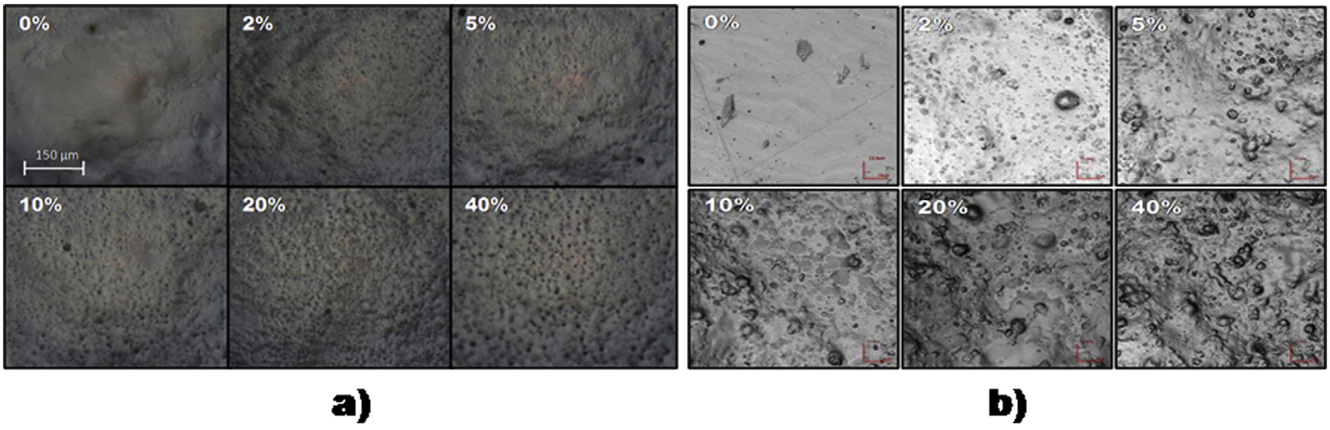

Fig. 6. Surface images of SPI-SNC films obtained with: a) optical microscope (1000×) and b) laser scanning confocal microscope (2132×).

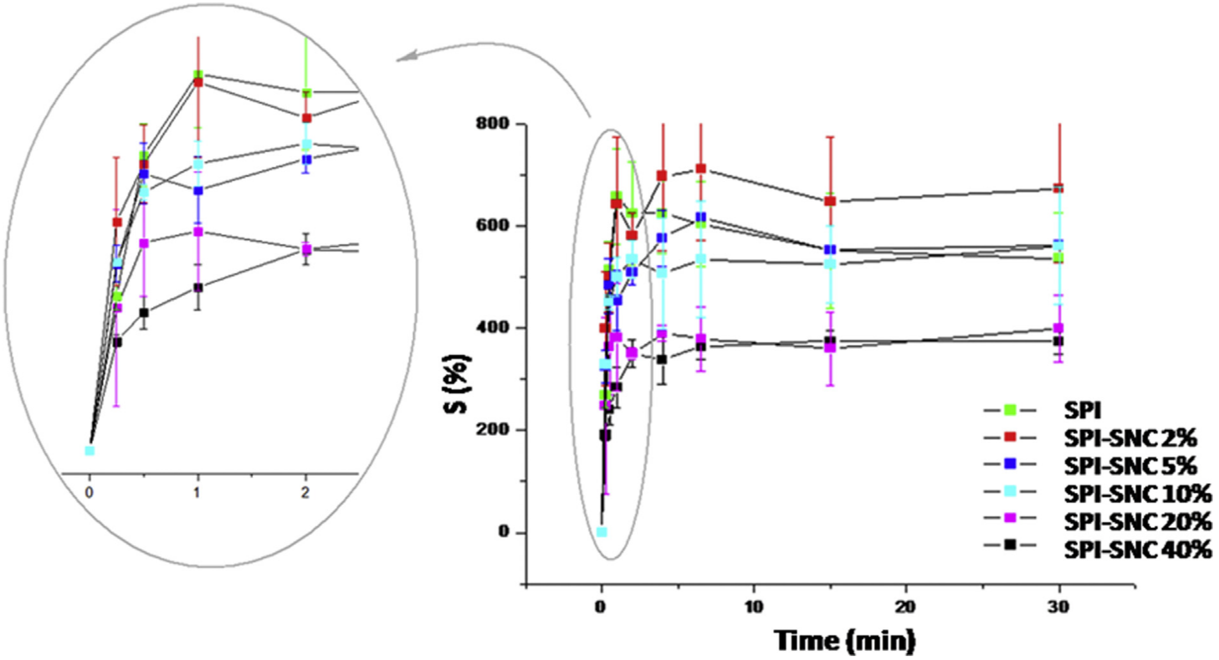

Fig. 7. Swelling ratio of the films as a function of time.

Table 2
WVP values of SPI and SNC-containing SPI films.

Films	WVP (x 10^{-10} g/Pa s m)
SPI	$(4.3 \pm 0.2)^{A}$
SPI-SNC 5%	$(4.8 \pm 0.3)^{A}$
SPI-SNC 20%	$(3.9 \pm 0.1)^{B}$
SPI-SNC 40%	$(3.57 \pm 0.08)^{C}$

Any two means in the same column followed by the same letter are not significantly ($P \geq 0.05$) different according to Turkey test.

Table 3
TS, EB and *E* values of SPI and SNC-containing SPI films.

Films	TS (MPa)	EB (%)	*E* (Mpa)
SPI	1.10 ± 0.20^{A}	65.95 ± 17.76^{A}	26.89 ± 11.21^{A}
SPI-SNC 2%	1.42 ± 0.22^{A}	53.79 ± 4.02^{AB}	55.31 ± 4.78^{AB}
SPI-SNC 5%	1.34 ± 0.07^{A}	58.67 ± 9.88^{A}	39.42 ± 9.93^{AB}
SPI-SNC 10%	1.79 ± 0.27^{A}	32.17 ± 8.00^{BC}	71.05 ± 13.01^{BC}
SPI-SNC 20%	2.61 ± 0.26^{B}	41.89 ± 8.61^{ABC}	102.23 ± 14.93^{C}
SPI-SNC 40%	5.08 ± 0.48^{C}	21.35 ± 10.54^{C}	310.34 ± 21.55^{D}

Any two means in the same column followed by the same letter are not significantly ($P \geq 0.05$) different according to Turkey test.

Table 4
Amount of cholesterol and its decrease in percentage (%) for milk samples after contact with the active films.

Films	Milk (mL)	Cholesterol (ppm)	Cholesterol decrease (%)
–	50	$(4.7 \pm 0.1)^{A}$	–
SPI-SNC 5%	50	$(4.7 \pm 0.2)^{A}$	−0.50
SPI-SNC 20%	50	$(4.6 \pm 0.2)^{A}$	0.15
SPI-SNC 5%-βCD 14%	50	$(4.6 \pm 0.4)^{A}$	1.31
SPI-SNC 20%-βCD 14%	50	$(4.6 \pm 0.1)^{A}$	0.77
SPI-SNC 5%-βCD 71%	50	$(3.7 \pm 0.2)^{B}$	20.10
SPI-SNC 20%-βCD 71%	50	$(3.5 \pm 0.3)^{B}$	25.76
SPI-SNC 5%-βCD 143%	50	$(2.8 \pm 0.1)^{C}$	40.52
SPI-SNC 20%-βCD 143%	50	$(2.6 \pm 0.0)^{D}$	43.30

Any two means in the same column followed by the same letter are not significantly ($P \geq 0.05$) different according to Turkey test.

was observed. In the other hand, those with higher amounts of β-CD, showed a marked decrease, being the effect more important in those with greater amounts of β-CD incorporated into the films. This effect can be explained by the fact that β-CD has cavities available to form inclusion complexes with cholesterol. This effect indicates that β-CD acts effectively as a cholesterol receptor through inclusion complex between the cavity and the hydrophobic tail of cholesterol. Furthermore, variation in the amount of the SNC into the films (5 or 20%) did not significantly affect the cholesterol retention.

The reduction of cholesterol using β-CD-containing films based on SPI-SNC proved best results in comparison with that yielded with poly(vinyl alcohol) films containing 20 and 30% of β-CD, which showed a decrease of 6.2 and 8.3%, respectively (López-de-dicastillo et al., 2011).

4. Conclusions

Biodegradable, homogeneous and transparent SPI-based films were obtained. The incorporation of SNC as nano-reinforcements was performed in order to improve physical and mechanical properties (decreasing water solubility, swelling and WVP and increasing TS and E) of the films. The films obtained showed important properties that could be useful in a variety of areas. In particular, they were assayed as active food coating by incorporation of β-CD, demonstrating the ability of sequestering cholesterol from milk. Substantial decreases in percentages of cholesterol were yielded in milk samples in contact with the active films. This behavior demonstrated the versatility that modified SPI films can acquire. This research establishes an important precedent to consider the replacement of synthetic petroleum-based plastics by biodegradable and environmental friendly materials useful in applications such as active food coating or active packaging containers.

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