Improvement of Water Resistance of Vegetable Proteins by the Use of Synthetic Origin Additives: Trials with Resins and Metal Ions

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Abstract: The adhesives industry is increasingly interested in products coming from natural and renewable resources. The aim of the present work was to improve the water resistance of soy-based proteins by using synthetic and formaldehyde-free additives. These include polyamide-amine epichlorohydrin (PAE), different types of isocyanates, and combinations of these cross-linkers between them and with other agents, including metal ions. In addition, the effect of both curing temperature and maturation time was assessed. Performances were evaluated by means of shear strength tests, solubility tests, and spectroscopic analysis. The obtained results showed that while isocyanates reacted completely but with water instead of proteins, tests with PAE were generally successful. In fact, the insoluble residue as well as the shear strength in wet conditions dramatically increased after PAE addition. Moreover, the wet performances of protein/PAE formulations appreciably increased gluing at 60 °C instead of room temperature. Furthermore, the maturation time had a positive effect on the formulations where metal ions were added, both for solubility and wet shear strength. Actually, for the very long conditioning time of 3 months, a significant and substantial increase of wet shear strength was observed for the series protein/PAE/aluminum.

Keywords: glues; bio-based adhesives; natural glues; soy; isocyanates; polyamide-amine epichlorohydrin; PAE; HDI; pMDI

1. Introduction

Research and market trends in the wood adhesives sector have led to an increasing search for alternatives to formulations containing formaldehyde due to their negative impact on the environment and human health. As a matter of fact, this product has been recently classified as a carcinogenic agent [1]. Within this framework, protein-based adhesives have received much interest due to their availability, excellent performances in dry conditions, and provenance from renewable sources [2–10].

Soy is the most popular crop for the development of biobased adhesives due to its relationship between cost, availability, biodegradability, and performance. A SWOT analysis (strengths, weaknesses, opportunities, and threats) carried out on soy protein-based adhesives evidenced that strengths are mainly based on their renewability and absence of formaldehyde in formulations; weaknesses include high viscosity with low solid content and long pressing time due to water excess; opportunities are the crop availability and stringent legislation on volatile organic compounds (VOCs) for wood products; threats are related to cost (compared with UF resins, which are relatively inexpensive) and climatic factors (such as droughts or floods) [11].

However, although it has been previously shown that several other crops have high potential as possible wood adhesives [12], there are still many aspects that appear critical...
in the use of such products. In particular, the poor resistance to moist conditions leads to the necessary use of crosslinking agents to improve resistance. The reasons of the poor resistance of pure vegetable proteins towards water are related to the presence of chains of groups capable of interacting with water molecules. Thus, when in contact with moisture, the formed film (even if dried) swells and loses mechanical characteristics, thus inducing failure of the bonded joint.

Thus, efforts are currently directed towards the use of curing agents, evaluating several products and combinations as possible cross-linkers and investigating the effect of these agents on the bonding performances in wet conditions.

The use of isocyanates to crosslink proteins has been reported in the literature. Lei et al. [13] used polymeric 4,4′-diphenylmethane diisocyanate (pMDI) to crosslink wheat gluten previously reacted with formaldehyde or glyoxal. Gao et al. [14] formulated adhesives for glulam with serum proteins, using pMDI as the crosslinking agent of these proteins. However, this study was limited to one single type of isocyanate, whereas there are several types that could be evaluated. In general, isocyanates are able to react with both the amine and the hydroxyl groups of proteins (Figure 1). Yet, the reactivity is greater for amines rather than for hydroxyls, and therefore, mainly the basic amino acids react with isocyanates to form the urea linkage (Path (a) in Figure 1).

Recently, a soy protein-based product providing the use of soy flour with a particular polyamide-amine epichlorohydrin (PAE) resin was developed [15]. It has received much attention in the wood industry because it is able to form a water-resistant adhesive. This soy/PAE adhesive is able to crosslink without the use of formaldehyde, and therefore, it is particularly suitable for the use in interior applications.

PAE is a resin normally used in the paper industry. The commercial product contains the resin in cationic form. The reactive group is the azetidinium ring (Figure 2). It is usually considered that it can give origin to reactions involving an amine group of PAE (homo-crosslinking) or some carboxylic acids of amino acid residues or (potentially) wood components [16] (Figure 2). Further potential curing agents (even in combination with the ones mentioned above) are continuously tested in the attempt to decrease the amount of cross-linkers (that are additives of synthetic origin) in the final formulation as possible wood adhesives.

It has been previously shown that guanidine hydrochloride is able to denature proteins, thus making potentially more reactive the protein groups toward the isocyanate [17,18]. Moreover, it is possible (at least in principle) to make PAE interact with other substances, such as tannins and ions of bi- or trivalent metals, as it is known that they are able to make complexes with amines and other functional groups of amino acids [19,20]. Thus, they could hypothetically interact with the amino groups present in the PAE.
The aim of the present work was improving the water resistance of natural proteins by using synthetic and formaldehyde-free additives. These include already tested products (such as PAE), different types of isocyanates, and combinations of these cross-linkers between them and with other possible agents capable of interacting with proteins, such as guanidine hydrochloride and metal ions. In addition, the effect of selected parameters (including both curing temperature and maturation time) on the bonding performances in wet conditions was assessed. Performances were evaluated not only with reference to mechanical strength but also carrying out solubility tests on dried films and spectroscopic analysis.

This work is part of a wider research plan aimed at modifying vegetable proteins to obtain high-performance wood adhesives of a semi-structural type.

2. Materials and Methods

2.1. Materials

Two kinds of soy (*Glycine max* L.) proteins were used in the present work:

- ProCote 5000 (PC5), pre-treated with alkali directly by the producer;
- ProCote E115702 (PCE), which needs to be treated with alkali by the user.

Both proteins were provided by DuPont (St. Louis, MO, USA) and had the same amino acidic profile. The only difference is that due to the elimination of NH₄OH during the manufacturing process, PCE needs a preliminary alkalinisation treatment to be solubilised in water [21].

Two families of formaldehyde-free synthetic cross-linkers were selected as protein additives:

- Several types of isocyanates, provided by Bayer AG (Leverkusen, Germany), whose main characteristics have been reported in Table 1;
- A resin based on polyamide-amine epichlorohydrin (PAE), which is normally used to enhance the water resistance of paper. It was provided by Mare Spa (Ossona, Italy) (commercial name Maresin VHP 200) and had a solid content of 20%.
Table 1. Commercial names and main characteristics of the isocyanates used in the present work.

| Commercial Name | Abbreviation | NCO Content (%) | Type                                                                 |
|-----------------|--------------|-----------------|----------------------------------------------------------------------|
| Bayhydur® 3100  | BAY          | 17.4            | Water-dispersible aliphatic diisocyanate (having hexamethylene diisocyanate, HDI, as the basis) |
| Desmodur® VK 10 | DVK          | 31.5            | Aromatic diisocyanate with highly functionalised isomers and homologues (polymeric diphenylmethane-4,4'-diisocyanate, pMDI) |
| Desmodur® DA/L  | DAL          | 20.0            | Water-dispersible hydrophilic polyisocyanate (having hexamethylene diisocyanate, HDI, as the basis) |
| Desmodur® DN    | DN           | 21.8            | Water-dispersible and low-viscosity hydrophilic aliphatic polyisocyanate (having hexamethylene diisocyanate, HDI, as the basis) |

Other additives were also used in combination with the previous ones. Guanidine hydrochloride (GH) was provided by Sigma-Aldrich (St. Louis, MO, USA); Quebracho tannin was provided by Silvateam SpA (San Michele Mondovì, Italy). Moreover, as regards the evaluation of metals to improve the water resistance, 30% solutions of CuSO₄ (corresponding to 1.8 M), Al₂(SO₄)₃ (2.4 M), and FeCl₃ (1.8 M) were used with the PCE plus PAE combination.

2.2. Formation of Films and Solubility of Dried Films

PCE protein mixtures were prepared by solubilising 20% of PCE in demineralised water and regulating the pH to 9 in order to increase the solid content. PC5 protein was prepared in a similar way, except for the value of pH, which was not adjusted. These films were considered as references.

2.2.1. Isocyanates

In order to select the best isocyanate among the considered ones, in a first phase, some films were formed by adding all of them to PC5. In a 10% mixture of proteins in deminerised water, the various isocyanate-based products were added at a 10% proportion with respect to the dry content of protein; then, the whole mixture was kept under stirring at 55 °C. After 2 h, each mixture was poured into Petri dishes and allowed to air dry for 7 days. In such a way, the general appearance of films was visually evaluated.

Furthermore, new dried films prepared with the selected cross-linkers were subjected to solubility tests. These new films were prepared by mixing the protein with water (20%) under stirring at 55 °C for 2 h and by adding the selected diisocyanate at 10% with respect to the dry weight of the protein. After drying in air for 7 days, films were kept for 1 h in boiling water under stirring and left to stay overnight. Then, the insoluble fraction was dried in an oven at 103 °C and weighed.

In addition to tests with the selected isocyanates, the combination of PC5 with BAY plus guanidine hydrochloride (GH) was considered as well. In this case, protein was first added to a 1 M aqueous solution of GH and kept under stirring for 6 h; then, BAY was added.

2.2.2. PAE

Tests with the PAE resin were carried out on both proteins, PC5 and PCE.

First, 5 g of protein was added to 30 mL of a 12% solution of PAE and to 6 g of water (with PCE the solution was correct to pH 9), and the mixture was left under stirring for 2 h at room temperature (ca. 24 °C), according to what was suggested by Li et al. [15]. In such a way, the ratio between protein and PAE was 1.33, and the solid content of the prepared mixture was 20%. This also constituted the reference formulation for all following PC5/PAE mixtures.
A part of that mixture was put into a Petri dish and dried under vacuum at room temperature in order to obtain a film. The remaining part was put in an oven at 103 °C. The solubility tests of dried aggregates were carried out for both the vacuum-dried and the oven-dried films, and the followed procedures were the same as for the addition of isocyanates.

The 30% solutions of the considered salts (CuSO$_4$, Al$_2$(SO$_4$)$_3$, and FeCl$_3$) were added to the mixture of PCE with PAE (ratio PCE:PAE of 1.33, total solid content 36%, brought to pH 9) with the proportion between solution and PCE/PAE of 7:93, as suggested in [22]. Moreover, in additional tests, the same formulations were heated for one night (16 h) at 60 °C before performing the solubility tests.

Some attempts to prepare formulations in which metal salt solutions were put directly into the protein suspension, in the absence of PAE, were also carried out. However, in these cases, the protein precipitated after the metal addition, leading to the mixture separation. This also prevented the application to wood surfaces for the measurement of the adhesive properties (Section 2.4).

Cu and Al ions were also used to assess the effect of the metal concentration on the water solubility of dried films. They were carried out in parallel on three types of films: one prepared according to the formulation described above, one with a double amount of the considered ion, and another with half amount, while keeping constant the amount of PAE. In addition, these series were heated at 60 °C for 16 h (overnight) before tests.

2.3. FT-IR Analysis

Samples for FT-IR spectroscopic measurements were obtained from small portions of the films prepared for the solubility tests. Spectra were acquired on a Bruker Optics Alpha FT-IR spectrometer (Ettlingen, Germany) with the following settings: 40 scans per sample, spectral resolution: 4 cm$^{-1}$, wave number range: 4000 to 400 cm$^{-1}$, by using a diamond single reflection Attenuated Total Reflectance (ATR) device. Spectra were acquired and processed using the software OPUS 6.5 (by Bruker Optics, Ettlingen, Germany), without any preliminary treatment of samples.

2.4. Preparation of Mixtures for Shear Strength Tests

Protein-based adhesives were prepared as specified in Section 2.2. PCE and PC5 protein mixtures were prepared by solubilising 20% of protein in demineralised water and regulating the pH to 9 only in PCE mixtures.

Mixtures with additives were prepared as specified in the following sections.

2.4.1. Isocyanates

Several combinations were tested, which are all reported in Table 2. The reference series (batches RD and RE) were prepared as specified in Section 2.2 for the solubility test: mixing the protein with water (20%) under stirring at 55 °C for 2 h and adding the selected diisocyanate at 10% with respect to the dry weight of the protein. Only BAY and DAL were selected among the considered isocyanates.

Table 2. Batches prepared to evaluate the shear strength of wood assemblies glued with protein PC5 and isocyanates.

| Series          | Reference | GH (1 M) | PAE |
|-----------------|-----------|----------|-----|
| PC5 BAY (10%)   | RD        | RA       | SD1 |
| PC5 DAL (10%)   | RE        | RB       | –   |

Series with GH (batches RA and RB) were prepared by hydrolysing an 18% mixture of PC5 directly into a 1 M solution of GH, in place of water, for 2 h at 55 °C.
Series in which PAE was also used (batch SD1) was prepared by mixing 4 g of PC5, 15 g of the 20% PAE solution, and 16 g of water. BAY (10% relative to the mixture solid content) was added just before spreading the whole mixture onto the wood surface.

2.4.2. PAE

Tests with the PAE resin were carried out on both considered soybean proteins, PC5 and PCE (Table 3). The reference series were prepared as specified in Section 2.2: first, 5 g of protein were added to 30 mL of a 12% solution of PAE and to 6 g of water (with PCE the solution was corrected to pH 9), and the mixture was left under stirring for 2 h at room temperature.

Table 3. Batches prepared to evaluate the shear strength of wood assemblies glued with soybean proteins (either PC5 or PCE) and PAE. For each batch, various pressing times, and/or curing temperature, and/or test conditions were adopted (see Tables 8 and 9 for details).

| Series          | No Other Additives | Al  | Cu  | Fe  |
|-----------------|--------------------|-----|-----|-----|
| PC5 + PAE       | QE, QI, QP, RU1, RV1 | –   | –   | –   |
| PCE + PAE       | TR2                | TQ2 | TS1 | TS2 |

Formulations with metal solutions added to PAE and PCE mixtures (batches TQ1, TQ2, TR1, TS1, TS2, and TT) were also prepared as for solubility tests: a 30% solution of the considered salts (CuSO$_4$, Al$_2$(SO$_4$)$_3$, and FeCl$_3$) were added to the mixture of PCE with PAE with the proportion between metal solution and PCE/PAE of 7:93.

2.5. Shear Strength Tests on Glued Wood Assemblies

Shear strength tests were carried out according to standard EN 205 [23], in which the specimen geometry is the same as EN 302-1 [24], which is intended for structural adhesives [25]. Gluing conditions were the following: glue quantity 200 g/m$^2$, spread on each side of the assemblies, open and closed times 0 min (nominal value), bonding pressure 1.5 MPa. This latter value was higher than those commonly used for wood. It was selected because it was previously shown that performances of wood joints glued with protein adhesives appreciably increased by using this value [14]. Assemblies were prepared by gluing beech (Fagus sylvatica L.) thin boards, thickness 5 mm, conditioned at 20 °C and 65% r.h. until equilibrium. The boards were sanded with paper 150 just before gluing [26]. All tests were carried out on a universal testing machine (Instron 5557, load capacity 30 kN, precision class 0.5%).

For assemblies glued at room temperature, two series of glued joints were tested:

- Test type $\tau$$_{\text{DRY}}$, where specimens were conditioned for 7 days in standard climate (20 °C and 65% r.h.) before measuring dry shear strength values;
- Test type $\tau$$_{\text{WET}}$, where specimens were tested in wet conditions after 4 days of immersion in water at room temperature, according to EN 204 [27].

For assemblies glued at 60 °C (series prepared with PCE + PAE), only the test type $\tau$$_{\text{WET}}$ was evaluated.

2.5.1. Evaluation of the Maturation Time (Series Prepared with PCE + PAE)

In general, for the samples of these series wood assemblies were kept for 16 h at 60 °C after 4 days from bonding (see Figure 3 for better clarity). Furthermore, to evaluate the “maturation” time (in fact, proteins are macromolecules, and therefore, their reactions and rearrangements could be slow processes), specimens were divided in two groups (Figure 3).
The first group (RW1) was soaked in water after one week from gluing (Figure 3), and samples were tested wet.

The second one (group RW2), instead, was soaked in water after two weeks from gluing, that is, assemblies were left an additional week at 20 °C and 65% r.h. in undisturbed conditions (Figure 3). These samples were also tested wet.

Furthermore, in one batch (TT), the conditioning phase at 20 °C and 65% r.h. after temperature treatment (Figure 3) was extended at 3 months before immersing the specimens in water, in order to evaluate the long-term effect of maturation on the mechanical performances.

2.5.2. Evaluation of the Curing Temperature (Series Prepared with PCE + PAE)

The effect of curing temperature was further assessed for the mixture between PCE and PAE to which only Al was added. Thus, the additional batches TS2 and TS1 (Table 3) were prepared keeping the assemblies at 80 and 100 °C, respectively, instead of 60 °C (batch TQ2).

3. Results and Discussion

3.1. Formation of Films and Solubility Tests of Dried Films

3.1.1. Isocyanates

The films formulated with the selected isocyanates are visible in Figure 4. It can be seen that:

- PC5 plus Desmodur DN (DN) formed a cohesive and transparent film;
- PC5 plus Desmodur DA/L (DAL) formed a cohesive film with lot of bubbles;
- PC5 plus Desmodur VK 10 (DVK) formed a cohesive film that, however, presented an insoluble part at the bottom;
- PC5 plus Bayhydur 3100 (BAY) formed a cohesive film.

Therefore, the most homogeneous films were obtained using PC5 with both BAY and DN, whereas DAL had apparently reacted with water, thus producing CO₂ that caused the formation of bubbles. It is worth noticing that the observed behaviour was not simplistically related to the NCO content of the used isocyanate (Table 1).

The results of the solubility tests evidenced how protein-isocyanate films partially dissolved in water; that is, they had the same behaviour as PC5 prepared without any additive addition (data not shown). Only the combination of PC5 with GH and BAY showed an appreciable amount of insoluble residue (36%) comparable to unmodified PC5. Therefore, in solubility tests, the addition of isocyanate did not bring the expected benefits in terms of water resistance. This behaviour is related to the higher reactivity of water compared to the amino group of proteins, at least under the reaction conditions used here.
3.1.2. PAE

Films of both PC5 and PAE, although not uniform, were rigid after drying. The results of the solubility tests of dried films (Table 4) showed that the presence of PAE dramatically increased the insoluble residue of PC5 as such (from 0% to 82%). Therefore, this occurrence demonstrates that PAE reacts at least in part with the proteins, thus limiting the access of their hydrophilic groups by water molecules during solubility tests. Moreover, the same PC5 increased its insoluble residue when dried in an oven at 103 °C compared to vacuum-dried films (series ‘PC5 103 °C’ in Table 4), passing from 0% to 52%. This is due to the increase of the cross-linking density of the film as the free groups of the proteins could react with each other [28]. On the other hand, oven drying the PC5/PAE mixtures (series ‘PC5 PAE 103 °C’ in Table 4) did not lead to insolubility increases of similar entity (that is, the insoluble residue only passed from 82% to 85%) compared to vacuum-dried film. Thus, the high-temperature treatment only influences the protein as such (not the progression of the reaction with PAE), at least in the short-term period.

Table 4. Insoluble residue after the high-temperature dissolution test in water for the films of PC5 added with PAE.

| Film                          | Insoluble Residue, % |
|-------------------------------|----------------------|
| PC5 vacuum dried              | 0                    |
| PC5 103 °C                    | 52                   |
| PC5 PAE vacuum dried          | 82                   |
| PC5 PAE 103 °C                | 85                   |

The addition of metals into the protein PCE/PAE implied values of the insoluble residue that did not substantially differ from those of the series ‘PCE PAE’ alone (Table 5): this can be also appreciated in Figure 5, where it is shown that all solutions were transparent, and the films well preserved, after one hour of boiling. It is worth adding that proteins in
which copper was used became appreciably green. This is due to the reaction of copper with the amine groups of both PAE and proteins (a complex with the biuret is formed). It can be also seen from Table 5 that moderate-term exposure to 60 °C did not appreciably change the solubility of PCE/PAE/metals series (the insoluble fraction was kept at a value of approximately 80%). Thus, the obtained results imply that even if a complex between metal and protein would have been formed, its effect is surpassed by the one inferred by PAE.

**Table 5.** Insoluble residue after the water solubility tests on PCE with the addition of PAE and different metal ions. Films dried at room temperature and for 16 h (overnight) at 60 °C.

| Films Dried at Room Temperature | Insoluble Residue, % | Films Kept at 60 °C Overnight | Insoluble Residue, % |
|--------------------------------|----------------------|-------------------------------|----------------------|
| PCE PAE                        | 81                   | –                             | –                    |
| PCE PAE Cu                     | 83                   | PCE PAE Cu                   | 82                   |
| PCE PAE Fe                     | 78                   | PCE PAE Fe                   | 79                   |
| PCE PAE Al                     | 78                   | PCE PAE Al                   | 76                   |

**Figure 5.** Appearance of the water solutions after one hour of boiling for the solubility tests carried out on samples to which metals have been added to PCE: the solutions are quite clear, meaning that the soluble fraction was very limited.

It is also interesting to look at the effect of the metal concentration in the formulation (Table 6). The results showed that the amount of insoluble residues did not vary very much between the minimum and the maximum value of the used metal, irrespective of the considered ion (similar results were obtained for both Cu and Al). This occurrence indicated that a metal excess (or lack) does not provide any benefits in terms of water resistance due to the limited contribution of the protein–metal complex (provided that it actually forms in the presence of PAE). Moreover, it can be suggested that the overabundant part of the salt was solubilised in water during the leaching procedure, and this slightly increased the apparent solubility of films (Table 6).

3.2. FT-IR Analysis

Spectra acquired on the PC5/BAY film and on the separate compounds are shown in Figure 6, whereas the related band assignments are summarised in Table 7.

Considering the reactivity of HDI, which is the basis of BAY, two different reactions could be theoretically expected in our conditions:

- When the isocyanate group reacts with a hydroxyl group from the protein, the urethane linkage is formed; see Path (b) in Figure 1. The characteristic bands of the urethane group fall at 3470 cm$^{-1}$ (NH str.), 1510 cm$^{-1}$ (NH str. amide II), and ca. 1720 cm$^{-1}$ (C=O group) [29]);
- If the isocyanate group reacts with an amine (either from the amino acid of the protein or from the isocyanate hydrolysis reaction), the urea group is obtained; see Paths (a)
and (d) in Figure 1. In this case, the characteristic vibrations are at 3300 cm$^{-1}$ (NH str.), 1640 cm$^{-1}$ (C=O group), and 1560 cm$^{-1}$ (NH str. amide II) [14].

Table 6. Insoluble residue after the water solubility tests for PCE added with PAE and with metal ions at different concentrations. In the table, the symbol “1” indicates the use of the amount provided for by the reference formulation, “2” indicates a double quantity of metal compared to the reference formulation, “0.5” indicates half the amount. All films were kept at 60 °C for 16 h (overnight) before performing the solubility tests.

| Films with Varying Cu Amounts and Kept at 60 °C Overnight | Insoluble Residue, % | Films with Varying Al Amounts and Kept at 60 °C Overnight | Insoluble Residue, % |
|----------------------------------------------------------|----------------------|----------------------------------------------------------|----------------------|
| PCE PAE Cu 0.5                                           | 78                   | PCE PAE Al 0.5                                           | 78                   |
| PCE PAE Cu 1                                            | 79                   | PCE PAE Al 1                                             | 76                   |
| PCE PAE Cu 2                                            | 74                   | PCE PAE Al 2                                             | 74                   |

Figure 6. Spectra of: pure protein PC5 (blue curve); pure isocyanate BAY (orange curve); the PC5/BAY aggregate (red curve).

Table 7. Main signals related to the IR spectrum of PC5/BAY.

| Wavenumber, cm$^{-1}$ | Band Assignment                                                                 |
|----------------------|---------------------------------------------------------------------------------|
| 3346                 | NH str., secondary ammine [30]                                                  |
| 3275                 | str. N–H, band A of proteins                                                    |
| 2900                 | CH str.                                                                          |
| 2260                 | C=O str. in CNO [31]                                                            |
| 1680                 | C=O str. in CNO isocyanurate ring (trimer) [31]                                  |
| 1630                 | amide I, C=O str.                                                               |
| 1530                 | amide II, N–H bend combined with C–N str. [30]                                  |
| 1460                 | deformation in CH$_3$ and CH$_2$                                                |
| 765                  | C=N=O in plane def. [29]                                                         |

Observing the spectrum of the film, no bands due to the urethane bond could be detected (Figure 6): in fact, at least the band corresponding to the C=O group, at ca. 1720 cm$^{-1}$ should have been clearly visible, and instead, it could not be observed. In contrast, the band of the NCO group at 2257 cm$^{-1}$ (present in the BAY as such, Figure 6 and Table 7) completely disappeared (Figure 6). This occurrence implied that the isocyanate group reacted, forming the urea group (see Paths (a) and (d) in Figure 1). However, in our
case, the absorption at 1640 cm\(^{-1}\) can be assigned to the C=O stretching mode of both urea and protein [14], so that the characteristic bands of urea were completely masked by the protein amide bands (Figure 6). A similar observation was also reported in [14], although in that case, the characteristic band of isocyanate was still present at 2260 cm\(^{-1}\), while in our case, it is completely absent, which is probably due to the complete reaction with water.

As mentioned previously (see the reaction scheme shown in Figure 2), PAE can react with carboxylic acids to form ester groups. The related band appears in fact in the aggregate PC5/PAE spectrum as a shoulder at ca. 1720 cm\(^{-1}\) (Figure 7), indicating that a crosslinking reaction has taken place. As in the case of isocyanates, basing on IR spectra, it is not possible to establish whether this is a homo-crosslinking between terminal carboxylic groups of PAE or between PAE and carboxyl groups of amino acids (e.g., glutamic acid and aspartic acid or chain terminal) of the protein.

![Figure 7](image_url)

**Figure 7.** Spectra of: pure protein PC5 (blue curve); the PC5/PAE aggregate (red curve).

Spectra of the PC5/BAY/PAE aggregate show that the main reaction comes from PAE since the spectra is quite similar to PC5/PAE (Figure 8). Again, any bands related to the polyurethane bond appeared, confirming that the isocyanate groups react faster with water and hydroxyl groups rather than amine groups also in case of higher amount of amine group due to PAE.
3.3. Shear Strength Tests on Glued Wood Assemblies

3.3.1. Isocyanate

Mechanical characteristics of the joints prepared with isocyanates are shown in Table 8. For all considered series, the average strength values in dry conditions were above the minimum threshold (10 MPa) given in [27]. The observed differences among the four series were statistically not significant (ANOVA analysis gave a probability >> 0.05 for the null hypothesis). However, for the series prepared with both BAY and DAL, during tests in wet conditions, most of the specimens broke in the course of the immersion phase in water or when put into the clamp of the dynamometer, and for this reason, the results for those series are not reported in Table 8. Therefore, tests in wet conditions were shown to be much more severe than the dry ones. It is worth noting that analogous results were obtained for PC5 without any additive (as also found previously [12]).

Table 8. Results of shear strength in both standard ($\tau_{\text{DRY}}$) and wet ($\tau_{\text{WET}}$) conditions for the protein PC5 to which isocyanates (BAY, DAL), guanidine (GH), and PAE were added.

| Series   | Batch | Additive | Pressing Time (h) | Test Type | Average Value (MPa) | Standard Deviation (MPa) |
|----------|-------|----------|-------------------|-----------|---------------------|--------------------------|
| PC5 BAY  | RD    | BAY      | 16                | $\tau_{\text{DRY}}$ | 12.5              | 0.8                      |
| PC5 DAL  | RE    | DAL      | 16                | $\tau_{\text{DRY}}$ | 11.3              | 0.6                      |
| PC5 GH BAY | RA    | GH + BAY | 4                 | $\tau_{\text{DRY}}$ | 12.8              | 2.1                      |
| PC5 GH DAL | RB    | GH + DAL | 4                 | $\tau_{\text{DRY}}$ | 11.8              | 1.3                      |
| PC5 PAE BAY | SD1   | PAE + BAY | 16              | $\tau_{\text{WET}}$ | 3.4               | 0.6                      |

Considering that the amount of isocyanates reported in the scientific literature is usually not more than 20% [32], it can be assumed that our results could be improved by using a higher amount of additive in the formulation. However, in the present case, it was decided to use a maximum amount of 10% in order to minimise the presence of extraneous substances deriving from non-renewable sources in the formulation of our bio-adhesives.

Mechanical tests confirmed film solubility results about the fact that the reactivity of BAY was not directed towards the protein but to water and perhaps guanidine, where
present. This latter molecule is in fact rich in amino groups and with a smaller size with respect to protein. In both cases (water and guanidine), the reaction pattern does not bring to a more water-resistant aggregate. Even the samples with the addition of Quebracho tannin (batch SF1) broke during the immersion phase in the bath (this series was only prepared for the test in wet conditions, and hence, it is not shown in Table 8). Therefore, the introduction of additional hydroxyl groups, present in tannins, did not improve the joint performance, which is probably due to the low reactivity of these compounds.

The only formulation where the mechanical performance in wet conditions was appreciably improved was that one making use of PAE, which had an average value of 3.4 MPa (Table 8). Thus, this fact, together with the evidence of IR measurements (Figure 7), shows that the presence of PAE helped in the decreased presence of free carboxyl groups in the aggregate, with the consequent increase of ester bonds. Therefore, the cured network made the adhesive less hydrophilic (and hence more water-resistant).

3.3.2. PAE

Formulations with PC5 and PAE were used to state the optimal press conditions. Shear strengths evidenced that while prolonging pressing times at room temperature did not bring any substantial improvement in the dry shear strength of the joints (already after 2 h, values were higher than 10 MPa, which was the threshold value prescribed in EN 204 [27]), a time of 16 h was needed to reach the value of 2.3 MPa in wet conditions (the limit threshold being 2 MPa). It is worth noting that this value could be further improved by adding BAY to the formulation (Table 8). However, in both cases (use of either PAE or PAE/BAY as additives), the limit threshold prescribed in EN 301 [33] (intended for structural uses) in wet conditions, 6 MPa, was never reached.

The wet performances of PCE/PAE assemblies were appreciably increased up to 5.2 MPa by gluing the same assemblies at 60 °C (Table 9). However, although the minimum level for non-structural wood adhesives (2 MPa) was largely attained, the prescribed limit of 6 MPa according to EN 301 [33] was still not reached. It is worth noting that an additional week of maturation in undisturbed conditions (group RW2 in Table 9) did not bring any appreciable improvement to the wet performances.

The presence of metals together with PAE did not practically improve the joint strength in wet conditions, and the series prepared with PCE and Al (batch TQ2), Cu (TQ1), and Fe (TR1) showed an average value of approximately 4 MPa for the RW1 group (Table 9). This value was apparently even lower than the reference value of 5.2 MPa for the PCE/PAE series. This occurrence is in agreement with the results of the solubility tests, where the addition of metal ions decreased the solubility of PCE/PAE films. This confirms that the two types of tests give comparable information, since a lower mechanical resistance corresponds to a higher solubility. However, in this case, the maturation time had a positive effect on the performances, and wet shear strengths almost reached the value of the reference series for the group RW2, with an average value of approximately 5 MPa for both Al and Fe ions (Table 9).

In contrast, the curing temperature did not substantially improve the joint performances in wet conditions. This parameter was evaluated with Al, and the wet strength was on average 4.7 MPa, irrespective of the temperature or the group (RW1 or RW2) (Table 9). This result confirms once again the results of solubility tests (Table 4).
Table 9. Results of shear strength in wet conditions (test type $\tau_{\text{WET}}$) for the protein PCE to which PAE and salts of the metals Cu, Fe, and Al have been added. In the table, the group indicates that joints have been soaked in water after one week (RW1) or two weeks (RW2) from gluing.

| Series       | Batch | Additive | Gluing Temperature | Group | Average Value (MPa) | Standard Deviation (MPa) |
|--------------|-------|----------|--------------------|-------|--------------------|-------------------------|
| PCE PAE 60 °C | TR2   | PAE      | 60 °C              | RW1   | 5.2                | 0.5                     |
| PCE PAE 60 °C | TR2   | PAE      | 60 °C              | RW2   | 5.2                | 0.8                     |
| PCE PAE Cu 60 °C | TQ1 | PAE + Cu | 60 °C              | RW1   | 3.7                | 0.6                     |
| PCE PAE Al 60 °C | TQ2 | PAE + Al | 60 °C              | RW1   | 4.4                | 0.7                     |
| PCE PAE Fe 60 °C | TR1 | PAE + Fe | 60 °C              | RW1   | 4.3                | 0.7                     |
| PCE PAE Cu 60 °C | TQ1 | PAE + Cu | 60 °C              | RW2   | 4.3                | 0.2                     |
| PCE PAE Al 60 °C | TQ2 | PAE + Al | 60 °C              | RW2   | 5.0                | 0.6                     |
| PCE PAE Fe 60 °C | TR1 | PAE + Fe | 60 °C              | RW2   | 4.9                | 0.2                     |
| PCE PAE Al 80 °C | TS2 | PAE + Al | 80 °C              | RW1   | 4.6                | 1.2                     |
| PCE PAE Al 80 °C | TS2 | PAE + Al | 80 °C              | RW2   | 4.9                | 1.3                     |
| PCE PAE Al 100 °C | TS1 | PAE + Al | 100 °C             | RW1   | 4.9                | 0.7                     |
| PCE PAE Al 100 °C | TS1 | PAE + Al | 100 °C             | RW2   | 4.4                | 0.9                     |
| PCE PAE Al 1\) | TT\) | PAE + Al | 60 °C              | RW2\) | 6.2                | 0.6                     |

\[1\] This batch was kept for 3 months at 20 °C and 65% r.h. in undisturbed conditions.

Nevertheless, for the very long conditioning time of 3 months in undisturbed conditions, a significant and substantial increase of wet shear strength was observed for series PCE/PAE/Al, which showed an average value of 6.2 MPa, which was higher than the prescribed threshold limit provided in [33] (Table 9). This outcome confirms the data obtained comparing RW1 and RW2 (batches TQ1, TQ2, and TR1), and evidences that the maturation time has a positive effect on the mechanical characteristics of proteins/PAE resins. Thus, it is possible to hypothesise that the reaction is relatively slow, and it is not complete when the usual conditions for testing (that is, usually after one week from bonding) are adopted; instead, they increase with the maturation time due to the progressing of the reaction.

4. Conclusions

The results reported in the present work showed that only some of the crosslinking agents among the considered ones were successful in improving the water resistance of protein-based adhesives. In detail, both solubility and shear strength tests evidenced that protein-isocyanate formulations behaved similar to proteins prepared without any additive; that is, they partially dissolved in water, and glued joints broke in the course of the immersion phase in water or when put into the clamp of the dynamometer. Neither the addition of guanidine hydrochloride nor of tannins improved this behaviour. FT-IR spectra showed that isocyanates reacted completely but with water instead of proteins, with no appreciable contribution to the aggregate. Moreover, with some isocyanates, the formation of bubbles was observed (due to CO$_2$ production), but this behaviour was not simplistically related to the NCO content of the used isocyanate.

In contrast, tests with PAE were generally successful. In fact, the presence of PAE dramatically increased the insoluble residue of proteins as such (from 0% to 82% in the case of PCE5), thus evidencing that PAE reacts at least in part with proteins, limiting the access of their hydrophilic groups to water molecules. FT-IR spectra also confirmed that a crosslinking reaction has taken place, although it is not possible to establish whether this is a homo-crosslinking between terminal carboxylic groups of PAE or between PAE and carboxyl groups of amino acids. Moreover, the wet performances of protein/PAE assemblies appreciably increased (up to 5.2 MPa in the case of PCE), gluing at 60 °C instead of room temperature, although the insoluble residue did not appreciably change after curing at higher temperatures.

In the case of PAE, the addition of metal ions to formulations did not practically improve the joint strength in wet conditions, as the insoluble residue was similar with the one of the series prepared with PAE alone. However, although the curing temperature...
did not substantially affect the mechanical behaviour of joints in wet conditions (similar to PAE alone), in formulations with metals, the maturation time had a positive effect on the performances (in contrast to series with PAE alone), both for solubility and wet shear strength. Actually, for the very long conditioning time of 3 months in undisturbed conditions, a significant and substantial increase of wet shear strength was observed for series PCE/PAE/Al. Thus, it is possible hypothesising that in the presence of metal ions, the reaction is relatively slow, and it is not complete in short-term tests (usually after one week from bonding).

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