Food Hydrocolloids 125 (2022) 107423

Contents lists available at ScienceDirect

Food Hydrocolloids

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

Sinapic acid impacts the emulsifying properties of rapeseed proteins at acidic pH

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ARTICLE INFO

Keywords:
Plant proteins
Rapeseeds
Sinapic acid
Purification
Emulsions
Interfacial rheology

ABSTRACT

Extensive purification of plant proteins is probably not a prerequisite for their emulsifying properties, however, details of the interfacial stabilization mechanism of less purified protein extracts are not sufficiently known. Phenolic compounds present in less purified plant protein extracts can interact with proteins, inducing protein aggregation, impeding their interfacial properties. Here, we show that when a rapeseed protein mixture (RPM) containing 40 wt% proteins and 6 wt% sinapic acid is used to form oil-in-water emulsions (10.0 wt% oil) at pH 3.8 and at different protein concentrations (0.2–1.5 wt%), emulsion droplets of 2.0–0.6 μm are formed and large protein aggregates are randomly attached to the droplet interface. By reducing the sinapic acid content to 2.5 wt % to produce a rapeseed protein concentrate (RPC) (65 wt% proteins), smaller emulsion droplets are formed (0.4–0.5 μm) at the same protein concentrations, and no large proteins aggregates are present at the droplet interface. According to our findings, in both RPM- and RPC-stabilized emulsions, napins primarily adsorb at the interface, while cruciferins form a secondary layer which protects the droplets against coalescence during homogenization. However, in RPM, the higher sinapic acid content possibly induces aggregation of cruciferins, which hinders the formation of a sufficient secondary layer. As a result, during homogenization, the colliding droplets coalesce, resulting in emulsions with larger droplets. Our findings show that sinapic acid affects the emulsification mechanism of rapeseed proteins at acidic pH, and recommend that plant protein purification might be necessary for the application of plant proteins in emulsion food products.

1. Introduction

The effect of protein purification on the functionality of plant proteins is currently a topic of intense research in food science, both in academia and in industry. To date, several studies using various plant protein sources (Fuhrmeister & Meuser, 2003; Geerts, Nikiforidis, van der Goot, & van der Padt, 2017; Karefyllakis, Octaviana, van der Goot, & Nikiforidis, 2019; Kornet et al., 2021; Ntone et al., 2021; Peng, Kersten, Kyriakopoulou, & van der Goot, 2020; Sridharan, Meinders, Bitter, & Nikiforidis, 2020a; (Yang, Faber, et al., 2021)) have shown that extensive purification of plant proteins is not a prerequisite for functionality, such as interfacial and emulsifying properties. Most of these studies have postulated that the functionality of less purified protein extracts is linked to the avoidance of extensive process-induced alterations in the physicochemical properties of the proteins.

Although the results of the above studies are promising for the use of less purified plant protein extracts as emulsifiers in food systems, there is a lack of understanding on the exact emulsification mechanism. The multicomponent nature of the less purified protein extracts includes various types of proteins co-existing with other non-protein molecules, which under various system conditions (e.g. pH) can impact the interactions between the molecules, the adsorption of proteins, and the interfacial stabilization.

In our previous research on rapeseed proteins, we showed that at pH 7, a less purified rapeseed protein extract (40 wt% protein), containing both storage proteins (napins and cruciferins) and non-protein

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https://doi.org/10.1016/j.foodhyd.2021.107423
Received 22 July 2021; Received in revised form 24 October 2021; Accepted 29 November 2021
Available online 30 November 2021

molecules (i.e. sinapic acid) can form stable oil-in-water emulsions (Ntone et al., 2021). At this pH, napins dominate the oil/water interface, due to their small size (estimated radius of 1.7 nm) and their concentrated hydrophobic domains on one side of the protein structure, resembling amphiphilic Janus particles. Cruciferins due to their larger size (estimated radius of 4.4 nm) and the wide distribution of their hydrophobic domains over their hexameric structure, form a secondary interfacial layer, providing stability to the emulsion droplets against coalescence (Ntone et al., 2021). Under these pH conditions, the free sinapic acid present in the protein extracts does not impact the interfacial properties of the proteins (Ntone et al., 2021; J.; Yang et al., 2021).

However, at pH 3.8, in which emulsion food products like salad dressings are produced, the emulsification mechanism of the less purified rapeseed protein extracts might differ, as pH can affect the interactions between proteins with sinapic acid, and subsequently the interfacial properties of the proteins (Ozdal, Capanoglu, & Altay, 2013; Rawel, Meidtner, & Kroll, 2005a). At acidic pH conditions, the free sinapic acid present in the less purified rapeseed protein extracts can bind to proteins through non-covalent bonds (Cao & Xiong, 2017; Ozdal et al., 2013; J. Yang, Lamochi Roozalipour et al., 2021). The protein-phenol interactions can alter protein hydrophobicity, structure and size (Karefyllakis, Altunkaya, Berton-Carabin, van der Goot, & Nikiforidis, 2017; Ozdal et al., 2013; Rawel et al., 2005a) affecting the protein interfacial properties (Bock, Steinhäuser, & Drusch, 2021; J.; Yang, Lamochi Roozalipour et al., 2021). The protein-phenol complexation has been reported to lead to protein-protein interactions through hydrophobic attractive forces and the formation of aggregates. As a result, the absorption rate of the proteins decreased and the same holds for the lateral interactions on the interface (Bock et al., 2021; J.; Yang, Lamochi Roozalipour et al., 2021). Hence, it can be suggested that potential interactions of proteins with sinapic acid as affected by pH can impact the emulsification mechanism of less purified rapeseed protein extracts.

This paper aims to establish the emulsifying mechanism of rapeseed protein extracts of different protein purities at pH 3.8. A less purified rapeseed protein mixture (RPM) containing 40 wt% proteins and 6 wt% free sinapic acid, as well as 12 wt% oleosomes, 8 wt% ash and 34 wt% carbohydrates was used as an emulsifier. RPM was further purified using diafiltration to obtain and a rapeseed protein concentrate (RPC) containing 65 wt% proteins, where sinapic acid is reduced to 2.5 wt%. The oleosome content in RPC increased to 15 wt%, while the ash and carbohydrate content reduced to 2.5 wt% and 14 wt% respectively (Ntone, Bitter, & Nikiforidis, 2020). Identifying the potential effect of non-protein compounds, such as phenols, on the interfacial stabilization mechanism of proteins at acidic pH, is essential to evaluate the necessity of protein purification for structuring plant-based food emulsions.

2. Materials and methods

2.1. Materials

Untreated Alize rapeseeds were used as raw material to extract the rapeseed protein extracts. The seeds were stored at −18°C until use. All chemical reagents were analytical grade and purchased from Sigma Aldrich (Zwijndrecht, Netherlands).

The rapeseed oil used in the experiments was kindly provided by Danone. The oil was stored in containers under nitrogen at −15°C for maximum of six months.

2.2. Extraction of rapeseed proteins

The rapeseed protein extracts with a different degree of protein purity-rapeseed protein mixture (RPM) and rapeseed protein concentrate (RPC)- were extracted using our previously developed extraction process (Ntone et al., 2020). Briefly, dehulled rapeseed particles were dispersed in deionized water (1:8 w/w) and kept at room temperature (around 20°C) for 4 h under continuous stirring using a head stirrer (EUROSTAR 60 digital, IKA, Staufen, Germany). The pH of the dispersion was maintained at 9.0 during the soaking time by adding NaOH (0.5 M). Afterwards, the dispersion was blended with a kitchen blender (HR2093, Philips, Netherlands). at maximum speed for 2 min. To separate the solids from the liquid phase, the slurry was filtered by using a twin-screw press (Angelia 7500, Angel Juicer, Naarden, The Netherlands). The filtrate was filtrated, the pH was re-adjusted to pH 9.0, and centrifuged (10,000 g, 30 min, 4°C) (Sorvall Legend XFR, ThermoFisher Scientific, Waltham, MA, USA). A cream layer (oleosome-rich), a serum (rapeseed protein mixture (RPM)) and a pellet (fiber-rich) were collected. The cream layer was carefully removed with a spatula, and the liquid phase (RPM) was filtered through a sieve to remove any floating cream pieces while the pellet was discarded. To obtain the rapeseed protein concentrate (RPC), RPM was first concentrated by ultrafiltration and then diluted 1:1 (v/v) with NaCl (0.08 M) to avoid protein precipitation. Thereafter, it was pumped through two coupled diafiltration cassettes (cut-off 5 kDa; membrane area 0.2 m^2) (Hydrosart, Sartorius, Göttingen, Germany) for 6 cycles until a transparent filtrate was obtained. The inlet and outlet pressure were adjusted to 1.4 and 0.6 bar, respectively. The conversion ratio (CR) was set at 35% (below 40%) to minimize membrane blocking. In the last cycle, we used deionized water to remove any remaining salt. Finally, RPM and RPC were freeze-dried (Alpha 2–4 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and stored at −18°C until further use.

2.3. Isolation of napins

Napins were isolated using a previously reported the method (Ntone et al., 2021). RPM was first diafiltrated through two coupled 100 kDa cut-off diafiltration cassettes (Hydrosart, Sartorius, Göttingen, Germany) to separate cruciferins (300 kDa) and other high molecular weight non-protein compounds. The filtrate (<100 kDa) containing napins was collected and concentrated by ultrafiltration and then diluted with NaCl (0.08 M) at a ratio 1:1 to avoid protein precipitation. The diluted filtrate was pumped through another diafiltration system (two coupled diafiltration cassettes; 5 kDa cut-off; membrane area 0.2 m^2) (Hydrosart, Sartorius, Göttingen, Germany) for 6 cycles to remove small molecular weight compounds. In the last cycle we used deionized water to remove the remaining salt. The retentate (>5 kDa) containing napins was freeze-dried (Alpha 2–4 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and stored at −18°C until further use.

2.4. Protein content analysis

The protein content of all the protein extracts was determined on dry-matter weight basis using the Dumas method (FlashEA 1112 Series, Thermo Scientific, Waltham, Massachusetts, US); We used D-methionine (≥98%, Sigma Aldrich, Darmstadt, Germany) as a standard and as a control. Cellulose (Sigma Aldrich, Darmstadt, Germany) served as blank. A nitrogen–protein conversion factor of 5.7 (calculated based on amino acid sequence) was chosen.

2.5. Emulsion preparation

RPM and RPC were used to stabilize 10.0 wt% oil-in-water emulsions. The protein extracts were dispersed in deionized water, standardized at different protein concentrations (0.2–1.5 wt%). The pH was adjusted initially to pH 3.8, and then was checked after 1 h and at the end of the solubilization time (2 h) and readjusted to pH 3.8 if necessary, before emulsification. The dispersions were stirred for 2 h at room temperature (around 20°C) with a magnetic stirrer at 300 rpm (2mag magnetic e motion, 2mag AG, Munich, Germany) to ensure hydration and solubilization of the proteins. Subsequently, we applied a pre-

homogenization step, where the dispersions were sheared using a disperser (Ultra-Turrax, IKA®, Staufen, Germany) at 8000 rpm for 30 s. Next, 10.0 wt% rapeseed oil was slowly added to the dispersion and sheared for 1 min at 10,000 rpm. The formed coarse emulsions were further processed with high-pressure homogenizer (GEA®, Niro Soavi NS 1001 L, Parma, Italy) 5 times at 250 bars.

2.5.1. Addition of sinapic acid to RPC before emulsification

Sinapic acid powder (Sinapic acid ≥98%, Sigma Aldrich, Darmstadt, Germany) was added in RPC protein dispersions standardized at 0.7 wt % protein, prior to protein solubilization. In order to achieve the same amount of sinapic acid in RPC emulsions as in RPM emulsions at 0.7 wt% protein concentration, 80 mg of sinapic acid powder were added to the RPC protein dispersion. The pH was adjusted to pH 9 and kept for 30 min under stirring with magnetic stirrer at 300 rpm (2mag magnetic e motion, 2mag AG, Munich, Germany), to allow solubilization of sinapic acid in the aqueous phase. Thereafter, the pH was adjusted to pH 3.8 and kept for another 2 h. Lastly, oil was added to prepare the emulsions as described in 2.5.

2.6. Emulsion characterization

2.6.1. Particle size distribution

The particle size distribution of the emulsions was measured with laser light scattering using a Mastersizer-2000 (Malvern Panalytical, Malvern, UK). The emulsions were diluted 10× before measurement. To measure the droplet size of the emulsions at the system conditions applied (pH 3.8) (droplet aggregate size) the pH of the dilution medium was adjusted to pH 3.8 prior to measurements. To determine the individual droplet size aliquots were diluted in 1 wt% SDS solution (1:1 v/v) prior to the measurements. The dilution medium (water) for these measurements was kept at pH values around 7, where also higher repulsive forces are induced, allowing to determine the actual individual droplet size instead of the droplet aggregates.

The refractive index was set at 1.47 for rapeseed oil and the density at 0.97 g/cc. The results were expressed as surface ($d_{3,2}$) and volume ($d_{4,3}$) mean diameter using:

$$d_{3,2} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2} \quad (1)$$

$$d_{4,3} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3} \quad (2)$$

Where n_i is the number of droplets with a diameter of d_i

2.6.2. Confocal laser scanning microscopy

The microstructure of the emulsions stabilized by RPM and RPC was visualized using Confocal laser Scanning Microscopy (CLSM) (Leica TCS CP5 X, Leica Microsystems, Wetzlar, Germany). Nile red (1 mg/mL in ethanol) and Fast green (1 mg/mL in deionized water) were used to stain the oil and protein respectively, at a sample to dye ratio of 1:200. Nile red was excited at 488 nm and the emission was captured between 500 and 600 nm and Fast green was excited at 633 nm and the emission was captured between 650 and 750 nm. The dyes were excited and emitted in a sequential mode using white light laser.

2.6.3. Interfacial protein composition

To qualitatively determine the type of proteins adsorbed at the interface electrophoresis was used (SDS-PAGE). The emulsions (10 g) were centrifuged (10,000 g, 30 min, 4°C) in 15 mL tubes to remove the unadsorbed proteins. To eliminate any interactions of the non-adsorbed proteins with the interface that could interfere with the results, the emulsions were centrifuged at two pH conditions; 1) at pH 3.8 (control) and 2) at pH 9, where the repulsive electrostatic forces between protein molecules become larger than at pH 3.8, which could eliminate possible interactions of the unadsorbed proteins with the primary layer.

After centrifugation a cream layer (interface) and a serum phase (continuous phase) were obtained. The serum was drained by making holes at the bottom of the tube. The cream was collected with a spatula and resuspended (1:10 w/w) in deionized water and stirred (200 rpm, 1 h, room temperature (around 20 °C)) (2mag magnetic e motion, 2mag AG, Munich, Germany). The centrifugation step was repeated one more time under the same conditions and the cream was collected and further analyzed with SDS-PAGE.

The resulting samples (interface) were mixed with sample buffer (NuPAGE LDS, ThermoFisher, Landsmeer, the Netherlands) and deionized water to achieve a final protein concentration of 1.0 mg/mL. The prepared samples (1 mL) were further heated for 15 min at 70°C in a heating block and centrifuged (1 min, 2000 g, 20°C) to remove any insoluble material. No oil removal was necessary prior to the SDS-PAGE analysis as the addition of the reagents for sample preparation break the emulsion droplets. During the small centrifugation step after the sample preparation the oil layer is separated in the top of Eppendorf tube, which is not collected and placed on the gel. Next, the gel (NuPAGE Novex 4–12% Bis-Tris Gel, ThermoFisher, Landsmeer, the Netherlands) was assembled and MES running buffer (NuPAGE MES SDS Running Buffer, ThermoFisher, Landsmeer, the Netherlands) was added into the chamber. 10 μl of protein marker (PageRulerTM Prestained Protein Ladder, 10–180 kDa, ThermoFisher, Landsmeer, the Netherlands) and 10 μl of samples were loaded onto the gel. The electrophoresis was carried out at 200 V for 30min. The gel was subsequently washed with distilled water and stained using Coomassie Brilliant Blue R-250 Staining Solution (Bio-Rad Laboratories B.V., Lunteren, the Netherlands) for 1 day under a mild shaking. Afterwards, the staining solution was discarded, and the gel was washed with distilled water and immersed in the destaining solution for 2 days under a mild shaking.

2.7. Interfacial properties

2.7.1. Dynamic interfacial tension

The interfacial tension of the oil/water interface at pH 3.8 was measured with an automated drop tensiometer (ADT, Tracker, Teclis-instruments, Tassin, France). An oil droplet with a surface area of 15.0 mm^2 was created at the tip of a rising-drop capillary. Stripped rapeseed oil was used. The oil was stripped with Florisil (Sigma-Aldrich, 20,281, Supelco, 100–200 mesh) to remove any surface-active impurities, following the process as described by (Berton, Genot, & Ropers, 2011). The droplet was immersed in the protein dispersion standardized at 0.01 wt% protein content for RPM and RPC. As napins composed 50% of the total proteins in the extracts (Ntone et al., 2021), the dispersion of the napin isolate was standardized at 0.005 wt% proteins. The protein dispersions were filtrated with a 0.2 μm filter before measurement to remove any insoluble material which could disturb the measurements. The interfacial tension γ was monitored for 12000 s (3.3 h) at 20°C.

2.7.2. Dilatational interfacial rheology

At the end of the interfacial tension measurements (after 3.3 h) when a plateau in the interfacial tension reduction was reached, oscillatory interfacial dilatational deformations were applied to determine the dilatational elastic moduli (E_d') and viscous (E_d'') moduli as a function of deformation amplitude. The oil droplet was subjected to sinusoidal deformations at amplitudes of 5–30% of its original surface area at a constant frequency (0.01 Hz). Each amplitude consisted of a series of 5 cycles followed by 5 cycles of resting period. The changes in area and interfacial tension were recorded during the oscillations, and the dilatational elastic (E_d') and viscous (E_d'') moduli were obtained using:

$$E_d' = \Delta\gamma\left(\frac{A_0}{\Delta A}\right)\cos\delta \quad (3)$$

$$E_d'' = \Delta\gamma\left(\frac{A_0}{\Delta A}\right)\sin\delta \quad (4)$$

Where $\Delta\gamma$ is the change of interfacial tension at each deformation, A_0 is the initial droplet surface area (15.0 mm^2), ΔA is the change in droplet surface area and δ is the phase shift of the oscillatory interfacial tension signal with respect to the oscillating area signal.

3. Results and discussion

3.1. Emulsifying properties of the rapeseed protein extracts

The two rapeseed protein extracts with a different degree of protein purity were extracted using a previously developed protocol (Ntone et al., 2020); The rapeseed protein mixture (RPM) -recovered as a serum after centrifugation-, composed of 39.4 ± 0.4 wt% proteins (on dry matter, as determined from different batches extracted for this study). The approximate composition of the non-protein compounds as determined from previous studies were 12 wt% oleosomes, 6 wt% phenolic compounds (i.e. sinapic acid), 8 wt% ash and 34 wt% carbohydrates. After we diafiltrated RPM and removed the low molecular weight compounds, the protein content of the rapeseed protein concentrate (RPC) increased to 66.0 ± 3.5 wt%. The oleosome content also increased to 15 wt%, while the phenolic compound, ash and carbohydrate content reduced to 2.5 wt%, 2.5 wt% and 14 wt% respectively (Ntone et al., 2020). Both RPM and RPC contained napins and cruciferins in a mass ratio of approximately 1:1.

Next, to assess the impact of composition on the emulsifying properties of the rapeseed protein extracts (RPM, RPC) at pH 3.8, 10.0 wt% oil-in-water emulsions were prepared, standardized at different protein concentrations (0.2–1.5 wt%). Aiming to provide a mechanistic understanding on the emulsifying and interfacial properties of rapeseed protein extracts, we chose to prepare 10.0 wt% oil-in-water emulsions, as a simplified non-jammed model emulsion food system. Thereafter the droplet size and microstructure were characterized. Droplet size is an important emulsion attribute that reflects 1) the ability of proteins to adsorb and stabilize the interface and 2) the impact of the non-protein compounds on the interfacial stabilization mechanism and droplet formation.

Fig. 1a shows the droplet aggregate size of the emulsions at different protein concentrations, at time zero and after storage for seven days. The emulsions stabilized by RPM, showed large droplet aggregates of around 40–60 μm, which increased during storage (around 50–100 μm). In contrast, the emulsions stabilized by RPC showed limited aggregation, with droplet aggregate size of around 2–3 μm, which remained constant during storage for the protein concentrations below 0.7 wt% (Fig. 1a). At protein concentrations higher than 0.7 wt% an increase in the droplet aggregate size was observed. At the measured pH, both RPM and RPC emulsions were slightly positively charged (0–15 mV) showing that the electrostatic forces present were weak, having a minor effect, and possibly hydrophobic interactions were responsible for the droplet aggregation

To determine the individual droplet size of the emulsions we added 1.0 wt% SDS. SDS is a low molecular weight surfactant whose role is to break protein hydrophobic interactions and it was added to prevent bridging between the emulsion droplets (Reynoldst & Tanford, 1970). This allowed us to determine the individual droplet size instead of the droplet aggregate size. The individual droplet size (Fig. 1b) of the emulsions stabilized with RPM decreased with increasing protein concentration, from 2.0 μm at 0.2 wt% protein concentration to 0.6 μm at

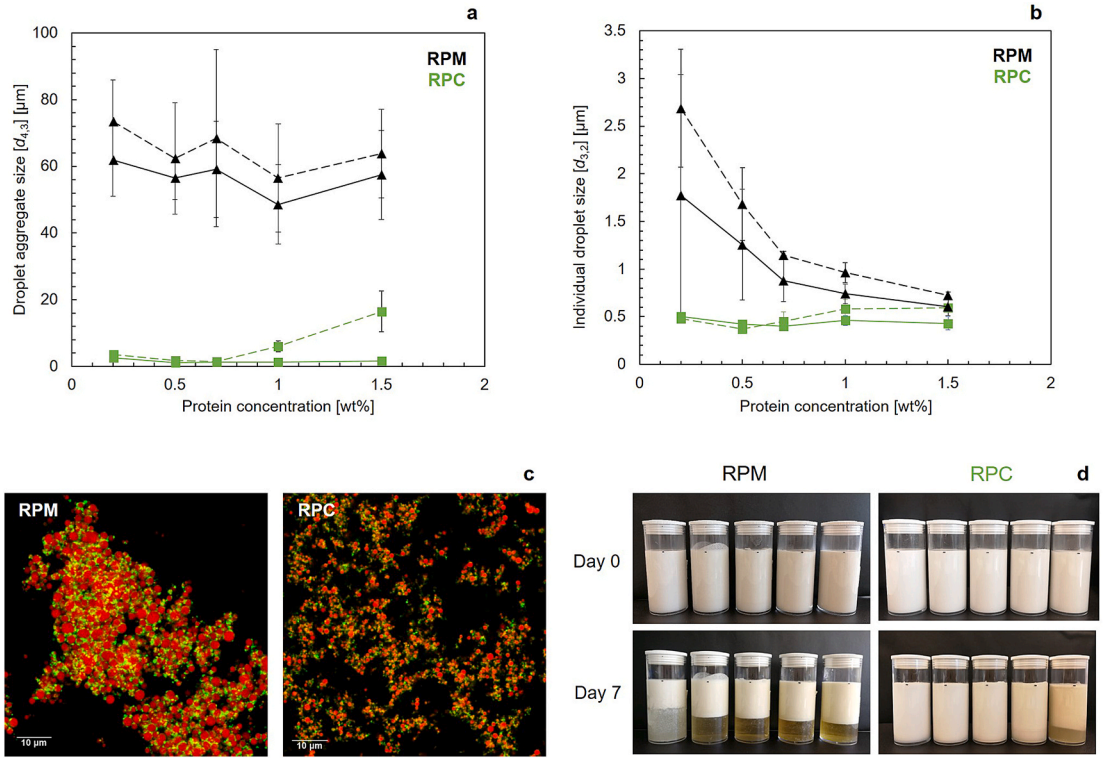

Fig. 1. Droplet aggregate size ($d_{4,3}$) of emulsions stabilized by RPM (black triangle symbol) and RPC (green square symbol) at time zero (continuous line) and after seven days of storage (dotted line), b) Individual droplet size ($d_{3,2}$) of 10.0 wt% oil-in-water emulsions at pH 3.8 stabilized with RPM (black triangle symbol) or RPC (green square symbol) at different protein concentrations (0.2–1.5 wt%) measured after the addition of 1% SDS, at time zero (continuous line) and after seven days of storage (dotted line), c) CLSM images of 10.0 wt% oil-in-water emulsions stabilized with RPM (left) or RPC (right) at 0.7 wt% protein concentration. The emulsions are stained with Nile red (shown as red) for the oil and Fast green (shown as green) for the proteins, Scale bar; 10 μm, d) images of emulsions made with RPM (left) or RPC (right) at time zero and after storage for seven days, showing the creaming behavior of the emulsions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

1.5 wt% protein concentration. An increase of the individual droplet size was observed during storage of seven days, especially at protein concentrations below 0.7 wt%, indicating droplet coalescence during storage. The individual droplet size of the emulsions stabilized with RPC showed almost no dependency on the protein concentration with $d_{3,2}$ values around 0.4–0.5 μm (Fig. 1b). The independence of droplet size on the protein concentration in RPC implies that a protein concentration of 0.2 wt% was sufficient to cover and stabilize the oil droplet interface. Therefore, the additional amount of proteins present does not lead to a significant change in the emulsion droplet size. The presence of excess unadsorbed proteins might be responsible for the droplet aggregation observed at protein concentrations above 0.7 wt% (Fig. 1a), due to depletion phenomena (Dickinson & Golding, 1997). The individual droplet size remained stable over time (seven days).

To confirm the droplet size measurements (both the individual and aggregate droplet size) and visualize the differences in the microstructure of the emulsions stabilized by RPM and RPC, we used confocal microscopy. Fig. 1c shows the confocal image of 10.0 wt% oil-in-water emulsions stabilized by RPM or RPC at 0.7 wt% protein concentration, where the oil is presented by red and the proteins by green. The images showed the presence of larger and aggregated emulsion droplets in RPM-stabilized emulsions compared to the RPC-stabilized emulsions where smaller and less aggregated droplets were present. Protein aggregates were present in both emulsions, however, in the RPM-stabilized emulsions, we also observed larger protein aggregates that were randomly attached to the oil droplets interface, bridging the droplets. Protein aggregation has been previously reported to induce emulsion droplet aggregation (Yerramilli, Longmore, & Ghosh, 2017), mainly due to dominating hydrophobic intermolecular interactions (Hinderink, Münch, Sagis, Schroën, & Berton-Carabin, 2019).

The bridging and aggregation of droplets in turn can affect the macroscopic visual appearance of the emulsions, such as the creaming stability during storage. The images provided in Fig. 1d show the creaming behavior of the emulsions at time zero and after storage for seven days. The RPM-stabilized emulsions showed higher creaming compared to the RPC-stabilized emulsions where almost no creaming was observed, which started already in the first hour after emulsification (data not shown). The creaming behavior of RPM-stabilized emulsions is dominated by the extensive aggregation of the emulsion droplets as shown in Fig. 1a, as the creaming velocity according to the Stoke's equation is proportional to the square of the droplet diameter and density (Robins, 2000). Accordingly, the creaming of the RPC-stabilized emulsions at concentrations above 0.7 wt% is probably related to the presence of the larger aggregates at these protein concentrations and depletion phenomena as discussed earlier.

The protein aggregates present in both RPM and RPC-stabilized emulsions could be related to the low solubility of the proteins in both extracts at pH $<$ 5 (protein solubility $<$50%) (Ntone et al., 2020). We hypothesize that the larger protein aggregates in RPM-stabilized emulsions could be correlated with the higher sinapic acid content in RPM. According to prior studies, the phenolic hydroxyl groups of phenolic compounds are protonated at low pH values and non-covalent hydrophobic interactions with proteins may occur (Bock et al., 2021; Jakobek, 2015). Hydrophobic interactions can occur between the hydrophobic amino acid residues of the proteins and the phenolic compounds. For instance, proline residues seem to play a key role by engaging in hydrophobic interactions with phenolic compounds, which are important for stabilizing the protein-phenol complexes formed (Rawel, Rohn, Kruse, & Kroll, 2002). Such interactions can potentially cause protein structural changes (Jakobek, 2015) and protein cross-linking (Ozdal et al., 2013) promoting protein aggregation (Bock et al., 2021; Czubinski & Dwiecki, 2017; Karefyllakis et al., 2017). We, therefore, hypothesized that the higher sinapic acid content in RPM affects the emulsifying properties of the proteins by inducing further protein aggregation. Other non-protein components, such as oleosomes, which are present in both RPM and RPC, are not expected to be responsible for the differences observed in the emulsions, as they have been reported as inert with no significant impact on the interfacial and emulsifying properties of the proteins (Karefyllakis, Jan Van Der Goot, & Nikiforidis, 2019; Karefyllakis, Octaviana, et al., 2019; Ntone et al., 2021; (Yang, Faber, et al., 2021)).

To validate our hypothesis, sinapic acid was added to RPC prior to emulsification to reach the same content as present in RPM and made emulsions (standardized at 0.7 wt% protein, 10.0 wt% oil). Fig. 2 shows the confocal images of the emulsions stabilized with RPC before and after the addition of sinapic acid. The confocal images showed that in RPC-stabilized emulsions where sinapic acid was added, larger and aggregated oil droplets with larger droplet aggregates in between the droplets appeared. The above result confirmed that sinapic acid content in rapeseed extracts can impact the emulsifying properties of rapeseed proteins at pH 3.8.

To further understand the impact of sinapic acid on droplet formation, we investigated in detail the mechanism of droplet stabilization. Our first hypothesis was that sinapic acid affects the ability of proteins to adsorb at the oil droplet interface in RPM-stabilized emulsions. To test this hypothesis, the protein profile at the droplet interface of the RPM- and RPC-stabilized emulsions was analyzed using electrophoresis (SDS-PAGE). For this analysis, the emulsions were centrifuged twice to remove the non-adsorbed proteins. At pH 3.8 in both RPM and RPC dispersions the repulsive electrostatic forces between proteins are weak (ζ potential around 5 mV) (Ntone et al., 2020) and attractive forces (i.e. hydrophobic and van der Waals forces) between the adsorbed and non-adsorbed proteins could become dominant (Roth, Neal, & Lenhoff, 1996), preventing the removal of the non-adsorbed proteins from the interface. Thus, the emulsions were also washed at pH 9 where the repulsive electrostatic forces between proteins is three times larger than at pH 3.8 (ζ potential around −15 mV) (Ntone et al., 2020).

Fig. 3 shows the electrophoregram of the interface of the RPM and RPC-stabilized emulsions washed at pH 3.8 and at pH 9. At pH 3.8 at both RPM and RPC-stabilized interface a protein band around 17 kDa associated with napins and protein bands of 30–70 kDa which associate with cruciferins (Perera, McIntosh, & Wanasundara, 2016; Wanasundara, 2011) were observed. However, when the interface was washed at pH 9, at both RPM- and RPC-stabilized interfaces only napins were present while cruciferins were washed out. This outcome shows that the interface stabilized by both RPM and RPC is dominated by napins, while cruciferins probably form an additional secondary layer which interacts with napins through non-covalent bonds. The above stabilization mechanism of the interface is similar to what we observed when we used RPM as an emulsifier at pH 7 (Ntone et al., 2021). This result highlights that independent of the pH conditions the free sinapic acid present in RPM does not affect the ability of napins to adsorb at the interface. The fact that napins dominated the interface can be explained by their small molecular size and amphiphilic properties, which contribute to their higher surface activity than cruciferins (Ntone et al., 2021).

As the higher sinapic acid content in RPM did not affect the ability of napins to adsorb at the interface at pH 3.8, we suggest that the larger oil droplets in RPM-stabilized emulsions emerge from the impact of the higher sinapic acid content on the secondary layer formed by cruciferins. A possible mechanism is that sinapic acid induces aggregation of cruciferins, with impact on the formation of a secondary cruciferin layer around the emulsion droplet interface. Cruciferins at low pH are reported to dissociate from hexamers into trimers (Folawiyo & Apenten, 1996; Perera et al., 2016; Withana-Gamage, Hegedus, Qiu, & Wanasundara, 2011). Although cruciferin dissociation can also induce further protein self-assembly and aggregation (Perera et al., 2016) (as also observed in the emulsions stabilized by both RPM and RPC), an equilibrium between trimers and aggregates is probably reached (Sridharan, Meinders, Bitter, & Nikiforidis, 2020b). This partial dissociation into trimers results in exposure of the buried hydrophobic residues of the inner face of the cruciferin trimers increasing the surface hydrophobicity

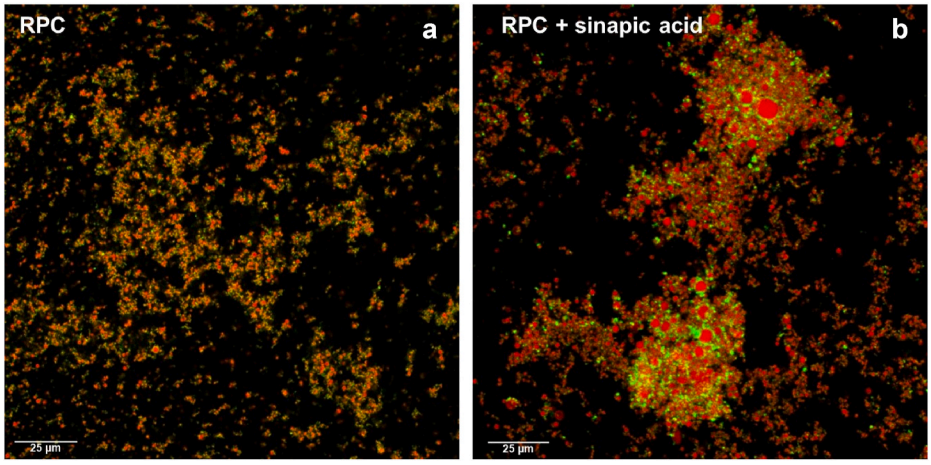

Fig. 2. CLSM images of 10.0 wt% oil-in-water emulsions stabilized with a) RPC or b) RPC after the addition of sinapic acid at 0.7 wt% protein concentration at pH 3.8. The emulsions were stained with Nile red (shown as red) for the oil and Fast green (shown as green) for the proteins, Scale bar; 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

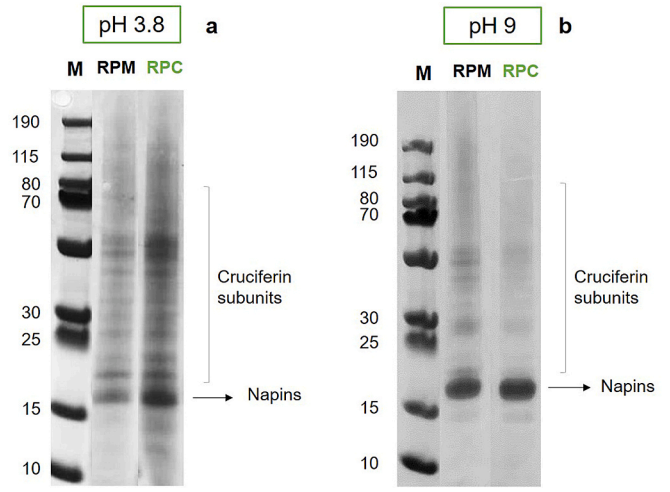

Fig. 3. Protein profile of the adsorbed proteins at the interface of the 10.0 wt% oil and 0.7 wt% protein RPM and RPC- stabilized emulsions after removal of the non-adsorbed proteins (referred as washing step) at a) pH 3.8 and b) pH 9. M: protein molecular weight markers.

of the protein (Perera et al., 2016; Withana-Gamage et al., 2011). Due to the exposure of hydrophobic residues at low pH, sinapic acid possibly has more binding sites at cruciferins (Ozdal et al., 2013). Additionally, the slightly positive charge of cruciferins at this pH also encourages electrostatic interactions with the negatively-charged sinapic acid (Rubino, Arntfield, Nadon, & Bernatsky, 1996). As plant phenols are generally small molecules (180–700 Da) and proteins are comparatively very large (14000–350000 Da), it is likely that more than one phenol molecule can bind to one protein molecule (Rawel, Meidtner, & Kroll, 2005b). Moreover, the sinapic acid molecules can bridge different cruciferin molecules together resulting to further protein aggregation. As a result of the sinapic acid-cruciferin interactions, large cruciferin aggregates are possibly formed. These large protein aggregates, as also shown in the CLSM images of the RPM-stabilized emulsions, probably cannot form a homogeneous secondary layer around napins to prevent droplet re-coalescence during turbulence and shear in the homogenizer (Mohan & Narsimhan, 1997). As a result, larger emulsion droplets are formed when RPM is used as emulsifier.

3.2. Interfacial properties of the rapeseed protein extracts

To further study the interfacial properties and lateral interactions on the interfaces stabilized by RPM or RPC, we applied interfacial dilatational rheology. Interfacial dilatational rheology is a powerful tool that provides further information on the intermolecular interactions at the interface, providing a deeper understanding of the emulsion properties at a macroscopic level. As our main hypothesis is that sinapic acid impacts the formation of a secondary layer formed by cruciferins, it was necessary to determine the contribution of cruciferins in the structure and rheological properties of the interfaces stabilized by RPM and RPC, which contain both napins and cruciferins. To do so, a pure napin sample was included in the measurements.

Fig. 4 displays the interfacial tension reduction using the RPM, RPC and napin isolate after 12000 s. Both RPM and RPC reduced the interfacial tension similarly, from 25 mN/m to around 9 mN/m. The interfacial tension decreased only to 13 mN/m when pure napins were used. The lower interfacial tension observed in RPM and RPC samples compared to single napins is possibly the result of the interaction of adsorbed napins with the cruciferins present in the bulk, which form a secondary layer around napins.

To gain more insights into the interactions between protein molecules at the oil/water interface, we employed interfacial dilatational rheology. In Fig. 4b we plotted the dilatational moduli of the interfaces stabilized by the protein extracts (RPM, RPC and napins) as a function of amplitude of deformation. The elastic modulus (E_d') was much higher than the viscous modulus (E_d") in all protein samples, which implies solid-like viscoelastic behavior. The elastic moduli of napins were around 20 mN/m and independent on the amplitude of deformation, which indicates that the interfacial microstructure did not change significantly during deformation. This response implies weak lateral interactions between napin molecules mainly driven by attractive interactions, such as van der Waals and hydrogen bonds after adsorption and no significant formation of covalent sulfur bonds. The result was similar to our previous research at pH 7 (Ntone et al., 2021), highlighting that the interactions between napins are not strongly influenced by pH changes.

The elastic moduli (E_d') of RPM and RPC stabilized interfaces were similar and higher than the single napin interface at small amplitudes (E_d' around 36 mN/m). This outcome suggests that cruciferins present in the bulk in RPM and RPC dispersions enhance the lateral interactions of napin molecules and result in a stronger viscoelastic interface than a single layer of napins. The moduli of the interface at pH 3.8 were relatively higher compared to pH 7 (E_d' around 36 at pH 3.8 vs E_d' around

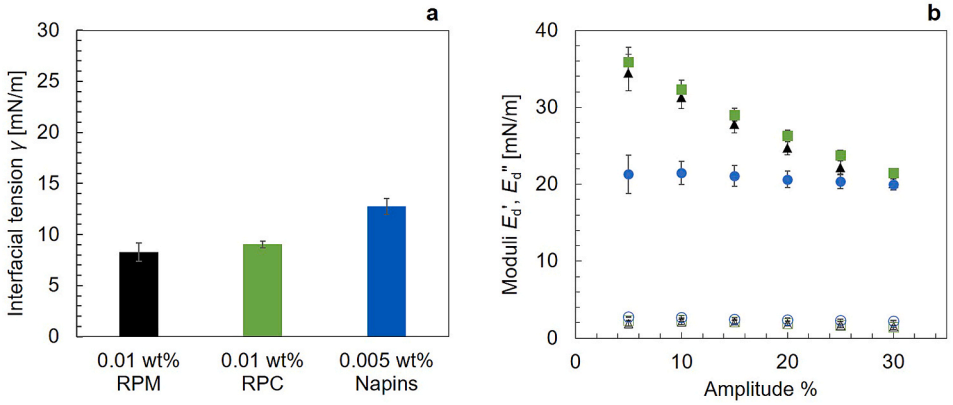

Fig. 4. a) Interfacial tension (γ) after 12000 s of RPM (black) and RPC (green) dispersions at 0.01 wt% protein concentration and napins (blue) dispersion at 0.005 wt% protein concentration at pH 3.8 (20°C). The dispersions were filtered with a 0.2 μm syringe filter prior to measurements, b) Dilatational elastic modulus (E_d': filled symbol) and viscous modulus (E_d'': hollow symbol) of RPM (black triangle symbol), RPC (green square symbol) and napins (blue circle symbol) as a function of amplitude of deformation at constant oscillatory frequency of 0.01 Hz. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

28 mN/m at pH 7 at 5% amplitude) (Ntone et al., 2021). The differences in the moduli between pH 3.8 and pH 7, suggest that at low pH (i.e. pH 3.8), the dissociation of cruciferin into trimers with increased hydrophobicity may allow stronger interactions of cruciferins with the adsorbed layer of napins compared to cruciferin hexamers. However, at pH 3.8, when the amplitude of deformation was increased, the moduli decreased from around 36 mN/m at 5% amplitude to 20 mN/m at 30% amplitude both in RPM- and RPC-stabilized interfaces, showing that the interfacial microstructure was weakened.

To further understand the behavior of the interfacial network upon

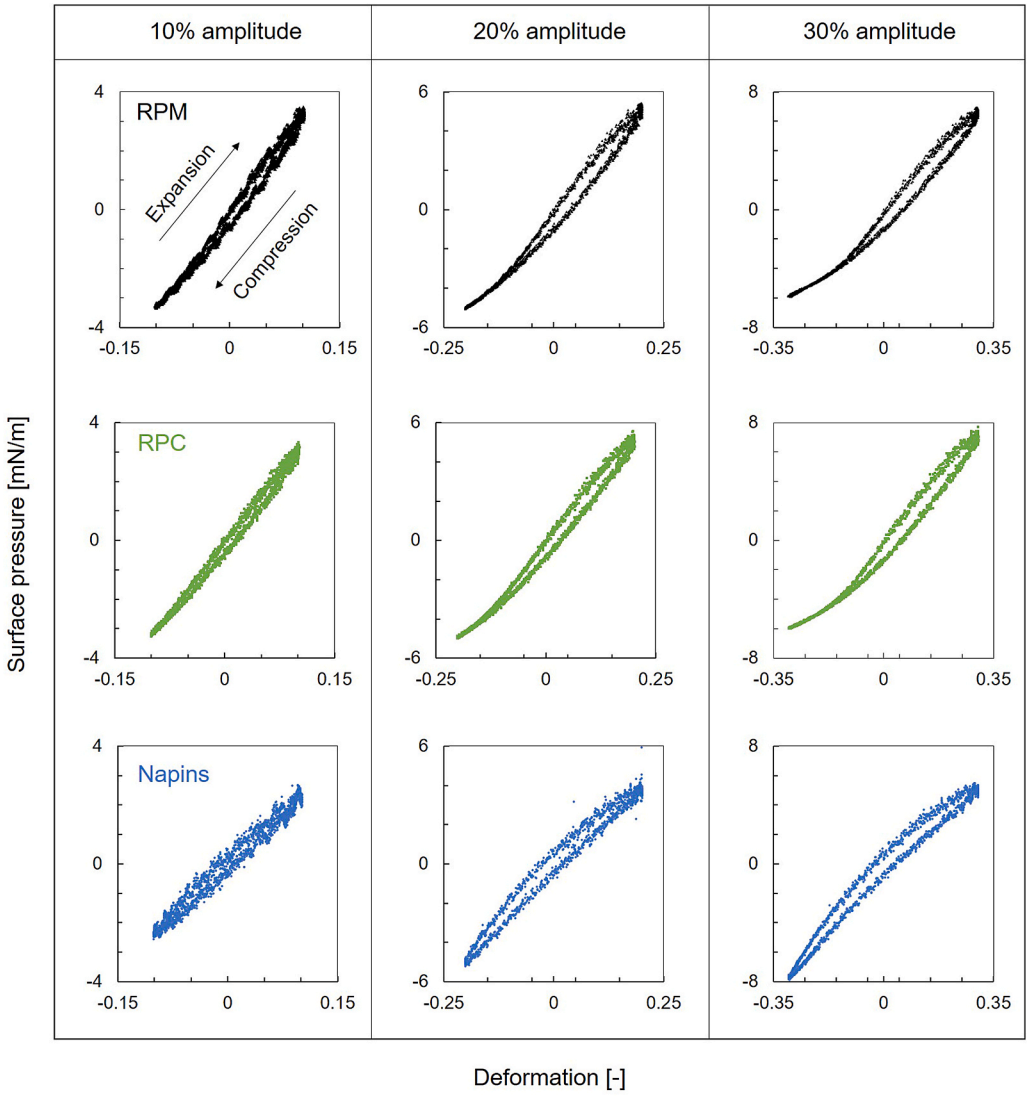

Fig. 5. Lissajous plots of the interface stabilized by RPM (black), RPC (green), Napins (blue) at 10%, 20% and 30% amplitude of area deformation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

extension and compression, we used Lissajous plots, where the surface pressure is plotted against the area deformation. Lissajous plots, are a more insightful way of analysing rheological data, especially in the nonlinear regime, where first harmonic based moduli are inaccurate. Fig. 5 shows the Lissajous plots of the interface stabilized by the protein extracts (RPM, RPC, napins). The Lissajous plots of RPM and RPC-stabilized interfaces were similar and displayed an asymmetrically narrow ellipse shape at all amplitudes. At 10% amplitude, upon extension the ellipse was slightly wide but becomes very narrow -almost a line-upon compression. By increasing the amplitude to 20% and 30% the curves start to level off on compression as the surface pressure almost does not change by increasing the amplitude (from -5 mN/m to −6 mN/m respectively). This levelling off shows a strain softening behavior which suggests that upon compression the interfacial network is jammed, and most-likely the interacting cruciferin molecules forming the secondary layer are expelled to the bulk (Sagis & Fischer, 2014). A schematic representation of the interactions occurring at the interface upon increasing the amplitude of deformation above 20% when RPM or RPC is used is given in Fig. 6.

To understand the effect of cruciferins in the structure and rheological properties of the interface, we compared the Lissajous plots of RPM and RPC, containing both napins and cruciferins, with those constructed for the interface stabilized only by napins. At 10% amplitude the plot showed a narrow ellipse shape without noticeable asymmetries. This shape indicates an almost linear, predominantly elastic response of the interface, indicating a weak solid structure (Sagis & Scholten, 2014). By increasing the amplitude of deformation to 20% and 30%, the surface pressure increased from −3 mN/m at 10% to −8 mN/m at 30%, at maximum compression (lower left corner of the plot). This increase in surface pressure can be explained by the increased density of napins on compression (increase in protein molecules/area). Additionally, upon compression the slope of the curve increase slightly which indicates a weak strain hardening behavior (Hinderink, Sagis, Schroën, & Berton-Carabin, 2020; Sagis & Fischer, 2014; Van Kempen, Schols, Van Der Linden, & Sagis, 2013). Towards maximum expansion (top right part), the slope of the curve slightly levels off, showing a strain-softening tendency. This behavior upon expansion and compression implies an interface with weak in-plane interactions with nonlinearities in the response, primarily related to changes in surface density (Sagis & Fischer, 2014; Sagis & Scholten, 2014).

The fact that there are no significant differences observed in the interfacial properties of RPM and RPC determined by the ADT, in contrast to the emulsion properties can be a result of the 1) limited diffusion of large protein aggregates to the interface or even their precipitation during the interfacial measurements 2) the absence of large aggregates in the system due to the necessary filtration step taken before the interfacial measurements 3) the higher surface area created during emulsification, where the large cruciferin aggregates possibly have a more drastic effect on droplet formation; at these significantly higher surface areas the number of large cruciferin aggregates is probably insufficient to form a homogeneous secondary layer which strongly interacts with the primary layer of napins and sufficiently cover the created interface. Moreover, as a result of turbulence and high shear forces in the homogenizer it is possible that these weakly bound aggregates could be expelled from the interface. As a result, during emulsification, the colliding oil droplets probably recoalesce, and larger emulsion droplets are formed when RPM is used as an emulsifier.

All the above results from the emulsion and interfacial measurements combined, show that the presence of sinapic acid present in RPM does not affect the ability of napins to adsorb at the interface, but suggest that sinapic acid affects the secondary layer formation by cruciferins by inducing aggregation. A schematic representation of the suggested emulsification mechanism of RPM and RPC-stabilized interface is illustrated in Fig. 7.

4. Conclusions

In this research, we investigated the mechanism of interface stabilization when a rapeseed protein mixture (RPM) containing 40 wt% proteins and 6 wt% free sinapic acid and a rapeseed protein concentrate (RPC) containing 65 wt% proteins in which sinapic acid is reduced to 2.5 wt% are used as emulsifiers at pH 3.8. The two extracts showed different emulsification properties; Larger emulsion droplets and larger protein aggregates randomly attached at the interface of the droplets were found in RPM-stabilized emulsions compared to RPC-stabilized emulsions. The higher sinapic acid content in RPM does not impact the interfacial composition, with napins being adsorbed at the interface. However, sinapic acid in RPM affects the secondary layer formed by cruciferins by inducing cruciferin aggregates. This aggregation probably results in a less homogenous secondary layer that cannot strongly interact with the primary adsorbed layer of napins and prevent recoalescence of the colliding oil droplets during homogenization. Thus, larger oil droplets are formed when RPM is used as an emulsifier. We anticipate that our insights into the effect of sinapic acid on the emulsification mechanism of less purified rapeseed protein extracts can be used to evaluate the need for protein purification for the application of these extracts in emulsion food products.

Author contributions

Eleni Ntone; Conceptualization; Investigation; Methodology; Formal analysis; Visualization; Validation; Writing - original draft.

Qiyang Qu; Investigation; Formal analysis.

Kindi Pyta Gani; Investigation; Formal analysis.

Marcel Meinders; Validation; Writing - review & editing; Funding acquisition.

Leonard M.C. Sagis; Methodology; Validation; Writing - review & editing.

Johannes H. Bitter; Writing - review & editing.

Constantinos V. Nikiforidis; Conceptualization; Supervision; Validation; Methodology; Project administration; Writing - review & editing.

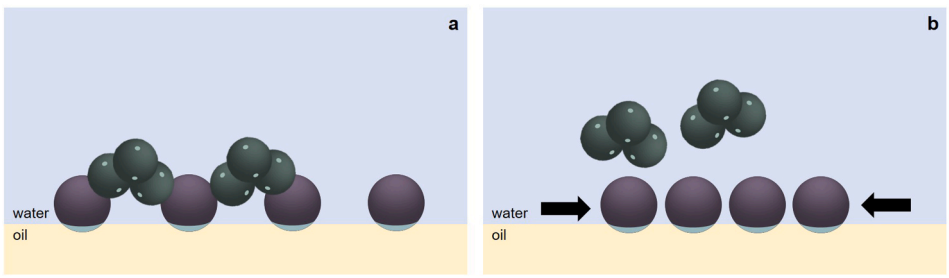

Fig. 6. Schematic representation of dynamic interactions of cruciferins with the adsorbed napins a) before and b) after compression at amplitudes ≥20%, when the RPM or RPC is used to stabilize the interface, showing expelling of cruciferins into the continuous phase.

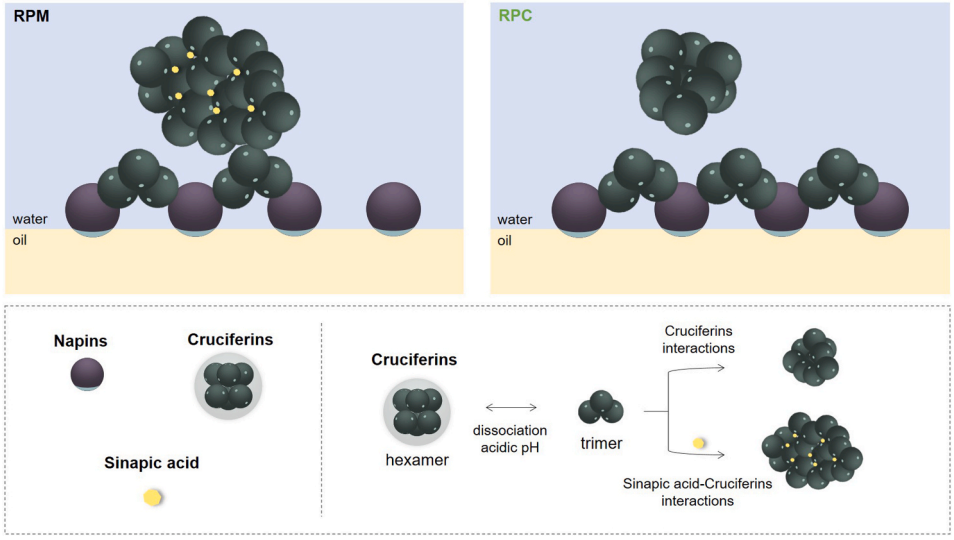

Fig. 7. Schematic representation of the suggested stabilization mechanism of RPM and RPC-stabilized interface at acidic pH. The size of molecules and the number of cruciferin trimers and sinapic acid in each protein aggregate are not to scale.

Declaration of competing interest

None.

Acknowledgments

The project is organized by and executed under the auspices of TiFN, a public-private partnership on precompetitive research in food and nutrition. The authors have declared that no competing interests exist in the writing of this publication. Funding for this research was obtained from Unilever Research and Development Wageningen., Nutricia Research B·V., Bel group, Pepsico Inc., the Netherlands Organisation for Scientific Research (NWO) (ALWTF.2016.024) and the Top-sector Agri&Food.

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