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# Combination modes impact on the stability of $\beta$ -carotene-loaded emulsion constructed by soy protein isolate, $\beta$ -glucan and myricetin ternary complex

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## Abstract

A  $\beta$ -carotene rich emulsion with improved physical and chemical stability was obtained in this study, using different types of protein-polysaccharide-polyphenol ternary complexes as novel emulsifiers. The ternary complexes were prepared by covalent or non-covalent binding of soy protein isolate (SPI),  $\beta$ -glucan (DG) and myricetin (MC), which were evidenced to be stable. It was indicated that the emulsion stabilized by covalent complex of SPI, DG and MC, exhibited higher zeta-potential and smaller particle size than those stabilized by non-covalent complex. Furthermore, the covalent complexes prepared from different addition sequences showed different efficiencies in stabilizing the emulsion, in which SPI-DG-MC and SPI-MC-DG-stabilized emulsions possess better stability, emulsifying activity and storage resistance under adverse environmental treatment, with CI values of 62.7% and 64.3% after 25 days, respectively. According to oxidative stability and rheology analysis of the emulsions, it was found that the SPI-MC-DG complex prepared at the ratio of 4:2:1 was more stable with relatively less lipid oxidation products and a tighter stacking structure, and the final LH value was 39.98 mmol/L and the MDA value was 6.34

mmol/L. These findings implied that the ternary complex has the potential to deliver fat-soluble active ingredient by means of emulsion, but which depends on the mode and sequence of the molecular interactions.

*Keywords:* Soy protein isolate Polysaccharide Polyphenol Covalent interaction,  $\beta$ -carotene-loaded emulsion Stability

## 1. Introduction

$\beta$ -carotene is a representative carotenoid, which is the most common and stable natural pigment in nature. It plays an important role in preventing chronic diseases and protecting cells from oxidation (W. Chen et al., 2022). However, the release of  $\beta$ -carotene in the small intestine of human is limited and the solubility in the micelle phase is low, resulting in extremely low and variable bioaccessibility for direct uptake. It has been reported that lipids can stimulate the secretion of bile and chylomicron to promote the formation of micelles. Therefore, it is recommended to add additional lipids to the diet to increase the bioaccessibility of  $\beta$ -carotene by intestinal epithelial cells (Mapelli-Brahm et al., 2019; Yi et al., 2019). Some studies have shown that the presence of specific types of lipids (such as linseed oil or soybean oil) can improve the bioavailability of  $\beta$ -carotene (H. Zhang et al., 2023), however, excessive fat in diet is unacceptable by people. Thus, a functional carrier emulsion has been produced with purpose of nutrition and health, which can also resist to the oxidation of  $\beta$ -carotene and improve its bioaccessibility in body as a promising fat replacement strategy. (J. Chen, Li, Li, McClements, & Xiao, 2017) developed an oil-in-water emulsion that improved the water dispersibility and chemical stability of the encapsulated  $\beta$ -carotene by optimized preparation process. (Xu et al., 2014) found that a conjugate prepared from whey protein and beet pectin could increase the stability of  $\beta$ -carotene during digestion.

However, the complicated microstructure of emulsion is thermodynamically unstable and easily decomposes during storage, and the extra additives must be incorporated to inhibit these processes in order to produce a product with the desired shelf-life. Therefore, it is necessary to develop composite or modified emulsifiers or dispersants for use in emulsions. (Pham, Wang, Zisu, & Adhikari, 2019) have explored the interface and emulsifying properties of flaxseed protein isolate and its phenolic complexes, developing a plant-based natural emulsifier with improved antioxidant performance. Furthermore, (Yan et al., 2020)

introduced a ternary conjugate covalently prepared from bovine serum albumin (BSA), chlorogenic acid (CA) and dextran (DEX) as a novel emulsifier. Compared with individual protein, the use of ternary conjugate could more effectively stabilize the emulsions with enhanced stability near the isoelectric point, and improve the chemical stability of the lutein embedded in the emulsion. The complexes of soluble starch and whey protein isolate (WPI) were prepared to stabilize high internal phase emulsion (B. Guo et al., 2021). It was found that the HIPE stabilized by starch-WPI complex was more stable in acidic environment, and it was proved that starch-WPI complex could improve the emulsifying ability of WPI and enhance the stability of high internal phase emulsion. These literatures all indicate that protein conjugate/complex is more suitable as an emulsifier to deliver hydrophobic nutraceutical.

As we know, soybean protein isolate (SPI) is a natural emulsifier or surfactant. Because of its amphiphathy, biocompatibility, biosafety, high digestibility and commercialization, it has been widely used to stabilize emulsions (Zhou et al., 2022). However, SPI always shows poor solubility and emulsification due to partial denaturation during spray drying. Therefore, the physical and chemical modification of SPI to improve emulsion stability has been regarded as a hot topic. It has been proved that the combination with typical polysaccharides or polyphenols can greatly improve the functional properties of protein. For example, (Quan, Benjakul, Sae-leaw, Balange, & Maqsood, 2019) found that the protein–polyphenol conjugate had higher thermal stability, antioxidant activity, better emulsifying property and enhanced gelling property, which can be used as a novel food additive. The use of protein-polysaccharide interactions in dairy formulation design was also described in (Corredig, Sharafbafi, & Kristo, 2011), highlighting that binding of charged polysaccharides to milk proteins was potent to produce functional complexes, novel components and delivery systems. Recently, a ternary complex of SPI,  $\beta$ -glucan (DG) and myricetin (MC) was prepared in our laboratory. We have previously evidenced that  $\beta$ -glucan (DG) has a high adsorption capacity on MC. Interestingly, it was further found that the non-covalent complexation between SPI, DG and MC could significantly increase the foaming and emulsifying properties of protein (Lei et al., 2022; Su et al., 2023). This indicates that the successful construction of ternary complex can significantly improve the interface properties of SPI. However, the delivery system stabilized by the ternary complex as a nutritional factor has not been studied.

The exploration of protein-polysaccharide-polyphenol complexes has been carried out by

many researchers, but the effects of action modes involving covalent and non-covalent have not been discussed, as well as the impact of their addition orders and proportions on the corresponding emulsions. Referring to our previous research, we propose that the ternary complexes with different binding modes differ in the stability of emulsions. Therefore, the covalent and non-covalent combinations of SPI, DG, and MC were primarily compared in this study. Subsequently, different interaction sequence of SPI, DG and MC was focused. And the most optimum emulsion with best performance on the stability and delivery of  $\beta$ -carotene was screened. This study will provide a theoretical basis for the further development of functional food ingredients using SPI and its complex, which will help to promote the delivery process of hydrophobic active substances.

## 2. Materials and methods

### 2.1. Materials and chemicals

Soybean protein isolate (SPI,  $\geq 96.0\%$ , CAS: 9010-10-0, Batch No.202016, MW:232.235) was purchased from Henan Zhongtai Food and Chemical Co., Ltd.  $\beta$ -glucan (DG,  $\geq 95.0\%$ , CAS: 9041-22-9, LOT: 20210306, MW:470.42) was obtained from barley and myricetin (MC,  $\geq 98.0\%$ , HPLC, CAS: 529-44 2, LOT: 200619, MW:318.24) from Shandong West Asia Chemical Co., Ltd.  $\beta$ -Carotene ( $\geq 96\%$ , CAS: 7235-40-7, Lot:C12705655) was purchased from Shanghai Macklin Biochemical Co., Ltd. Flaxseed oil (product standard code: GB/T8235, quality grade I) was purchased from Ningxia Junxingfang Food Technology Co., Ltd. Other chemical reagents were of analytical grade and purchased from Guoyao Chemical Reagent Co., Ltd.

### 2.2. Preparation of different SPI complexes

According to (Liu, Ma, McClements, & Gao, 2016; Y. Zhao et al., 2020) with appropriate modifications the binary and ternary covalent complexes of SPI were prepared by adding SPI (10 mg/mL), DG (2 mg/mL), and MC (2.5 mg/mL) in different volume ratios. **1)** The SPI and DG solutions were mixed and stirred at room temperature until completely dissolved and hydrated, allowed to stand for 12 h and swollen overnight. Adjusted the pH value of the solution to 7, carried out magnetic stirring reaction on the solution in a water bath at 95 °C for 6 h to enable the solution to generate a Maillard covalent reaction, and immediately stopped the Maillard reaction in an ice bath after 6 h to obtain a solution, namely an **SPI-DG** binary covalent complex solution. **2)** SPI and MC solutions were mixed and stirred at room temperature until complete dissolution. The pH of the solution was adjusted to 9, and

magnetic stirring was performed at room temperature for 15 h. Oxygen permeation condition was maintained during the reaction. The solution after the reaction was the **SPI-MC** binary covalent complex solution. **3)** The MC solution was added to the SPI-DG binary covalent complex to prepare the **SPI-DG-MC** ternary covalent complex. **4)** **SPI-MC-DG** ternary covalent complex was prepared by adding DG to SPI-MC binary covalent complex as described above. **5)** DG and MC were mixed, the pH of the solution was adjusted to 3.5, 0.05 g ascorbic acid and 2 mL of 5 M H<sub>2</sub>O<sub>2</sub> solution were added, followed by stirring in a 40 °C water bath for 15 h, followed by the addition of SPI solution after the reaction, and magnetic stirring at room temperature for 6 h to obtain the **DG-MC-SPI** ternary covalent complex. **6)** For corresponding non-covalent complexes, SPI, DG and MC were mixed in different adding sequences, after which they were transferred to the dialysis bag (1000 Da) at the pretreatment place, and the dialysis bag was placed in a 3 L beaker at 40 °C for 48 h until adsorption balance was reached. The free polyphenol was removed, and the solution in the dialysis bag was considered as **non-covalent complex**. All the resulting complexes were subsequently lyophilized.

### 2.3. Preparation of emulsion

Emulsions were prepared according to the method of (Wan Mohammad, McNaughton, Augustin, & Buckow, 2018).  $\beta$ -carotene was added into flaxseed oil at a concentration of 1%. The oil phase was magnetic stirring in a 50 °C water bath for 30 min and then ultrasonic stirring for 10 min to completely dissolve  $\beta$ -carotene in flaxseed oil. The prepared solution of SPI and complexes were mixed with oil in a mass ratio of 9:1, and the mixture was pretreated by a disperser at 1300 rpm for 3 min. The dispersed mixture was subjected to high pressure homogenization by a high-pressure homogenizer (ATS Engineering Limited, AH-2010, Canada) at a pressure value of 600 bar for three times to obtain stable  $\beta$ -carotene emulsions dispersed by different complexed compounds.

### 2.4. Zeta potential and particle size

The Zeta potential of different groups of emulsions were measured using a Zetasizer instrument (Brookhaven Corporation Holtville, USA) after 250-fold dilution with pure water of equal pH. The incident wavelength was 633 nm, and the values were measured three times at 25 °C to obtain the average value. Different groups of emulsions were taken and the average particle size of the emulsions was measured using a laser particle size analyzer

(Malvern Co., Ltd, UK). In the experiment,  $d_{43}$  volume average diameter was used to characterize the average droplet size. The samples were diluted with distilled water in the analyzer until the optimal opacity was in the range of 8–12%, and the refractive indices of oil and water were 1.45 and 1.33, respectively.

### 2.5. Emulsifying activity and stability

A total of 50  $\mu\text{L}$  of prepared emulsion was mixed with 5 mL of 0.1% SDS in mass fraction, and shaken vigorously by vortex oscillation. The absorbance ( $A_0$ ) was measured using a visible spectrophotometer at the wavelength of 500 nm. After placed for 30 min, the absorbance ( $A_{30}$ ) was measured again. The emulsifying activity (E) and emulsion stability (S) were calculated according to Equation (1)(2) (X. Chen et al., 2022). The milk analysis index (CI) was calculated according to Formula (3).

$$E = \frac{2 \times 2.303 \times A_0 \times D}{\rho \times L \times \varphi \times 10000} \quad (1)$$

$$S = \frac{A_0}{(A_0 - A_{30}) \times 30} \quad (2)$$

$$\text{CI}/\% = (H_1/H_0) \times 100 \quad \text{CI}/\% = (H_1/H_0) \times 100 \quad (3)$$

where: D is the dilution multiple, 100;  $\rho$  is the mass concentration of protein before dilution, mg/mL; L is the optical path, 1 cm;  $\varphi$  is the volume fraction of the oil forming the emulsion.  $A_0$  is that absorbance at 0 min and  $A_{30}$  is the absorbance at 30 min.  $H_1$  is the height at which water is precipitated from the emulsion, and  $H_0$  is the total height of the emulsion.

### 2.6. $\beta$ -carotene retention rate in emulsion

The measurement was carried out referring to (W. Chen et al., 2023; S. Li et al., 2022) with minor modifications. First, 1 mL of emulsion sample diluted 10 times was taken and extracted with anhydrous ethanol and n-hexane (the volume ratio of the former to the latter was 1:2) by shaking to achieve the purpose of fully demulsifying and dissolving the emulsion sample. Then, the emulsion sample was allowed to stand for a few minutes to fully separate the ethanol phase and the n-hexane. The extraction phase, i.e., the n-hexane phase, was taken out and collected. The extracts were repeated three times and the upper layers

were combined. The absorbance value was measured at a wave-length of 450 nm by a T6 ultraviolet–visible spectrophotometer. And the  $\beta$ -carotene content in the emulsion sample was calculated according to the drawn  $\beta$ -carotene concentration standard curve. Wherein the retention rate of  $\beta$ -carotene in the emulsion sample is expressed as:

retention rate of  $\beta$ -carotene =  $C_t / C_0 \times 100$ , where  $C_t$  is the concentration of  $\beta$ -carotene in the emulsion after storage for a certain time, and  $C_0$  is the concentration of  $\beta$ -carotene in the initial emulsion sample.

### 2.7. Storage stability

The storage stability of the emulsion was determined by periodically measuring the changes in Zeta potential, milking index,  $\beta$ -carotene retention rate and oxidative stability of the emulsions during storage for 25 days.

### 2.8. Effect of environmental stress on emulsion

Determination of acid tolerance of the emulsion: Fresh emulsion was adjusted to pH 3.0–7.0. Determination of thermal endurance property of emulsion: Fresh emulsion was heat-treated at pH 3.0, 30, 50, 70 and 90 °C for 30 min, and cooled immediately after treatment. Determination of the effect of ion concentration on the emulsion: after adding NaCl to the fresh emulsion, the ion concentrations in the emulsion were made to be 0.3, 0.6, 0.9 mol/L and 1.2 mmol/L, and the pH value was adjusted to be 3.0. All prepared emulsions were determined after 24 h at room temperature (W. Li et al., 2023).

### 2.9. Oxidation stability

The prepared emulsion was subpackaged into sample bottles, and the samples were placed at 45 °C in the dark for storage. The oxidation degree of emulsion was measured by regular sampling.

### 2.10. Primary oxidation products

The primary oxidation product content of the emulsions was determined by the method of (Y. Chen, Wang, Liu, Liu, & Kong, 2018; Xiao, Chao, Huang, & Qingrong) with slight modifications. After aspirating 200–300 mg of sample emulsion, 5 mL of a mixture of isooctane +isopropanol (2:1, v/v) was added and mixed equally, then the mixture was centrifuged at 8000 r/min for 2 min at 4 °C, pipetted 2 mL of supernatant, and added 20  $\mu$ L of potassium thiocyanate (3.94 mol/L) and ferrous chloride (0.072 mol/L) solutions, then



brought to volume of 5 mL with methanol + n-butanol (2:1, v/v) mixture solvent, mixed well and reacted in the dark at room temperature for 20 min, and the absorbance was measured at 510 nm with methanol + n-butanol (2:1, v/ v) mixed solvent as blank control. The concentration of hydrogen peroxide in the sample was calculated according to the standard curve made from hydrogen peroxide.

#### *2.11. Secondary oxidation products*

The concentration of secondary oxidation products in the emulsion was determined by reference to (Hu & Zhang, 2022). Firstly, 200 mg to 300 mg emulsion was dispersed in 1.8 mL deionized water and 4.0 mL TBA solution was added. The resulting mixture was then reacted in boiling water for 15–30 min and then rapidly placed in iced water for cooling. Finally, the mixture was centrifuged at 10,000 g for 15 min and the supernatant was collected for absorbance at 532 nm. The malondialdehyde content of the sample was calculated according to the standard curve prepared from 1,1,3,3- tetraethoxypropane. All reagents were ready for use and determined every 5 days.

#### *2.12. Rheological properties of emulsion*

##### *2.13. Apparent viscosity*

The apparent viscosity of the emulsion was determined using an MCR301 Advanced Rotational Rheometer (DHR-3, TA Instruments, USA). The emulsion was uniformly coated on a 50 mm plate, and the air bubbles generated during coating were removed. The upper and lower slits between the test plates were 0.5 mm, and the test was started after the plate was stabilized for 5 min and the test temperature reached 25 °C. The change in apparent viscosity of the emulsion at a shear rate of 0.1–100 s<sup>-1</sup> was recorded.

##### *2.14. Storage modulus and loss modulus*

The storage modulus( $G'$ ) and loss modulus ( $G''$ ) of the emulsion were determined using an advanced rotary rheometer MCR301. The test plates were 50 mm in diameter, 1 mm spaced above and below the plates, at 25 °C, 1% stress, and 0.1 to 100 rad/s angular frequency.

##### *2.15. Laser confocal microscope (CLSM)*

The protein particles and oil droplets in the emulsion were localized by laser confocal

scanning microscopy. The emulsions were stained and labeled with Nile Red methanol (15  $\mu$ L, 0.01% w/v) and Nile Blue methanol (15  $\mu$ L, 0.01% w/v) to a final dye concentration of 0.4  $\mu$ g/mL. The dyeing process should be protected from light, and the dyed emulsion should be protected from light and fully shaken to mix evenly, ensuring the full dyeing of the emulsion. Staining emulsion (40  $\mu$ L) was dropped onto and overlaid on the slide and observed under a confocal microscope with excitation wavelengths of 488 nm and 633 nm.

#### 2.16. Statistical analysis

The experiment was repeated for three times in each group, and the data were expressed as the mean  $\pm$  standard deviation. SPSS 21.0 software (IBM®, Chicago, IL, USA) was used to perform one-way analysis of variance and Duncan test for the obtained data. All figures were drawn by Origin 9 (Origin Lab Corporation, Northampton, MA, USA). All tests were set at the significant level of  $P < 0.05$ .

### 3. Results and discussion

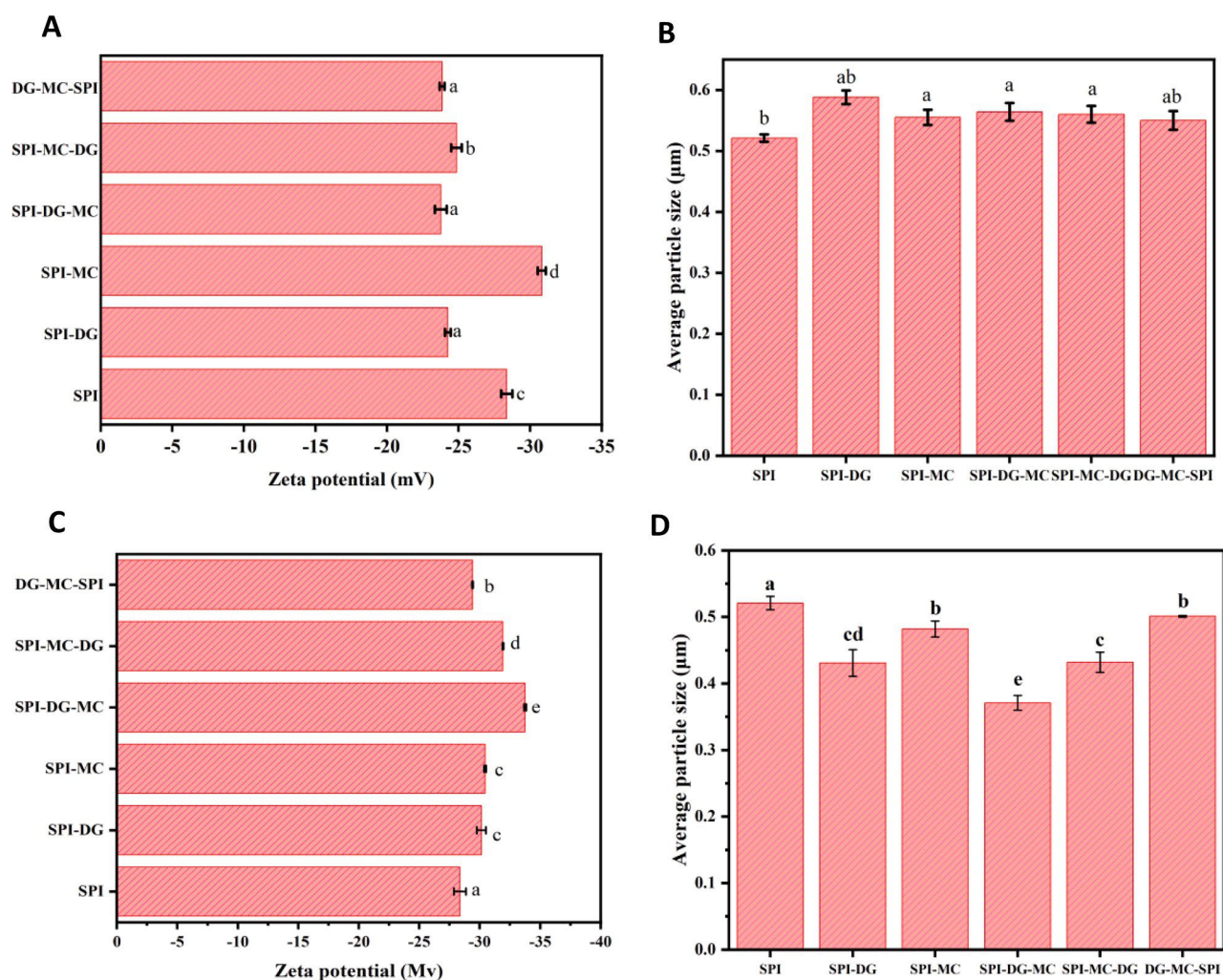
#### 3.1. Initial Zeta potential and particle size of different emulsions

The Zeta potential, an indicator of the surface charge density of the protein, provides an indication of the potential stability of the emulsion system (Shanmugam & Ashokkumar, 2014). The average particle size is also an important factor for emulsion stability (Feng et al., 2020). In this study, several different types of complexes and emulsions were prepared using different methods, namely binary non covalent complex, binary covalent complex, ternary non covalent complex under different addition sequences, ternary covalent complex as well as emulsion stabilized by individual complexes. As shown in Fig. 1, the Zeta potential of all emulsion was negative, because the isoelectric point of SPI was at about 4.5, and the pH of the emulsion was higher than it to allow protein to adsorb  $\text{OH}^-$  (J. Zhao, Wei, Wei, Yuan, & Gao, 2015). Compared to emulsions prepared with non-covalent complexes, the emulsions prepared by covalent complexes exhibited higher potential and smaller particle size, possibly because that the linkage of polysaccharides and polyphenols with SPI was mainly through noncovalent bonds via electrostatic attraction, hydrophobic interactions, or hydrogen bonding (Wang, Yang, Shao, Qu, et al., 2020). This will result in larger particle size of emulsion droplet and lower potential than that in control group (SPI only).

In the emulsions prepared by non-covalent complexes, the ternary complex group had lower potential and larger particle size than the SPI and binary complex group. These results indicated

that DG and MC were simply adsorbed on the surface of protein, and the addition of DG and MC did not occupy the spatial binding site of SPI (Feng et al., 2020; Lei et al., 2022). However, in the emulsions prepared by the covalent complex, the ternary complex group had larger electric potential and smaller particle size, while the emulsion stabilized by SPI had the largest particle size. It was likely that the covalent reaction products could provide strong steric hindrance to overcome the aggregation between the droplets, and the emulsion forms a larger interface thickness resulting in a protective layer around the droplets (Q. Guo et al., 2020; X. Zhang & Haque, 2015).

In the emulsions stabilized by covalent complexes, the covalent complexation between SPI, DG, and MC led to increase in potential of the composite nanoparticles. This may be due to the reduction of the isoelectric point of SPI after covalent binding to polysaccharide and/or polyphenol. The following institutions have reported similar findings (Wei, Yang, Fan, Yuan, & Gao, 2015), for instance, the potential of milk protein was increased when covalently conjugated with EGCG. Moreover, the absolute value of the potential of SPI-MC-DG-stabilized emulsion was found to be large because the combination with MC could increase the proportion of phenolic hydroxyl group on the surface of SPI, then increasing the absolute value of Zeta potential (Zhu, Lu, Zhu, Zhang, & Yin, 2019), so that the electrostatic repulsion prevented the aggregation between the emulsion oil drops. Once the binding site of SPI was occupied by MC, the excess polyphenols may interact with polysaccharides covalently, which were embedded into protein molecules by chemical crosslinking (L. Chen & Subirade, 2005). As a result, there could be less free polyphenols in the solution, which could change the secondary and tertiary structures of protein (Dai, Sun, Wei, Mao, & Gao, 2018), and alter the charge distribution and spatial size. In summary, **the covalent complex-stabilized emulsions** exhibited better stability when compared to non-covalent complexes. Therefore, different types of emulsions stabilized by different covalent complexes were investigated in the following sections.

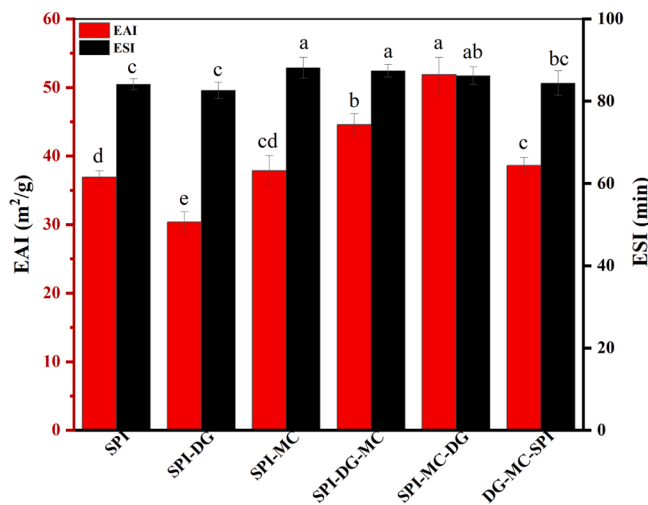


**Fig. 1.** Initial potential and particle size of emulsion, (A) initial potential of an emulsion prepared by a non-covalent complex, (B) initial particle size of an emulsion prepared by a non-covalent complex, (C) initial potential of an emulsion prepared by a covalent complex, and (D) initial particle size of an emulsion prepared by a covalent complex. Note: Soybean protein isolate (SPI),  $\beta$ -glucan (DG), myricetin (MC). Different lowercase letters represented significant differences between samples ( $P < 0.05$ ).

### 3.2. Emulsification performance of different emulsions

Emulsified total activity (EAI) refers to the ability of protein and polysaccharides to form an emulsified layer through adsorption at the oil–water interface. As shown in Fig. 2, the addition of DG and MC could significantly improve the emulsifying activity of SPI. This is because that modification by macromolecular substances can increase the surface hydrophobicity of protein and thus exhibit good emulsifying activity (Wang, Yang, Shao, Liu, et al., 2020).

The stability of the ternary complex-stabilized emulsion was improved to a certain extent, which was consistent with the result of Zeta potential. The SPI treated by high pressure had a high degree of emulsification, but the structure of protein was rapidly damaged. When the supramolecular structure was rearranged, the polysaccharide provided more surface charge, enhancing the electrostatic repulsion between emulsion droplets, while the polyphenols led to enhanced intermolecular interactions, and more hydrophobic sites of SPI will be exposed (Farooq, Ahmad, & Abdullah, 2022). However, significant differences ( $P < 0.05$ ) were found in the emulsifying activity of the ternary complexes prepared from different addition sequences of raw materials. That is to say, the molecular interaction between protein-polysaccharide and protein-polyphenol was diverse, producing different spatial conformation of complex and different degree of exposure on hydrophobic groups. Herein, it was assumed that more steric sites may be created when proteins preferentially interact with polyphenols. Nevertheless, the emulsion stability index (ESI) of the ternary complex-stabilized emulsions was not significantly improved compared to individual SPI.



**Fig. 2.** Emulsification activity and emulsion stability of emulsions stabilized with soy protein isolate and its covalent complex. Note: soy protein isolate (SPI),  $\beta$ -glucan (DG), and myricetin (MC). Different lowercase letters represented significant differences between samples ( $P < 0.05$ ).

### 3.3. Storage stability

#### 3.3.1. $\beta$ -carotene retention rate and potential change

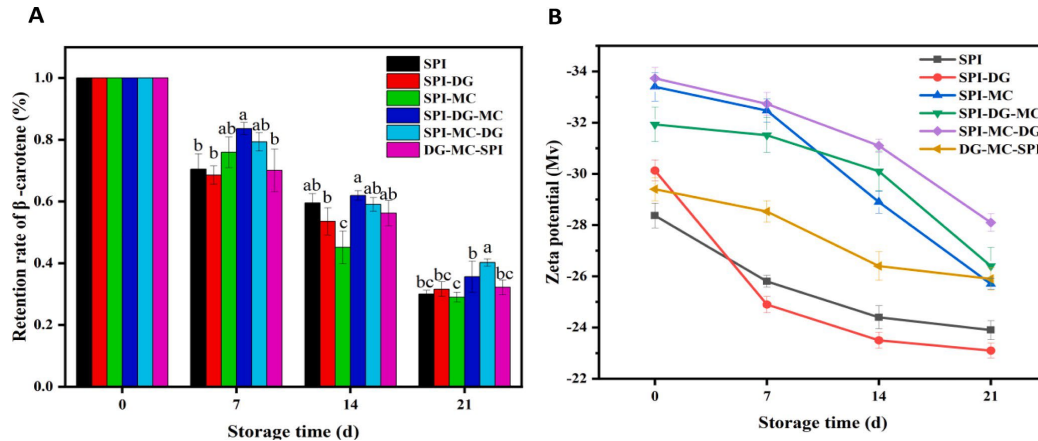
Generally,  $\beta$ -carotene was dispersed in the oil phase of the ternary complex-stabilized

emulsion. As shown in Fig. 3A, the retention rates of  $\beta$ -carotene in six different emulsions varied during 21 days of storage, which were all significantly decreased with the extension of time ( $P < 0.05$ ). Indeed,  $\beta$ -carotene in the flaxseed oil could contact with light and oxygen, which was gradually oxidized and degraded, making the retention rate of  $\beta$ -carotene in oil phase become low. However, the  $\beta$ -carotene retention rate in the emulsions stabilized by multi-component complex was ultimately higher than that in the emulsion stabilized by SPI alone. On one hand, some factors that promote the degradation of  $\beta$ -carotene can be prevented by the covalent complexes, such as the dense protective layer formed by the covalent complexes, to block the entry of oxygen and heat into the interior and prevent the further oxidative decomposition of  $\beta$ -carotene. On the other hand, a certain number of hydroxyl groups or reduced pyrrole groups in the Maillard conjugates of SPI and DG could provide hydrogen atoms, which also exert excellent antioxidant activity (Jimenez-Castano, Villamiel, & Lopez-Fandinno, 2007). In addition, emulsions stabilized by SPI-MC-DG showed the highest retention of  $\beta$ -carotene, probably due to the high grafting ratio of this complex, resulting in more Maillard reaction products and stronger antioxidant activity. Therefore, the covalent conjugation of SPI-MC-DG can improve the performance of SPI and make SPI have better structural stability, so that the SPI-MC-DG stabilized emulsion has improved both physical and chemical stability during storage.

The Zeta potential on the surface of emulsion was mainly dependent on the content of emulsifier on the surface of emulsion droplet. It was generally believed that the higher the absolute value of Zeta potential was, the more stable the system would be. It could be seen that the potentials of the six different types of emulsions were significantly decreased with the prolongation of storage time, indicating that the aqueous and oil phases in the emulsions polymerized during storage, leading to a gradual aggregation of droplets and decrease in surface repulsion. SPI-MC, SPI-MC-DG, and SPI-DG-MC stabilized emulsions maintained relatively high potential, indicating that the surface charges of the stabilized emulsions were relatively high, and the electrostatic repulsion between particles was relatively strong, so that emulsion breaking and aggregation were not easy to occur during storage. The emulsion stabilized by SPI-MC-DG exhibited the highest potential, which further implied that the ternary covalent binding in this sequence could inhibit droplet aggregation and enhance the stability of the emulsion.

### 3.3.2. *Change of milk analysis index (CI)*

It is necessary to measure the degree of delamination that occurs in the emulsion, in which CI is considered as an effective indicator. The difference in density between the lipid and aqueous phase is a key factor leading to the stratification of the emulsion. Table 1 showed the CI changes of the emulsions during 25 days of storage. The SPI and DG-MC- SPI group exhibited the worst stability with relatively high CI values.



**Fig. 3.**  $\beta$ -carotene retention rate and potential changes during storage of SPI and its covalent complex-stable emulsion. Note: soy protein isolate (SPI),  $\beta$ -glucan (DG), and myricetin (MC). Different lowercase letters represented significant differences between samples ( $P < 0.05$ ).

**Table 1**

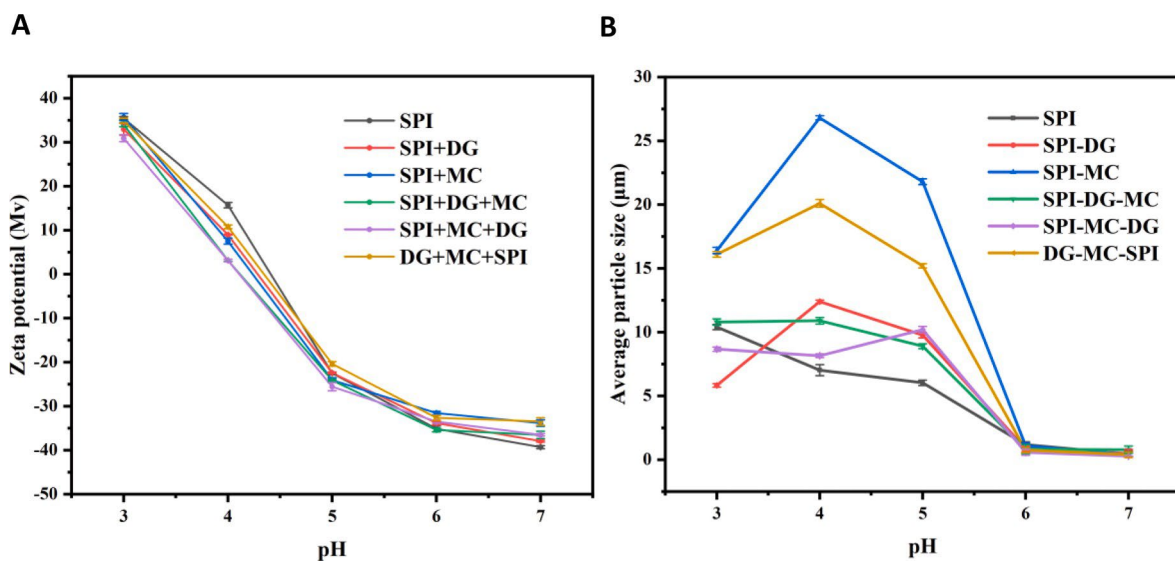
Changes of milk analysis index of different types of emulsions.

CI(%)	Sample					
	SPI	SPI-DG	SPI-MC	SPI-DG-MC	SPI-MC-DG	DG-MC-SPI
<b>0d</b>	0	0	0	0	0	0
<b>5d</b>	0	31.2	0	0	0	10
<b>10d</b>	11.2	62.2	0	0	0	13.5
<b>15d</b>	22.7	73.1	20.5	0	0	50.7
<b>20d</b>	55.8	75	46.5	20.4	41.2	80.6
<b>25d</b>	83.1	75	70.2	62.7	64.3	87

By contrast, the emulsions formed by SPI-DG-MC and SPI-MC-DG complex presented stronger stability during storage, in which SPI-DG-MC group exhibited the best stability with the lowest CI at about 62.7%. Thus, it was illustrated that the more stable complex generates greater spatio- temporal steric hindrance. These observed results were consistent with the previous conclusion, which can be assumed that the improvement in emulsion stability may be due to the formation of gel-like microstructures (Fu Liu & Tang, 2011; Tzoumaki, Moschakis, Kiosseoglou, & Biliaderis, 2011). Additionally, the increase in repulsion between the nanoparticles of ternary complex was considered as another important reason for the improvement of the emulsion stability.

#### 3.4. Effect of different environmental stresses on the physical stability of emulsion

In the emulsion transfer system, droplet aggregation, flocculation and phase separation will occur during the processing and storage, which will seriously affect the sensory results of the foods. Therefore, the emulsion-based delivery system should be well tolerated in the face of processing and storage conditions for commercial foods such as ionic strength, high temperature, pH, etc.



**Fig. 4.** Effect of pH value on emulsion potential and particle size, (A): Effect of pH value on emulsion potential, (B): Effect of pH value on emulsion particle size. Note: Soy protein isolate (SPI),  $\beta$ -glucan (DG) and myricetin (MC).

##### 3.4.1. Effect of pH value on the stability of emulsion

The outstanding problem of protein-stabilized emulsion is the flocculation of emulsion,



especially at the pH near the isoelectric point of protein. Many covalent protein complexes present good acid tolerance compared to natural proteins. However, the stability of the emulsion near the isoelectric point did not achieve the desired effect. As shown in Fig. 4, the particle size of different types of emulsions was significantly affected by pH. When pH was at 4.5, the absolute value of Zeta potential of the emulsion was close to zero, and the mutual repulsion between the liquid droplets was greatly weakened, resulting in the enhanced aggregation. There were two possible explanations for this effect: (I) the decreased electrostatic repulsion between the oil droplets wrapped around the protein, (II) a thinning of the droplet thickness, which led to a decrease in steric hindrance (Gumus, Davidov-Pardo, & McClements, 2016). Emulsion droplets prepared from SPI-MC-DG and SPI-DG-MC conjugates showed good stability at the same pH, indicating that there was an intermolecular repulsion between the complexes which prevented aggregation. The emulsion stabilized by SPI-MC complex was seriously affected by pH, which might be on account of its non-glycosylation degree, weak steric hindrance, and the presence of natural protein and hydrolysates that did not participate in the glycosylation process in the components (Jiménez-Castano et al., 2007). It was further found that the emulsions stabilized by different covalent complexes were significantly different when resistant to acid environment. As expected, the SPI-MC-DG and SPI-DG-MC complex, exhibited better stability.

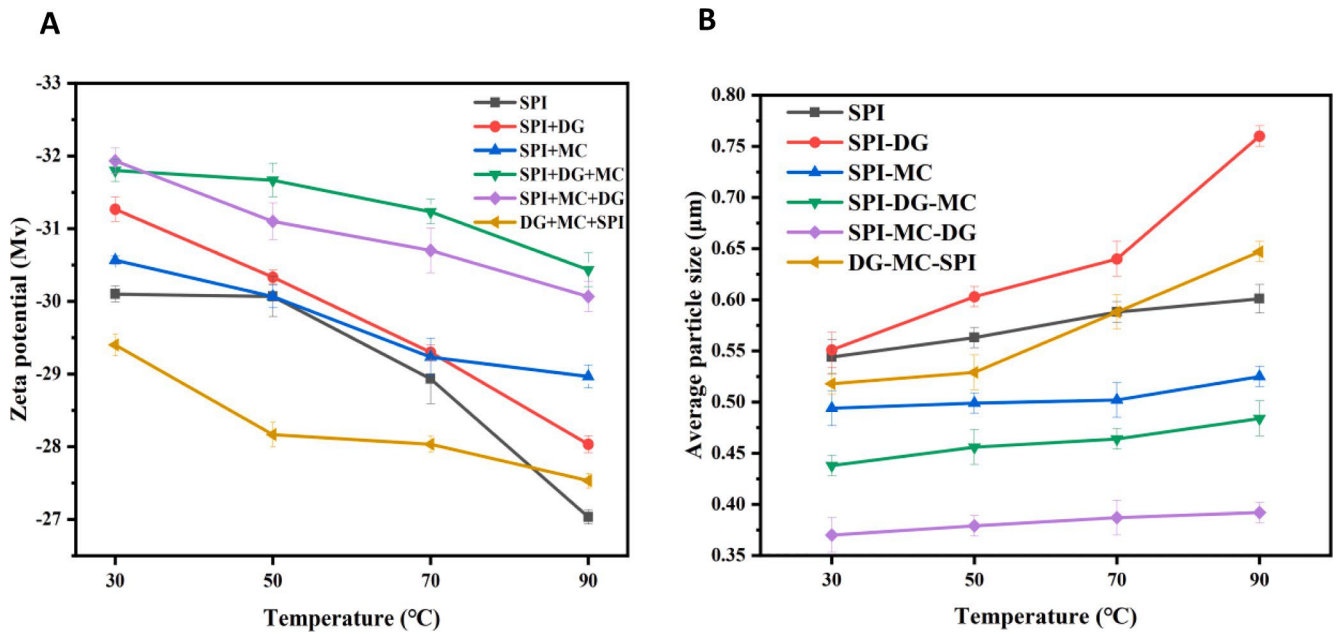
#### *3.4.2. Effect of heat treatment on the stability of emulsion*

Heat treatment can accelerate the movement of dispersed phase droplets, destroy the molecular structure of the emulsifier, and reduce the stability of the emulsion. The effects of different heat treatments on the mean droplet size and Zeta-potential of  $\beta$ -carotene emulsions were investigated at 30, 50, 70 and 90 °C. As shown in Fig. 5, the absolute value of Zeta potential of the emulsions prepared by natural SPI and SPI-DG was decreased significantly after heat treatment. Meanwhile, the average particle size of the emulsion was increased notably with the increase of temperature. This might be because that the heat treatment on globular protein resulted in changes in the spatial structure of protein molecules. The exposed non-polar molecules and disulfide bonds accelerated the droplet aggregation through hydrophobic interaction, and the non-polar groups on the surface of SPI-DG complex were easily exposed, thus enhancing the hydrophobic attraction between emulsion droplets (Salminen & Weiss, 2014). Moreover, the net charge of SPI-DG complex-stabilized emulsion was higher than that of SPI-stabilized emulsion. This phenomenon may be resulted

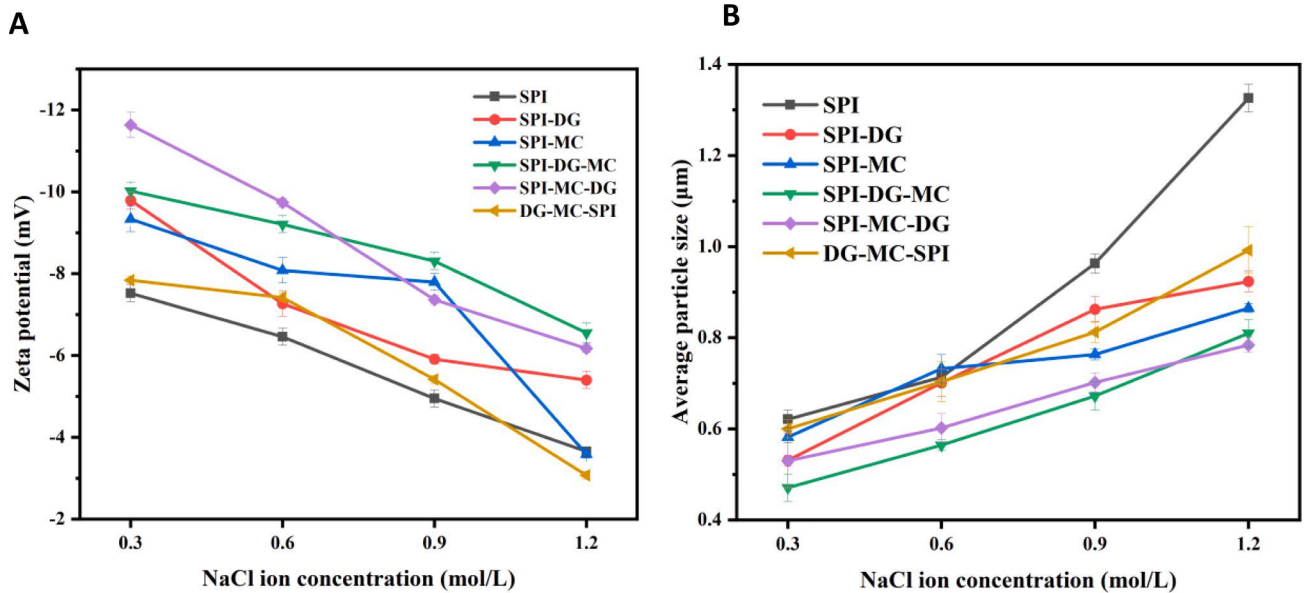
from the loss of ionizable amino groups and the conversion of cationic amino groups to anionic residues, when SPI was linked to DG (W. Liu et al., 2019). It was assumed that once SPI, DG and MC are covalently conjugated, DG and MC are wrapped on the surface of the protein to form a precise protective layer. Furthermore, the surface charge change of the emulsion liquid drop is small, therefore, the average particle size of the emulsion prepared from the SPI-MC-DG and the SPI-DG-MC complex showed good thermal stability. The results also indicated that the grafting degree of SPI-MC-DG and SPI-DG-MC was higher than DG-MC-SPI, so more free molecules were adsorbed on the surface of emulsion droplets, and the spatial repulsion force between the droplets was intensified (Bi, Yang, Fang, Nishinari, & Phillips, 2017).

#### *3.4.3. Effect of sodium ion on the stability of emulsion*

As exhibited in Fig. 6, with the increase of ion concentration, the surface charge of the emulsion droplets was significantly reduced, indicating that the salt ions had a certain shielding effect on the solution or emulsion. After addition of NaCl, the droplet net charge of the SPI- stabilized emulsion decreased significantly and the average droplet size increased sharply. In this case, the charge on the droplet surface was blocked by the salt ions, resulting in the droplet attraction overriding the electrostatic repulsion and droplet aggregation between the small droplets (H. Chen et al., 2018). Similar, but to a lesser extent, trends in particle size and potential charge have been found in emulsions prepared from SPI complexes. This finding further evidenced the stability of the ternary complex-stabilized emulsions. Furthermore, the SPI-DG-MC and SPI-MC-DG complex-stabilized emulsions had relatively small particle size and Zeta potential changes even at the presence of 1.2 mol/L of NaCl. This may contribute to the thick adsorption layer generated by SPI-DG-MC and SPI-MC-DG complexes, which increases the steric hindrance. Simultaneously, the complex can inhibit the electrostatic shielding of salt ions and prevent the decrease of electrostatic repulsion between the emulsion droplets. It was also implied that adding protein to emulsion preferentially may form a multi-layered interface to prevent oil permeation from one droplet to another, thereby inhibiting droplet aggregation.



**Fig. 5.** Effect of temperature on emulsion potential and particle size. (A): effect of temperature on emulsion potential; (B): effect of temperature on emulsion particle size. Note: Soybean protein isolate (SPI),  $\beta$ -glucan (DG), myricetin (MC).



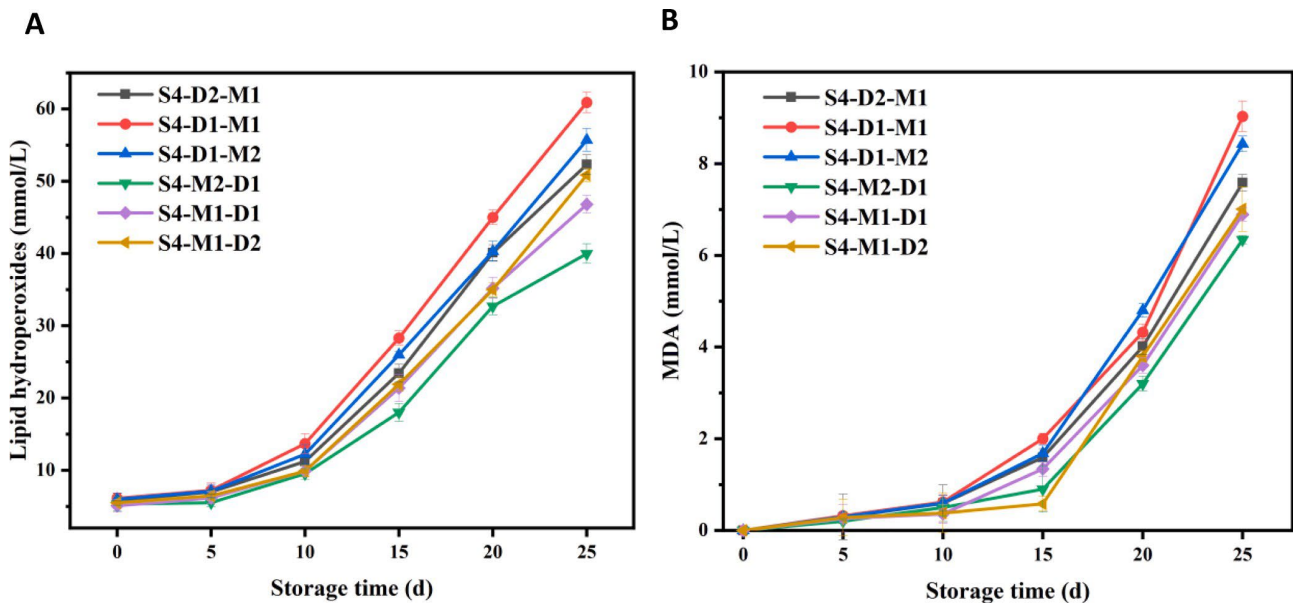
**Fig. 6.** Effect of sodium ion on emulsion potential and particle size. (A): effect of sodium ion on emulsion potential, (B): effect of sodium ion on emulsion particle size. Note: Soybean protein isolate (SPI),  $\beta$ -glucan (DG), myricetin (MC).

To be mentioned, SPI-MC-stabilized emulsion was more stable than SPI-DG, probably due

to the higher grafting yields of poly- phenol to protein, resulting in more grafted molecules extending into the continuous phase of the emulsion. At the same time, the efficient covalent grafting of polyphenol could change the physicochemical properties of protein, and thus having a positive effect on the stability of emulsion. The emulsion prepared with DG-MC-SPI complex was the most sensitive to the ion concentration. On one hand, it had a lower degree of glycosylation, and the modification effect of polysaccharide and polyphenol on protein was not significant. On the other hand, emulsions stabilized by this complex exhibit poor physical stability due to the presence of soluble protein aggregates in the components (Zhong et al., 2019).

In summary, the emulsions stabilized by SPI-DG-MC and SPI-MC-DG revealed relatively excellent storage stability and environmental tolerance. However, the effect of their addition ratio on the stability of emulsion still needs to be further verified. Therefore, different complexes were subsequently prepared and evaluated at ratios of 4:2:1, 4:1:1, and 4:1:2 (protein: polysaccharide: polyphenol, m/m/m), respectively.

### 3.5. Chemical storage stability



**Fig. 7.** Evolution of lipid hydroperoxide (A) and malondialdehyde (B) in the emulsion after 25 days of storage at room temperature under accelerated oxidation at 45°C. Note: The alphabetical order of soya protein isolate (S),  $\beta$ -glucan (D) and prunetin (M) indicates the different order of addition and the numbers indicate the percentage of addition.

Greases are typically present in foods such as dairy products, flavors, beverages and infant formulas as oils, fat bodies or oil-in-water emulsions. When these colloidal systems contain unsaturated lipids or other chemically unstable molecules, oils and fats are easily oxidized. The formation of oil and fat oxidation products will affect the quality and functional properties (especially the sensory properties) of these systems, and may even lead to the accumulation of potentially dangerous products (Zeng et al., 2017). Thus, the development of new stabilizers should not only stabilize the emulsion but also effectively inhibit the oxidation of oil in the product.

The oxidative stability of the ternary complex-stabilized emulsions at different proportions was focused by monitoring the content of lipid hydrogen peroxide (LH) and malondialdehyde (MDA) during storage. As shown in Fig. 7, the levels of LH and MDA in all groups increased, and significantly increased at the 15th day, suggesting that the oil was oxidized during storage. It was found that the emulsion stabilized by SPI-MC-DG exhibited better oxidative stability than SPI-DG-MC group, because when the protein-polysaccharide complex was formed by Maillard reaction, some of the amino groups, cysteine and tyrosine present in protein as well as the carboxylic acid groups in polysaccharide could react with the polyphenol, but the complex formed by this method may easily affect the functional activity of protein. In addition, the SPI- MC-DG emulsion prepared in the ratio of 4:2:1 had the lowest LH and MDA contents. Thus, the emulsion stabilized by the S4-M2-D1 complex has excellent oxidative stability. The formation of S4-M2-D1 complex blocked the chain reaction of lipid peroxidation to remove free radicals by better preventing the interaction of LH and co-oxidant, and thus acted as a barrier to lipid oxidation. Furthermore, the emulsion stabilized by S4-M2-D1 had a stronger, denser interfacial layer as a physical barrier that prevented water droplets from contacting the co-oxidizer in the aqueous phase, which was consistent with previous studies. It has been demonstrated that solid particles present at the water–oil interface blocked the contact between the emulsion and oxygen, so the emulsions with better stability possess better antioxidant properties (Waraho, McClements, & Decker, 2011). In a word, the application of S4-M2-D1 complex could delay the oxidation of lipid in the emulsion system.

### 3.6. *Rheological performance*

The relationship between the viscosity and the shear rate of emulsion was shown in Fig. 8(A). The viscosity of all emulsions decreased with the increase of shear rate and finally stabilized.

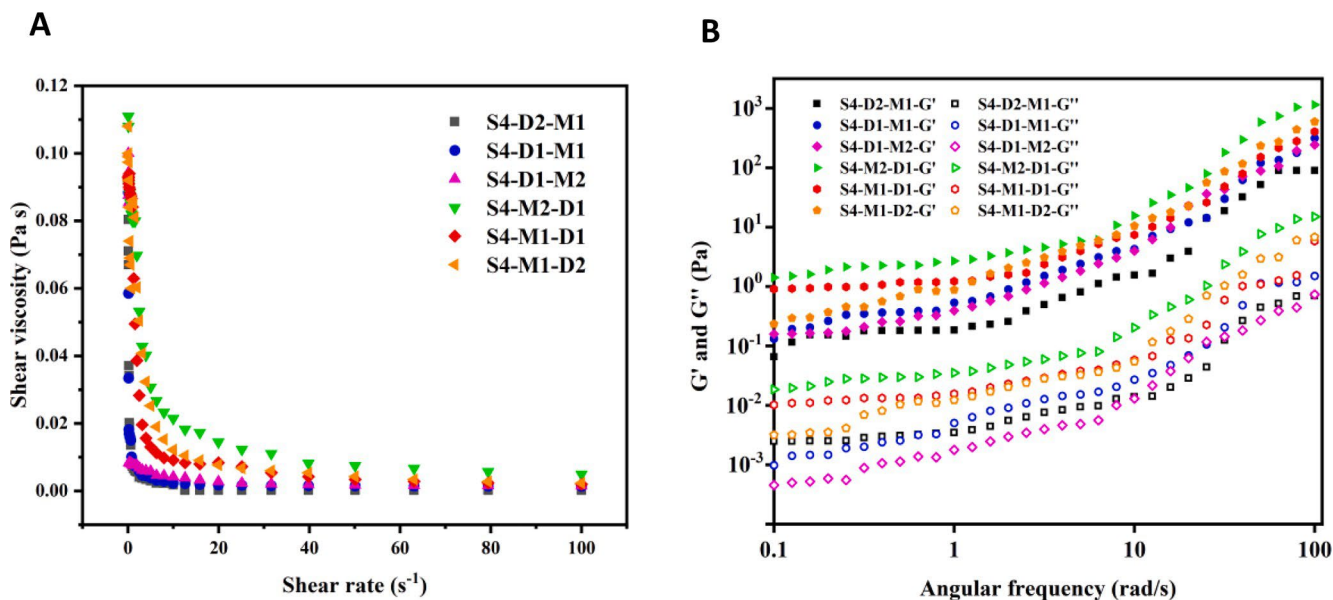
This demonstrated that all samples exhibited significant shear thinning as the internal structure of the droplets was destroyed at high shear rates and tended to rearrange to obtain a new balance in the flow direction. All the nanoemulsions prepared by this method were pseudoplastic fluids, indicating that the deflocculation of oil droplets was caused by the interaction of molecules adsorbed at the oil/water interface (Mohammadzadeh, Koocheki, Kadkhodae, & Razavi, 2013). In addition, the apparent viscosity of the nano-emulsion stabilized by S4-M2-D1 was slightly larger, which might be due to the interaction between protein, polyphenol and polysaccharide, better improving the interfacial properties of the emulsion. It also resulted in the formation of a more compact three-dimensional network film of protein at the interface of the nano-emulsion.

In order to better understand the relationship between the micro-structure and the macroscopic properties of emulsions, the dynamic viscoelasticity was determined. The result in Fig. 8(B) suggested that the storage modulus ( $G'$ ) and the loss modulus ( $G''$ ) of emulsion increased with the increase of angular frequency (0.1 to 100 rad/s) and implied a certain dependence. The  $G'$  of each component was higher than  $G''$ , indicating that the emulsion was less likely to deform under the external force. Furthermore, it was found that the  $G'$  and  $G''$  values of S4-M2-D1- stabilized nanoemulsion were relatively high, manifesting that the gel strength of emulsion was significantly enhanced, which helped to form a closely packed structure (Niu et al., 2018). It seemed that there were more MC molecules adsorbed on the oil/water interface, and the thickness of the interface film was increased, so that the accumulation between the liquid droplets became more compact, and its resistance to deformation was enhanced.

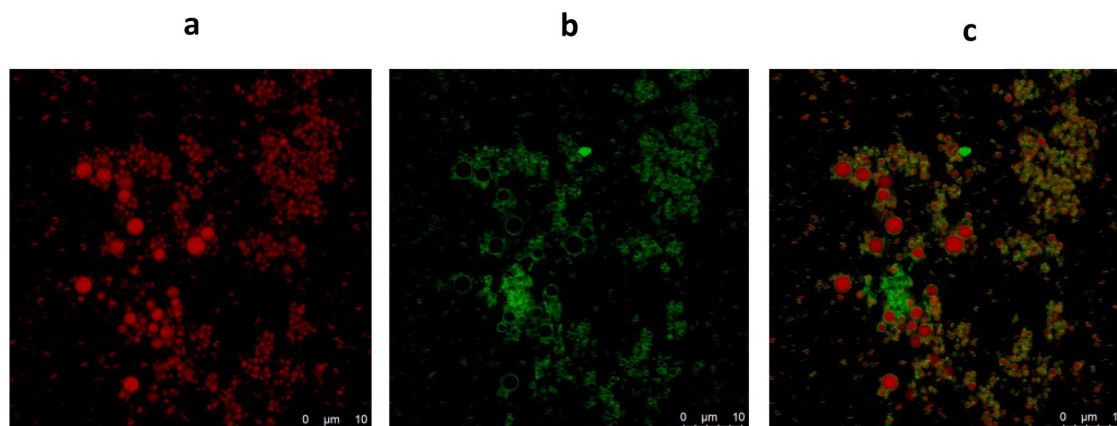
### 3.7. Emulsion microstructure

Laser confocal microscopy (CLSM) is a common tool to observe the interfacial structure and the distribution of proteins in the bulk phase. Fig. 9 revealed the microstructure of emulsion stabilized by S4-M2-D1. The oil and water phases were stained with Nile red and Nile blue, which were shown as red and green areas, respectively. It was obvious from Fig. 9(c) that the red oil droplets were surrounded by the green protein solution, proving that the O/W nanoemulsion was formed, and the droplets were well separated without significant agglomeration. In addition, the liquid droplets in the emulsion were presented as smaller sizes. The smaller liquid droplets have higher interface regions, so that more sites can be provided for the adsorption of protein. The binding effect of oil droplets to the composites

was thus enhanced, resulting in improved stability and elasticity of the emulsions. Moreover, the adsorption of complex on the oil–water interface formed a dense filler layer, which served as a physical barrier to improve the stability of emulsion (Hebishy, Buffa, Guamis, Blasco-Moreno, & Trujillo, 2015). Therefore, it could be concluded that the emulsion stabilized by S4-M2- D1 complex had smaller particle size and more uniform dispersion.



**Fig. 8.** Apparent viscosity diagram (A) and dynamic viscoelasticity diagram (B) of the emulsion with different addition ratios. Note: The alphabetical order of soya protein isolate (S),  $\beta$ -glucan (D) and prunetin (M) indicates the different order of addition and the numbers indicate the percentage of addition.



**Fig. 9.** Laser confocal microscope image of emulsion, oil distribution (a), protein distribution (b), overlapping image (c).

## 1. Conclusion

In this study, a kind of novel emulsion stabilized by protein- polysaccharide-polyphenol ternary complex was successfully prepared. It was demonstrated that the covalent complex-stabilized emulsions exhibited higher surface net charge and smaller particle size than the emulsions stabilized by non-covalent complex. Moreover, the SPI-MC- DG and SPI-DG-MC covalent complex had strong potential to encapsulate  $\beta$ -carotene, with the retention rates of 40.21% and 35.60% after 21 days, respectively. They were also effectively resistant to the environmental stress and had a protective effect on the oxidation of  $\beta$ -carotene. In the addition order and ratio at S4-M2-D1, the antioxidant capacity of the emulsion was significantly improved, with an LH value of 39.98 mmol/L and an MDA value of 6.34 mmol/L after 25 days, which presented better elasticity and distribution uniformity confirmed by its rheological properties and microstructures. Thus, it seems that the emulsion stabilized by S4-M2-D1 complex is an effective delivery system for bioactive components. It implies that the interaction between protein-polysaccharide-polyphenol gives the ternary complex a more stable steric hindrance, thus enhancing the force between the emulsion droplets and improving the overall stability of the emulsion. These results will contribute to the preparation and application of protein- polysaccharide-polyphenol ternary complexes as functional carrier, and further illustrating the interaction mechanism of these food components.

## CRedit authorship contribution statement

**Mengjiao Jian:** Conceptualization, Investigation, Software, Writing – original draft. **Shuyi Li:** Conceptualization, Writing – review & editing. **Zhenzhou Zhu:** Conceptualization, Funding acquisition, Supervision. **Na Zhang:** Data curation. **Qianchun Deng:** Formal analysis, Supervision. **Giancarlo Cravotto:** Supervision.

## Declaration of Competing Interest

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

## Data availability

Data will be made available on request.



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