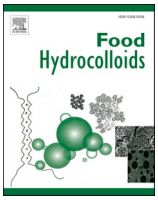
Food Hydrocolloids 131 (2022) 107800

Contents lists available at ScienceDirect

Food Hydrocolloids

journal homepage: www.elsevier.com/locate/foodhyd



Covalent and non-covalent modification of sunflower protein with chlorogenic acid: Identifying the critical ratios that affect techno-functionality

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ARTICLE INFO

Keywords:
Sunflower protein isolate
Chlorogenic acid
Non-covalent modification
Covalent modification
Techno-functional properties

ABSTRACT

Phenolic compounds are removed from plant protein extracts because their interaction with proteins can lead to undesirable sensory and techno-functional changes. The trend toward less refined plant protein fractions requires clarification of the degree to which removal is necessary.

Chlorogenic acid (CGA) was added to sunflower protein solutions to obtain apparent CGA-protein molar ratios between 1:10 and 10:1. The samples were incubated either at pH 7 to induce non-covalent interactions or at pH 9 to induce covalent interactions. The type and extent of protein modification, physicochemical properties, solubility, and gelling ability were evaluated.

Both binding modes of CGA had a positive effect on protein solubility. Covalently modified samples showed color changes upon a molar ratio of 1:1 and higher. All solutions were able to form gels (protein concentration 10% w/v). Maximum gel strength was obtained at a 1:1 ratio in case of covalent modification. Higher molar ratios led to lower gel strengths and this effect was more pronounced for covalently modified samples than for non-covalent ones.

Depending on the applications, complete removal of CGA is not necessary, since CGA improves the solubility of the sunflower proteins. However, CGA removal to a more favorable ratio between 5:1 and 1:1 is recommended if the material is used for gel formation. Under conditions that promote covalent binding with CGA, ratios above 1:1 lead to significant green coloration. It remains to be tested whether this observation also applies to less refined sunflower ingredients with multiple components.

1. Introduction

Sunflower protein is considered to be a promising source of protein for human consumption because of its nutritional value and techno-functional properties. Instead of purification into a protein isolate, modern food design aims at less refined fractions but high techno-functionality (Loveday, 2020; Van der Goot et al., 2016). Sunflower kernels naturally contain phenolic compounds. About 80% of the phenolic compounds is chlorogenic acid (CGA), which is present in a concentration of 2–4 g/100 g in the kernel. CGA can bind reversibly to various proteins by non-covalent interactions (Jiang, Zhang, Zhao, & Liu, 2018; Zhang et al., 2021) through hydrophobic interactions and hydrogen bonds (Prigent et al., 2003). The binding can alter the protein solubility (Ozdal, Capanoglu, & Altay, 2013). Upon oxidation, CGA transforms into *o*-quinones, which can form covalent interactions with proteins, resulting in the formation of green-colored adducts (Pierpoint, 1969). In contrast to non-covalent binding, covalent interactions are much stronger. CGA quinones bind covalently to the amino acid residues of lysine and cysteine, for example (Keppler, Schwarz, & van der Goot, 2020), and this interaction is often perceived negatively because it can reduce protein digestibility, alter protein solubility, and affect organoleptic properties negatively (Karefyllakis, Salakou, Bitter, Van der Goot, & Nikiforidis, 2018; Wildermuth, Young, & Were, 2016). The information above explains why CGA is generally removed when sunflower

Abbreviations: ATR-FTIR, Attenuated total reflectance-Fourier transform infrared spectroscopy; CGA, Chlorogenic acid; DSK, De-oiled sunflower kernel; LGC, Least gelling concentration; RP-HPLC, Reversed-phase high-performance liquid chromatography; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SFPI, Sunflower protein isolate; UV–vis, Ultraviolet–visible spectroscopy; WHC, Water holding capacity.

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https://doi.org/10.1016/j.foodhyd.2022.107800
Received 11 February 2022; Received in revised form 10 May 2022; Accepted 11 May 2022
Available online 14 May 2022

material is processed into ingredients for food applications.

Despite the negative effects on the proteins, CGA has a high antioxidant activity and possibly beneficial health effects (Lu, Tian, Cui, Liu, & Ma, 2020). Thus, it would not only be a loss in resource efficiency but also in bio-functionality when removing it completely. Furthermore, there are also reports about improved techno-functional properties: covalent interactions of whey protein isolate with caffeic acid in gelatine gels were found to improve the mechanical strength of the gel, as well as induce stable foam formation in protein-colloidal dispersion (Prigent, Voragen, Visser, van Koningsveld, & Gruppen, 2007; Strauss & Gibson, 2004). In addition, similar positive effects were found for non-covalent whey protein and CGA interactions (Jiang et al., 2018). Although the final techno-functionality of these modified proteins depends on the source of the reacting protein and the phenolic compound, a dose-dependent effect on the gelling properties was reported (Cao & Xiong, 2015; Cheng et al., 2021; Keppler et al., 2020). The results of improved techno-functionality are in contrast to the negative reports. The reason is that little is yet known about the impact of the relative importance of covalent and non-covalent interactions of phenolic compounds and proteins on various techno-functional properties.

It is essential to understand up to what extent the CGA can be retained in the material without compromising its potential use in food applications. The effects of CGA on the overall functionality of sunflower proteins (techno-functional, sensory and biological functionality) cannot be easily summarized. They depend on many factors including the type (covalent, non-covalent) (Ozdal et al., 2013), the extent of the interaction (molar ratio of CGA and protein) (Cao & Xiong, 2015; Keppler et al., 2020), and the properties of the sunflower proteins (nativity, protein fraction composition) (Alu'datt, Rababah, Kubow, & Alli, 2019; Prigent et al., 2003). It is possible to study the effect of protein modification using less refined sunflower material instead of protein isolates. However, a sound interpretation of the results is complicated by the fact that this material contains a large number of components (i.e. protein, oil, polysaccharides, phenolic compounds, and small sugars). That is why we will approach our question first using a simplified model mixture comprising CGA and pure sunflower protein, which is a common procedure for such studies (Jiang et al., 2018; Qie et al., 2021; Wang et al., 2021). The outcomes will then be used for a discussion on how the results could be translated to design rules for less refined fractions with multiple components. In this work, we will study the following aspects: (1) quantification of the modification degree of covalent and non-covalent binding under different CGA to sunflower protein molar ratios (from 1:10 to 10:1), (2) effect of the binding mode, and the modification degree on the physicochemical and techno-functionality of sunflower proteins, to identify the critical CGA-protein ratios in a model mixture. Gelling properties will be the main target functionality here because of their importance for many food applications, such as meat analogues (Kyriakopoulou, Keppler, & Van der Goot, 2021).

2. Materials and methods

2.1. Materials

De-oiled sunflower kernels (DSK) were provided by Avril Group (Bruz, France). DSK contain 52.7% protein (based on nitrogen content, with conversion factor of 5.6), 7.1% fat, 7.5% ash, 3.9% chlorogenic acid (CGA). The remaining part consists mainly of carbohydrates. The apparent CGA-protein molar ratio of 6.5:1 in DSK was calculated using the assumption of a mean molecular weight of 30,000 g/mol for sunflower proteins (Geneau-Sbartaï, Leyris, Silvestre, & Rigal, 2008), and 354 g/mol for CGA. Trifluoroacetic acid (purity of 99%), chlorogenic acid (CGA), sodium hydroxide (NaOH), and sodium chloride (NaCl) were purchased from Sigma Aldrich (St.Louis, USA). Acetonitrile ULC-MS (purity >99%) and hydrochloric acid (HCl) was obtained from Actu-All chemicals (Oss, the Netherlands). Ethanol (purity of 96%) and sodium carbonate (Na_2CO_3) anhydrous were purchased from VWR Chemicals (Leuven, Belgium). For protein extraction, demi-water was used, and for all other experiments, Milli-Q water was used.

2.2. Methods

2.2.1. Preparation of sunflower protein isolate

2.2.1.1. Dephenolization. DSK was washed to remove phenolic compounds and other soluble components. The procedure is based on the protocol described in Jia, Rodriguez-Alonso, Bianeis, Keppler, & van der Goot (2021). For this, 100 g DSK was mixed with an aqueous ethanol solution (ethanol content of 40%) in a 1 L centrifuge bottle at a solid to liquid weight ratio of 1:5 w/w. The dispersion was stirred with a magnet at 400 rpm for 10 min at room temperature. The mixture was centrifuged at 6000 rpm for 10 min at 20 °C. The wet pellet was redispersed with fresh solvent. The pH of the supernatant was adjusted to 11 with 2 M NaOH to detect a possible greenish color, which would indicate the presence of CGA in the dispersion. Up to 9 sequential washing steps were performed until no greenish color could be detected anymore. All supernatants were discarded after the washing process. The final pellet was kept overnight in a vacuum oven (Model VD 23, Binder GmbH) at 30 °C to allow for ethanol evaporation. The pellet was freeze-dried and kept at 4 °C for further fractionation. In total three individual batches were prepared.

2.2.1.2. Alkaline extraction. Sunflower protein isolate was fractionated using the protocol adapted from González-Pérez et al. (2002). The dephenolized DSK obtained after aqueous ethanol washing were evenly distributed over two centrifuge bottles of 1 L. Demi-water was added to the bottle at a solid-to-liquid ratio of 1:10 w/v. The pH value of the dispersion was adjusted to 9 with 2 M NaOH, after which it was further mixed with a magnetic stirrer at 400 rpm and continuously adjusted at pH 9 for 1 h at room temperature. Afterward, the dispersion was centrifuged for 20 min at 13,000×*g* at 20 °C. The supernatant was freeze-dried and stored at 4 °C for further experiments, these samples are referred to as sunflower protein isolate (SFPI).

The nitrogen content of the SFPI was determined with the Dumas combustion method by using a Nitrogen Analyzer (Flash EA 1112 Series, Thermo scientific, Delft, The Netherlands), with a protein conversion factor of 5.6 (Pickardt, Eisner, Kammerer, & Carle, 2015). Besides, it is assumed that the SFPI was completely dephenolized after the intensive washing and alkaline extraction, based on our previous publication (Jia, Kyriakopoulou, et al., 2021). The SFPI had a protein purity of 91.6 wt% after freeze-drying based on Dumas analysis. The protein content is slightly lower compared with the reported protein content of 94 wt% and 98 wt% in a purified sunflower isolate (González-Pérez et al., 2002; Karefyllakis, Altunkaya, Berton-Carabin, van der Goot, & Nikiforidis, 2017), which might be due to different fractionation processes.

2.2.1.3. Protein modification. A covalent or non-covalent interaction between sunflower protein and CGA was induced by incubating the SFPI protein solution with CGA for 24 h at pH 9 (covalent) or pH 7 (non-covalent) using 2 M of NaOH or 2 M of HCl, respectively (Ozdal et al., 2013; Prigent et al., 2007). Firstly, a protein stock solution (40 mg/mL) of 16.5 mL and a CGA stock solution (4.6 mg/mL) of 4.5 mL was prepared in falcon tubes. The pH value of both stock solutions was adjusted to either 7 or 9. Afterward, the protein stock solution of 2.5 mL at pH 7, or pH 9, was mixed with CGA stock solution at the respective pH using the volumes indicated in Table 1. Milli-Q water was added to reach the different mass ratios of 1:850, 1:450, 1:85, 1:15, and 1:10 between CGA and protein in the final mixtures with a protein concentration of 20 mg/mL. These mass ratios correspond to apparent molar ratios of 1:10, 1:5, 1:1, 5:1, and 10:1, based on the assumption of the mean molecular weight of dissociated sunflower proteins (30,000 g/mol) and CGA (354

Table 1
Modified protein solutions at different CGA-protein apparent molar ratios in terms of volume, protein, and CGA concentrations.

Modified protein solution CGA[a]-protein		Stock protein solution[c]	Stock CGA solution[c]	Milli-Q Water	Protein concentration	CGA concentration
Apparent molar ratios [b]	Mass ratios	mL	mL	mL	mg/mL	mg/mL
Reference	Reference	2.5	0	2.5	20	0
1:10	1:850	2.5	0.025	2.475	20	0.023
1:5	1:450	2.5	0.05	2.45	20	0.046
1:1	1:85	2.5	0.25	2.25	20	0.23
5:1	1:15	2.5	1.25	1.25	20	1.1
10:1	1:10	2.5	2.5	0	20	2.3

[a] CGA for chlorogenic acid.
[b] Assumption of molecular weight of protein (30,000 g/mol) and CGA (354 g/mol). All values are given as integers.
[c] Stock protein solution of 40 mg/mL and stock CGA solution 4.6 mg/mL.

g/mol) (Geneau-Sbartaï et al., 2008). The chosen ratios (especially 5:1) were close to the natural CGA-protein ratio (6.5:1) in the de-oiled sunflower kernel and were also studied in a previous publication by Karefyllakis et al. (2018). The upper limit was set at 10:1 because it showed intensive dark green color formation. We will mainly use the apparent molar ratios in the following sections for simplicity. All CGA and protein mixtures were vortexed. The pH value of the mixtures was checked and readjusted to either pH 7 or 9. Afterward, the mixtures were rotated (SB3 rotator, Stuart, UK) for 24 h at room temperature at a speed of 20 rpm to allow complete reactions (Karefyllakis et al., 2018). Subsequently, all pH 9 solutions (reference and incubated CGA-protein mixtures) were adjusted to pH 7 for comparison purposes. The same procedure was applied at a higher concentration of 10% w/v for the thermal measurements (Section 2.2.6).

2.2.2. Protein analysis

Quantification of CGA and modified proteins.

To elucidate if the presence of unbound CGA affects the protein structure, all samples were analyzed with FTIR before and after diafiltration. The CGA diafiltrates of the different mixing ratios were also used as a blank for the respective unfiltered CGA-protein solutions. For the diafiltration, 3 mL of the reference and modified protein solutions were filtered with centrifugal filters (10 kDa, Amicon® Ultra, 4 mL, Merck) at 4000 rpm. The filters were twice refilled with water to remove unbound CGA and other small molecules. The original sample, as well as the diafiltrated samples, were used for FTIR analysis as well as for RP-HPLC.

The amount of bound and unbound CGA in the reference, the covalently or non-covalently modified protein solution was determined based on the methods reported previously (Ali, Keppler, Coenye, & Schwarz, 2018). A reversed-phase high-performance liquid chromatography (RP-HPLC) (Ultimate 3000, Thermo scientific, Sunnyvale, CA, USA) was used for the measurement with a PLRP-S column (300°A, 8 m, 150 × 4.6 mm). The eluents were 0.1% trifluoroacetic acid (v/v) in water (A) and acetonitrile (B). The gradient was applied under the following conditions: 10%–18% B, 22 min; 18%–80% B, 8 min; 80% B, 3 min; 80%–10% B, 2 min; 10% B, 7 min. The run time was 42 min 50 μL of eluent was injected with a flow rate of 0.6 mL/min. All reference and modified protein solutions were filtered using syringe filters (Whatman®, Merck, 0.45 μL for RP-HPLC). The filtered samples of 10 μL were injected into the system and the flow rate was maintained at 0.6 mL/min. A calibration with a CGA standard between 0.05 and 0.35 mg/mL was conducted (Fig. S1). The diode array detector (Chromeleon Chromatography Data system, Thermo Fisher, the Netherlands) was set to the wavelengths of 280 and 330 nm for protein and CGA analysis, respectively.

RP-HPLC can also be used to semi-quantify the non-covalent modification degree of the protein (Ferraro et al., 2015). Three assumptions were made: (1) the unbound CGA was completely removed by the diafiltration (see 2.2.2), (2) at 330 nm, the absorption of CGA in the diafiltrated samples corresponds to non-covalently bound CGA, (3) the absorption of the CGA in the non-diafiltrated samples includes both unbound and non-covalently bound CGA. Thus, the percentage of the non-covalently bound CGA can be calculated by the amount of CGA in the diafiltrated solution divided by the total CGA detected with the non-diafiltrated solution in equation (1). Also, the apparent modification degree can be calculated with equation (2)

$$\text{Non-covalently bound CGA} = \frac{C_{\text{CGA,diafiltrated}} \times V_{\text{dilfiltrated}}}{C_{\text{CGA, total}} \times V_{\text{total}}} \times 100\% \quad (1)$$

$$\text{Apparent modification degree} = \frac{C_{\text{CGA,diafiltrated}} \times V_{\text{dilfiltrated}} \times MW_{\text{protein}}}{C_{\text{protein, total}} \times V_{\text{total}} \times MW_{\text{CGA}}} \quad (2)$$

where the $C_{\text{CGA, diafiltrated}}$ and $V_{\text{dilfiltrated}}$ indicates the concentration of CGA and the volume of the diafiltrated solution, and the $C_{\text{CGA, total}}$ and V_{total} indicates the concentration of CGA and the volume of the non-diafiltrated solution.

2.2.2.1. Protein molecular weight distribution by SDS-PAGE. The protein molecular weight distribution of the reference and modified protein solutions were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (Jia, Rodriguez-Alonso, et al., 2021). For this, a protein solution (2 mg/mL) was prepared in a falcon tube using Milli-Q water. The reference and modified protein solutions were prepared with Tris-buffer containing 2% w/w SDS, 10% glycerol, 0.5% w/v bromophenol blue, and 5% β-mercaptoethanol. The solutions were vortexed and heated at 95 °C for 10 min, after which the sample was centrifuged at 10.000 rpm for 1 min. The electrophoresis was performed at 200 V for approximately 40 min in a Mini-Protean II electrophoresis cell (Bio-rad, Veenendaal, Netherlands).

2.2.2.2. Protein secondary structure. The protein secondary structure of the reference and the filtered and unfiltered modified protein solutions was measured using a Fourier Transform Infrared (FTIR) spectrometer with a thermally controlled Bio ATR2 unit at 25 °C and a MCT detector (Confocheck™ system, Bruker Optics, Ettlingen, Germany). Interferograms were accumulated over the spectral range 4000–500 cm^{-1}, with a resolution of 4 cm^{-1}. Measurements were conducted at 25 °C against the respective solvent mixtures without protein (e.g. obtained by the filtration described above) as background and averaged over 60 scans at a resolution of 0.7 cm^{-1}. Independent duplicates were loaded for measurements. For each measurement, 20 μL of the sample was injected into the cell. For evaluation, the measured spectra in the frequency range of the amide band I (1580–1700 cm^{-1}) were vector-normalized using the Bruker OPUS software system (8.25, Ettlingen, Germany). The second derivative was calculated using 9 smoothing points. Difference spectra between the reference and the modified proteins were obtained for clarification of structural differences by subtracting each sample spectrum from its reference protein spectrum.

2.2.3. Physicochemical properties

2.2.3.1. Color measurement. The UV–vis absorbance of the reference and modified protein solutions were measured by a spectrophotometer (DR6000 UV/VIS, Hach, the Netherlands) using a multi-wavelength scan between 400 and 800 nm. The solutions were first centrifuged at 10.000 rpm for 10 min and the supernatant was further diluted 20 times to fit the absorbance range below 2 AU.

2.2.3.2. Water holding capacity and nitrogen solubility index (NSI). The water holding capacity (WHC) and nitrogen solubility index (NSI) of the insoluble protein pellet after centrifugation of a protein solution was measured using a method based on a protocol described previously (Jia, Kyriakopoulou, et al., 2021). A 2% w/v protein solution of reference or the modified protein samples was made in a falcon tube. Protein modifications upon CGA addition were prepared with the same method as described in section 2.2.1. The samples were vortexed and rotated overnight with a rotator (SB3 rotator, Stuart, UK) at a speed of 20 rpm. Afterward, the samples were centrifuged at 15,000×*g* at 25 °C for 20 min. The supernatant was removed with a pipette and the wet pellet was transferred into an aluminum tray and dried in an oven at 105 °C (Model E28, Binder, Germany) for 24 h. The weight of the wet pellet ($M_{wet\ pellet}$) and afterward dried pellet ($M_{dry\ pellet}$) was measured. The dry masses of the original sample and the pellet were measured and expressed as $M_{original}$ and $M_{dry\ pellet}$. The WHC of the dry pellet was calculated with equation (3). The protein content $Pro_{original}$ in the initial sample can be calculated from the Dumas result described in section 2.2.1. Thus, the remaining pellet indicates the insoluble protein $Pro_{dry\ pellet}$, and the NSI can be calculated in equation (4).

$$\mathrm{WHC} = \frac{M_{\mathrm{wet\ pellet}} - M_{\mathrm{dry\ pellet}}}{M_{\mathrm{dry\ pellet}}} \ [\mathrm{g\ water\ /g\ dry\ pellet}] \quad (3)$$

$$\mathrm{NSI} = \frac{Pro_{original} - Pro_{\mathrm{dry\ pellet}}}{Pro_{original}} \times 100\% \ [\%] \quad (4)$$

2.2.3.3. Protein thermal stability and nativity. The protein thermal stability and nativity of reference and modified protein samples with 10% w/v protein concentration (see section 2.2.1) were analyzed with differential scanning calorimetry (DSC) (TA instrument 250; TA Instruments, Newcastle, DE, USA). 40 μL samples were weighed and added to a high-volume pan and sealed. The pan was heated from 25 to 130 °C using a heating rate of 5 °C/min. After 1 min, the pan was cooled down to 25 °C using a cooling rate of 20 °C/min. This heating and cooling process was repeated for a second time to make sure the peak indicated protein denaturation. Duplicates were measured for each sample. The onset protein denaturation temperature (onset T_d), the peak temperature of denaturation (onset T_d), and the denaturation enthalpy (J/g protein) were collected by Trios data analysis software (TA Instruments).

2.2.3.4. Rheological properties. To identify the necessary protein concentration for gelling experiments, various concentrations of unmodified SFPI solutions of 5 mL were prepared in test tubes (4%, 6%, 8%, 10%, 16%, and 20% w/v at pH 7). All samples were subsequently heated at 95 °C for 30 min using a water bath. Afterward, the solutions were cooled to 4 °C overnight. The concentration above which the sample did not fall or slip when the tube was inverted was noted as the least gelation concentration (LGC). The gelling behavior of the modified protein solutions was then measured at 10% w/v, also for a comparison with other publications which often use 10% for the sunflower protein gels (González-Pérez & Vereijken, 2007; Malik, Sharma, & Saini, 2016).

The rheological properties of the reference and modified protein solutions were studied by monitoring the storage (G') modulus and loss modulus (G'') during thermal treatment. An Anton Paar MCR 502 rheometer (Graz, Austria), equipped with concentric cylinder CC17 geometry, was used for the measurement. The method was previously applied to other plant protein fractions as well (Kornet et al., 2021; Peng et al., 2021). First, the linear viscoelastic properties of the reference sample were evaluated. Subsequently, a temperature sweep was applied to the reference and the solutions with protein and CGA. The samples were heated from 20 to 95 °C using a heating rate of 3 °C/min, then held at 95 °C for 5 min and cooled to 20 °C using a cooling rate of 3 °C/min. Silicon oil was added on top of the solution inside the cylinder to avoid water evaporation during thermal treatment. The storage (G') and loss modulus (G'') dependency on temperature and frequency was recorded. After cooling, a frequency sweep was performed in the range of 0.1–10 Hz at a constant strain of 1%, and G' and G'' were recorded as a function of frequency. A strain sweep was performed on the gels in the range of 0.1–1000% at a constant frequency of 1 Hz at 20 °C. G' and G'' were recorded as a function of strain for 10 min to collect 100 data points. The analysis was done for all solutions, but in the SFPI solution with CGA at a 1:5 molar ratio, triplicates analysis was done to ensure the obtained result. Subsequently, the gels were subjected to a frequency sweep from 0.01 to 10 Hz (at a strain of 1%).

Additional experiments were performed with the dephenolized sunflower protein concentrate (protein content of 60% in dry base), which was prepared by an aqueous ethanol washing process (section 2.2.1 of dephenolization). The covalent modification was induced here in a solution of 10% w/v with CGA-protein molar ratios of 1:10, 1:5, 1:1, 5:1, and 10:1. It is noteworthy that protein concentration in the solution is only 6% w/v.

2.2.4. Statistic analysis

The data derived from the DSC, water holding capacity, and protein solubility measurement was analyzed using SPSS software (IBM statistical analysis Version 25.0). An univariate general linear model with least significant difference (LSD) test was performed to investigate significant differences between the different apparent CGA-protein molar ratios of 1:10, 1:5, 1:1, 5:1, and 10:1 and reference samples of pure protein but similar pH-treatments. Differences were considered significant if $P < 0.05$.

3. Results & discussion

3.1. Sunflower protein modification analysis

The covalently and non-covalently modified sunflower protein by chlorogenic acid (CGA) was analyzed by RP-HPLC. The sunflower protein isolate (SFPI) free of CGA was used as a reference benchmark.

3.1.1. Reference sample

The chromatograms of the CGA standard and reference samples at 330 nm are shown in Fig. 1. The according chromatograms at 280 nm are shown in the supplementary data of Fig. S1. Fig. 1A and C show the absorbance of CGA after ~10 min retention time (RT) at 330 nm and 280 nm (Prigent et al., 2003; Salgado, Molina Ortiz, Petruccelli, & Mauri, 2011). No CGA was detected in the reference sample at 330 nm (Fig. 1B), which indicated effective removal of CGA by the intensive aqueous ethanol washing and alkaline extraction. However, a small peak was evident at ~28 min for the SFPI reference sample at 330 nm. A similar chromatogram was obtained at 280 nm (Fig. 1D), here the peak at 28 min corresponded to sunflower proteins. Thus, the peak at 28 min detected at 330 nm wavelength (Fig. 1B) might be the weak absorbance of the proteins or the absorbance of covalently protein-bound CGA, which was probably created during the protein fractionation process.

3.1.2. Non-covalent modification

The chromatograms of the non-covalently modified samples with CGA-protein molar ratios at 1:10 and 10:1 are shown in Fig. 2. The chromatograms of all the modified samples are shown in Figs. S2–S3 (supplementary data). As expected, the peak height of free CGA at a RT

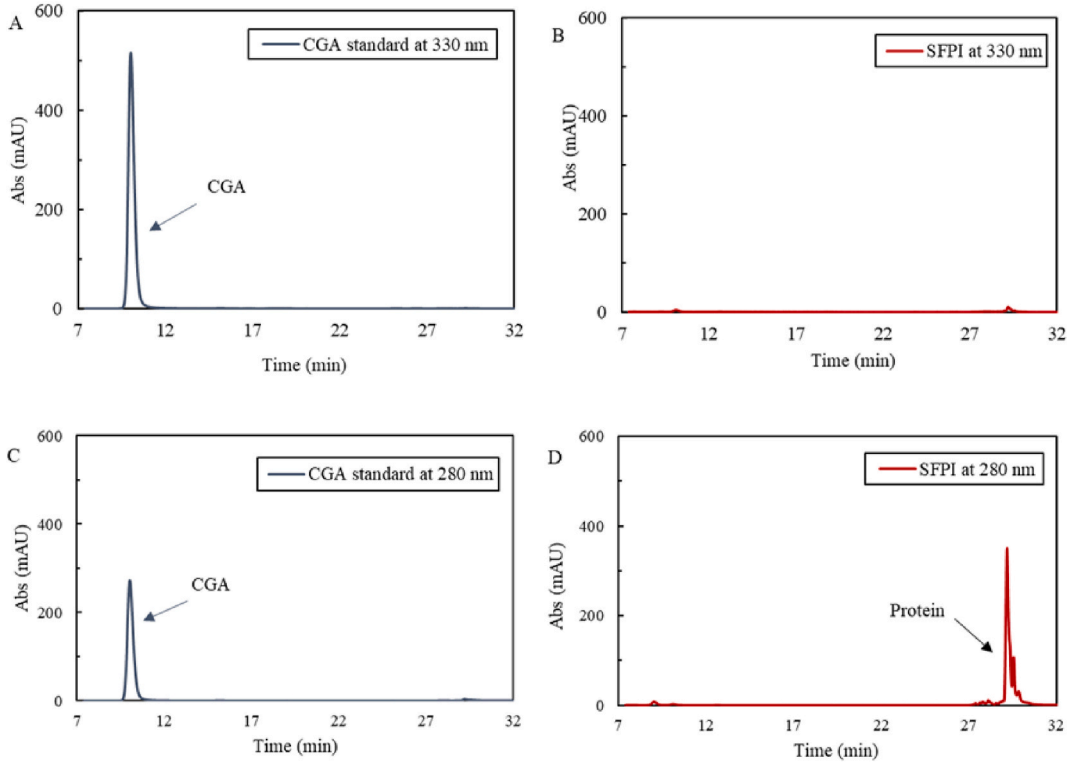

Fig. 1. RP-HPLC chromatogram at the wavelength of 330 nm of standard CGA sample (A) and the reference SFPI sample (B) at pH 7, as well as at 280 nm of standard CGA (C) and modified SFPI samples (D).

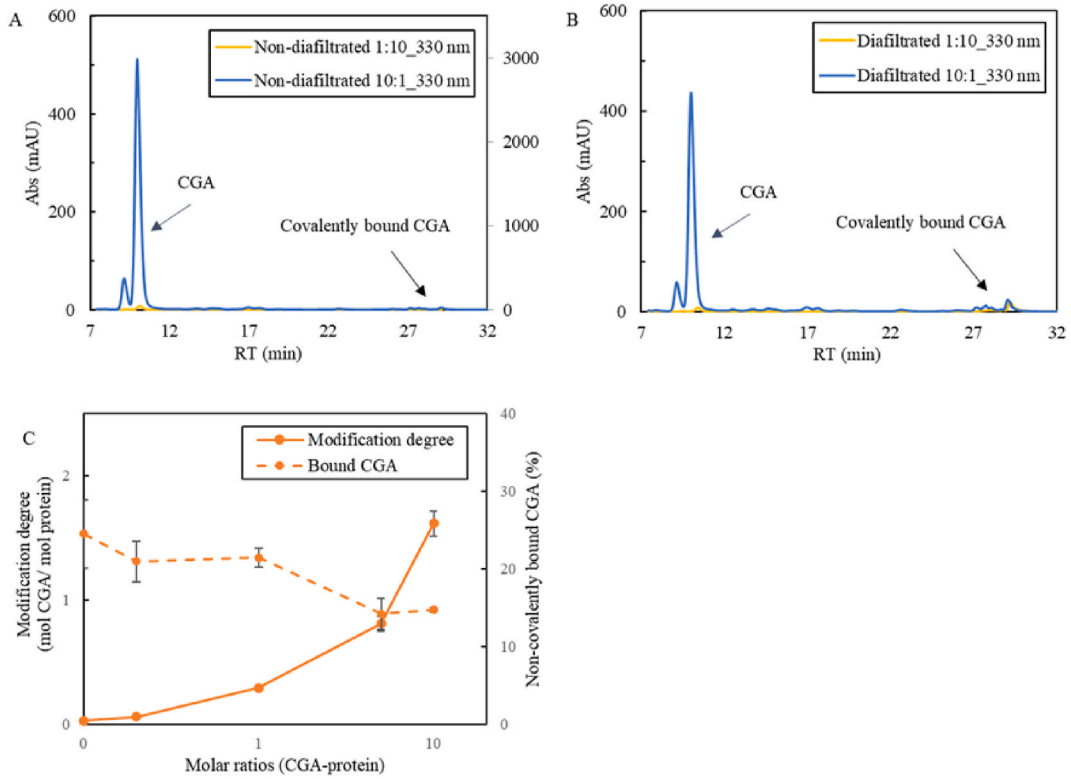

Fig. 2. RP-HPLC chromatogram of non-covalently modified protein samples at the apparent CGA-protein molar ratios of 1:10 and 10:1 (A), and the accordingly diafiltrated samples at 330 nm (B). The modification degree (mol CGA/mol protein) and the percentage of the non-covalently bound CGA of the modified samples at different molar ratios (X-axis in log) are shown in C.

of 10 min increased with increasing CGA-protein molar ratio (Fig. 2A). Covalent interactions with proteins can be excluded for the most part. Only at higher molar ratios of 5:1 and 10:1 is a slight peak area increase observed at 28 min RT (detected at 330 nm), from 10 to 18 mAU*min between reference and 10:1 modification. Non-covalent interaction cannot be directly seen in RP-HPLC as the interaction is reversible and those interactions might get lost due to the conditions and eluent used in the RP-HPLC measurement. However, diafiltration can be used to remove unbound CGA prior to RP-HPLC analysis. This CGA removal is shown in Fig. 2B as a lower CGA peak at 10 min RT of the diafiltrated sample compared with the non-diafiltrated modified protein samples. The peak area was converted into the CGA concentration with the CGA standard calibration curve (Fig. S1). Thus, the concentration difference between the remaining CGA in the diafiltrated samples and the non-diafiltrated protein samples roughly gives the percentage of non-covalently bound CGA (Carson et al., 2019) (Fig. 2C). Approximately 25% and 20% of the added CGA was non-covalently bound to the protein between the molar ratios of 1:10 and 1:1. This corresponds to an apparent modification degree of less than 0.2 mol of CGA per mol of protein. At higher mixing ratios of 5:1 and 10:1, about 16% of the added CGA was non-covalently bound. The degree of modification corresponds to 1–1.5 mol CGA per mol protein. However, the calculated modification degree is an underestimation of the actual modification level, since the non-covalent interaction is reversible and the binding equilibrium is likely to be affected by the diafiltration (Ozdal et al., 2013).

3.1.3. Covalent modification

The chromatograms of covalently modified protein samples with a CGA-protein molar ratio of 1:10 and 10:1 are shown in Fig. 3. The chromatograms of all the modified samples are shown in Fig. S4-S5 (supplementary data). The covalent protein modification can be detected using the same RP-HPLC method as used for the non-covalent interactions because both unbound CGA and irreversibly protein-bound CGA absorb at the 330 nm but elute at different retention times (Ali et al., 2018). Earlier elution of the covalently modified whey protein-bound CGA was reported than the unmodified ones. While unbound CGA elutes after 10 min RT, its various oxidized quinone derivatives are evident between 7 and 27 min, and protein-bound CGA elutes with the protein after 28 min. The second peak after 29 min could indicate that multiple CGA are covalently bound per protein (affecting the protein hydrophobicity and thus prolonging the retention time) (Keppler et al., 2014). The areas of both peaks at the RT of 28 and 29 min became larger with higher CGA-protein ratios (Fig.3C, Fig. S4), which suggests the degree of covalently modified proteins significantly increased above an apparent molar ratio of 1:1. A plateau was nearly reached at the CGA-protein molar ratio of 10:1 and the result suggested that most of the proteins (or available binding sites) were conjugated with the CGA (Liang & Were, 2020).

3.2. Effect of CGA modification on protein chemical properties

3.2.1. Protein molecular weight distribution

The SDS-PAGE profiles of unmodified and modified SFPI are shown in Fig. 4. The molecular weights of the proteins after adding a reducing agent were mainly in the range between 10 and 50 kDa. Sunflower proteins are known to consist of water soluble 2 S albumins with the molecular weight (MW) between 10 and 20 kDa and 11 S globulins named helianthinin with the MW between 19 and 50 kDa under reducing conditions (González-Pérez et al., 2002; Karefyllakis et al., 2017). In total three dissociated polypeptide groups of helianthinin in the presence of β-mercaptoethanol were reported: two acidic ones with the MW of 36.8–42.9 kDa and 31–35.3 kDa respectively, and the neutral polypeptide group had a MW of 21.0–29.6 kDa (Geneau-Sbartaï et al., 2008; González-Pérez et al., 2005). These polypeptides were also detected here, as shown in Fig. 4. Another band with 50 kDa was probably related to the helianthinin (Karefyllakis et al., 2018). Besides, the band found at 10 and 20 kDa can be related to the 2 S albumins and the rest of the bands are clustered as globulins (González-Pérez & Vereijken, 2007). The protein profile of all the modified samples remained similar to the reference, except for the covalently modified samples with CGA-protein ratios above 1:1. In those samples, the 50 kDa band disappeared and the bands between 25 and 37 kDa became less intense,

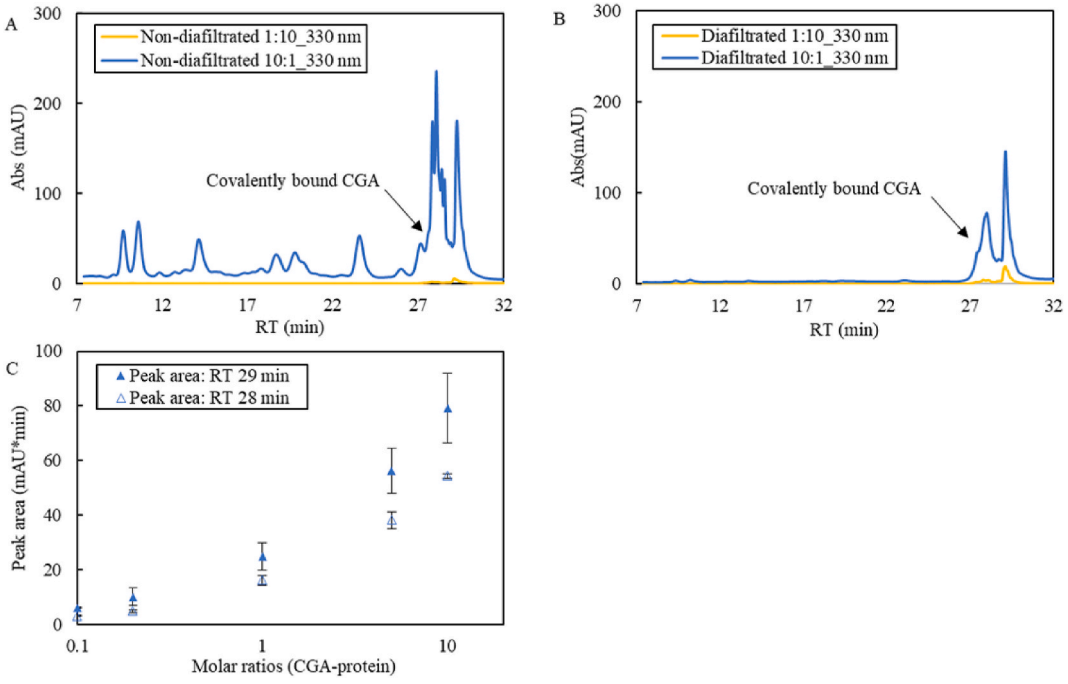

Fig. 3. RP-HPLC chromatograms of covalently modified protein samples at the apparent molar ratios of 1:10 and 10:1 (A), and the accordingly dialyzed samples at 330 nm (B). The peak area of the dialyzed modified SFPI samples with different molar ratios (X-axis in log) at the retention time of ~29 min (closed symbol) and ~28 min (open symbol) (C).

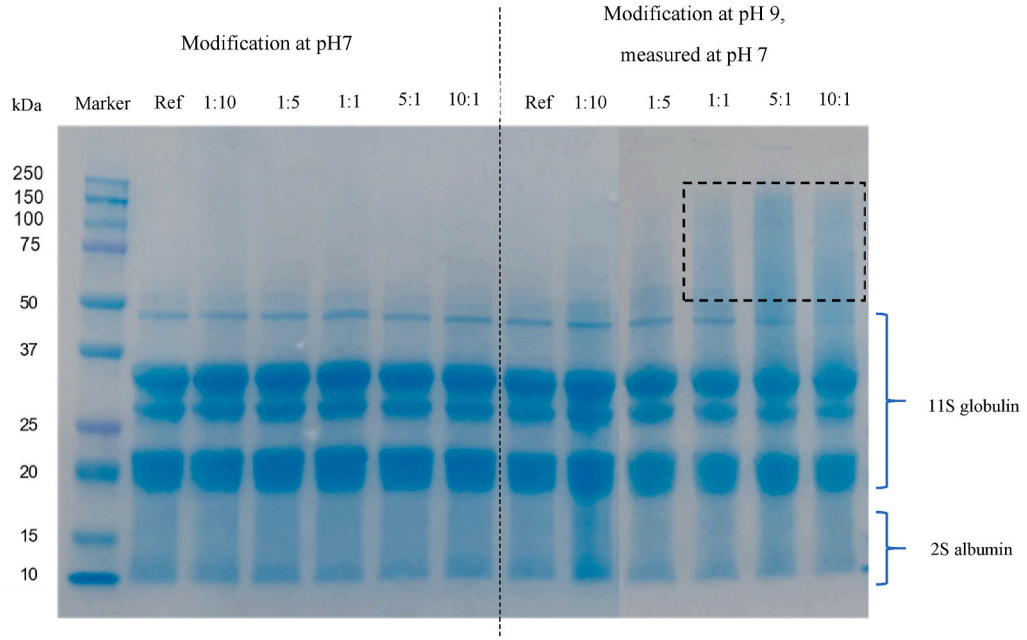

Fig. 4. Protein molecular profiles measured by SDS-PAGE, with respect to the reference and modified protein samples at pH7 and pH9.

whereas unclear bands between 50 and 250 kDa became visible.

Thus, it is most likely that covalent CGA-protein complexes were formed with large MW at higher molar ratios between 1:1 and 10:1. This aggregation is in line with previous research (Karefyllakis et al., 2018). Overall, the SDS-page results confirm the formation of covalent CGA-protein complexes as described above in the results of RP-HPLC, especially for higher ratios of 5:1 and 10:1.

3.2.2. *Protein secondary structure*

The protein secondary structures of the reference and modified protein before and after diafiltration were measured with ATR-FTIR (2nd derivative intensity in supplementary data of Fig. S9) For all the samples, the 2nd derivative intensity of β-sheets (1635 cm^{-1}) (Subaşı et al., 2020) was highest among all the other structures. This observation aligns with previous research stating a high β-sheet concentration in sunflower proteins (Malik et al., 2016). For the pH 9 reference sample (Figs. S9A and B), the pH-shift (24 h incubation at pH 9, then back to pH

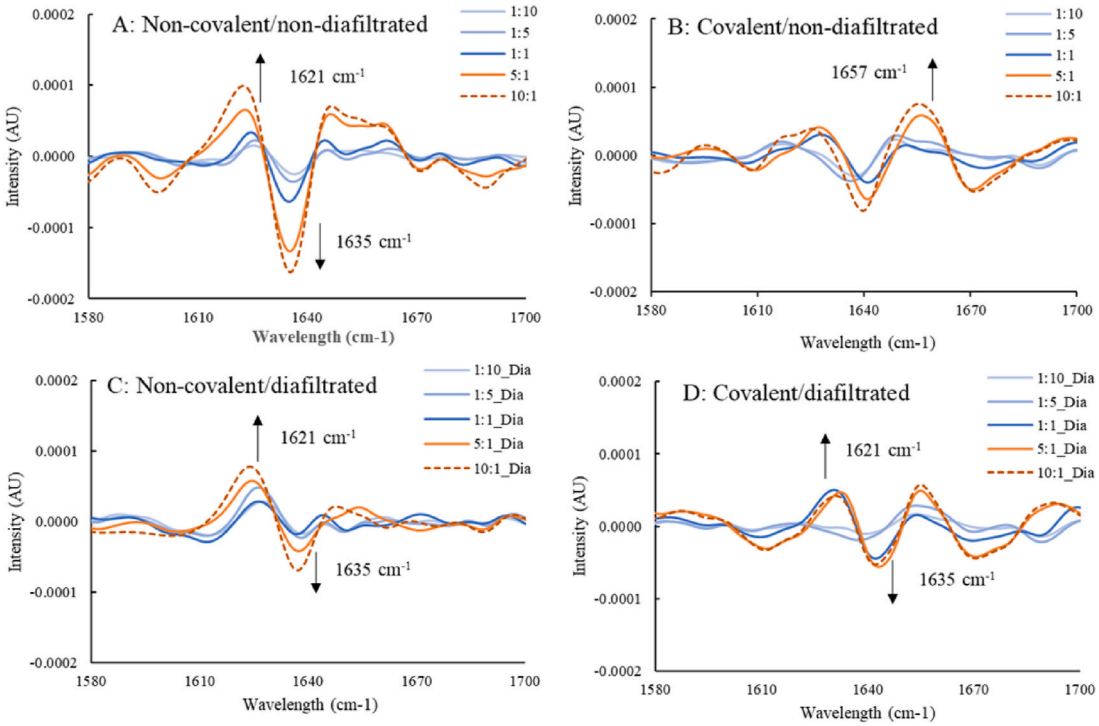

Fig. 5. FTIR difference spectra in the amide I region of the non-covalently modified and non-diafiltrated protein samples (A); the covalently modified and non-diafiltrated protein samples (B); the non-covalently modified and diafiltrated protein samples (C); the covalently modified and diafiltrated protein samples (D). The apparent molar ratios of the CGA-protein are 1:10, 1:5, 1:1, 5:1 and 10:1.

7) affected the secondary structure: the 2nd derivative intensity of intramolecular β-sheets (1635 cm^{-1}) increased while the intensity for intermolecular aggregates (1621 cm^{-1}) (Kayser, Arnold, Steffen-Heins, Schwarz, & Keppler, 2020) decreased compared with the reference sample incubated at pH 7 for 24 h. The secondary structure of the reference protein was not changed by the removal of salt by diafiltration (Fig. S9B). Thus, the pH-dependent change could be caused by the pH-shift, which is a known type of structural protein modification in which the numerous multiple subunits of globulins are broken down into their individual subunits and thus into soluble protein aggregates (Jiang et al., 2017).

The corresponding difference spectra of the modified samples minus the reference are shown in Fig. 5. In the non-covalently modified samples, the 2nd derivative intensity of intramolecular β-sheets (1635 cm^{-1}) increased as a function of added CGA (Figs. 5A and S9C). CGA addition resulted in a higher 2nd derivative intensity for the samples with a CGA-protein molar ratios of 5:1 and 10:1 (−0.00064 AU) than the lower CGA content (above −0.0005AU). Further, the 2nd derivative intensity of the α-helix structure (1657 cm^{-1}) and the random coil structures (1645 cm^{-1}) (Keppler, Heyn, Meissner, Schrader, & Schwarz, 2019) decreased for the CGA-protein ratios of 5:1 and 10:1. The 2nd derivative intensity at 1621 cm^{-1} was lower for these 2 molar ratios as well, suggesting increasing intermolecular interactions that typically occur in solutions with phenolic compounds and proteins. The diafiltration led to a smaller difference between the samples with molar ratio above 5:1 (Figs. 5C and S9E). This suggested that diafiltration affected the binding equilibrium between CGA and protein. In general, less structural changes were found for the covalently modified protein samples than for the non-covalent ones (Figs. 5B and S9D). The most pronounced change was the α-helix structure at 1657 cm^{-1}, and the intensity decreased with higher molar ratios of 5:1 and 10:1. The limited effect of the CGA addition on protein structure is probably a result of the fact that the covalently modified protein samples have underlying additional structural modifications caused by the pH-shift. The diafiltration showed only a small effect on the 2nd derivative Amide I intensity bands in these samples (Figs. 5D and S9F). Since covalent interactions are mostly irreversible, they are expected to be unchanged by the diafiltration step.

A similar effect was observed previously for covalently modified sunflower protein isolate (Karefyllakis et al., 2018). Besides, a likewise increase of α-helix elements was observed with covalently modified lactoferrin and CGA (Liu, Sun, Yang, Yuan, & Gao, 2015). However, the intensity of both β-sheets and α-helix increased after the binding of epigallocatechin gallate with the milk protein β-lactoglobulin (Kanakis et al., 2011). The difference in the protein secondary structure change is dependent on the type of protein and the modifying phenolic compounds.

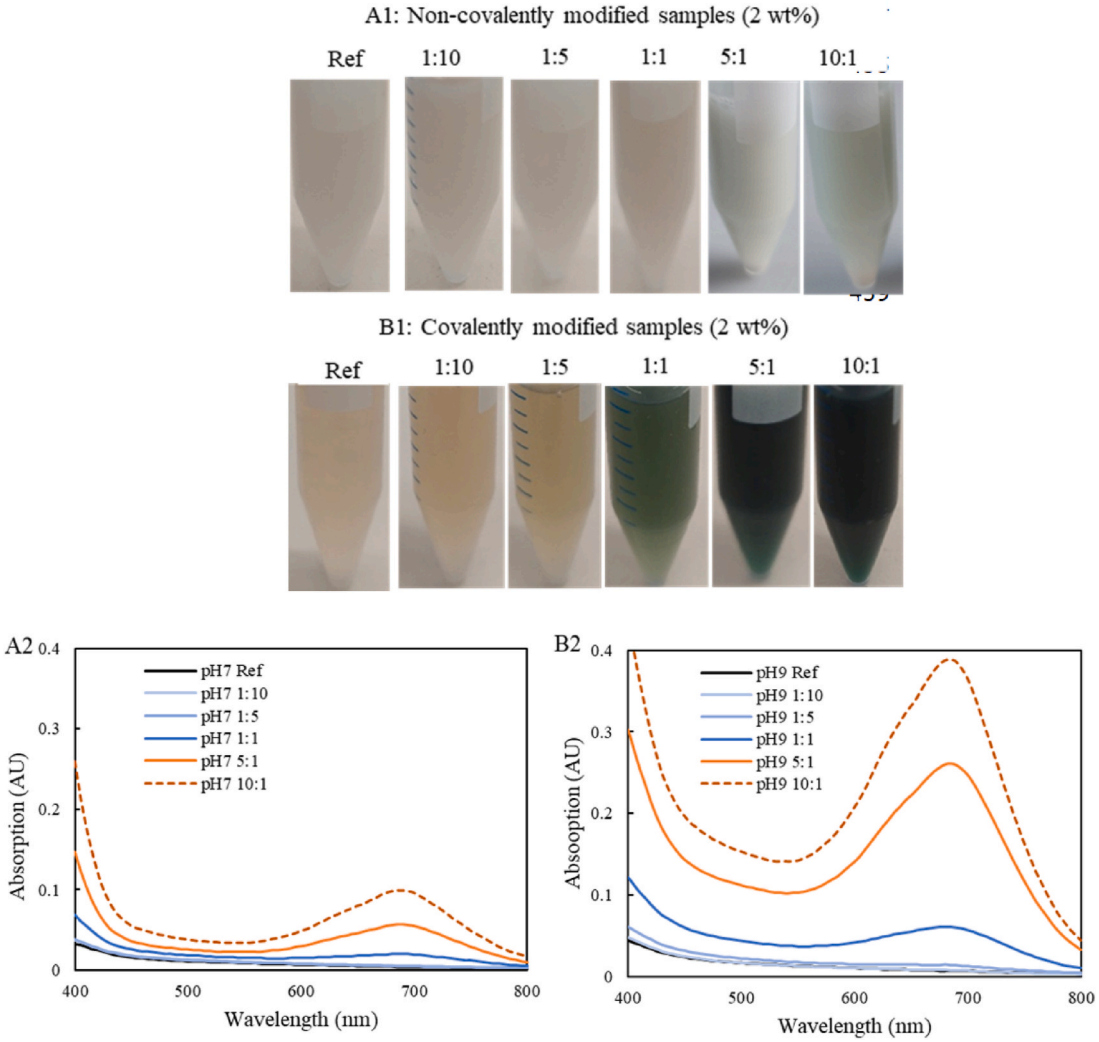

Fig. 6. Photos of the non-covalently modified sunflower protein solutions at 2 wt% (A1), UV–Vis spectra between the wavelength 400–800 nm for the non-covalently modified samples (A2) and photos of covalently modified sunflower protein solutions at 2 wt% (B1) and the corresponding UV–Vis spectra (B2). Different molar ratios between protein and CGA are shown as follows: Ref protein (1:0), 1:10, 1:5, 1:1, 5:1, 10:1.

3.3. Physicochemical properties

In the previous experiments all samples were measured before and after diafiltration to better characterize their binding behavior and elucidate how the pH-shift, and the presence of unbound CGA, affect the analysis and protein structure. The evaluation of the functional properties of the covalently and non-covalently modified SFPI was carried out without the diafiltration step.

3.3.1. Color measurement

The non-covalently modified samples were colorless at low apparent CGA-protein molar ratios, but some light green color was observed in solutions with a CGA-protein molar ratio of 5:1 and 10:1 (Fig. 6). The absorption peak found at the wavelength of 700 nm for these two samples also confirmed the absorption of red color (thus green transmission). An adduct e.g. with lysine side chains especially in proteins results in a green benzacridine derivative (Namiki, Yabuta, Koizumi, Yano, 2001; Yabuta, Koizumi, Namiki, Hida, & Namiki, 2001), which was confirmed with the aid of HPLC coupled with ESI-MSn (Schilling, Sigolotto, Carle, & Schieber, 2010). A potential CGA-protein adduct was also observed in the RP-HPLC-chromatograms, in which a small peak at ~28 min became visible at higher modification levels (Fig. 2B). In contrast, the solutions containing the covalently modified proteins with CGA-protein molar ratios of 1:10 and 1:5 and the reference solution had a light-yellow color. A green color was observed for the solution with higher molar ratios between 1:1 and 10:1. This observation was also confirmed with peak absorption in the range of 0.04 and 0.4 AU at 700 nm. This consecutive stronger occurrence of the green color formation is most likely associated with the increased rate of covalent modification observed in RP-HPLC (Fig. 3C), as well as the changes in protein secondary structure as observed with FTIR. The green color was also observed for covalently modified sunflower protein with CGA-protein molar ratio of 1:1 and onwards, or the amino acid/CGA molar ratio of 1:1 (Iacomino et al., 2017; Karefyllakis et al., 2018).

3.3.2. Nitrogen solubility index (NSI) and water holding capacity (WHC)

The protein solubility of the reference solution was about 67%. After applying a pH-shift the protein become almost completely soluble, which might be linked to the observed change in protein secondary structure (FTIR, Fig. 5). The addition of CGA at pH 9 did not reduce the high solubility of the solution. As a result, the WHC and NSI of this solution at pH 9 could not be measured.

Thus, only the effect of non-covalently bound CGA on protein solubility and WHC could be determined. The solubility of the protein was already largely increased above a molar ratio of 1:1 (Fig. 7), where 20% of the added CGA was protein-bound (which corresponds to more than 0.5 mol CGA per mol protein) (Fig. 2). An explanation for the increase in solubility could be the polar nature of CGA, which makes the protein more hydrophilic when bound to protein. Similar findings were reported previously that the CGA can positively affect the solubility of globulin from white bean both at pH 7 or pH 9 (Sczyk, Swieca, Kapusta, & Gawlik-Dziki, 2019). The WHC for all the non-covalently modified protein samples was approximately 3 g/g dry pellet except for a significantly higher value of 7 g water/g dry pellet obtained for the CGA-protein molar ratio of 10:1. The result was found to be in line with the reported 3 ml water/g sunflower protein isolate by previous studies (Khalil, Ragab, & Hassanien, 1985).

3.3.3. Protein thermal stability and nativity

The onset and peak denaturation temperature (T_d) was rather similar for the reference and all the modified protein samples (93–94 °C and 99–100 °C, respectively), Table 2. The results indicated that the protein modification by CGA hardly affected the onset and peak T_d of the protein. The denaturation enthalpy (E_d) of the reference sample was found to be 9.5 J/g protein when incubated at pH 7, and 11.8 J/g protein after the pH-shift. This difference in the E_d-value might be associated with the

Table 2
The onset denaturation temperature (T_d, °C), denaturation temperature (Peak T_d, °C) and enthalpy of denaturation (E_d, J/g protein) for the reference and modified protein solutions at pH 7 and pH 9 at varied apparent CGA-protein molar ratios of 1:10, 1:5, 1:1 5:1 and 10:1.

Non-covalent modification	Onset T_d (°C)	Peak T_d (°C)	Enthalpy E_d (J/g protein)
Ref	93.8 ± 0.3 ^{a}	100.0 ± 0.3 ^{b}	9.5 ± 1.6 ^{a}
1:10	93.0 ± 1.1 ^{a}	100.2 ± 0.3 ^{ab}	12.2 ± 0.4 ^{ab}
1:5	93.5 ± 0.2 ^{a}	100.1 ± 0.2 ^{b}	13.0 ± 1.5 ^{b}
1:1	93.8 ± 0.5 ^{a}	100.2 ± 0.3 ^{ab}	12.6 ± 0.7 ^{ab}
5:1	93.9 ± 0.5 ^{a}	100.8 ± 0.3 ^{a}	11.8 ± 0.8 ^{ab}
10:1	93.4 ± 0.5 ^{a}	99.9 ± 0.3 ^{b}	10.7 ± 1.4 ^{ab}
Covalent modification	**Onset T_d (°C)**	**Peak T_d (°C)**	**Enthalpy E_d (J/g protein)**
Ref	92.6 ± 0.5 ^{a}	99.3 ± 0.6 ^{ab}	11.8 ± 1.0 ^{b}
1:10	92.4 ± 0.5 ^{a}	98.7 ± 0.7 ^{b}	13.2 ± 1.3 ^{b}
1:5	92.2 ± 0.2 ^{a}	98.5 ± 0.3 ^{b}	10.2 ± 1.2 ^{b}
1:1	93.7 ± 0.3 ^{b}	98.7 ± 0.2 ^{b}	5.7 ± 1.2 ^{a}
5:1	94.6 ± 0.9 ^{b}	100.4 ± 0.5 ^{a}	6.4 ± 1.4 ^{a}
10:1	93.8 ± 0.7 ^{b}	99.6 ± 0.3 ^{ab}	5.1 ± 1.5 ^{a}

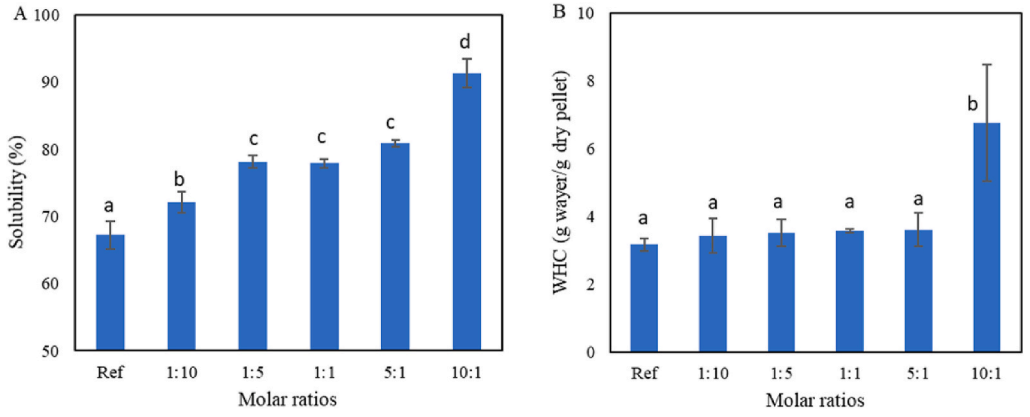

Fig. 7. Apparent protein solubility (%) (A) and apparent water holding capacity (WHC) (g water/g dry pellet) (B) of the non-covalently modified samples at different molar ratios.

differences observed in the protein secondary structure by FTIR (Fig. 5A, B). The energy value found here was slightly lower than the value of 14.9 J/g sunflower protein reported by González-Pérez et al. (2002), which might be due to a different fractionation process, experimental condition and protein composition. The E_d-value was found to increase slightly for the non-covalently modified proteins with lower molar ratios of 1:10 and 1:5, indicating a minor structural stabilization caused by the non-covalent modification. This was in line with the previous findings that some proteins became more thermal stable by non-covalently protein-phenol interactions (Rawel, Czajka, Rohn, & Kroll, 2002). Though a slightly lower E_d-value was observed for the samples with higher CGA-protein ratios, the E_d-value was still higher compared with the reference sample. Prigent et al. (2003) also reported that at high CGA-bovine serum albumin ratios, the non-covalent modification increased the energy needed for the protein denaturation. For the covalently modified protein samples, the highest E_d-value (13.3 J/g protein) was found at a low CGA-protein molar ratio of 1:10. The increase in the E_d-value was also reported for soy glycinin modified with CGA at pH 9 (Rawel et al., 2002). A decrease in E_d was found with a further increase of CGA-protein ratios from 1:5 till 10:1, while at the same time the onset T_d was slightly higher. These changes are in line with the more intense changes of the protein secondary structure observed in FTIR (Fig. 5E, F), and the evidence of multiple binding sites in RP-HPLC (Fig. 3B), as well as the large aggregate formation in SDS-PAGE (Fig. 4). To sum up, the use of low CGA-protein ratios slightly improved the protein thermostability in the case of non-covalent modifications at pH 7 while the thermostability of covalently modified protein largely decreased above the molar ratios of 1:1.

3.3.4. Rheological properties

The least gelling concentration (LGC) of the reference sample incubated at pH 7 was tested by heating, and subsequent cooling the solutions with concentration ranging from 4% to 20% w/v (Fig. 8). The LGC was 5% w/v, which is lower compared to the 8–10% w/v reported for the sunflower kernel protein isolate free of polyphenols in literature (González-Pérez & Vereijken, 2007; Malik & Saini, 2017). Fig. 8 shows that all the modified protein solutions of 10% w/v gelled, except for the covalently modified sample with the ratio of 10:1.

The rheological properties (G' and G'') of the references and covalently/non-covalently modified samples were further analyzed by temperature sweep (20-95-20 °C) in Anton Paar in Fig. 9 (complete data from rheological measurements are shown in Figs. S7 and S8 of supplementary data). For the reference sample, the G' and G'' value can be divided into three regions: a stable region from 20 to 90 °C (approximately 0.5 Pa for G' and 0.1 Pa for G''); a steep increase from 90 to 95 °C (0.5–22 Pa for G' and 0.1–2 Pa for G'') and a further increase region during cooling from 95 to 20 °C (22–411Pa for G' and 2–80 Pa for G''). The steep increase of G' indicated gel formation at 90 °C, which temperature is close to the onset T_d of approximately 93 °C (Table 2). For the reference sample after the pH-shift, the steep increase region of G' and G'' was found to start at a higher temperature, as expected from Table 1. Thus, the pH-shift of the reference sample influenced the gelling temperature. The G' value increased further upon cooling since the gel was known to become more firm upon cooling (Kornet et al., 2021). This G' values for both of the reference samples were similar (~400 Pa) after cooling (Fig. 9A4 and B4), which were slightly lower than the 500 Pa reported for the sunflower protein isolate (10% w/v) in a previous study (González-Pérez & Vereijken, 2007).

For the non-covalently modified samples at the two extreme molar ratios of 1:10 and 10:1, similar trends for the G' and G'' values were found as the reference sample. The G' values were lower (200–300 Pa) after cooling for the non-covalent modification compared to the reference sample (400 Pa), suggesting a decreased gel strength. The protein gel network might be disrupted due to the CGA binding (Malik & Saini,

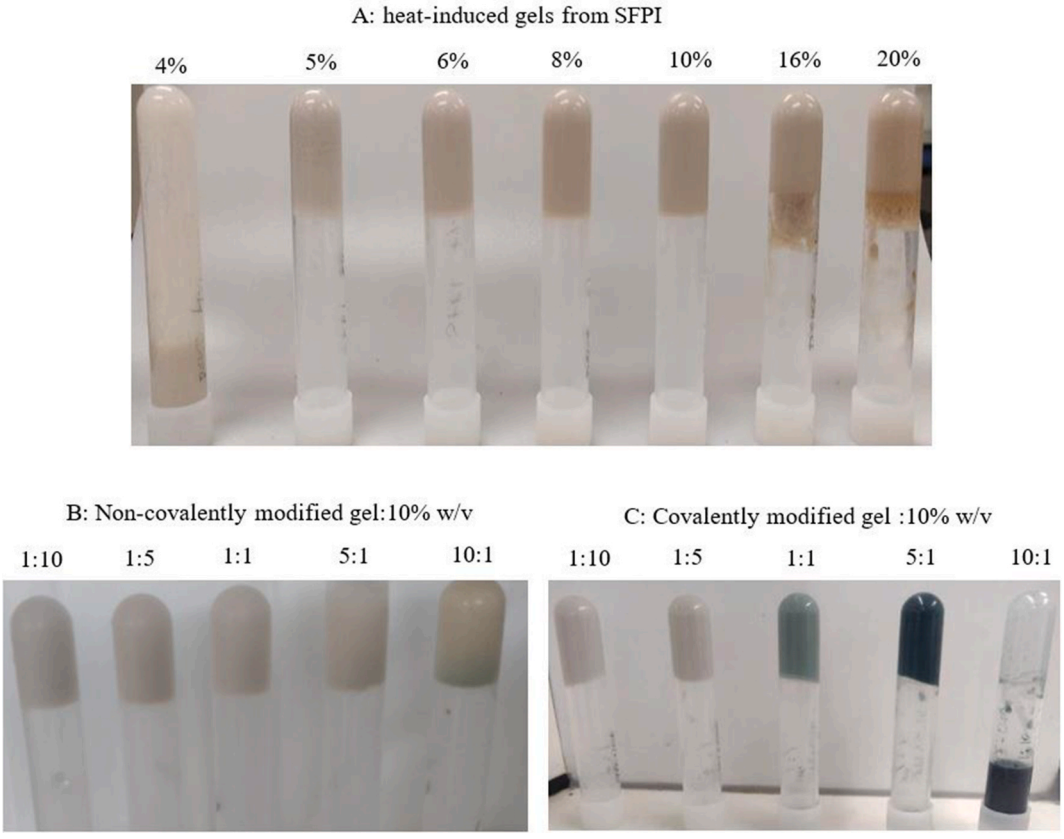

Fig. 8. Temperature induced gel from the SFPI solutions between 4 and 20% w/v (A). Non-covalently modified sunflower protein gels at 10% w/v (B), covalently modified protein gels at 10% w/v (C).

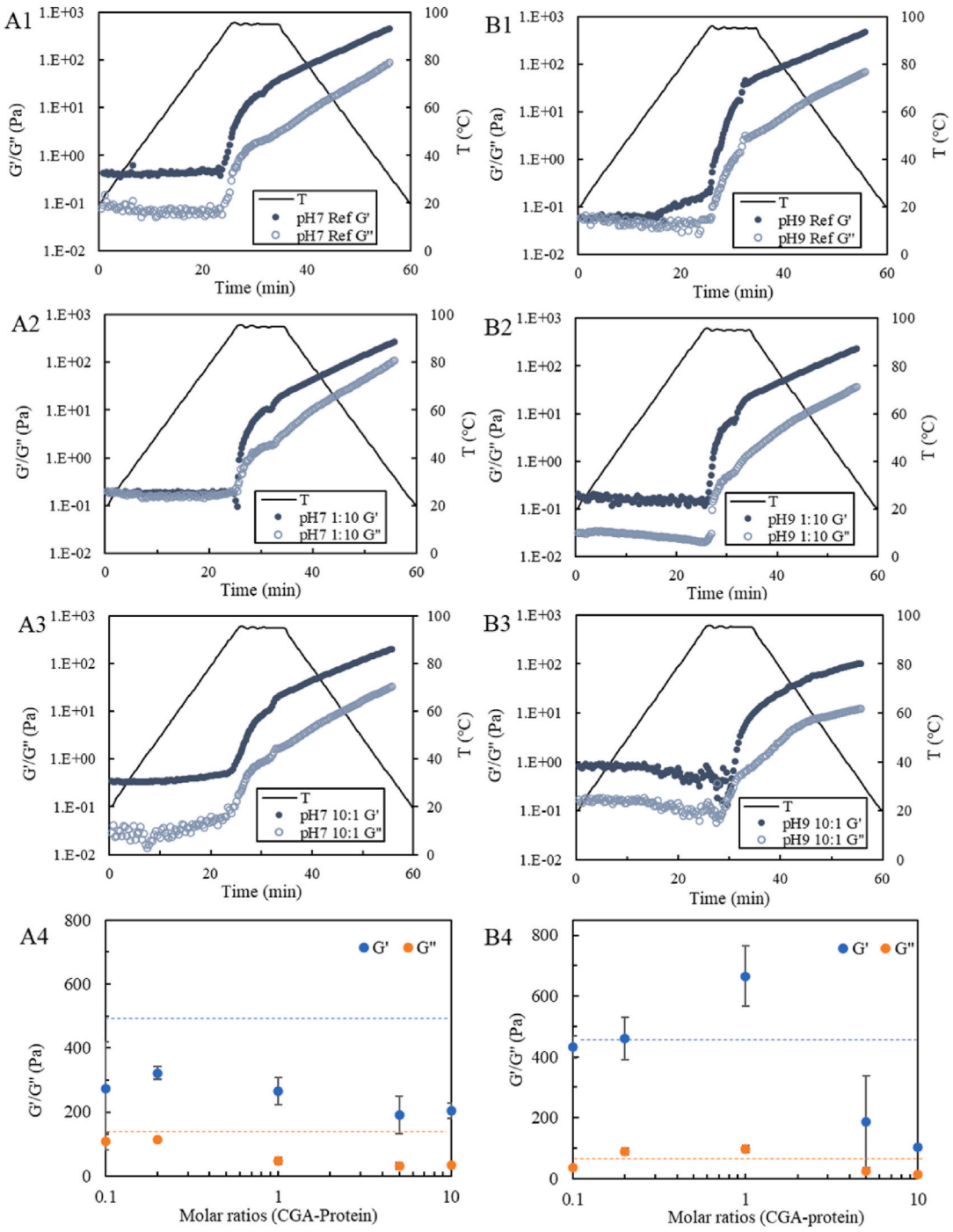

Fig. 9. The rheological properties of G′ and G″ for the non-covalently modified samples (10% (w/v)): reference (A1), 1:10 (A2) and 10:1 (A3); and the covalently modified samples (10% (w/v)): reference (B1), 1:10 (B2) and 10:1 (B3) by temperature sweep (20-95-20 °C) at 1% strain and 1 Hz. The G′ and G″ value at the end of cooling for the reference sample and the non-covalently modified protein samples or covalently modified protein samples are shown in A4 and B4 (dotted blue and orange lines are for the G′ and G″ value for the reference sample. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2017). Samples with covalently modified proteins required a longer holding time at 95 °C to show a steep increase region at a 10:1 ratio. It is noteworthy that the steep increase of the *G′* value with the 10:1 ratio upon heating indicated gel formation, although no gel was observed in Fig. 6C. A possible explanation is a weak gel formation at the 10:1 ratio, which might be broken when inverting the tube (Fig. 8). For covalently modified protein gels, the *G′* value at the end of cooling was increased with increasing CGA-protein ratios up to 1:1 (Fig. 9B4) indicating that CGA could have favored protein aggregation (as observed in SDS-PAGE, Fig. 4). A further increase of the CGA content resulted in a large decrease of the *G′* value and the lowest value was found to be 104 Pa for the gel with a CGA-protein molar ratio of 10:1. Such a dose-dependent reaction was also reported in a study on myofibrillar protein: the *G′* value of the CGA modified protein was enhanced at low CGA concentrations of 6 and 30 μM (approximate CGA-protein ratios of 1:10 and 1:2) (Cao & Xiong, 2015), while they found a large decrease of *G′* at a CGA concentration of 150 μM (approximate CGA-protein ratio of 2:1). It was explained that excessive covalent binding of CGA to proteins hinders the formation of a

protein gel network (Ali, Homann, Khalil, Kruse, & Rawel, 2013; Malik et al., 2016).

The G' and G'' dependencies on frequency and strain for the non-covalently and covalently modified protein gels after cooling were determined through a frequency and strain sweep (Figs. S7 and S8). For all the samples, the G' and G'' values slightly increased with frequency. The length of the linear viscoelastic (LVE) regime was determined by using a strain sweep at a constant frequency. The critical strain (γ_c) was taken as the end of the LVE regime. The γ_c values measured for the sunflower proteins were found to be even higher compared to the soy and pea protein gels (Kornet et al., 2021; Peng, Kyriakopoulou, Keppler, Venema, & van der Goot, 2022), suggesting stronger gels compared to soy and pea proteins. Overall, the functional properties assessed here showed a clear dose-dependent effect for covalent modifications, whereas the effect was less pronounced for the non-covalent modifications.

3.4. Outlook: CGA removal towards less refined protein concentrates

The results presented above showed that CGA affected the functional properties of sunflower protein. In general, the effects were limited and often in a positive direction. Only a large dose of CGA that is capable of binding covalently to the protein negatively influences the functional properties (i.e. the solutions turned green and the gels become much weaker).

Based on the above discussions, it becomes clear that a certain amount of CGA can be accepted in a protein isolate derived from sunflower. In case process conditions are applied that lead to covalent interactions (mainly high pH, high temperature), a CGA-protein molar ratio up to 1:1 is still acceptable when aiming at preserving high techno-functionality (i.e., solubility, gelling ability) without negative visual consequences (i.e., green color). In contrast, non-covalent CGA modifications have no clear negative effect on the sunflower proteins at the tested molar ratios (1:10–10:1) except a slight decrease for the G' and G''. The conclusion that a certain amount of CGA can be accepted in a protein isolate is relevant when considering the current discussion about the necessity for highly refined food ingredients. In modern food applications, the use of less refined protein fractions from plants is suggested. Thus, a future study should investigate whether a certain amount of CGA can also be permitted in less refined de-oiled sunflower kernels. These kernels contain a CGA-protein molar ratio of roughly 4.5:1–8:1 (Geneau-Sbartaï et al., 2008; Saeed & Cheryan, 1988). As a first step, we also evaluated an acceptable CGA-protein molar ratio (tested ratio between 1:10–10:1, results not shown) with dephenolized sunflower protein concentrate instead of isolate, where 28% carbohydrates were still present. The outcome was similar to the presented results with the isolate and a critical ratio of 1:1 was also concluded with covalent modification conducted at pH 9. The results indicated that the carbohydrate impurities in the concentrate do not affect the protein modification rate by CGA.

However, the question is now whether the added free CGA into the dephenolized protein isolate or concentrate represents the behavior of the original CGA in de-oiled sunflower kernels. Additional factors can play a role as well. First, the structure of the raw material can be relevant as CGA is mainly located inside the cell wall structure of the protein bodies (Sastry & Rao, 1990). Thus, an intact cell wall structure might limit the exposure of CGA to the proteins for reaction. Second, the process conditions for dephenolization can be relevant: the DSK was dephenolized in this study by an aqueous ethanol washing process, and the process conditions applied (solvent quality, temperature, and pH value) were already found to induce some protein-CGA interactions, thereby affecting the functional properties (Jia, Kyriakopoulou, et al., 2021). Finally, the presented study focused only on the techno-functionality of modified sunflower protein, while the sensory and biological properties were not taken into consideration. Clearly, a future study should focus on whether the critical ratio found in the simplified model blends also holds for less refined sunflower materials, and also for other than techno-functional properties.

4. Conclusion

The dose-dependent effects on functional properties of covalently and non-covalently modified sunflower protein isolate by chlorogenic acid (CGA) were studied using different molar ratios. It was found that CGA can bind both covalently and non-covalently, depending on the process conditions applied to induce the interactions. Non-covalent interactions induced structural changes in the protein, which also increased the denaturation temperature and solubility. The effects on the color formation and gelling properties were limited. The effect of covalent interactions on protein structure was less clear, mainly because the process conditions used to induce interactions (the pH-shift) had a much larger effect. Nevertheless, covalent interactions led to changes in functional properties: a dose-dependent effect was observed for the gelling capacity, with a maximum G' and G'' at 1:1 ratio. At higher CGA dosages, gelling properties were negatively influenced, and the green color was observed.

Overall, the presented results can be used for tailoring the techno-functionality of the sunflower protein fractions by identifying the critical CGA protein ratios, which retain or even improve techno-functional properties in less refined fractions irrespective of the mode of interaction that might later be induced by applications. This study was performed with a well-defined mixture of pure sunflower protein isolate and CGA. Experiments with a multi-component material such as de-oiled sunflower kernel are yet to be done to understand the effect of CGA on sunflower proteins in less refined ingredients.

Author statement

Wanqing Jia: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing-original draft, Data Curation, Visualization.

Divjyot Singh Sethi: Methodology, Formal analysis.

Atze Jan van der Goot: Funding acquisition, Supervision, Writing-Review & Editing.

Julia K Keppler: Conceptualization, Supervision, Methodology, Validation, Writing-Review & Editing.

Declaration of competing interest

None.

Acknowledgment

This research is part of the project Plant Meat Matters, which is co-financed by Top Consortium for Knowledge, The Netherlands and Innovation Agri & Food by the Dutch Ministry of Economic Affairs, The Netherlands; the project is registered under contract number TKI-AF-16011. The authors thank Maurice Strubel for the support in RP-HPLC experiment.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2022.107800.

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