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Water-in-oil Pickering emulsions stabilized by an interfacial complex of water-insoluble polyphenol crystals and protein

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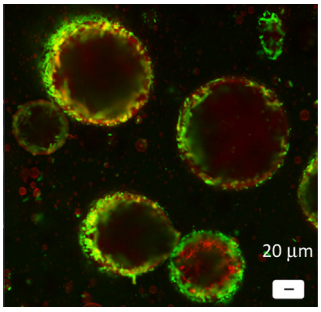
GRAPHICAL ABSTRACT



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ABSTRACT

Long term stabilization of water-in-oil (W/O) emulsions remains a particularly challenging problem in colloid science. Recent studies have shown that polyphenols act as Pickering stabilizers at the water-oil interface. In this work we propose a novel way to stabilize water droplets via interfacial complex formation. It was observed that polyphenol crystals (curcumin or quercetin) absorb at the interface and provide stabilization of water droplets for several days; however formation of a polyphenol- whey protein (WPI) complex at the water-oil interface revealed a pronounced improvement in the stabilization. The mechanism of complex formation was tested by subjecting the systems to different environmental conditions, such as ionic strength and temperature. The evidence suggests that the complex is probably stabilized by electrostatic attraction between the oppositely-charged polyphenol particles and protein at the interface, although hydrogen bonding between the two components may also contribute. The resulting stable water droplets have a Sauter mean diameter ($D_{3,2}$) of approximately ~22 and ~27 µm for curcumin and quercetin systems, respectively. Emulsions were more stable at pH 3 than at pH 7, due to either weaker complex formation at pH 7 and/or chemical degradation of the polyphenols at this more alkaline pH. Interfacial shear viscosity measurements confirmed that there was strong interfacial complex formation with aqueous WPI concentrations of ~0.5 wt.%.

1. Introduction

In many water-in-oil (W/O) emulsion systems (e.g., in food, pharmaceuticals, cosmetics, agriculture etc.), adsorbed solid particles can provide kinetic stability to the dispersed phase [1]. Such solid particles are known as 'Pickering' particles and create a steric barrier between adjacent water droplets, thereby hindering coalescence [1]. The use of Pickering particles as stabilizers for W/O emulsions is gaining significant attention owing to their ability to adsorb irreversibly at the interface and the lack of alternative, clean-label W/O emulsifiers [2,3]. Particularly, there is a huge

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driving force from food and pharmaceutical industries to replace the chemically-synthesized surfactants (e.g. PGPR) with some natural, biodegradable and affordable materials.

Combination of particles, biopolymers and/or surfactants have been reported in literature to stabilize W/O emulsions, however, the research is this area is very limited. Midmore [4] prepared W/O emulsions using hydrophobic silica particles in the oil phase and hydroxypropyl cellulose into the aqueous phase. It was found that the addition of hydroxypropyl cellulose allowed silica particles to absorb more readily at the interface owing to the improved the presence of hydrophobically modified cellulose in the aqueous phase [4]. Skelhon et al. [5] used fumed silica particles (dispersed in the oil phase) and positively charged chitosan in the aqueous phase under acidic conditions to stabilize W/O emulsions. They have observed that the addition of chitosan in the system promoted the silica to adsorb favourably onto the droplet surface due to electrostatic interactions between the oppositely charged chitosan and silica particles [5]. In addition, Rutkevičius et al. [6] studied the stabilization of W/O emulsions through the addition of zein particles present in the aqueous phase and lecithin in the oil phase, where the latter promoted adsorption of zein particles to the interface [6].

In our previous work, we have shown the ability of polyphenol crystals to stabilize water droplets via the Pickering mechanism [3]. Water-insoluble curcumin and quercetin crystals were proven to be hydrophobic, based on their three-phase contact angle measurements. Neither of these particle dispersions suppressed the interfacial tension significantly, indicating a Pickering stabilization mechanism. Micro-structural evaluation at various length scales revealed that quercetin crystals had a more rod-like shape than curcumin crystals, the latter being smaller and having a more polyhedral shape. The differences in the shape and size of the polyphenols were reflected in the Pickering stabilization efficiency of the two materials, perhaps explaining why other workers [7] were apparently not able to stabilize W/O emulsions as effectively with different polyphenols. Curcumin and quercetin particles at 0.14 wt. % imparted stability to coalescence of W/O emulsions for several days of storage, but the size (up to 6 μm) of the droplets resulted in significant sedimentation of both droplets and particles over this time period [3], most probably due to the large size range of the stabilizing particles, making efficient coverage of small water droplets difficult. Thus, although polyphenol crystals show potential as W/O emulsion stabilizers, improvements are required to extend the kinetic stability of such emulsions. In this paper we describe a novel strategy based on complex formation between polyphenols and protein at the interface - a patent has been recently filed on this technology [8]. This novel mechanism uses natural materials, such as polyphenol crystals and proteins to stabilize water droplets in the oil phase, allowing a "clean" label emulsifier in the final product.

Curcumin is a natural low-molecular weight polyphenolic compound found in the rhizomes of the perennial herb turmeric (*Curcuma longa*) [9]. It possesses two phenolic –OH groups and an α,β-unsaturated-β-diketone moiety in its chemical structure [10,11]. The polar hydroxyl/ketone groups are expected to impart hydrophilicity whilst the aromatic/aliphatic parts would be expected to make the molecule more hydrophobic. Quercetin is one of the most abundant flavonoids present in fruits and vegetables - particularly onions, kale, French beans, broccoli, etc. [12]. It is characterized by its C6-C3-C6 basic backbone [13]. Quercetin has hydroxyl groups that impart hydrophilic characteristics and ring structures that impart hydrophobicity [14]. The quercetin crystal structure can be described as layers of hydrogen bonded dimers. These dimers form a two-dimensional net held together via a network of hydrogen bonds with water molecules also present [15]. Thus, quercetin molecules can pack in a way that some of the –OH groups are not exposed to the continuous phase, potentially explaining the apparent hydrophobic character of the crystal particle surface.

Polyphenols have the ability to interact with proteins via hydrogen bonding, hydrophobic and ionic interactions [16,17]. However, the way proteins adsorbing from the aqueous side of a W/O interface might interact with polyphenol crystals adsorbing from the oil side of the interface has not been explored to date.

In this work, we provide evidence that the addition of protein to the aqueous phase of W/O emulsions can give significant improvement to their stability. Whey protein isolate (WPI) was the protein chosen, due to its surface activity (in water) and its ability to form strong viscoelastic adsorbed layers at the interface on its own, when adsorbed from an aqueous phase to a more hydrophobic phase. WPI is a mixture of proteins with numerous functional properties and is of a considerable importance to the food industry. The main proteins it contains are α-lactoglobulin and β-lactoglobulin, which represent ca. 70% of the total whey protein and are responsible for its main functional properties [18]. β-Lactoglobulin is a globular protein with a polypeptide chain of 162 residues stabilized by two disulfide cross-links and also contains an internal free sulfhydryl group which is sensitive to interfacial denaturation and heat treatment [18,19]. The monomeric molecular mass is 18.3 kDa. At pH 5–8, β-lactoglobulin exists as a dimer but at pH 3–5 the dimers associate to form octomers [18]. α-Lactoglobulin is a smaller protein with 123 amino acid residues and four disulfide bridges and a molecular mass of 14.2 kDa. It has a relatively low content of organized secondary structure for a globular protein and therefore has great molecular flexibility [18]. The conformation and physicochemical properties of both proteins naturally depends on the environmental conditions such as salt, pH and temperature treatments [18].

In this work, we show a unique stabilization mechanism of water droplets in oil via complex formation at the interface between polyphenol crystals (i.e., curcumin and quercetin) and WPI. It is hypothesized that polyphenol crystals in the continuous (oil) phase and WPI in the aqueous phase form complexes at the W/O interface via attractive electrostatic interactions and/or hydrogen bonding. The stability of the corresponding W/O emulsion droplets was evaluated as a function of different WPI concentrations and pH values and the mechanism of the interfacial interactions was probed by subjecting the systems to different ionic strengths and temperatures whilst using a range of complimentary physical and microstructural techniques.

2. Material and methods

2.1. Materials

Curcumin (orange-yellow powder) from turmeric rhizome (96% total curcuminoid content) was obtained from Alfa Aesar (UK). Quercetin (≥95%) in the form of a yellow crystalline solid was purchased from Cayman Chemicals (USA). Both polyphenols were used without further purification. Soybean oil (KTC Edibles, UK) was purchased from a local store. Aluminium oxide, (99%, extra pure) was used for soybean oil purification in some experiments and was purchased from Acros Organics (Belgium). Whey protein isolate (WPI) containing 96.5% protein was obtained from Fonterra (New Zealand). Water, purified by treatment with a Milli-Q apparatus (Millipore, Bedford, UK), with a resistivity not less than 18 MΩ cm^{-1}, was used for the preparation of the emulsions. A few drops of hydrochloric acid (0.1 M HCl) or sodium hydroxide (0.1 M NaOH) were used to adjust the pH of the emulsions. Sodium azide and Rhodamine B were purchased from Sigma-Aldrich (USA).

2.2. Preparation of W/O emulsions

Curcumin or quercetin dispersions were prepared by dispersing the polyphenol crystals (0.14 wt.%) in the continuous phase (soybean oil) using an Ultra-Turrax T25 mixer (Janke & Kunkel, IKA-Labortechnik) with a 13 mm mixer head (S25N- 10G) operating at 9400 rpm for 5 min. The aqueous phase was prepared without or with WPI (0.05–4 wt.%). The WPI (4 wt.%) was dissolved in aqueous phase for at least 2 h at room temperature to ensure complete hydration. The WPI solution was then diluted to the desired WPI concentration and 0.02 wt.% sodium azide was added as a preservative. The pH of the aqueous phase was adjusted to 3 or 7, depending on the experiment, by adding few drops of 0.1 M HCl or NaOH. Coarse W/O emulsions were prepared by homogenizing 5, 10 or 20 wt.% of this aqueous phase with soybean oil in an Ultra-Turrax mixer for 2 min at 13,400 rpm. Fine emulsions were prepared by passing the coarse emulsions through a high pressure Leeds Jet Homogenizer, twice, operated at 300 bar. The initial temperature of the particle dispersion was 21 °C. The temperatures of the dispersions were 23 and 26 °C after Ultra-Turrax mixing at 9,400 rpm for 5 min and 13,400 rpm for 2 min, respectively. The temperature of the particle dispersion after passing through the Jet homogenizer (two passes at 300 bar) was 33–34 °C. Note that these slight temperature increases were too low to have any significant impact on solubility of the particles or the proteins [3]. Immediately after preparation, emulsions were sealed in 25 mL cylindrical tubes (internal diameter = 17 mm) and stored at room temperature in a dark place.

2.3. Particle size measurements

The particle size distributions (*PSD*) of emulsions were measured using static light scattering (SLS) via a Mastersizer Hydro SM small volume wet sample dispersion unit (Malvern Instruments, UK). Average droplet size was monitored via the Sauter mean diameter, $D_{3,2}$, or volume mean diameter, $D_{4,3}$, defined by:

$$D_{ab} = \frac{\sum n_i d_i^a}{\sum n_i d_i^b} \quad (2)$$

where n_i is the number of the droplets of diameter d_i.

For water droplet size measurements, refractive indices of 1.33 and 1.47 were used, for water and soybean oil, respectively. Absorption coefficients of 0.01, 0.1 and 0.01 for curcumin, quercetin and water, were used, respectively. All measurements were made at room temperature on at least three different samples.

2.4. Confocal laser scanning microscopy (CLSM)

The microstructure of the W/O emulsions was observed using a confocal microscope (Zeiss LSM700 inverted, Germany). The emulsions were prepared as before but were deliberately not passed through the Leeds Jet homogenizer in order to maximize their size because with larger droplets it was easier to discern the absorbed layers of polyphenols and WPI at the interface. Approximately, 80 μL of sample were placed into a laboratory-made welled slide and a cover slip (0.17 mm thickness) was placed on top, ensuring that there was no air gap (or bubbles) trapped between the sample and coverslip. The samples were scanned at room temperature (25 ± 1 °C) using a 20×/0.5 objective lens. Auto-fluorescence from the particles was excited using 488 and 405 nm wavelength lasers for curcumin and quercetin crystals, respectively. Rhodamine B was used as a dye for whey protein and was excited using a 545 nm wavelength laser. The emission fluorescent light was detected at wavelengths of 525, 460 and 580 nm for curcumin, quercetin and Rhodamine B, respectively.

2.5. Interfacial tension

Interfacial tension (γ) measurements were performed using soybean oil, with or without the presence of polyphenol crystals and for Milli-Q water in the absence or presence of 0.5 wt.% WPI (pH 3), using the pendant drop method in a Dataphysics OCA tensiometer (DataPhysics Instruments, Germany). The apparatus includes an experimental cell, an optical system for the illumination and the visualization of the drop shape and a data acquisition system. An upward bended needle was used to immerse a drop of the lower density liquid (oil) into the higher density one (water). Thus, a drop of soybean oil or polyphenol suspension in oil (0.14 wt.% curcumin or quercetin) was formed at the tip of the needle and suspended in the cuvette containing Milli-Q water with or without 0.5 wt.% WPI, at pH 3. The contour of the drop was extracted by the SCA 22 software and fitted to the Young-Laplace equation to obtain γ. All measurements were carried out in triplicate and error bars represent the standard deviations.

2.6. Interfacial shear viscosity (η_i)

A two dimensional Couette-type interfacial viscometer, described in detail elsewhere [20,21] was operated in a constant shear-rate mode to measure interfacial viscosity. Briefly, a stainless steel biconical disc (radius 15.0 mm) was suspended from a thin torsion wire with its edge in the plane of the W/O interface of the solution contained within a cylindrical glass dish (radius 72.5 mm). The constant shear rate apparent interfacial viscosity, η_i, is given by the following equation:

$$\eta_i = \frac{g_f}{\omega} K(\theta - \theta_o) \quad (4)$$

where K is the torsion constant of the wire; θ is the equilibrium deflection of the disc in the presence of the film; θ_o is the equilibrium deflection in the absence of the film, i.e., due to the bulk drag of the sub-phase on the disc; g_f is the geometric factor and ω is the angular velocity of the dish. A fixed value of $\omega = 1.27 \times 10^{-3}$ rad s^{-1} was used, which aids comparison with measurements made on many other systems at the same shear rate.

For these measurements, 0.14 wt.% curcumin or quercetin particles were dispersed in purified soybean oil using the Ultra-Turrax mixer at 9400 rpm for 5 min. The oil was purified with aluminium oxide to eliminate free fatty acids and surface active impurities that may affect the measurements. A mixture of oil and aluminium oxide in proportion 2:1 w/w was stirred for 3 h and centrifuged at 4000 rpm for 30 min. For the experiments with salt, the aqueous phase consisted of 0.5 wt.% WPI dispersed in 0.001, 0.01 or 0.1 M NaCl at pH 3. For the experiments with thermal treatment, the systems were prepared as before but η_i was measured at 45 °C for 3 h and then the system was left to cool down overnight, at 25 °C and the η_i was measured again, at this temperature.

2.7. Optical microscopy

Curcumin or quercetin dispersions (0.14 wt.%) were heated at 45 °C under continuous stirring at 20–40 rpm using mixer (IKA 2830001 Compact Mixer, UK) for 30 min. A drop of each dispersion was placed on a microscope slide at (25 °C) before the heating, during the heating (to 45 °C) and after it had cooled back down to 25 °C (after 1 h). The slide was covered with a cover slip (0.17 mm thickness). The size and shape of the polyphenol crystals were observed using an LCD digital microscope (PentaView, Celestron, USA) using a 20×/0.4 objective lens.

3. Results and discussion

3.1. Stability measurements of W/O Pickering emulsions

3.1.1. Aqueous phase at pH 3

The main component of WPI, β-lactoglobulin, undergoes significant changes on adsorption, in terms of unfolding of its globular conformation [19,22,23]. WPI acquires a positive charge (zeta potential = +34 mV) at pH 3, whilst as the pH increases it becomes more negatively charged (e.g., zeta potential = −34 mV at pH 7) [24]. The isoelectric point (pI) of WPI is at around pH 5 [24]. It has been shown previously that at pH 3 curcumin crystals *dispersed in water* had a low positive zeta potential (+12 mV), whilst quercetin had a distinct negative charge (zeta potential = −26 mV) [3]. Both crystals *dispersed in water* had similar negative zeta potentials at pH 7 (−48 mV) [3]. Therefore, at pH 3, there is the possibility of electrostatic attraction between the negatively-charged quercetin crystals wetted by water from the oil side of the interface and the positively-charged WPI adsorbing from the aqueous side of the interface. For curcumin, weaker electrostatic repulsion might take place at pH 3, noting that proteins are polyampholytes and that WPI will bear some negatively-charged patches even at pH 3, whilst its net charge is still positive.

The *PSD* of the fine emulsions, prepared at pH 3 with 0.14 wt.% curcumin or quercetin dispersed in the oil phase plus varying concentrations of WPI in the aqueous phase, are shown in Fig. 1a and 1c, respectively. Some smaller peaks (≤1 μm) were observed in the *PSD*s for both curcumin and quercetin particles because of the presence of free particles in the continuous phase (the peaks are separated by the dashed line). Thus, the *PSD* of the water droplets is more correctly identified by the distribution >1 μm for both systems and these are shown in Fig. 1b and d. The $D_{3,2}$ values for the water droplets (i.e., >1 μm) were recalculated from this cut of the total *PSD*s for both systems. For both emulsions with curcumin or quercetin crystals, the mean size ($D_{3,2}$) of the water droplets with 0 and 0.05 wt.% WPI were smaller (~12 and 20 μm for curcumin and quercetin, respectively) than the $D_{3,2}$ for the systems containing a higher concentration of WPI (0.5, 1, 2 and 4 wt.%), at ~22 and 27 μm for curcumin and quercetin, respectively. However, over time (see Fig. 1b and d), the $D_{3,2}$ of emulsions with 0 and 0.05 wt.% WPI in the aqueous phase increased significantly ($p < 0.05$, see Supporting Information Tables S1 and S2) and phase

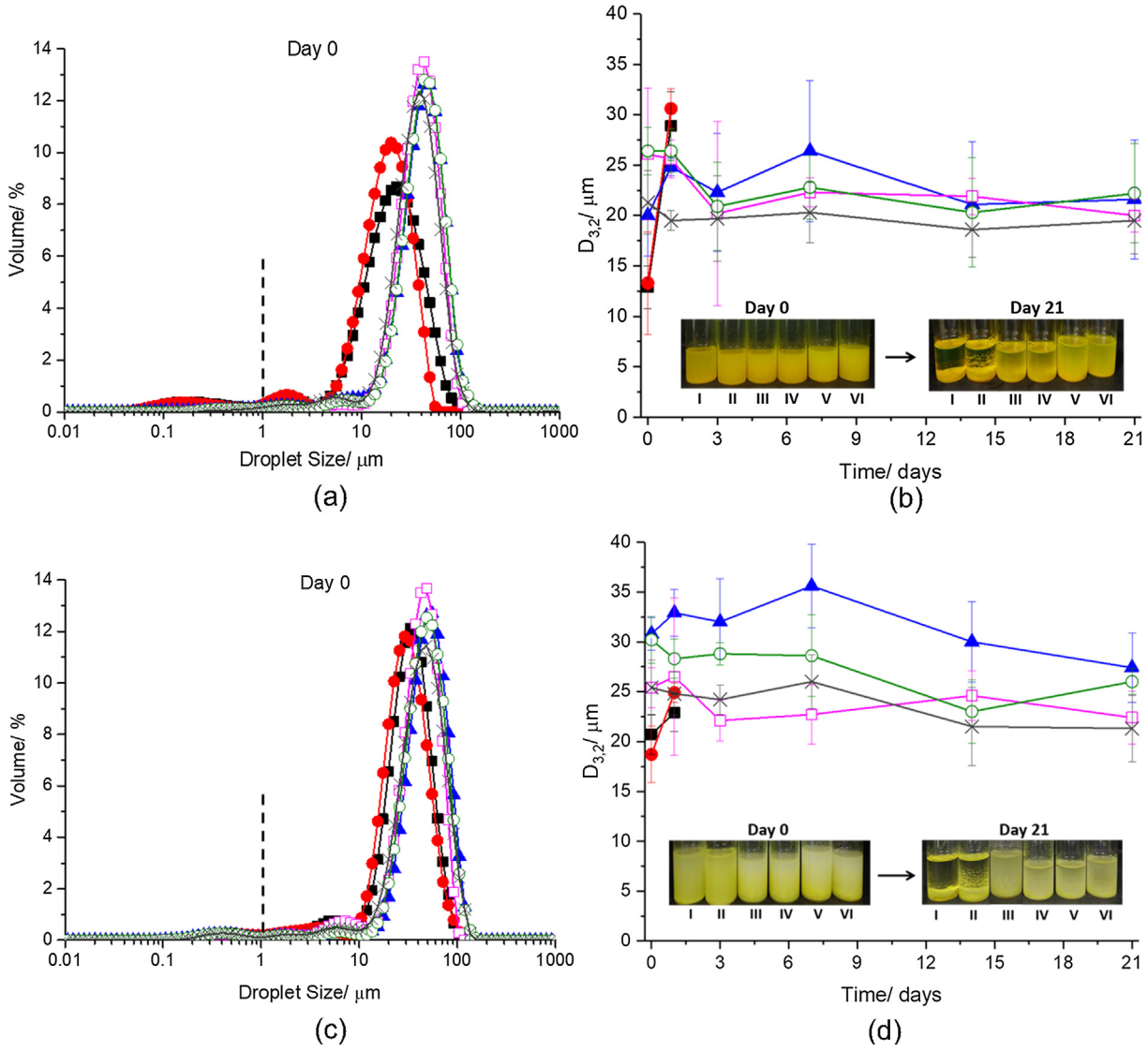

Fig. 1. *PSD* (a and c) and mean particle size ($D_{3,2}$) of water droplets over time with visual images (b and d) of 5 wt.% W/O emulsions stabilized by 0.14 wt.% curcumin (a and b) and quercetin (c and d) particles containing 0 wt.% [■] [I], 0.05 wt.% [●] [II], 0.5 wt.% [▲] [III], 1 wt.% [□] [IV], 2 wt.% [○] [V] and 4 wt.% [╳] [VI] WPI in the aqueous phase at pH 3, respectively. For statistical analysis according to Tukey's test see Supporting Information Tables S1 and S2 for curcumin and quercetin, respectively.

separation occurred within 24 h. On the other hand, $D_{3,2}$ of the emulsions with 0.5, 1, 2 and 4 wt.% WPI was stable ($p > 0.05$, see Supporting Information Tables S1 and S2), for more than 3 weeks. Sedimentation of particles and/or water droplets was observed, but no coalescence or obvious phase separation of a clear water layer was seen. From these visual observations, plus the changes in mean particle size, it is suggested that the presence of ≥0.5 wt.% WPI, improved significantly the stability of the emulsions, suggesting some synergistic interaction of WPI and polyphenol crystals at the W/O interface. Our previous work has shown that the dispersion and emulsification conditions had negligible effect on the size distributions of the polyphenol crystals themselves.

Confocal images of the emulsions stabilized by curcumin or quercetin plus WPI are shown in Fig. 2a and b, respectively, demonstrating that water droplets were surrounded by a dense layer of polyphenol particles (green), confirming their preferential location at the W/O interface. The green brightness in the images is due to the auto-fluorescence of polyphenol particles. Rhodamine B (red) was used to visualize the location of protein and so the intensity of the red color indicates a higher concentration of WPI on the inside of the droplets at the W/O interface, as expected. Thus, both polyphenol crystals and WPI appeared to be in close proximity at the interface. Note the size of the water droplets in these images does not reflect the size measured using the Mastersizer because these emulsions were prepared without passing through the Jet homogenizer, as mentioned above, in order to deliberately have larger water droplets and make it easier to visualize the location of the two types of surface active material at the W/O interface.

To further understand the interfacial stabilization mechanism, the interfacial tension (γ) was measured, at pH 3, and the results are shown in Table 1. Firstly, γ was measured between soybean oil and the aqueous phase (in the absence of particles) as a control. The equilibrium γ for this system was 25.8 ± 0.4 mN m^{-1}. The value of γ did not alter significantly on the addition of curcumin or quercetin particles in the oil phase, indicating Pickering stabilization [3]. However, upon addition of 0.5 wt.% WPI (to the aqueous phase) there was a significant decrease in γ ($p < 0.05$), presumably due to WPI adsorption, i.e., irrespective of whether polyphenol crystals also adsorbed from the other (oil) side of the interface.

These results support the idea that WPI and polyphenols adsorb together at the W/O interface at pH 3. This co-adsorption may explain the improvement in stability of the emulsions at pH 3, but to test whether such improvement was due to electrostatic complex formation between them, experiments were also conducted at pH 7, where both crystals and protein have the same (negative) sign of charge, which should inhibit such complex formation.

3.1.2. Aqueous phase at pH 7

Similar to the behavior at pH 3, freshly prepared W/O emulsions stabilized by curcumin or quercetin (Fig. 3a and c) at pH 7, with 0 and 0.05 wt.% WPI, had smaller initial droplet sizes, but their size increased ($p < 0.05$, see Supporting Information Tables S3 and S4) and gross phase separation occurred within 1 day. The emulsions with 0.5, 1, 2 and 4 wt.% WPI had similar $D_{3,2}$ compared to those prepared at pH 3 (Fig. 1a and c) but the emulsions at pH 7 were not as stable as at pH 3. Emulsions prepared with quercetin + WPI (≥0.5 wt.%) showed sedimentation and some degree of phase separation within 7 days (Fig. 3d) whereas those with curcumin crystals took 2 weeks to show phase separation (Fig. 3b). Therefore, in both cases the emulsions at pH 7 were not as stable as the corresponding emulsions at pH 3, with an obvious water layer appearing on the bottom of the samples. As observed at pH 3, some smaller peaks below 1 μm were seen for both particles (the peaks were separated by a dashed line, and the $D_{3,2}$ values were recalculated for water droplets as above (Fig. 3b and d)). In addition, the *PSD* of the emulsions with both curcumin and querce-

Table 1
Interfacial tension (mN m^{-1}) data, with or without the presence of curcumin or quercetin particles (0.14 wt.%) and WPI (0.5 wt.%), between the soybean oil and aqueous phases. Samples with the same letter do not differ significantly ($p > 0.05$) according to Tukey's test.

Oil Phase	Aqueous Phase (pH 3)	γ/mN m^{-1}
Soybean Oil	Milli-Q Water	26.2 ± 0.5^{a}
Curcumin	Milli-Q Water	25.4 ± 0.3^{a}
Quercetin	Milli-Q Water	25.8 ± 0.4^{a}
Curcumin	WPI solution (0.5 wt.%)	17.1 ± 0.7^{b}
Quercetin	WPI solution (0.5 wt.%)	18.0 ± 0.6^{b}

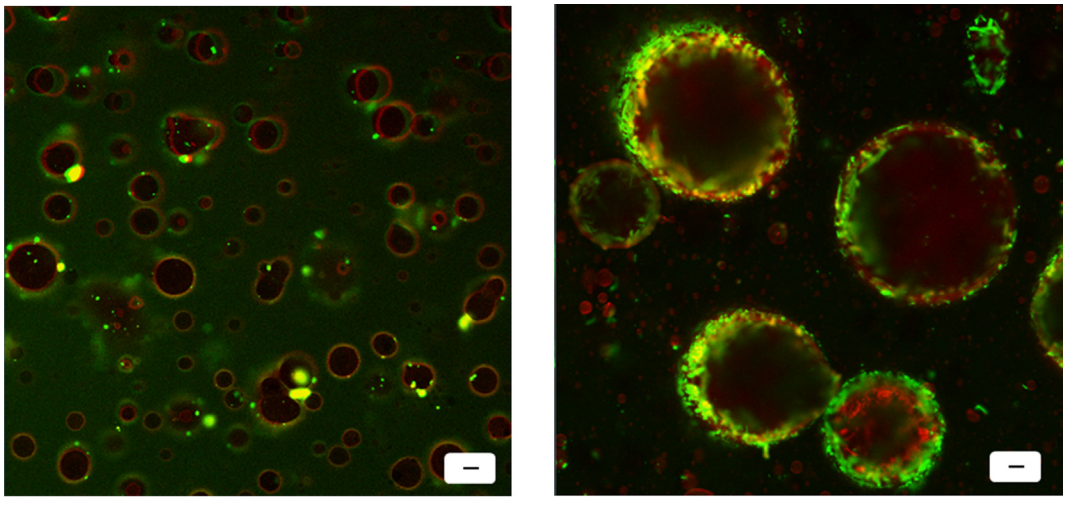

Fig. 2. Confocal images of 5 wt.% W/O Pickering emulsions stabilized by 0.14 wt.% curcumin (a) and quercetin (b) crystals with 0.5 wt.% WPI in the aqueous phase, at pH 3. The green brightness in the images is caused by auto-fluorescence of curcumin (488 nm excitation) or quercetin (405 nm excitation) crystals. The red brightness is due to WPI stained by Rhodamine B (568 nm excitation). The scale bar represents 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tin crystals + ≥0.5 wt.% WPI had a second peak at larger particle sizes (between 1 and 10 μm – see Fig. 3c) at pH 7, unlike those at pH 3. From the confocal images (Fig. 4), one can clearly observe considerably greater aggregation of polyphenol particles at pH 7 compared to at pH 3 (Fig. 2). Much of the extra aggregation at pH 7 seemed to occur at the interface, appearing to 'stick' droplets together. Therefore, the peaks at larger sizes at pH 7 probably represent these mixed polyphenol + droplet aggregates. Such flocculation might be attributed to particle aggregation in the continuous phase with consequently less coverage of the W/O interface, i.e., less primary particle coverage of the interface. In any case, the results suggest that if electrostatic complex formation contributes to emulsion stability, it is less effective at pH 7, where both protein and polyphenols have the same (negative) sign of charge.

3.1.3. Effect of increase in volume fraction of water droplets

Higher water:oil ratios (10 and 20 wt.% water) were tested at pH 3 for 0.14 wt.% curcumin or quercetin crystals +0.5 wt.% WPI as Pickering stabilizers, since this WPI concentration seemed to be the minimum required to give enhanced stability to 5 wt.% W/O emulsions and emulsions were more stable for both polyphenol crystals at this lower pH. As observed previously, some smaller peaks below 1 μm were seen for both particle types (the peaks are separated by a dashed line and the $D_{3,2}$ values were recalculated for water droplets, as above (Fig. 3b and d)). Fig. 5 shows that with curcumin the initial $D_{3,2}$ increased slightly from ~20 to ~25 μm ($p > 0.05$) as the wt.% water was increased from 5 to 20 wt.%, whilst with quercetin $D_{3,2}$ decreased from ~30 to ~22 μm as the wt.% water was increased from 5 to 20 wt.% ($p < 0.05$). However, with both curcumin and quercetin, emulsions containing 10 or 20 wt.% water showed an obvious water layer at the bottom of the samples after 1 day and the systems had completely phase separated after 3 days. This suggests that at these higher water volume fractions there were not enough polyphenols crystals to fully cover the interface. The $D_{3,2}$ of the 5 wt.% W/O emulsion stabilized by curcumin was slightly lower than that for the corresponding emulsions stabilized by quercetin and we believe this is probably due to the smaller size of the curcumin crystals ($D_{3,2}$ = 0.2 and 6 μm for curcumin and quercetin in oil, respectively), which also had a lower aspect ratio [3]. This would lead to more densely packed and even multilayers of curcumin particles at the interface. At 10 and 20 wt.% water there is more interface to cover and so both crystal types will be more evenly spread at the interface, which probably explains why there was relative little difference in the mean droplet size for the two systems at these higher water droplet concentrations.

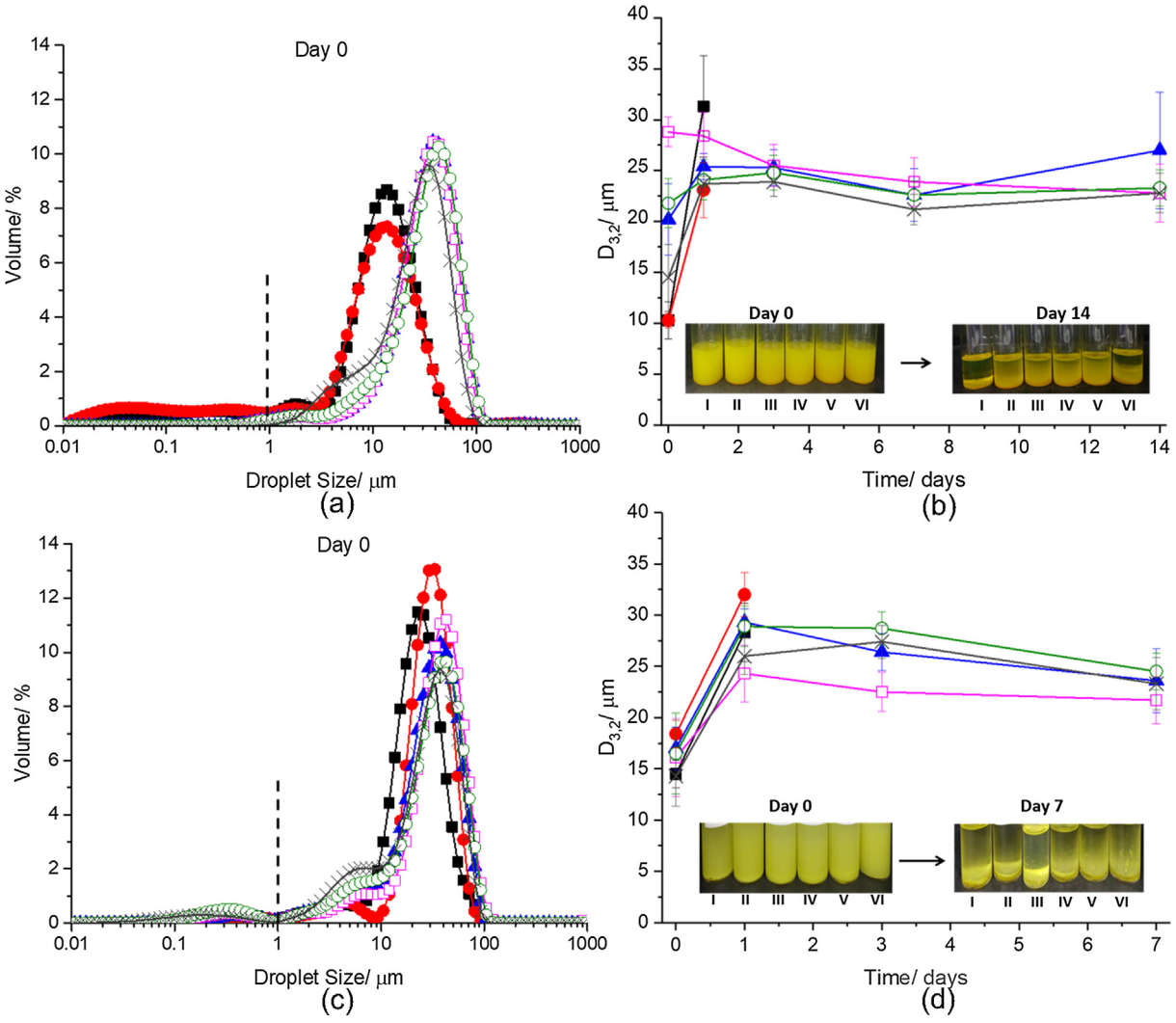

Fig. 3. *PSD* (a and c) and mean particle ($D_{3,2}$) size of water droplets over time with visual images (b and d) of 5 wt.% W/O emulsions stabilized by 0.14 wt.% curcumin (a and b) and quercetin (c and d) particles containing 0 wt.% [■] [I], 0.05 wt.% [●] [II], 0.5 wt.% [▲] [III], 1 wt.% [□] [IV], 2 wt.% [○] [V] and 4 wt.% [╳] [VI] WPI in the aqueous phase at pH 7, respectively. For statistical analysis according to Tukey's test see Supporting Information Tables S3 and S4 for curcumin and quercetin, respectively.

3.2. Interfacial shear viscosity

In order to test more directly for evidence of interfacial complex formation, measurements of interfacial shear viscosity (η_i) were undertaken. Interfacial shear viscosity is a particularly sensitive method for monitoring the formation and structuring of adsorbed films [25]. It can give insight into structural and compositional changes within adsorbed layers, and how interfacial properties can be related to aspects of the formation and stability of emulsions [26,20]. Fat crystals and mono- and di- glycerides that are present in vegetable oils can influence the interfacial rheology of adsorbed particle films [20]. The co-adsorption of mono and diglycerides tends to destroy high values of η_i of particle films because they tend to be more surface active than particles and displace them from the interface [20]. For this reason, experiments were first performed with soybean oil purified via aluminium oxide (as described in the Methods section) in order to remove any such low molecular weight surface active components. Fig. 6a and b show the values of interfacial shear viscosity after 24 h in the presence of 0.14 wt.% curcumin or quercetin particles dispersed in the oil with different concentrations of WPI in the aqueous phase, at pH 3 and 7. A control experiment with purified oil (i.e., containing no polyphenol crystals) and Milli-Q water (at pH 3 and 7) was performed but $\eta_i = 0$ mN s m^{-1} even after 24 h (results not shown). Addition of 0.5 wt.% WPI (pH 3) to the aqueous phase (but with only purified oil as the upper phase) also did not show a significant increase in η_i even after 24 h (maximum of 4.5 mN s m^{-1}).

Here it should be pointed out that there is an error in our previously published preliminary surface shear viscosity values [3]

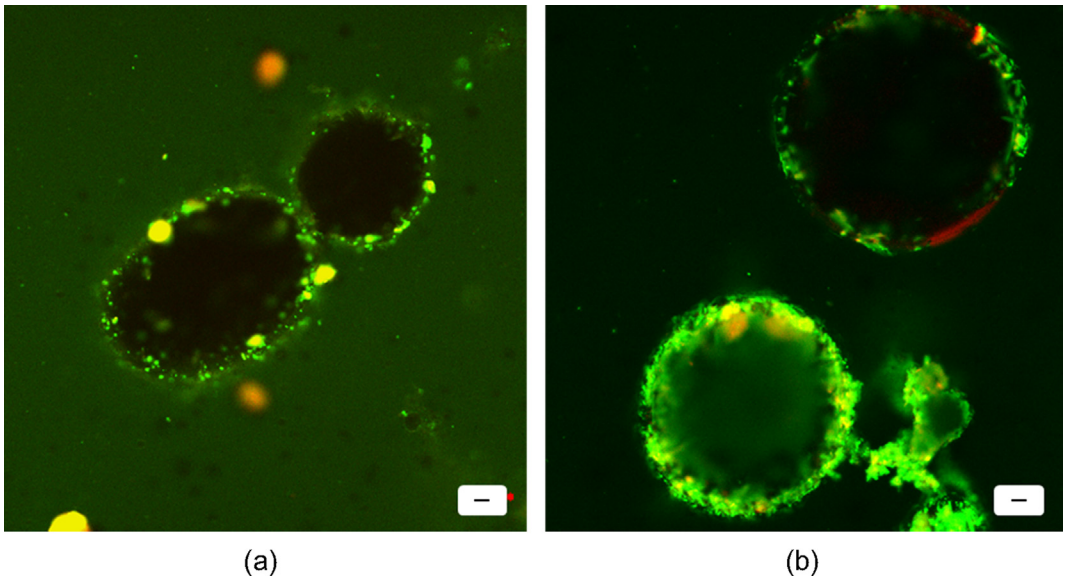

Fig. 4. Confocal images of 5 wt.% W/O Pickering emulsions stabilized by 0.14 wt.% curcumin (a) and quercetin (b) crystals with 0.5 wt.% WPI in the aqueous phase at pH 7. The green brightness in the images is caused by auto-fluorescence of curcumin (488 nm excitation) or quercetin (405 nm excitation) crystals. The red brightness is due to WPI stained by Rhodamine B (568 nm excitation). The scale bar represents 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

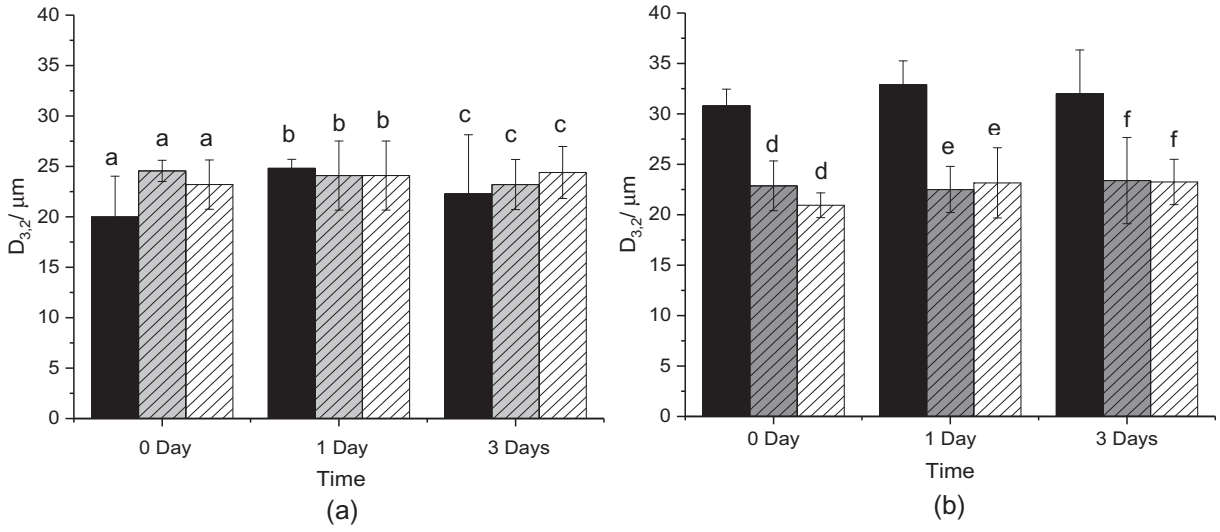

Fig. 5. Mean size of water droplets ($D_{3,2}$) over time of W/O emulsions containing 5 wt.% (black), 10 wt.% (grey) and 20 wt.% (white) water stabilized by 0.14 wt.% curcumin (a) and quercetin (b) particles at 0.5 wt.% WPI in the aqueous phase at pH 3, respectively. Samples with the same letter do not differ significantly ($p > 0.05$) according to Tukey's test.

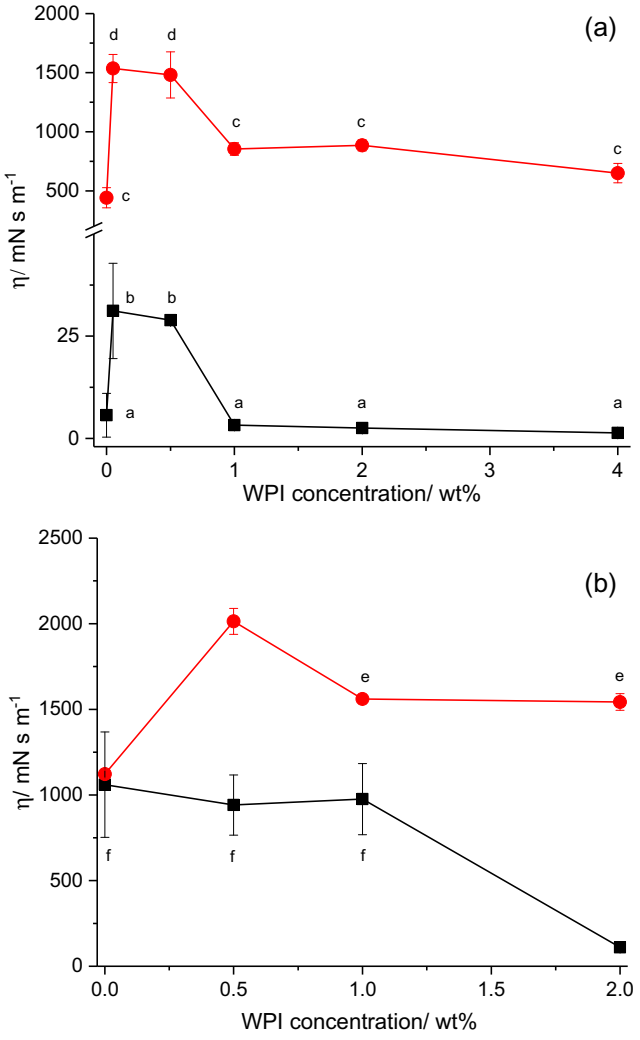

Fig. 6. Interfacial shear viscosity at W/O interface of 0.14 wt.% curcumin [■] and quercetin [●] particles dispersed in purified oil with different concentrations of WPI in the aqueous phase after 24 h of adsorption at pH 3 (a) and pH 7 (b), respectively. Samples with the same letter do not differ significantly ($p > 0.05$) according to Tukey's test for each polyphenol + WPI system at each pH value.

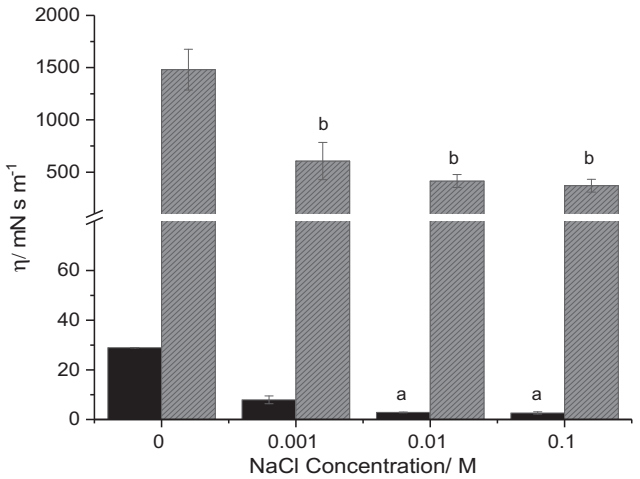

Fig. 7. Interfacial shear viscosity at W/O interface of 0.14 wt.% particles of curcumin [black] and quercetin [grey] dispersed in purified oil and 0.5 wt.% WPI at different NaCl concentrations in aqueous phase at pH 3, after 24 h of adsorption. Samples with the same letter do not differ significantly ($p > 0.05$) according to Tukey's test.

for 0.14 wt.% curcumin and quercetin alone at the same interface. This was due a unit error (radians versus degrees in the disk deflection). In the Supplementary Information Figs. S1 and S2 we have corrected and replotted all this old data and it is seen that, within experimental error there is good agreement between these old values for quercetin after 24 h and the ones presented in this current paper (Fig. 6 above). For curcumin there is more of a discrepancy, but as also discussed below the curcumin crystals are much smaller and the rate of accumulation of curcumin crystals at the interface is probably more sensitive to the exact *PSD* of curcumin crystals added to the system. Plus, as seen in Fig. 6, the quercetin results are far more reproducible.

As seen in Fig. 6, for both curcumin and quercetin the trend of η_i (24 h) versus WPI concentration at pH 3 was very similar. Below 0.5 wt.% WPI, there was a significant increase ($p < 0.05$) of η_i, whereas above 0.5 wt.% WPI, η_i decreased ($p < 0.05$). Such behavior is typical – at low concentrations of protein the particles and

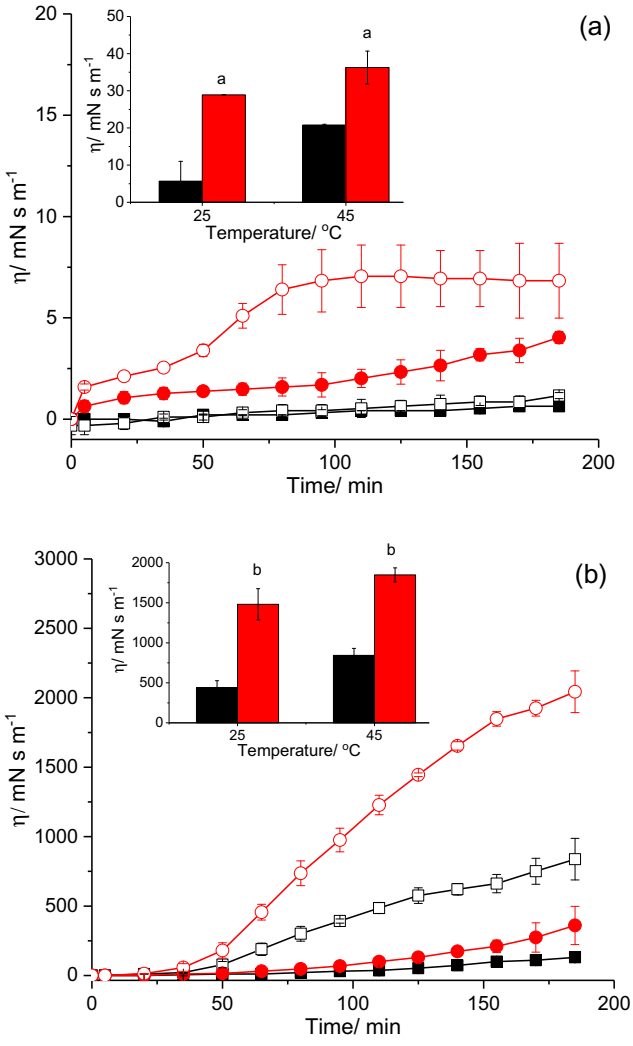

Fig. 8. Interfacial shear viscosity at W/O interface against time of 0.14 wt.% curcumin (a) and quercetin (b) particles with Milli-Q water [black], 0.5 wt.% WPI in aqueous phase [red] at 25 °C [filled symbols] or 45 °C [open symbols] at pH 3 for the first 3 h of adsorption. The embedded graph shows the interfacial shear viscosity at 25 or 45 °C, for curcumin (a) or quercetin (b) particles with Milli-Q water [black] and 0.5 wt.% WPI [red] at pH 3, after 24 h of adsorption. Error bars represent standard deviation of at least two independent experiments. Samples with the same letter do not differ significantly ($p > 0.05$) according to Tukey's test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

protein co-adsorb at the interface and so are able to form complexes, which often forms much stronger films than proteins on their own. For example, such behavior in η_i has been observed previously for WPI + cellulose particles, where electrostatic complexation between the protein and particles resulted in a significantly stronger interfacial film than the protein alone [21]. At higher concentrations of WPI, protein adsorption tends to dominate over particle adsorption, and so η_i values decrease to those more similar of pure protein films (Fig. 6a and b).

Curcumin particles gave lower η_i at all concentrations of WPI at pH 3 (Fig. 6a) compared to quercetin, suggesting stronger accumulation of quercetin particles at the interface. This could be due to the larger aspect ratio of the quercetin particles compared to those of curcumin, which were smaller in size and therefore would also require more time to settle at the interface [3]. On the other hand, this might also be due to stronger interactions between quercetin and WPI, noting again that quercetin (in water) has net negative charge whereas WPI net charge is positive at pH 3, as discussed above.

The η_i results with quercetin at pH 7 (Fig. 6b) were fairly constant across the WPI concentration range, but all higher than at pH 3. The results with curcumin at pH 7 (Fig. 6b) were similar to at pH 3 in that values of η_i increased initially and then decreased with increase in WPI concentration. However, the values at pH 7 were, like with quercetin, all considerably higher than at pH 3. The η_i of curcumin even without WPI at pH 7 was ca. 60× higher than at pH 3, indicating some other possible mechanism of strengthening of the adsorbed film. It is widely known that curcumin is more stable in acidic emulsions, existing in its enolic structure, but it is very unstable at higher pH values [27]. Oxidative and hydrolytic degradation reactions of curcumin could take place at the interface at pH $\geq$ 7 [28]. Some of these degradation products are more hydrophilic and therefore more likely to move into the aqueous phase [28]. To restore the equilibrium, more curcumin would migrate from the oil phase to the interface and then eventually to the aqueous phase, continuing the degradation process [28]. Some of the products, such as vanillin and ferulic acid, have been shown to covalently cross-link whey proteins, which might explain higher values of η_i at pH 7 [29,30]. Literature suggests that the center ring structure of quercetin at pH 7 is also unstable due to oxidation reactions, resulting in degradation of the ring structure [31,32]. These possible changes in the chemical nature of the polyphenols at pH 7 therefore make it more difficult to interpret the differences in emulsion stability and η_i at pH 7.

Another way of testing for the electrostatic origin of complex formation at the interface is to increase the ionic strength of the aqueous phase, which will screen attraction between opposite charges on the protein and the particles [33]. Measurements of η_i at pH 3 were therefore also conducted at different salt concentrations in systems with curcumin and quercetin (+0.5 wt.% WPI); values after 24 h as shown in Fig. 7. It was observed that addition of a very low concentration of NaCl (0.001 M) caused a two-fold decrease in η_i for both curcumin and quercetin particles. Higher concentrations (0.01 and 0.1 M) of NaCl did not change η_i significantly further ($p > 0.05$), but the inverse dependence of the Debye screening length on the square root of the ionic strength means that the biggest change in screening length would be expected between 'zero' and 10^{-3} M salt. Therefore, it seems that at pH 3, electrostatic attraction between the two components is probably important in strengthening the adsorbed film and increasing emulsion stability, although it is recognized that other interactions might also take place, discussed below.

Heat treatment at 45 °C was also used to test the nature of the interactions between polyphenols and WPI at the interface, but

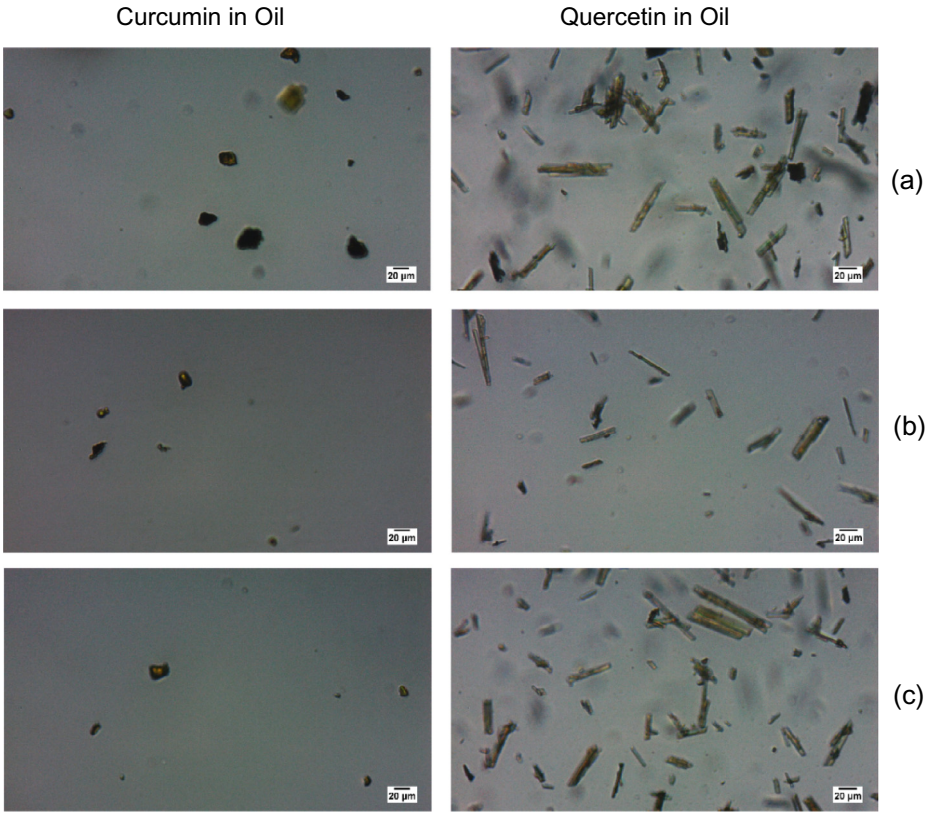

Fig. 9. Optical microscope images of curcumin or quercetin dispersions (0.14 wt.%) at 25 °C (a), during heating at 45 °C (b) and after cooling down to 25 °C overnight (c). The scale bar represents 20 µm.

also because of the importance of heat processing in practical applications. The η_i of the system was measured during heating at 45 °C for 3 h and then after cooling down to 25 °C and leaving overnight (Fig. 8). A control experiment was undertaken without any polyphenol crystals in the oil phase but with only 0.5 wt.% WPI in the aqueous phase at pH 3. It was observed that η_i of this system increased significantly, within the first 3 h, when the system was heated at 45 °C (from 0.4 to 3.0 mN s m^{-1}, results not shown) due to the formation of new covalent cross-links between the molecules, as observed previously [26,20]. However no change in η_i was observed after 24 h for the heated system: η_i remained the same as that of the non-heated system (4.5 mN s m^{-1}, results not shown). With curcumin crystals in the oil phase (without WPI in the aqueous phase), η_i increased slightly over the first 3 h, but after 24 h η_i had increased dramatically compared to the non-heated system at 25 °C (Fig. 8a). However, η_i with quercetin crystals (without WPI) increased very quickly over the first 3 h but after 24 h, η_i was just 2× higher compared to the non-heated system at 25 °C (Fig. 8b). The larger change over the first 3 h of heating for quercetin could possibly be due to the higher temperature accelerating the adsorption and rearrangement of the larger quercetin crystals at the interface. When WPI was added into the aqueous phase, η_i was much higher but approximately the same for both curcumin (~30 mN s m^{-1}, $p > 0.05$) and quercetin (~1600 mN s m^{-1}, $p > 0.05$) after 24 h, whether heated or not.

Optical images of the above systems described in Fig. 8 were also taken at 25 °C (before heating), during heating at 45 °C and 1 h after heating (at 25 °C), shown in Fig. 9. Curcumin crystals during heating at 45 °C (Fig. 9b) were much smaller in size than at 25 °C (before heating, Fig. 9a) but the size of quercetin crystals did not change significantly during heating. After cooling at 25 °C and 1 h later (Fig. 9c), curcumin crystals were smaller in size compared to before heating, whilst quercetin crystals showed a small increase in size (see Supporting Information Figs. S3 and S4). These changes suggest Ostwald ripening of quercetin crystal size due to its increased solubility at high temperature that perhaps explains the higher η_i after heating (24 h, Fig. 8b), since larger particles (of the same contact angle properties) are adsorbed more strongly.

4. Discussion

Drawing together the results of all these experiments, a possible mechanism of complex formation between the proteins and

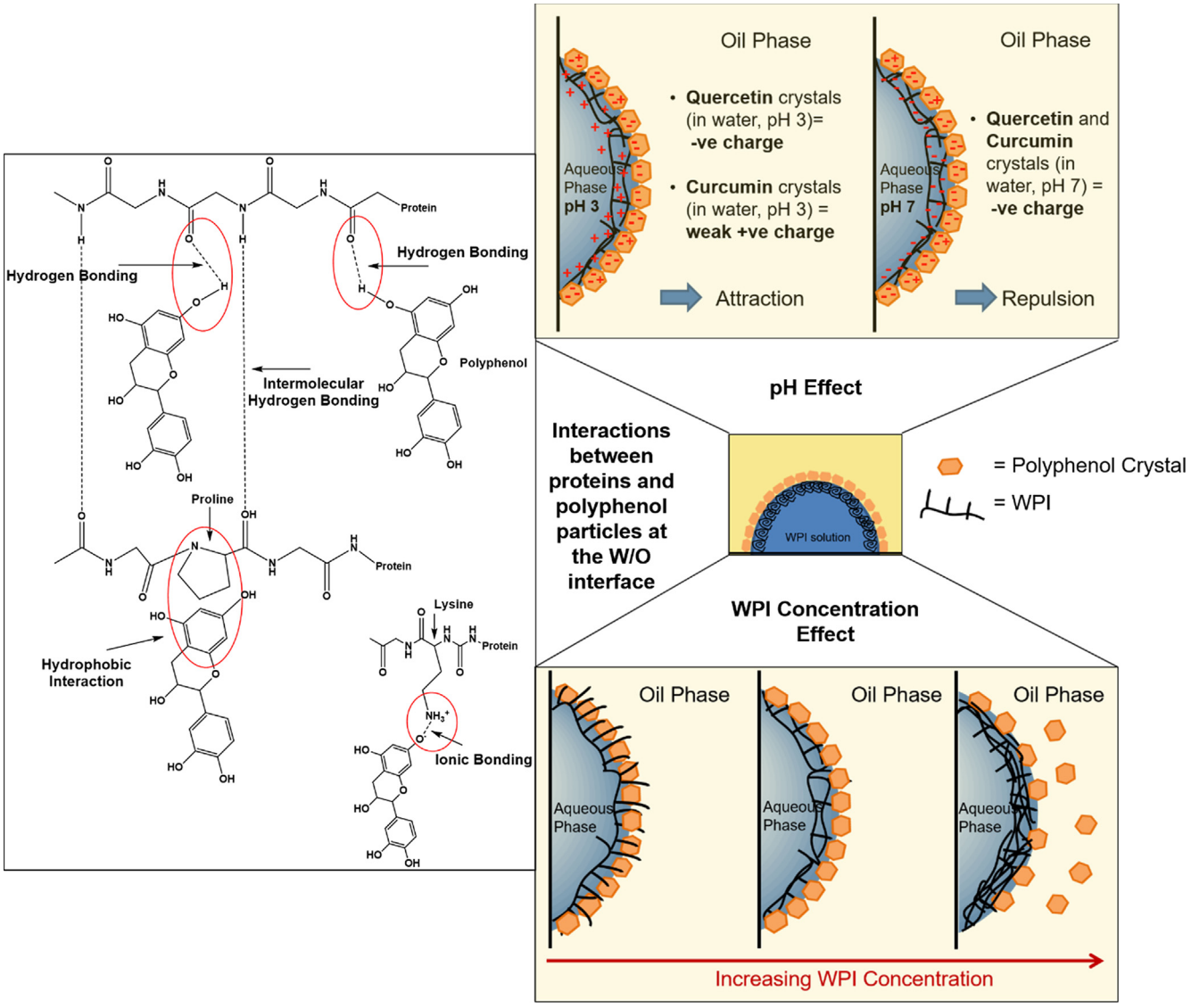

Fig. 10. Schematic representation (not to scale) of curcumin or quercetin + WPI-stabilized W/O emulsions, illustrating the effects of pH and WPI concentration and the possible mechanisms of interaction between the proteins and polyphenols at the interface.

polyphenols at the interface is schematically shown in Fig. 10. The phenolic nucleus of polyphenols is the most favorable part for molecular (non-covalent) interactions with proteins with a defined globular tertiary structure [16]. The interactions are affected by the relative concentration of polyphenol and protein, solvent composition, temperature, ionic strength and pH [34]. It has been suggested by many authors that polyphenol interactions give rise to complex formation mainly via hydrophobic interactions and hydrogen bonding [16,17,34]. Hydrophobic interactions arise from the association of aromatic rings of polyphenols and hydrophobic sites of proteins, such as the pyrrolidine rings of proline residues, plus phenolic rings tyrosine and phenylalanine, whilst hydrogen bonding can occur between the many H-acceptor sites of proteins and hydroxyl groups of the polyphenols [34,35]. Ionic interactions between positively charged groups on the proteins, such as the amino acid side chains of lysine and arginine and the negatively charged hydroxyl groups of polyphenols probably take place as well, although this has shown to have a minor effect on complex formation in the bulk [34].

Obviously, polyphenol-protein interactions are dependent upon the nature of the polyphenol and the protein, plus how the protein structure changes on adsorption in the case of the W/O interface [34]. Polyphenol reactivity is influenced by the size, the conformational mobility/flexibility and the solubility of polyphenols in water [36]. Higher solubility in water (lower logP) reduces the affinity with proteins [34]. High molecular weight polyphenols have the ability to precipitate or interact with proteins more effectively because they possess more functional groups [34], whilst greater conformational flexibility increases the capacity for interactions. Similarly, proteins with more open and flexible structures can more easily associate with polyphenols [37]: higher contents of charged or proline residues maintain the peptide in a more open conformation, inhibiting the formation of intramolecular hydrogen-bonded structures such as α-helix [38,39]. Available binding sites are then maximized and the carbonyl oxygens of peptide bonds are more exposed and available for hydrogen bonding than those of a compactly folded protein [38,40].

Riihimaki et al. [41] investigated the binding effect of different phenolic compounds, such as flavonols and isoflavonols, to the main constituent of WPI: β-lactoglobulin (β-LG). They showed the polyphenols formed complexes with β-LG that were stable under acidic conditions, indicating that phenolic compounds probably bind to the exterior of β-LG rather than the calyx. Moreover, it was shown that phenolic compounds are stable in basic conditions in the presence of β-LG, i.e., β-LG protects them from the sort of chemical degradation discussed above.

5. Conclusions

In this work, we propose a novel way to stabilize W/O emulsions via complex formation at the interface between Pickering polyphenol particles adsorbing from the oil side and biopolymers (proteins) co-adsorbing from the aqueous side of the interface, which strengthens the mechanical properties of the adsorbed film. In this case, addition of WPI up to 0.5 wt.% gave a significant increase in improvement in the stability of the emulsions over time. Based on the interfacial shear viscosity measurements, with and without added salt, we conclude that one of the main factors affecting complex formation and strengthening of the film is the electrostatic attraction between oppositely-charged polyphenol particles and proteins at the interface, which therefore also depends on the pH of the aqueous phase. This agrees with correspondingly more stable emulsions at pH 3 compared to pH 7, due to more disparate charges between the two stabilizers at pH 3, though greater chemical instability of the polyphenols at pH 7 may also have an influence. Higher concentrations of WPI do not improve the stability further due to protein adsorption dominating over polyphenol particle adsorption. Such particle + biopolymer interfacial complex formation could be utilized for designing water-in-oil emulsions for a variety of soft matter applications, in foodstuffs but also more widely, where there is a lack of biocompatible Pickering particles for stabilization of aqueous droplets in an oily (non-aqueous) phase.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcis.2019.04.010.

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