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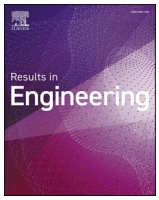

Research paper

Formaldehyde-free wood adhesives based on protein materials from various plant species

Bernardo Grossi [a], Benedetto Pizzo [a,*], Francesco Siano [b], Antonio Varriale [b], Rosanna Mabilia [c]

[a] CNR-IBE, National Research Council of Italy, Institute of Bioeconomy, via Madonna del Piano, 10. I-50019, Sesto Fiorentino, (FI), Italy
[b] CNR-ISA, National Research Council of Italy, Institute of Food Sciences, Via Roma, 64, I-83100, Avellino, Italy
[c] CNR-IBE, National Research Council of Italy, Institute of Bioeconomy, via dei Taurini, 19, I-00185, Roma, (RM), Italy

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ABSTRACT

This study investigated the use of different protein materials to produce formaldehyde-free wood adhesives for plywood production. Biomass from various sources offers a steady supply of raw materials that could encourage wider use. The aim of the study was therefore to find proteins that are naturally high performing without needing costly pre-treatment (e.g., purification or physical processes), in order to reduce costs. Materials from seven plants (soybean, cotton, hemp, carob, grape, maize, and jatropha) were analysed for water resistance and mechanical strength in dry and wet conditions using wood-wood joints (WWJ) and the tests used to characterise plywood. The proteins were tested alone and with polyamide-amine epichlorohydrin (PAE), and blends of different proteins were also evaluated.

The results showed that drying conditions affected the insoluble fraction (InFr) and shear strength in WWJ, with variations related to protein content and carbohydrate composition. Protein-rich materials (e.g., soybean isolate) generally exhibited higher dry shear strengths (>10 N/mm^2) compared to materials with lower protein content (<7 N/mm^2). Wet strengths were generally low or even absent for flours and concentrates. Jatropha concentrate was a notable exception, achieving D3 adhesive classification even without additives. PAE treatment significantly increased wet shear strengths, often exceeding 2 MPa (the minimum for D3 adhesives). Flours and concentrates (except soybean flour) showed the highest strengths (≥3 MPa). PAE probably interacted with both proteins and carbohydrates to improve performance. Moreover, the study showed that protein materials can be blended to enhance adhesive strength, potentially reaching the levels of the best-performing formulations.

1. Introduction

In recent years, formaldehyde has attracted increased attention due to its reclassification as 'H350: May cause cancer - Danger category: Carc. 1B' [21]. Formaldehyde is found in many materials such as paints, cosmetics and textiles, but the main source in indoor environments is wood-based composites [2,7,52]. In fact, this compound is one of the components of urea-formaldehyde (UF) glues used in the production of plywood and particleboard, which are widely used in buildings, while it is known that formaldehyde is released over time due to the slow hydrolysis of the cured resin [6,40]. Therefore, considerable efforts have been made in recent years to develop resins derived from natural products, mainly plant proteins [23,25,69]. Furthermore, the use of plants ensures the processing of renewable and low environmental impact products, which is particularly important for the construction industry [3,26].

To date, several plant proteins have been used as possible wood adhesives, such as soybean, cottonseed and corn [33,61,69]. This is particularly important for the applicability of these products as true substitutes for UF resins, which are currently used in very large quantities (several million tonnes per year) in the plywood and wood-based panel industries [37,55]. Therefore, the use of biomass from different sources for the production of wood adhesives could ensure a steady supply of raw materials, thus favouring their real and widespread application.

Plant proteins have a polar structure that makes them particularly suitable for binding to wood surfaces. However, in order to make this structure available for secondary interactions with the structural

* Corresponding author.
E-mail address: benedetto.pizzo@ibe.cnr.it (B. Pizzo).

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polysaccharides within the wood cell walls, several processes are usually carried out. In the case of wood adhesives, these include chemically induced unfolding [70,74] and the use of physical pretreatment techniques (such as microwave or ultrasound treatments, or high-pressure homogenisation), which can also unfold the protein and help to expose its active chemical groups or change the particle size, thereby improving adhesion [33,69]. However, all of these techniques increase the cost of the product, making proteins uncompetitive with petroleum-based adhesives. Instead, searching for proteins that inherently perform better than others, and investigating the possible reasons for this higher performance, would allow the properties to be optimised (e.g. by blending) at low cost, as the pre- and post-processing of these products would be minimised or eliminated. For this reason, 10 types of 7 different protein materials were evaluated in the present work (see Section 2.1 for full details). Some of these come from agricultural waste biomass, the production of which is expected to increase in the coming years [13].

In any case, the highly polar nature of proteins means that their interactions with wood are not sufficiently stable to be water resistant. Therefore, cross-linking agents that can make the polar groups of the protein less accessible to water molecules are usually required to improve water resistance. Many cross-linking agents have been investigated for protein materials, including epoxy resin [10], lignin-based resin [72], undecylenic acid [42], isocyanate [68], magnesium oxide [36] or a combination of sodium periodate treatment followed by citric acid [65]. However, one of the most effective crosslinkers is a water-soluble prepolymer of polyamide-amine epichlorohydrin (PAE), which has been successfully used under alkaline conditions to produce soy protein-based wood adhesives for interior use [11,53]. The chemical structure of PAE is characterised by a polyamidoamine backbone containing a four-membered salt ring, which is the main reactive site of the molecule. This reactive group is the azetidinium ring (Fig. 1), and it is generally believed that it can give rise to reactions involving an amine group of PAE or some carboxylic groups of amino acid residues or (potentially) wood components during the drying and heating process [48] (Fig. 1).

The aim of the present work is to investigate the possibility of using different protein materials without any purification or concentration process or physical pretreatment techniques to produce formaldehyde-free wood adhesives for the production of plywood for interior use. Several characterisation tests were carried out: dissolution tests, adhesion to a standard wood substrate in both dry and wet conditions, and bonding quality assessment in 3-layer poplar plywood. Protein blending was also considered. All these properties were evaluated both for the proteins used alone and for the proteins cross-linked with PAE. This has allowed a better understanding of the mechanisms underlying the reactivity of PAE towards the different components of protein materials. To the best of our knowledge, such a comprehensive approach has never been carried out on a similar number of material proteins considered in a single work.

2. Materials and methods

2.1. Materials

Several protein materials were selected based on their availability as agricultural waste or residues from other crops. All products were used as received without purification. Soy (*Glycine* max (L.) Merr.) protein isolate (SPI) was provided by DuPont, maize (*Zea mays* L.) protein concentrate (MPC) by Roquette Italia S.p.A., grape (*Vitis vinifera* L.) seed flour (GSF) by Vegea s.r.l., carob (*Ceratonia siliqua* L.) flour (CF) by Raft s.r.l., hemp (*Cannabis sativa* L.) seed flour (HSF) from ParodiNutra s.r.l. and cotton (*Gossypium* spp.) seed concentrate (CSC), soy (*Glycine* max (L.) Merr.) flour (SF) and two concentrates of *Jatropha curcas* L. from Agroils Technologies S.p.A. The latter were from two different processes of the same raw material and were named JV and JP. The defatted protein materials were all in powder form, except for the two Jatropha protein concentrates, which were two cakes with slightly different moisture contents (56.7 % and 61.0 % for JV and JP, respectively).

Maresin VHP200 was a 20 % w/v aqueous solution of polyamide-amine epichlorohydrin (PAE) and was provided by Mare S.p.A. NaOH (purity 97 %) was purchased from Sigma Aldrich.

2.2. Composition of protein materials

Kjeldahl analysis was used to determine the protein content of each analysed material. The total crude protein of the samples was determined by the Kjeldahl method using the Official Methods of Analysis of AOAC International [38]. Briefly, 1 g of raw material was hydrolysed with 15 mL of concentrated sulphuric acid (H_2SO_4) containing a catalyst tablet (3.47 g K_2SO_4 + 0.003 Se, VELP Scientifica, Italy) in a heating block at 300 °C for 2 h (DK Heating Digester, VELP Scientifica, Italy). After cooling, H_2O was added to the hydrolysates before neutralisation with NaOH (30 %) and subsequent distillation in a steam stream. The distillate was collected in 25 mL of H_3BO_3 (1 %), titrated with 0.1 M HCl and a mixture of bromocresol green/methyl red as indicator. The amount of total N in the raw materials was calculated and the protein concentration in the samples was extrapolated using a conversion factor

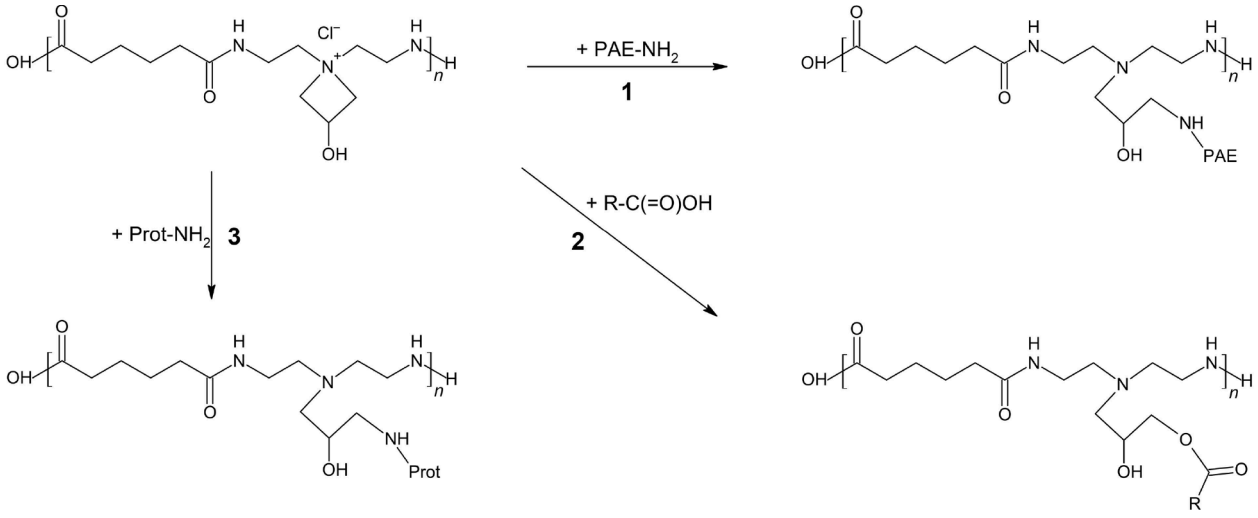

Fig. 1. Scheme of possible reactions of PAE. In the scheme, 'Prot' indicates a protein moiety.

of 6.25. All determinations were carried out in triplicate.

Total carbohydrates (including monosaccharides, disaccharides and polysaccharides) were determined by the phenol/sulphuric acid method developed by DuBois et al. [14]: 1 ml of each protein dispersion was mixed with 5 % aqueous phenol solution and concentrated (99 %) sulphuric acid. This solution was allowed to react for 20 min, cooled at RT for 10 min and then analysed spectrophotometrically (Varian Cary50 UV–visible spectrophotometer) at 488 nm. Soluble carbohydrates were determined by adding 100 mg of each protein material to 25 ml of distilled water in a test tube and mixing vigorously with a magnetic stirrer for 1 h. The dispersions were then centrifuged and the supernatants dried. The latter were used to prepare the solutions for the DuBois analysis, which was carried out as described above. The amount of lignin was determined by the Klason method: protein materials were Soxhlet-extracted first with ethanol-toluene (4 h) and then with deionised water (6 h); the residue was treated with concentrated sulphuric acid at RT, followed by treatment with diluted sulphuric acid at the appropriate boiling temperature.

All results are expressed on the anhydrous mass of the protein material.

2.3. Formulations

Each formulation was prepared in a 25 ml beaker using a magnetic stirrer. Raw materials were added in the following order: distilled water, NaOH in pellets, protein material and PAE (where appropriate). The composition of the formulations for the protein materials as such is given in Table 1. These were the reference for all evaluations. All dispersions were kept under stirring until lumps disappeared completely. The solid content (also given in Table 1) was calculated as follows:

$$\textbf{\textit{Solid content}} = \frac{\textbf{\textit{Protein material}}\ (\textbf{\textit{g}}) + \textbf{\textit{NaOH}}\ (\textbf{\textit{g}}) + [\textbf{\textit{PAE}}\ (\textbf{\textit{g}})]}{\textbf{\textit{Total weight}},\ \textbf{\textit{including water}}\ (\textbf{\textit{g}})} \times 100$$

This parameter was set as the maximum possible to ensure smooth spreadability on wood surfaces, and of course depended on the protein chosen. In general, the aim was to achieve a solid content > 20 %.

The viscosity of the formulations was determined at RT within 10 min after preparation using a rotational viscometer (ViscoQC 300 L, Anton Paar GmbH, Graz) equipped with spindle L4, at a speed of 20 rpm for 30 s.

2.4. Dissolution tests

Four aliquots of 4.00 ± 0.01 g each were taken from each liquid formulation, transferred to silicone moulds of approximately 7 cm^2 surface area and allowed to dry [49]. Two aliquots were dried at room temperature (RT, approximately 24 °C) to constant weight (≥ 1 week) and the other two were dried in an oven at 103 °C for two hours. These conditions were chosen to simulate the adhesive drying processes that would occur in industrial situations, cold and hot pressing respectively. Dissolution tests were carried out at RT and in an oven at 103 °C.

In this way, 4 combinations were tested (Fig. 2):

- samples dried at RT and tested either at RT or at 103 °C (series 'RT/RT' and 'RT/103 °C', respectively);
- samples dried at 103 °C and tested either at RT or at 103 °C (series '103 °C/RT' and '103 °C/103 °C', respectively.

For dissolution tests, the samples were placed in a 100 ml vessel and distilled water was added to achieve a 63:1 ratio with the solid fraction. Samples soaked in water at RT were placed in a metal capsule to avoid collisions with the magnetic stirring rod. Stirring was carried out for 3 h. On the other hand, high temperature tests were carried out by hermetically sealing the vessel and placing it in an oven for 3 h without stirring (Fig. 2). The samples were then removed from the water and dried in an oven to constant weight. Finally, the (InFr) fraction of the samples was calculated as follows and expressed as a percentage:

$$\textbf{insoluble fraction} = \textbf{InFr} = \frac{\textbf{\textit{final dry weight}}\ (\textbf{\textit{g}})}{\textbf{\textit{initial dry weight}}\ (\textbf{\textit{g}})}$$

2.5. Gluing and mechanical tests

The shear strengths of the formulations under consideration were evaluated in two ways (Fig. 3):

- on wood-wood joints (WWJ) prepared according to EN 205 [17] using 5 mm thick beech boards as the wood adherends,
- on three-layer plywood (PW) prepared according to EN 314–1 [18] using three 1 mm thick poplar veneers orthogonally bonded.

The WWJ test was chosen because it is specifically used to evaluate adhesives (in this case, those intended for use on wood). Therefore, it carefully regulates the condition of the wood adherends: wood species, density, ring orientation, surface quality etc., and the tests provide a little biased value. In contrast, the PW test is normally used to assess the bonding quality during the plywood production, and therefore it does not specify the substrate conditions, which are instead influenced by the veneer characteristics (e.g. the presence of lathe checks due to rotary cutting) [51]. However, to minimise the variability of the data, poplar veneers from the same production batch were used for all PW tests.

The protein formulations were applied at a total application rate of 90 g/m^2 (evaluated on a dry basis) and pressed at 1 MPa. A thermocouple was used to monitor the temperature reached in the bonded joints. In general, the assemblies were pressed for 15 min for WWJ and 10 min for PW. However, if the temperature rose to 125 °C or 120 °C for WWJ or PW respectively, the pressing was stopped earlier. A minimum pressing time of 4 min was set, so in one case (CF/PW) the minimum temperature was exceeded. It should be noted that no obvious correlations were found between higher temperatures (or shorter pressing times) and mechanical strength, so the reasons for the temperature differences found were not investigated further.

After hot pressing, the assemblies were stored in a climatic chamber at 20 °C and 65 % RH to constant weight (≥ 1 week). WWJ were tested both dry and wet, the latter after immersion in water for 96 h at RT. PW specimens were tested wet after immersion in water for 24 h at RT (Fig. 3). Wood failure percentage (WFP) was also visually assessed after fracture. All mechanical tests were performed on a universal testing machine (Instron, mod. 5567) equipped with a 30 kN load cell, accuracy 0.5 %.

Table 1
Reference formulations of protein materials.

	H_2O (g)	NaOH (g)	Protein material (g)	pH	Solid content (%)
Soybean isolate (SPI)	16.00	0.19	4.00	9.1	20.8
Soybean flour (SF)	19.00	0.11	6.0	7.0	24.3
Maize concentrate (MPC)	18.50	0.84	5.00	12.0	24.0
Carob flour (CF)	16.00	0.09	9.00	7.3	36.2
Jatropha P concentrate (JP)	9.00	0.21	13.15*	6.8	23.9
Jatropha V concentrate (JV)	9.50	0.13	12.70*	7.2	25.2
Grapeseed flour (GSF)	16.00	0.06	7.00	7.0	30.6
Cottonseed concentrate (CSC)	20.20	0.07	6.00	7.7	23.1
Hempseed flour (HSF)	18.00	0.11	7.00	11.0	28.3

* Moisture content of 61.0 % and 56.7 % for JP and JV, respectively.

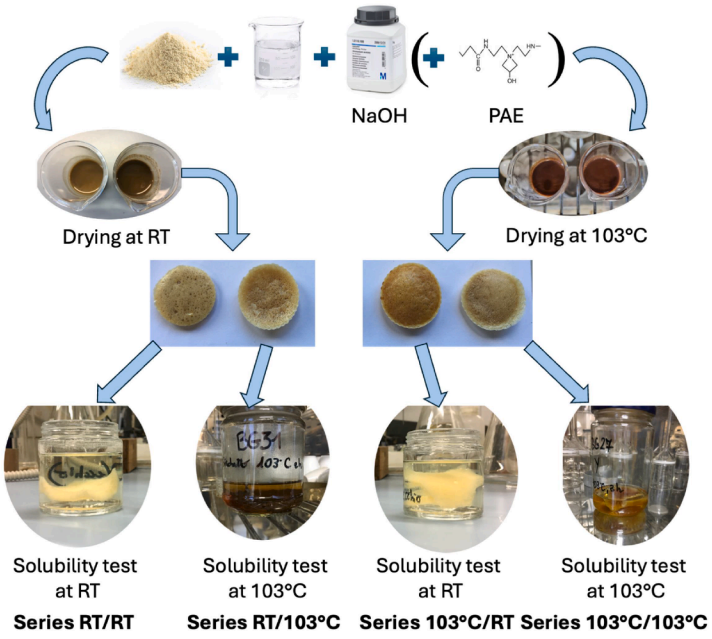

Fig. 2. Scheme of the material distribution in dissolution tests.

Fig. 3. Sequence of sample preparation and mechanical testing.

2.6. ATR-FTIR spectroscopy

Spectra were acquired on a Bruker Optics Alpha FT-IR spectrometer equipped with a diamond single reflection Attenuated Total Reflectance (ATR) instrument with the following settings: 40 scans per sample; spectral resolution: 4 cm^{-1}, wave number range: 4000 to 400 cm^{-1}. Spectra were collected without any preliminary sample preparation. Spectra were normalised in the 700–1800 cm^{-1} range using vector normalisation (OPUS 6.5, Bruker). Vector normalisation calculates the average y-value of the spectrum. The average is subtracted from the spectrum by lowering the mid-spectrum to $y = 0$. The sum of the squares of all the y-values is calculated and the spectrum is divided by the square root of this sum. The vector norm of the resulting spectrum is 1.

3. Results and discussion

3.1. Protein materials used as such

The composition of the material analysed, evaluated as described in Section 2.2, is shown in Table 2. The products were classified according to their protein content as isolate (> 70 %), concentrate (50–70 %) or flour (< 50 %). The results show the variability of the protein content of

Table 2
Composition of the protein raw materials. The column 'other components' has been calculated as the complement to 100 % of the sum of the crude protein content and the total carbohydrate content.

Protein material	Crude protein content (%)	Carbohydrate content			Other components (%)
		Total (%)	Soluble (%)	Insoluble (%)	
SPI	78.6	6.6	0.7	5.9	14.8
SF	48.3	32.7	5.4	27.3	19.0
CSC	52.7	32.9	3.1	29.8	14.4
GSF	12.7	36.9	5.8	31.1	50.4
MPC	61.3	23.9	2.0	21.9	14.8
JV	51.8	12.4	3.7	8.7	35.8
JP	55.9	11.1	2.8	8.3	33.0
CF	8.9	49.3	31.3	18.0	41.8
HSF	29.7	18.8	0.8	18.0	51.5

the biomass considered, which justifies the need to verify their physico-mechanical properties when used for wood bonding, i.e. their real suitability as substitutes for formaldehyde-based adhesives.

3.1.1. *Dissolution tests*

The results of the various dissolution tests are shown in Fig. 4a. It was observed that:

a) Materials with the highest protein content (SPI and MPC) showed the lowest values of InFr in the 'RT/RT' dissolution test (InFr < 25 %) and completely dissolved in water (InFr = 0) when tested at 103 °C ('RT/103 °C' and '103 °C/103 °C' series, Fig. 4a). Interestingly, SPI formulations achieved InFr of almost 50 % in the '103 °C/RT' tests;
b) The products characterised by a protein content < 60 % showed values of InFr > 50 % (except CF and SF) and therefore very low relative variations from one drying method to the other: the average InFr was 63 % for both the series prepared at RT ('RT/RT' + 'RT/103 °C') and those prepared at 103 °C ('103 °C/RT' + '103 °C/103 °C'), Fig. 4a;
c) In addition, also for products with protein content < 60 % (except CF and SF), InFr was 65 % for the series tested at RT ('RT/RT' and '103 °C/RT') and 61 % for those tested at 103 °C. SF was in line with the other flours and concentrates in the tests at RT, with an average InFr of 58 %, but showed much lower values in the tests at 103 °C (average InFr of 26 %).

The reason for the very low InFr values of the materials richer in proteins such as SPI and MPC is that protein / water interactions are in competition with protein / protein secondary bonds in these materials,

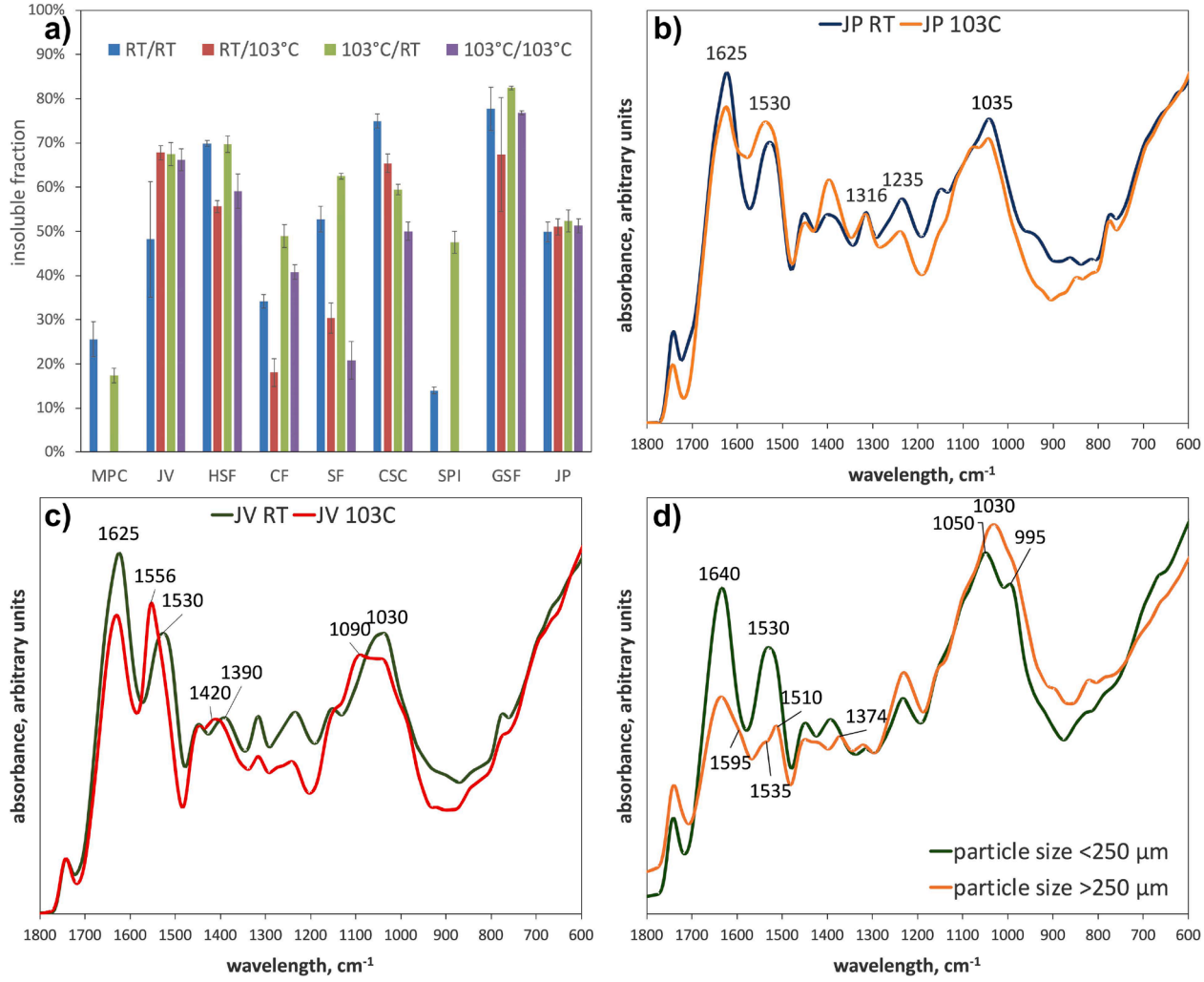

Fig. 4. Results obtained for the protein materials used alone; a) insoluble fraction for the different series: dried at RT and tested at either RT or 103 °C ('RT/RT' and 'RT/103 °C', respectively) and dried at 103 °C and tested at either RT or 103 °C ('103 °C/RT' and '103 °C/103 °C', respectively). The vertical bars represent the standard deviation of the data; b,c) FTIR spectra of JP (b) and JV (c) protein materials dried at either RT or 103 °C; d) FTIR spectra of HSF meal protein material separated into two fractions: particle size >250 µm (orange curve) and <250 µm (dark green curve).

where structural rearrangements due to the alkaline pH imply a high solvent-exposed surface area during setting. In this respect, the marked improvement of InFr for SPI set at 103 °C could be explained by the hypothesis of coalescence of soy protein globules at high temperatures [28], resulting in conformational changes strong enough to resist water molecules that have low energy (i.e. water molecules at RT). In contrast, MPC proteins change their secondary structures from mostly α-helix to mostly β-pleated sheets, turns and random coils upon temperature increase [5], but as prolamins they do not usually coagulate upon heating (instead they can be hydrolysed to proline and ammonia) [43].

The situation for the other products, characterised by a protein content < 60 % and the presence of carbohydrates and other compounds, is more complex to explain as it involves several potential mechanisms. One of them could be the development of Maillard reactions between sugars and proteins at high temperatures, which has been reported for soy-based adhesives [9,66,71]. However, in our case, possible evidence for the occurrence of this mechanism was only observed for some of the products. In this respect, it is interesting to compare the behaviour of JV and JP, both having the same protein materials. Between these two products, JV has a higher carbohydrate content than JP (Table 2). In the dissolution tests at RT, JV showed a greater difference in InFr between the 'RT/RT' and '103 °C/RT' series (from 52 % to 67 %, Fig. 4a, relative increase of 29 %). This behaviour could be explained by the increased cross-linking due to Maillard reactions.

The spectra of both JP and JV (Fig. 4b and c) show the typical bands associated with proteins at 1625 cm^{-1}, 1530 cm^{-1}, 1316 cm^{-1} and 1235 cm^{-1}, due to the vibrations of the bands of Amide I, Amide II and Amide III (the last two bands), respectively, to which is added a strong absorption at 1035 cm^{-1} due to the C—O—H stretching vibration in carbohydrates (Fig. 4b). The Amide I band is mainly associated with the C═O stretching (ca. 80 %) and both the C–N stretching and N–H bending (ca. 20 %) vibrations. Amide II is due to the N—H bending (ca. 50 %) and C—N stretching (ca. 30 %) vibrations. Amide III is a complex band whose signals fall in the range of 1350–1200 cm^{-1} and are associated with C—N stretching and N–H bending vibrations (ca. 30 % each), C—C stretching and C—H bending (ca. 30 % together) ([35,59]). Amide III can be found divided in two bands, the relative height of which depends on the secondary structure of the protein [56,59].

The modification of the spectra when JP and JV were subjected to the high temperature treatment can be seen in Fig. 4b and Fig. 4c respectively.

The intensity of the Amide I and Amide III bands decreased. These bands are very sensitive to changes in the secondary structure of proteins and so this indicates the expected conformational change due to the thermal treatment. Instead, the Amide II band is much less sensitive to protein conformational elements. This band increased in JP, whereas in JV a new sharp band appeared at 1556 cm^{-1}, very close to the original one (Fig. 4c). In JV the shift of the band at 1390 cm^{-1} to 1420 cm^{-1} was also observed. All these bands are related to N–H and C–N stretching vibrations. Therefore, in principle, the observed changes could be attributed to the formation of the different moieties involved in the Maillard reaction, for instance to the enaminols originating from Schiff bases [32,34,60]. However, FTIR is not the best technique for assessing the presence of Schiff bases. Other techniques, such as NMR, should be used to confirm this occurrence.

In JV, an additional band at 1090 cm^{-1} appeared close to the original carbohydrate band at 1030 cm^{-1}, which decreased. This confirmed the disappearance of C—O—H bonds in favour of C—O—C bonds, indicating a change in the protein structure, which can also be attributed to the formation of Maillard reaction products [31,34,73]. Thus, it is evident that the extent of protein modification was higher in JV than in JP (Fig. 4b,c), confirming the increased cross-linking in JV.

Therefore, in JP and JV, which have comparable (although not identical) amounts of proteins and carbohydrates, different evidence appeared in the spectroscopic analyses. This shows that the efficacy of heat-induced reactions and rearrangements in protein materials depends not only on the amounts of possible reagents (e.g. in the case of the Maillard reaction, the amount of proteins and carbohydrates), but also on their structural typology, and that this mechanism does not explain all the results observed in our tests.

For example, CSC and HSF showed high InFr values even in the RT-dried samples (average InFr of 72 % for the 'RT/RT' series and of 61 % for the 'RT/103 °C' series, Fig. 4a). In addition, CSC showed a significant decrease in InFr when dried at 103 °C compared to RT (60 % vs. 75 % in the tests at RT and 50 % vs. 65 % in the tests at 103 °C, Fig. 4a). This is likely due to the high arginine content, which is 11–12 % in cottonseed protein and 5–6 % in hemp compared to, for example, 1–2 % in maize [12,30]. In fact, arginine is known to reduce heat-induced protein aggregation [44,47,58], which may result in more soluble products.

3.1.2. Mechanical tests

The results of the mechanical tests on WWJ are shown in Table 3, together with the viscosity values. The latter were consistent with the values previously obtained in the case of soy meal [39,72]. However, no relationship between viscosity and shear strength was observed. SPI, MPC, CSC and JV with protein content > 50 % (Table 2) showed the highest average dry tensile shear strength (DTSS) of 14.5, 11.5, 10.6 and 10.6 MPa respectively. At the same time, the materials with lower protein content showed a DTSS < 7 MPa. This is because the DTSS is higher for higher molecular weight chains, such as proteins, provided they are able to interact efficiently with the wood surface. On the other hand, the presence of physical barriers, such as residues of hulls, could exclude HSF from the overall trend. In fact, the DTSS of HSF was as low as 4.5 MPa, even lower than that of CF and GSF, which contain the lowest amount of proteins (8.9 % and 12.4 %, respectively). To confirm the negative contribution of hull residues on adhesion, the HSF meal was sieved to separate it into two fractions with different particle sizes: below and above 250 μm (mesh 60). The fraction with a particle size >250 μm (37.4 %) appeared brownish and resistant to grinding, while the fraction with a particle size <250 μm (51.4 %) was a greenish powder. The IR spectra of the two fractions showed several differences (Fig. 4d): the fraction with a particle size <250 μm showed more intense vibrations at 1640 cm^{-1} and 1530 cm^{-1} (Amide I and Amide II bands of proteins, respectively), indicating a higher protein content [54]. Moreover, the bands in the 900–1100 cm^{-1} region, due to C–O–H and C–O vibrations in carbohydrates, were less intense and better resolved (two

Table 3
Results of shear strength tests carried out on wood-wood joints glued with protein materials used as such. DTSS = dry shear strength; WTSS = wet shear strength. The symbol '-' means that the specimens were broken before the tests, during the immersion phase.

	Solid content (%)	Viscosity (mPa·s)	T (°C)	Pressing time (min.)	DTSS (MPa)	WTSS (MPa)
SPI	20	6749	115	15	14.5 ± 2.3	0.5 ± 0.3
MPC	24	270	109	15	11.5 ± 1.4	–
JP	30	1350	111	15	7.7 ± 1.0	0.9 ± 0.1
JV	29	120	125	10	10.6 ± 1.4	2.4 ± 0.5
CSC	20	4140	110	15	10.6 ± 0.7	0.7 ± 0.1
GSF	30	4919	111	15	6.1 ± 0.5	–
SF	24	18,810	114	15	8.3 ± 1.5	–
HSF	28	3810	125	6	4.5 ± 1.2	0.7 ± 0.3
CF	35	960	113	15	5.7 ± 0.0	–

separate peaks were observed at 995 cm^{-1} and 1050 cm^{-1}). In contrast, in the fraction with particle size >250 μm, the band at 1510 cm^{-1} was evident and a shoulder at 1595 cm^{-1} appeared. Both bands are due to aromatic moieties. In addition, the new band at 1374 cm^{-1}, attributable to phenol OH vibration [22], also appeared. These occurrences indicate a higher aromatic content of this fraction, which could be related to the presence of hull residues in the original flour. In fact, polyphenols are mainly located in the hull rather than in the kernel of hempseed [24,50], and hemp hulls are composed of approx. 30 % lignin [62]. A higher lignin content in the coarser fraction was confirmed by the Klason lignin evaluation, which was 29 % for the flour as such and 33 % for the fraction >250 μm. Mechanical tests performed on the fraction <250 μm showed much higher DTSS than HSF (9.4 ± 1.0 MPa and 4.5 ± 1.2 MPa, respectively, Table 3) and comparable wet tensile shear strength (WTSS) (0.5 ± 0.2 MPa and 0.7 ± 0.3 MPa, respectively), demonstrating that the deviation observed in HSF was more related to contamination problems than to chemical aspects.

In the wet mechanical tests, the MPC, CF, SF and GSF prepared assemblies opened before the end of the water treatment and therefore no measurements were possible. The remaining series remained bonded after the wet phase, but for most of them the WTSS values were very poor and almost none of them reached the minimum threshold of 2 MPa required by EN 204 [16] for class D3, the same as the pre-treatment chosen in our tests. A notable exception was JV, which reached this threshold and could therefore in principle be classified as a D3 adhesive (suitable for interiors exposed to high humidity and exteriors not exposed to weathering). This behaviour is particularly interesting as it concerns the protein material alone, i.e. without the addition of an external hardener. As shown in Fig. 4a, the reason for this behaviour is not only related to the insolubility of the protein, which is of the order of 70 %. Rather, it is most likely related to the specific composition and structure of the protein. This will be investigated in a separate study.

The tests also showed that protein concentrates were more water resistant than flours, with the two exceptions of HSF (as mentioned above) and MPC, whose low water resistance has already been shown [54]. This behaviour confirms the results of the solubility tests and is due to the positive effect of the thermal treatment on the protein-protein interactions (SPI) and to the occurrence of the Maillard reaction at high temperature in the concentrates. On the other hand, in the case of flours, the low solubility of the adhesive is not sufficient to ensure significant adhesion in wet conditions, since the strength of the bonds is due to a combination of protection against water and resistance to external stresses, which is reduced in flours, as shown by the dry strength values (Table 3).

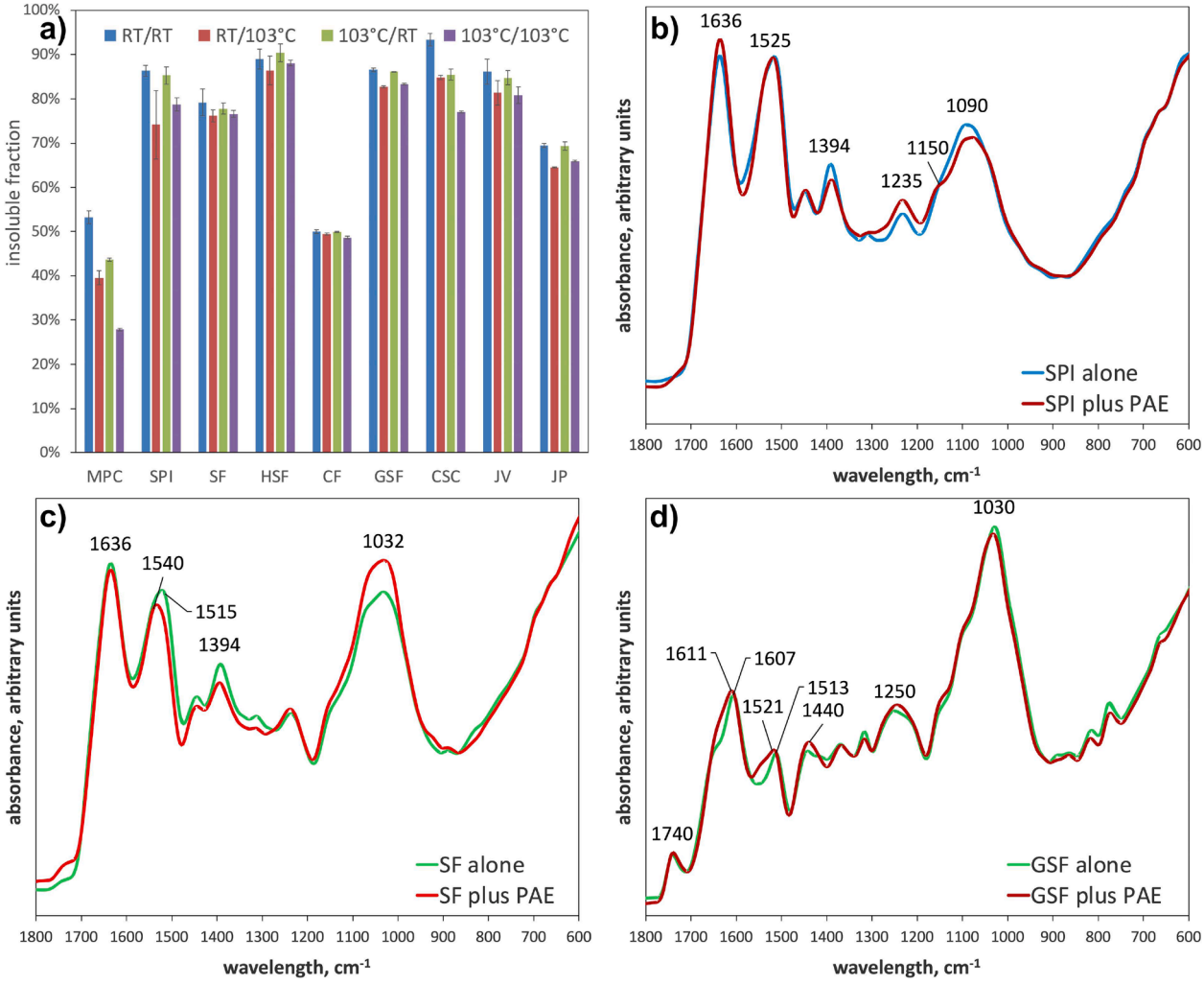

Fig. 5. Results obtained for the protein materials cured with PAE; a) insoluble fraction for the different series: dried at RT and tested at either RT or 103 °C ('RT/RT' and 'RT/103 °C', respectively) and dried at 103 °C and tested at either RT or 103 °C ('103 °C/RT' and '103 °C/103 °C', respectively). Vertical bars represent the standard deviation; b,c) FTIR spectra of SPI (b) and SF (c) protein materials cured at 103 °C in absence and in presence of PAE; d) FTIR spectra of the GSF protein material cured at 103 °C in absence and in presence of PAE.

3.2. Formulations with the cross-linker

The InFr values for all the materials tested are shown in Fig. 5a, demonstrating that PAE controls the solubility of the samples. In fact, the results of the dissolution tests were more homogeneous compared to the formulations without PAE (Fig. 4a).

In particular, GSF, CSC, HSF, JV, SPI and SF showed values of InFr between 80 % and 90 % in each type of dissolution test. JP showed values around 70 %, while CF and MPC showed the lowest values, around 50 % for CF and even less for MPC. The homogenising effect of PAE is due to the reaction characteristic of PAE, whose azetidinium ring can react with active hydrogen-containing groups such as the carboxyl and amine groups (Fig. 1). However, while the self-crosslinking of PAE (the reaction between the azetidinium group and the carboxyl and amine groups in the same PAE resin) has been observed several times [4,20,27,67], the reaction of PAE with protein amines is more speculative. For example, in our case, SPI had almost overlapping IR spectra before and after curing with PAE, indicating only minor chemical changes in the protein (Fig. 5b). The differences consist of the limited decrease of the band at 1394 cm^{-1} and the slight increase of the bands at 1636 cm^{-1} and 1235 cm^{-1}. In addition, the large band centred at 1090 cm^{-1} became slightly broader and lower, and a shoulder appeared at 1150 cm^{-1} (Fig. 5b). The increase in the bands of Amide I at 1636 cm^{-1} and Amide III at 1235 cm^{-1}, as well as the appearance of the band at 1150 cm^{-1}, are all due to the curing reactions of PAE [8], as these bands are due to the additional amide groups of PAE and the increased contribution to C–N stretching as consequence of the opening of the azetidinium ring (Fig. 1, pathway 1). Therefore, these changes indicate that PAE undergoes predominantly self-crosslinking in SPI. However, the limited decrease of the band at 1394 cm^{-1}, due to the carboxylate groups in the sugar units, which are also present in SPI (Table 2), indicates that PAE can react with these molecules, although to a more limited extent. Thus, in proteins, PAE forms a polymeric network that protects the existing bonds and interactions within the peptide chains, thereby reducing dispersion in water. A similar mechanism exists in pulp cellulose fibres [1,15,57]. This produces a network of material that is physically entangled with the major components of the protein material (proteins and carbohydrates) [15,53].

Considering that the amount of soluble components in MPC was very high (Fig. 4a), it can be concluded that the cured cross-linked network of PAE was not able to fully retain these soluble components of MPC. Similar considerations can be extended to CF due to its high soluble carbohydrate content (31.3 %, Table 2).

Viscosity measurements showed that all values decreased when PAE was added to the protein materials. This behaviour has been observed previously and is related to the smaller size of PAE molecules compared to proteins, thus acting as a plasticiser and reducing the internal friction in the adhesive formulations [71,72]. With regard to the shear strength tests carried out according to EN 205, it was observed that the addition of PAE caused a reduction in the DTSS of the materials with the highest protein content, such as SPI, MPC, CSC and JV, with the exception of JP.

On the other hand, the flours (SF, GSF, HSF and CF) all increased their DTSS (Table 4 and Table 3). This behaviour has no obvious relationship with viscosity and can be explained by the assumption that, in our systems, PAE reacts, at least partially, with carbohydrates as an additional pathway to the reaction with proteins, as also highlighted previously [46]. This was confirmed by IR analysis. Fig. 5c shows the representative case of SF, where it can be seen that SF had a clearly visible carbohydrate band at 1032 cm^{-1} (C–O–H stretching vibration), which is virtually absent in SPI. In SF the differences between the uncured and PAE-cured protein are more pronounced than for SPI. After curing, the Amide II band decreased and broadened from a peak at 1515 cm^{-1} to 1540 cm^{-1}, while the band at 1032 cm^{-1} increased. This was previously observed for SF+PAE [72] and indicated a denser cross-linked structure that is mechanically stronger and more water resistant. Similarly, the band at 1394 cm^{-1} (carboxylate groups in carbohydrates) decreased significantly, indicating carboxyl-azetidinium reactions and hence carboxyl consumption, confirming the reaction of carbohydrate carboxyl with the azetidinium ring [20,46]. Indeed, it can be observed as a general trend that the higher the amount of insoluble carbohydrates (Table 2), the higher the relative variation of DTSS ($\Delta\tau_{DTSS}$), defined as

$$\Delta\tau_{DTSS} = \frac{\tau_{PAE} - \tau_{prot.alone}}{\tau_{prot.alone}}.$$

For example, in CF (where total carbohydrates are 49 %) $\Delta\tau_{DTSS} = 82$ % (dry strength increased), in JV (total carbohydrates 12 %) $\Delta\tau_{DTSS} = -5$ % (dry strength essentially unchanged), in SPI (total carbohydrates 7 %) $\Delta\tau_{DTSS} = -21$ % (dry strength decreased).

WTSS showed the highest values for GSF, JV, HSF, CSC and CF (≥ 3 MPa), whereas the lowest values were associated with both soy products (SPI and SF). However, these low values were approx. 2.5 MPa, i.e. they were higher than the minimum threshold of 2 MPa specified in EN 204 [16]. MPC samples broke after the water immersion phase. The behaviour observed for flours can be explained by the same reasoning as for dry strength (i.e. reaction of PAE with carbohydrates in addition to proteins). However, the value measured on GSF was remarkable (its WTSS was the highest, 4.9 MPa, Table 4). In this case, the protein content (13 %) was the second lowest among the products considered, while the carbohydrate content (37 %) was similar to SF and CSC and lower than CF (Table 2). Therefore, an additional mechanism should be involved in this material.

It is known that *Vitis* seeds are particularly rich in proanthocyanidins (PAC), i.e. oligomeric flavonoids, including gallic acid esters of catechin and epicatechin, and more complex polyphenols such as condensed tannins [64]. The presence of these compounds in our case was confirmed by FTIR spectroscopy (Fig. 5d). The band at 1740 cm^{-1} is due to the C = O group in the ester groups. Moreover, the bands at 1607 cm^{-1} and 1513 cm^{-1} are both due to the aromatic rings, although they are slightly shifted, possibly due to the partial overlap with the Amide I and Amide II bands (due to the protein contribution). The region around 1513 cm^{-1} is particularly complex because it overlaps with semicircle stretching mixed with C − H bending in the aromatic rings. However, this last vibration has a second component at 1440 cm^{-1}, which is less affected by other signals and is clearly visible in the spectra. Also important are the bands at 1030 cm^{-1} and 1250 cm^{-1}, due to O − H and C − O stretching vibrations respectively. They are very intense because of the abundance of carbohydrates, but also because the same signals (O − H and C − O stretching) are present in phenolic compounds as well.

Table 4
Results of shear strength tests carried out on WWJ glued with protein materials/PAE blends. The values after the symbol '±' are the standard deviations. T is the temperature of glue lines monitored by a thermocouple during pressing; t is the pressing time. DTSS = dry shear strength; WTSS = wet shear strength. The symbol '-' indicates that the specimens were broken before testing, during the immersion phase.

	Solid content (%)	Viscosity (mPa·s)	T (°C)	t (min.)	DTSS (MPa)	WTSS (MPa)
SPI	20	360	110	15	11.4 ± 2.5	2.5 ± 0.3
MPC	24	120	124	15	4.6 ± 1.2	–
JP	30	1290	125	7.5	9.1 ± 1.0	2.8 ± 0.8
JV	29	90	125	10	10.1 ± 2.5	4.7 ± 0.6
CSC	20	1620	110	15	8.4 ± 0.5	3.0 ± 0.7
GSF	30	930	118	15	9.8 ± 0.8	4.9 ± 0.8
SF	24	10,680	114	15	12.9 ± 0.9	2.6 ± 0.3
HSF	27	960	125	6	10.7 ± 0.7	3.5 ± 0.7
CF	35	630	118	15	10.4 ± 1.1	3.0 ± 0.7

When PAE reacts with GSF, the spectra show limited changes (Fig. 5d), the most important of which are the broadening and shift at 1611 cm^{-1} and 1521 cm^{-1} of the bands mentioned above. These changes are due to the curing reaction of PAE, which increases the contribution to the Amide I and Amide II bands and makes the structure more densely linked. However, these changes are not directly related to chemical reactions involving PAC in GSF. Nevertheless, it must be taken into account that PAC has previously been reported to increase the strength of gluten proteins through complexation involving both their hydroxyl groups and hydrophobic aromatic rings [29]. This mechanism of action of PAC has also been confirmed for other proteins [41], as it depends on their multidentate character (larger molecules can simultaneously bind more than one site of the other involved molecule). At the same time, it has been shown that PAE and dendritic molecules of tannic acid can combine with a protein matrix to form a tough co-crosslinked network through strong intermolecular forces (ionic and hydrogen bonds) in adhesive systems [45,63]. Therefore, it can be suggested that in our case, the GSF PAC can form complexes (rather than chemical bonds) with both the polar and hydrophobic groups present in the system, the number of which was dramatically increased due to the curing of PAE.

Given the results of the solubility tests (Fig. 5a), it is also surprising that the values measured for both SPI and SF were so different from those of the best performing protein materials (around 2.5 MPa versus > 3.5 MPa, Table 4). It is reasonable to assume that the reaction of PAE with polysaccharides only contributes to wet strength when the latter have a complex and structured composition, whereas the reaction with simpler, unstructured carbohydrates does not contribute to strength but reduces the solubility of the materials in water. Indeed, among the other protein materials considered, SF had a high proportion of soluble (simple) carbohydrates and a low proportion of insoluble (structured) carbohydrates (Table 2).

3.3. Production of plywood

The results of the EN 314–1 shear strength tests (specimens tested wet after 24 h immersion in water) (WSS_PW) on plywood made from protein materials used alone are given in Table 5. While the SPI specimens broke after 18 h in water, the other materials at least showed a value, although in several cases it was quite low (< 0.4 MPa). On the other hand, the JV and CSC protein concentrates achieved significant WSS_PW values (around 0.8 and 0.6 MPa respectively). In particular, JV confirmed the very high value (2.4 MPa) already found in EN 205 tests. However, it should be noted that in similar cases, EN 314–2 [19] requires a minimum value of 40 % for WFP and this value was never achieved (Table 5).

The results of the tests carried out on plywood made from protein materials with the addition of PAE are shown in Table 6, where it can be seen that all the samples exceeded 0.7 MPa. In general, the WSS_PW values followed a similar trend to that observed for the samples analysed according to EN 205. In fact, the highest value (1.2 MPa) was obtained with JV, followed by HSF (1.1 MPa), CSC, CF and SF (around 1.0 MPa), JP and GSF (around 0.8 MPa) and finally SPI (0.7 MPa). The results for WFP highlighted a critical point: when it was important to consider this same parameter (i.e. for WSS_PW < 1 MPa), it was always lower than the minimum thresholds set by EN 314–2. Thus, the low WSS_PW obtained for GSF (which registered the highest WTSS in the tests carried out according to EN 205) could be explained by the limited penetration of the product in the preparation and configuration adopted for the tests on plywood. This is reflected in the low WFP.

Table 5
Results of shear strength tests carried out according to EN 314–1 (specimens tested wet after 24 h immersion in water) (WSS_PW) on plywood glued with protein materials used as such. T is the temperature of glue lines monitored by a thermocouple during pressing; t is the pressing time; WFP = wood failure percentage. The symbol '-' indicates that the specimens were broken before testing, during the immersion phase.

	Solid content (%)	T (°C)	t (min.)	WSS_PW (MPa)	WFP (%)
SPI	20	110	10	–	–
SF	24	117	10	0.21 ± 0.03	0
CSC	20	111	10	0.57 ± 0.08	0
CF	36	150	4	0.23 ± 0.07	10
HSF	28	120	6	0.46 ± 0.03	0
JV	29	119	6	0.77 ± 0.20	30
JP	30	120	5	0.31 ± 0.04	10

Table 6
Results of shear strength tests carried out in accordance with EN 314–1 (specimens tested wet after 24 h immersion in water) (WSS_PW) on plywood made with protein materials/PAE blends. T is the temperature of glue lines monitored by a thermocouple during pressing; t is the pressing time; WFP = wood failure percentage.

	Solid content (%)	T (°C)	t (min.)	WSS_PW (MPa)	WFP (%)
SPI	20	110	10	0.72 ± 0.14	0
SF	24	120	6	0.94 ± 0.14	0
CSC	22	125	10	0.97 ± 0.21	10
HSF	28	123	10	1.11 ± 0.24	50
JV	29	120	4	1.20 ± 0.29	50
JP	30	120	5	0.79 ± 0.21	10
GSF	30	120	5	0.77 ± 0.76	0
CF	35	120	5	1.01 ± 0.18	30

3.4. Blends of different protein materials

The combination of different protein materials is a valuable strategy to achieve possible scale-up for the use of a protein-based adhesive. Therefore, some of the better performing formulations were selected to investigate the effect of blending them on the bonding quality of wood preparations. The selected protein materials are listed in Table 7. In all blends, the ratio between the two protein materials was 1:1 (dry basis). As can be seen, HSF was present in all formulations. This choice was related to the fact that this product showed a marked improvement with the addition of the crosslinker and because flour, rather than isolate, is a low cost raw material that can reduce the final cost of the adhesive. SPI was also selected for blending to evaluate the effect of blending a flour with an isolate. The InFr results for the formulations considered are shown in Table 7 and were consistent with the values obtained with the individual formulations, which were already very similar between them (Fig. 5a).

Mechanical strengths according to both EN 205 (dry and wet) and EN 314–1 are shown in Table 8. It can be seen that the presence of HSF equalises the values for the other protein materials when tested according to EN 205 in both dry and wet conditions. For example, CSC increased the DTSS and WTSS from 8.4 and 3.0 MPa respectively (Table 4) to 11.5 and 3.4 MPa respectively (Table 8), while SPI confirmed the high DTSS and slightly increased the WTSS (from 2.5 MPa to 2.8 MPa). Results similar to ours were obtained by Cheng et al. [12], who mixed cottonseed and soybean isolates and concluded that blending seems to be a good way to regularise the adhesion strength to a value intermediate between the two pure materials.

Table 7
Insoluble fraction (InFr) for the selected blends of protein materials used together with PAE.

	InFr 'RT/RT' (%)	InFr 'RT/103 °C' (%)	InFr '103 °C/RT' (%)	InFr '103 °C/103 °C' (%)
SPI + HSF	87.3 ± 0.9	87.3 ± 0.9	87.3 ± 2.1	89.2 ± 1.5
CSC + HSF	83.6 ± 0.2	83.6 ± 0.2	82.6 ± 0.3	78.3 ± 0.1
SF + HSF	81.7 ± 1.5	81.7 ± 1.5	81.6 ± 1.4	80.5 ± 0.3

Table 8
Results of the shear strength tests carried out on material protein blends mixed with PAE. DTSS = dry shear strength (according to EN 205); WTSS = wet shear strength (according to EN 205); WSS_PW = wet shear strength carried out according to EN 314–1 (specimens tested wet after 24 h immersion in water).

	DTSS (MPa)	WTSS (MPa)	WSS_PW (MPa)	WFP (%)
SPI + HSF	11.6 ± 2.2	2.8 ± 0.7	1.0 ± 0.2	20
CSC + HSF	11.5 ± 0.6	3.4 ± 1.3	1.3 ± 0.3	50
SF + HSF	12.1 ± 0.5	2.6 ± 0.8	1.2 ± 0.1	0

This levelling effect was also obtained in tests carried out according to EN 314–1, where all the formulations considered showed values of WPSS_PW > 1 MPa. They could therefore be considered suitable for use in Class 1 without having to consider the WFP. Interestingly, these tests also confirmed that protein materials can be blended without problems in the production of plywood for interior use, and the strength values obtained can be adjusted to the level of the formulations that behave better.

4. Conclusions

The possibility of using 10 types of protein materials from seven different plants to produce formaldehyde-free wood adhesives for plywood production was investigated. Indeed, the use of biomass from different sources can ensure a steady supply of raw materials, facilitating their widespread application. These protein materials were investigated without any pre-treatment process, such as purification, concentration or other physical techniques (e.g. microwave or ultrasound treatments). This was done to reduce the cost of their use, to try to find proteins that inherently perform better than others, and to investigate the possible reasons for this higher performance.

The dissolution tests carried out showed that the materials with the highest protein content (SPI and MPC), used alone, showed low values of InFr (< 25 %) in tests at RT and even dissolved in hot water. Thus, the improvement of protein-protein interactions by heat treatment was limited. On the other hand, for flours and concentrates (protein content < 60 %), the high temperature favours the development of Maillard reactions with certain polysaccharides, resulting in bonds strong enough to resist water (InFr > 50 %), except for CF and SF. Mechanical tests according to EN 205 showed that dry strengths were higher in the protein-rich materials (> 10 N/mm^2) compared to those with a protein content of < 50 % (< 7 N/mm^2). In contrast, wet strengths were absent for MPC and flours (except HSF) and at least measurable for concentrates containing both protein and carbohydrates. This was due to the positive effect of thermal treatment and the development of Maillard reactions, although wet strength values were limited. A notable exception was JV, which could be classified as a D3 adhesive even when used alone. In any case, the addition of PAE to the protein materials changed this general picture.

The reactivity of PAE substantially homogenised both the values obtained in the dissolution tests and the dry shear strengths. On the other hand, the wet shear strengths increased significantly to values above the minimum threshold of 2 MPa specified in EN 204 for class D3 adhesives, with the exception of MPC. Interestingly, the highest values (≥ 3 MPa) were associated with flours and concentrates (except SF). However, the lower values measured for soy products (2.5 MPa) suggest that this reaction only contributes to wet strength when the polysaccharides have a complex, structured composition. In contrast, simpler, unstructured carbohydrates do not contribute to wet strength but do reduce water solubility.

Tests on plywood produced with the individual protein materials used alone confirmed the results of the EN 205 tests. Better values were observed for concentrates (≥ 0.6 MPa, except for JP) compared to both isolates and flours (≤ 0.2 MPa, except for HSF), whereas with the addition of PAE the strength values were > 0.7 MPa for all products and some of them (JV, HSF and CF) even exceeded the threshold of 1 MPa given in EN 314–2. In this case, there is no need to consider the WFP when evaluating the bonding quality of the panels.

When different protein materials were combined, it was shown that the addition of higher performing products, such as HSF, equalised the values of the other materials tested according to EN 205 in both dry and wet conditions, while still meeting the criteria of EN 314–2. This demonstrates that protein materials can easily be blended to bring strength values up to the level of the better performing formulations.

CRediT authorship contribution statement

Bernardo Grossi: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation. **Benedetto Pizzo:** Writing – review & editing, Visualization, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Francesco Siano:** Writing – review & editing, Investigation. **Antonio Varriale:** Writing – review & editing, Investigation, Funding acquisition. **Rosanna Mabilia:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Benedetto Pizzo and all the authors reports financial support was provided by National Institute for Insurance against Accidents at Work.

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Data availability

Data will be made available on request.

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