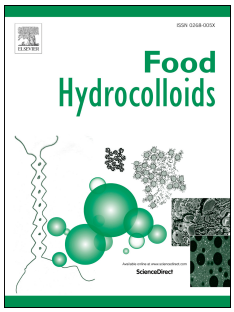


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# Author statement

Xiaobin Ma: experimental investigation, data processing, original draft preparation

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Song Miao: paper reviewing

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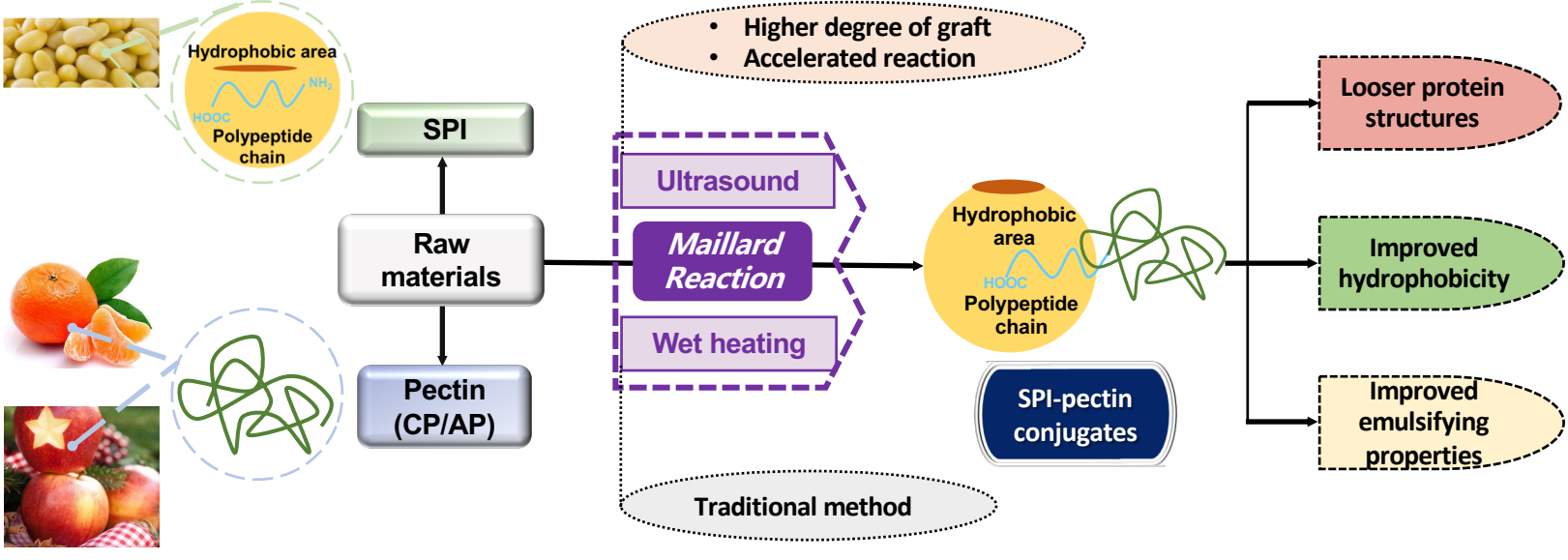
Hydrophobic area
NH2
HOOC
Polypeptide chain
SPI
Raw materials
Pectin (CP/AP)
Ultrasound
Maillard Reaction
Wet heating
• Higher degree of graft
• Accelerated reaction
Traditional method
Hydrophobic area
HOOC
Polypeptide chain
SPI-pectin conjugates
Looser protein structures
Improved hydrophobicity
Improved emulsifying properties

# Conjugation of soy protein isolate (SPI) with pectin by ultrasound treatment

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**Abstract**

The Maillard reaction in the aqueous system with and without ultrasound treatment was used to prepare conjugates between soy protein isolate (SPI) and citrus pectin (CP) / apple pectin (AP). Ultrasound treatment at a power of 450 W and a temperature of 70 ℃ significantly accelerated the conjugation processes between SPI and pectin samples and led to much greater grafting extents compared to the traditional wet heating. A higher degree of graft of the SPI-CP conjugates was achieved at a shorter ultrasound duration compared to the SPI-AP conjugates, possibly attributed to the larger molecular weight and the more flexible structure of AP. SDS-PAGE analysis confirmed the formation of SPI-pectin conjugates. Analysis of the protein secondary and tertiary structures suggested that the attachment of CP or AP changed the spatial conformation of SPI and led to a looser protein structure. In addition to the grafting process, ultrasound was also observed to play a marked role in unfolding the SPI resulting in more favorable structures for the Maillard reaction. Furthermore, the application of ultrasound to the conjugation process significantly increased the surface hydrophobicity and emulsifying properties of both conjugates, indicating that ultrasound can be a desirable method for protein-polysaccharide conjugation.

**Keywords:** ultrasound; wet heating; soy protein isolate; pectin; structure; emulsifying property

## 1. Introduction

In the past few decades, O/W emulsions stabilized by proteins have been extensively introduced as a useful delivery system (Nooshkam & Varidi, 2019). It is these emulsions that food and pharmaceutical scientists have used over the years to protect lipophilic active compounds against environmental stress or degradation, allow for controlled release, and cover up an unpleasant smell or taste (Bouyer, Mekhloufi, Rosilio, Grossiord, & Agnely, 2012). However, the main challenge raised by the use of such protein-stabilized emulsions is lack of stability, given that they are prone to coalescence, creaming and phase separation that can be induced by a series of factors such as extreme pH, temperature and ionic strength (Yang, et al., 2015). To overcome these techno-functional issues, recently scientists have focused on anchoring polysaccharides onto oil droplet surfaces by the use of a simple Maillard reaction (Nooshkam & Varidi, 2019).

The Maillard reaction is an umbrella term for a complex group of reactions between an amino acid and a reducing sugar, which can produce various colored and volatile products (Yu, Seow, Ong, & Zhou, 2017). The early stage of Maillard reaction involves an initial condensation of the carbonyl group of a reducing sugar with an amino group of the protein, resulting in the formation of Schiff base. It then undergoes Amadori rearrangements and a range of reactions and finally turns into melanoidins that gives the brown color to food matrices. These advanced products however can be very harmful, and have been reported to cause various diseases including diabetes and Alzheimer's (Silván, Assar, Srey, Del Castillo, & Ames, 2011).

This has led increasing research effort to the application of polysaccharides to the Maillard reaction. Compared to mono- and oligosaccharides, polysaccharides have relatively weaker reducibility and stronger molecular steric hindrance, which could restrict the advanced reactions and thus reduce the undesired products (de Oliveira, Coimbra, de Oliveira, Zuñiga, & Rojas, 2016; Zhang, et al., 2019). The formation of protein-polysaccharide conjugates also combines the merits of these two biomacromolecules together, resulting in unique characteristics such as the excellent solubility, improved emulsifying properties and higher stability against various environmental conditions (Nooshkam & Varidi, 2019).

Today, most protein-polysaccharide conjugates are simply prepared via dry heating without extra substrates. However, this method involves a lyophilization process prior to the reaction and requires controlled temperature and humidity, leading to the high cost for industrial applications (de Oliveira, et al., 2016; Zhang, et al., 2019). In addition, the relatively moderate conditions (60 ℃) and lack of medium generally result in very slow reactions that could last for several weeks (Zhang, et al., 2019). In this case, some researchers start to conduct Maillard reaction in aqueous mediums, where the conjugation time can be shortened by increasing the reaction temperature, though protein denaturation also easily occurs (Zhang, et al., 2019). This is why diverse non-thermal processing techniques, including ultrasound (Li, Huang, Peng, Shan, & Xue, 2014; Qu, et al., 2018), pulsed electric field (Guan, et al., 2010) and high pressure (Xu, et al., 2010), are increasingly introduced to the traditional Maillard reaction.

Among these non-thermal processing techniques studied thus far, ultrasound is of particular interest because of its unique cavitation effect, *i.e.* the rapid formation, growth and collapse of gas bubbles. It has been shown that ultrasound could accelerate the Maillard reaction and improve the grafting extent; further, it also proved effective in altering the structures of protein-polysaccharide conjugates resulting in more desirable functionality (Qu, et al., 2018). As supported by Li, et al. (2014), a degree of graft (DG) of peanut protein isolate (PPI)–glucomannan conjugates of 30.15% was achieved with ultrasound treatment for 80 min, whereas it took 40 h for classical wet heating to obtain a similar DG. Furthermore, ultrasound treatment was also observed to improve the solubility and emulsifying properties of the conjugates. Similar findings have also been reported for the rapeseed protein isolate (RPI)-dextran (Qu, et al., 2018) and PPI-maltodextrin (Chen, Chen, Wu, & Yu, 2016) conjugates prepared by ultrasound.

In this work, soy protein isolate (SPI) and two pectin samples, *i.e.* citrus pectin (CP) and apple pectin (AP), were selected as the raw material for conjugation. As an ideal protein product that contains more than 90% protein, SPI has received extensive research interest for its low cost and desirable nutritional and functional properties (Wang, et al., 2008). Likewise, pectin is also a low-cost plant material with 85.5% commercial source from citrus peel and 14.0% from apple pomace (Chan, Choo, Young, & Loh, 2017); its excellent gelling, stabilizing and emulsifying properties have made it a promising material for the food industry. In the previous work (Ma, et al., 2020) we have studied the Maillard reaction between SPI and CP/AP in the dry

state, where the SPI-CP and SPI-AP conjugates were obtained at 60 °C and a relative humidity of 79% for 5 days, achieving the DG of 25.00% and 21.85%, respectively. As ultrasound offers possibility of enhancing the conjugation process within shorter period of time, in this study we will employ the high-intensity ultrasound to prepare the SPI-pectin conjugates. On the other hand, currently, the systematic research involves in the Maillard reaction between protein and polysaccharide under an ultrasonic field, including the effects of different operational factors, structural changes, and relevant functionality improvements of the conjugates is still deficient, since only a small amount of studies regarding these aspects have been published (Qu, et al., 2018). Therefore in this study, our objectives are to investigate the effects of ultrasound conditions (e.g. power, temperature, time) on SPI-pectin conjugation, to analyze the quantitatively structural changes of conjugates that occurred at the secondary and tertiary levels, and to illuminate the implications of these changes on the emulsifying properties of the resulting conjugates. Furthermore, the performance of traditional wet heating and ultrasound treatment in preparing conjugates, as well as the properties of conjugates produced by different pectin samples, will be compared to provide references on both method and material aspects for innovative food emulsifier design.

## 2. Materials and Methods

### 2.1. Materials

Defatted soybean meal was obtained from Hengrui Food Ltd. (Guangzhou, China). Citrus pectin (P9135), apple pectin (93854) and

8-Anilino-1-naphthalenesulfonic acid (ANS) were purchased from Sigma–Aldrich (Shanghai, China). Sodium-dodecyl-sulfate polyacrylamide gel (SDS-PAGE) kit was purchased from Beyotime Biotechnology (Shanghai, China). All other reagents including o-Phthaldialdehyde (OPA), sodium azide, SDS, etc., were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

### 2.2. Preparation of SPI

Preparation of SPI was carried out according to our previous studies (Ma, et al., 2020; Ma, et al., 2019). The final protein content was 96.22 ± 0.48 (%).

### 2.3. Analysis of molecular information of pectin

The weight-average molecular weight (Mw), number-average molecular weight (Mn), polydispersity (Mw/Mn), z-average root mean square (RMS) radius of gyration (Rz) and intrinsic viscosity [η] were measured through a size exclusion chromatography (SEC) combined with a multi-angle laser light scattering (MALLS, Wyatt Dawn Heleos-II, USA) system, equipped with a refractive index detector (RID-10A, Shimazu Corporation, Kyoto, Japan) and an on-line differential viscometer (ViscoStarTMII, Wyatt Technology, USA). The mobile phase was 0.2 M NaCl solution (containing 0.02% $NaN_3$) and used to dissolve pectin samples to obtain a concentration of 3 mg $mL^{-1}$. Solutions were stirred overnight and then filtered through a 0.22 μm membrane before loading. The flow rate, elution time and dn/dc value were set at 0.75 mL $min^{-1}$, 90 min and 0.1355 mL $g^{-1}$ (Chen, et al., 2017; C. Y. Wei, et al., 2018), respectively.

### 2.4. Preparation of SPI-pectin conjugates

#### 2.4.1. Wet-heating conditions

SPI and pectin (1 : 1 w/w) were dissolved and the pH was adjusted with NaOH to 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0, respectively. The mixed solution was then reacted at different temperatures (50, 60, 70, 80 and 90 ℃) for different times (4, 8, 12, 16, 20 and 24 h). Then, the reactor was immediately cooled in an ice bath for 3 min to terminate the reaction. The conjugates were freeze-dried and ground into fine powders for measurements. The optimum pH and temperature were determined by the DG of the conjugates.

#### 2.4.2. Ultrasound treatment

SPI and pectin (1 : 1 w/w) were mixed at pH 10.0 and 100 mL of the mixtures were put in a cylinder reactor to be processed with a probe sonicator (JY92-IIDN, Ningbo Scientz Biotechnology Co., Ningbo, China) with a maximum output power of 900 W and an operating frequency of 22 kHz. The temperature of reactants was controlled with a water bath and monitored by a temperature probe. Samples were subjected to ultrasound at different powers (270, 360, 450, 540 and 630 W), durations (15, 30, 45, 60, 75, 90, 105 and 120 min) and temperatures (50, 60, 70, 80 and 90 ℃). Then, the reactor was immediately cooled in an ice bath for 3 min to terminate the reaction. The optimum power, duration and temperature were determined by the DG of the conjugates.

### 2.5. Measurement of degree of graft (DG)

The DG of conjugates were determined by the modified OPA assay as described in our previous study (Ma, et al., 2020) and was calculated according to Eqn (1):

$$DG = \frac{A_0 - A_t}{A_0} \times 100\% \quad (1)$$

where $A_0$ is the free amino groups content of the mixtures of SPI and pectin, $A_t$ is the free amino groups content of SPI-pectin conjugates prepared with or without ultrasound.

### 2.6. Sodium-dodecyl-sulfate polyacrylamide gel (SDS-PAGE) electrophoresis

SDS-PAGE electrophoresis was carried out as described in our previous work (Chen, et al., 2019; Ma, et al., 2020). The stacking gel (5%) and separating gel (12%) were prepared. Samples, including SPI, SPI and pectin mixtures, and SPI-pectin conjugates prepared under traditional wet heating or with ultrasound treatment, were dissolved with a constant protein concentration of 2 mg $mL^{-1}$, and then mixed with the protein loading dye. The mixtures were heated in boiling water for 5 min and then loaded in the gel slot. Electrophoresis for the stacking gel and the separating gel was conducted at 80 V and 120 V, respectively. Gel was stained with Coomassie Brilliant Blue R250 dye for 30 min followed by destaining in a solution comprising 40% methanol and 10% acetic acid to the proper color density level.

### 2.7. Measurement of circular dichroism (CD)

Solutions of SPI and SPI-pectin conjugates (prepared under traditional wet heating or with ultrasound treatment) were prepared at a protein concentration of 0.25 mg $mL^{-1}$ in the 0.01 M PBS at pH 7.0 and then put in a quartz cuvette of 1 mm optical path length. The CD spectra of samples were measured by a spectropolarimeter (JASCO J1500, Tokyo, Japan) at 20 °C ± 1 °C. Scanning was conducted in the wavelength range of 190 nm to 250 nm at a rate of 50 nm $min^{-1}$ with a bandwidth set

194 at 1 nm. The CD data were expressed in the form of mean residue ellipticity [$\theta$]
195 (deg $cm^2$ $dmol^{-1}$). The secondary structures of samples were analyzed using
196 DICHROWEB.

197 **2.8. Measurement of intrinsic fluorescence**

198 Samples were dissolved using 0.01 M PBS solution (pH 7.0) to obtain a protein
199 concentration of 0.25 mg $mL^{-1}$. The fluorescence spectra ($\lambda_{ex}$, 280 nm; $\lambda_{em}$, 300–500;
200 slit = 5 nm) were measured with a Model Cary Eclipse spectrophotometer (Varian Inc.,
201 Palo Alto, USA) at a scanning rate of 600 nm $min^{-1}$.

202 **2.9. Measurement of surface hydrophobicity ($H_0$)**

203 The $H_0$ was measured using ANS as a fluorescence probe. Lyophilized samples
204 were dissolved using 0.01 M PBS solution (pH 7.0) to obtain protein concentrations
205 ranging from 0.001 mg $mL^{-1}$ to 0.4 mg $mL^{-1}$. Then, 50 μL of 8 mM ANS was added to
206 4 mL of sample solutions. Fluorescence intensity ($\lambda_{ex}$, 365 nm; $\lambda_{em}$, 484) was
207 measured with a Model Cary Eclipse spectrophotometer (Varian Inc., Palo Alto, USA).
208 The slope of fluorescence intensity versus protein concentration was used as the $H_0$ of
209 protein.

210 **2.10. Analysis of emulsifying properties**

211 The emulsifying activity index (EAI) and emulsifying stability index (ESI) were
212 measured according to the method established by Pearce and Kinsella (1978) with
213 some modifications. Briefly, 5 mL of the blend oil and 15 mL of 2.5 mg $mL^{-1}$ samples
214 were mixed and homogenized at 13500 r for 2 min. The prepared emulsion (50 μL)
215 was then sampled from the bottom of the tube at regular time intervals and diluted

with 10 mL of 0.1% (w/v) SDS solution. The absorbance of the diluted emulsion was measured at 500 nm. EAI and ESI were calculated by Eqn (2) and (3), respectively:

$$\text{EAI}\ (m^2/g) = \frac{2 \times 2.303 \times A_0 \times D}{(1-\varphi) \times c \times 10^4} \quad (2)$$

$$\text{ESI}\ (\%) = \frac{A_{10}}{A_0} \times 100\% \quad (3)$$

where $A_0$ and $A_{10}$ are the absorbance measured at 0 and 10 min, respectively, $\varphi$ is the oil phase fraction, $c$ is the initial concentration of SPI (g $mL^{-1}$) and $D$ is the dilution factor.

### 2.11. Statistical analysis

One-way analysis of variance (ANOVA, $p < 0.05$) and Duncan's multiple range tests were performed using SPSS 17.0 to evaluate the differences among various samples. Data were represented as the means of triple measurements.

## 3. Results and discussion

### 3.1. Effect of initial pH and temperature on the conjugation of SPI and pectin

#### 3.1.1. Initial pH

SPI and pectin were conjugated at 70 ℃ for 24 h at a pH range of 6.0 to 12.0. Fig. 1 A and B illustrate the DG of the SPI-CP and SPI-AP conjugates, respectively, at each tested pH; Fig. 1 C and D show the corresponding appearance of these samples. The large molecular weight and complicated structures of both SPI and pectin make it difficult for Maillard reaction to take place, leading to the low DG values for all the samples as can be observed from Fig. 1 A and B. DG of both conjugates was increased as pH was increased from 6.0 to 10.0, possibly attributed to the increased proportion of unprotonated amino acid at higher pH (Lertittikul, Benjakul, & Tanaka,

238 2007). However when pH was further increased from 10.0 to 12.0, the DG of both
239 samples started to decrease, and the solutions turned to a dark brown color (Fig. 1 C
240 and D). This was considered to be related to the racemization and formation of
241 lysinoalanine under combined heating-alkaline treatment, which could cause great
242 damage to SPI (Schwass & Finley, 1984). As well, at an alkaline medium,
243 β-elimination and demethoxylation readily happened inducing pectin molecules to
244 degrade (Fraeye, et al., 2007), which could also influence the conjugation process. In
245 this study, the best pH for preparing both samples was determined at pH 10.0.

246 On the other hand, the conjugates prepared by CP can be seen to show an
247 obviously greater extent of conjugation than AP at each pH. For instance, DG of the
248 SPI-CP conjugates at pH 10.0 was 20% higher than that of the SPI-AP conjugates,
249 which was due to the structural differences of the two pectin samples. As can be seen
250 in Table 1, AP has a significantly higher Mw (1050.50 kDa) than CP (478.00 kDa),
251 with a larger polydispersity and Rz. The exponent α from Mark-Houwink equation
252 ($[\eta] = KM_w^{\alpha}$) was measured to provide information of pectin chain conformations. As
253 a characteristic constant, α indicates the stretching forms of polymers in solution; its
254 value within the range of 0 – 0.3, 0.5 – 0.8 and 1.0 – 2.0 indicates that polymers are
255 presented as spheres, flexible chains and rod-like rigid chains, respectively (C. Wei, et
256 al., 2018). As shown in Table 1, α for CP and AP was 0.85 and 0.71, respectively,
257 demonstrating that AP exhibited more flexible chains than CP in the 0.1 mol $L^{-1}$
258 $NaNO_3$ solution. These flexible chains can readily intertwine together in the aqueous
259 system and burry the reactive groups for Maillard reaction, impeding the conjugation

process and resulting in the low DG values.

### 3.1.2. Temperature

DG of the SPI-CP and SPI-AP conjugates prepared at different temperatures is illustrated in Fig. 2 A and B, respectively; the corresponding appearance of these conjugates is shown in Fig. 2 C and D. Despite the differences in pectin structures, the optimum temperature for Maillard reaction for both samples was 70 ℃, where the SPI-CP conjugates and SPI-AP conjugates showed the DG values of 8.25% and 6.60%, respectively. This suggested that the variation trend for DG was mainly influenced by changes in SPI at high temperatures. As reported in previous studies (Chen, et al., 2019; Mu, et al., 2010), an increase in temperature could accelerate the Maillard reaction and lead to higher DG of conjugates; however, German, Damodaran, and Kinsella (1982) have reported that the transition temperature for glycinin (11s) and β-conglycinin (7s) is 92 ℃ and 77 ℃, respectively, indicating that there is an “upper threshold”, beyond which the further increase in temperature will result in severe protein aggregation (Chen, et al., 2019) and in turn, the decreased DG. Looking at Fig. 2 C and D, both conjugates formed at 90 ℃ presented a deep brown color and precipitated a lot after being placed for a few minutes (not shown in pictures). Mu, et al. (2010) has reported that high temperatures could induce serious browning of conjugates, while the phase separation can be attributed to the insoluble protein aggregates formed at high temperatures as mentioned above.

## 3.2. Effect of ultrasound conditions on the conjugation of SPI and pectin

### 3.2.1. Ultrasound power

DG of SPI-pectin conjugates prepared at different ultrasound powers for 20 min is depicted in Fig. 3. As ultrasound power was increased from 270 W to 450 W, DG of SPI-CP and SPI-AP conjugates was significantly increased by 248.43% and 85.63%, respectively, indicating that more energy input accelerated the Maillard reaction and led to a higher grafting extent. This was in line with the previous observations (Chen, et al., 2019; Li, et al., 2014; Mu, et al., 2010). Li, et al. (2014) applied ultrasound to the Maillard reaction of PPI and glucomannan; it was found that when the ultrasound intensity was increased from 302.55 W $cm^{-2}$ to 786.62 W $cm^{-2}$ at 60 ℃, 70 ℃ and 80 ℃, the DG of PPI-glucomannan conjugates was increased by 90.66%, 55.25% and 65.84%, respectively. High-intensity ultrasound is able to induce local translational motions and strong micro-jets in liquid systems, which bring reactive groups into closer proximity and enhance their contacts, resulting in a more steady grafting process (Chen, et al., 2016). Furthermore, it has also been suggested that proper doses of ultrasound could generate certain amounts of shear forces and free radicals that favorably modify the structures of proteins and polysaccharides, causing more exposure of amino and carbonyl radicals for Maillard reaction (Chen, et al., 2016; Qu, et al., 2018). However, looking at Fig. 3, when the ultrasound power was further increased beyond 450 W, the DG of both conjugates started to decrease, indicating that after exceeding a certain level of energy input, proteins would be denatured by the shear forces and free radicals produced from the intense ultrasonic field, leading to the formation of protein aggregates and thus impeding the grafting process (Chen, et al., 2019; Resendiz-Vazquez, et al., 2017).

Similar phenomenon was also observed in our previous study (Chen, et al., 2019), where the DG of whey protein isolate (WPI)-gum acacia (GA) conjugates was firstly increased as the ultrasonic power was increased from 100 W to 500 W, whereas it decreased as the power was further increased to 700 W.

### 3.2.2. Temperature

The DG of SPI-pectin conjugates prepared at different temperatures under an ultrasonic field at 450 W for 20 min is depicted in Fig. 4. Similar to the routine wet-heating process, DG of SPI-CP and SPI-AP conjugates was observed to increase from 3.76% to 8.25%, and 2.93% to 6.60%, respectively, when the temperature was increased from 50 ℃ to 70 ℃; whereas it was decreased to 3.57% and 0.27%, respectively for CP and AP, as the temperature was further increased to 90 ℃. Qu, et al. (2018) has reported that ultrasound treatment could increase the optimum temperature for conjugation of RPI and dextran. Authors ascribed this to the ultrasound cavitation increasing the denaturation temperature of RPI and thus preventing protein aggregations at high temperatures. However in this study, DG of both conjugates prepared with and without ultrasound treatment peaked at 70 ℃, indicating that at 80 ℃ and 90 ℃, the effect of thermal denaturation of SPI might outweigh the positive effects brought by ultrasound irradiation.

### 3.2.3. Ultrasound duration

The DG of SPI-CP/AP conjugates prepared by traditional wet heating and ultrasound treatment at different times is shown in Table 2. Ultrasound treatment at short bursts of time can be seen to significantly accelerate the Maillard reaction

between SPI and pectin. For example, the DG of SPI-CP conjugates treated by ultrasound at 45 min was 24.06%, nearly treble the value obtained by wet heating for 24 h. As stated before, the favorable changes in structures of both macromolecules, as well as the enhanced contacts of the reactants under a high-intensity ultrasonic field could result in an improved grafting process. Similar observations have been reported for WPI-GA (Chen, et al., 2019), SPI-GA (Mu, et al., 2010) and PPI-glucomannan conjugates (Li, et al., 2014) prepared with ultrasound treatment. However, prolonged ultrasound irradiation was observed to be less beneficial for the grafting process. As shown in Table 2, when ultrasound duration was increased from 15 min to 45 min, the DG of SPI-CP conjugates was increased by 128.71%; whereas when the duration was further prolonged to 120 min, the increase in DG can be seen to hit the plateau and was only 10.85%. Similarly, when ultrasound was applied to the conjugation between SPI and AP, the DG of SPI-AP conjugates treated at a duration of 60 min was 261.44% higher than that at 15 min; while for the last 60 min (from 60 min to 120 min), the DG was increased by only 14.66%. These results suggested that long-time ultrasound treatment might denature the proteins (Zhu, et al., 2018) and lead to a decreased grafting efficiency. Considering both productivity and energy efficiency, the ultrasound durations for the preparation of the SPI-CP and SPI-AP conjugates were selected at 45 min and 60 min, respectively.

It is also worthwhile to note that the increase in the DG of SPI-AP conjugates with ultrasound treatment was higher than that of the SPI-CP conjugates. Table 1 lists the conformational parameters of CP and AP with and without ultrasound

348 treatment. After being treated with ultrasound for a certain duration, both pectin
349 samples showed significantly lower Mw and Rz, and their polysaccharide chains
350 tended to be more rigid (with an increased exponent α), which in combination
351 facilitated the conjugation process with SPI. Ultrasound mechanical breakage is
352 known to act on the midpoint of polymer chains (Koda, Taguchi, & Futamura, 2011)
353 and is more effective for long-chain polymers (Portenlanger, et al., 1997). Therefore,
354 AP with higher Mw was more accessible for ultrasound breakage, resulting in a
355 larger increase in the grafting extent than CP. Furthermore, under a high-intensity
356 ultrasonic field, CP would be degraded into small segments at a shorter treatment
357 time compared to AP, making ultrasound less effective for degradation. This also
358 explained why the optimum condition for SPI-CP conjugation was obtained at a
359 shorter ultrasound duration.

360 **3.2.4. SDS-PAGE analysis**

361 Fig. 5 shows the SDS-PAGE profiles of SPI, mixtures of SPI and pectin samples,
362 and SPI-pectin conjugates prepared by wet heating and ultrasound treatment. SPI
363 mainly consists of glycinin (11s globulin) and β-conglycinin (7s globulin), which are
364 known to contain a series of polypeptide chains with diverse Mw (Petruccelli & Anon,
365 1995). The two mixture samples in Lanes 2 and 3 showed a same pattern as the native
366 SPI, indicating that both pectin samples contained little protein impurities and did not
367 change the electrophoretic profiles of SPI. However, the Maillard reaction generated
368 various conjugated products with too large Mw, which can be seen to form a new
369 band on the top of the separating gel in Lanes 4 to 7. Meanwhile, compared with

Lanes 1–3, the featured bands of SPI at the Mw range of 15 kDa, 25–35 kDa and 55–70 kDa clearly faded out in Lanes 4–7, indicating that these proteins had participated in the grafting process. Furthermore, compared with the conjugated samples prepared by the traditional wet heating, the ultrasound-treated conjugates displayed darker new bands and lighter featured bands of the native SPI, indicating the greater grafting extent for these samples, which is in line with the results of DG analysis.

### 3.2.5. CD analysis

The far-UV CD spectrum was employed to identify the variations in the secondary structures of SPI during different conjugation processes. Fig. 6 depicts the CD spectra of SPI, mixtures of SPI and pectin samples and SPI-pectin conjugates prepared by wet heating and ultrasound treatment, and Table 3 summarizes the secondary structures of each sample. Huang, Ding, Dai, and Ma (2017) analyzed the secondary structures of SPI and found that the contents of α-helix, β-sheet, turn and random coil were 6.5%, 36.7%, 23.0% and 33.8%, respectively, which is very similar to our results. As shown in Table 3, the simple physical mixing with pectin samples did not change the secondary structures of SPI. However, the conjugation process by either traditional wet heating or ultrasound treatment reduced the contents of α-helix, β-sheet and turn in the protein conformation, whereas it increased the contents of the random coil. Similar results were reported by Mu, et al. (2010), where the contents of α-helix, β-sheet and turn in the SPI-GA conjugates were decreased by 73.58%, 76.69% and 46,79%, respectively, while the contents of the random coil were increased by 112.95%, compared to the original SPI. It has been reported that the α-helix and

β-sheet are generally buried inside the polypeptide chains (Mu, et al., 2010), therefore the decrease of these structures indicated that the attachment of CP or AP changed the spatial conformation of SPI and led to a looser protein structure (Qu, et al., 2018). The increase in the content of random coil further confirmed the unfolding of proteins during the Maillard reaction resulting in a more disordered molecular structure. However, the SPI-CP and SPI-AP conjugates prepared by the same conjugation process (*i.e.* traditional wet heating or ultrasound treatment) showed similar secondary structures, indicating that the grafting method rather than the different pectin samples played a more important role in changing the secondary structures of proteins. It was revealed that the conjugates prepared by ultrasound treatment lost more α-helix and β-sheet but gained more random coil compared to those obtained by traditional wet heating, suggesting that in addition to the grafting process, ultrasound also had a hand in breaking the peptide bonds and unfolding the proteins, which was reported to be related to the pressure alterations and turbulence generated from cavitation (Mu, et al., 2010). Such an extension in the protein structures possibly made SPI more favorable for binding to pectin, which could be a reason for the higher DG values obtained under an ultrasonic field as described in Section 3.2.3. These structural transformations led to a greater conformational flexibility of proteins, allowing for easier adsorption at the oil-water interface and could result in the improved emulsifying properties (Martínez, Sanchez, Ruíz-Henestrosa, Patino, & Pilosof, 2007).

### 3.2.6. Intrinsic fluorescence analysis

Alterations in the proteins' tertiary structures during conjugation were determined by the intrinsic fluorescence spectra based on the tryptophan (Trp) content. Fig. 7 depicts the intrinsic fluorescence spectra of SPI, mixtures of SPI and pectin samples and SPI-pectin conjugates prepared with and without ultrasound treatment. It was shown that the highest fluorescence intensity of the original SPI was obtained at 341 nm; and this maximum absorption wavelength ($\lambda_{max}$) remained unchanged for the two mixture samples. However, a slight decrease in the intensity can be observed for both mixtures, as a result of the steric-hindrance effect of pectin samples. As mentioned in Section 3.1.1, AP had a higher Mw and more flexible chains than CP, so it could induce a stronger steric-hindrance effect and in turn, a lower fluorescence intensity. Conjugation under the traditional wet heating conditions caused a further decrease in the fluorescence intensity, and it led the $\lambda_{max}$ to shift from 341 nm to 343 nm, suggesting that the Trp residues reached a more exposed and polar microenvironment (Sheng, et al., 2020). The transformation of SPI secondary structures, exposure of hydrophobic groups in proteins, and the shielding effects of pectin during the Maillard reaction were considered as the reasons for this phenomenon (Sheng, et al., 2017; Sheng, et al., 2020). As the DG of both conjugates were very low (as shown in Table 2), the steric-hindrance effect of pectin still played a main role in shielding the fluorescence signals and thus the SPI-AP conjugates showed a lower fluorescence intensity than the SPI-CP conjugates. Compared to the conjugates obtained by traditional wet heating, the conjugates prepared with ultrasound can be seen to have much less compact tertiary structures, with

436 significantly lower fluorescence intensities and a greater red shift to 345 nm. This was
437 possibly due to the unfolding of protein molecules and the destroyed hydrophobic
438 interactions during ultrasound treatment (Subhedar & Gogate, 2014). Despite the
439 more complex structures and the potentially stronger shielding effect of AP, the
440 ultrasound-treated SPI-CP conjugates showed an overall lower fluorescence intensity
441 than the SPI-AP conjugates, which can be ascribed to the greater grafting extent.

442 **3.2.7. $H_0$ analysis**

443 $H_0$ is one of the most important factors determining a range of functional
444 properties of proteins, such as the solubility, emulsifying ability and foaming ability
445 (Jiang, et al., 2015). Here we employed a hydrophobic fluorescent dye, *i.e.* ANS, to
446 evaluate the $H_0$ of SPI, SPI and pectin mixtures, and SPI-CP/AP conjugates prepared
447 by wet heating and ultrasound treatment; results are shown in Fig. 8. The addition of
448 CP or AP can be seen to block the way of ANS to the hydrophobic residues, resulting
449 in the 38.93% and 40.08% lower $H_0$ values for the SPI-CP and SPI-AP mixtures,
450 respectively, compared to the original SPI. Glycosylation under the traditional wet
451 heating conditions induced further reduction in the $H_0$, indicating that the Maillard
452 reaction has changed the SPI conformation and restrained the exposure of
453 hydrophobic groups (Sheng, et al., 2020); also, it might have produced advanced
454 glycation products with little surface hydrophobicity (Chen, et al., 2016). When
455 ultrasound was introduced to the conjugation process, a significant increase in $H_0$ can
456 be observed for both conjugates, which was in accord with the previous studies (Chen,
457 et al., 2016; Li, et al., 2014). This can be attributed to the unfolding of proteins and/or

458 dissociation of protein aggregates under a high-intensity ultrasonic field, leading to
459 the exposure of the previously buried hydrophobic groups (Chen, et al., 2016). The
460 increased $H_0$ is known to be able to increase proteins' adsorption rate to the oil/water
461 interface, which is helpful in improving the emulsifying properties (Delahaije,
462 Gruppen, Giuseppin, & Wierenga, 2014). Furthermore, despite the higher DG of the
463 SPI-CP conjugates, no significant differences were observed for $H_0$ of the SPI-CP and
464 SPI-AP conjugates under a specific conjugation condition (*i.e.* wet heating or
465 ultrasound treatment), which was possibly due to the stronger steric-hindrance effect
466 provided by AP.

467 **3.2.8. Emulsifying properties analysis**

468 Fig. 9 shows the EAI and ESI of SPI, SPI and pectin mixtures, and SPI-CP/AP
469 conjugates prepared by wet heating and ultrasound treatment. Both indices were
470 increased with the addition of CP or AP, possibly due to the emulsifying properties
471 provided by pectin samples. It has been recognized that the conjugation of protein and
472 polysaccharide combines the characteristic property of proteins to adsorb strongly to
473 the oil-water interface, with the excellent solubility of polysaccharides in the aqueous
474 medium (Dickinson & Galazka, 1991). As expected, conjugation under the traditional
475 wet heating conditions significantly improved the emulsifying properties of the
476 SPI-CP/AP conjugates when compared to the original SPI; however, due to the low
477 DG of both conjugates (less than 10%), there were no significant differences in EAI
478 and ESI as compared with the mixture samples. Nevertheless, ultrasound can be seen
479 to significantly improve the emulsifying properties of both conjugates. As shown in

Fig. 9, the EAI and ESI of the ultrasound-treated SPI-CP conjugates were increased by 147.59% and 102.76%, respectively, compared to those prepared by the traditional wet heating; as for the SPI-AP conjugates, the EAI and ESI were significantly increased by 104.42% and 111.56%, respectively, compared to those prepared by the traditional wet heating. As described in the above sections, the improved DG, enhanced surface hydrophobicity, together with the extended spatial conformations of proteins obtained with ultrasound treatment, have contributed to this significant enhancement in the emulsifying properties of the conjugates. As can be seen, the SPI-CP/AP conjugates prepared by ultrasound demonstrated satisfying emulsifying properties (with the EAI more than 30% and ESI more than 80%), suggesting that both pectin samples in conjugation with SPI can form excellent emulsions under a high-intensity ultrasonic field.

## 4. Conclusions

In this work, SPI was conjugated with CP and AP via traditional wet heating or ultrasound treatment. Ultrasound treatment at a power of 450 W and a temperature of 70 ℃ significantly accelerated the conjugation processes between SPI and pectin samples and led to much greater grafting extents compared to the traditional wet heating. However, it took a longer time for SPI-AP conjugates to achieve a desirable DG when compared to the SPI-CP conjugates, possibly attributed to the larger molecular weight and the more flexible structure of AP. Considering both productivity and energy efficiency, the ultrasound durations for preparing the SPI-CP and SPI-AP conjugates were selected at 45 min and 60 min, achieving the DG of 24.06% and

20.06%, respectively. SDS-PAGE analysis confirmed that the conjugates were formed between SPI and pectin samples. CD spectra showed that the ultrasound-assisted conjugation process led to lower contents of α-helix and β-sheet, and higher contents of random coil. Fluorescence spectra showed that the conjugates prepared with ultrasound had much less compact tertiary structures, with lower fluorescence intensities and a greater red shift. These results suggested that in addition to the grafting process, ultrasound also played a marked role in the unfolding of SPI, resulting in more favorable structures for Maillard reaction. Furthermore, the application of ultrasound to the conjugation process significantly increased the hydrophobicity and emulsifying properties of both conjugates. Despite the different structures of CP and AP and their different performance in conjugation with SPI, no significant differences were observed for the emulsifying properties of the two conjugates, indicating that both pectin samples were capable of forming excellent conjugates with ultrasound treatment.



## Declarations of interest

None.

## Acknowledgement

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**References**

Bouyer, E., Mekhloufi, G., Rosilio, V., Grossiord, J.-L., & Agnely, F. (2012). Proteins, polysaccharides, and their complexes used as stabilizers for emulsions: alternatives to synthetic surfactants in the pharmaceutical field? *International Journal of Pharmaceutics, 436* (1-2), 359-378.

Chan, S. Y., Choo, W. S., Young, D. J., & Loh, X. J. (2017). Pectin as a rheology modifier: Origin, structure, commercial production and rheology. *Carbohydrate Polymers, 161*, 118-139.

Chen, J. L., Cheng, H., Wu, D., Linhardt, R. J., Zhi, Z. J., Yan, L. F., Chen, S. G., & Ye, X. Q. (2017). Green recovery of pectic polysaccharides from citrus canning processing water. *Journal of Cleaner Production, 144*, 459-469.

Chen, L., Chen, J., Wu, K., & Yu, L. (2016). Improved low pH emulsification properties of glycated peanut protein isolate by ultrasound Maillard reaction. *Journal of Agricultural and Food Chemistry, 64* (27), 5531-5538.

Chen, W., Ma, X., Wang, W., Lv, R., Guo, M., Ding, T., Ye, X., Miao, S., & Liu, D. (2019). Preparation of modified whey protein isolate with gum acacia by ultrasound Maillard reaction. *Food Hydrocolloids, 95*, 298-307.

de Oliveira, F. C., Coimbra, J. S. d. R., de Oliveira, E. B., Zuñiga, A. D. G., & Rojas, E. E. G. (2016). Food protein-polysaccharide conjugates obtained via the Maillard reaction: A review. *Critical Reviews in Food Science and Nutrition, 56* (7), 1108-1125.

Delahaije, R. J., Gruppen, H., Giuseppin, M. L., & Wierenga, P. A. (2014). Quantitative description of the parameters affecting the adsorption behaviour of globular proteins. *Colloids and surfaces B: Biointerfaces, 123*, 199-206.

Dickinson, E., & Galazka, V. B. (1991). Emulsion stabilization by ionic and covalent complexes of β-lactoglobulin with polysaccharides. *Food Hydrocolloids, 5* (3), 281-296.

Fraeye, I., De Roeck, A., Duvetter, T., Verlent, I., Hendrickx, M., & Van Loey, A. (2007). Influence of pectin properties and processing conditions on thermal pectin degradation. *Food Chemistry, 105* (2), 555-563.

German, B., Damodaran, S., & Kinsella, J. E. (1982). Thermal dissociation and association behavior of soy proteins. *Journal of Agricultural and Food Chemistry, 30* (5), 807-811.

Guan, Y. G., Lin, H., Han, Z., Wang, J., Yu, S. J., Zeng, X. A., Liu, Y. Y., Xu, C. H., & Sun, W. W. (2010). Effects of pulsed electric field treatment on a bovine serum albumin–dextran model system, a means of promoting the Maillard reaction. *Food Chemistry, 123* (2), 275-280.

Huang, L., Ding, X., Dai, C., & Ma, H. (2017). Changes in the structure and dissociation of soybean protein isolate induced by ultrasound-assisted acid pretreatment. *Food Chemistry, 232*, 727-732.

Jiang, L., Wang, Z., Li, Y., Meng, X., Sui, X., Qi, B., & Zhou, L. (2015). Relationship between surface hydrophobicity and structure of soy protein isolate subjected to different ionic strength. *International Journal of Food Properties, 18* (5),

1059-1074.

Koda, S., Taguchi, K., & Futamura, K. (2011). Effects of frequency and a radical scavenger on ultrasonic degradation of water-soluble polymers. *Ultrasonics Sonochemistry, 18* (1), 276-281.

Lertittikul, W., Benjakul, S., & Tanaka, M. (2007). Characteristics and antioxidative activity of Maillard reaction products from a porcine plasma protein–glucose model system as influenced by pH. *Food Chemistry, 100* (2), 669-677.

Li, C., Huang, X., Peng, Q., Shan, Y., & Xue, F. (2014). Physicochemical properties of peanut protein isolate–glucomannan conjugates prepared by ultrasonic treatment. *Ultrasonics Sonochemistry, 21* (5), 1722-1727.

Ma, X., Chen, W., Yan, T., Wang, D., Hou, F., Miao, S., & Liu, D. (2020). Comparison of citrus pectin and apple pectin in conjugation with soy protein isolate (SPI) under controlled dry-heating conditions. *Food Chemistry, 309*, 125501.

Ma, X., Yan, T., Hou, F., Chen, W., Miao, S., & Liu, D. (2019). Formation of soy protein isolate (SPI)-citrus pectin (CP) electrostatic complexes under a high-intensity ultrasonic field: Linking the enhanced emulsifying properties to physicochemical and structural properties. *Ultrasonics Sonochemistry, 59*, 104748.

Martínez, K. D., Sanchez, C. C., Ruíz-Henestrosa, V. P., Patino, J. M. R., & Pilosof, A. M. (2007). Effect of limited hydrolysis of soy protein on the interactions with polysaccharides at the air–water interface. *Food Hydrocolloids, 21* (5-6),

589 813-822.

590 Mu, L., Zhao, M., Yang, B., Zhao, H., Cui, C., & Zhao, Q. (2010). Effect of ultrasonic
591 treatment on the graft reaction between soy protein isolate and gum acacia and on
592 the physicochemical properties of conjugates. *Journal of Agricultural and Food*
593 *Chemistry, 58* (7), 4494-4499.

594 Nooshkam, M., & Varidi, M. (2019). Maillard conjugate-based delivery systems for
595 the encapsulation, protection, and controlled release of nutraceuticals and food
596 bioactive ingredients: A review. *Food Hydrocolloids*, 105389.

597 Pearce, K. N., & Kinsella, J. E. (1978). Emulsifying properties of proteins: evaluation
598 of a turbidimetric technique. *Journal of Agricultural and Food Chemistry, 26* (3),
599 716–723.

600 Petruccelli, S., & Anon, M. C. (1995). Soy protein isolate components and their
601 interactions. *Journal of Agricultural and Food Chemistry, 43* (7), 1762-1767.

602 Portenlanger, G., Heusinger, H., Coimbra, M. A., Barros, A., Barros, M., Rutledge, D.
603 N., & Delgadillo, I. (1997). The influence of frequency on the mechanical and
604 radical effects for the ultrasonic degradation of dextranes. *Ultrasonics*
605 *Sonochemistry, 4* (2), 127-130.

606 Qu, W., Zhang, X., Chen, W., Wang, Z., He, R., & Ma, H. (2018). Effects of ultrasonic
607 and graft treatments on grafting degree, structure, functionality, and digestibility
608 of rapeseed protein isolate-dextran conjugates. *Ultrasonics Sonochemistry, 42*,
609 250-259.

610 Resendiz-Vazquez, J., Ulloa, J., Urías-Silvas, J., Bautista-Rosales, P.,

Ramírez-Ramírez, J., Rosas-Ulloa, P., & González-Torres, L. (2017). Effect of high-intensity ultrasound on the technofunctional properties and structure of jackfruit (Artocarpus heterophyllus) seed protein isolate. *Ultrasonics Sonochemistry, 37*, 436-444.

Schwass, D. E., & Finley, J. W. (1984). Heat and alkaline damage to proteins: racemization and lysinoalanine formation. *Journal of Agricultural and Food Chemistry, 32* (6), 1377-1382.

Sheng, L., Su, P., Han, K., Chen, J., Cao, A., Zhang, Z., Jin, Y., & Ma, M. (2017). Synthesis and structural characterization of lysozyme–pullulan conjugates obtained by the Maillard reaction. *Food Hydrocolloids, 71*, 1-7.

Sheng, L., Tang, G., Wang, Q., Zou, J., Ma, M., & Huang, X. (2020). Molecular characteristics and foaming properties of ovalbumin-pullulan conjugates through the Maillard reaction. *Food Hydrocolloids, 100*, 105384.

Silván, J. M., Assar, S. H., Srey, C., Del Castillo, M. D., & Ames, J. M. (2011). Control of the Maillard reaction by ferulic acid. *Food Chemistry, 128* (1), 208-213.

Subhedar, P. B., & Gogate, P. R. (2014). Enhancing the activity of cellulase enzyme using ultrasonic irradiations. *Journal of Molecular Catalysis B: Enzymatic, 101*, 108-114.

Wang, X. S., Tang, C. H., Li, B. S., Yang, X. Q., Li, L., & Ma, C. Y. (2008). Effects of high-pressure treatment on some physicochemical and functional properties of soy protein isolates. *Food Hydrocolloids, 22* (4), 560-567.

633 Wei, C., He, P., He, L., Ye, X., Cheng, J., Wang, Y., Li, W., & Liu, Y. (2018). Structure
634 characterization and biological activities of a pectic polysaccharide from cupule
635 of Castanea henryi. *International Journal of Biological Macromolecules, 109*,
636 65-75.
637 Wei, C. Y., He, P. F., He, L., Ye, X. Q., Cheng, J. W., Wang, Y. B., Li, W. Q., & Liu, Y.
638 (2018). Structure characterization and biological activities of a pectic
639 polysaccharide from cupule of Castanea henryi. *International Journal of*
640 *Biological Macromolecules, 109*, 65-75.
641 Xu, C. H., Yu, S. J., Yang, X. Q., Qi, J. R., Lin, H., & Zhao, Z. G. (2010). Emulsifying
642 properties and structural characteristics of β - conglycinin and dextran
643 conjugates synthesised in a pressurised liquid system. *International Journal of*
644 *Food Science & Technology, 45* (5), 995-1001.
645 Yang, Y., Cui, S., Gong, J., Miller, S. S., Wang, Q., & Hua, Y. (2015). Stability of
646 citral in oil-in-water emulsions protected by a soy protein–polysaccharide
647 Maillard reaction product. *Food Research International, 69*, 357-363.
648 Yu, H., Seow, Y.-X., Ong, P. K., & Zhou, W. (2017). Effects of high-intensity
649 ultrasound on Maillard reaction in a model system of d-xylose and l-lysine.
650 *Ultrasonics Sonochemistry, 34*, 154-163.
651 Zhang, Q., Li, L., Lan, Q., Li, M., Wu, D., Chen, H., Liu, Y., Lin, D., Qin, W., &
652 Zhang, Z. (2019). Protein glycosylation: a promising way to modify the
653 functional properties and extend the application in food system. *Critical Reviews*
654 *in Food Science and Nutrition, 59* (15), 2506-2533.

655 Zhu, Z., Zhu, W., Yi, J., Liu, N., Cao, Y., Lu, J., Decker, E. A., & McClements, D. J.
656 (2018). Effects of sonication on the physicochemical and functional properties of
657 walnut protein isolate. *Food Research International, 106,* 853-861.

658

## Figure captions

**Fig. 1** Effects of pH on the DG of (A) SPI-CP conjugates (70 ℃, 24 h) and (B) SPI-AP conjugates (70 ℃, 24 h); and the morphology of (C) SPI-CP conjugates and (D) SPI-AP conjugates.

**Fig. 2** Effects of temperature on the DG of (A) SPI-CP conjugates (pH 10.0, 24 h) and (B) SPI-AP conjugates (pH 10.0, 24 h); and the morphology of (C) SPI-CP conjugates and (D) SPI-AP conjugates.

**Fig. 3** Effects of ultrasound power on the DG of SPI-pectin conjugates (pH 10.0, 70 ℃, 20 min).

**Fig. 4** Effects of ultrasound temperature on the DG of SPI-pectin conjugates (450 W, pH 10.0, 20 min).

**Fig. 5** SDS-PAGE of SPI, mixtures of SPI and pectin, and SPI-pectin conjugates prepared by traditional wet heating and ultrasound treatment. Lane M, molecular weight markers (kDa); Lane 1, SPI; Lane 2, mixtures of SPI and CP; Lane 3, mixtures of SPI and AP; Lane 4, SPI-CP conjugates prepared by traditional wet heating (70 ℃, 24 h); Lane 5, SPI-AP conjugates prepared by traditional wet heating (70 ℃, 24 h); Lane 6, SPI-CP conjugates prepared with ultrasound treatment (450 W, 70 ℃, 45 min); Lane 7, SPI-AP conjugates prepared with ultrasound treatment (450 W, 70 ℃, 60 min).

**Fig. 6** CD spectra of SPI, mixtures of SPI and pectin, and SPI-pectin conjugates prepared by traditional wet heating (70 ℃, 24 h) and ultrasound treatment (450 W, 70 ℃; 45 min for the SPI-CP conjugates and 60 min for the SPI-AP conjugates).

**Fig. 7** Intrinsic fluorescence spectra of SPI, mixtures of SPI and pectin, and SPI-pectin conjugates prepared by traditional wet heating (70 ℃, 24 h) and

ultrasound treatment (450 W, 70 ℃; 45 min for the SPI-CP conjugates and 60 min for the SPI-AP conjugates).

**Fig. 8** $H_0$ of SPI, mixtures of SPI and pectin, and SPI-pectin conjugates prepared by traditional wet heating (70 ℃, 24 h) and ultrasound treatment (450 W, 70 ℃; 45 min for the SPI-CP conjugates and 60 min for the SPI-AP conjugates).

**Fig. 9** The EAI and ESI of SPI, mixtures of SPI and pectin, and SPI-pectin conjugates prepared by traditional wet heating (70 ℃, 24 h) and ultrasound treatment (450 W, 70 ℃; 45 min for the SPI-CP conjugates and 60 min for the SPI-AP conjugates).

# Tables

**Table 1** Conformational parameters of pectin samples with and without ultrasound treatment (450 W, 70 ℃).

| Parameters | CP | CP (with ultrasound for 45 min) | AP | AP (with ultrasound for 60 min) |
|---|---|---|---|---|
| $M_w$ (kDa) | 478.00 ± 0.60 $^{c}$ | 246.40 ± 1.80 $^{d}$ | 1050.50 ± 5.50 $^{a}$ | 575.50 ± 0.80 $^{b}$ |
| $M_n$ (kDa) | 188.00 ± 0.10 $^{c}$ | 115.60 ± 1.50 $^{d}$ | 314.10 ± 1.10 $^{a}$ | 249.00 ± 4.30 $^{b}$ |
| Polydispersity | 2.54 ± 0.00 $^{b}$ | 2.13 ± 0.04 $^{d}$ | 3.34 ± 0.03 $^{a}$ | 2.31 ± 0.04 $^{c}$ |
| $R_z$ (nm) | 36.80 ± 0.00 $^{b,c}$ | 34.60 ± 0.20 $^{c}$ | 40.70 ± 0.50 $^{a}$ | 38.00 ±1.20 $^{b}$ |
| α | 0.84 ± 0.00 $^{b}$ | 0.89 ± 0.01 $^{a}$ | 0.71 ± 0.03 $^{d}$ | 0.79 ± 0.00 $^{c}$ |

*Note:* values with different italic superscript letters (a–d) in the same column within each pectin indicate significant differences as estimated by Duncan's multiple range test ($P < 0.05$).

**Table 2** The DG of SPI-CP/AP conjugates prepared by wet heating and ultrasound treatment (450 W) at pH 10.0 and 70 ℃.

| Samples | Wet heating | | With ultrasound | |
|---|---|---|---|---|
| | Time (h) | DG (%) | Time (min) | DG (%) |
| SPI-CP conjugates | 4 | 1.92 ± 0.09 $^{f}$ | 15 | 10.52 ± 0.57 $^{f}$ |
| | 8 | 2.75 ± 0.18 $^{e}$ | 30 | 17.29 ± 0.16 $^{e}$ |
| | 12 | 3.85 ± 0.37 $^{d}$ | 45 | 24.06 ± 0.08 $^{d}$ |
| | 16 | 5.96 ± 0.27 $^{c}$ | 60 | 24.55 ± 0.41 $^{c,d}$ |
| | 20 | 6.78 ± 0.00 $^{b}$ | 75 | 25.12 ± 0.16 $^{c,d}$ |
| | 24 | 8.25 ± 0.18 $^{a}$ | 90 | 25.53 ± 0.57 $^{b,c}$ |
| | | | 105 | 26.51 ± 0.08 $^{a,b}$ |
| | | | 120 | 26.67 ± 0.08 $^{a}$ |
| SPI-AP conjugates | 4 | 1.92 ± 0.64 $^{c}$ | 15 | 5.55 ± 0.49 $^{f}$ |
| | 8 | 3.12 ± 0.18 $^{c}$ | 30 | 11.74 ± 0.33 $^{e}$ |
| | 12 | 4.58 ± 0.37 $^{b}$ | 45 | 16.64 ± 0.33 $^{d}$ |
| | 16 | 5.13 ± 0.18 $^{b}$ | 60 | 20.06 ± 0.49 $^{c}$ |
| | 20 | 5.50 ± 0.37 $^{a,b}$ | 75 | 20.55 ± 0.16 $^{b,c}$ |
| | 24 | 6.60 ± 0.37 $^{a}$ | 90 | 21.37 ± 0.49 $^{b}$ |
| | | | 105 | 22.76 ± 0.24 $^{a}$ |
| | | | 120 | 23.00 ± 0.16 $^{a}$ |

*Note:* values with different italic superscript letters (a–d) in the same column within each conjugate sample indicate significant differences as estimated by Duncan's multiple range test ($P < 0.05$).

**Table 3** Secondary structures of SPI, mixtures of SPI and pectin samples, and SPI-pectin conjugates prepared by traditional wet heating (70 ℃, 24 h) and ultrasound treatments (450 W, 70 ℃; 45 min for the SPI-CP conjugates and 60 min for the SPI-AP conjugates).

| | α-Helix (%) | β-Sheet (%) | Turn (%) | Random coil (%) |
|---|---|---|---|---|
| SPI | 6.70 | 38.30 | 22.10 | 32.90 |
| Mixtures of SPI and CP | 6.70 | 38.30 | 22.10 | 32.90 |
| Mixtures of SPI and AP | 6.70 | 38.30 | 22.10 | 32.90 |
| SPI-CP conjugates (wet heating) | 4.60 | 38.00 | 19.40 | 38.00 |
| SPI-AP conjugates (wet heating) | 4.41 | 37.68 | 19.21 | 38.70 |
| SPI-CP conjugates (with ultrasound) | 2.60 | 35.76 | 19.98 | 41.66 |
| SPI-AP conjugates (with ultrasound) | 3.70 | 35.97 | 20.37 | 39.96 |

**Fig. 1**

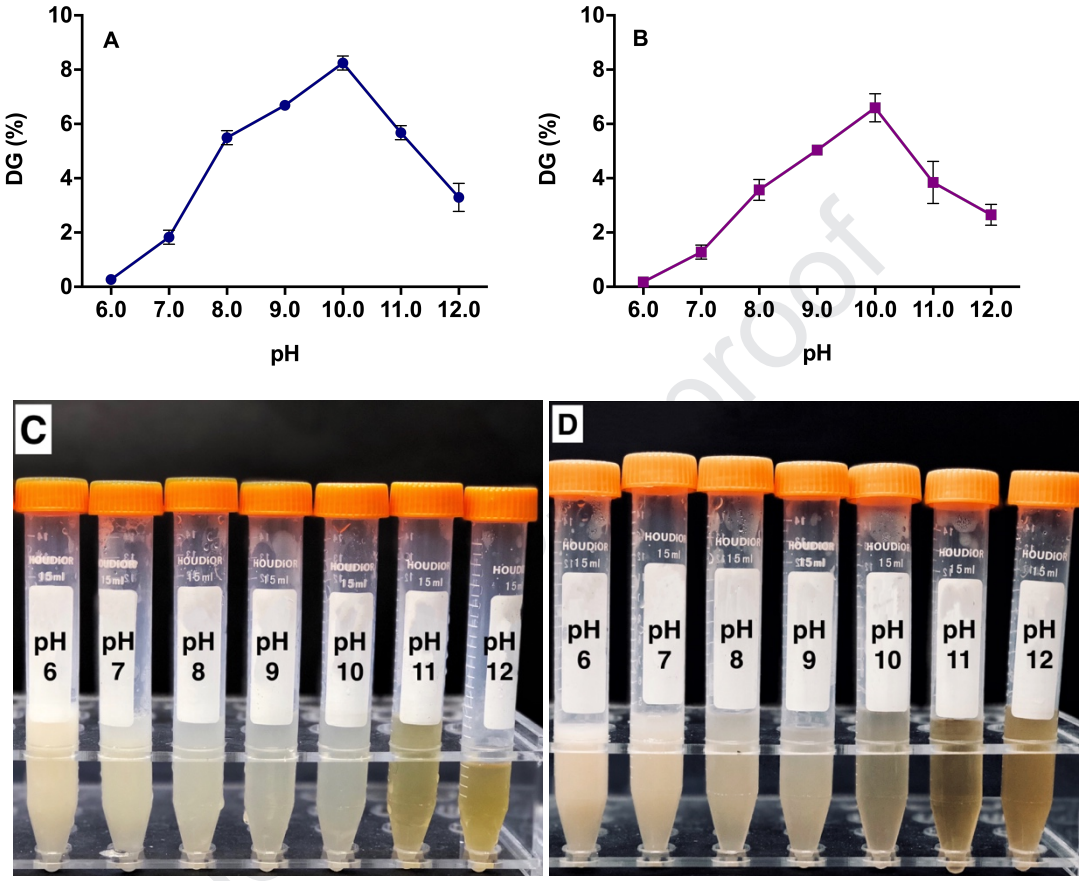


**Fig. 2**

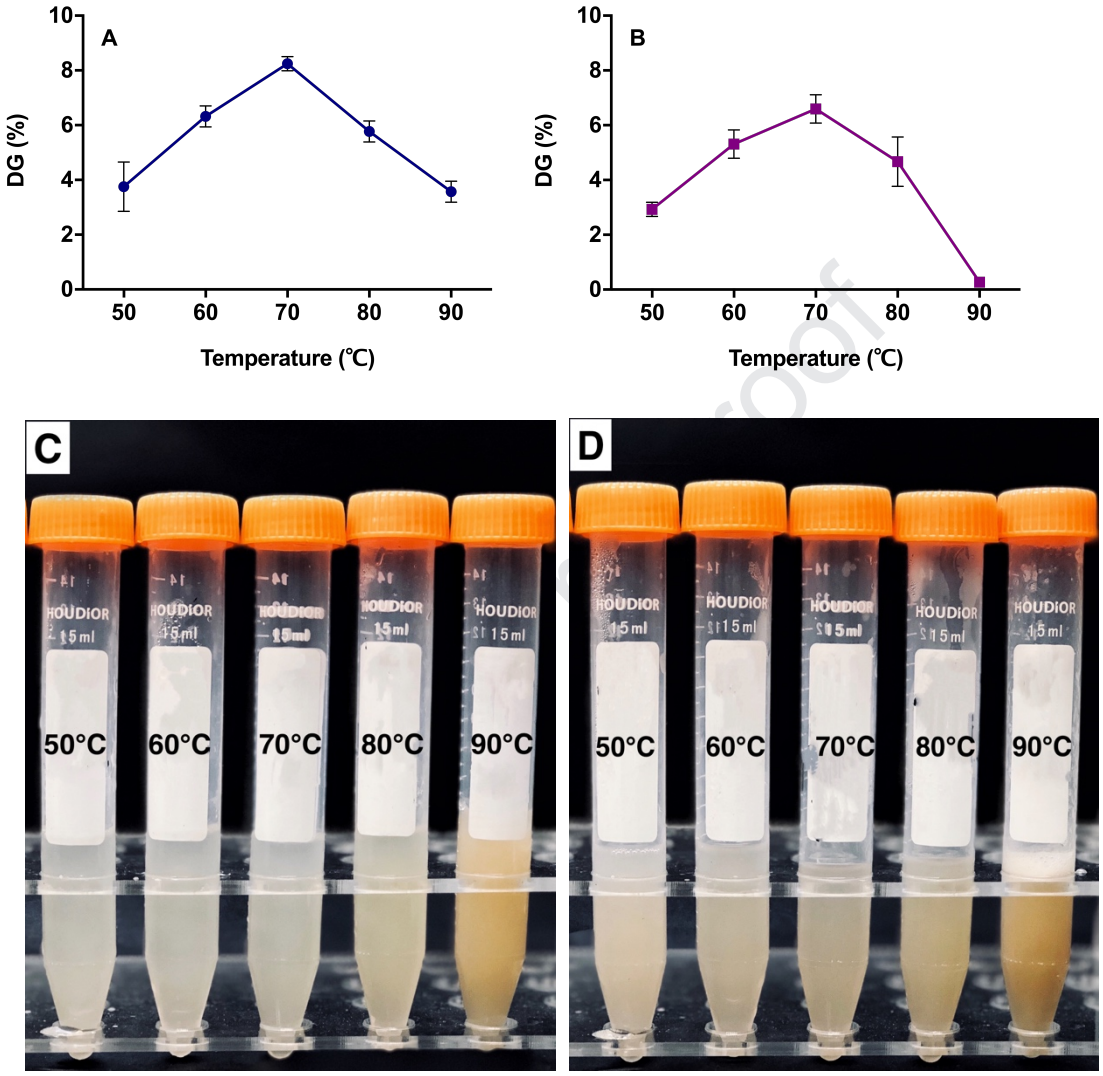
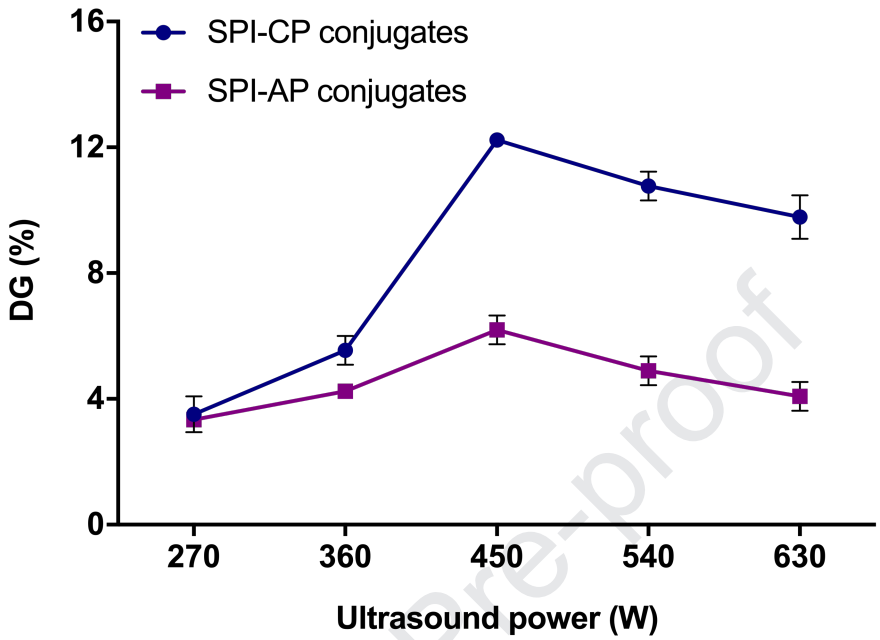


**Fig. 3**



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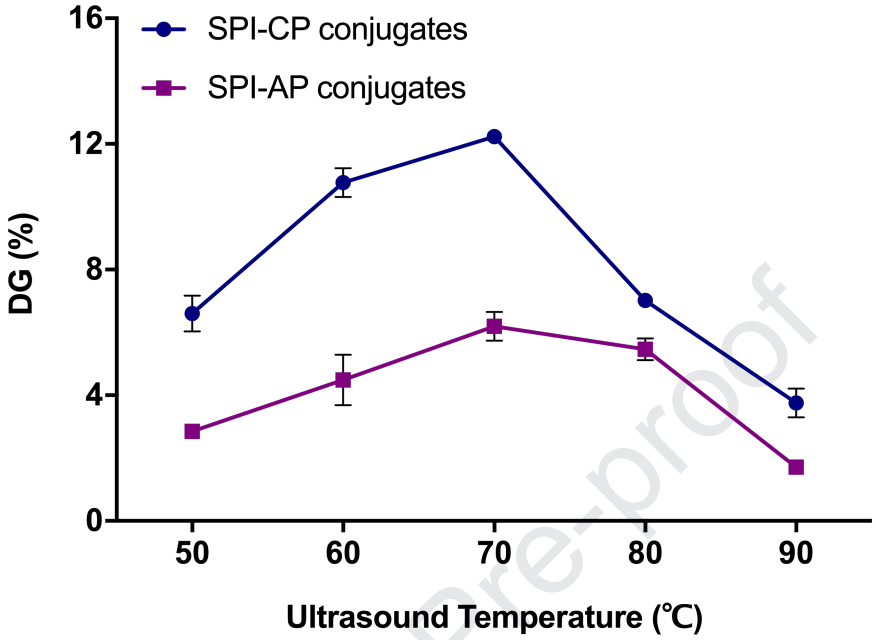
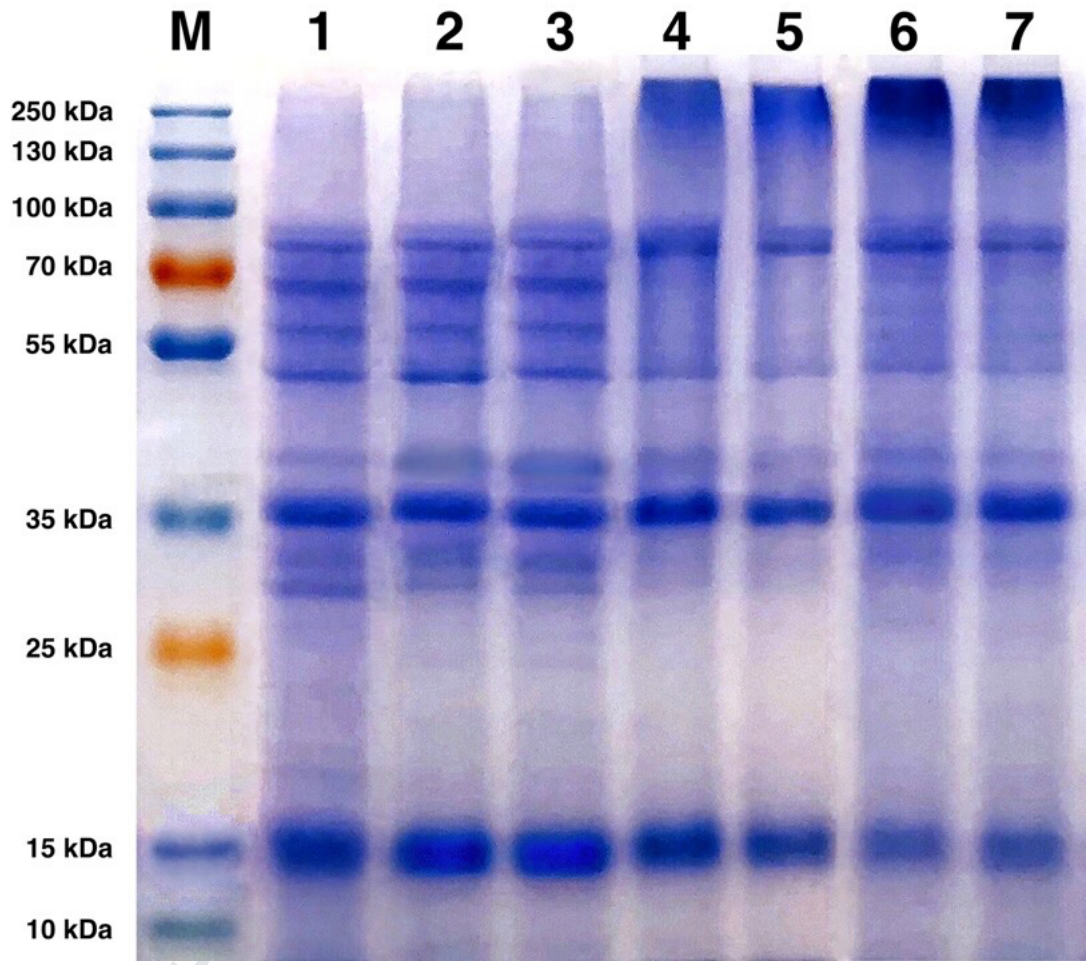


**Fig. 5**


M
1
2
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250 kDa
130 kDa
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70 kDa
55 kDa
35 kDa
25 kDa
15 kDa
10 kDa

**Fig. 6**

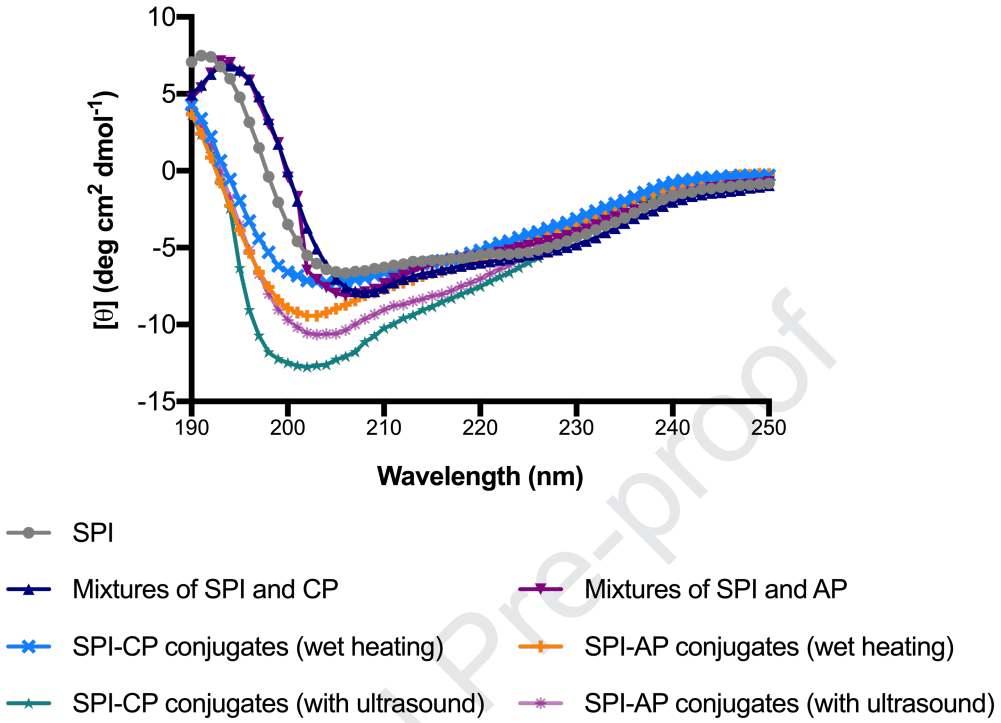


**Fig. 7**

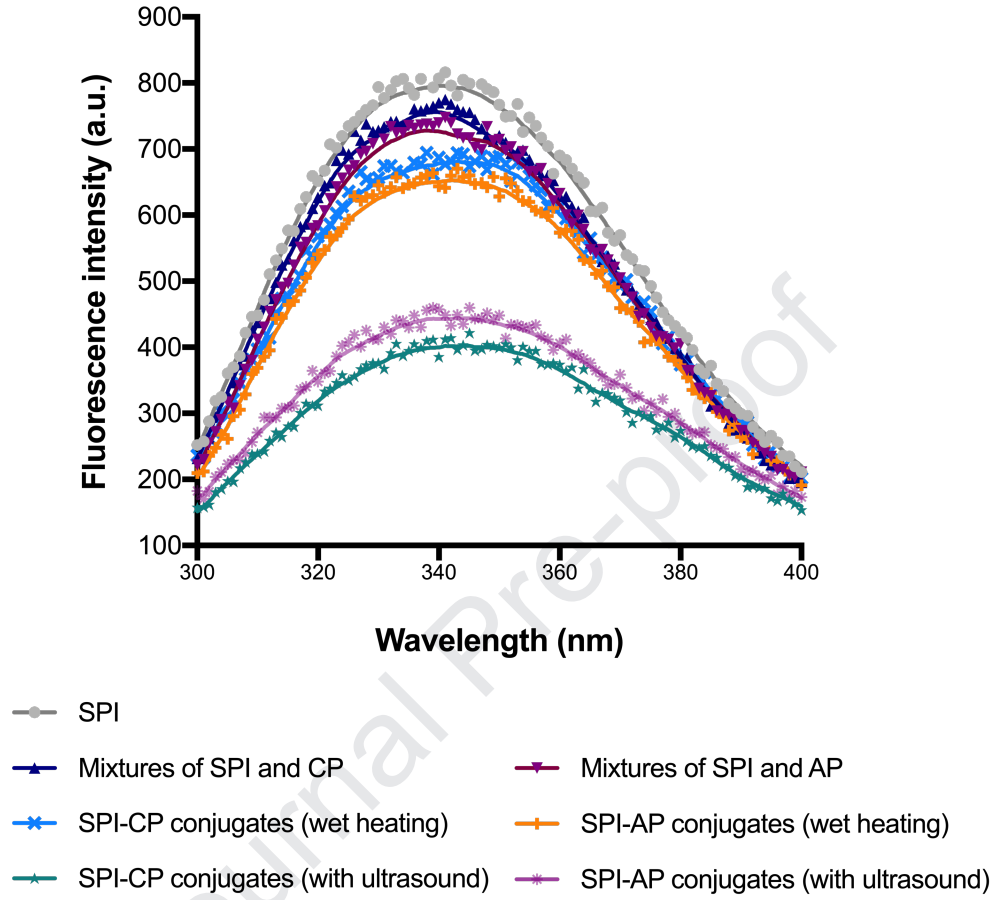


**Fig. 8**

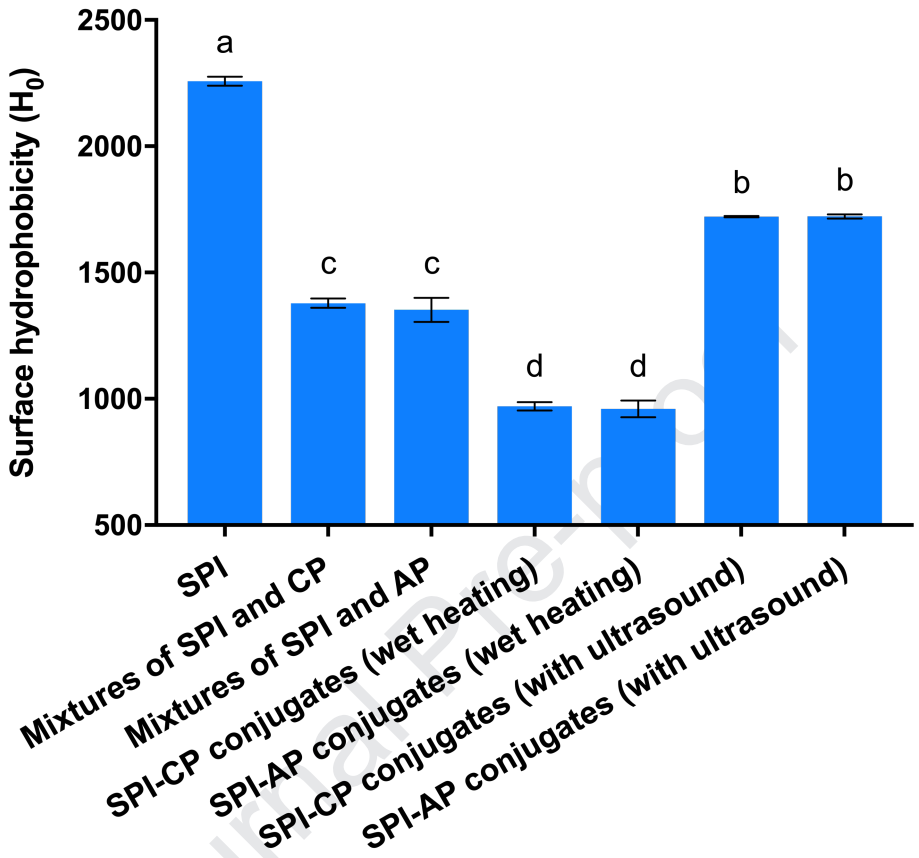
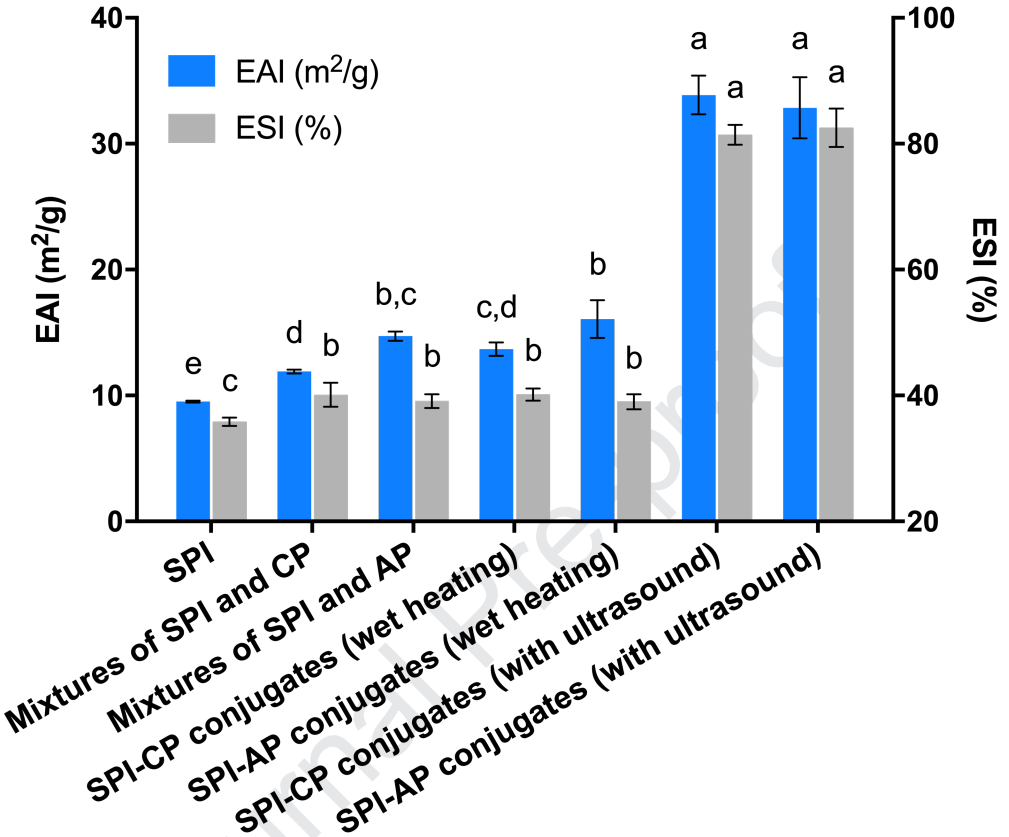


**Fig. 9**



# Highlights

1. Ultrasound enhanced the conjugation process resulting in higher grafting extents.
2. Formation of the conjugates was confirmed by SDS-PAGE analysis.
3. The ultrasound-assisted conjugation process led to looser protein structures.
4. Ultrasound increased the $H_0$ and the emulsifying properties of the conjugates.
5. Both pectin samples are capable of forming excellent conjugates by ultrasound.

**Declaration of interests**

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: