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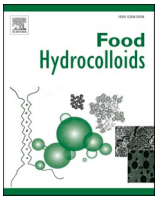

# Modulating commercial pea protein gel properties through the addition of phenolic compounds

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ABSTRACT

The use of pea protein in dense food is limited because of the low gel strength. Commercial pea proteins were modified with phenolics under alkaline conditions (pH 9, 24 h) that favour covalent bonding. Three phenolic compounds that differ in molecular size but contain similar structural units were selected (gallic acid, 0.17 kDa; epigallocatechin gallate, 0.458 kDa; tannic acid, 1.71 kDa) to better understand the role of molecular weight and added hydroxyl and aromatic groups on the gelling properties. The effect of the dose on gelling properties was studied by varying the phenolic concentrations (0–4 mM). The maximum changes were observed for conjugates prepared with tannic acid: colour, $\Delta E$ 38; decreased concentration of binding sites, 43%; solubility, 31%. The maximum increase in gel strength was 16-fold from 3.0 to 48 kPa. The result was positively correlated with the mass concentration of the added phenolic compounds, molecular weight and the approximate number of hydroxyl groups. Modification of pea proteins with phenolics can be as effective as adding thickening agents to increase the gel strength. To increase the elasticity of pea protein gel, the phenolic concentration added should not exceed 1.36 g/L, which is equal to 3.8 wt% of the protein mass. We demonstrated that pea protein modification with phenolics makes a useful tool to tailor gel strength and elasticity based on the molecular weight and the dose of phenolic compounds added.

## 1. Introduction

Pea (*Pisum sativum*) protein is increasingly used as an alternative to soy protein because pea plants can be grown in more moderate climates than soy. In addition, genetic modification is less of a concern with pea proteins and they are not listed as allergens (Lam, Can Karaca, Tyler, & Nickerson, 2018). Unfortunately, the low strength of pea protein gels compared with soy protein gels limits their use in foods (Batista, Portugal, Sousa, Crespo, & Raymundo, 2005; De Berardinis, Plazzotta, & Manzocco, 2023). For example, at pH 7, soy proteins formed gels that were almost 3.5-fold stronger than gels formed with pea proteins (De Berardinis et al., 2023) and the critical gelation concentration was higher for pea proteins than for soy proteins (Batista et al., 2005). Therefore, methods to increase gel strength are gaining attention, such as enzymatic crosslinking (Sun & Arntfield, 2011; Tang et al., 2006) and the addition of thickening agents (Uruakpa & Arntfield, 2004; Zhu et al., 2008). The functionality of proteins can also be modulated through conjugation with phenolic compounds (Keppler, Schwarz, & van der Goot, 2020).

Peas naturally contain phenolic compounds. Pea seeds are particularly rich in phenolic acids such as *p*-hydroxybenzoic acid, protocatechuic acid, gallic acid (GA), ferulic acid, and chlorogenic acid, and the pea pod is also rich in larger phenolics such as tannins (Nicolás-García et al., 2021). The phenolic content in pea pods ranges from 4.0 mg to 32 mg/g (Mejri et al., 2019; Pinchao-Pinchao, Ordoñez-Santos, & Osorio-Mora, 2019; Taha, Hetta, Ali, Yassin, & Guindi, 2011). The total protein content in pea pods accounts for 20%–27% of their weight (Wang & Daun, 2004), and the overall phenolic content ranges from 2% to 16% of the total protein mass. Phenolic compounds comprise an aromatic ring with one or more hydroxyl substituents. They can exist as simple phenolic molecules, polyaromatic molecules and highly polymerized compounds. Interactions between proteins and phenolic compounds can be noncovalent or covalent. Covalent bonds can be created by using alkaline conditions to induce deprotonation of the hydroxyl groups on the phenolic compounds forming quinones. Once these quinones are formed, they react covalently with other phenolic compounds, which results in dark-pigmented dimers and larger polyphenolics. In the presence of proteins, quinones react covalently

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with amino acid side chains, especially the free amino groups of lysine and the thiol groups of cysteine (Bittner, 2006; Li, Jongberg, Andersen, Davies, & Lund, 2016; Prigent, Voragen, Visser, Van Koningsveld, & Gruppen, 2007), leading to a deep brown, yellow or green colour (Bongartz et al., 2016; Iacomino et al., 2017). There are also reports of modification of tryptophan residues (Kroll, Rawel, & Rohn, 2003; Pham, Wang, Zisu, & Adhikari, 2019; Rawel, Czajka, Rohn, & Kroll, 2002; Seczyk, Swieca, Kapusta, & Gawlik-Dziki, 2019). The products resulting from covalent protein–phenolic interactions are referred to as conjugates.

Interactions between phenolic compounds and proteins are usually perceived negatively because they alter the expected properties of proteins. For example, covalent binding often decreases protein solubility, reduces the bioavailability of essential amino acids (Prigent et al., 2007) and changes the colour of the protein solution or dispersion (Jiang et al., 2019). However, a controlled change of protein properties by phenolic conjugation is an interesting approach to targeted applications. Under controlled conditions (such as choosing an optimum modification degree of the protein), the functionalities of plant proteins such as interfacial behaviour for emulsifying and foaming properties (Djuardi, Yuliana, Ogawa, Akazawa, & Suhartono, 2020; Pan et al., 2019; Sui et al., 2018; Tao et al., 2018; Wang et al., 2021; Yi, Wu, Yu, & Su, 2021) and gelation (Guo, Bao, Sun, Chang, & Liu, 2021; Zhang et al., 2023), were enhanced. In addition, harshly treated commercial protein isolates have relatively low functionality. Increasing the functionality of these proteins by covalent modification with phenolics can be especially interesting for industry.

So far, there is little information on the effects of covalent modification of plant proteins with phenolics on gel strength. In the case of sunflower proteins and chlorogenic acid, it was shown that noncovalent protein modifications reduced gel strength, but that covalent interactions increased gel strength in a dose-dependent manner (Jia et al., 2022). Studies on the gelling properties of pea protein–phenolic mixtures are scarce, although gelling properties are particularly important for new-generation plant-based products such as meat and dairy alternatives. In addition, systematic studies on the effect of both the molecular structure and the quantity of the phenolic compounds added on the physicochemical and gelling properties of proteins are limited.

It is hypothesized that the formation of protein–phenolic conjugates can increase the gel strength of protein gels because (1) of the formation of intermolecular covalent bonds between proteins, which can act as crosslinking agents (Ali, Keppler, Coenye, & Schwarz, 2018; Guo, Jiang, True, & Xiong, 2021) and (2) newly formed conjugates lead to additional hydrogen bonds and hydrophobic interactions. All these interactions are expected to contribute to the overall density of covalent and noncovalent bonds in the gel structure. Hence, it is expected that the strongest pea protein gels are obtained when they are conjugated with phenolic compounds that have the most hydroxyl groups and aromatic rings.

To prove whether phenolic compounds can modulate the gelling properties of commercial pea protein isolate, we studied the extent of the changes in gelling properties that can be achieved by creating protein–phenolic conjugates using pea protein isolate and phenolics in different ratios. This highlighted the dose-dependency effect on the gelling properties. To investigate the role of hydroxyl groups and aromatic rings on the gelling properties, we compared three different phenolic compounds with an increasing number of aromatic and hydroxyl groups but with similar structural units. Gelling properties are studied using rheology. The TNBS (2,4,6-trinitrobenzene sulfonic acid) method, Ellman's assay, and Folin Ciocalteu method were used to confirm the degree of modification and the number of hydroxyl groups. Changes in protein solubility were used to confirm the change in the overall hydrophobic character of the prepared pea protein isolate (PPI) conjugates because protein solubility in water can be enhanced when the size and hydrophobic character of the protein are reduced (Chabanon, Chevalot, Framboisier, Chenu, & Marc, 2007; Jones & Tung, 1983).

## 2. Materials and methods

### 2.1. Materials

Pea protein isolate (PPI) was supplied by Yantai Shuangta Food Co., 149 Ltd. (dry matter, 93%; purity, 73%; N factor, 5.7; lot no. 3700D04019DB). The proteins consisted of 37% legumins, 51% vicilins and 12% albumins following the method of Vreeke, Meijers, Vincken, and Wierenga (2023). Results from dynamic light scattering and differential scanning calorimetry revealed that the proteins in PPI were completely denatured and partly aggregated (Supplementary Material, Appendix A, Fig. 1A and B). Gallic acid (GA, product, no. G7384; purity, 98.5%; lot no. SLCJ8281), epigallocatechin gallate (EGCG; product no. PHR1333; purity, 92%; lot no. LRAC4432), tannic acid (TA; product no. 403040; purity, >99%; lot no. MKCH9318), sodium phosphate monobasic dihydrate (product no. 71500; purity, ≥99.0%; batch no. BCCG9521), sodium phosphate dibasic (product no. S7907; purity, ≥99.0%; batch no. BCCF510), sodium dodecyl sulphate (SDS; product no. L6026; purity, ≥99.0%; lot no. SLCB4247), picrylsulphonic acid solution (TNBS) 5 w/v% in $H_2O$ (product no. P2297; lot no. SLCK4178). L-leucine (purity, ≥98%; lot no. BCCG9176), L-cysteine (purity, ≥98%; lot no. BCBV4037), 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB, Ellman's assay) (product no. D8130; purity, ≥98%; lot no. SHBD2937V) were obtained from Sigma-Aldrich. Biodesign cellulose dialysis tubing was obtained from Fisher Scientific (8 kDa molecular weight cut-off; 49.55 mm diameter). Ethanol (product no. 20905.365; purity, 96%) was purchased from VWR Chemicals BDH. All other chemicals used were of analytical grade and purchased from Merck or Sigma-Aldrich. Ultrapure water was obtained from Milli-Q (Milli-Q IQ 7000 Ultrapure Lab Water System, Merck KGaA, Darmstadt, Germany) and was used for the preparation of all aqueous solutions.

### 2.2. Methods

#### 2.2.1. Preparation of PPI–phenolic conjugates

PPI was modified by alkaline treatment with phenolic compounds which favour covalent modification. Protein–phenolic mixtures were prepared with different molecular concentrations of phenolics normalized to the protein content. Eight grams of PPI was used following an estimated average molecular size of 73 kDa based on the proximate composition by SDS-PAGE. Three phenolic compounds with varying molecular weights were used: GA (170.1 g/mol), EGCG (458.37 g/mol) and TA (1701.1 g/mol). Stock solutions of the phenolic components were prepared in 96% ethanol and diluted further in 96% ethanol when required to obtain the specific phenolic molecular concentration. A total of 200 mL of protein–phenolic mixture was prepared for each phenolic molecular concentration, resulting in an aqueous dispersion with 1 wt% ethanol and 4 wt% PPI (0.08 mmol). Phenolic molecular concentrations ranged from 0 to 4 mM and these are described as the molar-to-molar ratio (Table 1).

The protein dispersions were prepared in ultrapure water after which the pH was adjusted to pH 9.0. The dispersion was stirred for 1 h. The phenolic–ethanol solutions were prepared separately and were also solubilized for 1 h. The phenolic–ethanol solution was added to the protein dispersion and the pH was adjusted back to 9.0. The protein–-phenolic mixtures were stirred at 200 rpm at room temperature with full exposure to air for 24 h. The mixtures were subsequently dialysed at 4 °C for 24 h to remove free phenolic compounds. Finally, the mixtures were frozen at −18 °C overnight and lyophilized into a powder which are referred to as the PPI–phenolic conjugates.

#### 2.2.2. Visual appearance: colour change

The change in colour of the prepared PPI–phenolic conjugates was measured using 0.8 g of powder. The colours were measured as CIE-LAB values using a port-up dual-beam benchtop spectrophotometer (Hunterlab Colorflex EZ; Hunter Associates Laboratory Inc., Reston, VA).

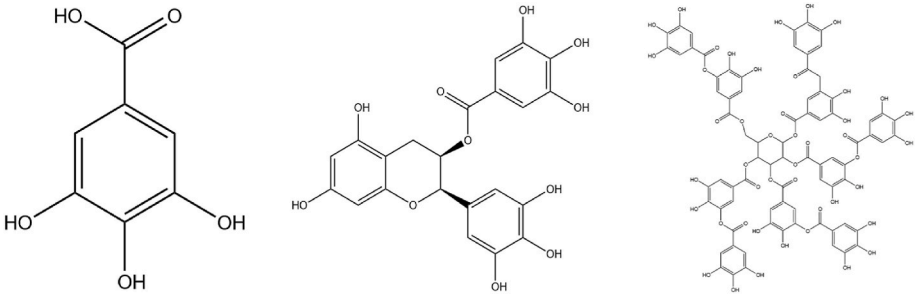

**Fig. 1.** Selected phenolic compounds from left to right: gallic acid (GA, 170 Da), epigallocatechin gallate (EGCG, 458 Da), and tannic acid (TA, 1700 Da).

**Table 1**
Overview of phenolics added in the preparation of pea protein–phenolic conjugates.

| Added phenolic molecular concentration (mM) | Molecular addition of phenolic (mmol) | Protein to phenolic ratio (mmol protein:mmol phenolic) | Added phenolic mass concentration (g/L) | | |
|---|---|---|---|---|---|
| | | | GA | EGCG | TA |
| 0 | 0 | 1:0 | 0 | 0 | 0 |
| 0.02 | 0.004 | 1:0.05 | 0.0035 | 0.0099 | 0.034 |
| 0.04 | 0.008 | 1:0.1 | 0.0069 | 0.020 | 0.068 |
| 0.08 | 0.02 | 1:0.2 | 0.014 | 0.040 | 0.14 |
| 0.2 | 0.04 | 1:0.5 | 0.035 | 0.099 | 0.34 |
| 0.4 | 0.08 | 1:1 | 0.069 | 0.20 | 0.68 |
| 0.8 | 0.2 | 1:2 | 0.14 | 0.40 | 1.4 |
| 2 | 0.4 | 1:5 | 0.35 | 0.99 | 3.4 |
| 4 | 0.8 | 1:10 | 0.69 | 2.0 | 6.8 |

The same molecular quantities were used for all used phenolic compounds gallic acid, epigallocatechin gallate and tannic acid.

$$\Delta E = \left[(L^* - L_0^*)(a^* - a_0^*)(b^* - b_0^*)^2\right]^{1/2} \quad (1)$$

where $\Delta E$ is the colour change, $L$ values stand for lightness (−), $a$ values stand for red/green hue (−) and $b$ values stand for blue/yellow hue (−). $L_0$*, $a_0$* and $b_0$* are the colour values of the control.

### 2.2.3. Chemical characterization

For the chemical characterization in Sections 2.2.3.1, 2.2.3.2. and 2.2.3.3. The lyophilized PPI–phenolic conjugate powders were dispersed in 5% SDS solvent to a 5 wt% dry matter concentration. SDS is a common denaturing agent for proteins and allows water-insoluble proteins to be solubilized. Screening was performed with various concentrations of SDS ranging from 0 to 10 wt% to find the concentration of SDS at which maximum pea protein solubilization took place. A 5 wt% SDS solution appeared to be the most efficient and was able to solubilize 100% of the pea proteins in water. This SDS concentration for pea protein solubilization was in accordance with the work of Wehrmaker et al. (2022). The samples were stirred overnight at room temperature and subsequently centrifuged at 15,000 RCF (relative centrifugal force) at room temperature for 20 min. The supernatant obtained was used for all chemical characterization analyses. The soluble protein content in the samples was determined with the Dumas method (N factor 5.7).

*2.2.3.1. Ellman's assay: free thiol groups.* The content of free thiol groups was determined with the DTNB method (Ellman's assay) from Ellman (1959) with slight modifications. First, 25 μL of sample or calibrant was added to 1 mL of 0.2125 M phosphate buffer (pH 8.2). Next, 500 μL of 10 mM DTNB reagent was added and incubated at room temperature in the dark for 15 min. The absorbance was measured at 412 nm with a spectrophotometer (DR3900 Laboratory VIS Spectrophotometer; Hach, Loveland, CO). The absorbance values were corrected for the absorbance of the samples that followed the same protocol without reagent. The calibration was created with L-cysteine with a concentration range between 0 and 3.3 mM.

*2.2.3.2. TNBS: free amino groups.* The content of free amino groups was determined with the TNBS method described by Adler-Nissen (1979) with slight modifications that were similar to the work of Wehrmaker et al. (2022). First, 65 μL of the sample solution was added to 0.5 mL of 0.2125 M phosphate buffer (pH 8.2). Subsequently, 0.5 mL of TNBS reagent was added and the samples were incubated at 60 °C covered from light for 1 h. The reaction was stopped by the addition of 0.1 M HCl and rested for 30 min in the dark before the absorbance was measured at 340 nm with a spectrophotometer (DR3900 Laboratory VIS Spectrophotometer). The absorbance values were corrected for the absorbance of the samples that followed the same protocol without reagent. The calibration curve was created using L-leucine with a concentration range between 0 and 1.5 mM.

*2.2.3.3. Folin Ciocalteu: total phenolic content.* The total phenolic content was measured with the Folin Ciocalteu method. The concentration of free phenolic hydroxyl groups can be estimated from the phenolic concentration. The reactivity with the reagent and the resulting absorbance can relate to the number of free phenolic hydroxyl groups that can react with the reagent and the molecular weight. However, the absorptivity per reactive group can differ for each phenolic compound and is difficult to predict (Singleton, Orthofer, & Lamuela-Raventós, 1999). For the reaction, 20 μL of sample or calibrant was used, 1.6 mL of ultrapure water was added followed by 100 μL of Folin reagent and 0.3 mL of sodium carbonate. The samples were incubated at 60 °C for 45 min. The absorbance was measured at 765 nm. The calibration was made using GA with a concentration range between 0 and 5 mM.

### 2.2.4. Rheology: gelling properties

Gelling properties were measured in an Anton Paar Modular Compact Rheometer (MCR302; Oosterhout, Netherlands). Dispersions of 15 wt% dry mass were prepared in ultrapure water and stirred at 4 °C overnight. Samples were brought back to room temperature and 1 h before the measurement, the pH was adjusted to pH 7. All rheological measurements were strain-controlled and performed in duplicate.

*2.2.4.1. Small deformations.* Linear viscoelastic properties of the gels were evaluated with small amplitude oscillatory shear (SAOS), as performed by Diedericks, de Koning, Jideani, Venema, and van der Linden (2019) and Kornet et al. (2021). The protein dispersion was gelled within the concentric cylinder in the rheometer. The samples were exposed to a temperature sweep with constant strain (0.1%). Protein-rich solutions (15 wt% dry matter, pH 7) were placed in a smooth concentric cylinder geometry (CC10) with a layer of paraffin oil to prevent water evaporation. The temperature was increased from 20 °C to 95 °C at a rate of 3 °C/min. The temperature was kept constant at 95 °C for 30 min after which it was lowered to 20 °C at a rate of 3 °C/min. The temperature was then kept constant at 20 °C for 20 min.

The dependence of the storage ($G'$) and loss ($G''$) moduli on temperature was measured. Samples were evaluated for gel strength, defined here as the elastic modulus ($G'$) at room temperature at the end of the temperature sweep.

*2.2.4.2. Large deformations.* Large oscillatory shear (LAOS) was performed as a continuation of the SAOS measurements. Hence, the same geometry and gels were used. The length of the linear viscoelastic regime for the gelled samples was studied with a strain sweep at a constant frequency of 1 Hz. The dependence of the storage ($G'$) and loss modulus ($G''$) were measured as a function of strain amplitude. Gelled samples were subjected to a logarithmically increasing strain (−) from 0.01 to 100 at 20 °C for 36 min to collect 80 data points. The end of the linear viscoelastic regime was defined as the strain at which the elastic modulus had decreased to 90% of its plateau value. The strain at which this occurred is referred to as the critical strain (−). This is the point at which the gel network is disrupted by internal fractures. Stress and strain data obtained from the LAOS measurements were analysed using the MITlaos software (version 2.2 beta, freeware distributed from MITlaos@mit.edu) in MATLAB R2022B. The odd harmonic n = 3 was used in the analysis which is deemed most relevant for semi-solid foods (Yazar, Caglar Duvarci, Yildirim Erturk, & Kokini, 2019). Only elastic Lissajous curves (stress vs strain) were constructed because viscous Lissajous curves (stress vs strain rate) gave almost perfectly mirrored outcomes and were not required to explain structure breakdown phenomena in this study. Elastic Lissajous curves were constructed for strain values 1.07, 11, 27.9, 49.9 and 89.7.

*2.2.4.3. Underpinning of phenolic binding mechanism in PPI–phenolic conjugate gels.* To study the effect of assumed noncovalent interactions of phenolics on the PPI protein gels, the effect of 'unoxidized' TA and 'pre-oxidized' TA was studied on the gelling properties.

In the first scenario, TA was not treated under alkaline conditions and was directly added in the concentration of 2 mM and 4 mM to the 15 wt% PPI reference dispersion in ultrapure water at pH 7 and stirred overnight at 4 °C. Samples were brought back to room temperature and 1 h before the measurement, the pH was adjusted to pH 7.

In the second scenario, 2 mM TA was pre-oxidized in the absence of pea proteins in similar conditions as described in Section 2.2.1. The phenolics were dispersed in an aqueous solution with 1 wt% ethanol. The pH of the dispersion was adjusted to 9.0 and was stirred at 200 rpm at room temperature with full exposure to air for 24 h. Afterwards, the pH was adjusted to 7. Subsequently, the phenolic dispersion was frozen at −18 °C overnight and lyophilized into a powder. This pre-oxidized phenolic powder was added to a 15 wt% dispersion of the PPI reference in ultrapure water at pH 7 and stirred overnight at 4 °C. Samples were brought back to room temperature and 1 h before the measurement, the pH was adjusted to pH 7.

Subsequently the gelling properties were recorded according the same protocols enlisted in Sections 2.2.4, 2.2.4.1 and 2.2.4.2.

*2.2.5. Confocal laser scanning microscopy (CLSM): visualization of microstructure*

The protocol of Janssen, Pouvreau, and de Vries (2024) was used for the visualization of the PPI and prepared gel structures. Protein dispersions (15% w/w protein, pH 7, 30 mM) were labelled non-covalently with a 0.005% Rhodamine B dye solution. The stained dispersions were transferred to sealed glass slides (Gene frame 25 μL adhesives, Thermo Fisher Scientific, UK) and heated in a water bath at 95 °C for 30 min after which they were cooled down to room temperature and stored at 4 °C overnight. The microstructures were visualized using a Nikon C2 Confocal laser scanning microscope (Nikon Instruments Inc., U.S.A). The excitation wavelength of 561 nm and a dry objective with 10× magnification was used. Images were processed using Fiji (Image J) software.

*2.2.6. Statistics*

XLSTAT (2023) add-in for Word software was used for the statistical analyses. All measurements were performed in triplicate unless stated otherwise. The mean values and standard deviations were calculated and used as a measure of error. Raw data was analysed on normality with the Shapiro-Wilk test. Significant differences in the measured values were determined by ANOVA followed by a Tukey's Honest Significant Difference (HSD) post hoc test. Significance was defined as $P < 0.05$.

## 3. Results and discussion

This section starts with a demonstration of the effect of phenolics on colour formation (Section 3.1). Following are a series of tests that confirm the modification of the PPI with the phenolic compounds by changes in the number of binding sites and changes in the free phenolic concentration (Section 3.2). Changes in the gelling properties as a consequence of pea protein modification with phenolic compounds of different molecular weights are discussed in Section 3.3. In Sections 3.4. and 3.5. All experimental outcomes are combined and underlying causes for changes in gel strength and deformability are discussed. Section 3.6. Places the overall increase in gel strength in perspective with alternative approaches (e.g. enzymatic crosslinking and the addition of thickening agents) typically used for increasing the gel strength of plant proteins.

### 3.1. Detection of colour change

The prepared samples showed a gradual change in colour ($\Delta E$) with increasing addition of phenolic compounds. Fig. 2 shows the lyophilized powders obtained after conjugation.

PPI powders were light in colour initially ($L$ values ≥ 70) and were almost white with a slight yellow hue ($a^*$ value close to 0, $b^*$ values ± 20). The PPI–phenolic conjugates were darker ($L$ values ≤ 45, no clear changes in $a^*$ and $b^*$ values), appearing brown (Supplementary Material, Appendix B). This brown colouring was also seen by Jiang et al. (2019) in soy proteins that were covalently modified with anthocyanins. When $\Delta E \geq 3.5$, an inexperienced observer can notice the difference in colour between the samples. When $\Delta E \geq 5$, the observer notices two different colours (Mokrzycki & Tatol, 2011). The maximum colour change ($\Delta E$) was 28 for GA, 31 for EGCG and 38 for TA. Thus, the largest colour differences occurred with TA, the largest phenolic compound (GA, 0.17 kDa; EGCG, 0.458 kDa; TA, 1.7 kDa) at the highest phenolic concentration added (4 mM). This can indicate that greater modification of PPI took place for the larger phenolic compounds and high phenolic concentrations. It could also indicate colouration caused by phenolic oxidation alone. To exclude the latter possibility, changes in potential binding sites on the proteins were measured.

### 3.2. Molecular characterization

#### 3.2.1. Detection of potential binding sites

Alkaline incubation of phenolics with proteins typically results in the oxidation of phenolics and can lead to subsequent covalent modification of the protein. However, the mode of binding (covalent and/or non-covalent) is difficult to assess for insoluble large plant protein aggregates as those present in this commercial PPI. Various attempts were made to get further information about the modification of the PPI used in this study. The covalent binding of phenolics to proteins is usually validated with chemical analyses. We focussed on the most reactive binding sites under alkaline conditions to confirm covalent modification; i.e. cysteine (SH groups) and lysine (NH groups) (Bittner, 2006; Li et al., 2016; Prigent et al., 2007). The concentration of free thiol groups (SH groups) in PPI was measured with Ellman's assay before and after incubation with increasing concentrations of phenolic compounds. Unfortunately, the concentration of cysteines was below or close to the detection limit of the assay (results not shown). The low cysteine

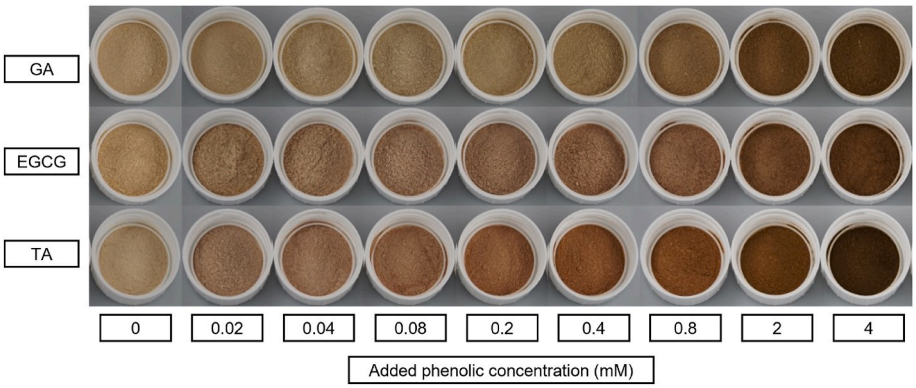


**Fig. 2.** Lyophilized powders of conjugated pea proteins. Pea protein conjugates formed with gallic acid (GA; top row), epigallocatechin gallate (EGCG; middle row) and tannic acid (TA; bottom row). From left to right: no modification with phenolics to modification with increasing phenolic concentration up to 4 mM.

concentration is probably because the PPI was already denatured and possibly aggregated (Section 2.1; Supplementary Material, Appendix A, Fig. 1A and B), which can block the exposure of free thiol groups. Furthermore, PPI is limited in cysteine residues to begin with (Gorissen et al., 2018). Therefore, we assumed that the number of binding sites is dominated by the free amino groups of lysine. Fig. 3 shows the change in concentration of free amino groups using the TNBS assay as a function of the added phenolic concentration.

The amount of free amino groups was reduced when PPI was modified with phenolics. This was most apparent when the high molecular weight phenolic compound TA was used. The addition of GA resulted in the lowest reduction of free amino groups. When normalizing the values for the PPI reference (i.e. 100% unmodified or 0% modified), the fraction of amino groups that had reacted could be estimated. It was found that conjugates prepared with GA could react with 23% of the binding sites, 33% for EGCG and 43% for TA. We conclude from these results that a larger phenolic compound binds multiple amino groups (either of the same protein or between different proteins). This is in line with the overview of Masoumi, Tabibiazar, Golchinfar, Mohammadifar, and Hamishehkar (2022), whose findings indicate that phenolics with higher molecular weight generally can bind with more hydroxyl groups to proteins. This increases the possibility of TA reacting with multiple binding sites (multidentate) simultaneously (Czubinski & Dwiecki, 2022; Dubeau, Samson, & Tajmir-Riahi, 2010; Huang et al., 2011; López-Yerena, Perez, Vallverdú-Queralt, & Escribano-Ferrer, 2020; Sekowski et al., 2018). The exact number of occupied binding sites per mass of pea protein could not be determined because the solubility of PPI in the 5% SDS solvent decreased after modification with phenolic compounds. The loss in solubility increased with increasing modification and with increasing phenolic size up to a loss of 12% for GA, 20% for EGCG and 31% for TA. However, the loss in binding sites as a function of phenolic addition exceeds the reduction in solubility as a function of phenolic addition (Supplementary Material, Appendix D, Fig. 3A and B). This confirms that the reduction in binding sites is not only caused by a loss in solubility but also by covalent PPI modification in solution.

Another aspect that should be considered is the possible formation of lysinoalanine (LAL) because proteins alone can be oxidized upon alkaline treatment as well (Friedman, Levin, & Noma, 1984; Karayiannis, MacGregor, & Bjeldanes, 1979; Struthers, 1981; Wehrmaker et al., 2022). In this study, little change in free amino groups (7.4% reduction) and thiol groups (3.0% increase) was seen between the starting material and alkaline-treated pea protein (PPI reference). However, analysis such as liquid chromatography-mass spectrometry would be required to completely exclude the formation of LAL, which is suggested for future studies.

### 3.2.2. *Detection of modification with free phenolic OH groups*

As stated in Section 3.2.1, the binding of phenolics to protein can be related to the molecular weight and the number of free hydroxyl groups on the phenolic used. The Folin Ciocalteu method can be used to measure the phenolic concentration, which can be correlated to the concentration of free phenolic hydroxyl groups (Section 2.2.3.3). The molar absorptivity per reactive OH group of the phenolics and the exact oxidation products are not known. Therefore, the phenolic concentration was not converted into the free phenolic OH concentration. However, a relative increase in the phenolic concentration (and approximate concentration of free phenolic OH groups) in the conjugates compared

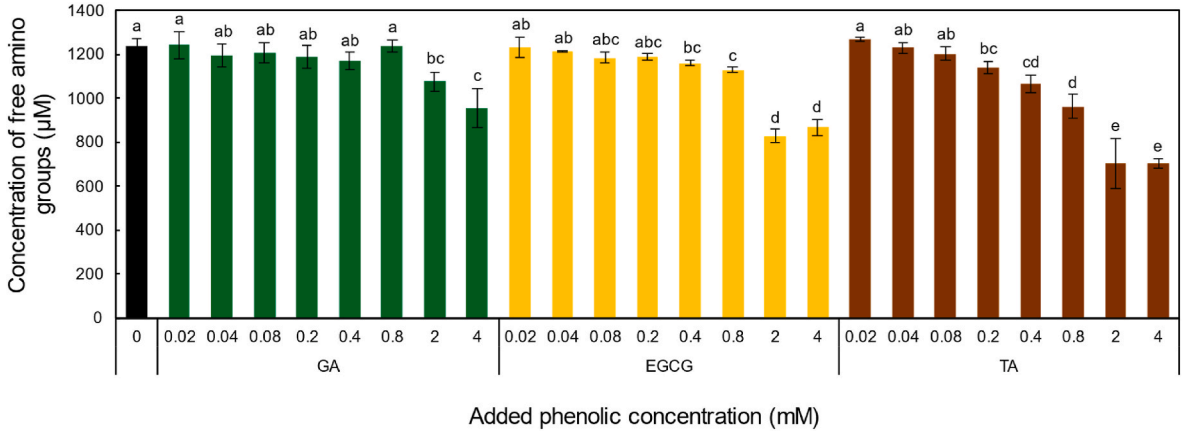


**Fig. 3.** The concentration of free amino groups (μM) compared with the added phenolic concentration (mM) for pea proteins modified without phenolics (reference, black), with gallic acid (GA, green), epigallocatechin gallate (EGCG, orange) and tannic acid (TA, brown).

with the unmodified PPI can indicate covalent attachment since all samples were extensively dialysed to remove unbound phenolics. Therefore, the total phenolic concentration was measured for each of the conjugates (Fig. 4). It is expected that the phenolic containing the most free phenolic hydroxyl groups per molecule will have higher absorbance with the Folin reagent at similar molecular concentrations. Before alkaline treatment, GA (0.17 kDa) has 3 free hydroxyl groups on the benzene ring, whereas EGCG (0.458 kDa) has 8 and TA (1.7 kDa) has 25 free hydroxyl groups (Fig. 1). As a result, the highest measured phenolic concentrations were expected for PPI conjugates prepared with the phenolics in the following order TA > EGCG > GA when added at the same molecular concentration.

An increase in the phenolic concentration, and hence in free phenolic hydroxyl groups, was seen for conjugates prepared with TA and EGCG with increasing added phenolic concentration (Fig. 4). At the same added phenolic concentrations, starting from 0.08 mM, a higher phenolic concentration was measured for TA than for EGCG and GA. At the highest added phenolic concentration (4 mM), the highest measured phenolic concentration was 9137 μM for TA followed by 4305 μM for EGCG and 2337 μM for GA. For the conjugates prepared with GA, no increase in phenolic concentration was seen compared with the PPI reference concentration (2623 μM). This confirms that with the same molecular concentration of TA, more free phenolic hydroxyl groups were detected compared with EGCG and GA and suggests that the molecular structure of the phenolic compounds influenced the absorbance of the assay. All three phenolic compounds most likely underwent oxidation reactions to quinones, and subsequent condensation and protein conjugation reactions, which decreased the content of OH groups participating in the Folin reaction. Thus, the slight decrease in phenolic content for PPI–GA conjugates was caused by the loss of free OH groups on oxidation. The relative loss of free OH groups is bigger for small phenolics than for larger ones, which is why this effect was not seen for PPI–EGCG and PPI–TA conjugates. This was confirmed by calibration curves made from the oxidized phenolics alone. The absorbance was highest for the phenolics in the following order: oxidized TA > oxidized EGCG > GA > oxidized GA (Supplementary Material, Appendix C) and is in line with the study of Singleton et al. (1999). Thus, the Folin method appears to be more suitable for detecting covalent conjugation for larger phenolic compounds than for smaller ones.

However, subsequent methanol extraction of PPI–phenolic conjugates showed that residual free phenolic compounds were still present and likely also contributed to the Folin signal (Supplementary Material, Appendix E). The highest concentrations of unbound phenolics were measured for 4 mM PPI–TA conjugates followed by PPI–EGCG conjugates and PPI–GA conjugates. This also explains the lower concentration of phenolics measured for PPI–GA conjugates. With the 4 mM PPI–TA conjugates, 4.02 g/L (2.36 mM) of initially added phenolic mass was noncovalently attached, even after dialysis. For the 2 mM PPI–TA conjugates this was only 0.07 g/L (0.04 mM). For the PPI–GA conjugates this was maximally 0.059 g/L (0.34 mM) and for the PPI–EGCG conjugates this was 0.339 g/L (0.68 mM). Therefore, the observed large increase in measured phenolic content between the 2 mM and 4 mM PPI–TA conjugate in Fig. 4 can be mainly attributed to residual noncovalently bound phenolics. The quantity of covalently attached phenolics is probably similar, especially when comparing the content of free amino groups (Section 3.2.1). It suggests that noncovalently bound phenolics were greatly involved with the 4 mM TA conjugates but were more limited for any of the other formed PPI–phenolic conjugates. In addition, the loss in protein solubility with an increasing degree of modification can result in an underestimation of the concentration of phenolics. Therefore, these results cannot substantiate the actual binding of phenolics on the conjugates.

In conclusion, overall changes for pea proteins in colour (Section 3.1), binding sites (Section 3.2.1) and detected phenolic concentration (Section 3.2.2) were enhanced when increasing the added molecular concentration of phenolics and the phenolic size. The results of an individual chemical characterization analysis didn't allow for strong concluding remarks of actual covalent binding of phenolics onto the pea proteins. However, observations for the three analyses combined suggest that covalent modification of PPI took place, albeit with the presence of some unbound or non-covalently bound oxidized phenolics.

### 3.3. Effect of PPI modification on gelling properties

PPI–phenolic conjugate dispersions in water were exposed to a so-called temperature sweep with a maximum temperature of 95 °C and the gelation behaviour was monitored over time (Section 3.3.1). The resulting gel strength after heating and subsequent cooling is discussed in Section 3.3.2. The structure breakdown behaviour of the PPI–phenolic conjugate gels is discussed in Section 3.3.3.

#### 3.3.1. Effect of modification on gelation kinetics

The effect of heating and subsequent cooling on PPI and modified PPI dispersions is illustrated in Fig. 5. The gelation behaviour of unmodified PPI and the 4 mM PPI–TA conjugate, the most extensively modified conjugate, are shown as examples. Looking at the gelation behaviour in Fig. 5, we see that $G'$ is always larger than $G''$, no moduli cross-over occurs. This indicates dominant solid-like behaviour and suggests an physical gel was formed from the start, before heating. For pea protein gels, network formation mainly relies on physical bonding e.g. hydrogen bonding and hydrophobic interactions between protein molecules (Sun & Arntfield, 2012), which is enhanced when proteins are unfolded through heating. Therefore, one generally observes the strengthening of the gel structure during applied heat for pea proteins (Kornet et al.,

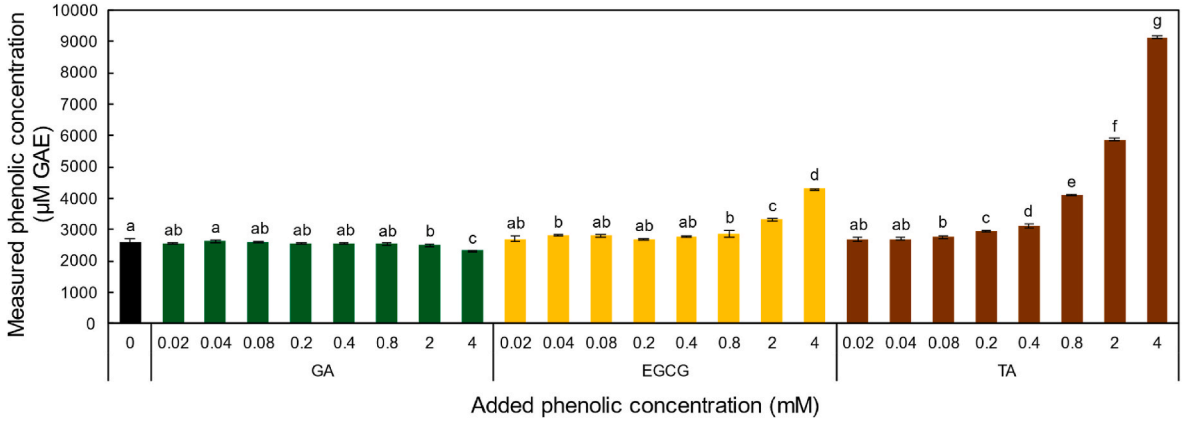


**Fig. 4.** Measured concentration of phenolic compounds (μM) compared with the added phenolic concentration (mM) for pea proteins modified without phenolics (reference, black), with gallic acid (GA, green), epigallocatechin gallate (EGCG, orange) and tannic acid (TA, brown).

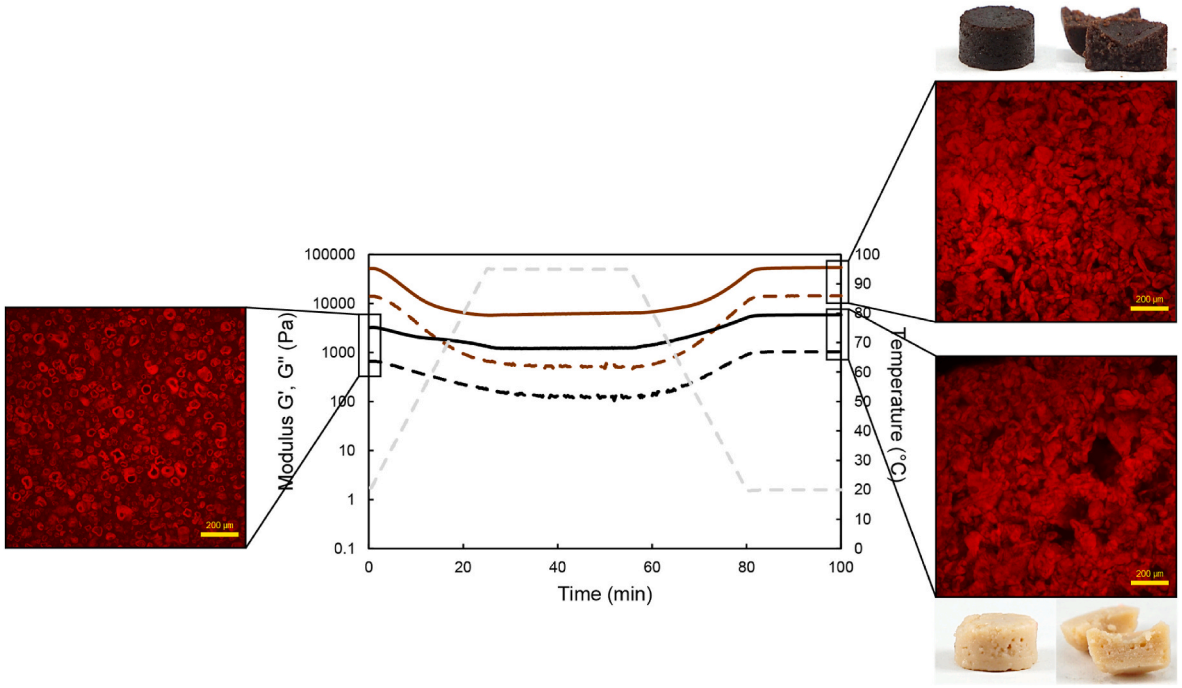


**Fig. 5.** Moduli $G'$ (continuous line) and $G''$ (dashed line) in Pa are indicated on the left *y* axis as a function of time in minutes. The given temperature (grey dotted line) as a function of time is indicated on the right *y* axis in °C. Samples were measured in duplicate, and representative curves of unmodified pea protein (reference, black) and pea protein modified with 4 mM tannic acid (brown) are shown. CLSM images were made with rhodamine B colouring at 10x magnification of the unmodified pea protein (reference, black) before (left picture) and after heating (right bottom picture) and of pea protein modified with 4 mM tannic acid (brown) after heating (right top picture). The visual appearance of corresponding gels are shown together with the CLSM figures.

2021; Sun & Arntfield, 2010). Spherical and hollow protein aggregates and particles were seen in the PPI before heat treatment (Fig. 5), which is in line with previous observations (Section 2.1) and suggests limited additional unfolding of the PPI is possible. Fusion of the protein aggregates and particles was observed for the PPI after heat treatment (Fig. 5), but did not increase already existing physical interactions between proteins. Instead, heat treatment weakened the protein gel temporarily, and subsequent cooling resulted in the regain of its original strength. The regain of the gel strength on cooling suggests there is a reformation of attractive forces between protein aggregates. In addition, for proteins rich in free thiol groups, the moduli can increase over time when gel structures are fully cooled because of the formation of disulphide bridges (Alting, Hamer, De Kruif, & Visschers, 2003). This effect was also seen in other studies with pea proteins (Kornet et al., 2021; Yang, Zamani, Liang, & Chen, 2021). For the PPI gels formed in this study, no further strengthening at 20 °C was seen. Presumably the lack of free SH groups (Section 3.2.1) prevented the possibility of the formation of additional S–S bonds. The results suggest that the gelation process of the PPI used in this study primarily involved noncovalent interactions (i.e. hydrogen bonding and hydrophobic interactions) between aggregated proteins.

No changes in gelation kinetics were seen for modified PPI, which suggests that the gelation mechanism was unchanged. Yet, the gel structures formed before and after the temperature sweep were stronger when more phenolics were bound to proteins. For instance, observed $G'$ values for the gels formed with 4 mM TA (Fig. 5) after cooling down were increased 16-fold compared with the gels from the unmodified PPI. The formed gel of the 4 mM TA conjugates was dark in colour which is in line with the results of Section 3.1. Confocal laser scanning microscopy showed a denser microstructure for the 4 mM PPI–TA conjugate gel than for the PPI reference. This agrees with visual observations where more defects (inhomogeneities such as incorporated air bubbles) were seen in the gels formed for the PPI reference. In addition, the PPI reference gel released serum from its structure and a smoother surface was observed after slicing. This was not seen for the 4 mM PPI–TA conjugate gel which had a more dense and gritty appearance at the surface after slicing.

### 3.3.2. *Effect of modification on gel strength after heat treatment*

A gradual increase in gel strength ($G'$ obtained at the end of the temperature sweep) can be seen with increasing modification of PPI (Fig. 6A). The largest increase in average gel strength was 48 kPa for TA conjugates which was significantly increased as compared to unmodified PPI (3.0 kPA). The highest average gel strength for EGCG conjugates was 22 kPa and 9.7 kPa for GA conjugates. Similar results were seen for other studies where the larger phenolics were more effective in increasing the gel strength of myofibrillar proteins, soy proteins and ginkgo seed proteins than mono phenolics (Guo, Jiang, et al., 2021; Y. Guo, Jiang, et al., 2021; Zhang et al., 2023). When the gel strength was plotted against the mass concentration of phenolic compounds (Fig. 6B), a master curve was obtained that shows a general pattern of the phenolic addition in mass on the elastic modulus irrespective of the type of phenolic selected. Therefore, it seems that the mass ratio is more relevant than the molar ratio of phenols and proteins to describe the effect on gel strength. This is only possible when one molecule of the larger phenolics can bind with several binding sites in the protein. That means that for the phenolics used in this study one molecule of TA would be able to bind most sites followed by EGCG and last GA.

No detrimental effect of phenolic compound conjugation on the gel strength was observed within the concentrations used in this study (GA, 1.7 wt%; EGCG, 5.0 wt%; TA, 17 wt% of protein mass), which is in contrast to other studies performed on plant protein–phenolic conjugates (Jia, Singh Sethi, Van der Goot, & Keppler, 2022; Zhang et al., 2023) and fibrillar protein–phenolic conjugates (Cao & Xiong, 2015; A. Guo, Jiang, et al., 2021). Zhang et al. (2023) described an increase in gel strength when ginkgo seed protein gels were modified with GA, EGCG or TA. However, a detrimental effect was seen for gel systems with EGCG at 4 wt% of the protein mass. Guo, Jiang, True, and Xiong (2021) showed that soy protein–tannic acid conjugates also exhibited a detrimental

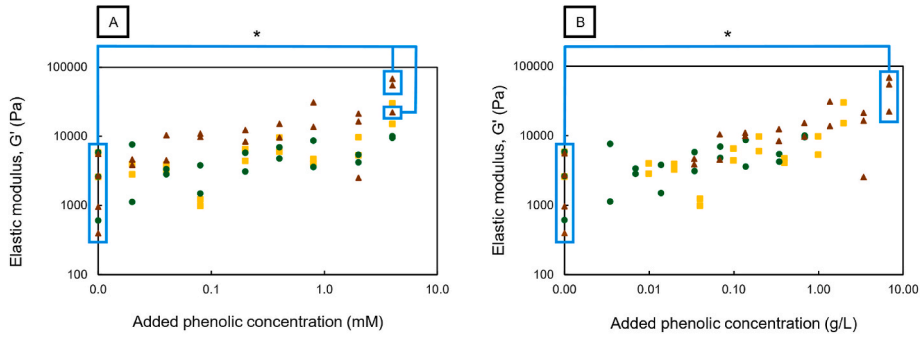


**Fig. 6.** Measured $G'$ modulus expressed as gel strength (Pa) after imposed temperature sweep compared with (A) added phenolic concentration (mM) and (B) (g/L) for pea proteins modified with gallic acid (green/dot), with epigallocatechin gallate (orange/square) and with tannic acid (brown/triangle). PPI gels modified without phenolics are manually added on the line of the y-axis. Statistical significant difference ($P < 0.05$) is indicated with * between the highlighted sample groups by blue frames.

effect on the gel strength at higher TA concentrations (25 wt% of protein mass); below this concentration, increases in gel strength were observed. It is possible that, in this study, the concentration at which a detrimental effect can be seen was not yet reached. Differences can also be attributed to variations between protein sources (e.g. amino acid composition, protein folding) and treatment history (native versus denatured protein) which greatly affects phenolic binding and functionality (Keppler et al., 2020; Yan et al., 2023). For instance, various studies attributed observed changes in gel strength partly to protein unfolding caused by the attachment of phenolics. At lower phenolic additions, unavailable SH groups became exposed and could participate in the formation of S–S bridges, thereby strengthening the gel. Extensive modifications blocked the thiol groups and hindered protein–protein interactions which resulted in weakening of the gel (Ali, Homann, Khalil, Kruse, & Rawel, 2013; Cheng, Lin, Tang, Yang, & Liu, 2022). We did not observe such an effect because little to no free thiol groups were available (Section 3.2.1), to begin with, because of the denatured state of the pea proteins (Section 2.1; Section 3.3.1). In addition, the increase in gel strength obtained for relative highly modified pea proteins could be attributed to both covalent and noncovalent attachment of phenolics. For example, the 2 mM PPI-TA conjugates had a similar degree of covalent modification as the 4 mM PPI-TA conjugates (Section 3.2.1), while the gel strength still increased. However, the measured residual unbound phenolic concentration greatly increased (Section 3.2.2; Supplementary Material, Appendix E). This confirms the role of noncovalent protein-phenolic interactions on the gel strength by residual unbound phenolics. These were most relevant for the 4 mM PPI-TA conjugates and did not play such an apparent role for the other PPI-phenolic conjugates as mentioned in Section 3.2.2. Hence, an increase in gel strength can be attributed to both covalent attachment of phenolics to the PPI as well as by noncovalent PPI–phenolic interactions.

### 3.3.3. *Effect of conjugation on gel deformability and fracture behaviour*

Subsequently, the gel was exposed to large deformations to study the deformability. The calculated critical strain, the strain at which structural breakdown was initiated, is shown in Fig. 7A and B. Structure breakdown occurs when all bonds between structural elements of a material in a certain macroscopic plane break. It ultimately leads to the falling apart of the material because the breakdown of the structure occurs over larger length scales than the structural elements themselves (Van Vliet & Walstra, 1995). In Fig. 7A, the critical strain is depicted as a function of the added phenolic concentration (mM). In Fig. 7B, the critical strain is plotted as a function of the added phenolic concentration based on mass (g/L). In addition, elastic Lissajous curves were constructed to further reveal the effects of phenolic addition on the structure breakdown patterns of the gels (Fig. 7C). Apart from the breakdown patterns, the dissipation ratio ($\varphi$) can be calculated to summarize the overall essential nonlinear behaviour (Ewoldt., Hosoi, & Mckinley, 2008; Klost, Brzeski, & Drusch, 2020). When $\varphi = 0$, the rheological response is purely elastic, whereas $\varphi = 1$ suggests that the material shows perfect plastic behaviour. We speak of a purely viscous or Newtonian fluid when $\varphi = \pi/4 \approx 0.785$ (Ewoldt et al., 2010). The dissipation ratio values are presented in a heatmap for comparison (Fig. 7D).

A higher critical strain is associated with stronger gel structures that require higher deformation before structure breakdown occurs (Song, Kuk, & Chang, 2006). An increase in the number of bonds and a higher strength of the bonds can increase the overall gel strength and resistance to deformation. In Fig. 7A, the critical strain increases with increasing added phenolic concentration. For the larger phenolics, the maximum deformation was seen at molar concentrations of 0.8 mM for TA (strain 11.2) and 2 mM for EGCG (strain 12.4). The addition of more phenolics after this point resulted in more brittle gels. For conjugates prepared with GA, this optimum was not reached. This relationship of the critical strain as a function of phenolic addition in mass concentration (Fig. 7B) was more apparent, showing that the phenolic concentration should not exceed 1.36 g/L to see an increase in deformability.

Constructed elastic Lissajous plots (Fig. 7C) showed similar breakdown patterns for all samples, which indicates inherent gel structures formed with the conjugates were similar to unmodified pea protein gels. The area enclosed by the Lissajous curve is a measure of the energy dissipation in the gel structure (Ptaszek, 2014). The elastic Lissajous curves showed linear viscoelastic behaviour at small strain amplitudes, which can be observed from small enclosed areas from the curves, the elliptical shape, the tilting and the straight line for the viscous stress contribution. As the deformation increases, the elliptical shape of the curves distorts and becomes wider, which indicates increasing viscous energy dissipation, and thus more liquid-like behaviour due to gel structure breakdown. From a strain of 27.9 onwards, the elastic stress contribution deviates from a linear line, indicating that viscous behaviour becomes more relevant. Deflections of the initial elliptical shape at maximum deformation into an inverted sigmoidal shape were seen, suggesting a mild intra-cycle strain stiffening behaviour. However, results of the full strain sweep suggest overall strain softening behaviour (Supplementary material, Appendix F). It suggests that the apparent strain stiffening behaviour observed in the elastic Lissajous plots is small. Similar observations were made for pea protein gels by Kornet et al. (2021). The results of the essential nonlinear behaviour of the PPI gels in the heatmap in Fig. 7D also clearly indicate increasingly viscous behaviour ($\varphi$ closer to 1), with increasing deformation. The gel structures formed with PPI–TA conjugates depict the most viscous energy dissipation at lower deformations (1.07–27.9) suggesting that structure breakdown sets in earlier than for the other gel structures formed, making the gel structures more brittle.

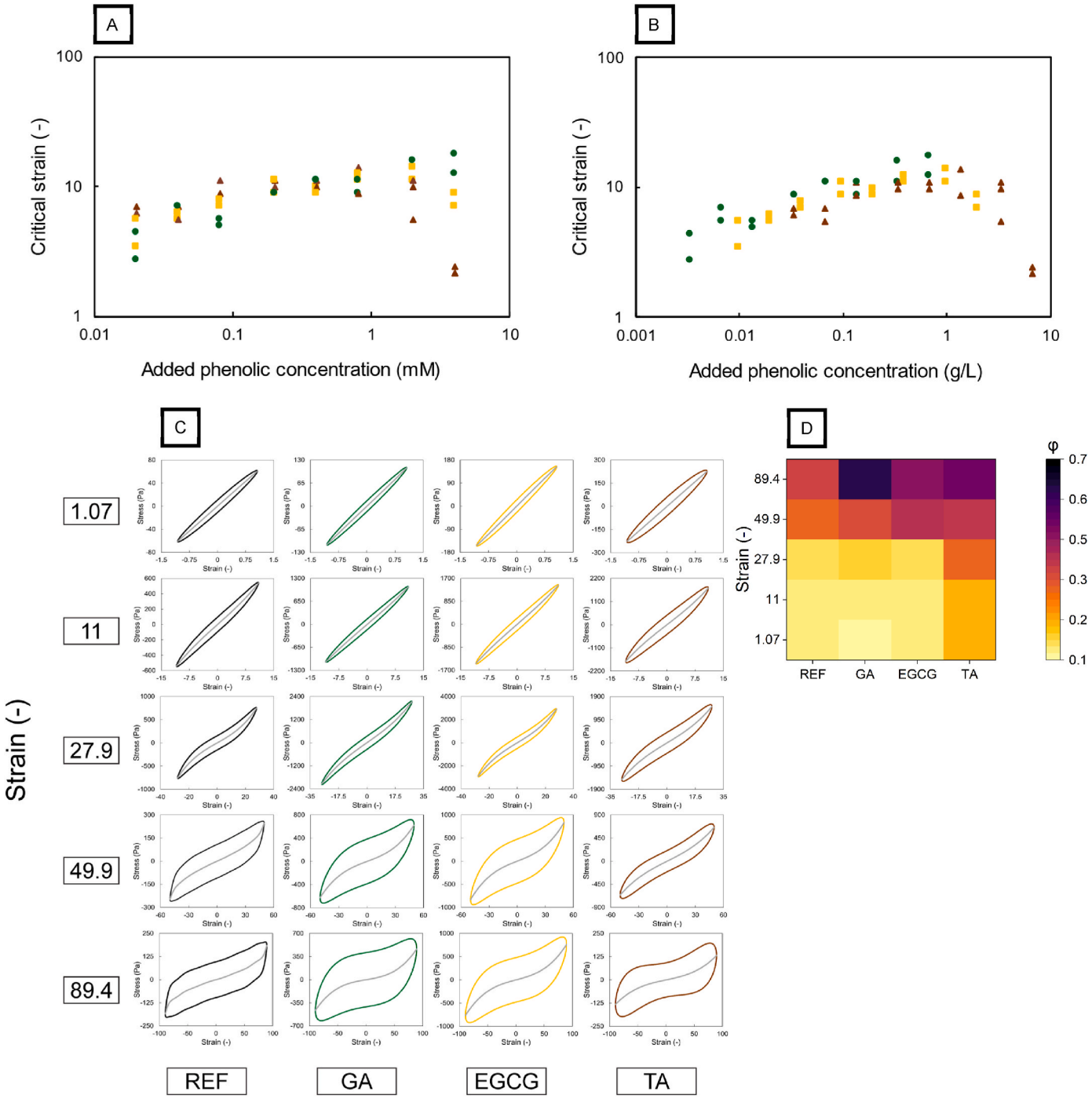


**Fig. 7.** Large oscillatory shear measurements for 15 wt% PPI gels modified without phenolics (reference [REF], black), with gallic acid (GA, green/dot), epigallocatechin gallate (EGCG, orange/square) and tannic acid (TA, brown/triangle). Established critical strain as a function of added phenolic concentration (A) (mM) and (B) (g/L). (C) Elastic Lissajous curves of stress versus strain amplitude and (D) dissipation ratios φ (−) at five different strain amplitudes (1.07, 11, 27.9, 49.9 and 89.4).

### *3.4. Efficiency in gel formation*

The decrease in free amino groups corroborates that primarily covalent modification took place (Section 3.2.1), but we also found an increasing concentration of unbound or non-covalently bound phenolics with increasing phenolic addition (Section 3.2.2). Covalently attached phenolics can increase covalent crosslinking and additional noncovalent interactions between formed conjugates as hypothesized in Section 1. The increase in gel strength was positively correlated with the added phenolic concentration and the phenolic molecular size (Section 3.3.2). A similar relation was seen for the modification of pea proteins which confirmed increased covalent binding of phenolics onto the pea proteins (Section 3.2.1) and the increasing number of free phenolic hydroxyl groups on protein–phenolic conjugates (Section 3.2.2). As described in Section 3.3.1, when the number of bonds (covalent or physical) between protein aggregates is increased, stronger gel structures are formed. These findings corroborate the hypothesis that indeed more bonds (covalent crosslinking, hydrophobic interactions and hydrogen bonding) could be formed in the gel structure between PPI aggregates when modified with phenolics and indicate the significance of the increased

density of bonds on the gel strength. The fact that this relationship was more apparent for mass concentration than for molar concentration (Fig. 6A and B) also confirms the importance of the increased density of these bonds. At a similar molar concentration, smaller phenolics possess fewer free hydroxyl groups than larger phenolics, whereas at the same mass, this is almost equal. This was confirmed by almost perfectly interchangeable curves when correlating the gel strength as a function of the molar concentration of free phenolic hydroxyl groups before the reaction (results not shown). Here, the molecular phenolic concentration was corrected for the number of hydroxyl groups on the molecule before oxidation. The decrease in free binding sites (Section 3.2.1) could be linked to the denser packing of protein aggregate strands for the highly modified 4 mM PPI-TA conjugate gel as opposed to the PPI reference gel (Fig. 5). However, the decrease in free binding sites could not be directly linked to cross-bridging of pea proteins by the phenolics because SDS-PAGE results under non- and reducing conditions could only indicate marginal additional protein aggregation. The comparison was particularly difficult due to the decreasing solubility of the PPI–-phenolic conjugates. Therefore, underestimation of the role of covalent cross-bridging by the phenolics in the gel structure is possible. However, the decrease in solubility of the PPI–phenolic conjugates does indicate increased hydrophobic character which can also positively contribute to the gel strength.

Whether contribution to the strong increase in gel strength could be attested by solely covalent or noncovalent attachment of phenolics to the pea proteins was additionally tested (Table 2). In scenario 1, TA did not undergo alkaline treatment and was directly added to the 15 wt% PPI reference dispersion in ultrapure water at pH 7, which is typically done to induce noncovalent interactions. In scenario 2, TA was oxidized under alkaline treatment according to the protocol in Section 2.2.1. And afterwards added as powder to the 15 wt% PPI reference dispersion in ultrapure water at pH 7. Subsequently the samples underwent heat treatment and the gelling properties were recorded.

The addition of TA that was unoxidized and did not form a quinone before it was added to PPI did result in a relative increase in the gel strength of 1.1 for 2 mM and 11 times for 4 mM. For the 2 mM pre-oxidized TA, the effect on the gel strength was more pronounced because the gel strength was increased 2.2 times. We assume only noncovalent interactions in these samples. However, this apparent noncovalent modification did not reach the same relative increases obtained for the protein PPI–TA conjugates primarily studied in the presented work (4.4 times for 2 mM TA and 16 times for 4 mM TA). From these results, it becomes clear that noncovalent interactions between the pea proteins and phenolics also contribute to an increase in gel strength. Hence, the increases in gel strength seen in this study for prepared PPI–phenolic conjugates, particularly for the high 4 mM modifications (Section 3.2.2), cannot be solely dedicated to the covalent binding of phenolics. However, the magnitude of the increases suggests that some level of covalent attachment has occurred. A denser microstructure was seen for the gel prepared with 4 mM PPI–TA conjugate as compared to the PPI reference (Fig. 5). This difference was less apparent for the gel structures formed with the PPI reference combined with 'unoxidized' or 'pre-oxidized' TA (Supplementary Material, Appendix G), which could confirm that covalent binding was involved in the PPI-phenolic conjugate gel structure formation. In addition, 15 wt% dispersions of unmodified PPI and of 4 mM PPI–TA conjugate in 0.1% SDS solvent showed that no gel could be formed for the unmodified PPI, but still resulted in a gel for the PPI–TA conjugate (Supplementary Material, Appendix H). This demonstrates that the gel structure formed by the PPI reference without added phenolics mainly exists from noncovalent protein–protein interactions. A gel could still be formed when prepared with 4 mM PPI–TA conjugates in the presence of SDS, even though it was a soft gel from which serum was expelled. This indicates that covalent crosslinking was more relevant for modified PPI.

For direct comparison, quantification of the degree of covalent protein modification is necessary. However, due to the loss of solubility on modification, PPI–phenolic conjugates are not easily characterized. Quantitative characterization of insoluble plant proteins for covalent modification should be investigated further in the future.

### 3.5. Large deformation

The gel deformability (Section 3.3.3) was affected in the same way as the gel strength by the modification of pea proteins with phenolics up to a 2 mM addition for EGCG and 0.8 mM for TA (Fig. 7A). Increased density of covalent, hydrogen bonds and hydrophobic interactions allowed for energy dissipation at higher deformations while staying intact. A similar relationship was seen by Balange and Benjakul (2009) for surimi protein–phenolic gel systems where the gel strength and deformability were increased when modified, particularly when larger phenolics were used. However, after the optimum at 2 mM for EGCG and 0.8 mM for TA (Fig. 7A), the interactions between PPI aggregates were so strong because of the increased density of either covalent, hydrogen bonds and/or hydrophobic interactions that energy dissipation was not possible without breaking the bonds when larger deformations were applied, which made the gel structures more brittle. In addition, upon increasing pea protein modification PPI–phenolic conjugates became more insoluble. It is also possible that extensive additional protein aggregation by crosslinking led to an inhomogeneous gel structure or even local phase separation between soluble components and insoluble protein aggregates. These insoluble protein aggregates might then act as inactive fillers and weaken the gel structure as they form 'fracture points' (Britten & Giroux, 2001). Therefore, increasing pea protein modification can initially result in tougher protein gel structures (stiffer and more resistant to deformation), but surpassing the optimal added phenolic molecular concentration results in more brittle gel structures. This is confirmed by the increasing viscous behaviour of the gels at high–strain deformation (Fig. 7D). The presence of noncovalently bound phenolics, which were highest for conjugates made with EGCG and TA at high modification (Section 3.2.2), is likely to have contributed to the local phase separation. Although such effects would be in small scale and could not be visualized with confocal laser scanning microscopy (Fig. 5;

**Table 2**
Measured gel strength of 15 wt% PPI dispersions with various phenolics in ultra-pure water after heat treatment as compared to pea proteins modified without phenolics (REF).

| | REF | TA 2 mM conjugate[a] | TA 2 mM unoxidized[b] | TA 2 mM pre-oxidized[c] | TA 4 mM conjugate[a] | TA 4 mM unoxidized[b] |
|---|---|---|---|---|---|---|
| Average gel strength, G' (Pa) | 3010[a] ± 2232 | 13387[a] ± 9727 | 3361 | 6487 | 48279[b] ± 23543 | 32200 |
| Increase in gel strength as compared to REF (−) | 1.0 | 4.4 | 1.1 | 2.2 | 16 | 11 |

[a] These samples are the PPI–TA conjugates as prepared according to Section 2.2.1.
[b] These samples were prepared by dispersing the PPI reference in ultrapure water to which unoxidized tannic was added at pH 7. These conditions are typically used for non-covalent interactions. This experiment demonstrates the effect of these types of interactions on the gel strength.
[c] This sample was prepared by dispersing the PPI reference in ultrapure water to which oxidized tannic acid (pre-oxidized at pH 9) was added at pH 7. This experiment demonstrates the effect on the gel strength if proteins and TA are oxidized separately, which typically does not lead to covalent interactions.

**Table 3**
Comparison of the maximum gel strength (G') obtained in this study after performing the strain sweep (as described in Section 2.2.4.1) for the most extensively modified pea proteins with tannic acid (TA) compared with the maximum measured gel strengths of plant proteins after modification by enzymatic crosslinking or fortification with additives.

| Protein | Protein concentration | Minor constituent | Modification | Maximum gel strength, $G'$ (kPa) | Maximum change in gel strength, $\Delta G'$ (kPa) | Maximum relative change in gel strength | Reference |
|---|---|---|---|---|---|---|---|
| Pea protein | 11 wt% | tannic acid (TA) | Conjugation | 48 | 45 | 16 fold | This study |
| Soy protein | 6 w/v% | none | Enzymatic crosslinking | 0.2 | 0.2 | 7 fold | Tang et al. (2006) |
| Pea protein | 10.5 wt% | None | Enzymatic crosslinking | 2.2 | 1.9 | 7.6 fold | Sun and Arntfield (2011) |
| Soy protein | 10.5 wt% | None | Enzymatic crosslinking | 1.6 | 0.7 | 1.8 fold | Sun and Arntfield (2011) |
| Canola protein | 15 w/v% | κ-carrageenan | None | 95 | 68 | 3.5 fold | Uruakpa and Arntfield (2004) |
| Soy glycinin | 10 wt% | κ-carrageenan | None | 38 | 36 | 22 fold | Zhu et al. (2008) |

GA, gallic acid; EGCG, epigallocatechin gallate; TA, tannic acid.

Supplementary Material, Appendix G). Similar to the gel strength, correlating the deformability with the number of OH groups per phenolic molecule (results not shown) results in an almost identical response to that shown in Fig. 7B (Section 3.3.3). This confirms the importance of the molecular structure of the selected phenolic before oxidation on the gelling properties. Therefore, it seems that the main factor to consider is the total number of free phenolic hydroxyl groups before oxidation. It means that when using the same mass, it does not matter whether a small or large phenolic compound is selected. The clear relationship between the gelling properties and the phenolic mass also allows for a more concrete recommendation for the use of the optimal phenolic concentration. Here, up to 1.36 g/L, which is equal to 3.8 wt% of the protein mass, can result in both stiffer and more deformable gel structures. This is within the range of the total phenolic concentration naturally present in field peas (Nicolás–García et al., 2021; Wang et al., 1998).

### 3.6. Placing the experimental outcomes in context

To place the outcomes in perspective, an overview of studies using alternative approaches to increase the gel strength of plant proteins (e.g. enzymatic crosslinking or the use of additives) as mentioned in Section 1 is provided in Table 3. The maximum gel strength, the G′ obtained at the end of the temperature sweep (Fig. 6) after cooling, was compared with the maximum gel strength obtained in other studies.

For our gels, prepared with a 15 wt% dispersion of PPI–phenolic conjugate (i.e. 11 wt% protein based on protein content measured in PPI), the relative gel strength could be maximally increased 16 times with TA at 4 mM addition (Section 3.3.2). This is much more than the maximum relative increase seen for gel structures made after enzymatic crosslinking of soy proteins at 10.5 wt% in the study from Sun and Arntfield (2011) or for canola protein gels fortified with κ-carrageenan in the research from Uruakpa and Arntfield (2004). Only fortification of soy protein gels with κ-carrageenan resulted in a larger maximum relative increase in gel strength (Zhu et al., 2008) than the increase in strength for gels obtained after modification of pea proteins in this study. Therefore, modification with phenolics can result in the same, if not superior, increases in gel strength than other approaches and can close the gap between protein sources that are traditionally known as superior gelling agents such as soy protein (Section 1).

The addition of phenolics to PPI resulted in brown colouring, which may be undesired for some applications (Section 3.1). The free amino groups on the pea proteins, which function as binding sites for the phenolics, originate mainly from the essential amino acid lysine (Gorissen et al., 2018). Covalently attaching phenolics to these groups potentially reduces their bioavailability and nutritional value (Prigent et al., 2007). In addition, phenolics that are naturally present in plant sources such as canola are perceived as bitter compounds (Naczk, Amarowicz, & Shahidi, 1998). Another drawback could be the potential formation of lysinoalanine because of protein oxidation at high pH. Therefore, for future studies, conjugation at milder pH levels or using enzymes such as polyphenol oxidases should be investigated. In addition, upscaling, which is deemed viable for the conjugation process, is required for industrial application. Removal of unreacted phenolics using membranes is suggested for upscaling. The removal of unreacted phenolics has also been investigated by Jia, Rodriguez-Alonso, Bianeis, Keppler, and van der Goot (2021). It is costly to add purified phenolics to a purified protein isolate. Once knowledge on plant protein–phenolic conjugation has been established, costs can be reduced by inducing conjugation in unpurified plant protein flours where phenolics are already inherently present. In this study main focus lays on increasing functionalization of commercial pea proteins with modification with phenolics. Therefore, the main considered drawbacks of covalent pea protein modification are the potential colour change, a reduction in the nutritional value of the proteins and bitterness. However, it is possible to select a moderate modification that results in mild colour changes and only partial loss of the nutritional value of the essential amino acids, while still gaining gel strength and deformability.

## 4. Conclusions

Unequivocal confirmation of the mode of binding is difficult with commercial PPI due to the low solubility. We obtained some indication of covalent modification, although in the presence of increasing levels of non-covalent or unbound phenolics when increasing the added phenolic concentration. Nonetheless, alkaline modification of commercial pea protein with phenolic compounds has shown to be a useful method to alter the gelling properties of pea protein. The phenolic mass concentration and the concentration of free phenolic hydroxyl groups are relevant to the increase in gel strength and deformability. This was explained by the fact that the binding affinity of a phenolic compound is directly related to its size or the number of free phenolic hydroxyl groups, which can induce both covalent crosslinking and noncovalent hydrogen bonding. A clear increase in gel strength was obtained at 4 mM. The deformability showed an optimum modification up to the addition of 1.36 g/L phenolic compounds. This research demonstrates that protein–phenolic conjugation can function as a tool to target a specific gel strength and/or deformability. It provides insights into the trade-off between the gain in gelling behaviour and loss of nutrition and shows promise for the use of less refined protein fractions where phenolic compounds are already naturally present.

## CRediT authorship contribution statement

**Iris Faber:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Laurice Pouvreau:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Atze Jan van der Goot:** Writing – review & editing, Supervision, Conceptualization. **Julia Keppler:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2024.110123.

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