

Impact of high hydrostatic pressure on the micellar structures and physicochemical stability of casein nanoemulsion loading quercetin

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ABSTRACT

Natural casein is a highly structured protein and the characteristic of self-assembly makes the formation of micelles, thus negatively limiting the applications. High hydrostatic pressure (HHP), as a novel non-thermal process, can modify the structures of protein and improve the related functionalities. In this study, micellar casein was subjected to HHP treatment from 100 to 500 MPa, which then loaded quercetin and formed the nanoemulsion. The thermal, pH, ions and physical stability of nanoemulsion were comprehensively investigated. The results showed 300–500 MPa could effectively disintegrate the micellar structures of natural casein by dissociating colloidal calcium phosphate, which significantly improved the emulsifying activity and encapsulation efficiency. However, 500 MPa caused the nanoemulsion loading most quercetin and subsequently showed the better physical and ions stability in comparison with control and 100–400 MPa. Therefore, HHP is expected to modify the high-order structure of casein, which becomes the ideal nano-vehicles for hydrophobic bioactive substances.

1. Introduction

Casein is the most abundant kind of protein in bovine milk, accounting for about 80% of the total protein content (Dagleish, 2011). It consists of α_1 -, α_2 -, β - and κ -casein, with an average diameter of about 150–200 nm (de Kruif, 1998). Casein can be considered as a highly structured protein with discrete hydrophobic and hydrophilic units, as it tends to show the characteristic of self-assembly, which spontaneously combine to form micelles (Moitzi, Portnaya, Glatter, Ramon, & Danino, 2008). These strong interactions between α - and β -caseins as well as the presence of charged κ -casein contribute to the structural integrity and make them stable against aggregation. Thus, casein micelles are believed to be the natural nano-vehicles and potentially used as the delivery system for drugs and bioactive substances (Ranadheera, Liyanaarachchi, Chandrapala, Dissanayake, & Vasiljevic, 2016). However, the loading capacity of casein is generally lower than expected, especially for embedding those water-insoluble nutrients, even though the interior of the micelle is fairly hydrophobic (Menendez-Aguirre, Kessler, Stuetz, Grune, Weiss, & Hinrichs, 2014). It is due to the micellar integrity maintained by hydrophobic attractive forces and colloidal calcium phosphate (CCP) acting as a bridge, which largely limits the

encapsulation efficiency (EE) of casein. Therefore, artificial caseinates (e.g. sodium caseinate and calcium caseinate) without micelles are more commonly used in the dairy and food industry as an emulsifier instead of natural casein. But with the high demands of food processing and clean label of products, less chemical modifications have been introduced into the natural protein. Hence, it is necessary to explore green physical technologies aiming to improve the encapsulation capacity of casein without using any chemical methods.

In recent years, a large number of studies focused on the applications of high hydrostatic pressure (HHP) technique on protein structural modification (Cepero-Betancourt, Opazo-Navarrete, Janssen, Tabilo-Munizagab, & Perez-Won, 2020; Tran, Lafarge, Pradelles, Perrier-Cornet, Cayot, & Loupiac, 2019; Yao, Jia, Lu, & Li, 2022). HHP is a novel non-thermal and environmentally friendly process, which could break up non-covalent bonds, e.g. ionic, hydrophobic and hydrogen bonds, without affecting the covalent bonds of small molecular compounds. Thus, it is believed to just modify the high-order three-dimensional structures of a protein as well as keeping the original nutrition, color and flavor of nature foods. In a previous study, Bai et al., (2021) demonstrated that HHP treatment (150 MPa) of myosin could improve its emulsifying activity by inducing moderate depolymerization and

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spiral unfolding of myosin structure. Qin et al., (2013) showed that HHP treatment could result in gradual unfolding of walnut protein isolate structure, further enhancing its emulsifying and foaming properties. Besides, the current research has found that the HHP process could largely improve the rehydration behaviors of micellar casein powders via dissociating the CCP connections (Ni et al., 2021). 500 MPa treatment was used to increase the free calcium content of casein solution by two times, demonstrating the effective disintegration of casein micelles. As mentioned above, the integrity of casein micelle might limit its encapsulation capability. This is due to the presence of CCP preventing the exposure of the interior hydrophobic groups of casein micelle, making the interior hydrophobic groups difficult to combine with those water-insoluble nutrients. In that case, HHP shows great potential in restructuring the micelles and creates the possible amphipathic nanoparticles to load the bioactive substances. Nassar et al. (2021) recently used the 100–500 MPa HHP on the micellar casein produced from caprine milk and it found the emulsifying activity of treated sample was significantly ameliorated. However, there still lacks enough evidence to prove the effect of HHP on the structure of micellar casein when used in the delivery system to protect the water-insoluble nutrients.

Quercetin is a natural flavonoid compound present in a variety of fruits and vegetables (Maheshwari, Kumar, Bhadauria, & Mishra, 2022). Studies have shown that quercetin exhibits antioxidant, anti-inflammatory, antiviral, antibacterial and anti-cancer activities, which makes it have a broad application prospect in food, medicine and other fields (Hai et al., 2020; Shanmugam, Ganguly, & Priya, 2022; Xu, Hu, Wang, & Cui, 2019). However, quercetin is classified in Biopharmaceutics Classification System (BCS) Class II, indicating the poor aqueous solubility and the low oral bioavailability greatly limit its commercial value. Therefore, oil–water emulsions or nanoparticles are often used to encapsulate quercetin aiming to enhance the solubility and stability (Cao et al., 2021; Shanmugam, Rengarajan, Nataraj, & Sharma, 2022). Currently, micellar casein has been used to encapsulate quercetin to improve its bioavailability (Ghayour et al., 2019; Penalva, Esparza, Morales-Gracia, Gonzalez-Navarro, Larraneta, & Irache, 2019). However, due to the existence of highly structured self-assembly system, the EE of quercetin is quite poor. Casein, as a nano-sized protein with the hydrophobic nucleus, is expected to be used as the ideal carrier of quercetin after proper HHP processing and regulation (Tang, 2021). Therefore, this study attempts to use the HHP technique to prepare a stable casein nanoparticle system containing quercetin as a model hydrophobic bioactive substance, which is of great significance for expanding the application scope of casein in the food industry.

In this study, micellar casein solution was firstly treated by HHP process from 100 to 500 MPa with a holding time of 15 min. The emulsification activity index (EAI) and the emulsification stability index (ESI) were used to evaluate the casein nanoparticles, which subsequently loaded with quercetin to form the nanoemulsion. Then the particle size, polydispersity index (PDI), Zeta-potential and EE were determined to characterize the nanoemulsion and compare the effect of different HHP. In addition, the thermal, pH, ions and physical stability of nanoemulsion were also investigated, which finally gave the strong evidence of natural casein acting as the nano-vehicles of quercetin by only using a green physical process.

2. Materials and methods

2.1. Materials

Micellar casein (protein content 86.98%, total fat content 1.41% and lactose content 2.85%) was obtained from Ingredia Dairy Experts (Ingredia, Inc., Wapakoneta, OH, USA). Soybean oil was obtained from Shanghai Macklin Biochemical Co., Ltd (Shanghai, China). Quercetin (purity \geq 98%) was obtained from Beijing Jinming Biotechnology Co., Ltd (Beijing, China). Calcium ion standard solution and calcium ionic strength adjustment solution were obtained from Mettler Toledo

International Trade Co., Ltd (Shanghai, China). BSA standard solution (5 mg/mL) and Coomassie brilliant blue G250 were obtained from Solarbio Biotechnology Co., Ltd (Beijing, China). Sodium dodecyl sulfate (SDS), hydrochloric acid (HCl), sodium hydroxide (NaOH) and sodium chloride (NaCl) were all analytical grade reagents, obtained from Shanghai Macklin Biochemical Co., Ltd (Shanghai, China).

2.2. Micellar casein solution preparation and HHP treatment

The micellar casein powders were reconstituted with 40 °C deionized water to produce suspensions with the target concentrations of 5% (w/w) and then magnetically stirred at $14 \times g$ for 12 h. After that, the suspensions were centrifuged at $3000 \times g$ for 10 min, which achieved the micellar casein solution with a solid content of $2.50 \pm 0.02\%$. The solution was sub-packed in polyethylene bags and sealed with a vacuum sealer for HHP treatment (FB-110G5, Litu Ultra High Voltage Equipment Co., Ltd, Shanghai, China). The samples were put into the pressure chamber of the equipment and completely immersed by the pressure transmission medium (distilled water). Five pressure treatments were carried out at 100, 200, 300, 400 and 500 MPa for a holding time of 15 min at 25 °C. The pressure rise rate was 100 MPa/min and the pressure was released instantaneously. The final obtained micellar casein solutions treated with HHP were labeled as MC-100, MC-200, MC-300, MC-400, MC-500, respectively.

2.3. Emulsifying activity and emulsifying stability analysis

The emulsifying activity and stability were analyzed according to the method of Han, Wang, Wang, and Tang (2020) with a slight modification. 80 mL of six casein solutions (control sample, MC-100, MC-200, MC-300, MC-400, MC-500) were mixed with 20 mL of soybean oil (oil volume fraction was 0.2), respectively. The mixtures were subsequently sheared at $5595 \times g$ for 5 min with a temperature of 25 °C to obtain the coarse emulsions. 50 μ L of the coarse emulsion was added into 5 mL of SDS solution (1 g/L) at 0 and 10 min after shearing, respectively. The absorbance of the mixture was recorded at 500 nm. The blank reading was measured in the same way using the SDS solution. The EAI and ESI were calculated by Eq. (1) and Eq. (2), respectively.

$$EAI \left(\frac{m^2}{g} \right) = \frac{2 \times 2.303 \times A_0}{C \times (1-\phi) \times 10000} \times \text{dilution factor} \quad (1).$$

$$ESI (\%) = \frac{A_{10}}{A_0} \times 100 \quad (2).$$

Where, A_0 and A_{10} are the absorbance values at 500 nm at 0 min and 10 min, respectively; C is the volume fraction of oil in the experiment (0.2); ϕ is the concentration of protein (g/mL), and the dilution factor is 100.

2.4. Characterization of micellar casein solution

2.4.1. Turbidity measurement

The turbidity of samples was measured by turbidimeter (WGZ-2000, Youke Instrumentation Co., Ltd, Shanghai, China). Prior to the test, all samples were diluted 50 times with deionized water. Approximately 3 mL of the sample was then added to the glass cuvette for turbidity measurement. Deionized water was used as a blank reference.

2.4.2. Determination of dissociation degree of CCP

The content of free calcium in HHP-treated casein solution was determined by multifunctional pH meter (S220, Mettler Toledo International Trade Co., Ltd, Shanghai, China) and composite calcium ion selective electrode (perfectION™, Mettler Toledo International Trade Co., Ltd, Shanghai, China). Before measurement, a series of concentrations (10, 100, 1000 ppm) of calcium ion standard solutions were used for equipment calibration in order. 50 mL of six casein solutions (control sample, MC-100, MC-200, MC-300, MC-400, MC-500) were mixed with 1 mL of calcium ionic strength adjustment solution, respectively. Following that, the electrode was immersed in the mixture and the

content of free calcium was read from the equipment screen. The content of total calcium in micelle casein was determined by inductively coupled plasma spectroscopy (ICAP 6300, Thermo Fisher Scientific Co., Ltd, Shanghai, China). The dissociation degree of CCP was presented as the percentage of free calcium content to total calcium content.

2.4.3. Determination of nitrogen soluble index (NSI)

The Measurement of NSI was based on a previous study with some modifications (Bradford, 1976). 5 mL of micelle casein solution with HHP treatment was centrifuged at $5000 \times g$ for 10 min. The protein content in the supernatant was determined by the Bradford method and the specific steps were as follows: a series of BSA standard solutions with different concentrations (0, 0.02, 0.04, 0.06, 0.08, 0.12, 0.16, 0.20 mg/mL) was prepared firstly, then a 20 μ L of BSA standard solution or sample supernatant was mixed with a 200 μ L Coomassie brilliant blue G250 staining solution, after the reaction was maintained at room temperature for 3–5 min, the absorbance at 595 nm was recorded. The protein content of the supernatant was calculated according to BSA standard curve, which was made with protein concentrations as abscissa and absorbance values as ordinate. The NSI was expressed as the percentage of protein content in the supernatant to total protein content of the sample.

2.4.4. Solubility measurement

The HHP-treated micelle casein solution was lyophilized into a powder, which was then sieved with a stainless-steel sieve (100 μ m aperture) and stored in a dryer until analysis. 2 g of powder was added into 100 mL of deionized water and then stirred at $14 \times g$ for 2 h. Subsequently, the obtained suspension was centrifuged at $3000 \times g$ for 10 min to separate the undissolved micelle casein solid, which was dried at 105 °C until its mass remained unchanged. The solubility of micelle casein powder was shown as the content of casein dissolved in deionized water, which was calculated based on the mass of the undissolved micelle casein solids.

2.5. Preparation of casein nanoemulsion loaded with quercetin

Soybean oil was used to prepare oil/water nanoemulsion loaded with quercetin. The dispersed phase was prepared by pure quercetin with a loading of 0.08% (w/w) in soybean oil under mild heating (<5 min, 50 °C), and magnetically stirring at $14 \times g$ for 1 h to ensure complete dissolution of quercetin. The continuous phase consisted of six casein solutions (control sample, MC-100, MC-200, MC-300, MC-400, MC-500). The dispersed phase was slowly added to the continuous phase, in which the ratio of continuous phase to dispersed phase was 9:1. After uniform stirring, the mixture was sheared at $5595 \times g$ for 5 min and homogenized for three times at 600 bar to form the oil/water nanoemulsion. The final obtained casein nanoemulsions loaded with quercetin were labeled as MCQ-100, MCQ-200, MCQ-300, MCQ-400, MCQ-500, respectively.

2.6. Characterization of casein nanoemulsion

2.6.1. Particle size, PDI and Zeta-potential

The average particle size, PDI and Zeta-potential of the nanoemulsion were determined using a particle size analyzer (Zetasizer ZEN 3700, Malvern Instruments Ltd, Worcestershire, UK). The refractive index of oil droplets and aqueous solution were set as 1.460 and 1.330, respectively. All samples were diluted 1000 times with deionized water before testing.

2.6.2. EE of quercetin

The EE of quercetin in nanoemulsion was measured following the method described by Tan et al. (2014). The aqueous suspension containing the quercetin nanoemulsion was centrifuged at $5595 \times g$ for 20 min to separate the free quercetin crystals. Then the free quercetin

crystal was dissolved in absolute ethanol and the absorbance of the solution was recorded at 373 nm. Quercetin solutions (0–200 μ g/mL) were used to generate a standard curve. The encapsulation efficiency was calculated according to Eq. (3).

$$EE (\%) = \frac{m_E}{m_I} \times 100 \quad (3)$$

Where, m_E is the mass of quercetin in the nanoemulsion; m_I is the initial mass of quercetin.

2.7. Effects of environmental factors on the stability of casein nanoemulsion

2.7.1. Thermal stability analysis

The nanoemulsion was heated in a boiling bath (100 °C) for 20 min and then cooled naturally to room temperature (20 °C). The sample was diluted 1000 times with distilled water before the analysis. A particle size analyzer (Zetasizer ZEN 3700, Malvern Instruments Ltd, Worcestershire, UK) was used to measure the droplet size, PDI and Zeta-potential of treated samples.

2.7.2. pH stability analysis

The pH value of the casein nanoemulsion was adjusted to 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 with 0.1 M HCl or NaOH solution, respectively. After 12 h of equilibration, the samples were diluted 1000 times with the corresponding pH buffer solution. Subsequently, a particle size analyzer (Zetasizer ZEN 3700, Malvern Instruments Ltd, Worcestershire, UK) was used to measure the droplet size, PDI and Zeta-potential of treated samples.

2.7.3. Ionic strength tolerance analysis

The nanoemulsion was mixed with different concentrations of NaCl buffer to achieve salt concentrations of 100, 200, 300, 400 and 500 mM, respectively. After 12 h of equilibration, the samples were diluted 1000 times with a buffer solution of the corresponding NaCl concentration. Subsequently, a particle size analyzer (Zetasizer ZEN 3700, Malvern Instruments Ltd, Worcestershire, UK) was used to measure the droplet size, PDI and Zeta-potential of treated samples.

2.8. Physical stability analysis

The physical stability was investigated by a stability analyzer (LUMiFuge 111, LUM Instruments Ltd, Berlin, GER), where the sample was centrifuged during the measurement process aiming to accelerate the particle movement and phase separation. Parallel near-infrared waves (880 nm) were used as the penetrating medium. The changes of transmitted light intensity as a function of time and position were recorded to determine the entire process of particle migration behavior. Briefly, 400 μ L of nanoemulsion was uniformly injected into a rectangular container (2 \times 8 mm). The temperature was set to 25 °C and the centrifugal speed was set to 4000 rpm. The characteristic lines of the transmittance of the sample were recorded every 10 s for a total of 360 times. The instability of the nanoemulsion was analyzed by using the “perspective integral” and “phase interface layer tracking” of the detected data.

2.9. Statistical analysis

In this study, all measurements were performed in triplicate, and the results were expressed as mean \pm standard deviation. Origin 2019 (OriginLab Corporation, Northampton, MA, USA) and SPSS 25.0 software (SPSS Corporation, Chicago, IL, USA) were used for plotting and data analysis, respectively. Significant difference analysis was carried out with analysis of variance followed by the Duncan test, and a level of $p < 0.05$ was considered as a significant difference between the two treatments.

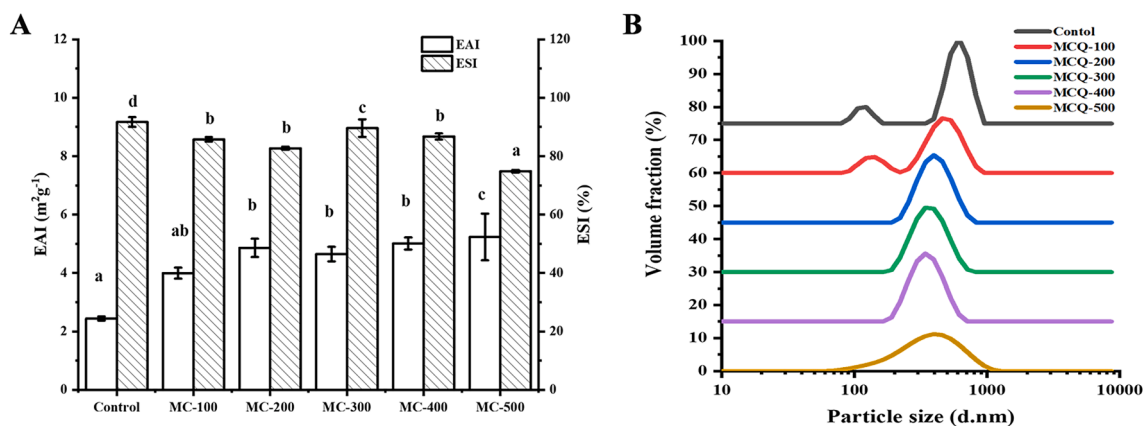


Fig. 1. A: Emulsification activity index (EAI) and emulsification stability index (ESI) of casein treated with different pressure treatments; B: Particle size distribution of six nanoemulsions.

Table 1
Physical properties of casein solutions and casein nanoemulsions.

Sample	Turbidity (NTU)	Dissociation degree of CCP (%)	NSI (%)	Solubility (%)
Control	106.37 ± 0.82 ^f	14.31 ± 0.41 ^a	69.43 ± 0.12 ^a	38.17 ± 0.25 ^a
MC-100	96.53 ± 1.25 ^e	16.44 ± 0.05 ^b	71.36 ± 0.25 ^b	71.60 ± 1.12 ^b
MC-200	61.93 ± 0.80 ^d	21.50 ± 0.42 ^c	73.46 ± 0.18 ^c	82.73 ± 1.13 ^c
MC-300	35.57 ± 0.93 ^c	24.03 ± 0.14 ^d	83.03 ± 0.26 ^d	92.07 ± 0.49 ^d
MC-400	30.62 ± 0.47 ^b	24.06 ± 0.09 ^d	88.45 ± 0.13 ^e	89.60 ± 3.57 ^d
MC-500	23.63 ± 0.44 ^a	28.51 ± 0.96 ^e	90.96 ± 5.46 ^f	83.60 ± 2.21 ^c
Sample	Particle size (d.nm)	PDI	Zeta-potential (mV)	EE (%)
Control	723.24 ± 28.78 ^d	0.540 ± 0.012 ^c	-15.87 ± 0.55 ^a	48.62 ± 1.65 ^a
MCQ-100	499.60 ± 5.73 ^c	0.551 ± 0.060 ^c	-16.13 ± 0.63 ^a	60.00 ± 0.65 ^b
MCQ-200	365.03 ± 13.42 ^b	0.302 ± 0.360 ^b	-17.87 ± 0.42 ^b	72.19 ± 1.45 ^c
MCQ-300	372.00 ± 16.57 ^{ab}	0.277 ± 0.067 ^{ab}	-17.72 ± 0.66 ^b	70.17 ± 0.98 ^c
MCQ-400	356.43 ± 19.30 ^{ab}	0.207 ± 0.068 ^a	-18.03 ± 0.47 ^b	76.49 ± 1.71 ^d
MCQ-500	345.08 ± 9.78 ^a	0.322 ± 0.061 ^b	-18.10 ± 0.79 ^b	79.50 ± 2.08 ^d

The data are shown as mean ± standard deviation. Different lowercase super-script letters in a column indicate a significant difference ($p < 0.05$).

3. Results and discussion

3.1. Emulsifying activity and stability

The EAI and ESI are effective indicators to evaluate the emulsifying properties of protein at the oil–water interface (Xu, Wang, Fu, Huang, & Zhang, 2018). As shown in Fig. 1A, when the pressure increased from 0 to 500 MPa, the EAI of casein samples increased significantly ($p < 0.05$) from 2.4 to 5.2 m^2g^{-1} , which indicated that HHP could improve the emulsifying activity of the casein. This was mainly due to the partial dissociation of casein micelles under high pressure, allowing more amphiphilic micellar fragments access to water and oil, in which hydrophilic residues were oriented to the aqueous phase and lipophilic residues were oriented to the oil phase, so as to reduce the surface tension at the interface. Meanwhile, the decrease in the ESI of HHP-treated casein was also observed. This was attributed to the breakdown of casein micelles induced by HHP treatment, causing the

formation of submicelles with smaller size and larger specific surface area. It could give rise to a thinner interfacial film and less space repulsion, thus reducing the emulsifying stability of casein (Anema, Lowe, & Stockmann, 2005; Cadesky, Walkling-Ribeiro, Kriner, Karwe, & Moraru, 2017; Knudsen & Skibsted, 2010).

3.2. Characterization of micellar casein solution

Table 1 displays the changes in turbidity, dissociation degree of CCP, NSI and solubility of micellar casein solution during pressure treatment. With the increase of pressure to 500 MPa, the turbidity of micellar casein solution decreased significantly ($p < 0.05$) from 106.37 to 23.63 NTU, while the dissociation degree of CCP increased significantly ($p < 0.05$) from 14% to 28%. CCP, as an important component of micellar casein, plays a crucial part in maintaining the structure of casein micelles (McMahon & Oommen, 2013). The dissociation of CCP caused by HHP treatment could lead to the disintegration of casein micelles, resulting in the reduction of casein particle size, which is manifested in the decrease of turbidity of micellar casein solution. The NSI of protein represents the solubility of protein in solvent. In comparison with the NSI of the control sample, the NSI increased by 21% after 500 MPa treatment, indicating that HHP treatment increased the casein content in the supernatant after centrifugation. In addition, the solubility of casein powder pretreated with HHP was significantly ($p < 0.05$) improved, and MC-300 powder showed the highest solubility, which was about 2.5 times that of the control sample. These observations were mainly due to the disintegration of casein micelles caused by the dissociation of CCP, which increased the surface area of casein in contact with water molecules, thus enhancing the hydration of casein.

3.3. Characterization of casein nanoemulsion loaded with quercetin

3.3.1. Particle size, PDI and Zeta-potential

The particle size, PDI and Zeta-potential of six nanoemulsions are listed in Table 1. As the pressure increased, the size of the stabilized emulsion droplet decreased significantly ($p < 0.05$) from 723 nm to 345 nm, PDI decreased remarkably ($p < 0.05$) from 0.54 to 0.32 and the negative value of Zeta-potential increased from -15.87 mV to -18.10 mV. The particle size distribution of the six nanoemulsions is shown in Fig. 1B. When the pressure reached 200 MPa, the particle size of the nanoemulsion changed from a bimodal distribution to a unimodal distribution. However, although the particle size distribution of MCQ-500 showed a single peak, its distribution became wider. These observations suggested that the nanoemulsion prepared from the casein treated with 200–400 MPa was able to form a stable system with smaller particle size and better uniformity. The change in particle size of nanoemulsion may be related to the decrease of bound calcium content in the casein

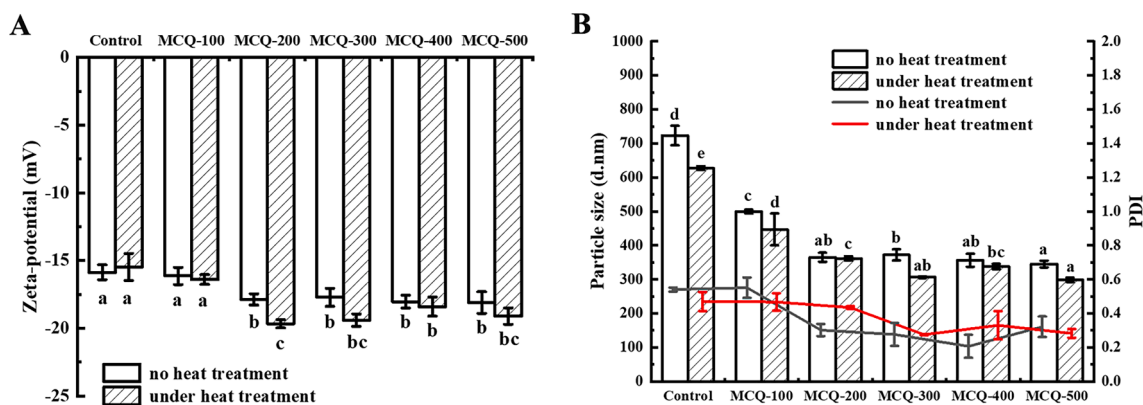


Fig. 2. The effect of heat treatment on six nanoemulsions: A: Zeta-potential; B: Particle size and PDI.

micelles. The study reported by Ye (2011) demonstrated that when the casein micelles in milk protein concentrates were dissociated owing to a decrease in bound calcium content, the emulsifying ability was improved by forming a fine emulsion with a smaller droplet size. Other studies had also shown that casein with lower calcium content could form a more stable emulsion with smaller emulsion droplets (Euston & Hirst, 1999; Singh & Ye, 2020; Ye & Singh, 2001). In this study, it has been proved that HHP treatment could increase the dissociation degree of CCP (Table 1), which meant HHP could reduce the bound calcium content in casein micelles. Therefore, the decrease of particle size of nanoemulsion was closely associated with the dissociation of CCP in casein micelles after HHP treatment.

3.3.2. EE of quercetin by nanoemulsion

The EE of six nanoemulsions on loading quercetin is illustrated in Table 1. The EE of the control sample was only 48.9%, which indicated that the natural casein could be used in the emulsion to load quercetin to a certain extent, but the EE was not satisfactory. However, this kind of efficiency was remarkably improved after HHP treatment, and the EE of MCQ-500 was increased into nearly 80%, which indicated that HHP process could help casein to bind more quercetin into the emulsion droplets. As a nano-scale protein with the hydrophobic core, casein can be potentially used as an ideal delivery carrier for hydrophobic active ingredients after proper processing and regulation (Tang, 2021). Menendez-Aguirre et al. (2014) investigated that the content of vitamin D2 loaded in casein micelles was about five-fold higher after being treated at 600 MPa and 50 °C. Chevalier-Lucia, Blayo, Gracia-Julia, Picart-Palmade, and Dumay (2011) found that high-pressure homogenization (100–300 MPa) enhanced the binding of α -tocopherol acetate to casein micelles, due to the micellar size decreased significantly as the

pressure increased, while the binding rate to α -tocopherol acetate increased. These observations were similar to our results, revealing that HHP pretreatment could induce the partial dissociation of micelles and thus improve the EE of casein on loading hydrophobic active substances.

3.4. The stability of casein nanoemulsion

3.4.1. The thermal stability of nanoemulsion

The food emulsion system may be affected by thermal treatment such as heat sterilization during processing, so it is necessary to study the changes of the six nanoemulsion systems under thermal treatment. Fig. 2 shows the changes in Zeta-potential, particle size and PDI of six nanoemulsions after treated in a boiling water bath (100 °C) for 20 min. As can be seen from Fig. 2A, there was no significant change in the Zeta-potential of the control sample and MCQ-100 after heat treatment, while MCQ-200 to MCQ-500 showed an increase, suggesting that strong electrostatic repulsion existed between emulsion droplets. It might prevent aggregation between droplets and kept the emulsion stable (Zhou, Zheng, & McClements, 2021). Besides, the particle size of the nanoemulsion decreased slightly after heat treatment, and the PDI of the nanoemulsion was mostly kept in the range of 0.2–0.5 (Fig. 2B), indicating that no flocculation occurred between the droplets. These results all confirmed that the casein nanoemulsion had good thermal stability. This was mainly because casein itself had a high thermal denaturation temperature of about 140 °C due to the lack of tertiary structure (Wang et al., 2013; Broyard & Gaucheron, 2015). Therefore, when casein was used as a delivery system, it could withstand high temperatures to some extent.

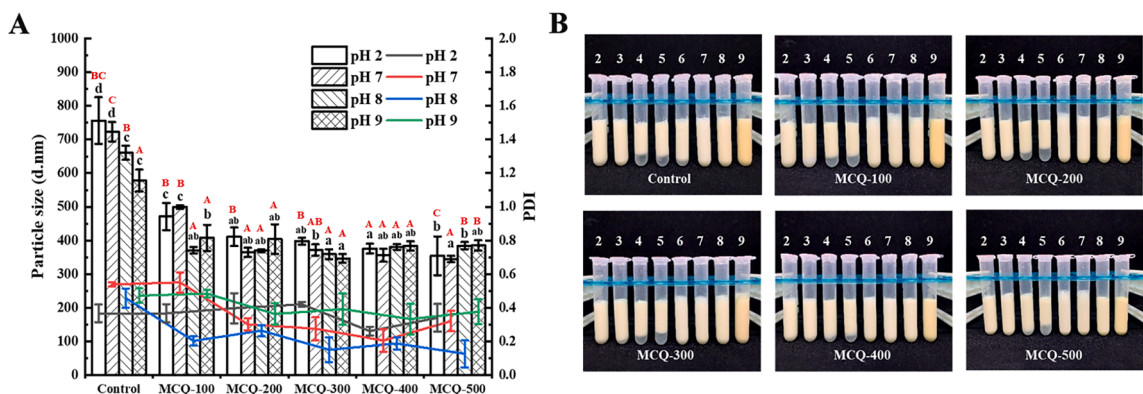


Fig. 3. The effect of pH on six nanoemulsions: A: Particle size and PDI; B: Appearance. Each picture in Fig. 3B corresponds to the appearance of samples at pH 2, 3, 4, 5, 6, 7, 8, 9 from left to right. Different uppercase letters indicate significant differences ($p < 0.05$) among different pH under the same pressure. Different lowercase letters indicate significant differences ($p < 0.05$) among different pressures in the same pH.

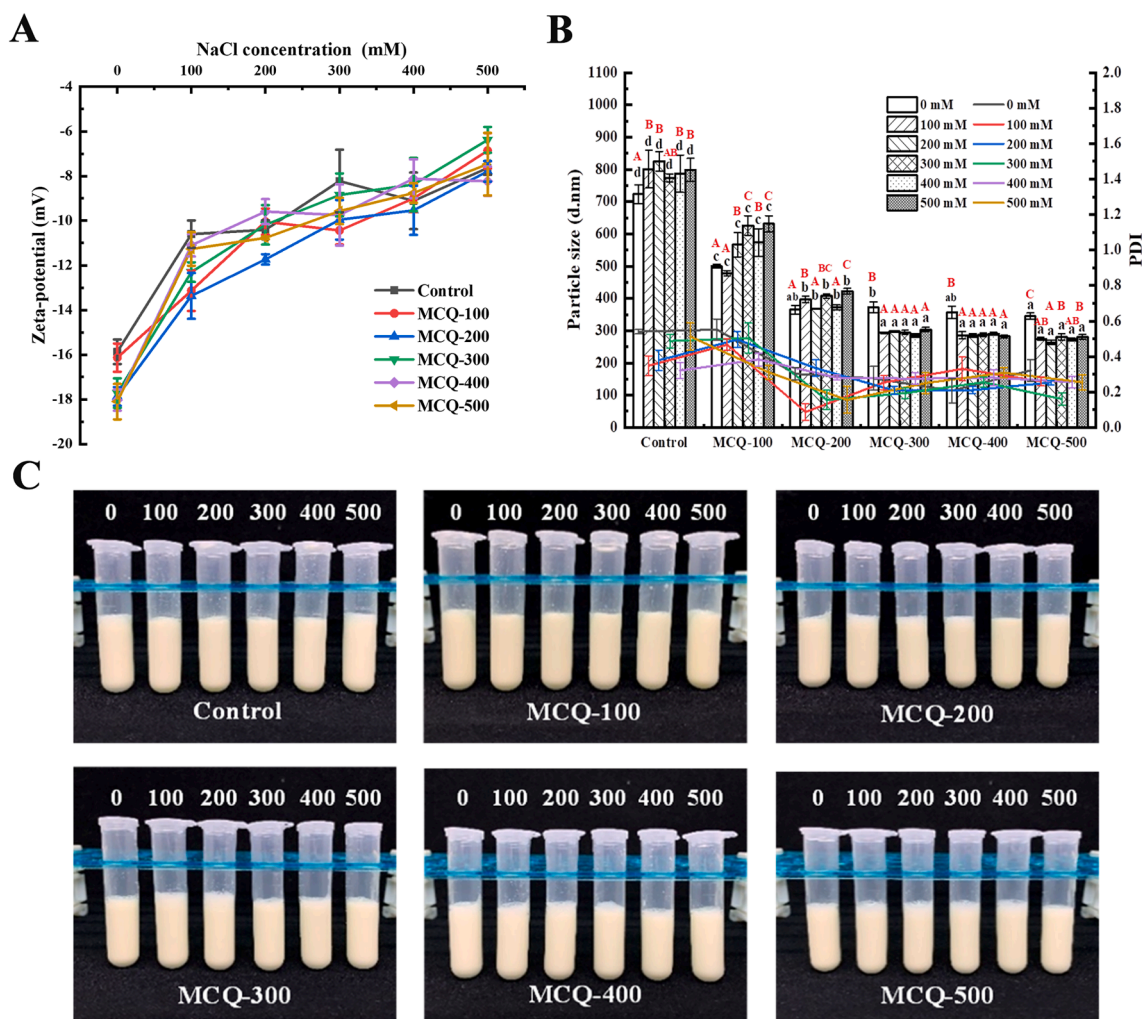


Fig. 4. The effect of ionic strength on six nanoemulsions: **A:** Zeta-potential; **B:** Particle size and PDI; **C:** Appearance. Each picture in Fig. 4C corresponds to the appearance of samples with sodium chloride buffer concentrations of 0, 100, 200, 300, 400, 500 mM from left to right. Different uppercase letters indicate significant differences ($p < 0.05$) among different ionic strengths under the same pressure. Different lowercase letters indicate significant differences ($p < 0.05$) among different pressures in the same ionic strength.

3.4.2. The pH stability of nanoemulsion

Food emulsion systems are usually subjected to different pH environments during food processing, so it is of great significance to investigate the stability of casein nanoemulsions at different pH values. Fig. 3 presents the corresponding particle size, PDI and appearance of six nanoemulsions. As shown in Fig. 3A, the particle size of the control sample had an obvious downward trend with the increase of pH value. Conversely, there was no remarkable change in the particle size of nanoemulsion prepared from HHP-treated casein, showing that HHP was conducive to improving the pH stability of casein nanoemulsion. According to Fig. 3B, the emulsion stratified when pH value was 4 and 5, and flocculated when pH was 3 and 6. This was because the pH was close to the isoelectric point of casein (pH 4.6), resulting in the weakening of electrostatic repulsion between the emulsion droplets. Furthermore, the color of nanoemulsion darkened when pH was adjusted to 9.0, which was related to the rapid dissolution and degradation of free quercetin. It was reported that the phenolic hydroxyl group of quercetin would be deprotonated and negatively charged under strongly alkaline conditions, which could greatly improve the water solubility of quercetin (Peng, Zou, Zhou, Liu, Liu, & McClements, 2019). In addition, compared with the color of the control sample under pH 9.0 conditions, the color of MCQ-300 to MCQ-500 became lighter, indicating that more quercetin was embedded in casein micelles after HHP treatment, which was

consistent with the EE measured earlier (Table 1).

3.4.3. The ionic strength stability of nanoemulsion

Studying the stability of emulsions at different ionic strengths is an important basis for further application in different food matrices. Fig. 4 shows the effects of different ionic strengths on the Zeta-potential, particle size, PDI and appearance of six nanoemulsions. The absolute value of Zeta-potential decreased with the increase of ionic strength (Fig. 4A), which was attributed to the electrostatic shielding effect caused by the addition of NaCl buffer. According to Fig. 4B, the particle size of the control sample and MCQ-100 increased as the ionic strength increased. It was also due to the addition of salt ions shielding the surface charge of the emulsion droplets, resulting in a decrease in electrostatic repulsion between droplets, and causing droplet aggregation. Nevertheless, for MCQ-300 to MCQ-500, even if there existed a strong electrostatic shielding effect when the ionic strength was 500 mM, the particle size showed a decrease instead of an increase. This was mainly because the particle size reduction effect resulting from the dissociation of CCP was greater than the particle size increase effect resulting from electrostatic shielding. In this study, it has proved that the dissociation of CCP of casein could be caused by HHP treatment. Meantime, the study of Famelart, Le Graet, and Raulot (1999) showed that the increase in NaCl concentration of casein micelle suspension could also lead to the

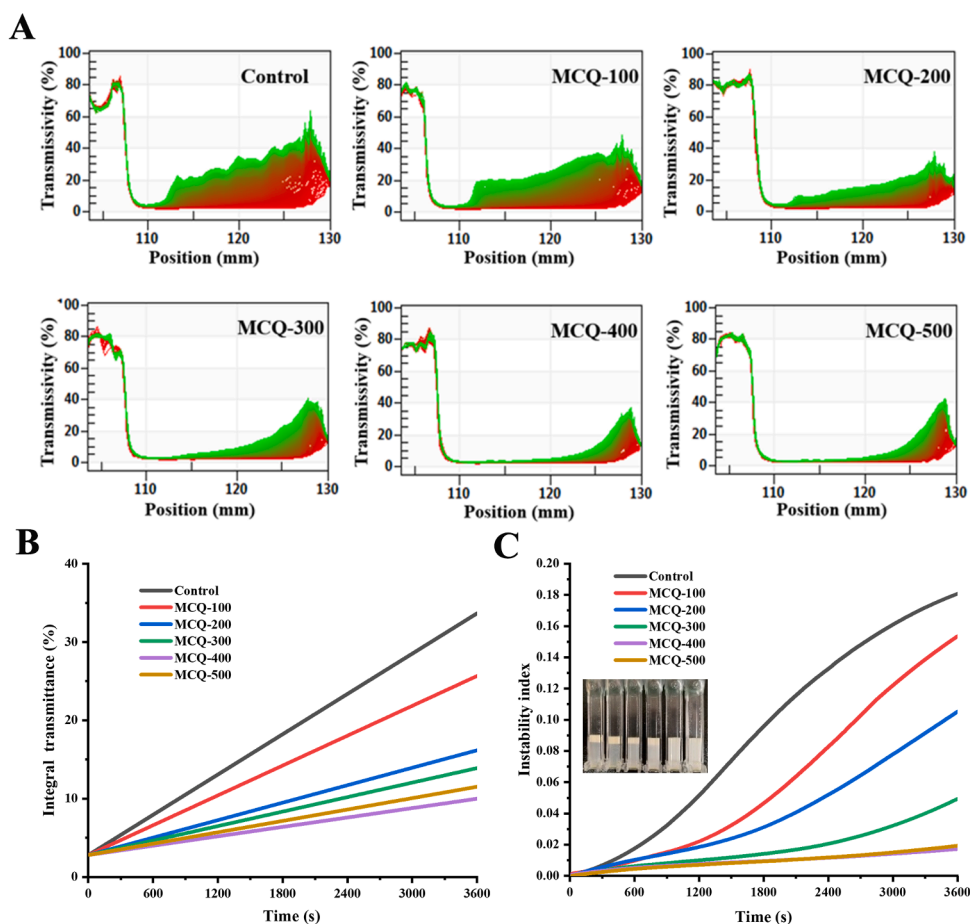


Fig. 5. The effect of different pressure treatments on the physical stability of six nanoemulsions: **A:** Transmission distribution; **B:** Integral transmittance; **C:** Instability index.

further dissolution of calcium and phosphorus in the micelles. Moreover, when the NaCl concentration increased from 100 to 500 mM, no significant change in the particle size of MCQ-300 to MCQ-500 was observed, and the PDI value was below 0.35, indicating that the nanoemulsion can remain stable under different ionic strengths. These observations were consistent with the appearance of nanoemulsions shown in Fig. 4C, all of which could maintain a uniform and stable opaque state.

3.5. Physical stability of nanoemulsion

Based on the food industrial application, it is of great necessity to evaluate the physical stability of the nanoemulsion, which largely affects the shelf life of the product. In this study, the dynamic changes in light transmittance of nanoemulsion during the centrifugation process were continuously recorded. The physical stability was quantified and evaluated by the Integral Transmittance and Instability Index that related to centrifugation time. As exhibited in Fig. 5A, the changes in transmittance detected at any point of the sample cell were small in the initial stage of measurement. This revealed that nanoemulsion could maintain strong stability at first. Nevertheless, with the increase of centrifugal time, the transmittance at the bottom of the sample cell gradually increased. It was due to the stratification of nanoemulsion, wherein the water phase with higher density moved to the bottom and the oil phase with lower density moved to the top. In addition, it could be further found that the degree of change in transmittance of MCQ-300 to MCQ-500 was smaller than that of the control sample, MCQ-100 and MCQ-200. This indicated that nanoemulsions prepared from casein

treated with 300 MPa and above had better physical stability. This observation was consistent with the results shown in Fig. 5B and 5C that MCQ-300 to MCQ-500 had lower integral transmittance and instability index. Meanwhile, the stratification degree of MCQ-300 to MCQ-500 significantly reduced (Fig. 5C), which could also further prove that HHP treatment (≥ 300 MPa) can significantly enhance the stability of the casein nanoemulsion.

4. Conclusion

In this study, HHP has been demonstrated to show a significant impact on the micellar structure and emulsifying ability of natural casein. It could effectively disintegrate the micellar structures of natural casein by dissociating colloidal calcium phosphate, which largely improve the emulsifying activity and encapsulation efficiency. 300 and 400 MPa treatments caused the formation of casein nanoparticles with the uniformed particle size distributions, while 500 MPa lead to the nanoemulsion loading most quercetin and subsequently showed the best physical, ions and thermal stability. This was conducive to improving the shelf life of casein nanoemulsion and expanding the application prospect of casein-based products.

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.

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