Near-infrared chemical imaging and its correlation with the mechanical properties of chitosan-gelatin edible films

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ABSTRACT

Plasticizers influence the physical properties of edible films by their interaction with the film-forming polymers. Using near-infrared chemical imaging, it is possible to characterize the interaction between compounds through the analysis of their relative presence throughout the film (abundance) and their variability. These parameters and standard mechanical properties were used to characterize the interaction between gelatin, chitosan and several plasticizers, pure or in binary combinations. Triacetin showed the least interaction with the polymers, while polyethylene glycol 400 and glycerol showed high interaction with them. In addition, we observed that the tensile strength of the film was well correlated with the variability of gelatin and chitosan.

Keywords

Hydrocolloid; near-infrared spectroscopy; plasticizer; molecular interaction; tensile strength

1. INTRODUCTION

Edible films are thin layers of a polymer material that control the solute exchange between the matrix and the environment, thus extending the shelf life of a food (Pavlath & Orts, 2009). To achieve this, the films' polymer matrix should have the necessary physical-chemical properties for the specific food with which it will be used. Edible films can be created to have the necessary mechanical and structural properties with the use of hydrocolloid molecules, which are hydrophobic biopolymers with high molecular weight and long polymeric chains (Falguera, Quintero, Jiménez, Muñoz, & Ibarz, 2011). The most used compound for the manufacturing of edible films is gelatin, mainly due to its ability to form films at low temperatures (Gómez-Estaca, Gómez-Guillén, Fernández-Martín, & Montero, 2011), and chitosan, for being antibacterial, biodegradable, biocompatible, non-toxic and renewable (van den Broek, Knoop, Kappen, & Boeriu, 2015).

Edible films are made up of molecular interactions between the polymers used in the film, including covalent bonds (i.e. disulfide interactions and cross-linking) as well as ionic bonds, hydrogen bonds and electrostatic interactions. These interactions generate films that are rigid and brittle, providing less protection to the food (Cao, Yang, & Fu, 2009; Rivero, García, & Pinotti, 2010). It is possible to improve the stability and performance of the edible films with the addition of plasticizers (Talja, Helén, Roos, & Jouppila, 2008). Plasticizers are compounds that reduce inter-molecular forces and increase the mobility of the polymeric chains of the film (Sothornvit & Krochta, 2005). The plasticizers interact with the polymers of the matrix through van der Waals forces, hydrogen bonds and weak electrostatic interactions (Marcilla, García, & Beltrán, 2012). The amount of oxygen atoms per molecule, the molecular weight and polarity (dielectric constant) are the characteristics of the plasticizer that determine the type and level of interaction with the film-forming polymers (Immergut & Mark, 1965). Also, the addition of high concentrations of hydrophilic plasticizers (i.e., over 20% in chitosan films, (Suyatma, Tighzert, & Copinet, 2005)) could over-plasticize the films, producing phase separation (Arvanitoyannis, Nakayamab, & Aibab, 1998). Over-plasticized films are sticky, weak and extend more than required. Sometimes exudate drops can be observed in

the surface of over-plasticized films (Taylor, Taylor, Belton, & Minnaar, 2009; Wilson, 1995).

Infrared spectroscopy (IR) is a good option to study the chemical interaction between compounds (Lefevre & Subirade, 2001). IR spectroscopy provides information about the internal chemical structure of the sample, for instance, the type or quantity of a given bond that is present in the sample (Zapf, 2009). This is achieved by detecting chemical bonds through the rotation and vibration that they show in a given spectral range of the electromagnetic spectrum ($13000\text{--}10\text{ cm}^{-1}$ or $0.76\text{--}1000\text{ }\mu\text{m}$, depending on the units used) (Gendrin, Roggo, & Collet, 2008). One type of IR is near-infrared spectroscopy (NIR), used mainly to study the overtones and combinations of CH, OH, NH bands and stretching, as well as bond vibration modes that provide highly specific molecular information for species identification (Furukawa, Sato, Shinzawa, Noda, & Ochiai, 2007).

Near-infrared chemical imaging (NIR-CI) techniques apply conventional near-infrared spectroscopy, together with a system that captures multiple images through many wavelengths. The resulting image is a tri-dimensional (3D) matrix or hyperspectral data cube, with two spatial dimensions and one spectral dimension (Gendrin et al., 2008). The conventional NIR technique only provides the average spectrum of the surface of each sample; however, NIR-CI provides a spectrum of each pixel of the samples' entire surface, allowing a complete chemical characterization with a quick and non-invasive process.

With the information obtained by the NIR-CI, together with the use of chemometric tools, a characterization can be created with the distribution of the samples' compound of interest (Sacré et al., 2014), obtaining the histogram parameters of the compounds' intensities: mean, standard deviation, kurtosis and skew. The mean of the distribution values corresponds to the abundance of the compounds on the surface of the sample (de Juan et al., 2004). The standard deviation of the distribution is defined as the variability of the abundance of the samples' compounds (Puchert, Lochmann, Menezes, & Reich,

2010). Kurtosis measures the symmetrical tailing or ‘peakedness’ of the distribution, where zero indicates a normal distribution. Finally, skew is the measurement of the asymmetry of the distribution, with zero corresponding to a symmetric distribution (E. N. Lewis, Schoppelrei, Lee, & Kidder, 2007; Montgomery & Runger, 2003). Viewed together, the variability of abundance, the degree of unimodal distribution and the asymmetry of the distribution are descriptors of the homogeneity of the sample. Moreover, these parameters depend on the spectral information of the sample, which changes in response to the interaction between the components. (Lefevre & Subirade, 2001).

In pharmaceuticals, there are studies that relate physical properties of drug matrices with the distribution and abundance of ingredients for the NIR-CI analysis. For example, Ellison, Ennis, Hamad, and Lyon (2008) showed that NIR-CI can be an effective tool for monitoring physical quality, distribution of active pharmaceutical ingredient in the tablets, and also their uniform compaction force. Y. F. Wang et al. (2014) reported correlation models that compared tablet hardness and spatial distribution, obtained using NIR-CI. Jérez Rozo et al. (2011) used NIR-CI to characterize the spatial distribution and abundance of the film-forming polymer and the drug contained in the film used in a pharmaceutical application. Both parameters were correlated with the size of the particle and the agglomeration of the drug, allowing them to determine the dissolution speed and drug uniformity obtained with the film. This suggests that the abundance and distribution of the polymeric film compounds obtained by NIR-CI, may be related to the films’ physico-chemical properties.

Since the mechanical properties of edible films are determined by the interaction between the plasticizers and the film-forming polymers (Bergo & Sobral, 2007), these properties could be analyzed with NIR-CI. This paper investigates the relationship between the abundance of the compounds and their variability, as obtained with NIR-CI, and the mechanical properties of the gelatin and chitosan edible films. Our objective is to research the potential of NIR-CI to qualitatively and quantitatively characterize, in a non-

- 154 invasive way, the homogeneity of the edible films and its impact on the films'
- 155 mechanical properties.

2. MATERIALS AND METHODS

2.1 Materials

The film forming solutions were fabricated using gelatin from cold water fish skin (Sigma-Aldrich Lot# SLBG7701V, average molecular mass 60 kDa; bloom number: 225 – 325; viscosity at 24°C, 31500 cps), and chitosan from *Pandalus borealis* (Sigma-Aldrich Lot# MKBD4275V; medium molecular weight; 77% deacetylation, viscosity 450 mPas at 1% concentration in 1% acetic acid). The plasticizers used were glycerol (≥99%, Sigma-Aldrich), sorbitol (≥99.5%, Sigma-Aldrich), polyethylene glycol-400 (PEG400) (EMD Millipore), and triacetin (99%, Acros Organics).

2.2 Methods

2.2.1 Film forming solutions

The film-forming solutions were fabricated by dissolving chitosan in acetic acid at 1% v/v to reach a concentration of 1.55% w/w. The dissolved chitosan was mixed with liquid state gelatin at 2.3% w/w. In each case, a plasticizer was added at 1.16% w/w (Hosseini, Rezaei, Zandi, & Ghavi, 2013), or two plasticizers at 0.58% w/w each (Table 1). Gelatin and chitosan film, without the addition of plasticizers, was used as control film. Each film-forming solution was stirred until dissolved, and then was heated and maintained at 45°C for 1 h, being constantly stirred. The resulting solution had a pH of 4.566 ± 0.007 and was filtered using vacuum and stored at room temperature until the edible films' fabrication.

Table 1. Formulation of edible films used in this study

Compound	(g/100 g sol.)	Percentage ⁽¹⁾
Gelatin	2.30	46
Chitosan	1.55	31
Plasticizers ⁽²⁾	1.16	23

⁽¹⁾ Percentage corresponds to composite grams/grams of total compounds in the film multiplied by 100.

⁽²⁾ Plasticizers correspond to glycerol, sorbitol, PEG400, and triacetin, either as a single plasticizer or as a combination of two of them in a proportion 1:1.

2.2.2 Fabrication of edible films

Solvent casting was used to produce edible films. Aliquots of 5.4 g of a film-forming solution were poured into petri dishes with surface areas of 24 cm², and dried at 30°C and 25 ± 5% relative humidity in a system of forced air until a stable mass was reached (15 h). The average films' thickness was 100 ± 23 µm.

2.2.3 Near-infrared chemical images (NIR-CI)

A Malvern Instrument Spectral Dimension SyNIRgi spectrometer chemical imaging system (Malvern, UK) was used. The Malvern system includes a focal plan array (FPA) mode with a tunable liquid-crystal filter that allowed full field analysis.

The edible films were placed over a white ceramic disk with a 28 mm diameter, which was used as a reference for the acquisition system. Spectra and images were obtained in transreflectance mode. In this mode, the radiation passes through the bulk of the film, bounces back in the highly reflective ceramic, passes through the bulk of the film again and then to the detector (Li Yoon et al., 1999). Therefore, the transreflectance mode evaluates not only the surface but also part of the bulk of the film.

The images were analyzed with a 40X objective and composed of 320 x 256 continuous pixels (1 pixel ≈ 1600 µm²) with a spectral range from 1200 to 2400 nm (81920 pixels in a spectral area). The image analysis was performed using the Malvern ISys 5.0 software for cross-platform chemical imaging analysis (Susarla et al., 2013). The data obtained was arranged in a hyperspectral three-dimensional cube: *X and Y* with spatial information, and *λ* with spectral information. For this analysis, the information contained in the hypercube was subjected to a baseline correction and a normalization to correct the scattering effects (Sacré et al., 2014). The pre-treatments used to normalize all of the spectral pixels included a standard normal variable (SNV) and second derivative Savitzky-Golay with a length of 11 and third order.

The spectral quantitative treatments that allowed the prediction of the compounds' abundances were obtained by Malvern ISys 5.0 software with cross-platform chemical

imaging analysis, using Matlab code routines. The software allows the construction of algorithms to determine the distribution of the film compounds and their abundance through partial least square regression analysis (PLS-DA). The term abundance is used to indicate that the signal related to an individual compound is analyzed at a specific sampling area on the surface of the film (de Juan et al., 2004). The abundance refers to the presence of a compound in a certain area, and differs from the term concentration that is based on volume instead of area. Pure compound spectra were used to create a library so as to quantify the abundance of each of the edible film's compounds (de Juan et al., 2004). The spectrum of pure chitosan was acquired from a film made with chitosan dissolved in acetic acid, the spectrum of pure gelatin from a film made with gelatin in solution, and the spectrum of pure plasticizers from the plasticizers in liquid state. In these models, each new factor or latent variable added to the model (variables that are not correlated with each other) indicates a lower systematic variation of the spectra but higher random variation. Each library was composed of the same number of elements that are in the film being analyzed, which allowed setting the optimal number of factors used in the calibration of the models.

The threshold for adjusting the limits to distinguish between the different compounds of the film is based on the similarity between the spectra of the pure compounds and the spectra of the film, along with the number of factors previously selected (Palou et al., 2012). The application of the resulting model normalizes the spectral data with an image based on a scale from 0 to 1. This scale represents abundance from 0 to 100% for each compound for each pixel of the image. As the final result of this methodology, the chemical distribution image of the film components, their abundance and histograms are obtained. The standard deviation of the component's abundance, skew (measure of the distribution asymmetry) and kurtosis (measure of the shape of the distribution) were reported.

2.2.4 Determination of the water content

We determined the water content gravimetrically. The films were weighed before and after being dried. The amount of water was calculated subtracting the weight of the initial film to the dried one, and then dividing by the weight of the dry film.

2.2.5 Mechanical Properties

A universal testing machine (MTS Sintech/5D Universal equipment, MTS SYSTEMS, MN, USA) was used to evaluate the tensile strength (TS), elongation percentage (%E) and the elastic modulus (EM) according to Bedoui et al. (2012). We prepared 5 x 55 mm strips of each film, which were placed and adjusted on the testing machine. The separation of the grips was set at 34 mm and the films were stretched at a constant speed of 10 mm/min at 25°C until they break. A load cell of 100 N was used to reduce the relaxation effects during the test.

The TS was calculated dividing the maximum weight required to break the sample by the area of the transverse section of the edible film. To obtain the %E, we divided the longitude at the breaking point by the initial length of the trial, and then multiplied by 100. Finally, the EM was obtained by dividing tensional stress over extensional strain. Each measurement was repeated 5 times.

2.2.6 Statistical analysis

The analysis of the effect of plasticizers on the mechanical behavior of edible films and the variance analysis (factorial) of the response variables with p values ≤ 0.05 were obtained using the Statgraphics® Plus software for Windows, version 4.0 (Stat Point Technologies, Inc., Herndon, VA). Multiple linear regression (MLR) was used to evaluate the correlation between the abundance of compounds, the abundance variability and the mechanical properties of the edible films. The correlation coefficients (R^2), the model's p-values and models' coefficients were calculated with StatPlus®: Mac version 5.8.2.0/Universal (AnalystSoft Inc).

3. RESULTS AND DISCUSSION

3.1. Method calibration

Partial Least Squares – Discriminant Analysis (PLS-DA) calibration models were developed to study the abundance of film-forming compounds in the films prepared. The first step in this process consisted in a careful evaluation of the NIR spectra obtained. Figure 1a shows the spectra of the pure compounds, whereas Figure 1b shows spectra pre-treated with standard normal variable (SNV) and the Savitzky-Golay second derivative with a length of 11 third order.

Figure 1a. NIR spectra of pure compounds used to fabricate edible films. **b.** NIR spectra of pure compounds with the average of the second derivative after normalization. NIR spectra were acquired by averaging the spectra of each pixel in an area of the hyperspectral image. Ge: gelatin, Ch: chitosan, G: glycerol, S: sorbitol, P: PEG400, T: triacetin.

There was a marked difference between the pure spectra of gelatin and chitosan, a necessary condition to predict the edible films' abundance (Figure 1a). In general, NIR spectra of proteins in ranges below 2200 nm are associated with NH stretching and bond; however, ranges below 2200 nm correspond to CH stretching and bond types (Segtnan & Isaksson, 2004). Gelatin showed three bands: 1720 nm, associated with the first overtone

of the stretching vibrations of the CH group (Segtnan & Isaksson, 2004); 2160 nm corresponding to NH stretching of the amide-B groups plus the amide-II group; and 2260 nm from the stretching of groups CH₂ and CH₃ (J. Wang, Sowa, Ahmed, & Mantsch, 1994). Moreover, chitosan showed relative intensity bands at 1520, 1720, and 2020 nm. All of these bands are related since chitosan possesses three bonds that contribute to the NIR spectrum: the hydroxyl group, the amino group and the end of the polymeric chain (Cervera et al., 2011). The difference between the spectra was corroborated with the low correlation coefficient between the second derivatives of the spectra ($R^2 = 0.323$ evaluated in the entire spectral range. Data not shown). Thus it was possible to generate scores of images related to the abundance of gelatin and chitosan using calibration models (E. Neil Lewis & Kidder, 2010).

Glycerol and sorbitol are both added and have very similar spectra (Figure 1a). The OH groups are responsible for the intense ranges of overtones between 1400 – 1450 nm and combination ranges between 1900 – 1940 nm of wavelength of the NIR spectra (Chen, Hu, Shao, & Su, 2004). These ranges are mainly attributed to the symmetrical and asymmetrical stretching of the OH groups (Boada-Lopez et al., 2013). The structures of glycerol and sorbitol are formed by a carbon's skeleton with three and six OH groups, respectively; these groups are also found in the structure of chitosan. The similarity between glycerol and sorbitol could influence the determination of the abundance values of chitosan. This challenge was overcome through spectral pretreatment with the standard normal variable (SNV) and second derivative transformations, to diminish the correlation coefficient between both spectra ($R^2 = 0.705$, evaluated for the entire spectral range; data not shown). Table 2 shows that it was possible to obtain values of abundance of chitosan, overcoming the similarity of the glycerol and sorbitol spectra.

3.2. Abundance distribution

After using PLS-DA models to obtain the total abundance of the films, this was then corrected for water content (Table 2). As a result, the abundance of these plasticizers was quantified including the water present in the film. When the total abundance values of the

edible films are corrected for water content, abundance values close to 100% are obtained. However, the glycerol, sorbitol, PEG400 and the water molecules already have OH groups in their chemical structures, therefore the abundance of those plasticizers were calculated including the water contained in the film.

Table 2. Values of total component abundance corrected and un-corrected for water content in edible films.

Film	Total abundance un-corrected		Abundance corrected				Total abundance corrected	
			Gelatin		Chitosan			
	(%)	STD	%	STD	%	STD	(%)	STD
Control	86.9	5.3	74.9	3.9	12.1	3.6	103.0	3.6
G	106.6	5.6	73.5	3.1	5.9	3.8	97.1	3.0
S	111.9	6.1	76.2	3.5	12.3	4.2	105.7	3.6
P	107.1	7.2	78.3	4.9	10.0	4.5	98.7	4.7
T	95.0	3.4	62.7	2.0	18.9	2.4	100.5	2.0
G + S	92.6	10.2	66.6	3.7	4.1	4.3	84.9	3.5
G + P	112.1	6.4	78.6	3.2	4.7	3.8	103.1	3.2
G + T	106.8	4.6	64.1	2.8	16.3	2.7	106.8	2.6
S + P	115.0	6.0	79.4	3.3	9.0	3.5	99.0	3.3
S + T	107.3	4.9	66.7	3.1	18.4	2.8	107.3	2.9
P + T	103.6	4.2	65.4	2.4	20.5	2.1	103.6	2.3
Total	105.2	5.2					100.9	3.2

Film without plasticizer (Control); Film plus glycerol (G); Film plus sorbitol (S); Film plus PEG400 (P); Film plus triacetin (T). Nomenclature which includes the "+" sign involves adding two plasticizers in a gelatin and chitosan edible film. The total abundance value of each film corresponds to the sum of component abundances in the film. The total standard deviation of each film was calculated by the sum of the standard deviations squared multiplied by the percentage of each compound present in the film.

The standard deviations shown in Table 2 describe the spread of abundance values about the mean. However, the mean and the standard deviation do not show how the components are distributed throughout the film. This important information is provided by the chemical images and the histograms of film components shown in Figure 2. The strong red color in the images is indicative of areas of high abundance of components. The deep blue color is indicative of areas of lower abundance of components. The intermediate color represents areas of intermediate abundance. These color images are an

important first step to visualize the distribution of film components, and are based on the abundance values of the 81,920 pixels. Histograms were then used to evaluate the distribution of the abundance values obtained, providing an estimate of the heterogeneity of each sample. The maximum value observed in the histogram is the abundance most observed throughout the film. The kurtosis and skew values describe the deviation from a normal distribution. The gelatin and chitosan in films without plasticizers showed negative values of kurtosis and skew but they are close to zero (Figure 2a), which implies that the histograms of both compounds have a distribution near to a normal distribution. A normal distribution would have a kurtosis of zero. A large value of kurtosis would be indicative of a higher peak with sharp declines and significant tailing, while a negative kurtosis indicates a flat peak obtained as a result of highly varying abundance values (E. N. Lewis, Schoppelrei, Makein, Kidder, & Lee, 2010). A negative skew indicates a large number of areas with low abundance values, while a positive skew indicates a large number of areas with high abundance values. The skew values indicate that gelatin presents more low abundance domains than chitosan. In addition, the standard deviation value of gelatin is slightly higher than the standard deviation value of chitosan; hence, gelatin had a less homogeneous distribution.

As mentioned in the methodology, the spectra and chemical images were obtained by NIR-CI in transreflectance mode. Thus, if a film presents phase separation due to over-plasticization, this will not necessarily interfere with the abundance measurements because they give information of the components throughout the film, not only of its surface.

2a. Formulation Control

(Edible films without plasticizer)

2b. Formulation G

(Edible films plus glycerol)

2c. Formulation S

(Edible films plus sorbitol)

2d. Formulation P

(Edible films plus PEG400)

2e. Formulation T

(Edible films plus triacetin)

Figure 2. Chemical images of abundance distribution and histograms for film-forming polymers and plasticizers of edible films studied. Kurtosis (g^2) and skew (g^1) values of the histograms were used to study the distribution of the film's compounds. The side bar in each image shows the intensity of the compounds of each pixel, where red indicates

higher intensity of the image. Each image corresponds to an edible film of triplicate per condition studied.

By adding glycerol to the film, the gelatin and chitosan kurtosis values varied slightly, maintaining their negative sign and therefore their homogeneity (Figure 2b). Moreover, the skew value of gelatin increased, implying a change of sign, while the skew of chitosan was maintained with a negative sign. In the presence of glycerol, the abundance variability increases for gelatin, however it decreases for chitosan (Table 2).

Therefore, the addition of glycerol increases the number of areas with high abundance values of gelatin and varies slightly the number of these areas of chitosan.

In the films where sorbitol was added, the kurtosis and skew values of gelatin and chitosan were greater than zero; however, the greatest changes were in the gelatin (Figure 2c). Specifically, the changes in the skew values indicate that the polymers showed an increase in the high abundance domains, particularly in gelatin. On the other hand, the changes in the kurtosis of both polymers showed that the addition of sorbitol creates distributions that are less homogeneous than the control film. The homogeneity reduction in the distribution of the polymers suggests that sorbitol interacts with both polymers.

The gelatin histograms of the films that included PEG400 showed the most negative skew values and greatest kurtosis values (Figure 2d). This indicates that the gelatin exhibits low presence of high concentration domains, generating less uniformity in the component's distribution (E. N. Lewis et al., 2007). Specifically, the negative skew indicates a reduction of high content fields of gelatin. The positive kurtosis of the film shows that the gelatin has a heterogeneous distribution with respect to the chitosan on the same film, while the positive skew value of chitosan indicates an increase of high abundance domains of chitosan. This information suggests that PEG400 presents a high interaction with chitosan and a low interaction with gelatin.

Compared with the other films analyzed in this study, the kurtosis of gelatin and chitosan of the films treated with triacetin show the closest to zero values (Figure 2e). This implies that these films presented the most symmetrical and narrow distribution of their

compounds (E. N. Lewis et al., 2007). Triacetin films show the lowest variability in total abundance (Table 2). Moreover, films with triacetin show the lowest variability of the total abundance. Triacetin is a molecule with low polarity, whereas gelatin and chitosan are polar polymers; which implies that triacetin will have a low interaction with gelatin and chitosan.

The abundance of gelatin and chitosan varied according to the plasticizer added, even though films contained the same plasticizer concentration (Figure 3). The NIR-CI method uses the similarity of the spectrum of the pure compounds to identify (quantitatively and qualitatively) each compound in each sample (Palou et al., 2012). This implies that when there is a conformational change of the compound, triggered by the interaction with another compound, a change in the spectrum is produced. We observed the greatest reductions of the abundance of gelatin in the films that were treated with triacetin, suggesting that triacetin interacts mainly with gelatin. Correspondingly, we observed that glycerol interacts mainly with chitosan.

Figure 3. Normalized abundance values of chitosan and gelatin, corrected to the abundance values of both polymers present in the control film.

3.3 Mechanical properties

Table 3 illustrates that the control film (without the addition of plasticizers) showed high levels of TS and EM (19.2 MPa and 2192 MPa, respectively) and low values for %E (1.2%). These values are typical of rigid, brittle materials, due to the high interaction between the film's molecules (Cao et al., 2009; Rivero et al., 2010). Given that the plasticizers reduce their intermolecular strengths due to the interaction between the polymeric chains (Sothornvit & Krochta, 2005), it shows the need for plasticizers to improve the mechanical properties of films.

The addition of glycerol, sorbitol or PEG4000 in edible films significantly changed the TS, %E and EM values versus the control film (Table 3). Specifically, the TS of films with glycerol and sorbitol diminished by 67.7 % and 43.2 % respectively in comparison with the control film. The incorporation of plasticizers in protein and polysaccharide films is generally associated with a reduction of TS, given the reduction of intermolecular forces between the polymeric chains (Matet, Heuzey, Pollet, Ajji, & Averous, 2013; Sothornvit & Krochta, 2005). However, the addition of PEG400 in the film increases TS by 28.6 % versus the control film. An increase in the tensile strength in gelatin films with the addition of PEG400 has been attributed to the strong interaction in the polymeric network that operates in protein films (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2006). This strong interaction in the polymeric network is also observed in the higher variability of the total abundance of the films observed in the chemical imaging experiments (Table 2).

The EM values show that glycerol reduces the rigidity of the film 10 times more than sorbitol. These results illustrate the strong plasticizer effect that polyols (glycerol and sorbitol) have on the polymeric mixtures of chitosan and gelatin; glycerol is the component with the greatest modulation of the mechanical properties. The pronounced effect that glycerol and sorbitol exerted on the mechanical properties of edible films was also observed when plasticizers were used in mixtures (Table 3). In films that had both plasticizers added, the films' TS values were reduced by up to 70% versus the control.

The edible films treated with triacetin showed mechanical properties similar to the films without plasticizers, showing that triacetin did not have a plasticizer effect on the films (Table 3). The reduction of TS in gelatin films with triacetin was reported before (Jiang, Liu, Du, & Wang, 2010); however, in this case the reduction was not significant. This may be due to the plasticizer effect of chitosan when creating gelatin films (Hosseini et al., 2013). Films with triacetin showed the lowest variability of the total abundance value obtained by NIR-CI (Table 2). Additionally, the kurtosis value of gelatin and chitosan was closest to zero (Figure 2e). Triacetin has three double links, and these are responsible for the formation of rigid bonds with other molecules. These bonds would cause the formation of films with similar characteristics to the control film or low interaction.

TS values obtained for edible films with different plasticizers were within the range of edible gelatin and chitosan films (Supplementary material), where no phase separation in the system was observed.

Table 3. Tensile strength (TS), Elongation at break (%E), and Elastic modulus (EM) of gelatin-chitosan edible films with plasticizers

Film	Tensile strength (MPa)	Elongation (%)	Elastic Modulus (MPa)
Control	19.2 ^e	1.2 ^a	2192 ^d
G	6.4 ^a	86.8 ^f	2.9 ^a
S	10.9 ^b	67.5 ^{cd}	30.3 ^a
P	24.7 ^f	53.3 ^b	304.4 ^b
T	17.2 ^{de}	1.3 ^a	1582 ^c
G + S	5.8 ^a	74.8 ^{de}	6.2 ^a
G + P	10.3 ^b	72.8 ^{de}	9.0 ^a
G + T	12.9 ^{bc}	80.0 ^{ef}	4.1 ^a
S + P	14.8 ^{cd}	64.3 ^c	38.2 ^a
S + T	14.8 ^{cd}	62.1 ^c	44.9 ^a
P + T	25.9 ^f	61.6 ^c	21.6 ^a

Different letter superscript means significant differences ($p < 0.05$)

Film without plasticizer (Control); Film plus glycerol (G); Film plus sorbitol (S); Film plus PEG400 (P); Film plus triacetin (T). Nomenclature which includes the "+" sign involves adding two plasticizers in a gelatin and chitosan edible film.

3.4 Correlation between variables

The variability of the abundance of gelatin and chitosan presented the highest correlation with the films' TS (Tables 4 and 5). In our study, the best model yielded a correlation coefficient (R^2) of 0.69 and a p-value of 0.009. The increase to tensile strength for gelatin and chitosan films is related to the creation of a more stable matrix due to interactions between chitosan and gelatin (Benbettaieb, Kurek, Bornaz, & Debeaufort, 2014). The variability of abundance estimates the degree of distribution of the films' compounds (Puchert et al., 2010). Likewise, the degree of distribution is obtained by the ability of the compounds to form molecular interactions that govern the tridimensional structure of the edible film (Skurtys et al., 2010). Therefore, the variability of the abundance of the films' compounds is related to the TS of the edible films.

The MLR models showed that the variables including chitosan (abundance or its variability) always showed the lowest p values (Tables 4 and 5). This indicates that chitosan is a factor that has a statistically significant effect on the modulation of the films' TS. The chitosan involvement in modulating the TS can be explained since glycerol, a compound that interacts primarily with chitosan, modified the mechanical properties of the films. However, triacetin, which interacts mainly with gelatin, slightly modified its mechanical properties. These findings suggest that a polymeric network of gelatin forms the structural conformation of the edible film whereas chitosan plays an anchoring role. This role will allow chitosan to interact with the polar plasticizers, reducing the rigidity and fragility of the film, as suggested by other authors (Hosseini et al., 2013; Jridi et al., 2014; Liu et al., 2012).

The variability of gelatin abundance showed a positive correlation with TS, whereas the chitosan variability of abundance was negative (Table 4). The increase of the distribution of chitosan suggests that chitosan increases the cross-linking of gelatin and decreases the amount of hydrogen bridges in the films (Hosseini et al., 2013). This produces a

519 weakening of interactions between polymers and a decrease in the distribution of gelatin.
520 Therefore, the reduction of gelatin distribution would be responsible for the increase of
521 the film's TS. This reinforces the evidence that chitosan plays an anchoring role and that
522 the structure of this film is made up of a polymeric network of gelatin.
523

524 **Table 4.** Tensile strength correlating with standard deviation of component abundance.

	TS v/s				TS v/s					
	[STD _(Ge) ,		STD _(Ch) ,		STD _(Plas)] ⁽¹⁾			[STD _(Ge) ,		STD _(Ch)] ⁽²⁾
Graph
R ²	0.692				0.691					
p-value model	0.033				0.009					
p-value variable	0.010	0.007	0.884		0.005	0.003				
Model coefficient ⁽¹⁾	D = 21.2	a = 11.6	b = -13.1	c = 0.2	D = 21.0	a = 11.6	b = -12.9			

525 ⁽¹⁾ $y = D + a \text{ STD}_{(\text{Ge})} + b \text{ STD}_{(\text{Ch})} + c \text{ STD}_{(\text{Plas})}$; ⁽²⁾ $y = D + a \text{ STD}_{(\text{Ge})} + b \text{ STD}_{(\text{Ch})}$.
526 $\text{STD}_{(\text{Ge})}$ correspond to gelatin abundance standard deviation; $\text{STD}_{(\text{ch})}$ correspond to chitosan abundance standard deviation; and
527 $\text{STD}_{(\text{plas})}$ correspond to plasticizer abundance standard deviation.
528 Ge: gelatin; Ch: chitosan; and Plas: plasticizer.

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534 **Table 5.** Tensile strength correlated with the abundance of the edible film's compounds.

	TS v/s				TS v/s			TS v/s								
	[A _(Ge) ,		A _(Ch) ,		A _{(Plas)]⁽¹⁾}			[A _(Ge) ,		A _{(Ch)]⁽²⁾}			[A _(Ch) ,		A _{(Plas)]⁽³⁾}	
Graph
R ²	0.718				0.568			0.669								
p-value model	0.02				0.03			0.01								
p-value variable	0.3		0.005		0.09		0.09		0.01		0.004		0.03			
Model coefficient ⁽¹⁾	D = 10.8		a = 0.3 b = 1.2		c = - 0.7		D = -40.1		a = 0.6 b = 1.1		D = -17.7		a = 1.0 b = - 0.9			

535 ⁽¹⁾ y = D + a A_(Ge) + b A_(Ch) + c A_(Plas); ⁽²⁾ y = D + a A_(Ge) + b A_(Ch); ⁽³⁾ y = D + a A_(Ch) + b A_(Plas).

536 A_(Ge) correspond to gelatin abundance; A_(ch) correspond to chitosan abundance; and A_(plas) correspond to plasticizer abundance.

537 Ge: gelatin; Ch: chitosan; and Plas: plasticizer.

538

Finally, we were not able to find correlations between %E or EM that would describe the elastic behavior of the film (Hosseini et al., 2013), and the values of abundance or its variability. The addition of plasticizers implies a relaxation of the polymeric network that modifies the films' %E and EM (Sothornvit & Krochta, 2005). In this research the control film didn't have plasticizers; the film with triacetin showed similar mechanical behavior to the control film however. In fact, the %E and EM of the control and triacetin films are similar, yet they are different, in magnitude, from the other films. For example, the EM of the control film and the film with triacetin were three orders of magnitude larger than the EM of the film with glycerol. This shows that the parameters obtained with NIR-CI are related to the resistance of the edible films, but not to rigidity and elasticity.

4. CONCLUSIONS

This study is the first to report the use of NIR-CI to characterize gelatin and chitosan edible films, with the goal of understanding their mechanical properties and improving their design. NIR-CI reveals that the abundance of gelatin and chitosan varies in relation to the added plasticizer. Based on the observed mechanical properties and NIR-CI analysis, it was possible to show that chitosan interacts mainly with glycerol and gelatin mainly with triacetin. Even though the edible films showed uniform distribution of their compounds, the distribution of gelatin and chitosan change in response to the plasticizer that was added; PEG400 is the plasticizer that modifies the distribution of gelatin and chitosan the most, when compared to the control, whereas triacetin is the one that has the least impact on the distribution. Chitosan interacts mainly with the polar compounds (glycerol and sorbitol) that affect the mechanical properties of the films and relax their tridimensional structure. This indicates that the structural conformation of the edible film consists of a polymeric network of gelatin, while chitosan fulfills an anchoring role, interacting with plasticizers to diminish the rigidity and fragility of the film. The variability of the abundance of gelatin and chitosan showed a significant statistical correlation with the tensile strength of the films, showing that it is possible to determine the mechanical resistance of films with a rapid and non-invasive analysis.

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