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Research papers

Development of pH indicator films based on chitosan and beeswax

Desarrollo de películas indicadoras de pH a base de quitosano y cera de abejas

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ABSTRACT

A pH-sensitive polymeric dye was synthesized by grafting phenol red onto chitosan using a simple methodology. Successful grafting is confirmed by physical-chemical techniques. From the polymeric dye obtained, pH indicator films were developed with the addition of beeswax. The influence of the concentration of chitosan and beeswax on the humidity and water vapor permeability of the films was evaluated. The lowest moisture values of the formulated films were obtained for lower concentrations of wax and chitosan. However, the permeability was lower at higher wax content, regardless of the chitosan content. The colorimetric capacity of the best film obtained changed in the expected pH range and in contact with shrimp used as model food. Therefore, the films obtained suggest their possible food applications.

Keywords: chitosan; phenol red; Bee wax; polymer dye; pH indicator films.

RESUMEN

Se sintetizó un tinte polimérico sensible al pH, injertando rojo de fenol en quitosano, utilizando una metodología sencilla. El injerto exitoso se confirmó mediante técnicas físico-químicas. A partir del tinte polimérico obtenido, se desarrollaron películas indicadoras de pH con la adición de cera de abejas. Se evaluó la influencia de la concentración de quitosano y cera de abejas sobre la humedad y la permeabilidad al vapor de agua de las películas. Los valores de humedad más bajos de las películas formuladas se obtuvieron para concentraciones bajas de cera y quitosano. Sin embargo, la permeabilidad fue de menor a mayor contenido de cera, independientemente del contenido de

quitosano. La capacidad colorimétrica de la mejor película obtenida, cambió en el rango de pH esperado y en contacto con camarones utilizados como alimento modelo; por lo tanto, las películas obtenidas sugieren su posible aplicación en alimentos.

Palabras clave: quitosano; rojo fenol; cera de abejas; tinte polimérico; películas indicadoras de pH.

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Introduction

Currently, pH is an important factor in food products, because it indicates microbiological deterioration, a key to food quality and safety.[1] For this reason, colorimetric materials capable of changing color due to their sensitivity to pH have been developed.[2] These sensors generally have a pH sensitive dye and are supported on a proton permeable matrix.

In recent years, natural and synthetic dyes have been used for this purpose. Natural dyes are biocompatible, but are generally unstable under heat, light, highly alkaline conditions, and it´s color chance depending on the extraction source, which restricts its reproducibility.[3] On the other hand, synthetic dyes can be toxic and represent contamination for consumers. To overcome this limitation, polymer grafting by covalent bonding is an interesting approach, since it provides the system with stability, biocompatibility, low toxicity, and high color permanence.[4] For this purpose, chitosan (CHI), a biopolymer derived from chitin, has been used due to its biodegradability and non-toxicity.[5,6,7,8,9,10]

Scientific investigations about CHI grafts with different synthetic dyes have been carried out using the Mannich reaction. The results showed that sometimes (e.g. phenolphthalein) the pH range of the derivatives was not appropriate for use as food sensors.[11] However, color transitions with phenol red (pH 6,8-8,4) and with rosolic acid (pH 6,6-8,0) were in the ranges indicating bacterial infections and food spoilage.[12] The cytotoxicity of these dyes was evaluated and it was shown that the polymeric dyes did not exhibit cell toxicity.[13]

Previous studies using polymeric dyes, although convenient, are scarce and not always suitable for countries with high values of temperature and relative humidity in the atmosphere. For this reason, recently, beeswax (BW) has been used as a hydrophobic constituent of the system. Materials that include BW in their composition offer greater resistance to pathogenic microorganisms that affect food.

Hence, films based on the chitosan (CHI), phenol red (FR) and beeswax (BW) were developed in the present work as new pH sensor materials for food applications. The polymeric dye between CHI

and FR (CHI/FR) was obtained by Mannich reaction and characterized by FTIR, UV-VIS, XRD and elemental analysis. Then, BW was incorporated into polymeric dye for developing a colorimetric pH-sensing films (CHI/FR/BW). The influence of the concentration of chitosan and beeswax on humidity and water vapor permeability of the CHI/FR/BW films was also evaluated. The film color in different pH media and in contact with shrimp were also determined.

Materials and methods

Chitosan (CHI, DA= 21,3 %, Mv= $1,4 \cdot 10^5$) was purchased from Aldrich, UK. Phenol red (PR) was purchased from Acros Organic (USA). Formaldehyde (37 % w/w) was provided by BDH Chemicals Ltd Poole, England. Dimethylformamide (DMF), pure for analysis, supplied by Chemicals Ltd. Poole, England. Beeswax, pure for analysis, supplied by "Apicuba", Cuba. All other reagents were pure for analysis and their solutions were prepared with distilled water.

Polymeric chitosan-based dyes (CHI/FR) were synthesized by the Mannich reaction between CHI and FR[13]. Briefly, a 1 % (w/v) CHI solution in 1 % (v/v) HAc solution was prepared. After complete dissolution, 50 mL of dye solution (934 mg of FR dissolved in DMF) and 40 µL of formaldehyde were added to the CHI solution. The mixture was continuously stirred at 60 °C for 24 h. Subsequently, a 0,5 M (w/v) NaOH solution was added dropwise to allow the precipitation of the products. The obtained precipitate was filtered to remove the remaining unreacted dye, and then washed with distilled water and ethanol. The polymeric dye was obtained after drying at 60 ºC in an oven.

For the experimental design and processing of the results of the colorimetric pH-sensing films, the Design Expert 11.1.0.1 program (Stad-Ease Inc., Minneapolis, USA) was used through the numerical optimization method an I-Optimal response surface design. Table 1 shows the factors and conditions tested, and table 2 shows the experimental design matrix.

Table 1- Experimental conditions

Factors	Symbol	Units	Type	Minimum	Maximum
Chitosan (CHI)	A	w/v-%	Numeric	- (0,5)	+ (1,5)
Beeswax (BW)	B	w/v-%	Numeric	- (0,0)	+ (0,5)

Colorimetric films were obtained by a modification of the technology reported by Chalitangkoon and Monvisade.[7] In details, 0,5, 1, 1,5 % (w/v) CHI solutions and three CHI/FR polymeric dye solutions at 1 % (w/v) in 1 % (v/v) acetic acid were prepared. So, each polymeric dye solution was added to the different CHI solutions for preparing three CHI/polymeric dye systems (1,5; 2 and 2,5 w/w) with magnetic stirring. Then Tween 80 was added at a fixed concentration of 0,1 % (v/v). After an hour, the system was filtered to remove impurities and insoluble material. Subsequently,

definite quantities of beeswax (BW) previously melted in a water bath at 80 ^{0}C were added according to experimental design (table 2).

Table 2- Matrix of the experimental design

Runs	1	2	3	4	5	6	7	8	9	10	11	12
Factor A: CHI % (w/v)	0,5	1,5	1,5	1	0,5	1,5	1,5	1	1,5	0,5	1	0,5
Factor B: BW % (w/w)	0,00	0,33	0,00	0,00	0,50	0,50	0,00	0,17	0,50	0,17	0,50	0,50

The emulsions were stirred at room temperature using a digital Ultra-Turrax IKA T25 homogenizer (Mod. T25 D S25, USA) at 12 000 min^{-1} for 5 min. The solution was poured into Petri dishes and dried in an oven at 60 ºC with air circulation for 24 h. The dried films were neutralized by immersing in 1M NaOH solution. The films were finally dried at room temperature. The films with polymeric dyes (CHI/FR) and BW, were labelled as CHI/FR/BW.

Polymeric dyes were characterized by various physic-chemical techniques as Fourier transform infrared spectroscopy (FT-IR), UV–Vis's analysis, X-ray diffraction (XRD) and elemental analysis (EA). IR spectra were obtained on a FT/IR-6300 type A spectrophotometer, serial number: A010561024, in a range of 4 000-600 cm^{-1} with a resolution of 4 cm^{-1}, Jasco, Spain, using the ATR for sample preparation.

The UV-Vis spectra of the polymeric dye solutions were recorded from 200 to 700 nm, using a Rayleigh UV 2601 UV-VIS spectrophotometer, China. Solutions of polymeric dye and 0,01 % (w/v) CHI were prepared by dissolving them in 1 % (v/v) HAc, pH ~ 4. To evaluate grafting of dye onto CHI, CHI solutions were also measured at 0,01 % (w/v) and the free dye FR at 3 µM (CHI+FR).

The crystallinity of the polymeric dyes was evaluated by XRD, using a Smartlab SE Diffractometer (RIGAKU, Japan). XRD patterns were recorded in 2θ with a step size of 0,04º from 5º to 30º.

The carbon, hydrogen, nitrogen and sulfur fraction of CHI and polymeric dyes was analyzed on a Flash Smart elemental analyzer (Thermo Fisher Scientific, USA). The degree of substitution (% DS_{EA}) was determined by (1):

$$\% DS_{EA} = \frac{(C/N)_D - (C/N)_o}{n} \cdot \frac{14}{12} \cdot 100 \qquad (1)$$

where: $(C/N)_D$ is the carbon to nitrogen ratio of the polymeric dye and $(C/N)_O$ is the carbon to nitrogen ratio of the starting chitosan, *n* is the number of carbons introduced into the amino group.

Additionally, an elemental analysis was performed by X-Ray Fluorimetry (XRF) using an X-Supreme Model Energy Dispersive X-Ray Spectrophotometer, using a 3-watt power, SDD-type Si semiconductor detector at 169 eV resolution at Kα of Zn in an air environment and He.

The humidity and water vapor permeability of CHI/FR/BW films were determined by indirect gravimetry by volatilization by separating water from the films by drying in an oven

(MLW TS 400 stove, Germany) at 110 ºC for 24 h, and by permeation cell containing distilled water (100 % relative humidity) with water vapor pressure values as a function of temperature (table 3) respectively.

Table 3- Water vapor pressures at different temperatures of interest

Temperature (°C)	26	27	28	29
Vapor pressure (KPa)	3,361	3,565	3,780	4,005

The cells will be placed in a desiccator between 26 and 29 °C and 0 % relative humidity R (~ 0Pa water vapor pressure) with silica gel. The water transferred through the film will be absorbed by the desiccant and determined by the mass loss of the permeability cell. The cells will be weighed at 2 h intervals for 10 h.

The permeability was determined by the formula (2):

$$Water\ Vapor\ Permeability\ (WVP) = (VTVA * L)/\Delta P \qquad (2)$$

where: VTVA is the water vapor transmission speed (g mm/m^2 h kPa) through the film; L is the average thickness of the film (m) measured by a Palmer millimeter with digital readout (COMECTA SA, Spain) with a sensitivity of 0,001 mm and ΔP is the difference in partial pressure of water vapor (kPa) at both sides of the film. The slope of mass loss vs. Timeline was obtained by linear regression.

With the best results in thickness, humidity and water vapor permeability, a film with the most appropriate (optimal) formulation was selected according to the application for which it was designed. The optimal films were immersed in pH regulating solutions (2,2-11,5) to visually observe the color changes with the variation of pH and to potentially relate it to those experienced by foods during their deterioration. To confirm the color response of these pH indicator films, a food model was used. In this sense, the film obtained was used to monitor the deterioration of shrimp after 24 h of storage at 25 ^{0}C. Four small shrimps and a film fragment of approximately 2 cm² were placed in 11 cm diameter Petri dishes. The plates were covered and hermetically sealed to ensure that the volatile nitrogenous compounds that are generated during deterioration were kept inside.

All experiments were performed in three replicates and data were analyzed by one-way analysis of variance (ANOVA) using SPSS statistical software version 25.0. Duncan's multiple range test was considered, and differences were significant for $p < 0,05$. The data obtained were presented as mean (standard deviation). Statistical analysis for the experimental design was performed using Design-Expert software version 11.0.1 (Statease Inc., Minneapolis, United States). To establish the

regression coefficients, the lack of fit, and the efficiency of the model (analyzed using the R^2 value) and obtaining the optimal conditions of the process.

Results and discussion

To obtain polymeric dyes to be used as pH-dependent colorimetric sensor materials based on CHI and FR, the FR dye was added to the CHI matrix through the Mannich reaction. In this study, a molar ratio of CHI:formaldehyde:dye of 1:0.1:0.5 was used to avoid insoluble products and for promoting the formation of a methylene bridge between the amino group of CHI and the ortho position in the aromatic rings of FR. The final CHI/FR powder is red and dissolved easily in acidic solutions.

The FTIR spectra of CHI and the respective polymeric dye CHI/FR are shown in figure 1. In the CHI spectrum, the characteristic absorption bands of the biopolymer appear at 1 652 cm^{-1} corresponding to the narrowing bands of the C=O bond of the amide I group, at 1 567 cm^{-1} the band belonging to the N-H deformation of the primary amino group appears and at 1 321 cm^{-1} the band of narrowing of the C-N bond of the amide III is present. The bands at 1 417 cm^{-1} and 1 376 cm^{-1} were assigned to the doubling reflections of the $-CH_2$ group and the symmetric deformations of the $-CH_3$ groups.

Unlike the CHI spectrum, the FTIR corresponding to the CHI/FR dye additionally showed a new absorption band at 1 340 cm^{-1} assigned to asymmetric S=O narrowing of the sulfonate groups. Furthermore, a broader band was observed between 1 525 cm^{-1} and 1 653 cm^{-1} and a small shoulder around 1 452 cm^{-1} approximately. This is due to the stretching of the C=C group of the aromatic rings of the dye and the possible interaction of the latter with the amino groups of CHI. The FTIR results suggest that the polymeric dye contains a CHI structurally modified by the aromatic rings of the FR, which is indicative of the possible mechanism previously proposed.

The UV-VIS spectra of CHI and CHI/FR polymeric dye dissolved in HAc (pH= 2-3), are also shown in figure 1. The spectrum of the polymeric dye showed a characteristic signal at 440 nm, while CHI did not show any absorption. This confirmed the appearance of new auxochromes in the dye molecules.[11] The structural features and bathochromic changes mentioned above suggested that FR was successfully grafted onto the CHI backbone through the Mannich reaction.

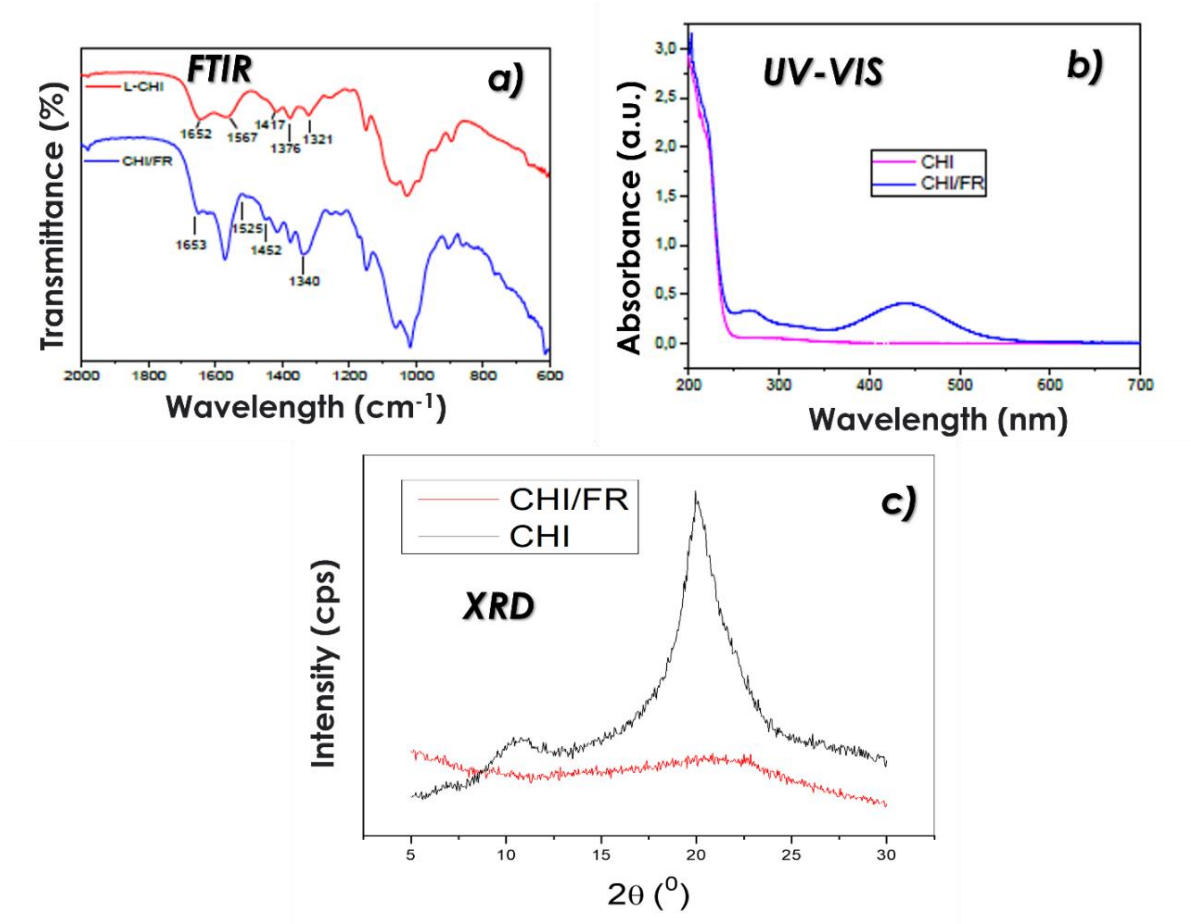

Fig. 1- a) FT-IR spectra, b) UV-VIS spectra and c) XRD patterns of CHI and the CHI/FR polymer dye

The XRD patterns of the CHI and the polymeric dye are displayed at bottom of figure 1. The XRD pattern of the CHI shows a peak of higher intensity at 2θ =20,00º (plane 200 of the CHI) and a second one of lower intensity at 2θ = 10,80º (plane 020 of the CHI), as reported in the consulted database JCPDS No. 39-1894 of hydrated CHI.[13] For its part, the XRD pattern of the polymeric dye shows a single widened band between 15,0 and 30,0 (2θ) corresponding to the main diffraction peak of CHI with a visibly decreased crystallinity in relation to the XRD pattern of the CHI. The decrease in crystallinity of diffraction patterns is consequence and evidence of the grafting of the phenol red dye into the chitosan polymer matrix.

The CHI modification by the grafting with phenol red was also confirmed by elemental analysis. The contents of C, N, H and S are listed in table 4. The fraction of C in the polymeric dye increased, while the fraction of N decreased, which caused an increase in its C/N ratio due to the additional carbon atoms provided by the dye grafted onto the CHI chain. The calculated degree of substitution percentage (DSEA) was 13,89 % for CHI/FR. The value obtained in our work is slightly higher than previous works also grafted phenol red into chitosan. A great sensitivity to colorimetric changes could then be expected, according to the application for which these materials have been designed.[13]

Table 4- Content of the C, N, H and S elements of the polymeric dyes

Sample	Element Content					
	N (%)	C (%)	H (%)	S (%)	C/N (%)	DS_{EA} (%)
CHI	6,76	37,19	5,92	-	5,50	-
CHI/FR	4,93	38,88	5,04	2,24	7,88	**13,89**

Figure 2 shows the elemental analysis using fluorimetry carried out for chitosan and the chitosan polymeric dye.

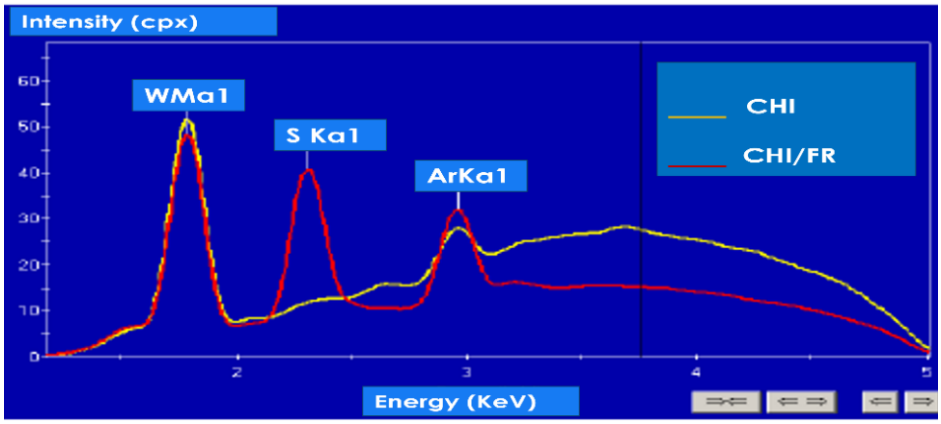

Fig. 2- Elemental analysis by fluorimetry of CHI and CHI/FR dye

From this study, the presence of sulfur (S) can be observed from the sulfonate groups of the phenol red grafted to the chitosan, whose element is completely absent in the biopolymer. This result is another indicative of the successful methodology used to obtain polymeric dye. This technique complements the previous results that confirmed the successful grafting of FR onto CHI with Mannich reaction.

The influence of the studied factors on the humidity of the films developed revealed in the statistical design is showed in figure 3. The humidity values ranged between 1,0 and 15,9 %, being the lowest for the films with chitosan concentration of 0,5 and 1,0 % and incorporated beeswax. It was expected that the addition of wax would decrease the moisture content of the films, especially if it is considered that in obtaining them the addition of a constant volume of film-forming solution to the plate is maintained, but it seems the hydrophilic characteristic from CHI has more influence. Other authors reported a directly relation .between a humidity CHI content.[(14)]

The quadratic model applied to the humidity results was significant with a confidence level of 95,0 %. The statistical R^2 indicated that the fitted model explained 95,11 % (adjusted R^2 of 90,21 %) of the variability in humidity content in the films. The lack of adjustment was not significant with respect to the pure error. Of the factors under study, the concentration of chitosan and its quadratic homologue were significant. Although the beeswax did not have a significant influence, an interaction between it and chitosan was observed. The statistical program used provided an equation displayed in figure 3. From the modular value and the sign of the estimated coefficient, the concentration of chitosan and its quadratic counterpart are the factors that influence most the

humidity. These results confirmed that the lowest humidity is obtained for the lowest concentrations of wax and chitosan and that the humidity was always higher for a concentration of 1,5 % chitosan, regardless the wax content.

Runs	Chitosan (%)	Polymeric dye (%)	Beeswax (%)	Humidity (%)
1	0,5	1	0,00	1,0 (0,5) a
10	0,5	1	0,17	5,2 (0,3) b
12	0,5	1	0,50	4,0 (1,0) b
5	0,5	1	0,50	7,0 (1,0) c
4	1	1	0,00	11,0 (1,0) d
8	1	1	0,17	3,0 (2,0) ab
11	1	1	0,50	5,0 (1,0) bc
3	1,5	1	0,00	15,9 (0,1) f
7	1,5	1	0,00	14,0 (2,0) def
2	1,5	1	0,33	14,5 (0,2) e
9	1,5	1	0,50	11,0 (1,0) d
6	1,5	1	0,50	12,0 (1,0) d

Mean (Standard deviation); n=3
Different letters mean significant differences for p≤0.05

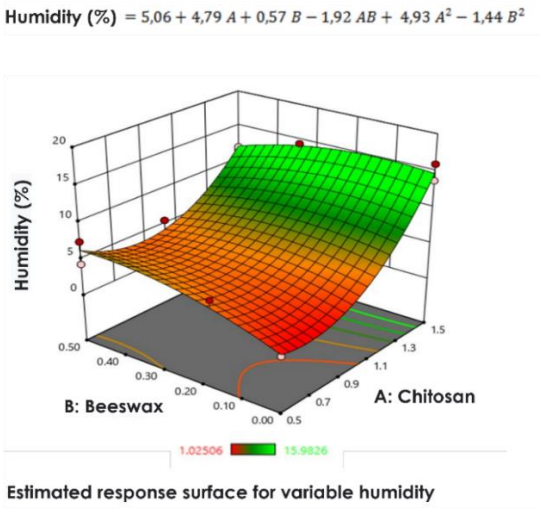

Fig. 3- Humidity results of the films obtained according to the experimental design matrix

The water vapor permeability (WVP) results of the films obtained according to the design matrix experimental is showed in figure 4. As a trend, it is observed that the incorporation of beeswax decreases this property, but the lowest values are shown for the 1 % chitosan concentration, so that there is an influence of the chitosan/beeswax ratio. It was observed that the quadratic model was also significant with a confidence level of 95,0 %. The statistical R^2 indicated that the model explained 90,0 % (adjusted R^2 of 80,0 %) of the observed variability. According to the equation displayed in figure 4 provided from statistical program, the presence of beeswax in the obtained films led to a marked decrease in WVP (negative sign of the B factor and modular value). Chitosan also had a marked effect (quadratic effect) on this important property and in contrast to what was analyzed for beeswax, its presence led to an increase in permeability.

Figure 4 shows in detail the behavior of the WVP variable according to the concentration of wax and chitosan in the films. According to the contour surface estimated from a concentration of 0,20 % (w/w) beeswax and 1 % (w/v) chitosan, the lowest permeability values are obtained. Regardless of the chitosan content, the higher the wax content, the lower the permeability. Velickova *et al*. reported a decrease in water vapor permeability in chitosan films from 4,15 x 1 011 mol m/m^2 sPa to 2,66 x 1 011 and 3,66 x 1 011 molm/m^2sPa for chitosan films with beeswax.[15] Also Santo *et al*. reported a decrease in water vapor permeability in chitosan films with beeswax incorporated at concentrations of 15, 30, 40 and 50 % (w/w), with values ranging from 2.73 g.mm/kPam^{2}h (0 % wax) up to 0,7 g . mm/hkPam^2 (40 % wax), behavior similar to the one obtained in the present study.[16]

Once the model was properly adjusted and validated, the surface described by the model was explored to find the combination of levels in the factors that result in an optimal value of the response. This was possible since the models found explain more than 70 % of the response behavior, in terms of the adjusted R^2. An optimum was obtained for concentrations of 0,66 % chitosan and 0,33 % beeswax respectively, with a statistical convenience of 100 %. Under these conditions, a theoretical PVA of 0,77 g mm/m^2hkPa was obtained.

Runs	Chitosan (%)	Polymeric dye (%)	Beeswax (%)	WVP (g mm/m² h kPa)
1	0,5	1	0,00	2,16 (0,68) e
10	0,5	1	0,17	1,31 (0,11) abc
12	0,5	1	0,50	0,85 (0,37) a
5	0,5	1	0,50	1,00 (0,08) ab
4	1	1	0,00	3,46 (0,39) g
8	1	1	0,17	0,81 (0,03) a
11	1	1	0,50	0,80 (0,07) a
3	1,5	1	0,00	2,68 (0,47) f
7	1,5	1	0,00	2,06 (0,23) de
2	1,5	1	0,33	1,78 (0,25) cde
9	1,5	1	0,50	1,53 (0,11) bcd
6	1,5	1	0,50	1,34 (0,09) abc

Mean (Standard deviation); n=3
Different letters mean significant differences for p≤0.05

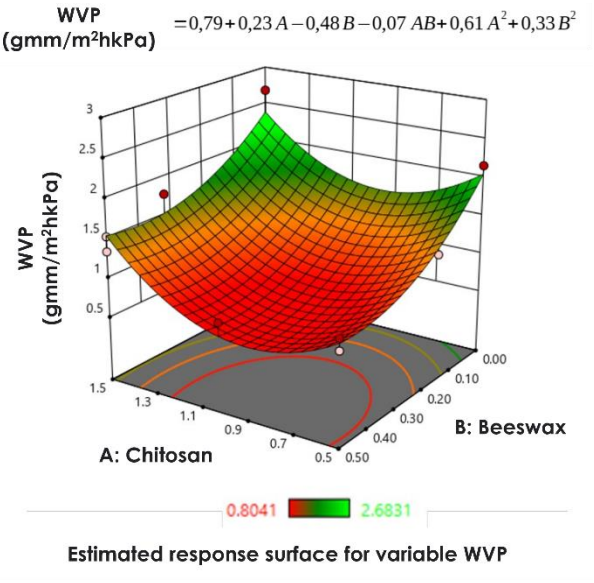

Fig. 4- Water vapour permeability of the films obtained according to the experimental design matrix

The closer optimal composition films of CHI/FR/BW immersed in different pH buffer solutions are shown in figure 5 (A-F). The visible color changes of the indicator samples are evident in the images shown. The initial color of these is red due to the grafting of phenol red into the polymeric chitosan matrix, which was previously confirmed by the physical-chemical characterization of the polymeric dye. This initial red color changes to brownish yellow at a pH between 2,2 to 4,1, however, for pH higher than 7,1. The tone begins to change to mauve, maintaining its original color between pH 6,6 and 7,1, which is in accordance with the literature report.[13]

Figure 5 (G, H) shows the images of the optimal films obtained in contact with the shrimp before and after 24 h of storage at 25 ^{0}C. A slight variation in the color tone of the films was noted due to the deterioration of shrimp. The color of the films changed from red (initial) to mauve, confirming the increase in pH of the food. This fact can be explained due to microbial deterioration could decompose protein, increase total volatile basic nitrogen (TVB-N) and modify the pH value, which is capable of indicating the deterioration of certain foods.[17] This experimental evidence confirms what was previously observed for pH regulating solutions and is in correspondence with the scientific literature consulted where a similar technique is carried out for films based on

anthocyanins.[18-20] Thus, CHI/FR/BW films may have the potential as smart indicator food packaging films to monitor food freshness through direct visual inspection.

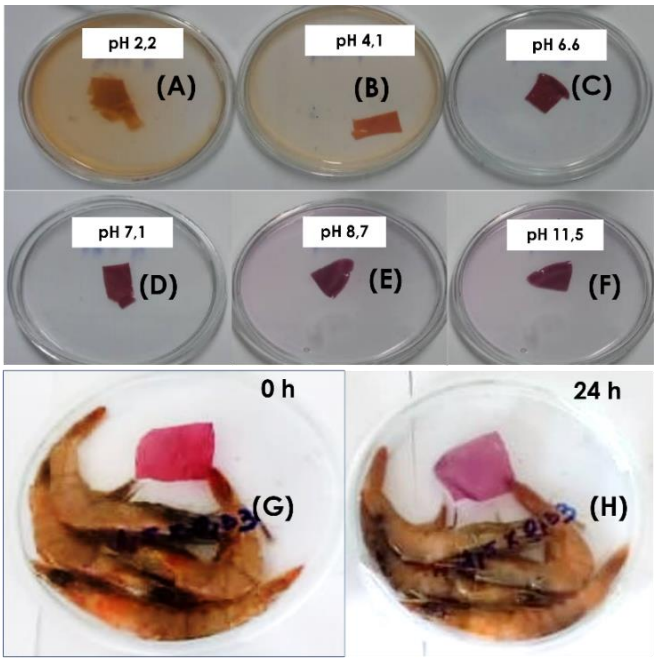

Fig. 5- Color change of CHI/FR/BW film after immersion in different pH buffer solutions: pH 2 (A), pH 4 (B), pH 6 (C), pH 7 (D), pH 9 (E), pH 11 (F) and to monitor the freshness of shrimp kept at room temperature (25 ºC): at 0h (G) and at 24h (H)

Conclusions

A polymeric dye was obtained from phenol red (FR) and chitosan (CHI) through the Mannich reaction. The results of FTIR, UV-VIS, XRD and elemental analysis confirmed that the dye used (FR) was grafted onto the CHI main chain. The lowest humidity values of the CHI/FR/BW formulated films from polymeric dye were obtained for the lowest concentrations of beeswax and chitosan and the humidity was always higher for a concentration of 1,5% of chitosan regardless of the wax content. The films formulated with a concentration of 0,20 % (v/v) beeswax and 1 % (w/v) chitosan presented the lowest permeability values. Regardless of the chitosan content, the higher the beeswax content, the lower the permeability. CHI/FR/BW films immersed in pH buffer solutions and in contact with shrimp changed color, suggesting their potential application as smart indicator food packaging films to control food freshness through direct visual inspection.

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Interests conflicts

The authors express that there are no conflicts of interest in the submitted manuscript.

Authors' contribution

Roberto Sáez-Gastón: data curation, formal analysis, research, methodology, resources, software, validation, visualization, writing the original draft.

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Yaimara Solís-Moré: data curation, formal analysis, funding acquisition, project administration, research, methodology, software, supervision, validation, visualization, writing the original draft and finally writing.