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1 **Understanding of the key factors influencing the properties of**  
2 **emulsions stabilized by sodium caseinate**

3

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18 **Abstract**

19 Emulsions can be easily destabilized under various conditions during preparation and  
20 storage. Therefore, it is necessary to understand the factors that influence the stability  
21 of emulsions, which is essential for their subsequent studies. Sodium caseinate (CAS)  
22 is a well-used nutritional and functional ingredient in emulsion preparation due to its  
23 good solubility and emulsifying properties. CAS-stabilized emulsions can be  
24 considered good food emulsion delivery systems, but their applications are still  
25 limited under certain conditions due to their instability to creaming and aggregation.  
26 Therefore, the purpose of this review is to provide a complete overview of how  
27 different environmental stresses and processing conditions affect the stability of  
28 CAS-stabilized emulsions and how to improve their stability. Initially, the general  
29 properties of CAS as emulsifiers and the characterization of CAS stabilized  
30 oil-in-water (O/W) emulsions were summarized. Secondly, the major instability  
31 mechanisms that operate in CAS-stabilized emulsions were presented. Furthermore,  
32 the general factors like pH, emulsifier concentration, ionic strength, oxidation and  
33 processing conditions, affecting the stability of CAS-stabilized O/W emulsion were  
34 discussed. On this basis, the commonly used methods for evaluating emulsion  
35 stability are introduced. Finally, state-of-the-art strategies to improve CAS-based  
36 emulsion stability are also described and summarized. This review is expected to  
37 provide a theoretical basis for the future applications of CAS in food emulsions.

38 **Keywords:** Sodium caseinate; Emulsion characterization; Emulsion stability;

39 Instability mechanisms

## 40 **1. Introduction**

41 Food emulsions, a dispersion systems composed of two or more incompatible  
42 phases, play an essential role in food systems since they directly affect the color, the  
43 texture, and the mouthfeel of food products (McClements, 2015). Typically,  
44 emulsions can be prepared in the presence of emulsifiers, which are usually composed  
45 of hydrophilic and hydrophobic parts to reduce the interfacial tension at the O/W or  
46 W/O interface. Until now, synthetic emulsifiers food additives, like sorbitan esters,  
47 sugar esters, and polysorbates, are still intensively applied in the most frequently  
48 consumed foods although some reports showed their possible harmful effects on  
49 intestinal barriers and microbiota (Csáki & Sebestyén, 2019). Currently, there is a  
50 growing worldwide trend in the food field to replace commercial synthetic emulsifiers  
51 with natural “label-friendly” ones. Consequently, food manufactures have focused on  
52 identifying and characterizing natural emulsifiers, such as proteins, lipids,  
53 polysaccharides, saponins, etc. (Dammak et al., 2020), that can be labeled as “clean”  
54 in emulsion-based food products. Some food proteins from different sources like milk,  
55 whey, eggs, legume, oilseed, and so on (Kim et al., 2020) have been shown to act as  
56 natural emulsifiers by adsorbing at oil droplets because of their good solubility and  
57 amphiphilic ability.

58 At present, the most widely applied commercial protein-based emulsifiers are  
59 casein proteins (Wang et al., 2013), which are generally sold industrially in the form  
60 of sodium caseinate (CAS). CAS are composed of all casein fractions:  $\alpha_{s1}$ - and

61  $\alpha_{s2}$ -caseins,  $\beta$ -casein, and  $\kappa$ -casein, which have strong amphiphilic properties and  
62 have ability to rapidly reduce the O/W interfacial tension during emulsification  
63 process (Walstra & Jenness, 1984). Different techniques, such as high-pressure  
64 homogenization (Liao et al., 2021), high ultrasonic (Wan et al., 2022) and  
65 microfluidizer (Ebert et al., 2017), etc. have been employed to produce stable  
66 CAS-based O/W emulsion systems. Nevertheless, CAS-based emulsions are prone to  
67 instability at certain unfavorable compositional and environmental conditions, such as  
68 different solution conditions (pH, protein concentration, ionic strength,  
69 homogenization conditions) and different environmental stresses (thermal processing  
70 and oxidation). These specific conditions limit the application of CAS-based  
71 emulsions in certain high-value fields. In recent years, several reviews have  
72 summarized various formulations of nanoemulsions, preparation methods, food  
73 applications, and methods to improve stability through using mixed emulsifiers,  
74 protein-polysaccharide complexation or conjugates (Dammak et al., 2020; Evans et al.,  
75 2013; Kim et al., 2020; McClements, 2007; Ribeiro et al., 2021). In this context, it is  
76 necessary to understand the factors that influence the stability of emulsions.  
77 Nonetheless, there are still few review papers that have systematically explored the  
78 factors affecting CAS-stabilized emulsions. In the present review, we will provide  
79 general factors affecting the stability of CAS-stabilized O/W emulsions, such as  
80 environmental stress and process conditions, and the relative importance of the  
81 various instability mechanisms of CAS-based emulsions as well as the relationships  
82 between them. In addition, the methodology for detecting emulsion stability and how

83 to improve the stability of CAS-stabilized food emulsions will be discussed.

## 84 **2. Sodium caseinate and its emulsifying ability**

85 CAS shares similar composition with casein micelles, which is a partially  
86 aggregated mixture of four individual caseins,  $\alpha_{s1}$ - and  $\alpha_{s2}$ -caseins,  $\beta$ -casein, and  
87  $\kappa$ -casein. In industry, CAS is generally manufactured by acid precipitation of casein  
88 micelles and then neutralized with alkali for spray-drying (Wusigale et al., 2020). By  
89 adding the cation of the alkalizing agent, various types of caseinate could be produced,  
90 such as sodium, calcium, potassium, and ammonium caseinate. Among those, sodium  
91 caseinate (CAS) is more commercially available. The aqueous suspension of CAS has  
92 been reported to be a particle suspension with a weight-average molar mass of  
93 1200–4700 kDa and a diameters range of 50 to 120 nm (Wusigale et al., 2020). As an  
94 important dairy ingredient, CAS can endow food with certain functionalities in terms  
95 of water solubility, viscosity, emulsification, foamability and encapsulation ability.  
96 The application of CAS as an alternative source of food emulsifiers in the food  
97 industry has attracted considerable attention, such as coffee whiteners, cream liqueurs  
98 and whipping ingredients (Euston & Goff, 2019).

99 Compared to the most other food proteins, CAS is more disordered and  
100 hydrophobic with an extremely open and flexible conformation (Dickinson, 1999).  
101  $\alpha_{s1}$ -casein and  $\beta$ -casein are the two main individual caseins that account for  
102 approximately 75% of the total milk casein. Both casein proteins have been shown to  
103 be the main contributors to the emulsifying properties of CAS and they can rapidly

104 adsorb at the O/W interface during emulsification to protect newly formed droplets  
105 against incipient flocculation and coalescence. This is the fact that the high proportion  
106 of proline residues disrupts  $\alpha$ -helix and  $\beta$ -sheet secondary structures and there are  
107 almost no cysteine residues in  $\alpha_{s1}$ - and  $\beta$ -casein (Dickinson, 1994). Even though both  
108 caseins proteins exhibited strong tendency to adsorb at hydrophobic surface, the  
109 mechanisms of  $\alpha_{s1}$ -casein and  $\beta$ -casein acting on the oil-water interface are  
110 significantly different. Dickinson (1988) compared the adsorption of  $\alpha_{s1}$ - and  $\beta$ -casein  
111 in oil-water emulsion and the data indicated that  $\beta$ -casein predominates at the  
112 interface since its equilibrium interfacial free energy is slightly lower than  $\alpha_{s1}$ -casein.  
113 The train-loop-tail model has been used to explain the adsorbed layer structure by  
114 CAS (Dalglish, 1996). As shown in Fig. 1a,  $\beta$ -casein develops a highly charged tail  
115 dangling away from the droplet surface as the N-terminal of the chain is the  
116 hydrophilic charged residue, and the remaining parts adsorbed on the surface in the  
117 shape as “trains” and small “loops”, which are predominantly hydrophobic. The  
118 adsorbed layer thickness is mainly determined by the distribution of “loops” and the  
119 dangling “tails” which also provide steric repulsions in the emulsion systems. The  
120 “tails” parts are very sensitive to the solution environmental parameters like pH and  
121 ionic strength, but the hydrophobic parts “loops” of the chain are relatively insensitive  
122 (Dickinson, 1999).  $\alpha_{s1}$ -casein adsorbed layer also develops the “loops” (Fig. 1a) on  
123 the oil-water interfaces as it contains three discontinuous hydrophobic regions  
124 (Shimizu et al., 1986). It was reported that  $\alpha_{s1}$ - casein provides less surface coverage  
125 and thinner layer during emulsification than  $\beta$ -casein (Dalglish, 1996), which



126 inefficiently provides steric repulsions. However,  $\alpha_{s1}$ -casein provides higher  
127 electrostatic repulsions than  $\beta$ -casein since the amino-acid sequence is significantly  
128 different. On the other hand, the hydrophobic plots of amino acid residues in  $\alpha_{s2}$ - and  
129  $\kappa$ -casein indicated that they have surface-active properties (Su & Everett, 2003) and  
130 these caseins could also be adsorbed on the oil-water interfaces and contributes to the  
131 surface stability. Kim et al. (2020) drew a schematic diagram (Fig. 1b) of the  
132 adsorption of 4 casein proteins at the oil-water interface based on the research of  
133 Dickinson (1989), which is mainly divided into 3 steps: (1) Simultaneous adsorption  
134 of  $\alpha_{s1}$  and  $\kappa$ -casein in the initial diffusion stage; (2) Subsequent displacement of  $\alpha_{s1}$   
135 and  $\kappa$ -casein by  $\beta$ -casein from the interface; (3) Formation of a mixture with  $\beta$ -casein  
136 as the main component film. In fact, due to the interactions between casein molecules  
137 and other food ingredients, the characteristics of the CAS layer adsorbed in many  
138 food emulsions are much more complicated than just described.

### 139 **3. The major instability mechanisms in CAS-stabilized oil-in-water emulsions**

140 Generally, a food emulsion system is prepared using a high shear device (e.g.,  
141 colloidal grinding, high-speed agitator and high-pressure homogenizer) in which the  
142 emulsion mixes oil and water in the presence of emulsifiers (McClements, 2015). For  
143 instance, the basic process of preparing a CAS-based emulsion by high-pressure  
144 homogenization is to force a coarse mixture of oil and CAS aqueous solution through  
145 narrow crevices, resulting in cavitation and intense laminar shear to disperse well. In  
146 this process, the casein molecules are adsorbed to the oil-water interface through

147 unfolding and rearrangement, forming a stable interface layer around the oil  
148 molecules. The degree of protein molecules unfolding rely on the flexibility of their  
149 molecular structure, which depends on the strength of the forces that maintain the  
150 secondary and tertiary structures. As CAS has a fairly flexible structure, it can expand  
151 rapidly at the interface and then form an extended layer of about 10 nm (Dalgleish,  
152 1990). The colloidal stability of the CAS-stabilized emulsion systems is eventually  
153 determined together by electrostatic repulsions and steric repulsions caused by all the  
154 interactions between adsorbed casein layers.

155       It is well established that emulsions are thermodynamically unstable, so various  
156 physical and chemical mechanisms (including creaming, flocculation, coalescence,  
157 Ostwald ripening and phase separation, as shown in Fig. 2) occur over time  
158 (Dickinson, 2019). Indeed, these instability mechanisms can occur simultaneously in  
159 CAS-stabilized emulsion systems, and the emergence of one instability mechanism  
160 encourages other mechanisms. When the free energy of surface interactions is  
161 repelling at all droplet surface-to-surface separation values, the emulsion will be  
162 stable. However, when the free energy of interactions has an attraction within a  
163 certain separation range, the droplets will tend to flocculate, which may adversely  
164 affect coalescence and creaming. These instability mechanisms occur in CAS-based  
165 emulsion depending on the overall balance amongst electrostatic forces, steric forces,  
166 and van der Waals forces. Generally, CAS-based emulsions are unstable under  
167 unfavorable compositional and environmental conditions, such as a pH value close to  
168 the isoelectric point (pI) of CAS (pH 4.6) (Bhat et al., 2016), high processing (Mao et

169 al., 2010), high temperatures (Mao et al., 2010), freeze-thaw cycles (L. Wang et al.,  
170 2020) and high ionic strength (Sharma et al., 2017). In the following part, we discuss  
171 several common instability mechanisms in CAS-based emulsion.

172       **Flocculation:** Flocculation is a process in which two or more droplets combine  
173 with each other, but each remains intact. When attractive interactions between  
174 droplets outweigh repulsive interactions, droplets tend to flocculate (Dickinson, 2019).  
175 Due to the Brownian motion, droplets are usually close to each other. The occurrence  
176 of flocculation will depend on the forces acting on the droplets and the time whether  
177 they stay close together for long period time. Under the certain situation, the presence  
178 of flocs is related to visible changes in the appearance of emulsion (Martínez-Padilla  
179 et al., 2021). The concentration of emulsifiers plays an essential role in the stability  
180 of CAS-based emulsions. For example, to avoid flocculation, even emulsions  
181 prepared at pH 7, the oil/protein ratio must be neither too low nor too high. At low  
182 caseinate content, casein molecules are generally shared by two or more oil droplets  
183 in emulsion systems which can lead to bridging flocculation, apparently accompanied  
184 by some coalescence. This type of flocculation is, at least partly, irreversible. In the  
185 presence of an excess of non-absorbent caseins in the continuous phase, depletion  
186 flocculation will occur which is reversible with no measurable change in droplet-size  
187 distribution after dilution (Dickinson et al., 1997). Typically, only strong shear forces  
188 like high-speed homogenization could destroy the bridging flocculation, while  
189 depletion flocculation can be simply disrupted by gentle stirring or dilution. Our  
190 recent study (Liao et al., 2022) supported that depleted flocculation-induced droplet

191 aggregation is reversible. The high content of pectin promoted extensive aggregation  
192 of CSA-based droplets (observed by microscopy), but the droplet size of the emulsion  
193 did not change significantly. In addition, caseinate molecules are sensitive to both pH  
194 and ionic strength. Different pH values and ionic forces in the continuous phase can  
195 affect the dissociation of ionized groups, thus determining the states of flocculation in  
196 emulsion systems. The macroscopic properties of CAS-based emulsions like  
197 appearance, rheology, and sensory property are strongly influenced by the degree and  
198 nature of emulsion flocculation. Generally, the occurrence of flocculation is harmful  
199 which leads to an increase in particle size to speed up gravity separation in some  
200 foods like emulsion-based soft drinks. However, in some specific cases, flocculation  
201 can be used to control the creation of an ideal texture feature. On the other hand, the  
202 flocculation state has a significant influence on other instable phenomena, such as  
203 droplet coalescence, phase separation and Ostwald ripening (Ma & Chatterton, 2021)

204       **Coalescence:** Coalescence is defined as the process of two or more droplets  
205 coming into contact and merging, creating larger droplets. This can happen if two  
206 droplets are very close for a while. As described above, the occurrence of flocculation  
207 in the emulsion is usually accompanied by coalescence. Moreover, the mechanism  
208 and the intermolecular forces of flocculation and coalescence are similar. One is  
209 considered reversible (flocculation), which a strong force can be used to separate the  
210 aggregates. The other is irreversible (coalescence). The occurrence of coalescence in  
211 emulsion systems significantly affects the processing or shelf life of O/W emulsions.  
212 It was reported that appropriate content of CAS-based emulsion systems was very

213 stable towards creaming and coalescence due to the thicker interfacial film formed by  
214 casein proteins (Dickinson et al., 1997). The adsorbed film formed by the emulsifier  
215 may rupture spontaneously during storage. The formation of a thicker water phase  
216 film at the oil-water interface helps prevent droplet coalescence (droplet melting) and  
217 achieve reasonable stability, thus providing the appropriate life for the final emulsified  
218 product (Costa et al., 2019). The larger the droplet diameter, the thinner the film  
219 thickness and the lower the oil-water interfacial tension, the easier the film ruptures to  
220 lead to irreversible coalescence (Fredrick et al., 2010). However, under certain  
221 circumstances, CAS-based emulsions have a tendency to coalesce. An recent study  
222 (Lamothe et al., 2019) reported that, under gastric conditions, CAS-stabilized  
223 emulsions showed extensive fat droplet coalescence, whereas coalescence was very  
224 limited in the whey protein-stabilized emulsion. They attributed this difference to the  
225 fact that CAS has highly flexible and disordered molecules that are more sensitive in  
226 the gastric environment. In an oil-in-water emulsion, the coalescence eventually forms  
227 a layer of oil on top of the solution, called demulsification. In the manufacture of  
228 some products, partial coalescence is desired to obtain the specific properties in  
229 products like ice cream. It contributes to the structure, physical and sensory flavors  
230 like fattiness and creaminess of the final products (Chen et al., 2019).

231 **Gravitational separation:** Emulsions tend to be divided into different layers of  
232 oil and water due to the different densities and the different polarities between oil and  
233 water molecules. Under the effect of gravitational separation, if the density of the  
234 droplets is lower than that of the surrounding liquid, they tend to move upwards,

235 which is termed creaming. Conversely, if they are denser than the surrounding liquid,  
236 they tend to move downward, which is termed sedimentation. This phenomenon is  
237 usually accompanied by emulsion separation, resulting in a droplet-rich cream layer  
238 and a droplet depleted watery layer. Gravitational separation can be used to measure  
239 emulsion stability by visual or optical imaging, but it usually takes a long time. In  
240 addition, the correlation between creaming and bridging flocculation was observed in  
241 the CAS stabilized O/W emulsion (Dickinson et al., 1997). In a dilute O/W emulsion,  
242 the occurrence of bridging flocculation can speed up the creaming of emulsion since  
243 the larger buoyance forces acting on flocs are stronger than those acting on individual  
244 droplets (Dickinson et al., 1997). On the contrary, in a higher concentrated O/W  
245 emulsion, bridging flocculation can retard creaming as a network structure is formed  
246 in emulsion system. Meanwhile, the creaming generally occurs before coalescence,  
247 followed by phase separation, and its degree in O/W emulsion can be described by  
248 creaming index (Onsaard et al., 2006). So, the creaming index can predict the degree  
249 of droplet aggregation: the higher the index, the more the droplet aggregation.

250 **Ostwald ripening:** Ostwald ripening manifests itself as a time-lapse, as the oil  
251 molecules diffuse through continuous phases, smaller droplets in the emulsion system  
252 increase the size of larger droplets at the expense of themselves (Jiao & Burgess,  
253 2003). The driving force in this process is the curvature effect, in which oil molecules  
254 near smaller droplets are more soluble than larger droplets. Prof. Laplace pointed out  
255 that the concave pressure of the curved interface is higher than the convex pressure,  
256 which is proportional to the tension of the interface multiplied by the curvature. For a

257 sphere, the curvature is given by the reciprocal of its radius. As a result, the dispersion  
258 of the smaller particles increases their solubility in the continuous phase, the smaller  
259 particles shrink (eventually disappear), and the larger particles become larger.  
260 Generally, Ostwald ripening is negligible in food emulsions containing low  
261 water-soluble long chain triglycerides. However, when more water-soluble oils  
262 (essential oils, flavor oils, short-chain triglycerides) are used in O/W emulsions,  
263 Ostwald ripening is a major obstacle that should be carefully controlled during  
264 long-term storage (Park et al., 2020). Our previous study indicated that altering the oil  
265 composition by adding medium or long-chain carrier oil before homogenization can  
266 significantly inhibit Ostwald ripening of CAS-based emulsions containing essential  
267 oils (Liao et al., 2021). In addition, to prevent or delay Ostwald ripening in food  
268 emulsions, another effective method is to combine protein emulsifiers with some  
269 amphiphilic polysaccharides to form a thicker interfacial film around the oil droplets  
270 (Artiga-Artigas et al., 2020; Dickinson, 2019).

#### 271 **4. Main factors determining the stability of CAS-stabilized emulsions**

272 Physical instability refers to changes in the spatial arrangement or size  
273 distribution of the emulsifier droplets over time, such as gravitational separation,  
274 flocculation and aggregation, while chemical instability involves changes in the  
275 composition of the emulsion, such as oxidation and hydrolysis (McClements, 2015).  
276 To meet the requirements of industrial applications, researchers must understand the  
277 factors that determine emulsion stability. As reported by McClements (2007),

278 effective methods to prevent adverse changes in food emulsions need to be  
279 determined based on the physical and chemical properties of the ingredients used to  
280 stabilize these emulsions. As we described above, fine O/W emulsions prepared by  
281 CAS as the only emulsifier are very sensitive to the influence of various factors such  
282 as temperature, pH value, ionic strength, calcium ion content, protein / oil ratio, etc.  
283 These most important factors influencing the stability of CAS-stabilized O/W  
284 emulsions will be discussed in the following section.

#### 285 **4.1. Effect of pH**

286 One disadvantage of using CAS as an emulsifier is known to be its limited  
287 applicability in acidified foods (Perugini et al., 2018). It is well known that the  
288 environmental pH has a great influence on protein solubility, surface charge (zeta  
289 potential), and surface hydrophobicity. Casein proteins in milk are very stable systems  
290 that can withstand the stringent conditions imposed during commercial processing  
291 (Bhat et al., 2016). However, they are still destroyed under certain temperature and  
292 pH conditions, especially acid pH (near their isoelectric point: ~ 4.6), resulting in  
293 reduced stability, which is reflected by visible flocculation or serum separation.  
294 Perrechil and Cunha (2010) studied the effect of pH on the stability of CAS stabilized  
295 water-in-oil emulsions and their results showed that the creaming behavior of  
296 emulsions was largely influenced by the pH of the emulsions.

297 As expected, the weakest emulsifying property of CAS was observed around its  
298 isoelectric point (pI) (Wang et al., 2019). When the pH value is far from its pI, the



299 absolute value of zeta potential of CAS-coated droplets increases and then it can  
300 provide enough electrostatic repulsions between droplets against flocculation. This is  
301 the fact that more negatively or positively charged proteins allow for more  
302 protein-solvent interactions and greater diffusion rates to the interface, which also  
303 contributes to increased interfacial tension of particles and the stability of emulsions.  
304 The interfacial tension can also be changed by pH. Amine e et al. (2014) reported that  
305 the interfacial tension of CAS particles is usually lower at alkaline pH than at pH 7,  
306 which contributes to a more expansive structure of the protein at the interface and  
307 then leads to a smaller emulsion size. Dickinson (1999) described that the “tail”  
308 extension around oil droplet formed by  $\beta$ -casein and the maximum extent of the  
309 loop-like region formed by  $\alpha_{s1}$ -casein are diminished as pH is reduced from 7 to 5.5,  
310 resulting in lower steric repulsion. Nevertheless, by mixing other emulsifiers such as  
311 adsorbed polysaccharides (Shi et al., 2021; Surh et al., 2006) and non-ionic  
312 surfactants (Perugini et al., 2018), CAS can also be used in acidic environments to  
313 stabilize emulsions. Perugini et al. (2018) used a non-ionic surfactant (Tween 20) in  
314 combination with CAS as blending emulsifiers of oil/water nanoemulsions to prepare  
315 nanoemulsions and their results showed that the addition of Tween 20 effectively  
316 enhanced the stability of the emulsion under acidic conditions. As shown in Fig. 3, the  
317 effects of pH and pectin type on the zeta potential and creaming stability of O/W  
318 emulsion stabilized by CAS were well investigated (Surh et al., 2006). The addition of  
319 pectin to CAS-based emulsions lead to an obvious change in the pH dependence of  
320 the surface droplet charge. At pH value around the pI of caseinate, the blending

321 emulsifier is negatively charged. Optical microscope measurements from their results  
322 also showed that CAS stabilized emulsion is dispersed well at a pH value higher than  
323 5.0 and aggregates near the pI.

#### 324 **4.2. Effect of ionic strength**

325 As the ionic strength is able to screen the electrostatic repulsions between the  
326 droplets, the presence of certain ions, especially calcium, may affect the interactions  
327 between protein stabilized droplets and induce flocculation (Dickinson, 2019).  
328 Targeted screening can promote aggregation and reduce adsorption at the emulsion  
329 interface, depending on the type and concentration of ions. Generally, protein-based  
330 food products usually need to be used in combination with various minerals in  
331 practical applications to meet different needs. Thus, the effect of different minerals on  
332 emulsion stability should be better taken into account. It has been shown that the  
333 addition of 15 mM  $\text{CaCl}_2$  to a CAS stabilized emulsion caused destabilization  
334 (Dickinson, 2010). Qin et al. (2018) investigated the effect of ionic strength on the  
335 diameter and zeta potential of CAS stabilized emulsion by sodium and calcium ions,  
336 respectively. As shown in Table 1, the addition of NaCl in different concentration (0.0  
337 - 1.1 mg/mL) exhibited no obvious effect on the size and zeta potential of  
338 caseinate-stabilized emulsions, while the addition of  $\text{CaCl}_2$  showed a higher influence.  
339 With the increase of  $\text{CaCl}_2$  concentration, the zeta potential was significantly  
340 decreased which may be caused by higher ionic strength shielding the surface charge  
341 of CAS stabilized droplets. The reduction of electrostatic repulsions between droplets

342 promoted flocculation and then increased the size of droplets. A similar phenomenon  
343 was reported in the study of caseinate stabilized clove oil nanoemulsion (Sharma et al.,  
344 2017). Multivalent counteracting ions such as  $\text{Ca}^{2+}$ ,  $\text{Fe}^{2+}$  or  $\text{Fe}^{3+}$  are more effective  
345 than monovalent ions in promoting emulsion instability, because they are shielded,  
346 electrostatic interactions are more effective, and they can be combined with the  
347 droplet surface, thus reducing the zeta potential (Liang, Matia-Merino, et al., 2017).  
348 Moreover, Dickinson and Davies (1999) found that the addition of calcium salts  
349 before or after homogenization had significantly different effects on the stability of  
350 CAS stabilized emulsions under the same preparing process. These different  
351 behaviors of electrostatic force can be regarded as the changes of charge density and  
352 protein aggregation state, and these changes will further work on the adsorption layer  
353 structure and protein surface coverage.

354 It is worth noting that, in the presence of excessive unabsorbed CAS, the addition  
355 of appropriate amounts of  $\text{CaCl}_2$  to emulsions can eliminate the effect of depletion  
356 flocculation (Ye & Singh, 2001). This is the fact that the addition of ionic molecules  
357 in the aqueous phase results in an increased size of casein aggregates due to the screen  
358 effect, thereby decreasing the amount of depleting unabsorbed proteins.

### 359 **4.3. Effect of emulsifier concentration**

360 The protein concentration plays an important role in the stability, color, nutrition  
361 and taste of the final emulsion. To improve stability against aggregation, it is  
362 significant to make sure that the protein/oil ratio is in the appropriate range even for

363 an emulsion formed at neutral pH and low ionic strength. Otherwise,  
364 protein-stabilized emulsions are susceptible to different types of flocculation during  
365 storage, leading to increased creams or serum separation. As reported by Singh (2011),  
366 with sufficient CAS molecules to cover the oil droplets during homogenization,  
367 emulsions are usually stable for long-term storage against coalescence. However,  
368 when the concentration is relatively low, casein proteins are insufficient to completely  
369 cover the oil-water interfaces during the homogenization process. As a result, casein  
370 molecules are shared by two or more droplets, leading to bridging flocculation. The  
371 effect of CAS concentration on the stability of emulsion through high-pressure  
372 homogenization was carried out by Sánchez & Patino (2005). As shown in Fig. 4,  
373 their results indicated that there is a critical concentration of CAS in the aqueous  
374 phase : when CAS concentrations were above 1% (w/w), the emulsifying capacity  
375 was constant and the size distribution of droplets was monomodal, however, at CAS  
376 concentrations below 1% (w/w), the size distribution of droplets in the emulsion was  
377 bimodal and the emulsions exhibited some degree of flocculation, which was  
378 inhibited to some extent by the presence of Sodium Dodecyl Sulfate (SDS). It is  
379 worth noting that excess of caseinate (>2%, w/w) increased the rate of creaming  
380 which may be attributed to the presence of depletion flocculation. Dickinson (1997)  
381 similarly reported that attractive depletion interactions may exist between pairs of  
382 dispersed particles when a colloidal system contains a large amount (>3.0 wt.%) of  
383 smaller size of unabsorbed molecules. However, a recent rheological study of  
384 nanoemulsions prepared with CAS as the sole emulsifying agent found no evidence of

385 depletion flocculation over a wide range of protein contents (Roulet et al., 2019).  
386 This could be attributed to the relatively small droplet size ( $d_{43} \sim 130$  nm). It has been  
387 reported that sufficiently high protein concentrations may lead to a stronger droplet  
388 network and this network can lead to partial restabilization of the emulsion against  
389 creaming and serum separation (Dickinson, 2019). Liang et al. (2014) investigated the  
390 relationship between creaming behavior and depleted flocculation of CAS-stabilized  
391 O/W emulsions with different contents of CAS (1–10% w/w). At low CAS  
392 concentrations (less than 3% (w/w)), depletion-induced droplet networks formed and  
393 showed fast phase separation. At middle CAS concentrations (3–5% (w/w)), the  
394 increase of non-adsorbed casein concentration increases the number of droplets  
395 involved in the network, thus stabilizing the emulsion structure dynamically to  
396 prevent creaming. Surprisingly, at higher caseinate concentrations ( $\geq 6\%$  (w/w)), the  
397 formation of droplet network shows obvious delay which was probably caused by the  
398 higher continuous phase viscosity decreasing the formation of the droplet network.  
399 These results show that there is an optimal protein concentration for CAS that can  
400 produce good, saturated surface coverage, and maximum emulsion stability against  
401 these instability mechanisms.

#### 402 **4.4. Effect of homogenization process**

403 The stability of an emulsion during storage is closely related to its homogeneity,  
404 especially its size distribution. Indeed, nanoemulsions (diameter  $< 200$  nm) generally  
405 have better physical and chemical stability than conventional emulsions ( $>1$   $\mu\text{m}$ )

406 because they are kinetically stable which are more stable to gravitational separation,  
407 coalescence, and flocculation than conventional emulsions (Sneha & Kumar, 2021).  
408 There are two types of emulsification techniques used to produce emulsified systems:  
409 a top-down approach and a bottom-up approach (Liao, Badri, et al., 2021). The  
410 top-down methods generally need large energy input for breaking up the dispersed  
411 phase into small structures by reducing the size and shaping the structure. The  
412 bottom-up methods generally include self-assembly, phase inversion, and spontaneous  
413 emulsification, which require the use of organic solvents or the addition of industrial  
414 surfactants. For food emulsion-based products, a top-down approach was normally  
415 selected for security reasons. High-speed homogenization, high-pressure  
416 homogenization, ultrasonic emulsification, membrane emulsification,  
417 microfluidization and so on have been widely used in different experimental  
418 laboratories for the preparation of nanoemulsions. The advantages and disadvantages  
419 of these techniques are summarized in previous reports (Saffarionpour, 2019; Sneha &  
420 Kumar, 2021). There are significant differences in droplet size and distribution  
421 available in different homogenization devices, and these differences will further affect  
422 the stability of the emulsions (Jacobsen, 2016). For example, it has been reported that  
423 high-pressure homogenizers can be used to produce nanoemulsions, but excessive  
424 ultrasonic power or ultrasonic time course can lead to increased temperature, resulting  
425 in flocculation or coalescence (Mao et al., 2010). Hebishy et al. (2017) compared  
426 different methods to prepare CAS stabilized emulsions. Their results showed that  
427 nanoemulsions produced by high-pressure homogenizers exhibited lower size and

428 much higher stability than those produced by conventional homogenization. At the  
429 same time, different pressure settings in high-pressure homogenization also have a  
430 significant effect on the stability of the emulsion. When sufficient CAS concentrations  
431 are used, pressure higher than 200 MPa resulted in physically and oxidatively stable  
432 emulsions, while the use of lower homogenization pressure (100 MPa) resulted in  
433 significant creaming. A similar trend was observed in Carpenter and Saharan's (2017)  
434 study where mustard oil nanoemulsions were prepared by different ultrasound powers,  
435 and they found that time and power parameters of ultrasonic can lead to increases or  
436 reductions in mean particle size. Another study (Let et al., 2007) indicated that less  
437 lipid oxidation of milk-based emulsions was obtained at high pressure (22.5 MPa)  
438 than at lower pressure (5 MPa).

#### 439 **4.5. Effect of heating process**

440 Food products based on emulsions generally undergo heat treatment at higher  
441 temperatures such as cooking and sterilization. One of the reasons why CAS has been  
442 widely used in food emulsions is that it has a relatively higher heating resistance  
443 ability than other common protein emulsifiers such as whey proteins and pea proteins  
444 (Liang, Matia-Merino, et al., 2017). This is the fact that relatively flexible casein  
445 molecules do not undergo significant heat-induced conformational changes like  
446 globular proteins (Srinivasan et al., 2002). Nevertheless, dephosphorylation,  
447 proteolysis and polymerization of CAS solutions can occur at higher temperatures and  
448 longer heating time (Liang, Gillies, et al., 2017). Therefore, it is still important to fully

449 understand the physical and chemical changes in CAS stabilized emulsions caused by  
450 heating and to develop strategies to control the destabilizing processes. Qin et al.  
451 (2018) studied the effect of different heating temperatures on CAS stabilized  
452 emulsions, and their results indicated that CAS has an excellent heating tolerance. The  
453 average diameter of CAS-based emulsions did not show obvious change even when  
454 the emulsions were heated to 90 °C. McSweeney (2004) reported that higher heating  
455 can affect the particle size of milk protein stabilized emulsions. The particle size  
456 distribution curve of the model emulsions (at pH 6.8) gradually increased from 1 µm  
457 before heating to ~10 µm when heated at 140 °C for 80 s. In this case, the unadsorbed  
458 caseinate protein fraction acts as "glue", causing the emulsion droplets to entangle  
459 into large aggregates. On the other hand, the disulfide bond between the milk proteins  
460 during the heating process can also grow the particle size due to the flocculation of  
461 the droplets. Liang, Gillies, et al. (2017) found that the phase separation of  
462 CAS-stabilized emulsions was markedly dependent on the duration of the heat  
463 treatment. The heat treatment at 120 °C caused casein proteins degradation and  
464 changed the intact CAS concentration in the continuous phase, thereby resulting in a  
465 greater extent of flocculation. Srinivasan et al. (2002) found a very interesting  
466 phenomenon: after heating treatment (121 °C for 15 min), the stability ratings of  
467 heated emulsions were higher than for unheated systems. In fact, at high temperatures,  
468 the molecular structure of the protein will fully unfold, and all hydrophobic residues  
469 will enter the oil phase, making the droplets less likely to aggregate. However, it was  
470 reported that adsorbed CAS molecules are more susceptible to thermally induced



471 degradation than unadsorbed ones in solution (Liang, Gillies, et al., 2017). This  
472 difference has been attributed to protein structure and conformational differences  
473 between adsorbed and unadsorbed caseinate. In some cases, especially when the final  
474 product is a gelatinous system (such as the formation of a gel matrix for casein  
475 emulsions), thermally induced emulsion destabilization is ideal (Silva et al., 2019).

#### 476 **4.6. Effect of oxidation**

477 Preventing oxidation is an important consideration when designing food emulsion  
478 systems since oxidation of an emulsion can greatly affect flavor, sense, and color of  
479 the food products during storage. The factors that cause lipid oxidation in an emulsion  
480 are very complex because they may include oxidation or electron transfer events in all  
481 the different phases of the system (Jacobsen, 2016). CAS has been reported to be very  
482 effective in protecting coated oils from oxidation, due to its unique iron-chelating  
483 properties and ability to generate thick interfacial layers around small oil droplets  
484 (McClements & Decker, 2018). For this reason, CAS has been used as an emulsifier  
485 to design delivery systems for omega-3 fatty acids, as well as for real food emulsified  
486 systems such as milk, yogurt, and cheese (Walker et al., 2015). Currently, a variety of  
487 methods and techniques can be used to detect the oxidation process of lipids in  
488 emulsions, such as peroxide value, *p*-Anisidine (*para*-anisidine) value, and  
489 thiobarbituric acid reactive substances (TBARS) assays (Jacobsen, 2016). O' Dwyer  
490 et al. (2013) measured the lipid hydroperoxide values and *p*-Anisidine values to  
491 monitor the oxidative stability of CAS stabilized emulsions containing various oils

492 (sunflower, camelina and fish). Their results showed that CAS, as emulsifier, was able  
493 to improve their oxidative stability compared to pure oils. Hebishy et al. (2017)  
494 reported that higher oxidative stable emulsions were prepared by ultra-high-pressure  
495 homogenized emulsions with sufficient CAS concentration (5 g/100 g). The oxidative  
496 stability of O/W emulsions is also related to the size of the droplets, the interfacial  
497 properties of the droplets, and the type of emulsifier. Ries et al. (2010) found that  
498 CAS and whey protein stabilized emulsions with small droplet size had greater  
499 oxidative stability than emulsions with large droplet size. Meanwhile, the antioxidant  
500 capacity of CAS stabilized emulsion was proportional to the concentration of CAS in  
501 the preparation. Higher concentrations of protein molecules can even offset the effects  
502 of milk droplet size and protein type (Ries et al., 2010). This may be because higher  
503 concentrations of unabsorbed casein molecules in the continuous phase inhibit free  
504 radical invasion of the O/W emulsion. In general, oxidation occurs faster in O/W  
505 emulsions than in bulk oils (Jacobsen, 2016), which may be due to the relatively harsh  
506 processing conditions required to produce the emulsion, such as exposure to air  
507 (oxygen), higher heating temperatures, or direct production through ultrasonic  
508 cavitation. These processes will promote the oxidation of the emulsion. In addition,  
509 the higher the unsaturated lipid content in O/W emulsion, the more it is sensitive to  
510 lipid oxidation in general (Chen et al., 2022).

## 511 **5. General technologies for detecting and evaluating CAS emulsion stability**

512 To meet specific applications needed in food industries, detailed information on

513 the emulsion matrix and droplet properties should be obtained to understand and  
514 estimate the stability of O/W emulsions. Essential emulsion characteristics such as  
515 droplet size, droplet charge, morphology, and rheological behavior could be measured  
516 and monitored by analytical instruments and standardized methods. These  
517 characteristics could be used to represent the physicochemical, appearance, and  
518 sensory properties of emulsion systems. For this reason, researchers can achieve  
519 information about instability mechanisms by combining several techniques such as  
520 particle size and surface charge with microscopic observations. Table 2 summarizes  
521 the advantages and limitations of the various techniques most commonly used to test  
522 the stability of O/W emulsions. In the following section, these techniques for  
523 evaluating the stability of O/W emulsions stabilized by CAS will be discussed.

#### 524 **5.1. Visual observation**

525 The stability of an emulsion substantially affects the quality, appearance, color,  
526 and odor of food products. In turn, changes in appearance, color, and odor of  
527 emulsion-based products can also be used to determine whether the emulsion is still  
528 stable. In some cases, the instability of the emulsion, such as creaming or precipitation,  
529 can be directly observed by the naked eye. Visual observation is probably the simplest,  
530 cheapest, and fastest method of evaluating emulsion gravity separation without the  
531 need for expensive and complex analytical devices. From the viewpoint of gravity  
532 separation, the macroscopic changes of emulsions may have two mechanisms:  
533 creaming and sedimentation. As shown in Fig. 5 (a), creaming can be observed clearly

534 as the density of the dispersed phase is lower than that of the continuous phase.  
535 Conversely, sedimentation occurs when a dispersed phase with a higher density than  
536 the continuous phase moves upwards and produces a thicker separation layer. In this  
537 regard, the degree of precipitation or creaming generally can be assessed by observing  
538 the thickness of the cream or precipitation layer with the naked eye, and then by  
539 instrumental measurement and recording. However, visual observation, though  
540 convenient, is not suitable for studying the mechanisms of instability phenomena such  
541 as flocculation, coalescence and Ostwald maturation. In addition, the emulsion layer  
542 is usually visible only when the degree of instability is quite high. Therefore, the  
543 initial stage of emulsion stability usually needs to be observed accurately with the  
544 help of other analytical instruments.

## 545 **5.2. Microscopy observation**

546 Visual observation is insufficient to study most instability mechanisms and  
547 smaller droplets, such as individual droplets, flocs, fat crystals, bubbles, and ice  
548 crystals. Several microscopy techniques have been established to observe these fine  
549 features of emulsions. Currently, the most widely used are optical, confocal  
550 fluorescent, scanning electron (SEM), transmitting electron (TEM), and atomic force  
551 microscopy (AFM). These techniques provide detailed information about complex  
552 systems in the form of "images" that are often relatively easy to understand.  
553 Meanwhile, each technique is based on a different physicochemical principle and can  
554 be used to further study the stability mechanism of emulsions with different particle

555 sizes and types.

556       Among the developed techniques, the optical microscope is the most commonly  
557 used emulsion observation equipment, because it is very convenient in handling and  
558 sample preparation, especially suitable for the observation of micron-scale emulsions.  
559 However, the main components of emulsion droplets captured by optical microscopy  
560 (the location of the oil/water phase) are often difficult to detect when using bright  
561 light fields since they have similar refractive indices or colors (McClements, 2007).  
562 The good news is that various methods using chemical stains have been established to  
563 enhance the contrast between different components, resulting in improving the overall  
564 image quality and providing spatial information about component locations (Malvehy  
565 et al., 2020). Emulsion components can be pre-stained using various types of  
566 chemical stains that bind to specific components of the emulsion (such as proteins,  
567 polysaccharides or lipids). After homogenizing, different colors are distributed in the  
568 oil phase or water phase, which therefore can be observed by using a conventional  
569 optical or fluorescent microscope because some chemical dyes are fluorescent. As an  
570 example, the photographs of CAS-based emulsion obtained for optical microscopy  
571 (Xu et al., 2020) and fluorescent microscopy (Y. Li & Xiang, 2019) are presented in  
572 Fig. 5b and 5c. Very clear droplet distribution can be observed as the oil droplets were  
573 stained with Nile red fluorescent dye. Confocal Laser Scanning Microscopy (CLSM)  
574 is another important innovation in microscopy for studying the interfacial structure of  
575 emulsions. The device focuses a very narrow laser beam on a specific point on the  
576 sample being analyzed, and the resulting laser signal is then sent through an

577 adjustable pinhole before reaching the detector to prevent defocusing. This equipment  
578 allows to obtain cross-section images of emulsions with basic morphology, structure  
579 and interface information (Kwok & Ngai, 2016). Álvarez Cerimedo et al. (2010) used  
580 CLSM to observe CAS-stabilized emulsions as shown Fig. 5d. Their results showed  
581 that the droplet distribution profiles of confocal images obtained after 24 hours were  
582 consistent with the emulsion stability data obtained from Turbiscan<sup>LAB</sup> stability  
583 analyzer (a technique that is able to quickly and sensitively identify instability  
584 mechanisms of emulsions (such as creaming, sedimentation, flocculation,  
585 coalescence...) (Jiang & Charcosset, 2022)).

586       However, from the perspective of magnification factor (as low as about 1  $\mu\text{m}$ ),  
587 the image obtained from the optical microscope cannot provide clear information  
588 about the structural characteristics of the emulsion (such as particle aggregation,  
589 interfacial layer thickness, etc.) (Low et al., 2020). Electron microscopy is an  
590 alternative to optical one and uses electron beams with much smaller wavelengths  
591 than light to help visualize material structures that are much smaller than visible light.  
592 Two types of electron microscopes, scanning electron microscopy (SEM) and  
593 transmission electron microscopy (TEM), are used to study the structure and  
594 physicochemical properties of nanoparticles, nanoemulsions, or Pickering emulsions.  
595 SEM allows observing the surface morphology of a sample by measuring the  
596 secondary electrons produced when the sample is bombarded with an electron beam  
597 (McClements, 2015). Typically, SEM sample preparation requires stringent conditions  
598 (fixation and dehydration) because electrons are easily scattered by atoms or

599 molecules in the gas. Unfortunately, the sample preparation procedures are generally  
600 not applicable to emulsions, as dehydration of emulsions usually results in droplet  
601 rupture and oil leakage. The recent development of Cryo-SEM enables us to visualize  
602 the microstructure of an emulsion in a frozen state. High-pressure freezing could help  
603 inhibit ice crystal formation and preserve emulsion integrity. Xu N. et al. (2021)  
604 observed the complete structure of CAS-coated nanoemulsions by cryo-SEM (Fig. 5e),  
605 through rapid emulsion freezing in a liquid nitrogen environment. The morphology,  
606 particle size and thickness of solid particles on the surface of the emulsion can be  
607 clearly calculated by the corresponding software. TEM has also been widely used to  
608 characterize the structure and morphology of CAS emulsions. For TEM, the electron  
609 beam can pass directly through the sample to be analyzed, where the electron beam  
610 transmitted to the sample is amplified and captured as an image, as shown in Fig. 5f  
611 ( Zhang et al., 2020). It should be noted that the droplet distribution of emulsion  
612 samples can be visualized by TEM without additional sample preparation steps.  
613 However, in some cases, the difference between the dispersed and continuous phases  
614 of emulsion in the resulting image remains unclear. This problem can be solved by the  
615 staining of emulsion (Zhai et al., 2018).

### 616 **5.3. Particle size analysis**

617 Under certain storage conditions, the stability of the original emulsion can be  
618 studied by measuring the droplet size of the sample. Meanwhile, by monitoring  
619 changes in the particle size distribution (PSD) of the emulsion system, the incidence

620 of flocculation between droplet interactions can be determined. As described by  
621 Stokes's Law, the stability of an emulsion increases with the decrease of droplet size :

$$V_{Stokes} = -2gr^2(\rho_2 - \rho_1)/9\eta$$

622 where  $V_{Stokes}$  is separation rate,  $r$  is the radius of the particles,  $g$  is the action of gravity  
623 acceleration,  $\rho_1$  and  $\rho_2$  are the density of the two phases, and  $\eta$  is the shear viscosity of  
624 the target system. According to the droplet size, the emulsion was further divided into  
625 three types, namely conventional emulsion ( $d > 200\text{nm}$ ), nanoemulsion ( $d < 200\text{nm}$ )  
626 and microemulsion ( $d < 100\text{nm}$ ). As stated by Piorkowski and McClements (2014),  
627 microemulsions are usually a combination of specific components (high levels of  
628 synthetic surfactants) through simple mixing and they are considered to be  
629 thermodynamically stable systems, so they usually have good long-term stability.  
630 Both conventional emulsions and nanometer emulsions are thermodynamically  
631 unstable systems and are consequently decomposed during storage by various  
632 instability mechanisms. In addition to microscopy techniques, a variety of  
633 commercially available analytical instruments have been developed to provide more  
634 information about the size and droplet distribution of emulsions, such as light  
635 scattering (static or dynamic light scattering), electrical pulse counting, and ultrasonic  
636 spectrometry, which are commonly used (McClements, 2015). Fig. 6a summarizes the  
637 measurement ranges of several commonly used particle size analysis techniques  
638 (Shuaifeng Zhang et al., 2021). These particle size analysis techniques are suitable for  
639 emulsion with different droplet sizes and concentrations and have the following



640 advantages: easy to use; fully automatic; and large amount of information about  
641 droplet size provided in a short time. Even so, since different instruments operate on  
642 different physical principles, they must be chosen carefully.

643 Light scattering (also called laser diffraction) particle sizing instrument is a  
644 well-established technique to investigate properties of particles in solutions, as shown  
645 in Fig. 6b. The basic principle is that the laser beam is irradiated on the sample, and  
646 the scattered intensity is detected by the detector at a certain angle  $\theta$  (Blott et al.,  
647 2004). The evaluation of fluctuation is often referred to “dynamic light scattering  
648 (DLS)”, and the analysis of absolute mean intensity is known as “static light  
649 scattering (SLS)”. DLS based devices usually operate with a specific scattering angle  
650 (fixed or variable), and then convert the flocculation strength math to PSD, which is  
651 used to detect smaller particles between 3 nm to 5  $\mu\text{m}$  (Shuaifeng Zhang et al., 2021).  
652 SLS is assessed by measuring the angle change of scattering light intensity when laser  
653 passes through emulsion medium. The droplet size of emulsions directly affects the  
654 diffraction angle, and the emulsion with larger particle size will lead to a lower  
655 diffraction angle, and *vice versa* (Blott et al., 2004). Meanwhile, the laser diffraction  
656 systems generally use the precise input particle characteristics (such as refractive  
657 index, shape, and size) to determine the particle size between 100 nm to 1000  $\mu\text{m}$   
658 (Shuaifeng Zhang et al., 2021). Our previous studies have successfully used DSL and  
659 laser diffraction to detect the average droplet size of CAS-based nanoemulsions and  
660 conventional emulsions (Liao et al., 2021). Multiple scattering is a common problem  
661 in light scattering measurements which lead to overestimation of particle size. This

662 problem can generally be solved by diluting before the measurement process.

663 The electrical pulse counting (i.e. electro-zone sensing) technique is based on  
664 measurements of the change of electrical conductivity as the diluted emulsion passes  
665 through a small orifice (Church et al., 2019). Since oil has a lower conductivity than  
666 water, the current decreases with each drop of emulsion passing through the pores.  
667 The emulsion droplet size can be calculated since larger particles move more slowly  
668 and it will generate larger electrical pulses. The droplet concentration and particle size  
669 distribution are determined by calculating the collected information from these  
670 electrical pulses. Commercial pulse counting techniques are currently available for  
671 measuring particles with diameters ranging from 0.4  $\mu\text{m}$  to 1200  $\mu\text{m}$ . However, this  
672 measurement is limited by the size of the holes in the glass tube and may require  
673 constant modification in the device to accommodate the measurement range. Brakstad  
674 et al. (2018) measured 2.0 ~ 60  $\mu\text{m}$  and 5.6 ~ 100  $\mu\text{m}$  oil droplets by 100  $\mu\text{m}$  and 280  
675  $\mu\text{m}$  aperture, respectively. A recent study (Fang & Nakagawa, 2020) applied tunable  
676 resistive pulse sensing with micropores (2-10  $\mu\text{m}$ ) to analyze the particle size of CAS  
677 suspensions. Notably, electrical pulse counting instruments are usually only suitable  
678 for relatively low droplet concentrations (< 0.1 wt %) so that only one particle passes  
679 through the hole each time. Consequently, this method is particularly appropriate for  
680 particle size analysis, but is not fit for studying flocculation in the system, because  
681 dilution will destroy the aggregated particles and lead to misunderstanding of sample  
682 stability (Church et al., 2019).

683 Ultrasonic spectroscopy is another technique that relies on measuring ultrasonic  
684 attenuation and/or changes in ultrasonic velocity as a function of frequency. The  
685 attenuation level of ultrasonic velocity frequency is related to concentration and  
686 particle size distribution in emulsion samples. Unlike other techniques, it is usually  
687 not necessary to dilute the emulsion before measurement as electroacoustic  
688 spectroscopy can be applied to high concentrated emulsions ( $\leq 50\%$ ). Nevertheless,  
689 the strength of the received signal is related to the density ratio between the particle  
690 and the surrounding liquid, so the application of electroacoustic measurements in  
691 some systems should be limited. The droplet size of CAS-based emulsions by DLS  
692 and ultrasonic attenuation has been compared (Gülseren & Corredig, 2011). The  
693 results indicated the mean diameter from DLS was larger than that measured by  
694 ultrasonics. This different is attributed to the differences in the size dependence of the  
695 ultrasonic and light scattering signals.

#### 696 **5.4. Charge analysis**

697 The strength and sign of the surface charge on the dispersed droplets are derived  
698 from the ionization properties of the absorbing CAS, so the surface charge is another  
699 important factor for detecting emulsion stability (McClements, 2015). The droplet  
700 charge of emulsion is subject to a variety of environmental factors including pH, ionic  
701 strength, and chemical reactions, which may ultimately lead to changes in surface  
702 charge and stability throughout the emulsion system. Electrostatic repulsions occur  
703 between droplets with the same surface charge symbol, which prevents particle

704 flocculation and inhibits the propagation of most emulsion instable paths. On the  
705 contrary, electrostatic attractions occur when particles with opposite charges come  
706 into contact, depending on the degree of attraction, and may cause droplet instability  
707 (McClements, 2015). It should be noted that when the attraction is weak, the droplets  
708 tend to gather close to one another but do not physically coalesce into larger particles,  
709 while when the attraction is greater, the droplets coalesce, resulting in larger droplets  
710 at the expense of smaller ones. As we described above, the charge characteristic of  
711 CAS is really sensitive to the pH and the presence of ions as well as other charged  
712 species in the aqueous phase. Zeta potential ( $\zeta$ ) is the potential at the "shear plane"  
713 which is a good reflection of the electrical properties of emulsion droplets because it  
714 stems from the fact that ions tend to adsorb onto the droplets (McClements, 2007). In  
715 our previous study, measuring the  $\zeta$ -potential of the CAS-coated droplets can reflect  
716 the stability of the emulsion to a certain extent (Liao et al., 2021).

717 A variety of analytical instruments have been used to measure the  $\zeta$  - potential on  
718 emulsion droplets and the most widely used analytical systems include  
719 microelectrophoresis and electroacoustic spectroscopy. Microelectrophoresis  
720 measures the electrochemical properties of the surface of a charged drop based on the  
721 velocity and direction at which it moves in the emulsion when an electric field is  
722 applied. In the development of recent technologies, the combination of  
723 microelectrophoresis and dynamic light scattering was used to measure  
724 electrophoretic mobility, from which zeta potential can then be calculated. The  
725 emulsion sample is placed in the measuring cell and an electric field is applied to it

726 through a pair of electrodes. Any charged particles in the emulsion will then move  
727 toward the opposite charge at different speeds, depending on their charge. Thus, the  
728 symbol and magnitude of the charges on the particles can be collected and then  
729 converted into particle potentials by fitting software. Similarly, samples analyzed by  
730 light scattering techniques should be diluted to a lower concentration to avoid  
731 multiple scattering effects. Moreover, special care should be taken when diluting  
732 samples, as the electrical properties are sensitive to changes in the surrounding water  
733 environment (Li et al., 2017).

734 Electroacoustic spectroscopy can also be used to determine the  $\zeta$  - potential of the  
735 emulsion (Gülseren & Corredig, 2011). Unlike other techniques, it is usually not  
736 necessary to dilute the emulsion before measurement because electroacoustic  
737 spectroscopy can be applied to high concentrated emulsions ( $\leq 50\%$ ). The  
738 measurements using electroacoustic spectroscopy can be designed in two operating  
739 modes: Electro-Sonic Amplitude (ESA) and Colloid Vibration Potential (CVP). For  
740 both modes, the measurements use an electrical or acoustic signal applied to the  
741 emulsion and record the acoustic or electrical signal produced by the oscillating  
742 particles, which are then converted to zeta potential by software fitting. Nevertheless,  
743 the strength of the received signal is related to the density ratio between the particle  
744 and the surrounding liquid, so the application of electroacoustic measurements in  
745 some systems should be limited.

## 746 **5.5. Rheological properties**

747 The rheological properties of emulsions strongly influence the processing  
748 behavior and shelf-life stability of liquid foods, as well as their appearance, texture,  
749 taste, and flavor release. As reported by Tadros (1994), the rheological properties of  
750 emulsions are related to pH, electrolyte concentration in the continuous phase, shape,  
751 size distribution, internal viscosity and concentration of oil droplets, as well as their  
752 ability to participate in various interactions with other components. Therefore,  
753 studying the rheological properties of CAS emulsions can provide more information  
754 for studying their instability mechanisms.

755 Currently, the most commonly used instruments for characterizing the rheological  
756 properties of food emulsions are rheometer and viscometer. The viscometer is a very  
757 convenient system to obtain viscosity information by applying a fixed rate of shear  
758 force in a short period, but the information obtained is limited and cannot be used to  
759 deeply study the instability mechanism and rheological properties of emulsions.  
760 According to Stokes Law, as viscosity increases, O/W emulsions are generally more  
761 resistant to instability caused by particle flocculation. The rheometer is another device  
762 designed to apply shear deformation forces to study liquids, solids, or other  
763 viscoelastic samples which are the most widely used instruments for measuring the  
764 rheological properties of fluids (Tavares et al., 2022). It can provide detailed  
765 information on viscosity, yield stress, elastic modulus, and fracture behavior of  
766 emulsion systems by controlled-stress and controlled-strain modes. A typical method  
767 for measuring rheological properties of emulsions is to measure the system's  
768 resistance to applied stresses, such as rotary or oscillating stresses, where stress can

769 cause emulsion system deformation (Tavares et al., 2022). Briefly, the greater the  
770 deformation resistance, the higher the viscosity of the system, and the linearity of the  
771 deformation depends on the interactions between its components. Sosa-Herrera et al.  
772 (2008) studied the relationship between rheological properties of CAS stabilized  
773 emulsions (30 wt% sunflower oil) and the physicochemical characteristics of the  
774 water phase. They found that the addition of gellan gum into emulsion can help to  
775 prevent both flocculation and creaming, as the rheological behavior strongly increased.  
776 This is the fact that the polysaccharides act as filler particles between the oil droplets  
777 and the casein aggregates. A recent study also reported that the presence of  
778 polysaccharides (inulin and konjac glucomannan) has the potential to modulate the  
779 stability and rheological properties of CAS-based emulsions (Xu et al., 2020).

## 780 **6. Strategies to improve the stability of CAS stabilized emulsions**

781 CAS stabilized emulsions are considered excellent systems for providing  
782 protection and transportation of nutrients in the food industry, but instability under  
783 certain conditions still limits their further applications. For example, at pH values  
784 close to their pI, the charge of casein proteins adsorbed at the oil-water interface is  
785 gradually lost, and the thickness of the adsorbed layer becomes thinner as the “loops”  
786 and “tails” change, resulting in droplet instability. Based on the characteristics of CAS  
787 stable emulsions, this protein can be used in combination with other emulsifiers to  
788 deal with these unstable conditions, ensuring effective control of the emulsion,  
789 thereby reliably producing products with a consistent appearance, uniform consistency,

790 and satisfactory shelf life. In this section, the main methods that can be used to  
791 improve CAS stabilized emulsion properties will be discussed and some recent cases  
792 are summarized in Table 3.

### 793 **6.1. Optimize emulsion formulation**

794 Firstly, optimizing the process of producing the emulsification is one of the most  
795 and safest ways to improve emulsion stability. During this process, several factors  
796 such as protein concentration, homogenization conditions, protein solubilization, ionic  
797 strength, and pH conditions can be optimized at the laboratory level to achieve better  
798 emulsion stability. At the same time, optimization of the formulation, selection of the  
799 right type of emulsifying equipment, addition of antioxidants, and optimization of  
800 packaging and storage conditions will also contribute to the stability of emulsions and  
801 prolong the shelf life of food products.

802 Hebishy et al. (2017) studied the effects of protein concentration and pressure on  
803 the stability of CAS stable emulsions and demonstrated that high-pressure treatment  
804 with sufficient CAS content can produce nanoscale emulsions with unimodal peaks  
805 and high physical properties. Compared with emulsions produced by conventional  
806 homogenization, oxidation stability of emulsion produced by high-pressure  
807 homogenization was improved. In another study by O' Dwyer et al. (2013), the effects  
808 of CAS concentration on the emulsion stability of O/W emulsions were examined.  
809 The authors reported that lipid hydroperoxide and p-Anisidine values of emulsions  
810 generally decreased as CAS concentration increased. Erxleben et al. (2021)



811 investigated the effect of ethanol on the stability of sodium caseinate stabilized  
812 emulsions and they observed a slower destabilization mechanism for these emulsions  
813 when ethanol was added after the processing step.

## 814 **6.2. Non-covalent bond**

815 Compared with emulsions prepared with CAS as the sole emulsifying agent,  
816 emulsions prepared from mixed emulsifiers (Cuomo et al., 2019; McClements &  
817 Jafari, 2018) and protein-polysaccharide complexes (Cheng et al., 2020; Cheng &  
818 McClements, 2016) have been reported with increased resistance to environmental  
819 stresses such as pH, ionic strength, and temperature. This is because CAS can  
820 non-covalently bond with other biopolymers on the droplet surface, such as  
821 electrostatic interactions, hydrophobic bonds, hydrogen bonds, and van der Waals  
822 interactions, among which electrostatic interactions are widely used.

823 A recent review (McClements & Jafari, 2018) detailed the many advantages of  
824 using mixed emulsifiers in improving emulsion formation, stability and performance.  
825 Generally, proteins, small molecule surfactants and phospholipids are the most used  
826 emulsifiers in food applications. Ji et al. (Ji et al., 2015) used CAS and soy protein  
827 isolate (SPI) as natural emulsifier to successfully develop a enteral nutrition system  
828 with enhanced stability. The results showed that the flexible protein CAS and the rigid  
829 globular protein SPI were adsorbed on the oil-water interface. Another study  
830 (Yerramilli et al., 2017) also showed that by using CAS and pea protein, the mixed  
831 protein based nanoemulsion was more stable than individual ones. They confirmed

832 the presence of both pea protein and casein at the interface by SDS-PAGE. Perugini et  
833 al. (2018) used a non-ionic surfactant (Tween 20) in combination with CAS as  
834 blending emulsifiers to prepare nanoemulsions and their results showed that the  
835 addition of Tween 20 effectively enhanced the stability of the CAS-based emulsion  
836 under acidic conditions. Another study (Cuomo et al., 2019) also used a blend of CAS  
837 and Tween 20 to prepared O/W nanoemulsions. The results showed that *in vitro*  
838 simulated digestion process, the mixed emulsifier system provided higher amounts of  
839 lipophilic compounds and lower fat intake compared to the sodium  
840 caseinate-stabilized nanoemulsion. In addition to proteins and small molecule  
841 surfactants, soy lecithin (Chung et al., 2019; Wu et al., 2022; Xue et al., 2015) are also  
842 used as a co-emulsifier with sodium caseinate in preparing food emulsions. A study by  
843 Xue et al. (2015) reported that the combination of CAS and soy lecithin facilitate the  
844 formation of transparent and stable thyme oil nanoemulsions when compared with  
845 treatments applying the two emulsifiers individually. Nonetheless, there may be  
846 adsorption competition between the different emulsifier components, which may  
847 adversely affect emulsion formation and stability (McClements & Jafari, 2018).

848 Unlike other emulsifiers, polysaccharides are generally not used as the only  
849 emulsifiers in food emulsions because typical hydrophilic polysaccharide molecules  
850 are nearly surface-inactive unless they contain protein moieties responsible for their  
851 emulsifying ability, such as gum arabic (Ishii et al., 2018). However, polysaccharides  
852 are widely used as stabilizers in emulsion preparation, usually in combination with  
853 proteins, which can effectively improve the viscosity, texture, and appearance of food

854 products as well as for introducing some nutritional components. As Ghosh and  
855 Bandyopadhyay (2012) described, the relative thickness of the liquid films between  
856 two approaching droplets lies in the order of hydrocolloids (5–10 nm) > proteins (1–5  
857 nm) > surfactants (0.5–1 nm). Thus, the stability is expected to be higher when the  
858 emulsion is stabilized by protein-polysaccharide complexes or conjugates compared  
859 to the same systems stabilized by protein or surfactants alone. Effective binding of  
860 CAS and polysaccharides can be achieved by utilizing electrostatic attractions  
861 (Non-covalent binding) through complexation at acidic pH (Artiga-Artigas et al.,  
862 2020; W. Xu et al., 2020) or by covalently binding polysaccharides (Consoli et al.,  
863 2018; Sato et al., 2015).

864 The prerequisite for the formation of electrostatic complexes is naturally the  
865 presence of opposite charges on the different polymers. Our previous study (Wang et  
866 al., 2019) has investigated the formation of CAS and low methoxyl pectin (LMP)  
867 complexes at different pH values (Fig. 7). The charge density of CAS is strongly  
868 dependent on the suspension pH, which can induce the formation of complexes with  
869 LMP through strong electrical interactions at pH 3. The complexes formed by CAS  
870 and anionic polysaccharides could be used to prepare emulsions containing oil-soluble  
871 active molecules. The effect of complexation on CAS emulsion has been reported in  
872 many literatures by either one or two-step complex coacervation techniques. As  
873 shown in Fig. 8a, the one-step method uses a mixture of two biopolymers to form a  
874 complex coacervate before preparing the emulsion, while the two-step method (Fig.  
875 8b) uses only CAS to form the emulsion and then adds the polysaccharide as a second

876 layer. Yesiltas et al. (2018) used CAS and commercial sodium alginate as combined  
877 emulsifiers to prepare fish O/W emulsions. Their results indicated that mixed  
878 emulsifiers performed better in terms of physical stability compared to emulsions  
879 produced with only CAS. Cheng et al. (2020) also used the one-step method to  
880 prepare emulsions with CAS/ pectin or gum Arabic. Both polysaccharides exhibited  
881 the ability to enhance the accumulation and stability of resveratrol at the oil-water  
882 interface, while also increasing the absolute value of the droplet zeta potential.  
883 However, one-step methods tend to promote droplet flocculation or coalescence in the  
884 process, and aggregation is generally irreversible (Burgos-Díaz et al., 2016). Liu et al.  
885 (2012) prepared multilayer emulsions using carboxymethyl cellulose (CMC) and CAS  
886 by the two-step method that can result in more uniform droplets. Firstly, CAS  
887 emulsion containing CMC in an aqueous phase was prepared under neutral pH, in  
888 which two biopolymers were negatively charged, and then the pH was adjusted to  
889 acid values. A complex interface layer can be formed at a pH around the pI of proteins,  
890 consisting of a first layer generated by the direct adsorption of CAS molecules on the  
891 exposed interfaces, and a second layer composed of CMC molecules interacting with  
892 CAS molecules through attractive electrostatic interactions. Emulsions containing  
893 drops prepared by CAS/CMC exhibited fairly stability since polysaccharide outer  
894 layers increase the net surface charge and create steric hindrance (Liu et al., 2012). A  
895 recent study compared the effect of different anionic polysaccharides on the stability  
896 of CAS-based emulsion by the two-step method (Shi et al., 2023). All four  
897 polysaccharides tested in this study can adsorb to the surface of CAS-based lipids

898 when the pH of the mixture was reduced from neutral to 5, 4, and 3, and enhanced the  
899 performance of the multilayer emulsion. In particular, multilayer emulsions prepared  
900 from  $\kappa$ -carrageenan could accelerate digestion in the gastrointestinal tract.

901 Furthermore, it was also reported that the presence of free polysaccharides can  
902 improve the salt stability of protein-stabilized emulsion even without interactions with  
903 proteins (Qiu et al., 2015). This result was attributed to the fact that the unadsorbed  
904 xanthan gum increased the viscosity of the emulsion and formed a thickening network  
905 around the protein, which reduces the particle movement rate and thus improves the  
906 emulsion stability. However, improving the stability of CAS-stabilized emulsions by  
907 electrostatic complexing (sequential adsorption or mixed emulsification) still suffers  
908 from a major disadvantage: improvements may be limited to narrow pH or ionic  
909 strength ranges.

### 910 **6.3. Covalent bond (Conjugation)**

911 The chemical modifications of proteins, such as acetylation, deamidation,  
912 succinylation and glycation, is another considering modification method to improve  
913 physicochemical and functional properties of protein stabilized emulsions. In food  
914 industries, the most common protein-polysaccharide conjugation method is the  
915 Maillard reaction, which is a series of non-enzymatic browning reactions that occur  
916 naturally between reducing sugars and amino acids (Evans et al., 2013). Unlike other  
917 protein modification methods, Maillard reaction is usually triggered by temperature  
918 elevation without other chemical reagents. In recent years, protein-polysaccharide

919 conjugates as emulsifiers provided more diverse possibilities for the improvement of  
920 emulsifying, stabilizing, solubility and thermal stability as well as better stability  
921 against pH variations. Casein and sodium caseinate have been reported to bound to a  
922 variety of galactose, lactose, and dextran under controlled conditions and these  
923 glycoconjugates showed improved emulsifying properties compared to natural CAS  
924 (Barbosa et al., 2019; Consoli et al., 2018; Liu et al., 2018). In fact, the open and  
925 hydrated random structure in caseins makes them more likely than globular proteins  
926 to benefit from glycosylation *via* the Maillard reaction (Consoli et al., 2018).  
927 Furthermore, as mentioned above, CAS has higher stability to temperature, which is  
928 required for glycosylation. A recent study formulated the Maillard conjugates  
929 biopolymers by sodium caseinate-locust bean gum (Barbosa et al., 2019). These  
930 conjugates appear to be a promising alternative of emulsifiers to address the  
931 application deficiencies of sodium caseinate in acidic situations. Furthermore, the  
932 emulsions prepared from the conjugates showed a reduction in droplet size, which can  
933 be attributed to the formation of the conjugates that promotes steric repulsion between  
934 droplets and avoids attractive droplet interactions. Similar explanations was also  
935 reported by the study of CAS-sugar beet pectin conjugates (Juyang & Wolf, 2021).  
936 Improved antioxidant stability and creaming stability of emulsions has been also  
937 reported when CAS Maillard reaction products were applied as emulsifiers (Consoli  
938 et al., 2018; Lee et al., 2015). Li et al. (2017) investigated the effect of pH variation  
939 on the emulsion stability of CAS-lactose conjugates formed *via* the Maillard reaction  
940 for better oil encapsulation in spray-dried powder. They found that conjugates

941 formulated at pH 11, exhibited better emulsifying properties and stability, and  
942 enhanced conjugate adsorption on oil droplets. This was due to the change of the  
943 hydrophilic/hydrophobic balance of emulsion systems and the resulted strong steric  
944 repulsions allowing to slow droplet flocculation. Finally, although Maillard reaction  
945 products can demonstrate these improved physicochemical properties of emulsions,  
946 rigorous safety testing should be performed before using these products as ingredients,  
947 as Maillard reaction might produce advanced glycation end-products (AGEs), causing  
948 side-effects in healthy individuals (Wei et al., 2018).

949 In addition, modifying the protein by breaking down its structure has been  
950 investigated as an alternative strategy to improve the emulsification properties, which  
951 can be done enzymatically (Sato et al., 2015) or by heating treatments (Liang, Gillies,  
952 et al., 2017), where controlled hydrolysis can increase the solubility, emulsification,  
953 and foaming properties of protein emulsifiers.

## 954 **7. Conclusions**

955 CAS provides interesting emulsifying properties that allow it to meet current  
956 consumer demands for the stabilization of food emulsions, but instability under some  
957 specific conditions limits its applications. Therefore, there is an urgent need to better  
958 understand the effects of different environmental and processing conditions on  
959 emulsion stability, and how to improve the stability of CAS-formed food emulsions.  
960 This review presented the structural properties of CAS and its emulsifying ability,  
961 summarized the recent applications of CAS for stabilizing food emulsions. From our

962 results and others discussed in literature, it is clear that the stability of emulsions is  
963 strongly affected by pH, protein concentration, ionic strength, heating process and  
964 oxidation. The instability mechanisms occurred in CAS-stabilized emulsions can be  
965 recorded and tracked by some instruments and methods, such as microscopy  
966 observation, droplet size distribution, droplet charge, and rheological measurements.  
967 Those technologies have advantages and disadvantages and provide different insights  
968 into the destabilization mechanisms. According to the general factors that control the  
969 stability of CAS stabilized emulsions, some general strategies for preventing  
970 instability are pointed out: using enough protein emulsifier to fully saturate the  
971 interface during emulsification; avoiding any large excess of non-adsorbed protein ;  
972 working away from pH values around the protein's pI ; keeping the ionic strength as  
973 low as possible; and using high energy methods in order to reduce particle size. In  
974 addition to the above proposals, a reliable strategy for preventing instability in CAS  
975 stabilized emulsions can be improved by binding proteins to other biopolymers,  
976 especially polysaccharides by using complexation or conjugation. In summary, this  
977 review can serve as a template for researchers to understand the fundamental  
978 properties of CAS-stabilized emulsions and study their stabilization methods.



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982 **CONFLICTS OF INTEREST**

983 The authors declare that there is no conflict of interest that could be perceived as  
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- 1404

1405 **Tables**

1406

1407 **Table 1.** Influence of ionic strength on the mean particle diameter and  $\zeta$ -potential of the CAS

1408 stabilized emulsions (Qin et al., 2018).

Concentration (mg/mL)		Z-average diameter (nm)	$\zeta$ -potential (mV)
NaCl	0	199.27 $\pm$ 1.56 <sup>bc</sup>	-36.7 $\pm$ 0.7 <sup>a</sup>
	0.1	197.76 $\pm$ 1.59 <sup>c</sup>	-36.9 $\pm$ 0.4 <sup>a</sup>
	0.35	202.25 $\pm$ 2.51 <sup>ab</sup>	-36.8 $\pm$ 1.1 <sup>a</sup>
	0.60	203.65 $\pm$ 1.92 <sup>a</sup>	-36.9 $\pm$ 0.7 <sup>a</sup>
	0.85	202.62 $\pm$ 1.65 <sup>ab</sup>	-37.5 $\pm$ 0.8 <sup>a</sup>
	1.10	204.20 $\pm$ 2.82 <sup>a</sup>	-37.2 $\pm$ 0.3 <sup>a</sup>
CaCl <sub>2</sub>	0	199.27 $\pm$ 1.56 <sup>e</sup>	-36.7 $\pm$ 0.7 <sup>d</sup>
	0.25	197.50 $\pm$ 0.75 <sup>e</sup>	-32.6 $\pm$ 1.6 <sup>c</sup>
	0.55	210.94 $\pm$ 1.94 <sup>d</sup>	-31.7 $\pm$ 0.9 <sup>c</sup>
	0.85	230.37 $\pm$ 2.38 <sup>c</sup>	-29.3 $\pm$ 0.6 <sup>b</sup>
	1.15	254.43 $\pm$ 1.74 <sup>b</sup>	-26.6 $\pm$ 0.5 <sup>a</sup>
	1.45	265.18 $\pm$ 4.39 <sup>a</sup>	-25.9 $\pm$ 0.4 <sup>a</sup>

1409 Mean values within the same column followed by the same lowercase letters are not significantly  
1410 different ( $P > 0.05$ ) between NaCl (or CaCl<sub>2</sub>) levels.

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**Table 2.** Advantages and limitations of various techniques for detecting the instability of emulsions

	Techniques	Advantages	Disadvantages
Visual observation	Appearance, color, and odor of emulsions	Simple and rapid; The extent of creaming or sedimentation can be observed with naked eyes	Long measuring period; Unable to study other instability phenomena, such as flocculation, coalescence, and Ostwald ripening
Microscopy observation	Optical microscopy	Easy sample preparation; Easy to operate and available in most research facilities; Quick view at the shape and integrity of emulsions	Unable to provide comprehensive information about the physical status of the dispersed phase; The contrast between the different components of an emulsion is usually poor
	Confocal laser scanning microscopy (CLSM)	Three-dimensional images and surface profiles can be available; The ability to eliminate or reduce background information far from the focal plane and result in better optical resolution and contrast	The ingredients in the emulsion need to be stained prior to analysis; Not suitable for light sensitive samples; Depending on the target sample and its stain agent; The dyeing process is complex
	Scanning electron microscopy (SEM)	Possible to in-depth study of the structure and morphology of the emulsion; Able to obtain detailed the size, 3D images and surface structure as well as multi-functional information	The emulsion needs to be dried and gold/platinum coating before visualization; Detailed information on the pellet layering and internal structure cannot be provided; Hard to operate
	Transmitting electron microscopy (TEM)	Very high magnification and resolution of emulsion droplet are available; Provides detailed information on morphology, size distribution, homogeneity and surface structure	Staining and dehydration can result in structural artifacts; Hard to construct 3D image from 2D slices; Tedious operating procedure

	Atomic Force Microscope ( AFM )	High resolution; Easy sample preparation; accurate height information; works in vacuum, air, and liquids; 3D images and surface roughness;	Limited vertical range; small scan size and slow scan speed; data not independent of tip; tip or sample can be damaged
Particle size analysis	Laser diffraction measurement	The measuring range for static light and dynamic light scattering are 100 nm to 1000 $\mu\text{m}$ and 3 nm to 5 $\mu\text{m}$ , respectively; Easy to operate; Easy sample preparation; Provides detailed information about droplet size and homogeneity in a short time	The sample needs to be diluted, which may damage the sample structure; The instrument should be flushed frequently to ensure the accuracy of the results
	Electrical pulse counting	The measuring range is 0.4 $\mu\text{m}$ and 1200 $\mu\text{m}$ ; Easy to operate; Rapid and accurate; Volume distribution and particle account are available	The samples should be diluted before analyzing; Pores are easily clogged
	Ultrasonic spectrometry	The measuring range is 10 nm to 1000 $\mu\text{m}$ ; Samples with high concentration (>50%) can be measured; Suitable for measuring opaque emulsions; Non-destructive	Not suitable for sample with concentration less than 1%; Tedious operating procedure
Surface charge	Microelectrophoresis	Easy to operate; Easy sample preparation; Provides detailed information about the surface charge of particle	The sample needs to be diluted; The instrument should be flushed frequently to ensure the accuracy of the results
	Electroacoustic spectroscopy	Samples with high concentration (>50%) can be measured; Non-destructive	Not suitable for sample with concentration less than 1%

Rheological properties	Rheometer	A wider dynamic range of control and measurement parameters are available; Detailed information about flow, deformation and the tackiness are available	Tedious operating procedure; Equipment cleaning is complicated; Device is large and less portable
	Viscometer	Simple and rapid; Small and portable; Accurate viscosity for a wide variety of fluids available	Speed and torque capabilities are limited; Difficult to clean the capillary tube

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**Table 3.** Summary of recent progress in improving the stability of CAS stabilized emulsions

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Methods	Biopolymer addition	Core material	Results	References
Optimize emulsion formulation	\	Sunflower and olive oils	Pressures (200–300 MPa) and 5 g/100 g CAS increased emulsions stabilities and the droplet size of CAS-based emulsions has an effect on the oxidation rate	(Hebishy et al., 2017)
	\	Sunflower, camelina and fish oil	Lipid hydroperoxide and p-Anisidine values of emulsions generally decreased as CAS concentration increased	(O' Dwyer et al., 2013)
	\	Sunflower oil	When ethanol is added after processing emulsions, CAS-based emulsions suffer from a slower destabilization mechanism	(Erxleben et al., 2021)
	\	Cinnamaldehyde MCT oil and sunflower oil	Mixing medium or long-chain carrier oil before homogenization can significantly inhibit Ostwald ripening of CAS-based emulsions containing essential oils	(Liao et al., 2021)
	\	Soybean oil	Ultrasound treatment improved oxidative stability of CAS based emulsion containing high level of soybean oil and enhanced the adsorbed CAS concentration	(Ke Li et al., 2020)
	\	Canola oil	Depletion interaction energy calculations showed droplet size reduction makes nanoemulsions more resistant to flocculation than conventional emulsions	(Yerramilli & Ghosh, 2017)
Mixed emulsifiers	Soy protein isolate	MCT oil	Mixed proteins contributed to good stability of the encapsulated liposoluble nutrient	(Ji et al., 2015)
	Pea protein	Canola oil	Mixed proteins nanoemulsions were more stable than the individual ones and SDS-PAGE confirmed presence of both proteins at the O/W interface	(Yerramilli et al., 2017)
	Whey protein	Canola oil	Mixed protein-based emulsions had better oxidative stability to protect volatile compounds	(Loi et al., 2019)

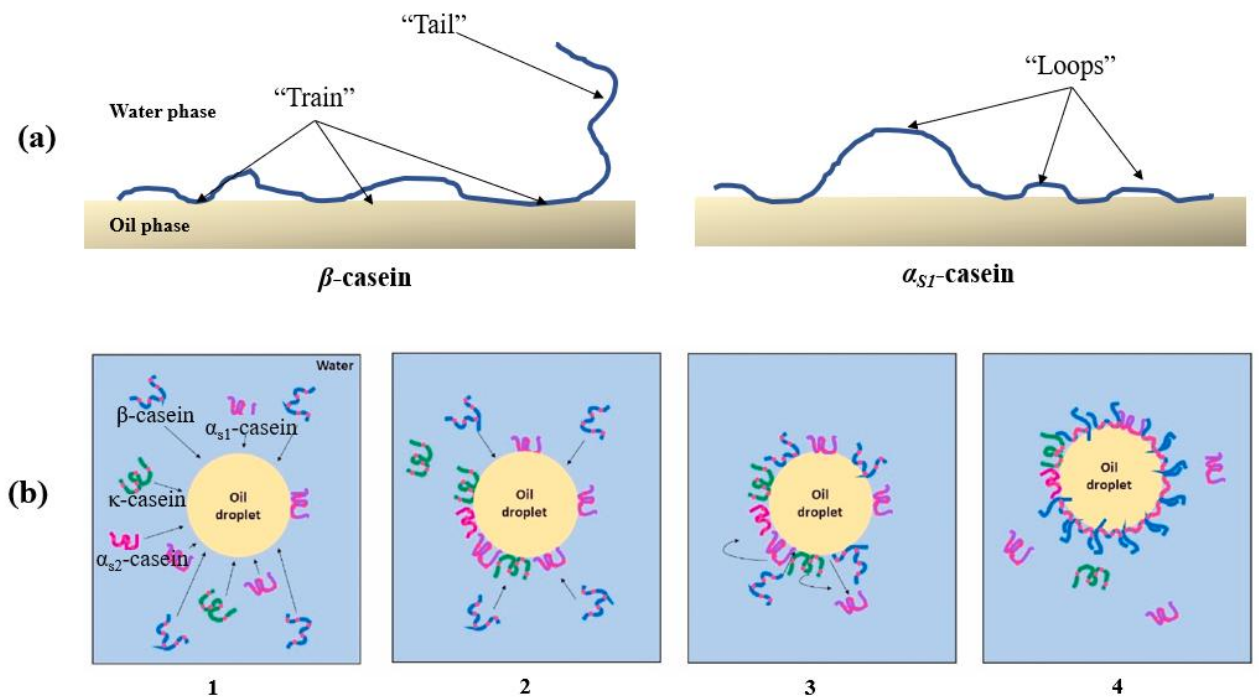
	Sucrose stearate	Thymol	Interaction between the two emulsifiers enhances exposure of tryptophan and tyrosine residues, favoring binding to thymol during heating	(D. Su & Zhong, 2016)
	Tween 20	Rice bran oil	Mixed emulsifier system offered a suited solution to absorption because they allow the intake of a high amount of curcumin together with a low-fat content	(Cuomo et al., 2019)
	Tween 20	Rice bran oil	The presence of Tween 20 ensured the steric stabilization promoting the use of CAS as an emulsion stabilizer at acid pH	(Perugini et al., 2018)
	Phosphatidylcholine (PC)	Fish oil	The substitution of some of the CAS with PC increased oxidative stability of the emulsions while maintaining the physical stability	(Yesiltas et al., 2019)
	Soy lecithin	MCT	Blends of CAS and lecithin produced O/W emulsions with similar appearance, color, particle size and electrical properties as those stabilizers used alone	(Chung et al., 2019)
	Soy lecithin	Coconut oil	The addition of amphiphilic lecithin could help decrease the droplet size of emulsion and increase the solubility of microcapsules	(Wu et al., 2022)
Complexation	Konjac glucomannan	Canola oil	The increase in the concentration of konjac glucomannan is beneficial to strengthen the emulsion gel network and prevent creaming of the emulsion	(Martínez-Padilla et al., 2021)
	Low methoxyl pectin (LMP)	Sunflower oil	At low LMP concentrations, complex-stabilized emulsions were more stable than layer-by-layer (LBL)-stabilized emulsions, while at high LMP concentrations, LBL-stabilized emulsions exhibited better stability.	(Jian Wang et al., 2018)
	Lactoferrin	Sunflower oil	The formation of heteroaggregates by mixing two emulsions stabilized by proteins with opposite charges, varying the emulsion volume ratio and ionic strength allowed producing systems with distinct properties	(de Figueiredo Furtado et al., 2016)
	Four different polysaccharides	Fish oil	Multilayer emulsion with targeted functions was formulated using polysaccharides; Especially, $\kappa$ -Carrageenan could reach the demand of rapid digestion	(Shi et al., 2023)
	Sulfated fucan	Fish oil	The sulfated fucans with higher molecular weights could significantly increase the initial digestibility rate of CAS-based emulsions	(Shi et al., 2021)

	$\gamma$ -cyclodextrin and alginate	Corn oil	$\gamma$ -CD/CAS/alginate-stabilized nanoemulsions exhibited nanoscale droplet diameter and high stability even under acid, alkali, and high temperature conditions	(C. Wang et al., 2022)
	<i>Quillaja</i> Saponin	MCT oil	The emulsifying performance of the mixed binary emulsifier and the subsequent stability of the emulsion are highly dependent on the concentration ratio of the emulsifier	(Salminen et al., 2019)
	Pectin and gum Arabic	$\alpha$ -tocopherol and resveratrol	Both polysaccharides enhance the accumulation and stability of resveratrol at the oil-water interface	(Cheng et al., 2020)
	Trehalose	Fish oil, sunflower oil, or olive oil	The addition of trehalose diminished the volume-weighted mean diameter and the interactions protein–sugar played a key role in creaming and flocculation processes	(Álvarez Cerimedo et al., 2010)
	Carboxymethyl-cellulose	Corn oil	The results indicated that the complex formed at O/W interfaces can protect the oil droplets against flocculation and coalescence upon acidification	(L. Liu et al., 2012)
	Dextran sulfate (DS)	n-tetradecane	CAS-based Emulsions containing 1 wt% DS exhibited good shelf-life stability on quiescent storage for 3 weeks, especially when the emulsion was prepared at low pH	(Jourdain et al., 2008)
	Inulin and konjac glucomannan	Tea seed oil	Above the critical inulin/KGM ratio of 2:0.1, the droplet size of CAS-based emulsions was significantly decreased, and the creaming stability was greatly enhanced	(Xu et al., 2020)
	Gellan gum	Sunflower oil	CAS-based Emulsions containing gellan gum were stable against both flocculation and creaming	(Sosa-Herrera et al., 2008)
	Succinylated alginate	Fish oil	The modified alginate increased the stability of the emulsion, and the emulsion had a lower creaming index	(Yesiltas et al., 2018)
Conjugation	Propylene glycol alginate (PGA)	Soybean oil	PGA and CAS undergo transacylation during pH changes. This reaction imparts a gel-like structure to the emulsions and improves thermal stability of emulsions	(Sun & Zhong, 2022)

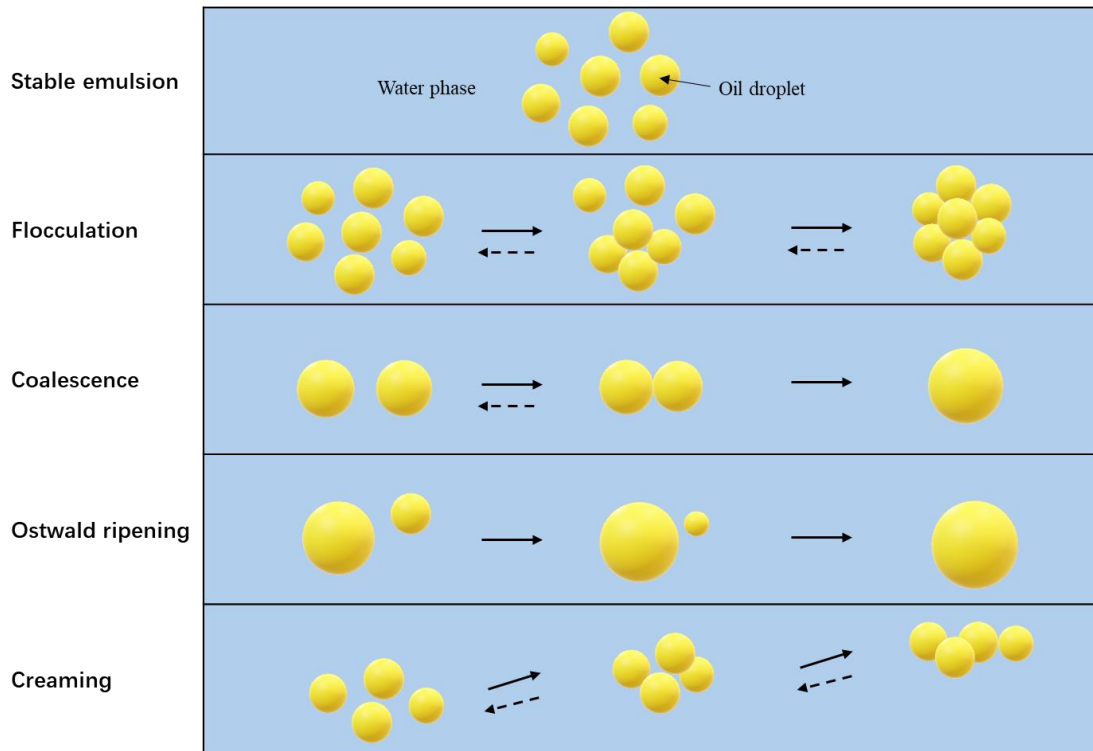
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Maltodextrin	Palm-based triacylglycerol	Glycation has the ability to reduce moisture and surface oil content and produce larger particle size of spray-dried powders	(Lee et al., 2015)
Dextran	Fish oil	Glycoconjugates of CAS to dextrans were explored and glycoconjugate of CD70 displayed the best emulsifying antioxidant activity and emulsion stability with improved activity	(Liu et al., 2018)
Locust bean gum (LBG)	Soybean oil	Emulsions prepared by CAS-LBG biopolymers presented high stability and reduced particle size at pH 3.5 and 7, which could be regarded as promising alternative of emulsifying agent in neutral or acidified products	(Barbosa et al., 2019)
Sodium alginate	Corn oil	The conjugates produced stable emulsions with low surfactant to oil ratios, and the content of CAS played a major role in determining droplet size	(N. Li & Zhong, 2022)
Corn starch hydrolysates	Palm oil	Protein-polysaccharide conjugates have lower interfacial tension, and the conjugates formed by heating improve their ability to stabilize emulsions	(Consoli et al., 2018)
Pectin	Conjugated linoleic acid	The aggregation and creaming stability of the emulsions formulated by conjugates increased as the pectin-to-caseinate ratio increase	(Cheng & McClements, 2016)
Maltodextrin	MCT-oil	CAS and maltodextrin conjugates stabilized $W_1/O/W_2$ double emulsions had improved general stability when compared to CAS stabilized $W_1/O/W_2$ double emulsions	(O'Regan & Mulvihill, 2010)

Bitter almond gum	Fish oils	The conjugate-based emulsions showed similar physical stability to CAS-stabilized emulsions, and lower antioxidant capacity.	(Rahimi et al., 2021)
Lactose	Canola oil	Better CAS-lactose interactions were achieved at pH 11, with enhanced adsorption of the conjugates at the oil droplet particles and stability.	(Li et al., 2017)
Soybean soluble polysaccharides	Sunflower oil	Conjugated based emulsions exhibited a decreasing trend in droplet size and emulsification index and have better performance at low pH and higher heat conditions.	(Tavasoli et al., 2022)
Maltodextrins and pectin	Eugenol-olive mixed oil	Higher stability of nanoemulsions were obtained by Maillard conjugate compared to protein or polysaccharides alone	(Nagaraju et al., 2021)



1419 **Fig. 1.** (a) Schematic diagram of typical adsorption configurations of  $\alpha_{S1}$ -casein and  $\beta$ -casein  
 1420 at oil/water interface; (b) Mechanisms for adsorption of CAS at oil/water interface.  
 1421 Adapted from Kim et al. (2020).  
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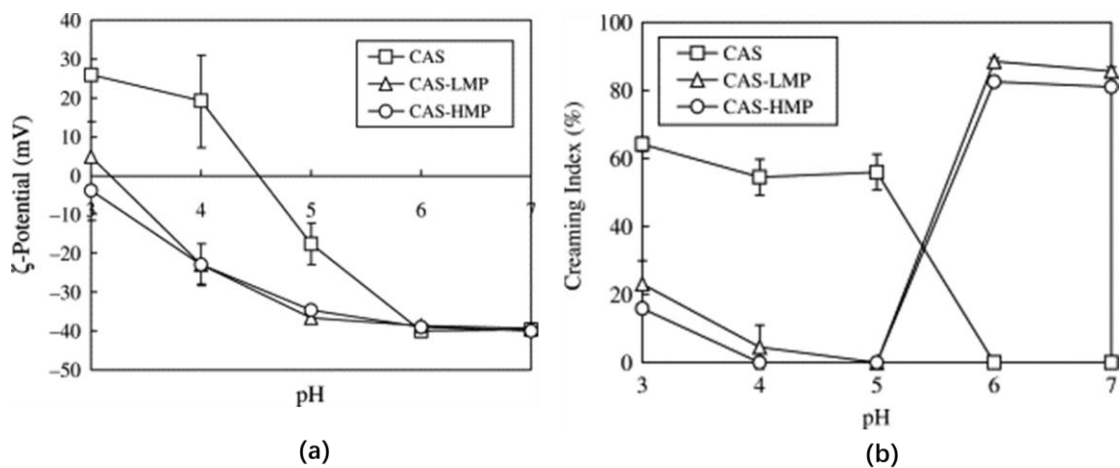
**Fig. 2.** Schematic diagram of the major instability mechanisms undergone by oil-in-water

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emulsions.

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**Fig. 3.** Dependence of (a) electrical charge of droplets ( $\zeta$ -potential) and (b) creaming index on pH

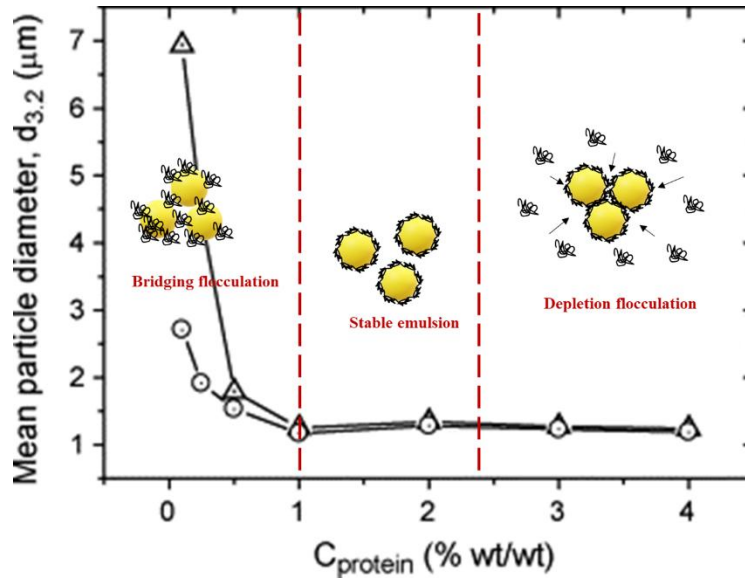
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and pectin type for emulsion encapsulated by CAS (Surh et al., 2006).

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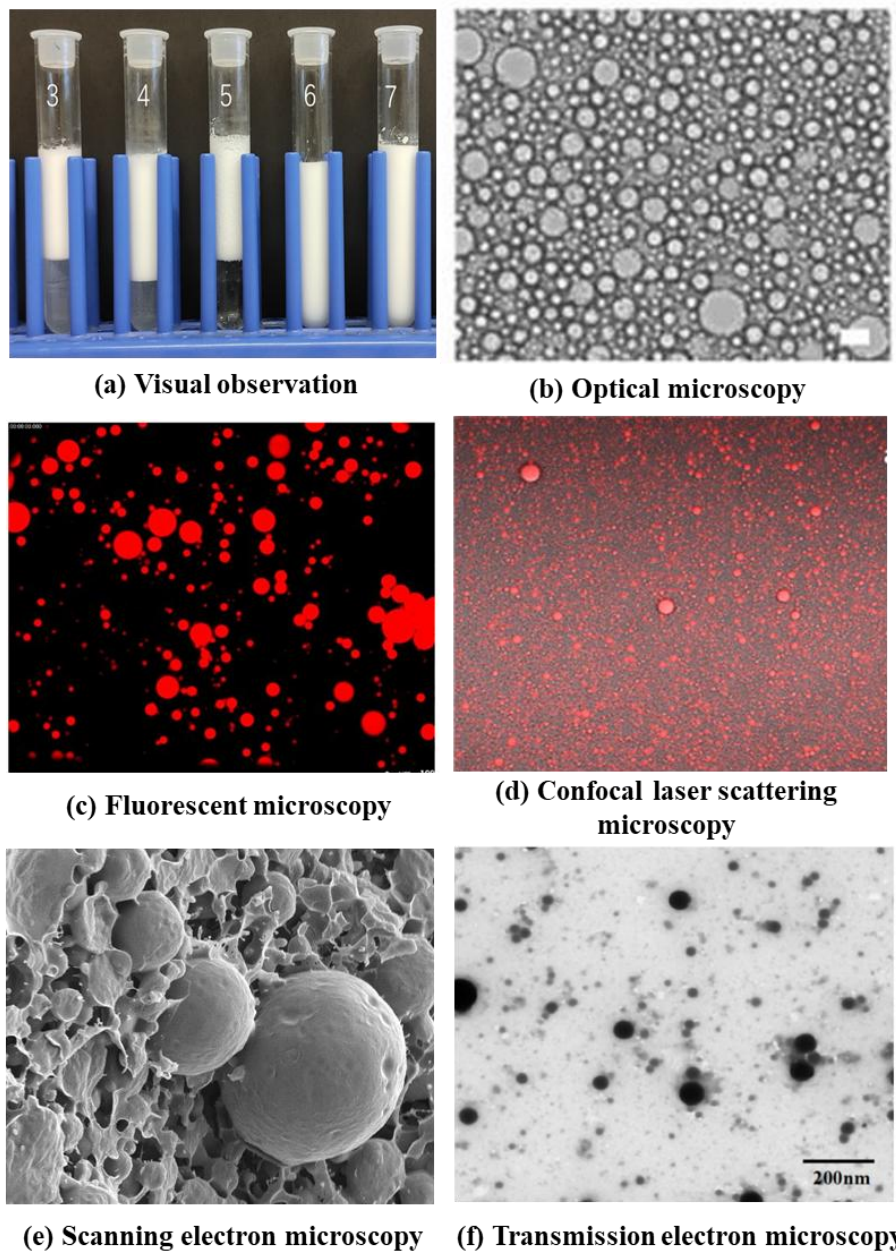
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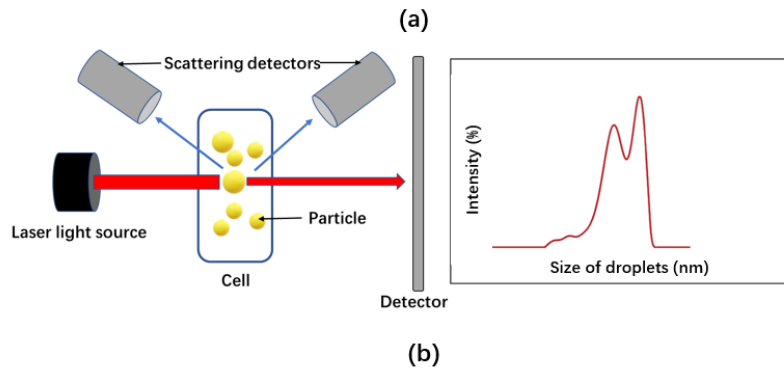
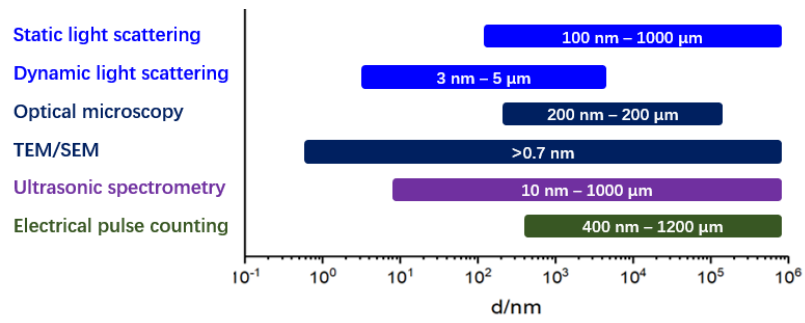
1434 **Fig. 4.** The effect of CAS concentration (% w/w) on mean particle diameter of O/W emulsions in  
 1435 the absence of SDS ( $\Delta$ ) or in the presence of SDS (O) with the schematic diagram of bridging  
 1436 flocculation and depletion flocculation in emulsion systems.  
 1437 Adapted from Sánchez and Patino (2005).  
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**Fig 5.** (a) Appearance of CAS-stabilized nanoemulsions at different pH (3-7); (b) Optical image of a tea seed oil emulsions stabilized by CAS (Xu et al., 2020); (c) Fluorescent microscopy of a coconut o/w emulsion stabilized by CAS (Li & Xiang, 2019); (d) Confocal laser scattering microscopy of a fish oil emulsion stabilized by 0.5 wt.% CAS (Álvarez Cerimedo et al., 2010); (e) Scanning electron microscopy of a soybean oil emulsion stabilized by CAS (Xu et al., 2021) and (f) Transmission electron microscopy of a CAS stabilized nanoparticles (Zhang et al., 2020).



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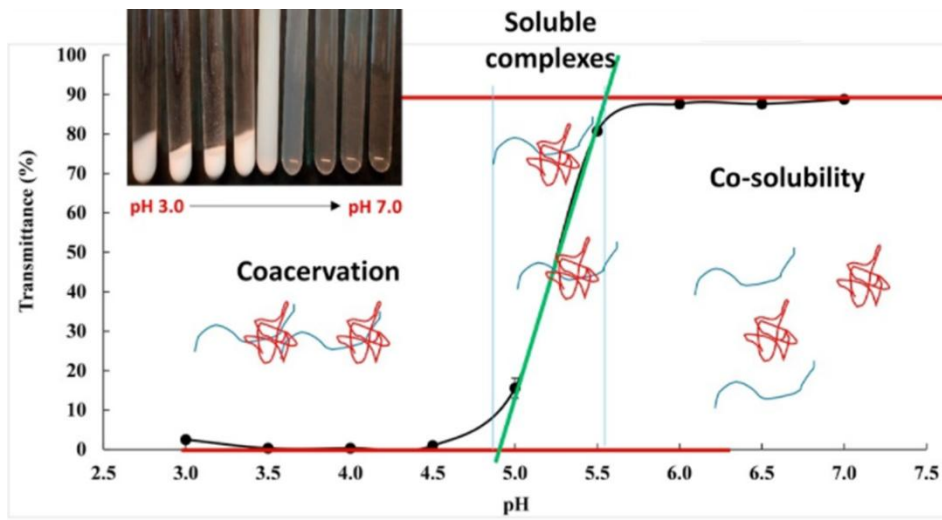
**Fig 6.** (a) The measuring range of common droplet size analysis techniques and

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(b) schematic diagram of particle size distribution detected by light scattering.

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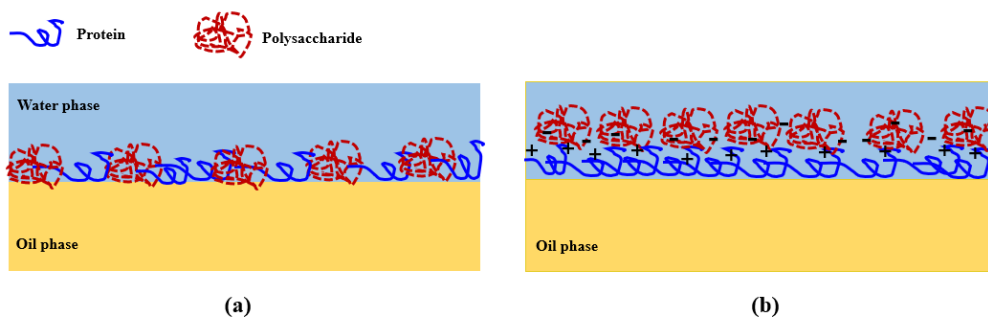
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**Fig. 7.** Transmittance (%) at 850 nm and photographs of CAS and pectin suspensions at

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different pHs (3.0–7.0) with ratio 2.5 (Wang et al., 2019).

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1455 **Fig. 8.** Schematic representation of two different methods for formulating oil-water  
 1456 emulsions with protein-polysaccharide complexes as emulsifiers; (a) Mixed protein and  
 1457 polysaccharide before emulsification; (b) Double layer emulsion, polysaccharide added to the  
 1458 emulsion, stabilized with protein first.