

# Tuning the Mechanical and Functional Properties of Pea Protein-Based Bioplastics via Different Physical and Chemical Cross-Linking Methods

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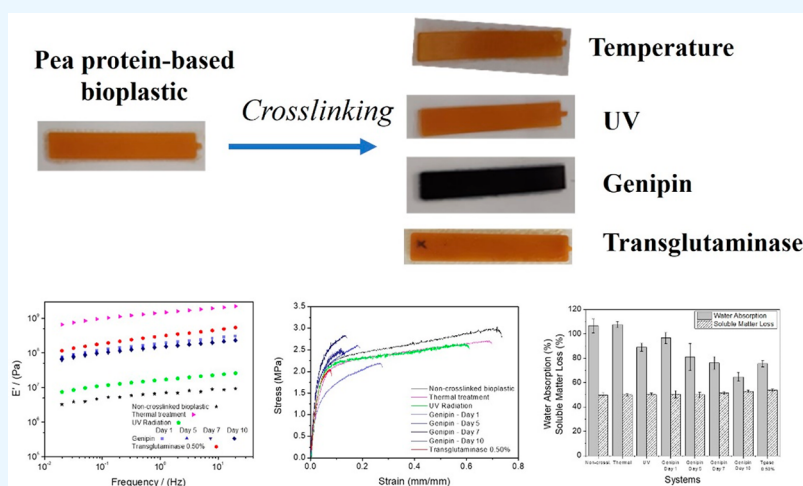
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**ABSTRACT:** Protein-based bioplastics have been highly investigated in order to substitute conventional plastics. This is due to their biodegradability and natural origin, which reduce well-known plastic pollution. Nevertheless, their low mechanical properties and high environmental sensitivity have slowed their advance to the market. One solution to this problem is the use of additional cross-linking methods, which improve the bioplastics' properties. In this sense, the main goal of this study was to evaluate the influence of different cross-linking methods (physical, chemical, and enzymatic) on the mechanical and functional properties of pea protein-based bioplastics. According to the results obtained in this work, an additional cross-linking stage could tune the properties of protein-based bioplastics.

**KEYWORDS:** Pea Protein, Cross-linking, Bioplastics, Injection molding, Biodegradability

## 1. INTRODUCTION

The vast majority of plastic waste is found in landfills or abandoned in the environment since 95% of the products are designed to be discarded after a single use.<sup>1</sup> In this way, one of the main challenges nowadays is related to exploring and using more sustainable and eco-friendly materials. The European Commission adopted the Circular Economy Action Plan (CEAP)<sup>2</sup> as one of the main pillars of the European Green Deal, Europe's current agenda for sustainable growth and employment.<sup>3</sup> This plan, together with the United Nations Environment Program (UNEP) and Zero Pollution Action Plan for Air, Water and Land, promises to identify the value chain and establish environmentally and economically sustainable production business models.<sup>4</sup> All this is developed in order to meet the Sustainable Development Goals (SDGs) promulgated by the United Nations to end poverty, protect the planet, and ensure that everyone enjoys peace and prosperity

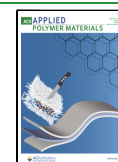
by 2030.<sup>5</sup> Many of its goals promote the reduction of the production of waste.<sup>6</sup> To this end, polymers and biopolymers have been used for several applications.<sup>7</sup> Considering the different SDGs, it significantly affects objective 13 of climate action, since it advocates combating climate problems generated by the plastic accumulation; objectives 14 and 15 of life below water and on land, respectively; and objective 12 of responsible consumption and production, which seeks to

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find more sustainable alternatives that do not harm the planet.<sup>8,9</sup>

Another of the many contributions that must be made to achieve fully sustainable advancement is the development of materials that can replace conventional plastics. In recent years, waste and byproducts from the agri-food industry are one of the most potential sources to obtain biobased raw materials due to their low value as well as their high content in proteins and polysaccharides.<sup>10</sup> Besides, the use of proteins or polysaccharides as raw materials is encouraged since they can be used to fabricate bioplastics. Bioplastics are biodegradable, have a natural origin, or meet both qualities.<sup>11</sup> In other words, they are more sustainable plastics than conventional petroleum-based plastics. However, the production of bioplastics faces challenges due to the high cost of obtaining the raw material or the lack of industrial waste management systems capable of handling bioplastics appropriately. In this sense, more research on these issues is necessary for the correct implementation of these materials in the industrial sector.<sup>12</sup>

Currently, the main field of application of bioplastics is food packaging products. These containers must perfectly preserve the organoleptic properties of food, not giving them harmful substances.<sup>13</sup> In this sense, they must have good humidity and atmospheric permeability to avoid the rapid decomposition of the materials, which can be achieved with the use of cross-linking agents. In addition, some additives can also act as antimicrobial agents that give interesting functional properties to these packages. Another bioplastic application is the substitution of poorly degradable materials, such as plastic bags or filters.<sup>14</sup> Nevertheless, they have to be reinforced in order to improve their mechanical properties. In addition, due to the high content of hydrophilic groups present in proteins, these materials can become superabsorbent; that is, they can absorb more than 10 times their weight in water without dissolving and can be used in personal hygiene applications and in agriculture and horticulture. In this way, they are used in the production of biodegradable diapers<sup>15</sup> and for the encapsulation and controlled release of agrochemical agents.<sup>16</sup>

The fabrication of bioplastics from biopolymers consists of the modification of the biopolymeric chains of these raw materials.<sup>17</sup> This modification exposes the hydrophobic groups that produce the formation of aggregates between the biopolymers and, therefore, a protein framework. This network is reinforced through the application of temperature and pressure (generally by thermomechanical methods) that generate covalent bonds (especially disulfide bridges) between the chains. However, the properties obtained by these materials still do not make them competitors of conventional plastics,<sup>18</sup> representing only 0.6% of the current plastic market.<sup>19</sup> Therefore, it would be interesting to add and combine different reinforcement techniques in order to continue advancing alternatives that favor their potential application for a future market.

In this sense, a more in-depth study of these materials is necessary to improve their properties. To this end many researchers have implemented additional mechanisms to promote the formation of new bonds in the polymer chains that may modify these types of materials.<sup>20–22</sup> These additional mechanisms are called cross-linking and allow the properties of the materials to be tuned. There are three methods of cross-linking, by physical, chemical, or enzymatic treatment.<sup>23</sup> Table 1 shows different alternatives carried out in previous studies for bioplastic materials.

**Table 1. Different Alternatives To Carry out a Cross-Linking Reaction to Bioplastic Materials**

| cross-linking method | cross-linking agent | raw material                  | ref    |
|----------------------|---------------------|-------------------------------|--------|
| Physical             | UV radiation        | Polybutylene succinate/lignin | 24     |
|                      | Thermal treatment   | Soy protein                   | 25     |
|                      |                     | Gluten                        | 26     |
| Chemical             | Citric acid         | Cellulose/PVA                 | 27     |
|                      |                     | Starch                        | 28, 29 |
|                      |                     | Agarose                       | 21     |
|                      | Glyoxal             | Wheat protein                 | 30     |
|                      |                     | Gelatin                       | 31     |
|                      |                     | Chitosan                      | 32     |
|                      | Genipin             |                               | 33     |
|                      |                     | Gelatin                       | 34     |
|                      |                     | Cellulose                     | 35     |
|                      | Calcium             | Cellulose                     | 35     |
|                      | Cinamaldehyde       | Gelatin                       | 36     |
|                      | Formaldehyde        | Gluten                        | 37     |
|                      | Succinic acid       | Agar                          | 38     |
|                      | Polyphenol          | Proteins                      | 39     |
|                      | Glutaraldehyde      | Plasma                        | 40     |
| Zein/gluten          |                     | 41                            |        |
| Polysiloxane         | Cellulose           | 42                            |        |
| Isocyanate           | Agar                | 43                            |        |
|                      | Zein                | 44                            |        |
| Enzymatic            | Transglutaminase    | Gluten                        | 45     |
|                      |                     | Hemp seed                     | 46     |

Regarding the different methods of cross-linking, physical cross-linking generates new bonds by applying an additional physical process such as UV radiation or ultrasound.<sup>47</sup> As shown in Table 1, several authors have carried out different physical treatments on polymer-based materials. For example, Zhang et al. (2019) tuned lignin-based materials with UV radiation, and the studies of Nagasawa et al. (2017) dealt with polyamide 11 under an electron beam radiation.<sup>24,48</sup> However, a thermal treatment is the most used physical cross-linking method, highlighting the studies of Jiménez-Rosado et al. or Jansens et al. with soy-based and gluten-based materials, respectively.<sup>26,49</sup>

On the other hand, chemical and enzymatic cross-linking is the process of combining two molecules through a covalent bond with specific functional groups, such as primary amines or thiols.<sup>50</sup> Among the different compounds, the most commonly used for chemical cross-linking are aldehydes,<sup>51,52</sup> while transglutaminase is the most used for enzymatic cross-linking.<sup>23,53</sup> Interestingly, both chemical and enzymatic cross-linking can obtain good results in improving the mechanical properties and thermal stability of the resulting material. As shown in Table 1, there are many possibilities for performing chemical cross-linking. Examples are the studies of Zárate-Ramírez et al. with formaldehyde in gluten-based systems,<sup>54</sup> the use of glutaraldehyde in protein-based systems,<sup>40,41</sup> or the use of glyoxal to cross-link gelatin films.<sup>31</sup> There are other chemical agents that can also be used to produce cross-linking. Different acids have been widely used, such as citric acid in starch,<sup>28</sup> agarose,<sup>21</sup> and wheat protein-based materials.<sup>30</sup> Belay et al. reported the effects of succinic acid on agar,<sup>38</sup> whereas Riyajan et al. and Suhag et al. studied the effect of maleic and boric acids on polymeric materials, respectively.<sup>55,56</sup> Nevertheless, recent studies opted for using natural substances to conduct chemical cross-linking due to their nontoxicity and

eco-friendly aspects. In this sense, the study of Lee et al. with calcium to tune the properties of cellulose films can be highlighted.<sup>35</sup> On the other hand, the most used enzymes are amylose, as in the work of Sagnelli et al., or transglutaminase in gluten or hemp seed films.<sup>45,46,57</sup>

Most of the studies worked on the effect of one type of cross-linking method on polymer-based materials, and there is no compilation on the various methods of cross-linking or comparison between them. Thus, the main innovation of this work is to investigate the effects of different types of cross-linking, comparing physical, chemical, and enzymatic cross-linking. The principal objective is the analysis of several cross-linking agents on the physicochemical, mechanical, and functional properties of pea protein-based bioplastics.

## 2. EXPERIMENTAL SECTION

**2.1. Materials.** Pea protein isolate (PPI, ~90 wt % of protein) was used as raw material, purchased from Roquette (France). Furthermore, glycerol, which was provided by Panreac Quimica S.A. (Spain), was used as plasticizer.

Considering the cross-linking agents, genipin ( $\geq 98\%$  (HPLC), powder) was supplied by Guangxi Shanyun Biochemical Science and Technology Co., Ltd. (China) and commercial enzyme transglutaminase ProbindTX (TG), with an enzymatic activity of 100 units/g, was supplied by BDF Ingredients (Spain).

**2.2. Processing of Bioplastics.** Pea protein-based bioplastics were obtained by means of an injection molding process, which comprises two stages: first, a homogenization stage was performed in a HaakePolylab QC batch mixer (ThermoHaake, Germany) to produce a homogeneous blend. The blends were prepared with a 60/40 protein/glycerol ratio. Second, the blends were injected in a MiniJet Piston Injection Molding System (ThermoHaake). The processing conditions were 50 and 130 °C (as cylinder and mold temperatures) and 500 bar for 20 s and 200 bar for 200 s (as injection and postinjection conditions). Two different specimens were fabricated: (1)  $60 \times 10 \times 1$  mm<sup>3</sup> rectangular-shaped specimen and (2) dumbbell-shaped specimen (type V) according to ISO 527-1:2012.

**2.3. Cross-Linking Methods of Bioplastics.** **2.3.1. Physical Cross-Linking.** As previously commented, a physical cross-linking is carried out by adding an extra stage to the fabrication process of a material. As can be seen in Figure S1A, the application of an external energy (thermal, UV radiation, ultrasound, etc.) may induce a higher denaturation, exposing the reactive groups of the protein chains. These groups promote the formation of new intra- and interchain bonds that are mostly physical. Among them, hydrophobic interactions or hydrogen bonds are the most common.<sup>17</sup> Nevertheless, some chemical cross-linking through disulfide bonds can also be induced.<sup>58</sup>

Specifically for this work, two different physical cross-linking methods were used: UV radiation using a microprocessor-controlled UV cross-linker XLE-1000 (Select, USA) with an intensity of 120 mJ/cm<sup>2</sup> and a wavelength of 254 nm for 30 min, and a thermal treatment introducing the bioplastics in an oven at 50 °C was conducted as physical cross-linking. UV radiation facilitates protein–protein covalent interactions at amino acid level in the range between 200 and 300 nm.<sup>59,60</sup> On the other hand, thermal treatment promotes the protein–protein interactions by improving the movement and unfolding of the chains.<sup>61,62</sup>

**2.3.2. Chemical Cross-Linking.** Chemical cross-linking requires the addition of a cross-linking agent in order to carry out the reaction. This type of cross-linking generally produces strong binding sites via covalent bonds. In general, aldehydes are widely used since they can react with primary amines to induce inter- and intracross-linking within the polymer chains (Figure S1B). In the same line can be placed the studies that use genipin as a cross-linking agent. Genipin is a natural substance that induces a reaction between lysine and arginine (Figure S1B) and that presents the singularity to dye the

resulting material into a blueish hue, as reported by Whitehead et al. (2020).<sup>33</sup>

Specifically for this work, genipin at 0.50 wt % was used as a chemical cross-linking agent. For this purpose, it was included in the initial formulation prior to the mixing stage (similar protein:glycerol ratio). It is important to take into account that the addition of genipin produces a blueish color in the materials in which it is used over time.<sup>63</sup> For this reason, the aging time of the bioplastics was also studied (day 1, day 5, day 7, and day 10 after the injection molding process).

**2.3.3. Enzymatic Cross-Linking.** The enzymatic cross-linking is similar to the chemical one but with the use of an enzyme to induce the cross-linking reaction. Its main difficulty lies in the specific conditions that enzymes generally require. As an example, the mechanism of enzymatic cross-linking produced with transglutaminase is shown in Figure S1C. This enzyme catalyzes the formation of peptide bonds between protein chains, generating inter- and intracross-linking.<sup>64</sup>

Specifically for this work, transglutaminase at 0.50 wt % was used as enzymatic cross-linking. For this purpose, it was also included in the initial formulation prior the mixing stage (similar protein:glycerol ratio).

**2.4. Characterization of Bioplastics.** **2.4.1. Physicochemical Properties.** **2.4.1.1. Cross-Linking Degree.** The protocol described by Zárate-Ramírez et al. was used to determine the cross-linking degree of the different samples.<sup>37</sup> Briefly, 170–180 mg of bioplastic was denatured with 5 mL of sodium dodecyl sulfate (SDS) solution, which solubilizes the non-cross-linked protein or those physically cross-linked. Then, Lowry's method was used to determine the solubilized protein.<sup>65</sup> The cross-linking degree was calculated with respect to a reference system. In this way, pea protein bioplastic without any cross-linking stage was selected as reference (0% cross-linked). In addition, a blank (system without bioplastic) was used as 100% cross-linked (no dissolved protein).

**2.4.1.2. Color Measurements.** Color analysis was performed using a Konica Minolta CM-700D colorimeter (Japan). The values of  $L^*$  (lightness) and  $a$  and  $b$  (red, green, blue, and yellow in CIELAB color space) were determined for each system as the average of five scans. The parameter  $a$  drifts from negative values (green) to positive values (red), whereas  $b$  drifts from negative values to positive values when the sample is blue or yellow, respectively.

**2.4.2. Mechanical Properties.** **2.4.2.1. Dynamic Mechanical Analysis (DMA).** DMA tests were performed with a RSA3 analyzer (TA Instruments, USA) with a three-point bending geometry in a flexural mode. For this, rectangular probes were used. First, strain sweep tests (between 0.002–2% of strain at 1 Hz and 20 °C) were performed to obtain the linear viscoelastic range. In addition, frequency sweep tests were carried out between 0.02 and 20 Hz within the linear viscoelastic range of 20 °C.

**2.4.2.2. Tensile Tests.** Tensile tests until break were performed in a universal machine Insight 10 kN Electromechanical Testing System (MTS, USA). For this, dumbbell-shaped probes were used. Samples were subjected to an increased uniaxial force at 10 mm/min and room temperature, according to the ISO 527-2:2012 standard for tensile properties of plastics.<sup>66</sup> The parameters obtained were Young's modulus ( $E$ ), the maximum tensile strength ( $\sigma_{\max}$ ), and the strain at break ( $\epsilon_{\max}$ ).

**2.4.3. Functional Properties.** **2.4.3.1. Water Uptake Capacity and Soluble Matter Loss.** The ability to absorb water by the bioplastic was determined with the ASTM D570 standard (ASTM "Standard test method for plastic", Annual Book of ASTM Standards; American Society for Testing and Materials: Philadelphia, PA, 2005).<sup>67</sup> For this, rectangular bioplastics were first dried in an oven at 50 °C for 1 h to obtain the initial dry weight. Later, they were immersed in distilled water for 24 h in a closed vessel (wet weight). Finally, the bioplastics were dried again in the oven to obtain the final dry weight. Water uptake capacity (WUC) and soluble matter loss were determined by eqs 1 and 2:

$$\begin{aligned} & \% \text{ Water uptake capacity (WUC)} \\ &= \frac{\text{Wet Weight} - \text{Initial Dry Weight}}{\text{Initial Dry Weight}} \times 100 \end{aligned} \quad (1)$$

$$\begin{aligned} & \% \text{ Soluble material loss} \\ &= \frac{\text{Initial Dry Weight} - \text{Final Dry Weight}}{\text{Initial Dry Weight}} \times 100 \end{aligned} \quad (2)$$

**2.4.3.2. Biodegradability.** The biodegradability of the systems was measured by burying the bioplastics in a composting medium (2:1 farmland:compost, the same inert/organic materials ratio specified by ISO 20200:2004<sup>68</sup>). Bioplastics from each system were visually evaluated by unearthing them and taking pictures to be visually evaluated on different days. The trial is complete when the bioplastic could not be unearthed (total biodegradation time).

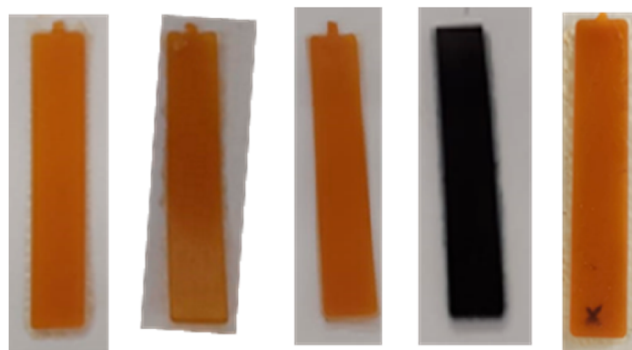
**2.5. Statistical Analysis.** Each measurement was presented as the mean of at least three replicates with their standard deviation (mean  $\pm$  SD). In addition, statistical analyses were carried out through *t* test and one-way analysis of variance (ANOVA), with selection of a confidence level of 95% ( $p < 0.05$ ). These analyses were performed in the statistical package Excel 2013 (Microsoft, Redmond, USA). Standard deviations from some selected parameters were calculated.

### 3. INFLUENCE OF CROSS-LINKING ON THE PROPERTIES OF BIOPLASTICS

In this section, the evaluation of the effect of different cross-linking methods on the properties of bioplastics is carried out.

**Table 2. Cross-Linking Degree Obtained for the Different Crosslinking Methods Carried Out**

| cross-linking method | systems                | cross-linking degree (%) |
|----------------------|------------------------|--------------------------|
| Physical             | Thermal treatment      | 16.8 $\pm$ 2.9           |
|                      | UV radiation           | 19.0 $\pm$ 6.5           |
| Chemical             | Genipin 0.50% day 1    | 47.9 $\pm$ 12.0          |
|                      | Genipin 0.50% day 5    | 52.2 $\pm$ 0.3           |
|                      | Genipin 0.50% day 7    | 49.1 $\pm$ 6.3           |
|                      | Genipin 0.50% day 10   | 46.4 $\pm$ 10.5          |
| Enzymatic            | Transglutaminase 0.50% | 30.7 $\pm$ 7.4           |



**Figure 1.** Macroscopic images of protein-based bioplastics under different cross-linking methods. From right to left: non-cross-linked, thermal, UV, genipin, and transglutaminase cross-linked bioplastics.

Their effect on the physicochemical properties (cross-linking degree and color), mechanical properties (static and dynamic), and functional properties (water uptake capacity and biodegradability) was analyzed and compared.

**3.1. Physicochemical Properties.** Table 2 shows the chemical cross-linking degree (through covalent bonds) obtained after denaturing the different pea protein-based

bioplastics, which were subjected to the different cross-linking methods.

Physical cross-linking resulted in bioplastics with a lower cross-linking degree than the chemical cross-linking studied. In the case of genipin, there were no significant differences between the bioplastics at different aging times. Considering the obtained results for genipin, all the systems presented around 50% cross-linking with respect to the reference system without any cross-linking treatment. Similar cross-linking percentages have been obtained by other authors using other chemical cross-linking agents.<sup>36</sup> On the other hand, the enzymatic cross-linking conducted with transglutaminase produced an intermediate cross-linking degree, higher than the one obtained by physical cross-linking but lower than those obtained by chemical cross-linking. These results are a culmination of the results mentioned above. In this sense, although physical cross-linking reinforces the protein matrix, this is usually due to physical interactions (hydrophobic interactions or disulfide bonds). On the contrary, chemical or enzymatic cross-linking favors the formation of covalent bonds between biopolymeric chains to a greater extent.

The changes derived from the cross-linking methods could also be visible in the morphology of the final bioplastics. Color is one of the most applied techniques in marketing, causing emotional reactions that affect consumer behavior. In fact, packaging could be the main connection between a product and the customer, communicating the qualities and benefits of the product.<sup>69</sup> Product characteristics, such as size, color or shape, provide a useful tool for marketers to differentiate their brands, enhancing the capacity to engage consumers.<sup>70</sup> Therefore, the characterization of the color of the final bioplastics is important to consider them as an engaging product. In this sense, a preliminary image of the bioplastic obtained is shown in Figure 1, verifying how the bioplastics cross-linked with transglutaminase or through a thermal treatment showed very slight changes with respect to the non-cross-linked system. However, the genipin-cross-linked system resulted in a bluish-colored material.

In this sense, the specific changes observed in the color of a material is usually measured with a colorimetry analysis evaluating  $L^*$  (lightness),  $a^*$  (negative values indicate green and positive values indicate red), and  $b^*$  (negative values indicate blue and positive values indicate yellow) in the CIELAB color space. The values obtained are summarized in Table 3, showing how the physical and enzymatic cross-linked bioplastics presented similar colorimetric values ( $a^*$ ,  $b^*$ , and  $L^*$ ) than the non-cross-linked bioplastic due to the lack of morphological changes, as shown in Figure 1. Nevertheless, the addition of genipin produced blueish systems, as shown in Figure 1. These changes were quantified as the decrease in parameter  $a^*$  and the negative values of parameter  $b^*$ . In fact, apart from the color change, there was a significant darkening of the bioplastics, as shown by the decrease of the  $L^*$  parameter.

**3.2. Mechanical Properties.** The formation of new bonds in the polymer chains as a consequence of the cross-linking processes may induce structural modifications that produce changes in the structuration of the system. Therefore, it is important to evaluate the mechanical properties of the resulting materials in order to analyze the effect of the cross-linking. In this case, the effect on the mechanical properties was studied under dynamic and static tests, as plotted in Figures 2 and 3.

Table 3. Color Parameters of Protein-Based Bioplastics Modified with Different Cross-Linking Methods<sup>a</sup>

| cross-linking method | systems                     | $a^*$         | $b^*$          | $L^*$          |
|----------------------|-----------------------------|---------------|----------------|----------------|
| Physical             | Non-cross-linked bioplastic | $3.7 \pm 0.2$ | $13.1 \pm 0.8$ | $31.5 \pm 0.6$ |
|                      | Thermal treatment           | $4.5 \pm 0.3$ | $14.8 \pm 1.3$ | $33.8 \pm 0.7$ |
|                      | UV radiation                | $3.9 \pm 0.2$ | $13.9 \pm 0.9$ | $31.6 \pm 0.2$ |
| Chemical             | Genipin 0.50% day 1         | $1.8 \pm 0.6$ | $-0.6 \pm 0.3$ | $19.2 \pm 0.1$ |
|                      | Genipin 0.50% day 5         | $2.4 \pm 0.1$ | $-0.4 \pm 0.1$ | $18.5 \pm 0.3$ |
|                      | Genipin 0.50% day 7         | $2.1 \pm 0.1$ | $-0.5 \pm 0.1$ | $20.8 \pm 0.2$ |
|                      | Genipin 0.50% day 10        | $2.1 \pm 0.1$ | $-0.5 \pm 0.2$ | $19.5 \pm 0.6$ |
| Enzymatic            | Transglutaminase 0.50%      | $3.9 \pm 0.2$ | $13.9 \pm 0.8$ | $31.6 \pm 0.2$ |

<sup>a</sup>Non-cross-linked pea protein-based bioplastic was also included as reference.

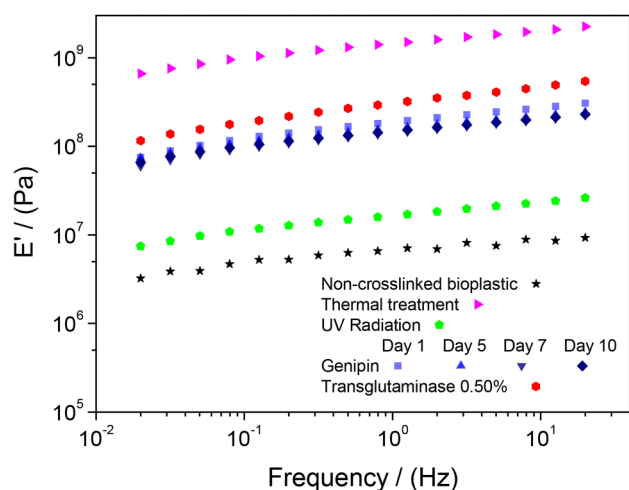


Figure 2. Frequency sweep tests of bioplastics reinforced by different cross-linking methods.

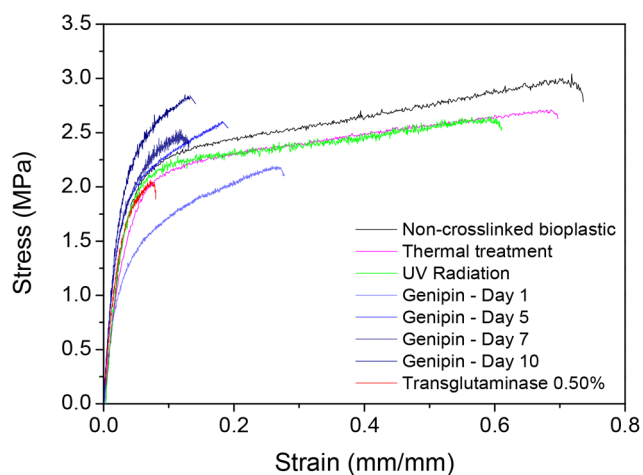


Figure 3. Tensile tests of bioplastics reinforced by different cross-linking methods.

First, dynamic frequency tests were carried out to evaluate the stability of the systems, and the results are shown in Figure 2.

Independent of the cross-linking method used, there was a reinforcement of the structure of the bioplastics obtained. This reinforcement was visible as an increase in the elastic modulus ( $E'$ ) values found in Figure 2, although the profile shown is similar to the non-cross-linked system with a slight increase in  $E'$  as the frequency increases in the range studied. Comparing the different cross-linking methods (Table 4), the addition of

either transglutaminase or genipin (0.50 wt %) produced a significant increase in the elastic modulus values at 1 Hz ( $E'_1$ ) of the cross-linked bioplastics. However, the inclusion of a heat treatment improved the flexural mechanical properties of the bioplastics, obtaining systems with  $E'$  values 1 order of magnitude higher than those chemically cross-linked. On the other hand, UV radiation increases the  $E'$  values obtained by the reference system, although not to a great extent.

On the other hand, mechanical analyses in static mode were also performed by studying tensile tests on the different cross-linked bioplastics. The stress–strain profiles can be seen in Figure 3, with the characteristic initial high-slope linear elastic region followed by a plastic region with a decreasing slope until sample breakage.

According to the results (Figure 3), the main difference lay in the length of the plastic zone, that is, in the deformability of the systems, being quite lower when transglutaminase or genipin is added to the initial formulation. This lower deformability is quantified by the strain at break (Table 4), observing how the stiffness of the cross-linked systems increased with the cross-linking reaction. Other authors have obtained similar results when applying an additional cross-linking stage to the fabrication of protein-based bioplastics, with a marked decrease in the strain at break with respect to the reference system.<sup>71,72</sup> Interestingly, the physical cross-linking (both heat treatment or UV radiation) did not cause a significant change in the strain at break of the samples compared to the chemical and enzymatic cross-linking.

On the other hand, Young's modulus and the maximum stress were also calculated from the tensile profiles shown in Figure 3. These results were also included in Table 4, showing that transglutaminase, genipin, and UV radiation increased the Young's modulus, but no significant differences were obtained in the maximum stress of the bioplastics processed under different cross-linking methods.

Thus, physical interactions seem to cause a certain structuring of the bioplastic since they cause an increase in the elastic flexural modulus, although no significant differences were observed in tensile tests. On the other hand, the covalent bonds induced by chemical or enzymatic cross-linking cause a strengthening of the bioplastics that makes them more rigid but less deformable.

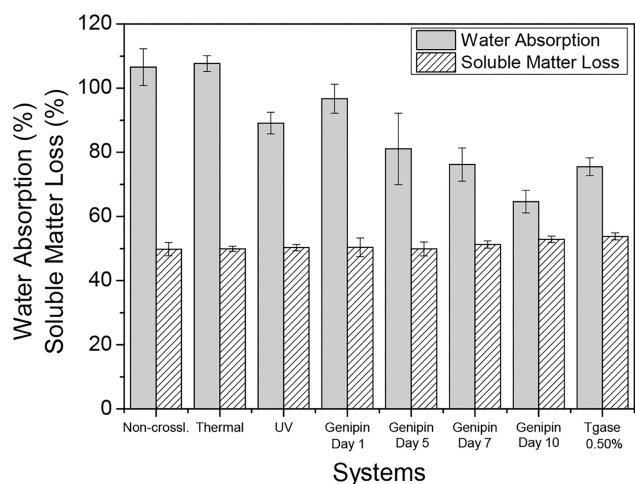
**3.3. Functional Properties.** In addition to the mechanical properties, the functional properties, of the cross-linked bioplastics were also evaluated. In this sense, their water uptake and biodegradability were measured and compared. Figure 4 shows the water absorption capacity of bioplastics after different cross-linking methods.

Comparing the different results, UV cross-linking produced significant differences in water absorption compared to the

**Table 4. Dynamic Flexural Parameters (Elastic Modulus at 1 Hz:  $E'_1$ ) and Tensile Parameters of Protein Bioplastics Modified with Different Cross-Linking Methods<sup>a</sup>**

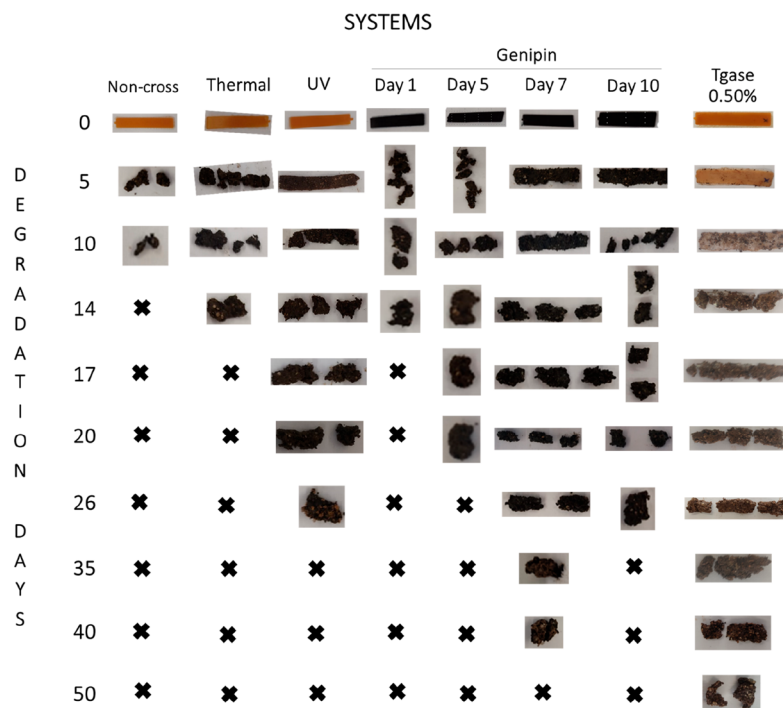
| cross-linking method | systems                     | $E'_1 \times 10^{-8}$ (Pa) | $E$ (MPa)           | $\sigma_{\max}$ (MPa) | $\epsilon_{\max}$ (mm/mm)    |
|----------------------|-----------------------------|----------------------------|---------------------|-----------------------|------------------------------|
| Physical             | Non-cross-linked bioplastic | $0.1 \pm 0.1^a$            | $59.6 \pm 8.9^A$    | $2.8 \pm 0.3^{\#}$    | $0.7 \pm 0.1^c$              |
|                      | Thermal treatment           | $26.1 \pm 0.7^b$           | $58.7 \pm 10.4^A$   | $2.6 \pm 0.2^{\#}$    | $0.7 \pm 0.1^c$              |
|                      | UV radiation                | $0.8 \pm 0.2^c$            | $70.2 \pm 7.9^{AB}$ | $2.2 \pm 0.1^{\#}$    | $0.6 \pm 0.1^{\beta}$        |
| Chemical             | Genipin 0.50% day 1         | $1.9 \pm 0.1^d$            | $56.6 \pm 10.8^A$   | $2.2 \pm 0.1^{\#}$    | $0.3 \pm 0.1^{\gamma}$       |
|                      | Genipin 0.50% day 5         | $1.6 \pm 0.1^e$            | $79.9 \pm 9.6^{AB}$ | $2.6 \pm 0.2^{\#}$    | $0.2 \pm 0.1^{\gamma}$       |
|                      | Genipin 0.50% day 7         | $1.5 \pm 0.1^e$            | $84.1 \pm 10.1^B$   | $2.4 \pm 0.2^{\#}$    | $0.1 \pm 0.1^{\gamma\delta}$ |
|                      | Genipin 0.50% day 10        | $1.5 \pm 0.1^e$            | $89.9 \pm 6.8^B$    | $2.5 \pm 0.4^{\#}$    | $0.1 \pm 0.1^{\gamma\delta}$ |
| Enzymatic            | Transglutaminase 0.50%      | $2.3 \pm 0.6^d$            | $90.9 \pm 17.6^B$   | $2.3 \pm 0.6^{\#}$    | $0.1 \pm 0.1^{\delta}$       |

<sup>a</sup>Non-cross-linked pea protein-based bioplastic was also included as reference. Different symbols were included as superscripts to denote significant differences between the values for each column.

**Figure 4.** Water absorption and soluble matter loss obtained for bioplastics under different cross-linking methods.

non-cross-linked bioplastic ( $\sim 90\%$  for the cross-linked system and  $\sim 110\%$  for the reference one). However, for the chemical and enzymatic cross-linking, a progressive decrease was observed, going from  $\sim 110\%$  for the reference system to  $\sim 65\%$  for the cross-linked bioplastic after 10 days with genipin or  $75\%$  when transglutaminase is used. However, the soluble matter loss remained constant at  $\sim 50\%$ , which means that apart from glycerol, a small amount of protein was also lost during immersion. These results are corroborated by other authors, such as Jia et al., who reported that the addition of cinnamaldehyde as cross-linking agent produced a decrease in water absorption.<sup>36</sup> This behavior could be due to the greater rigidity observed in these bioplastics (shown in the mechanical analysis), which does not allow them to swell to absorb and retain water inside. In the same sense, the physically cross-linked bioplastics do not show any differences from the reference ones.

The biodegradability of the cross-linked bioplastics was also evaluated. The results obtained can be seen in Figure 5. A

**Figure 5.** Biodegradation of bioplastics modified with different cross-linking methods. The soil adhering to the surface of the bioplastics has not been removed for these photos to not alter the biodegradation process.

complete degradation after 10 days was achieved by the reference non-crosslinked bioplastic. Nevertheless, as shown by Awadhiya et al., the inclusion of a cross-linking stage extended their degradation time.<sup>21</sup> In this study, the degradation time was 14–26 days for the physical cross-linking (thermal and UV radiation, respectively) and 45–50 days for chemical cross-linking. Indeed, the addition of genipin produced a delay in the degradation of the samples, which extended with the cross-linking time. In this sense, in terms of biodegradation, bioplastics after 7 days of cross-linking are the ones that presented the best results with a degradation time of 50 days. It should be noted that although the time to biodegrade increases in the cross-linked bioplastics, they are still biodegradable and compostable according to the ASTM D64000 standard.

#### 4. CONCLUSIONS

Bioplastics with enhanced properties can be obtained by carrying out an additional cross-linking stage to the fabrication process. For example, physical cross-linking methods improved the mechanical properties and biodegradation of the bioplastics. Furthermore, the addition of genipin produced systems with a bluish color, higher Young's modulus, and lower deformability, being more significant with the cross-linking time. On the other hand, the addition of transglutaminase increased the rigidity of the systems (and thus lowered their deformability) and decreased their water uptake capacity. Furthermore, it delayed their biodegradability up to 45–52 days. Thus, cross-linking can improve the properties of bioplastics, expanding their versatility in the market.

#### ■ ASSOCIATED CONTENT

##### SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsapm.3c02737>.

Example of a physical cross-linking using UV radiation or temperature (Figure S1A); example of a chemical cross-linking reaction using aldehydes or genipin as cross-linking agent (Figure S1B); example of an enzymatic cross-linking reaction using transglutaminase as cross-linking agent (Figure S1C) (PDF)

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#### Notes

The authors declare no competing financial interest.

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