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pH-indicating properties and storage stability of a smart edible film based on nopal-mucilage/gellan gum and red cabbage anthocyanins

Propiedades indicadoras de pH y estabilidad de almacenamiento de una película comestible inteligente basada en nopal-mucílago/goma gellan y antocianinas de col roja

L.G. Solano-Doblado[1], F.J. Heredia[2], B. Gordillo[2], G. Dávila-Ortiz[1], L. Alamilla-Beltrán[1], A. Maciel-Cerda[3], C. Jiménez-Martínez[1]*

[1]*Departamento de Ingeniería Bioquímica, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Mexico City, Mexico.*
[2]*Food Colour and Quality Laboratory, Universidad de Sevilla, 41012, Sevilla, Spain.*
[3]*Instituto de Investigaciones en Materiales (IIM), Universidad Nacional Nacional Autónoma de México, Mexico City, Mexico.*

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Abstract

Nopal mucilage is a complex polysaccharide with great potential in the preparation of films for food packaging. In this work, a smart edible film was developed with pH-indicating capabilities based on nopal mucilage/gellan gum, and red cabbage anthocyanins (70:20 w/w, and 10%, respectively). Physical properties, appearance, and colorimetric functionality were characterized at a pH range 2.5-5.9. Color stability, measured by CIELAB, was tested with different storage conditions and time, and interpreted by differential tristimulus colorimetry. Results showed films with uniform appearance, flexibility, easy-handling, and excellent color response within the pH range studied. The evolution of color difference (ΔE*ab) confirmed that the colorimetric functionality of films was stable at short storage periods (8 days) under natural conditions of sunlight and oxygen exposure, whereas a more extended storage period affected the films. Results showed that the film is an excellent barrier and presents color changes triggered by pH changes.

Keywords: edible films, nopal mucilage, acidic-basic indicators, red cabbage anthocyanins´ differential colorimetry.

Resumen

El mucílago de nopal es un polisacárido complejo con gran potencial en la preparación de las películas para el envasado de alimentos. En este trabajo, se desarrolló una película comestible inteligente con capacidades indicadoras de pH basadas en mucílago de nopal/ goma gellan y antocianinas de col roja (70:20 p/p, y 10%, respectivamente). Las propiedades físicas, la apariencia y la funcionalidad colorimétrica se caracterizaron a un intervalo de pH de 2.5 a 5.9. La estabilidad del color, medida por CIELAB, se probó con diferentes condiciones de almacenamiento y tiempo e interpretada por colorimetría diferencial de triestímulo. Los resultados mostraron películas con apariencia uniforme, flexibilidad, fácil manejo y excelente respuesta de color. La evolución de la diferencia de color (ΔE * ab) confirmó que la funcionalidad colorimétrica de las películas era estable en cortos períodos de almacenamiento (8 días) en condiciones naturales de exposición a la luz solar y al oxígeno, mientras que se vio afectada cuando el almacenamiento era por mayor tiempo. Los resultados mostraron que la película es una excelente barrera y presenta cambios de color provocados por los cambios de pH.

Palabras clave: películas, mucilago de nopal, indicador acido base, colorimetría diferencial de antocianinas de col.

1 Introduction

Fresh and minimally processed foods are active physicochemical systems susceptible to natural deterioration. During food storage, several factors such as the ripening stage, natural microbiota, environmental conditions, or rough handling can contribute to a faster deterioration, especially in fresh fruits and vegetables (Dhall, 2013; Fitch-Vargas *et al.*, 2019). Those factors induce changes that favor the growth of pathogenic microorganisms, diminishing shelf-life and compromising the consumer?s safety.

* *Corresponding author. E-mail*: crisjm_99@yahoo.com
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For these reasons, the development of innovative packaging technologies for extending shelf-life, monitoring freshness, or improving safety is of significant interest to the food industry (Musso *et al*., 2016; Treviño-Garza *et al*., 2020). The edible film is a preformed thin layer made of edible material, which, once formed, can be placed on or between food components (Falguera *et al*., 2011). They are made from proteins, polysaccharides, lipids, or their mixtures, whose biodegradable nature makes them safe materials for a sustainable food packaging industry in comparison to conventional plastics. In the last decades, renewable agricultural resources have attracted attention in search of new bio-based packaging materials to improve the efficiency and functionality of edible films (Calva-Estrada *et al*., 2019). The mucilage of nopal (*Opuntia* spp.) is a hetero-polysaccharide with interesting rheological properties due to its polymeric conformation and high capacity to absorb large amounts of water (Contreras-Padilla *et al*. 2016; López-García *et al*. 2017). Nopal is one of the most widespread and economically essential cactus crops, especially in Mexico, with more than 3 million Ha of wild species (Espino?Díaz *et al*. 2010). Due to its high concentration (5-19%), low cost, and high availability, nopal mucilage is a potential natural source of industrial packaging material. However, the application of nopal mucilage to produce edible films is still scarce (Allegra *et al*., 2017; Del-Valle *et al*., 2005; Espino Díaz *et al*., 2010). Besides the main hydrocolloid, other components should be incorporated into the polymeric matrix to expand the film's applications. Among them, gellan gum (linear anionic polysaccharide) has demonstrated to improve the water solubility and humidity stability of edible films, when used as a gelling agent (Kim *et al*., 2015).

Likewise, bioactive compounds having antimicrobial or sensorial properties can act as indicators of physicochemical and microbiological changes in foods (Treviño-Garza *et al*., 2020). Natural pigments capable of indicating pH changes through visual colorimetric variations represent a safe alternative to synthetic acidic-basic indicators in food packaging (Poyatos-Racionero *et al*., 2018; Balbinot-Alfaro *et al*., 2019). One class of natural pigments sensitive to pH changes is anthocyanins, a large group of flavonoids widely spread in nature. As water-soluble and innocuous compounds, anthocyanins may be used safely in food packaging as indicators of food spoilage produced by pH changes (Pereira *et al*., 2015; Pourjavaher *et al*., 2017; Silva-Pereira *et al*., 2015). This study aimed to assess the physicochemical, mechanical, and pH-indicating properties of edible films based on a combination of nopal-mucilage (*Opuntia robusta*) with gellan gum, incorporating red cabbage anthocyanins as a natural acid-base indicator. In addition, we compared the colorimetric functionality and stability at different pHs, light, and oxygen exposure over time.

2 Materials and methods

2.1 Chemical and solvents

Nopal mucilage was extracted from *Opuntia robusta* cladodes (two-year-old), and gellan gum was purchased from Plant Media (bioPLUS, Jiutepec, Morelos, Mexico). All chemical reagents used for the preparation of the films were analytical grades: glycerol was from Acofarma (Terrassa, Barcelona, Spain) and absolute ethanol, 37% hydrochloric acid, and sodium hydroxide were from Merck (Darmstadt, Germany). Purified water was obtained through a Milli-Q plus water purification system (Millipore Corp., Bedford, MA, USA). All chromatographic solvents (acetonitrile and formic acid) were of HPLC grade (Merck, Darmstadt, Germany).

2.2 Extraction of anthocyanins from red cabbage

Fresh red cabbage (*Brassica oleracea*), purchased from a local supermarket, was carefully washed, dried, manually peeled, and cut into small pieces. Samples of 20 g were extracted, each with 100 mL of distilled water at 40 °C for 4 h (maceration). After extraction, the supernatant was centrifuged (4190 g, 10 min) to remove the fine suspended particles and filtered through 0.45-μm Millipore-AP20 filters (Bedford Corp.). The anthocyanin aqueous extract obtained was analyzed to determine anthocyanin content, according to the method of Abdel-Aal, Young, and Rabalski (2006), and used in the preparation of the film as a visual pH indicator, adding 1.8 mg of anthocyanin/film

2.3 Preparation of films based on nopal-mucilage/gellan gum dispersions with red cabbage anthocyanins

Two aqueous dispersions based on nopal-mucilage and gellan gum, respectively, were prepared separately

using a thermoregulated magnetic stirrer, Nahita-Blue® 692 (Auxilab, Navarra, Spain). Nopal-mucilage dispersion consisted of a mixture of mucilage from *O. robusta*, glycerol, and distilled water (2:1:100 w/v/v), the emulsion was formed using an Ultra-Turrax homogenizer (T25 digital, IKA®, Germany) operating at 20,412 g for 1 min intervals at 25 ºC. Gellan gum dispersion was prepared by dissolving 1.6 g of gellan gum with 1.6 mL of glycerol and 100 mL distilled water at 70 ºC under constant magnetic stirring.

Edible films were prepared by a hot casting method as follows: Nopal-mucilage and gellan gum dispersions were mixed (70:30, v/v) using a magnetic stirrer for 10 min at 45 ºC (until complete homogenization of all components); afterward, the anthocyanin aqueous extract used as an acid-base indicator (10% of the total volume) was added. The pH of samples (liquid mixtures) was adjusted accordingly with 1 M HCl or 0.1 M NaOH solutions, to obtain films with different pH values: 2.5, 3, 3.5, 4, 4.5, 5, and 5.5. The natural pH of the edible films was 5.9 ± 0.1. Finally, 5 mL-aliquots of film solutions at each pH value were poured into Teflon dishes (5.3 × 2 cm diameter) and dried in an oven with air-flow circulation (Raypa Terrassa, Barcelona, Spain) at 60 ºC for 4 h. Films were preconditioned for 30 min in desiccators with saturated solutions of NaBr before being peeled from the casting surface. For each pH value, 12 replicates of films were produced.

Films were stored under different conditions of natural sunlight and oxygen exposure for 22 days at room temperature (20 ± 1 ºC). For each pH value (n = 12 films), four storage treatments were carried out in triplicate as follows: 1) Three films stored separately in open transparent plastic bags, exposed to light and oxygen. 2) Three films stored separately in closed transparent plastic bags, exposed to light and protected from oxygen. 3) Three films stored separately in open transparent plastic bags, in darkness and exposed to oxygen. 4) Three films stored separately in closed transparent plastic bags, in darkness protected from oxygen. Films were analyzed at the initial time (1 h after preconditioning) and at 8, 15, and 22 days during storage. Every measurement was made in triplicate.

2.4 Determination of physical characteristics of edible films

The pH of the films was measured in the formulation of the solution, prior to obtaining the film, using the pH-meter, Meter GLIPR2 Multimeter (CRISON, Barcelona, Spain), adjusting it to each point of the pH scale with and 0.01 M NaOH and 0.01 M HCl. To determine the moisture content, 1 g of the edible film was weighed in a thermobalance (OHAUS mb45, Switzerland) before and after drying to 100 ºC under an argon flow at a heating rate of 10 ºC/min. Moisture content values were calculated as the percentage of weight loss relative to the original weight.

Film thickness was measured by a 200-mm electronic digital caliper (Digital Caliper, Stainless hardened, SINAT, Zhejiang, China) with an accuracy of ± 0.001 mm according to the ASTM (2001). Measurements were done at five different positions of each sample and the average thickness was calculated. Film density was evaluated by dividing the weight of film by volume, while the film volume was determined by multiplying the film area by the thickness.

Water activity was measured at 25 ºC using an AQUALAB Dew Point Water Activity METER 4TE analyzer (Pullman, USA) with an accuracy of ± 0.003 aw. Opacity was determined using a UV-Vis spectrophotometer (Thermo Scientific Multiscan Go, Vantaa, Finland). The films were cut into rectangles approximately the size of the spectrophotometric cell. An absorbance scan was performed from 400 to 700 nm for each sample. Opacity was defined as the area under the absorbance curve between the nanometer range, being expressed as A × nm (Pérez-Gallardo *et al*., 2012).

For solubility measurements, the films were cut into 2 × 3 cm^2 rectangles and stored in a desiccator (about 0% RH) for 7 days. The samples were weighed and placed in beakers with 80 mL of distilled water, maintained under constant agitation for 1, 3, 6, 12, 24 h at room temperature. After the stirring time, the pieces of films were dried in an oven at 60 ºC for 2 h. The percentage of the soluble matter was calculated as follows:

Water solubility (%) = (wt. of initial dry matter - wt. of dry matter not solubilized) / (wt. of initial dry matter) × 100

Finally, to determine the structural properties of films, the films were cut into 3 × 3 cm^2 and placed on a slide covered by a coverslip without any contrast dye, using an optical microscope Eclipse Ci (Nikon HSS05, Tokyo, Japan). Micrographs were captured under the 20× and 40× objectives. A morphological analysis of surface and homogeneity was carried out using a JSPM4210 atomic force microscope under normal pressure conditions with a silicon tip in tapping mode at a frequency in the range of 120-190 kHz and using ultra-sharp cantilevers of silicon, obtaining

topographic, phase and deflection images.

2.5 *Physicochemical characterization of films*

The color of the films was measured with a CAS140B spectroradiometer (Instrument Systems, Munich, Germany) fitted with a top 100 telescope optical probe, a Tamron zoom mod. SP 23A (Tamron USA, Inc., Commack, NY, USA), and an external light source (a white light 150 W-metal-halide Phillips MHN-TD Pro lamp, 12900 lumens) as a source of illumination. An optical glass cuvette (45 × 10 × 10 mm^3) was used for the measurements and the blank was obtained with the cuvette filled with $BaSO_4$, as a reference. Reflectance spectra were taken by placing the films directly on the surface of the glass cuvette. The entire visible spectrum (380-770 nm) was recorded with a bandwidth of 1 nm, and the Illuminant D_{65} and a 10º observer were taken as references (Schanda, 2007). The CIELAB parameters (L*, a*, b*, C*ab, and H*ab) were obtained directly from the apparatus through the Specwin v.1.8.1.6 software provided by the manufacturer. The color data reported were the average of three sequential measurements (5 s exposure each).

Color differences between pairs of samples were computed employing the CIE76 color difference formulae: $\Delta E^*ab = [(\Delta L*)^2 + (\Delta a*)^2 + (\Delta b*)^2]1/2)$, as well as from the lightness, chroma, and hue angle differences (ΔL*, ΔC*ab, and ΔH*ab, respectively).

The relative contribution of the lightness, chroma, and hue that make up the color difference parameter (ΔE*ab) was calculated as follows:

- Relative contribution (%) of lightness: $(\%\Delta L = [(\Delta L*)^2 / (\Delta E * ab)^2] \times 100)$

- Relative contribution (%) of chroma: $(\%\Delta C = [(\Delta C * ab)^2 /(\Delta E * ab)^2] \times 100)$

- Relative contribution (%) of hue: $(\% \Delta H = [(\Delta H)^2/ (\Delta E * ab)^2] \times 100)$

being ΔH calculated from: $(\Delta H= [(\Delta E * ab)^2 - ((\Delta L)^2 + (\Delta C)^2)]1/2)$

2.6 *Statistical analysis*

Statistical analysis was performed using the Statistica software v. 8.0 (StatSoft, 2007). Univariate analyses of variance (Tukey test, ANOVA) were applied to discriminate among the means of physical and colorimetric data. Significant differences among samples were established at a p-value ≤ 0.05.

3 Results and discussion

3.1 *Physical appearance and color characterization of films at natural pH*

Natural pH (5.9 ± 0.1) of edible films based on blends of nopal-mucilage with both gellan gum and red cabbage anthocyanins (70:20:10, w/w/w) was considered as the setpoint to establish the color response over the pH range studied (2.5-5.5). We chose to cover possible variations in the pH in foods due to spoilage by microorganism growth or metabolism disorders. Twelve replicates of films were developed for each pH value.

The physical characteristics of the films at natural pH are shown in Table 1. The results for the average dry thickness (0.0925 ± 0.2 mm), density (1.243 ± 0.006 g/cm^3), % moisture (14.0 ± 2.7%) and water activity (0.35 ± 0.01) were similar to those reported in the literature for edible films based on nopal mucilage, gelatin, or proteins (Cian *et al*., 2014; Espino?Díaz *et al*., 2010; Musso *et al*., 2016). Small differences

Table 1. Physicochemical and colorimetric characteristics of nopal mucilage/gellan gum films incorporated with red cabbage anthocyanins at natural pH (5.9).

Physicochemical parameters		***Colorimetric parameters***	
pH	5.9 ± 0.1	L*	93.99±0.43
Weight (g)	0.21 ± 0.1	a*	0.35±0.09
Density (g/cm^3)	1.243 ±0.006	b*	14.94±0.84
Thickness (mm)	0.0925 ± 0.2	C*ab	14.67±0.58
Moisture (% w/w)	14.04 ± 2.7	hab	95.01±0.91
aw	0.35 ± 0.01		
Opacity (AU x nm)	19.4±2		

All values are the mean of 12 replicates of films.

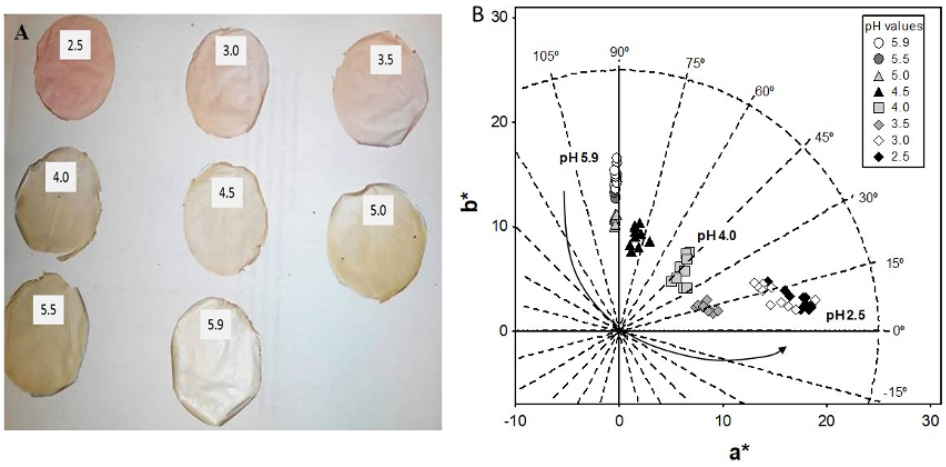

Figure 1. A) Appearance and visual color of nopal mucilage/gellan gum films enriched with red cabbage anthocyanins at different pH values (2.5-5.9), B) Evolution of the color of films within the CIELAB (a*b*) diagram according to pH changes.

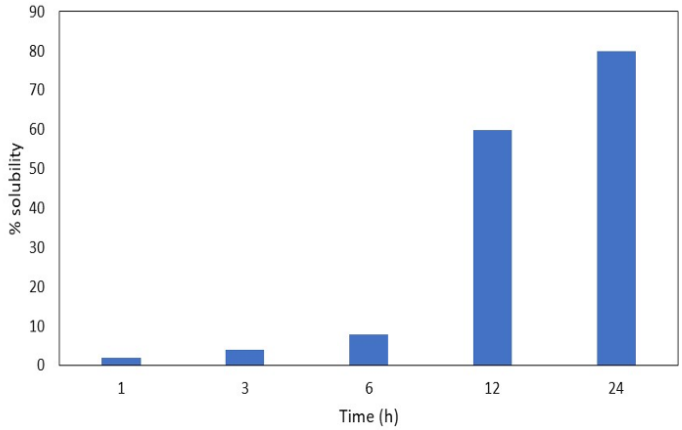

Figure 2. Changes in the solubility of the film (pH 5.9) over time (0-24h).

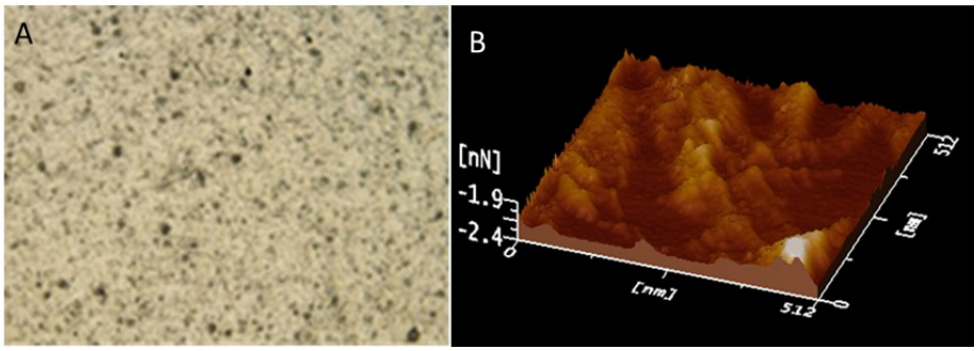

Figure 3. Micrographs of the film microstructure at natural pH (5.9): A) Optical microscopy (600x total magnification), B) Atomic force microscopy.

in these parameters could be due to the intrinsic viscosity/density of the main polymer of the films as well as to the presence of other components like plasticizers (Yoshida *et al*., 2014). However, no significant modification in the physical characteristics was observed among films associated with the pH.

Visual analysis (Figure 1a) revealed films were uniform in appearance independently from the pH, except for the color. The selected proportions of different gelling and colorant agents resulted in a flexible and easily handled edible film without phase separation among multi-components. Regarding colorimetric properties at natural pH (Table 1), films exhibited high lightness values (L* = 93.99 ± 0.43) and a slight yellow coloration (C*ab = 14.67 ± 0.58 and H*ab = 95.01° ± 0.91). These values were like those described by Espino-Díaz *et al*. (2010), who developed edible films based on nopal mucilage under different pHs without adding acid-base indicators. Notwithstanding, a typical yellow, purple, or pale blue coloration has been reported for tin gelatin film formulations at similar pH values (6.0), depending on the nature of the acid-base indicator (Figure 1b). Film solubility was lower than 10% during the first 6 h and increased significantly over time until 24 h (Figure 2). This is probably due to the association of the plasticizer with water, which disrupts the intermolecular interactions among polymer molecules (Silva-Pereira *et al*., 2015).

Regarding the film's microstructure, micrographs (Figure 3A) confirmed that it was uniform without visible remnants of fiber or pigments. Besides, the topographic image of films evaluated by atomic force microscopy showed smoothed peaks and a flattened appearance (Figure 3B). The results for the average peak height (Ra) and the roughness (Rz) (height = 30.6 nm, Ra = 3.17 nm; where Rz represents the average height of the 10 highest peaks and deepest valleys and the results show Rz = 21.4), indicating a polished surface (Hinojosa Rivera and Reyes Melo, 2001).

3.2 Effect of pH changes on color films at the initial time

The color response of the developed edible films to pH variations was first assessed by differential tristimulus colorimetry through the changes in the CIELAB parameters (L*, a*, b*, C*ab, H*ab) over the pH range studied (2.5-5.9). Table 2 shows the mean values for the parameters at each pH value and the significant differences found among them respect to the color of the film at the natural pH (5.9). Results showed that all colorimetric parameters significantly changed as a function of pH. This color variation can be observed graphically in the (a*b*) colorimetric diagram, where the color coordinates of films were represented (Figure 1b). As the pH decreases from 5.9 to 2.5, an important displacement of samples can be noticed in the (a*b*) diagram due to an increase of the a* values simultaneously to a decrease in b*. This displacement was consistent with the evolution of the parameter H*ab from 90º toward 0º, which indicates a clear trend in the qualitative attribute of color (Figure 1b). Thus, according to the location in the (a*b*) diagram and hue evolution, films exhibited a yellow color (around 90º) from pH 5.9 to 4.5, red-orange color at pH 4.0, while becoming red (around 0º-15°) at more acidic pH values (lower than 3.5).

As mentioned previously, the natural pH of the film is 5.9; this presents high lightness (L* 93.99), chroma, C*ab 14.67, and hue (H*ab 95.01) indicating that it is in the yellow region. Analysis of the color of the films when the pH was adjusted revealed that the lightness (L*) is higher on the pH close to 6, and it darkens as the pH diminishes (Table 2). The chroma (C*ab) is intense at low pH and close to the natural one; at pH 4 and 3.5 this parameter diminishes (8.14 and 9.02, respectively). As known, color variations of the film are due to the anthocyanin extracts, and these depend on the pH. At acidic pH, anthocyanins exist predominantly in the form of the flavylium cation, presenting a red color and a purple color; at neutral pH, colorless carbinol pseudo-base and chalcone structures are formed, followed by the formation of anionic quinoidal species. This is due to the kinetic and thermodynamic competition in the hydration reaction of flavylium ion (Khoo *et al*., 2017).

The color can be reversed changing anew the pH to acidic; this reacidification can be achieved before they are observed in most colorless chalcones (Khoo *et al*., 2017; Yoshida *et al*., 2014). Films based on chitosan and grape extract (1 g/100 g) have been made, as well as of red cabbage (25% of the total volume of the film) (Pereira *et al*., 2015), obtaining similar results regarding color changes in the presence of acidic or alkaline pH.

A similar trend was observed for the parameter, lightness (L*), which progressively decreased in the samples with pH diminution. However, the changes in lightness were only significant at pH values lower than 3.5, indicating that the red tonality of films appears consequently darker.

Table 2. Changes in colorimetric parameters (L*, C_{ab}*, h_{ab}) of films at each pH value (5.9-2.5) from the beginning (1 day) to the end (22 days) of storage under different light and oxygen conditions.

		Light + oxygen	**Light + absence of oxygen**	**Darkness + oxygen**	**Darkness + absence of oxygen**
Day	1	22	22	22	22
Lightness (L*)					
5.9	94.0±0.4 a	94.9±0.1 ^{b}	90.1±4.6 a	90.5±2.7 a	91.4±3.4 a
5.5	95.0±0.9 a	93.4±0.1 ^{b}	93.9±0.5 ^{b}	92.3±1.5 a	91.8±0.1 ^{b}
5	94.1±1.2 a	94.1±1.6 a	92.4±2.2 a	92.3±2.3 a	91.9±0.7 ^{b}
4.5	94.0±0.9 a	91.3±0.3 ^{b}	92.6±1.7 ^{b}	93.7±0.8 a	93.8±2.2 a
4	93.3±1.2 a	92.1±0.2 ^{b}	94.6±0.9 a	92.0±1.3 a	93.2±0.1 ^{b}
3.5	93.0±0.8 a	95.0±1.3 ^{b}	95.0±0.3 ^{b}	94.0±0.4 ^{b}	94.0±0.7 a
3	89.0±3.5 a	90.1±0.2 ^{b}	92.2±4.6 a	88.6±4.0 a	87.3±0.4 ^{b}
2.5	88.0±0.8 a	91.4±0.4 ^{b}	92.8±1.3 ^{b}	88.7±0.1 ^{b}	88.3±0.5 a
Chroma (C*a^b)					
5.9	14.7±0.6 a	11.5±0.6 ^{b}	12.1±0.8 ^{b}	17.0±0.6 ^{b}	16.8±0.6 ^{b}
5.5	13.7±0.9 a	12.3±0. 4 ^{b}	12.7±0. 5 ^{b}	14.0±0. 5 a	16.9±0. 3 ^{b}
5	10.6±0.5 a	12.4±0.2 ^{b}	11.8±0.5 ^{b}	15.8±0.9 ^{b}	15.7±0.3 ^{b}
4.5	9.4±0.8 a	15.1±0.4 ^{b}	14.4±1.8 ^{b}	14.8±2.0 ^{b}	14.3±0.6 ^{b}
4	8.4±0.9 a	9.3±0.1 ^{b}	9.2±1.5 a	11.1±1.0 ^{b}	12.6±0.3 ^{b}
3.5	9.0±0.4 a	6.7±0.6 ^{b}	6.7±0.1 ^{b}	8.6±0.6 a	8.6±0.5 a
3	15.1±1.8 a	10.3±1.3 ^{b}	8.2±1.4 ^{b}	11.9±0.4 ^{b}	11.9±1.4 ^{b}
2.5	18.0±0.8 a	9.5±0.7 ^{b}	7.6±2.0 ^{b}	13.3±0.9 ^{b}	12.5±0.9 ^{b}
Hue angle (ha^b)					
5.9	95.0±0.9 a	88.5±0.1 ^{b}	88.5±0.9 ^{b}	89.4±0.3 ^{b}	90.3±0.1 ^{b}
5.5	91.3±0.5 a	89.1±0.5 ^{b}	88.7±0.8 ^{b}	88.4±0.3 ^{b}	91.3±0.2 ^{b}
5	92.0±0.5 a	89.1±1.6 ^{b}	89.6±0.5 ^{b}	91.3±0.3 ^{b}	91.9±0.1 ^{b}
4.5	79.5±3.1 a	90.0±0.4 ^{b}	92.1±1.2 ^{b}	90.0±1.2 ^{b}	90.1±0.2 ^{b}
4	42.7±5.5 a	84.3±0.4 ^{b}	87.2±2.2 ^{b}	84.7±2.5 ^{b}	89.4±0.3 ^{b}
3.5	15.9±3.0 a	86.6±0.6 ^{b}	87.9±1.0 ^{b}	82.5±1.2 ^{b}	84.67±0.2 ^{b}
3	14.1±4.8 a	32.3±6.9 ^{b}	43.4±9.0 ^{b}	25.2±1.6 ^{b}	40.3±1.8 ^{b}
2.5	8.8±2.3 a	28.8±6.7 ^{b}	25.15±1.2 ^{b}	23.4±0.9 ^{b}	21.9±0.3 ^{b}

Different letters in the same row mean significant differences (Tukey test, $p < 0.05$) between day 1 and day 22 for each storage condition, at different pH values (5.9-2.5).

On the other hand, the chroma response of films was pH-dependent (Table 2), when the pH changed from 5.9 to 4.0 the chroma values significantly decreased from 14.7 to 8.1 CIELAB units. In contrast, a net rise of the chroma occurred from pH 3.5 to 2.5 (from 8.5 to 18 CIELAB units). The trend of the changes on the chroma was like those of the total color (Figure 4), representing a colorimetric parameter based on ΔE*ab that provides useful quantitative information of color (Gordillo *et al*., 2012; Gordillo *et al*., 2015). Thus, these findings mean that the yellow and red-orange tonalities of the films were less intense in the range of pH 5.9-4.0; but, at more acidic pH values, they showed a much higher red color intensity.

Notwithstanding, independently of the intensity of the different tonalities, the color changes previously described can be easily detected by visual analysis. This means that under the assayed anthocyanin concentration, edible films based on nopal mucilage and gellan gum dispersions exhibited a good color response over the pH range studied and could act effectively as a colorimetric pH monitoring system. Similar results were found by (Pourjavaher *et al*., 2017), who found that pH indicator labels, containing

diluted anthocyanins from red cabbage, showed a clearer response to pH variation, resulting even in lighter colors.

3.3 Color response to pH changes by differential colorimetry

To establish whether the observed changes in the CIELAB parameters were visually relevant, differential colorimetry was applied to color data. For this purpose, the color difference (Δ*E**ab) was calculated among pairs of samples as a function of the pH (Figure 4). The relative contribution of the lightness (%ΔL), chroma (%ΔC), and hue differences (%ΔH) were also calculated by the mean of the quadratic variations of each color attribute for a given Δ*E**ab (Gordillo *et al*., 2015). This differential approach is very useful for food industrial applications in which the main objective is to detect color variations visually. In this study, the quantitative evaluation of the Δ*E**ab allowed knowing the extent of the color variation of films due to pH modifications and whether these changes could be visually detected, by monitoring the %ΔL, %ΔC, and %ΔH. It was also possible to establish which color attribute (lightness, chroma, or hue angle) had a greater impact on the perceptible color differences.

The color of films at natural pH (5.9) was considered as the set point to assess the color differences over the pH range studied (2.5-5.5). Results showed that Δ*E**ab values among films ranged from 1.3 to 23 CIELAB units (Figure 4a). The lowest color variation was found between the natural pH (5.9) and pH 5.5 and the largest with pH 2.5. According to (Martínez *et al*., 2001), ΔE*ab around 3 units or higher indicates color differences appreciable to the human eye (as an average observer). Thus, it was confirmed that the color variations exhibited by films at decreasing pH values can be visually differentiated in the pH range from 5.0 to 2.5 (Δ*E**ab values > 4.5). These values were found to be higher than those described by Musso *et al*. (2016), who tested the effectiveness of different synthetic dyes (methyl orange, neutral red, and bromocresol green) as colorimetric pH indicators in gelatin-based films. Also, Δ*E**ab values obtained in this work, were higher than those reported by Silva-Pereira *et al*. (2015), who developed a system for pH monitoring based on chitosan, corn starch, and red cabbage extract. Thus, it was confirmed that, under the anthocyanin concentration assayed, the developed films show more perceptible color variability than other colorimetric

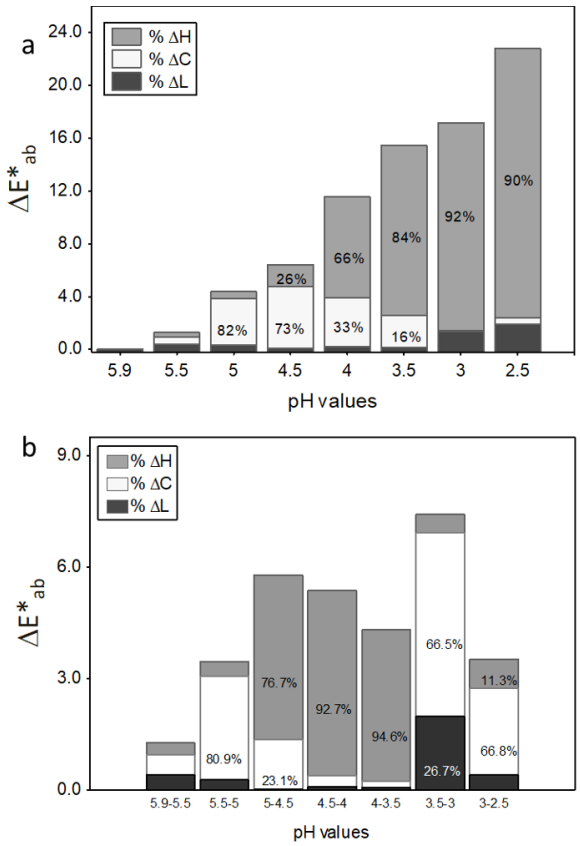

Figure 4. Color differences (?E*ab) with the quadratic variations of lightness (%Δ L), chroma (%ΔC), and hue (%ΔH) calculated by pairs of samples as a function of pH: (a) ΔE*ab among films at each pH (5.5- 2.5) respect to natural pH (5.9), (b) ΔE*ab among films at consecutive pH values (5.9-5.0, 5.0-4.5, 4.5-4.0, 4.0-3.5, 3.5-3.0, 3.0-2.5).

pH indicators (Silva-Pereira *et al*., 2015). When the role of each color attribute with respect to ΔE*ab was calculated (%ΔL, %ΔC, %ΔH), results evidenced that the perceptible color differences between the films at natural pH and those at pH 5.0 and 4.5 were mainly quantitative, with higher values of quadratic variations of chroma (%ΔC = 82 and 73 %, respectively). In contrast, in the pH range from 4.0 to 2.5, the color differences respect to natural pH were mainly qualitative, with increasing values of quadratic variations of hue at decreasing pH values (% Δ*H* = from 66 to 90%).

Likewise, the same color differences (ΔE*ab) were compared among films at consecutive pH values (pairs 5.9-5.5, 5.5-5.0, 5.0-4.5, 4.5-4.0, 4.0-3.5, 3.5-3.0, 3.0-2.5). As can be observed in Figure 4b, except for the pH 5.9-5.0 pair, all Δ*E**ab values were higher than 3

CIELAB units. Thus, it could be asserted that the color variability of the obtained films at consecutive pH values can be visually discriminated. These findings confirm the effectiveness of the developed films to sense pH modifications in the order of 0.5 units from more neutral to more acidic pH values in the 5.0-2.5 range, by means of perceptible color variations. The color changes were more noticeable among the pH pairs of 3.5-3.0, 5.0-4.5, and 4.5-4.0 (ΔE*ab = 7.4, 5.7, and 5.4, respectively). However, for the pH variations between 5.0-4.5 and 4.5-4, the perceptible color modification was due mainly to qualitative changes, evidenced by the higher values of quadratic variations of hue (%?H = 77 and 93%, respectively), whereas at acidic pH (3.5-3.0), color variation was due to quantitative changes (higher quadratic variations of chroma, % ΔC = 66.5).

3.4 Impact of storage conditions on the color stability of films

The effect of storage conditions on the shelf-life of the produced films was assessed from a colorimetric point of view during 22 days at 20 °C (Table 2). Different combinations of natural sunlight and oxygen exposures were considered in this study to know the conditions that maximize or minimize the colorimetric functionality of the films as visual indicators of food deterioration during storage and product consumption.

For this purpose, the assessment of the color differences over time (ΔE*ab, comparing day 1 and 8, 15, and 22) was used as a colorimetric index of the color stability of the films at each pH as a function of the storage conditions (Table 2). As observed, ΔE*ab had an ascendant tendency from the initial point to the end of the storage period in virtually all cases. However, the ΔE*ab evolution pattern was pH-dependent and influenced by storage conditions. In general, during the whole period of storage and in all conditions, the lower ΔE*ab corresponded to films in the range of pH 5.9-4.5 indicating higher color stability of films at higher pHs. These color modifications could be considered almost non-detectable during the first 15 days of storage (ΔE*ab values around or lower than 3 CIELAB units). Whereas between 15 and 22 days of storage, the color changes of films started to be appreciable, mainly at pH 4.5 in all conditions.

In contrast, the color evolution of films was more noticeable at pH from 4.0 to 2.5. After 8 days of storage, films maintained better their original color in the pH range of 2.5-3.0 since no detectable color modification was generated at more intense colorations. After 15 days of storage, the progressive color changes resulted in appreciable color differences in all cases. Regarding the influence of storage conditions at more acidic values of pH (2.5-3.0), films maintained better their original color when stored in darkness than under sunlight exposure (significantly lower ΔE*ab mean values: 6.2 versus 9.3, respectively). On the other hand, the exposure to oxygen led to a higher color modification (higher ΔE*ab values) in films stored under sunlight, but no significant influence was observed when stored in darkness.

Considering the largest perceptible color modifications, the changes in colorimetric parameters (L*, C*ab, H*ab) were investigated from the beginning to the end of the storage period to understand better the significance of the color variations in films. Results show significant ($p \leq 0.05$) quantitative (L* and C*ab) and qualitative (H*ab) color changes during storage, being H*ab the most altered parameter. However, the evolution pattern of color attributes differed as a function of the pH, like the ΔE*ab values, which were also influenced by the storage conditions.

From pH 5.9 to 4.0, the L* values slightly decreased over time whereas C* values increased in most cases. In this pH range, the differences in the hue were significant ($p \leq 0.05$) in all cases, but much more marked at pHs 4.0 and 4.5 (increases of $\Delta H*$ab about 40º or 10º, respectively). Thus, the appreciable color differences observed at these pH values during storage were due mainly to an important loss of the original orange tonality of films, which became more yellowish over time and under all storage conditions.

On the other hand, at pH lower than 3.5, C* values tended to decrease while hue values tended to increase, indicating that the color of films became less intense and, simultaneously, lost its original red tonality. Changes in hue were more noticeable at pH 3.5, which increased from 16º to 80-85º, this measured in the chromatic circle, that is, the films turned yellowish.

For the pH range 2.5-3.0, films tended to exhibit an orange tonality with time. At more acidic pH, the changes in C* and H* were less intense when films were stored in darkness, whereas oxygen does not seem to have a significant influence on their colorimetric functionality.

Conclusions

The developed edible film based on blends of nopal-mucilage with gellan gum and dispersions of red cabbage anthocyanins, as a visual indicator, was able to detect pH variations as little as 0.5 over a wide pH range. These variations could be related to microbiological growth and changes in metabolism in food products due to changes in storage conditions and post-harvest handling. The shelf-life studies over time suggest that the original colorimetric functionality of films is stable during short storage periods (8 days) under natural conditions of light and oxygen. However, at longer storage periods, perceptible color variations can significantly affect the film functionality. In these cases, it is better to use the films in food products stored in darkness than under sunlight with oxygen exposure. Although further research is needed to optimize the pigment concentration that maintains better the colorimetric functionality of films, this study could be of great interest for quality control purposes the food packaging industry. Its application as an intelligent edible film capable of modifying its color through pH changes appears to be promising for the food packaging industry.

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