This is the accepted manuscript of the article that appeared in final form **International Journal of Biological Macromolecules** 120(Part B) : 2131-2136 (2018), which has been published in final form at https://doi.org/10.1016/j.ijbiomac.2018.09.081. © 2018 Elsevier under CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

Assessment of gallic acid-modified fish gelatin formulations to optimize the mechanical performance of films

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Abstract

In this study, a response surface methodology (RSM) using a Box-Behnken design was applied to optimize the mechanical response (tensile strength, elongation at break and Young’s modulus) of fish gelatin films. These responses were analyzed as a function of glycerol content (0-10% on gelatin basis), added as a plasticizer, gallic acid content (5-15% on gelatin basis), used as crosslinker, and solution pH (4.5-10). Second order polynomial models were adjusted for the three responses, and they were found to be reliable according to the standard statistical analysis. The values of the independent factors that maximize the responses were also determined. In order to relate mechanical performance to material structure, Fourier transform infrared (FTIR) analysis was carried out and this revealed that a reaction occurs between gelatin and gallic acid through a process that releases water and provides a plasticizing effect. The performed time-, material- and cost-saving optimization of the formulation based on biodegradable compounds from abundant renewable resources enabled a sustainable approach to the development of new materials.

Keywords: bio-composites; response surface methodology; mechanical properties.

1. Introduction

In the current consumer society, the massive use of plastic products in the daily life of the world population poses a serious challenge to the environmental sustainability of the planet. By 2005, plastic made up at least 10% of solid waste by mass in 58% of countries with available data [1,2]. The range of estimated flux of plastic waste entering the ocean was as high as 4.8 to 12.7 million metric tons in 2010 [2], which negatively affect marine wildlife [3].

A substantial part of plastic production (39% in Europe) is used for packaging [4]. Conventional plastic packages are produced from fossil fuels, whose resources are finite and non-renewable. Being the consumption patterns and the need of one-use packages difficult to revert, finding renewable and recyclable alternatives is of paramount importance. In this scenario, the abundance of bio-waste converts this bio-waste into an environmentally friendly option to be exploited. In this context, the biopolymers that can be extracted from bio-waste, such as polysaccharides, proteins, and lipids, show a high potential because of their non-toxicity, biodegradability and biocompatibility. Among proteins, gelatin is the most employed one because of the vast number of sources from which gelatin can be obtained. Furthermore, due to religious and economic issues, fish-derived gelatins have gained relevance in the last years.

With the aim of developing new bio-based and biodegradable materials, the optimization of their mechanical properties is of great interest. Hence, plasticizers and other additives intended to be incorporated into the formulations must be carefully selected in order to enhance the mechanical performance of the final products, maintaining the bio-based origin and the biodegradable character. In this regard, plasticizers act as internal lubricators, weakening intra- and inter-molecular forces among molecules and easing polymeric chain movements. Among plasticizers, glycerol is widely used to plasticize biopolymeric films since its small size allows it to penetrate among the polymeric chains to form new physical interactions by hydrogen bonding, reducing brittleness and improving flexibility [5,6]. Furthermore, glycerol can be obtained as a by-product from the biodiesel production [7]. Additionally, other additives are used to interact chemically with gelatin. In this regard, gallic acid could be considered as an interesting option since it can be extracted from natural and renewable sources [8], such as tea leaves [9,10] or fruits [11, 12]. Besides gallic acid bio-based origin and appropriate chemical structure to react with gelatin, gallic acid displays antioxidant [13], antimicrobial [14,15], antitumoral [16,17], anti-inflammatory [18,19], and antiviral [20,21] properties, which are of high relevance when films are intended to be used as controlled drug delivery carries for food, pharmaceutical, and biomedical applications [22, 23].

The consciousness about sustainability must be reflected not only on the materials, but also on the methods and processes used to analyze and produce them. An inefficient protocol in the laboratory when developing new materials can lead to unnecessary costs of time, energy and/or raw materials. In order to address this problem, the Design of Experiments (DoE) theory and, particularly, the surface response methodology (RSM) can be applied [24]. Within this approach, the number of the experimental combinations and repetitions needed to analyze a system can be reduced through rational design.

With the use of one-variable-at-a-time experimental designs in the past, the analysis of the influence of many variables on a system property was time-consuming, and it did not allow detecting the simultaneous cross-effect of two or more variables. The modern Design of Experiments framework and, particularly, the sort of mathematical and statistical techniques known as RSM importantly reduce the number of experimental combinations of variables that must be tested in order to provide good knowledge of a complex response function. From the n^m variants needed in principle for studying m variables at n levels, only a reduced amount of them is required in fractional designs such as the Box-Behnken design [25], which is the one chosen in this work. This design was selected due to the few experimental combinations of the variables required for the adequate estimation of the complex response functions compared to other similar designs [26].

The goal of this work was to adopt a sustainable approach towards the development of novel packaging systems, including not only the selection of more environmentally friendly materials but also the design of the experiments carried out to characterize the developed materials, with the aim of minimizing the use of resources and energy. The behavior of the resulting materials was analyzed as a function of glycerol content, gallic acid content and solution pH. Empirical models were adjusted, and a posterior maximization of the tensile strength (TS), elongation at break (EB) and Young's modulus (E) allowed finding the optimal work region of the system.

2. Materials and methods

2.1. Materials

Fish gelatin was purchased from Healan Ingredients (East Yorkshire, UK). The fish gelatin used in this study was a type A gelatin with a 240 bloom value and an average molar mass of 125-250 kDa. Glycerol and gallic acid were obtained from Panreac (Barcelona, Spain). All chemicals were used as received without further purification

2.2. Film preparation

Fish gelatin films were prepared by mixing gelatin and gallic acid in distilled water. The acid contents employed in this work were 5, 10 and 15 wt. % on gelatin basis. Solutions were heated at 80°C for 30 min and stirred at 200 rpm. Then, 0, 5, or 10 wt. % glycerol (on gelatin basis) was added as a plasticizer and solution pH was adjusted with

1 N NaOH; the pH values used in the present work were 4.50, 7.25 and 10.00. The heating procedure was repeated and finally, solutions were poured into Petri dishes and allowed to cool for 48 h at room temperature. All films were conditioned in a controlled environment chamber at 25°C and 50% relative humidity before testing.

2.3. Experimental design

The effect on a mechanical response variable (TS, EB, E) of three independent factors was studied. These three inputs were glycerol content, gallic acid content and pH, each of which was coded at three levels: low (-1), medium (0), and high (+1). The analyzed glycerol content values were 0% (no glycerol), 5% and 10%, while gallic acid contents were 5%, 10% and 15%, all of them based on gelatin content. Finally, the pH values were 4.50, 7.25 and 10.00. RSM was used to determine if there was any relationship between response variables and independent factors, and to eventually quantify it through the application of multiple regression theory. Herein, an empirical full-quadratic polynomial model was fitted to estimate each response function:

$$y = b_0 + \sum\nolimits_{i=1}^{n} b_i x_i + \sum\nolimits_{i=1}^{n} b_{ii} x_i^2 + \sum \sum\nolimits_{i<j=2}^{n} b_{ij} x_i \, x_j$$

where b_0 was the constant coefficient or intercept, b_i were the first order linear coefficients, b_{ii} were the quadratic coefficients, and b_{ij} (with $i \neq j$) were the second order interaction coefficients. With the coefficients obtained from the expression with non-coded factors, the equation allowed predicting easily response variables by selecting the specific combinations of glycerol, gallic acid, and solution pH. In contrast, standardized coefficients were obtained from coded variables. When these coefficients were significant, they expressed a measure of the relevance of an influence and, thus, the higher the absolute value of a coefficient, the more important the effect of its corresponding factor on the response [27,28]. The response function corresponded to a hypersurface in a (n+1) dimensional space, where n was the number of factors or independent variables (three, in this case). Consequently, in this work the data was visualized with surface and/or contour graphs as a function of two factors and fixing the third one.

2.4. Film characterization

Fourier transform infrared (FTIR) spectra were recorded on a Nicolet 380 FTIR spectrometer equipped with horizontal attenuated total reflectance (ATR) crystal (ZnSe). The spectra were collected in absorbance mode on sample films. The measurements were recorded between 4000 and 800 cm^{-1}. A total of 32 scans were performed at a resolution of 4 cm^{-1}.

Tensile tests were performed in an electromechanical testing system (MTS Insight 10) in order to determine, tensile strength (TS), elongation at break (EB), and Young's modulus (E). The films were cut into dog-bone shape (4.75 mm×22.25 mm) and five samples were tested for each composition. Film thickness was measured to the nearest 0.001 mm with a hand-held digimatic micrometer (QuantuMike Mitutoyo). Tests were carried out according to ASTM D638-03 [29].

2.5. Statistical analysis

To evaluate the quality of the fitted model, an analysis of variance (ANOVA) was conducted. The central idea of ANOVA was to compare the variation due to the treatment (change in the combination of variable levels) with the variation due to random errors inherent to the measurements of the generated responses [30]. Performing the ANOVA, the significance ($p < 0.05$) of the regression coefficients was assessed by determining the F value. Non-significant terms were eliminated by backward elimination method, simplifying the model until its final form [31]. For the validation of the model, the coefficient of determination, R^2, as well as the significance values of the model and of the lack of fit were calculated. R^2 gave the percentage of variation explained by the factors, whereas adjusted R^2 referred to the percentage explained by the factors that actually affected the response, penalizing the extra factors. To be consistent, the model should be statistically significant to a 95% confidence level ($p < 0.05$), which means that there is only a 0.05% chance that the agreement between the predicted values and the experimental points (to be precise, the observed F-value) could occur owing to noise [32]. The lack of fit should be non-significant ($p \geq 0.05$) to be reliable and describe properly the observed behavior. In a subsequent analysis, the function provided by the empirical model of a response was maximized or minimized, determining the optimal response as well as the values of the factors that led to it. When several response variables were involved in a simultaneous optimization, these values were calculated according to the criterion that ensured a compromise. The desirability function approach introduced by Derringer and Suich [33] has been employed for that purpose [34] and was also used in this work.

Data analysis, ANOVA and linear regression including responses optimization were performed by using Minitab 17 software.

4. Results and discussion

The design variables selected in this study with actual and coded levels along with response variables are shown in **Table 1**. In this case, a basic Box-Behnken design demands 12 runs, and 3 additional runs were included at the center of the design to improve the quality of the statistics.

Table 1. Box-Behnken experimental design and responses for gelatin-based films.

Runs	Factors			Coded factors			Response variables		
	Glycerol	Gallic acid	pH	Glycerol	GA	pH	TS (MPa)	EB (%)	E (MPa)
1	0	5	7.25	-1	-1	0	77.909	3.460	3627.994
2	10	5	7.25	1	-1	0	77.475	3.579	3236.650
3	0	15	7.25	-1	1	0	86.371	3.188	4050.056
4	10	15	7.25	1	1	0	72.135	2.671	3411.436
5	0	10	4.5	-1	0	-1	78.664	3.846	3604.143
6	10	10	4.5	1	0	-1	81.775	3.072	3756.966
7	0	10	10	-1	0	1	56.347	2.661	2858.771
8	10	10	10	1	0	1	57.853	2.623	2989.918
9	5	5	4.5	0	-1	-1	80.488	3.949	3336.536
10	5	15	4.5	0	1	-1	83.374	2.673	4247.408
11	5	5	10	0	-1	1	70.809	3.127	3186.486
12	5	15	10	0	1	1	62.300	2.618	3138.438
13	5	10	7.25	0	0	0	81.399	3.144	3962.046
14	5	10	7.25	0	0	0	82.630	3.035	4077.392
15	5	10	7.25	0	0	0	79.623	3.165	3944.825

Glycerol content, gallic acid content, and pH are expected to affect the mechanical performance of gelatin films. Obtained regression equation coefficients are presented in **Table 2**. The experimental data were analyzed using a multiple regression technique to develop a response surface model. The results in **Table 3** revealed that *F* values for the model were significant ($p < 0.05$) for all responses. Moreover, lack of fit values were non-significant in all cases ($p \geq 0.05$), confirming the validity of the models.

Table 2. Regression analysis for the full quadratic model of mechanical responses.

	TS (MPa)				EB (%)				E (MPa)			
	Coef.	Std. Δ	*t*-value	*p*-value	Coef.	Std. Δ	*t*-value	*p*-value	Coef.	Std. Δ	*t*-value	*p*-value
b_0 (constant)	81.22	2.46	33.05	0.000*	3.1145	0.0995	31.29	0.000*	3995	127	31.38	0.000*
b_1 (Gli)	-1.26	1.50	-0.83	0.442	-0.1513	0.0610	-2.48	0.056	-93.2	78	-1.20	0.285
b_2 (Aci)	-0.31	1.50	-0.21	0.844	-0.3708	0.0610	-6.08	0.002*	182.5	78	2.34	0.066
b_3 (pH)	-9.62	1.50	-6.39	0.001*	-0.3138	0.0610	-5.15	0.004*	-346.4	78	-4.44	0.007*
b_{11} (Gli*Gli)	-4.16	2.22	-1.88	0.119	0.0344	0.0897	0.38	0.718	-294	115	-2.56	0.051
b_{22} (Aci*Aci)	1.42	2.22	0.64	0.550	0.0757	0.0897	0.84	0.437	-119	115	-1.04	0.346
b_{33} (pH*pH)	-8.39	2.22	-3.79	0.013*	-0.0985	0.0897	-1.10	0.322	-398	115	-3.47	0.018*
b_{12} (Gli*Aci)	-3.45	2.13	-1.62	0.166	-0.1589	0.0862	-1.84	0.125	-62	110	-0.56	0.599
b_{13} (Gli*pH)	-0.4	2.13	-0.19	0.858	0.1838	0.0862	2.13	0.086	-5	110	-0.05	0.963
b_{23} (Aci*pH)	-2.85	2.13	-1.34	0.238	0.1919	0.0862	2.23	0.077	-240	110	-2.17	0.082

Coef. : Standardized regression coefficients; Std. Δ: standard error of the coefficients; *t*-value: statistic of the *t*-test; *p*-value: significance value of the *t*-test (*) significant at $p < 0.05$.

Table 3. Analysis of variance (ANOVA) of the full quadratic model.

		TS (MPa)				EB (%)				E (MPa)			
	DF	SS (adj)	MS (adj)	*F*-value	*p*-value	SS (adj)	MS (adj)	*F*-value	*p*-value	SS (adj)	MS (adj)	*F*-value	*p*-value
Model	9	1161.25	129.028	7.12	0.022*	2.52065	0.28007	9.42	0.012*	2402864	266985	5.49	0.038*
Linear	3	754.40	251.466	13.88	0.007*	2.07066	0.69022	23.22	0.002*	1296004	432001	8.89	0.019*
Quadratic	3	326.13	108.711	6.00	0.041*	0.06645	0.02215	0.75	0.570	861574	287191	5.91	0.042*
Interaction	3	80.72	26.908	1.48	0.326	0.38354	0.12785	4.30	0.075	245286	81762	1.68	0.285
Error	5	90.60	18.120			0.14864	0.02973			243064	48613		
Lack of fit	3	86.03	28.677	12.55	0.075	0.13885	0.04628	9.45	0.097	232672	77557	14.93	0.063
Pure error	2	4.57	2.285			0.00979	0.00490			10392	5196		
Total	14	1202.93				2.66929				2645927			

TS (MPa) R^2 (%)	TS (MPa) R^2 (adj) (%)	EB (%) R^2 (%)	EB (%) R^2 (adj) (%)	E (MPa) R^2 (%)	E (MPa) R^2 (adj) (%)
92.76	79.74	94.43	84.41	90.81	74.28

DF: degrees of freedom; SS: sum of squares; SS (adj): adjusted sum of squares; *F*-value: statistics of the *F*-test; *p*-value: significance value of the *F*-test (*) significant at $p < 0.05$; R^2: coefficient of determination; R^2 (adj): adjusted coefficient of determination.

The relationship between the non-coded independent variables, plasticizer content (x_1), acid content (x_2) and pH (x_3), and TS dependent variable was established by the following second order polynomial equation:

$$\mathrm{TS} = 28.7 + 3.01\,x_1 + 0.99\,x_2 + 14.81\,x_3 + 0.1666\,x_1^2 + 0.0568\,x_2^2 - 1.110\,x_3^2 - 0.1380\,x_1x_2 - 0.029\,x_1x_3 - 0.207\,x_2x_3$$

As long as TS is concerned, the R^2 value was 0.93 whereas adjusted R^2 value was 0.80, indicating that the model explained the 80% of the total variation. As can be observed, linear and quadratic terms of the selected pH for film preparation had a significant effect ($p < 0.05$) on the TS of the film. Performing a backward elimination process of the non-significant terms, the ensuing reduced model equation was obtained:

$$\mathrm{TS_{red}} = 48.0 + 12.22\,x_3 - 1.084x_3^2$$

leading to a R^2 value of 0.79 and an adjusted R^2 of 0.76.

In reference to EB, the following second order equation showed the relation between the studied non-coded independent variables and EB:

$$\mathrm{EB} = 5.666 - 0.0733\,x_1 - 0.2042\,x_2 - 0.132\,x_3 + 0.00137\,x_1^2 + 0.00303\,x_2^2 - 0.013\,x_3^2 - 0.00636\,x_1x_2 + 0.01337\,x_1x_3 + 0.01396\,x_2x_3$$

The variables that showed significant ($p < 0.05$) effects on EB were gallic acid content and pH. Only the linear terms of these variables were significant ($p < 0.05$). R^2 value was 0.94 while adjusted R^2 value was found to be 0.84. The corresponding reduced model was:

$$\mathrm{EB_{red}} = 4.69 - 0.0742\,x_2 - 0.1141\,x_3$$

with a R^2 value of 0.70 and an adjusted R^2 of 0.66.

Regarding E, the second order model was significant ($p < 0.05$) and the relation between the response and the studied non-coded variables was:

$$\mathrm{E} = 305 + 126.5\, x_1 + 271\, x_2 + 814\, x_3 - 11.76\, x_1^2 - 4.77\, x_2^2 - 52.7\, x_3^2 - 2.47\, x_1x_2 - 0.39\, x_1x_3 - 17.43\, x_2x_3$$

The values of R^2 and adjusted R^2 were 0.91 and 0.74 respectively. Eliminating the non-significant terms, a reduced model was calculated:

$$\mathrm{E_{red}} = 2109 + 581x_3 - 48.8\, x_3^2$$

This model showed a R^2 value of 0.88 and an adjusted R^2 of 0.79. The factor that showed a significant ($p < 0.05$) effect on E was pH, through its linear and quadratic terms. Wang et al. also observed that pH has a considerable impact on gelatin-based films mechanical properties using RSM [35].

As can be followed from the reduced equations of the mechanical variables, in the studied range, glycerol content (x_1 factor) is not significant in any of them and, thus, it could be removed from an optimal formulation for the sake of economy and simplicity. Aloui et al. [36] analyzed the effect of glycerol on different biopolymer-coated papers through RSM, and their results revealed that the plasticizer had no significant effect on the mechanical properties of half of the studied coatings. A system of gelatin films with glycerol and gallic acid as additives was studied by Limpisophon and Schleining [37]. According to their results, the addition of plasticizer resulted in an increase in EB and a concomitant decrease in TS. This significant effect could be related to the use of an unequal pH and a different range of the amount of glycerol in the film formulation. The removal of glycerol from our model simplifies the landscape of the response variables as a function of the factors, as it becomes three-dimensional (response vs. gallic acid and pH). Furthermore, only pH appears to be significant for TS and E and, therefore, in these cases the search of the optimal formulation was reduced to find the maximum or minimum in the parabola of the response vs. pH. This can be appreciated in **Fig. 1a** and **b**. The curves showed that there was maximum response for TS and E in the studied range.

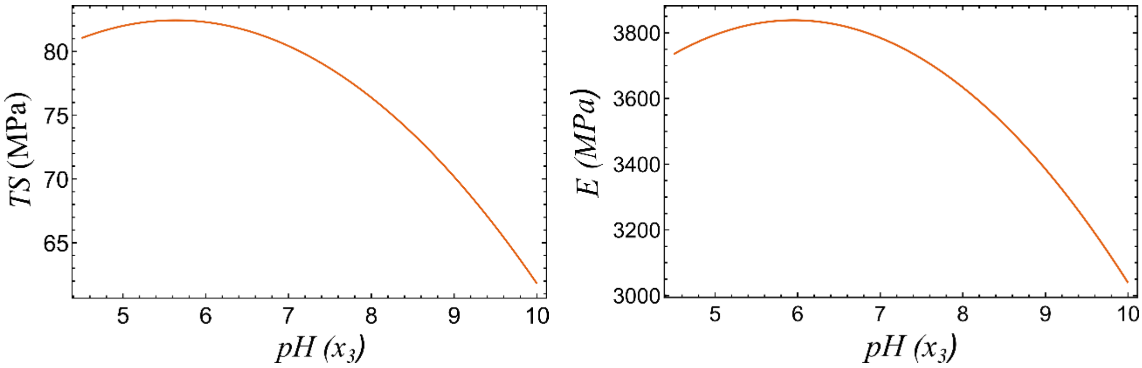

Figure 1. Two-dimensional response plot for the reduced model of a) TS and b) E.

For EB, the hypersurface had the form of an inclined plane, as shown in **Fig. 3**, since only linear terms of x_2 and x_3 were found to be significant. It is worth noting that EB increased when both acid content and pH decreased. As can be seen in **Fig. 2**, adjusting the solution pH in the manufacture process is vital to find an optimum TS. Regarding the acid content, even though its addition did not improve the film EB, its presence in the formulation of the film plays a vital role in the stability of the material.

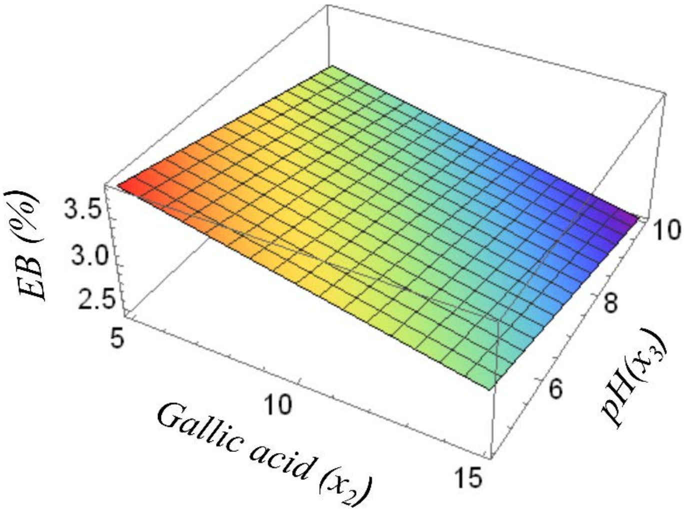

Figure 2. Three-dimensional response surface plot for the reduced model of EB.

In this study, the mechanical properties of gelatin films were optimized by maximizing TS, E and EB simultaneously, allowing the same weighting for the three magnitudes. The obtained result for the optimal values of factors was $x_1 = 0$; $x_2 = 5$; $x_3 = 5.28$ considering that the addition of glycerol can be avoided. With this combination, the expected theoretical responses were $\mathrm{TS}_{\mathrm{theo}}^{\mathrm{opt}} = 82.3\ \mathrm{MPa}$; $\mathrm{EB}_{\mathrm{theo}}^{\mathrm{opt}} = 3.72\%$; $\mathrm{E}_{\mathrm{theo}}^{\mathrm{opt}} = 3817.39\ \mathrm{MPa}$. This experiment was conducted in the lab with a view to comparing predicted and experimental values. The agreement was satisfactory, confirming the validity of the model: $\mathrm{TS}_{\mathrm{exp}}^{\mathrm{opt}} = 82 \pm 3\ \mathrm{MPa}$; $\mathrm{EB}_{\mathrm{exp}}^{\mathrm{opt}} = 3.8 \pm 0.6$; $\mathrm{E}_{\mathrm{exp}}^{\mathrm{opt}} = 3850 \pm 70\ \mathrm{MPa}$.

For a better understanding of the mechanical performance of films, FTIR analysis was carried out to analyze the interactions among the components of the film. The FTIR bands corresponding to gelatin materials are summarized in Table 4. The most characteristic bands of gelatin are related to C=O stretching (amide I) at 1632 cm^{-1}, N-H bending (amide II) at 1527 cm^{-1}, and C-N stretching (amide III) at 1238 cm^{-1} [38].

Table 4. Characteristic band position and assignment for gelatin materials.

Region	**Wavenumber (cm^{-1})**	**Assignment**
Amide A	3286	NH and OH stretching
Amide B	2926	CH_2 asymmetrical stretching
	2878	CH_2 symmetrical stretching
Amide I	1625	C=O stretching
Amide II	1521-1540	CN stretching
	1449	CH_2 bending
	1336	CH_2 wagging of proline
Amide III	1237	NH bending

Regarding glycerol, the main absorption bands appear at the 800–1150 cm^{-1} region and are related to the vibrations of C-C and C-O bonds [38]. Gallic acid shows the absorption band of the carboxylic group at 1664 cm^{-1}, which is overlapped by the gelatin amide I band, and absorption bands of hydroxyl groups at 1428 cm^{-1}, 1320 cm^{-1}, and 864 cm^{-1} [39]. In the films under study, increasing pH led to changes in the relative intensity between amide I and amide II bands, as illustrated in **Fig. 3**.

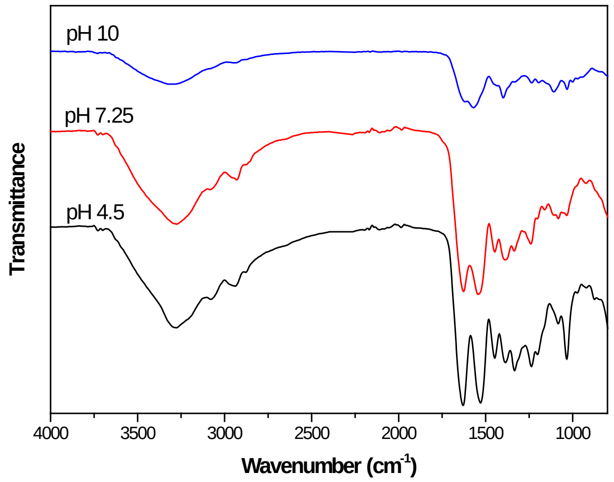

Figure 3. FTIR spectra of gallic acid-modified films depending on pH.

At pH 4.5, the intensity of the amide I band was slightly higher than that of amide II, which became more intense at pH 7.25; at pH 10, both bands became into a single one. This is indicative of the reaction between the amine group of gelatin and the carboxylic group of gallic acid, in a similar way as Uranga et al. reported for the reaction between gelatin and citric acid [40]. From the results obtained, it is clear that pH is the factor that most prominently affects the mechanical properties of films. This might be due to the promotion of the crosslinking reaction between gelatin and gallic acid at basic pHs. This chemical reaction, expected to cause an increase of TS, also caused the formation of water molecules, which led to a plasticizing effect that compensated the crosslinking effect. This plasticizing effect would make the addition of glycerol unnecessary, since this additive is actually employed as a plasticizer.

5. Conclusions

The analysis revealed the variables that significantly affect the film mechanical properties, and empirical models were adjusted in order to predict the responses in the studied range. Solution pH was found to be the factor that exhibited the most determinant effect, in accordance with the role of pH promoting the crosslinking reaction between gelatin and gallic acid. It was also found that glycerol can be removed from the formulations without any damage on the mechanical properties. In addition to this saving in raw materials, energy costs and manufacturing time could be reduced with a proper design of the matrix of experiments to be conducted by using the Design of Experiments theory. Moreover, film mechanical properties can be optimized using the developed models, maximizing TS, E and EB.

Acknowledgements

The authors thank the University of the Basque Country (PPG17/18), the Provincial Council of Gipuzkoa (Department of Economic Development, the Rural Environment and Territorial Balance), and the Basque Government (Department of Quality and Food Industry) for financial support.

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