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Stability of Water-in-Oil Emulsions Co-stabilized by Polyphenol Crystal-Protein Complexes as a Function of Shear Rate and Temperature

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Abstract

The process stability of water-in-oil (W/O) Pickering emulsions (10 or 20 wt.% water), co-stabilized by crystals of polyphenols (curcumin or quercetin, (0.14 wt.%)) dispersed in soybean oil phase, plus whey protein isolate (WPI, 2.0 wt.%) or whey protein microgel (WPM, 0.1 - 2.0 wt.%) particles present in the inner aqueous phase, was assessed by measuring the apparent viscosity (η), water droplet size (light scattering) and microstructural changes (confocal laser scanning microscopy, CLSM). Stability was measured as a function of temperatures (25 to 50 °C), using a shear rate cycle between 0.1 to 100 s^{-1} to highlight shear- and time-dependent hysteresis (if any) of η. All the emulsions showed shear thinning to some extent, but those without added WPI or WPM particles in the aqueous phase exhibited coalescence at increasing shear rate, that was more pronounced at higher temperatures. Emulsions containing WPI in the dispersed phase were stable, whilst those containing WPM particles showed a decrease in mean droplet size ($D_{4,3}$) on shearing due to the disruption of the aggregates of droplets, polyphenol crystals and/or WPM particles in the continuous oil phase, but with no droplet coalescence. The low shear rate (0.1 s^{-1}) viscosity showed an increase with increasing WPM particle concentration. This increase, plus confocal laser scanning microscopy (CLSM) of the emulsions, suggested that the WPM particles increased W/O emulsion stability not only via their adsorption to the inner surface of the water droplets, but also due to them promoting the formation of mixed weak flocs of polyphenol crystals + WPM particles + small water droplets in the oil phase attached to the surface of the main population of water droplets.

Keywords

Pickering emulsions; water-in-oil emulsions; polyphenols; shear/process stability; microgels; viscosity

1. Introduction

Emulsions are metastable colloids where one liquid phase is dispersed into another immiscible liquid as droplets, which are created via external shear energy and the droplets can be stabilized by surface-active molecules or particles [1]. Emulsions therefore tend to revert to their two-phase state, usually via a combination of creaming/sedimentation, aggregation, coalescence and Ostwald ripening, as well as due to various chemical and biological actions [2-3]. Apart from creaming and sedimentation, all these other instability mechanisms can be accelerated by shearing and mixing occurring during emulsion transportation and processing.

Food emulsions are often complex systems containing many different surface active ingredients and stabilizers – lipids, proteins, polysaccharides, particles, etc. [4]. Pickering emulsions, stabilized by solid particles that strongly adsorb at the interface between two fluid phases, were largely ignored even after their re-discovery by Pickering in 1907 [5]. However, in the past decades there has been renewed interests in Pickering stabilization, partly because of the increasingly novel and wide ranging types of particles available. As far as applications in foods is concerned, a continuing challenge is to find effective Pickering particles that are acceptable for use in the food industry [6-11]. In particular, there are relatively limited number of biocompatible particles that have been used so far in literature to stabilize water-in-oil (W/O) emulsions [12-17]. Although Pickering emulsions are extraordinarily stable to coalescence and Ostwald ripening, their process stability have attracted rare attention in literature to date, which is part of what this work aimed to investigate particularly in W/O emulsions.

In our previous work, we demonstrated the ability of water-insoluble polyphenol crystals, such as curcumin and quercetin, to stabilize water droplets via the Pickering mechanism [12]. Micro-structural evaluation at various length scales revealed that quercetin crystals ($D_{3,2}$ ~ 5.9 μm and ratio of length to diameter (L/D) = 2.5:1 – 7:1) had a more rod-like shape and curcumin crystals were smaller ($D_{3,2}$ ~ 0.2 μm) having a more polyhedral shape [12]. It was observed that both polyphenol crystals were able to adsorb at the water-oil interface and provide stabilization of water droplets for up to 2 – 3 days. However, formation of a hybrid polyphenol-biopolymer complex at the water-oil interface revealed a pronounced improvement in the kinetic stability of the emulsions (up to 21 days) [13]. This complex formation occurred between the Pickering polyphenol particles adsorbing from the oil side and whey protein isolate (WPI) biopolymer co-adsorbing from the aqueous side of the interface, which strengthened the mechanical properties of the adsorbed composite film [13]. Furthermore, by changing the physical nature of the protein – forming it into WPI-based microgel (WPM) particles via controlled shearing of heat set whey protein gels[18] (mean hydrodynamic radius of ~ 90 nm and polydispersity index of ~ 0.3 at pH 3.0) the stability of W/O emulsions was further improved (up to 90 days) and higher stable water volume fraction was possible (up to 20 wt.%) [19]. In this case, the water droplets were stabilized by a sort of 'double Pickering mechanism', where one type of particle (polyphenol crystals) adsorbed from the oil side and another (WPM particles) co-adsorbed from the aqueous side. Microgels represent a relatively new type of food-stabilizing ingredient,[20] whereby the functional properties of biopolymers (*i.e.*, proteins or polysaccharides) are improved by forming them into gel particles, either by bulk gelation then comminution into particles (as in this case), or controlled aggregation up to the microgel particle size then truncation of the process to avoid bulk gelation. The double Pickering stabilization was shown previously[19] to be strongly dependent on the concentration of WPM particles. At low WPM particle concentration, both polyphenol crystals and WPM particles were present at the interface and synergistically improved the W/O emulsion stability. At higher WPM particle concentrations, mixed weak flocculation in the oil phase of polyphenol crystals, WPM particles and possibly very small water droplets (which are almost impossible to distinguish from the latter) occurred. In the presence of these aggregates, which were usually attached to the surface of the main population of water droplets, the resilience of the emulsions to coalescence increased even further [19]. Evidence was also presented that the principal origin of the complex formation (for both WPI and WPM particles) was electrostatic attraction, at pH 3.0, between the oppositely charged polyphenol particles and protein, although hydrogen bonding between the two components may have also contributed to the stability [13, 19]. Emulsions were also prepared at pH 7.0, but at this pH value, the polyphenol crystals tend to degrade chemically, and the emulsion stability was significantly impaired. The results also suggest that electrostatic complex formation is less effective in contributing to emulsion stability at pH 7.0 because both WPI or WPM particles and the polyphenols have the same (negative) sign of charge [13]. However, in none of the afore-mentioned studies, emulsions were tested for their process stability to shear, which is best done using a combination of controlled rheological tests combined with particle sizing and confocal laser scanning microscopy (CLSM).

As well as rheological stability being used as a measure of colloidal stability [21], rheological control is, of course, vital in the manufacture of foods, *e.g.*, design of material handling systems plus maintenance of quality control and the desired sensory aspects of products [22-23]. In general, emulsion viscoelasticity depends on droplet size distribution, rheology of the continuous phase and inter-particle interactions, all of which are strongly influenced by processing conditions, such as energy input during emulsification, residence time, application of thermal treatments, mixing efficiency, etc. [21,4]. In order to test the likely process stability of the Pickering emulsions created using polyphenol crystals alone and/or in combination with WPI and WPM particles as stabilizers, the apparent viscosity (η), water droplet size and microstructural changes were measured as function water volume fractions, plus cycles of shear rate and temperature. Therefore in this manuscript the process stability of the W/O emulsions stabilized by curcumin or quercetin crystals dispersed in the oil phase, with or without the presence of WPI or WPM particles in the aqueous phase, will be characterized. This characterization will be undertaken by measuring the rheology and particle size distributions of the emulsions on subjecting them to a well-defined range of shear rates and temperatures, combined with confocal laser scanning microscopy of the systems. To our knowledge, this is the first study that has systematically characterized the process stability of W/O Pickering emulsions stabilized by complex interfaces.

2. Materials 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 polyphenol crystals were used without further purification. Soybean oil (KTC Edibles, UK) was purchased from a local store. 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 was purchased from Sigma-Aldrich (USA).

2.2. Preparation of oil dispersion of curcumin or quercetin crystals

Curcumin or quercetin dispersions were prepared by dispersing these 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-10 G) operating at 9,400 rpm for 5 min.

2.3. Preparation of aqueous dispersion of whey protein isolate (WPI)

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 3.0, by adding few drops of 0.1 M HCl or NaOH.

2.4. Preparation of aqueous dispersion of whey protein microgel (WPM) particles

An aqueous dispersion of WPM particles was prepared based on a slight modification of the methods previously described by Murray and Phisarnchananan [24, Sarkar, et al. [25,] Araiza-Calahorra and Sarkar [26]. Whey protein solution (10 wt.%) was prepared by dissolving WPI powder in 20 mM phosphate buffer solution at pH 7.0 for 2 hours. The WPI solution was then heated at 90 °C for 30 min and cooled at room temperature for 30 min followed by storage at 4 °C overnight to form whey protein hydrogels. The gels were mixed with 20 mM phosphate buffer solution (1:1 w/w) at pH 7.0 and were pre-homogenized using a blender (HB711M, Kenwood, UK) for 3 min before homogenization using two passes through the Leeds Jet homogenizer (University of Leeds, UK) operating at a pressure of 300 ± 20 bar. Sodium azide (0.02 wt.%) was added to the final 5 wt.% WPM particles stock solution.

2.5. Preparation of W/O emulsions

W/O emulsions were prepared at room temperature (21 to 26 °C) based on the procedure described in our previous work [12-13, 19]. Curcumin or quercetin dispersions were prepared, as described before, 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 9,400 rpm for 5 min. The aqueous phase was prepared with Milli-Q water, WPI (2.0 wt.%, prepared as discussed before) or WPM particles (0.1 - 2.0 wt.%, prepared as discussed before). Coarse W/O emulsions were prepared by homogenizing 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 the Leeds Jet Homogenizer, twice, operated at 300 bar. Immediately after preparation, emulsions were sealed in 25 mL cylindrical tubes (internal diameter = 17 mm) and stored at room temperature in a dark place. All the emulsions were in liquid form; by simply inverting the tubes the emulsions flowed.

2.6. Rheology

A modular compact rheometer, MCR-302 (Anton Paar, Austria) was used to measure the viscosity of soybean oil, curcumin and quercetin dispersions, WPI and WPM aqueous dispersions and emulsions at different temperatures (25, 35 and 50 °C). Cone-and-plate geometry (CP50-2, diameter: 50 mm cone angle: 2°) was used for all measurements. The rheometer was initialized with 0.2 mm gap between the cone and plate. The shear rate was set in the range of 10^{-1} to 10^{2} s^{-1}. For each measurement, a small amount of sample was pipetted onto the top of the plate, excluding any air bubbles. Samples were left in the rheometer for approximately 2 min to achieve a steady state. The viscosity was measured at shear rates of 10^{-1} to 10^{2} s^{-1} for 15 min, where subsequently the shear rate was kept constant at 10^{2} s^{-1} for 30 min. Then, the shear rate returned back to 10^{-1} s^{-1} (within 15 min) to check for any hysteresis. Although the normal force was nominally set to zero, during measurements it typically fluctuated between 0.3 and 0.5 N. Viscosity at each concentration was measured three times on separate samples.

2.7. Particle size measurements

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

$$D_{3,2} = \frac{\sum n_i A i d_i}{\sum n_i A_i} \qquad (2)$$

$$D_{4,3} = \frac{\sum n_i V_i d_i}{\sum n_i V_i} \qquad (3)$$

where n_i is the number of particles, A_i is the particle surface area, V_i is the particle volume and d_i is the diameter of the ith particles.

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.

2.8. 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 discussed above 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 polyphenol crystals and WPI/ WPM particles 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 crystals 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 emitted fluorescent light was detected at wavelengths of 525, 460 and 580 nm for curcumin, quercetin and Rhodamine B, respectively.

2.9. Statistical analysis

Significant differences between samples were determined by one-way ANOVA and multiple comparison test with Tukey's adjustment performed using SPSS software (IBM, SPSS statistics) and the level of confidence was 95 %.

3. Results and Discussion

3.1. Control Experiments

Before measuring the viscosity of the emulsions, it was necessary to check the effects of the particles on the viscosity of the dispersed and continuous phases alone.

a) Viscosity (η) of curcumin and quercetin dispersions in soybean oil

The viscosity (η) of curcumin and quercetin dispersions (0.14 wt.%) in soybean oil was measured at a range of shear rates ($\dot{\gamma} = 10^{-1} – 10^{2}\ s^{-1}$) at different temperatures (25, 35 and 50 °C) as shown in Figure 1. The η of soybean oil alone (*i.e.*, without added polyphenol crystals) was also measured as a control. All the samples showed Newtonian behavior at all temperatures, *i.e.*, the viscosity remained stable as the shear rate increased. The η results for soybean oil, curcumin and quercetin dispersions were indistinguishable at 25 °C indicating that the concentration of particles (0.14 wt.%) added to the oil did not cause any significant change in the η of the oil. As the temperature increased from 25 to 50 °C, the viscosity of all the samples decreased slightly, but remained Newtonian as expected for most oils [27-29], showing again that the η values of curcumin and quercetin dispersion were very similar to the control (soybean oil alone).

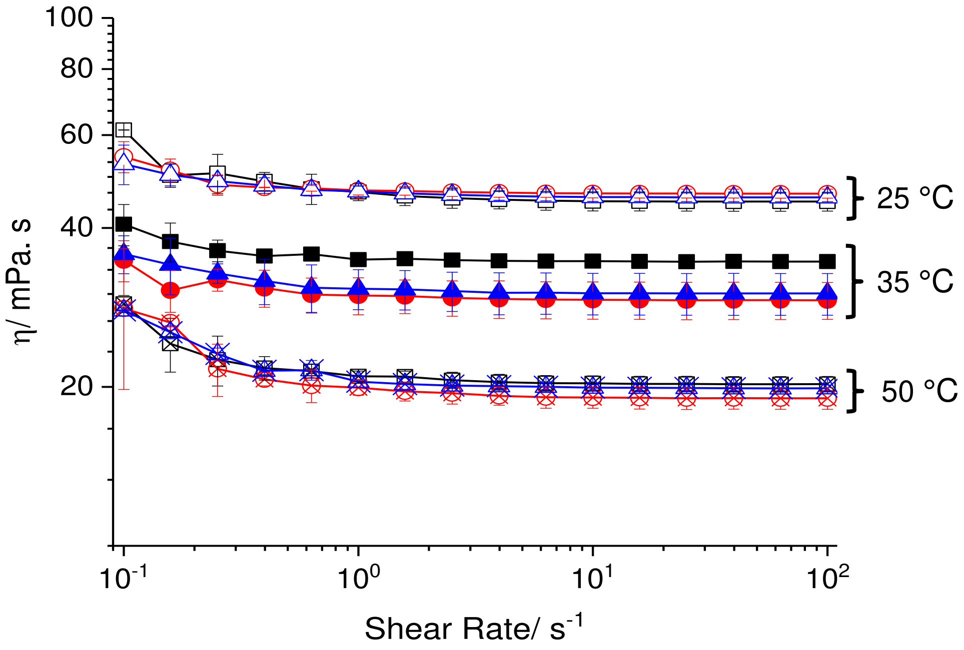


Figure 1. Viscosity (*η*) against shear rate ($\dot{\gamma}$) curves of soybean oil (control, ■), 0.14 wt.% curcumin (●) and 0.14 wt.% quercetin (▲) dispersions in soybean oil at different temperatures 25 (open symbols), 35 (closed symbols) and 50 °C (crossed symbols).

b) Viscosity (*η*) of 2.0 wt.% WPI or WPM particle dispersions in aqueous phase at pH 3.0

The *η* of 2.0 wt.% WPI or WPM particles in aqueous phase (pH 3.0) at different shear rates and temperatures (25, 35 and 50 °C) are shown in Figure 2. The solutions/dispersions were sheared from 10^{-1} to 10^{2} s^{-1} and then kept at this high shear rate for 30 min to check their stability (as described in the Methods section (2.6.)). The solution of 2.0 wt.% WPI did not show strong shear thinning behavior, indicating biopolymer entanglements were not formed, as expected for globular proteins at this concentration. In addition, the change in the temperature from 25 to 50 °C did not significantly affect the viscosity of the WPI solutions at low shear rates (< 1 s^{-1}) where the flow curves were indistinguishable. At higher shear rates (> 1 s^{-1}) *η* decreased slightly with the increase of the temperature. No hysteresis was observed in the WPI solutions at 25 °C but a hysteresis loop was identified at higher temperatures (35 and

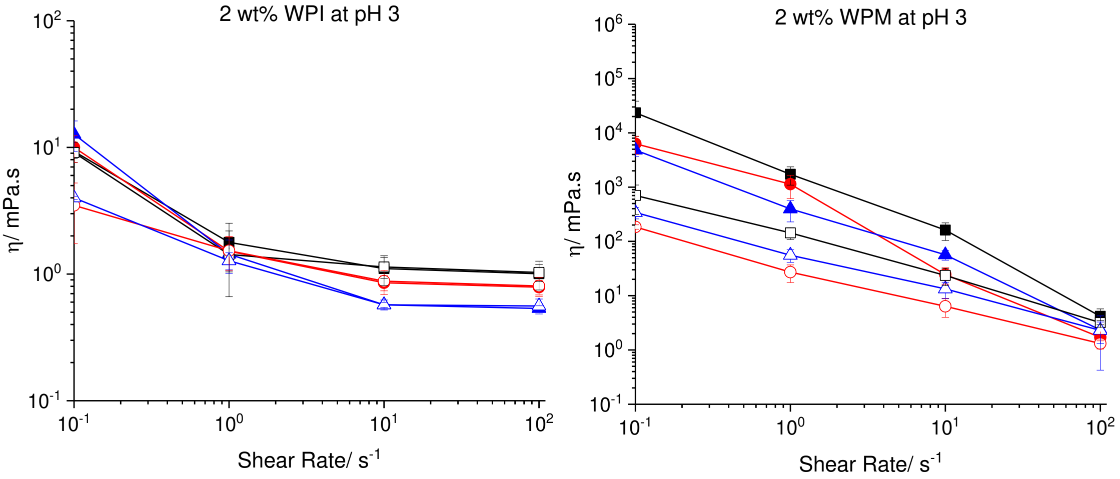
50 °C), where the η values, as $\dot{\gamma}$ was decreasing (Figure 2 (a), open symbols), were slightly lower at low shear rates (< 1 s^{-1}) than those when $\dot{\gamma}$ was ramped up (Figure 2 (a), closed symbols). This was probably due to the start of unfolding of WPI molecules at higher temperatures [13, 30].

WPM particles (2 wt.%) at all the 3 temperatures showed strong shear thinning behavior, typical of microgel dispersions. This is due to inter-particle entanglements and other interactions that occur at relatively low particle volume fraction but that are disrupted by shear [25, 31]. The effect of temperature on η of the WPM particle dispersions was more pronounced than on that of the WPI solutions. The initial η of the WPM particle dispersions (at $\dot{\gamma}$ = 10^{-1} s^{-1}, 25 °C) was higher (η ~ 10^4 – 10^5 mPa s) than that at 35 or 50 °C (η ~ 10^4 mPa s) and remained higher at all the shear rates applied (10^{-1} – 10^2 s^{-1}). The η values decreased with increasing temperature, suggesting either shrinkage of the microgel particles and/or a reduction in entanglements between them [25]. The viscosity curves of the WPM particle dispersions at 35 and 50 °C were very similar at all the shear rates. All the WPM particle dispersions at any temperature showed hysteresis, where the η values on decreasing $\dot{\gamma}$ (Figure 2 (b), open symbols) were much lower than those when $\dot{\gamma}$ increased (Figure 2 (b), closed symbols).

Protein microgels are soft colloidal particles that exhibit complex surface [20] and bulk rheological behavior [19, 25] since they do not have a true surface in the usual sense, but consist of particles of a gel network. Thus, their surface is expected to be porous and “fuzzy”, while the particles may be deformable or even be able to interpenetrate to some extent [25]. The interaction between microgel particles in the bulk and at the interface – the electrostatic repulsion between them, their interpenetration and/or deformation – are factors that are still not fully understood [20, 32]. It has been suggested that at interfaces both bulk phases interpenetrate such particles [33], illustrating that it is misleading to conceive of microgels as having a fixed and finite contact angle like true Pickering particles [20, 34]. Nevertheless, such particles can act as excellent stabilizers of emulsions and foams.

The η of the WPM particle dispersions (at 25 °C) was three orders of magnitude higher than that of WPI (at 25 °C) even though the overall protein concentration in these samples was 10× lower (*i.e.*, 0.2 wt.% whey protein isolate), taking into account the water content of the microgel particles themselves. In previous work Murray and Phisarnchananan [24] identified a strong dependence of WPM particle dispersion rheology on pH, attributed to changes in the protein charge and thence WPM particle aggregation. WPM particles display polyampholyte character in line with their constituent proteins, with an isoelectric point (IEP) at pH 4.7, where their overall charge is zero [35]. Below and above this pH value, WPM particles are positively and negatively charged, respectively [13, 26, 36]. Such particles are generally stable against aggregation when the ζ-potential exceeds the absolute value of 20 mV [37]. Electrostatic repulsion plays an important role in determining the rheology of concentrated dispersions in general [38]. At low shear rates, particles are not able to approach closely because of the electrostatic repulsion and their effective volume fraction is greater. At high shear rates the stresses are large enough to overcome the electrostatic repulsion between the particles and force them closer together, exhibited as a shear thinning effect [38]. However, charge effects are magnified with ampholytic microgel particles, due to the expansion and contraction of their diffuse surface polymer layers as they become more charged or uncharged, respectively.

Finally, it should be emphasized that the much higher η values of the 2 wt.% WPM particle dispersions compared to the 2 wt.% WPI solutions persisted after application of $\dot{\gamma} = 10^2$ s^{-1}, even though the WPM particles were highly shear thinning. Thus the WPM may have aggregated or interpenetrated as a function of shear rate to some extent, but they were certainly not completely destroyed by subjecting them to these conditions, so such interactions must be reversible, *i.e.*, the WPM particles are resilient under these conditions of shear.



(a) (b)

Figure 2. Viscosity (η) against shear rate ($\dot{\gamma}$) curves of 2.0 wt.% WPI (a) and 2.0 wt.% WPM particles (b) at different temperatures 25 °C (■, □), 35 °C (●, ○) and 50 °C (**▲, Δ**). All the aqueous dispersions have been prepared at pH 3.0. Viscosity values are shown for ramping up (closed symbols) at shear rates from 0.1 - 100 s^{-1} and ramping down (open symbols) at shear rates from 10^2 – 10^{-1} s^{-1}.

3.2. Stability of 10 wt.% W/O emulsions stabilized by curcumin or quercetin crystals with or without 2 wt.% WPI or WPM particles

Figure 3 shows η against $\dot{\gamma}$ for emulsions stabilized by 0.14 wt.% curcumin or quercetin crystals dispersed in the oil phase + water (*i.e.*, 0 wt.% protein) or 2.0 wt.% WPI or WPM particles at different temperatures; 25, 35 and 50 °C. The emulsions were prepared at pH 3.0 due to the formation of stronger interfacial complexes between the oppositely charged polyphenol crystals present in the oil phase and WPI or WPM particles in the aqueous phase, as shown earlier [12]. All the systems showed shear thinning behavior; they had a higher η at low shear rates which decreased dramatically as the shear rate increased. The trends for the emulsions stabilized by either curcumin or quercetin were very similar, with the latter having slightly higher η values, possibly due to the rod-like shape and larger mean size ($D_{3,2}$ ~ 5.9 µm) [12] of quercetin crystals than curcumin (polyhedral shape, $D_{3,2}$ ~ 0.2 µm) [12], at all temperatures. For both curcumin and quercetin systems at all temperatures, the initial η (at 10^{-1} s^{-1}) decreased in the order WPM particles > WPI > water. The η of the emulsions with or without WPI decreased slightly (from 10^4 to 10^3 mPa s) as the shear rate increased from 10^{-1} to 10^2 s^{-1} at 25 °C (Figure 3 (a_1) and (b_1)) reaching a final η value of ~ 10^2 mPa s at $\dot{\gamma} = 10^2$ s^{-1}. This was 1 - 2 orders of magnitudes higher than the η of the polyphenol dispersions in oil (~ 5×10^1 mPa s, Figure 1) or the 2.0 wt.% WPI solutions (1 mPa s, Figure 2 (a)) at this shear rate, respectively. As the temperature increased from 25 to 50 °C (Figure 3 ($a_2 - a_3$) and ($b_2 - b_3$)) the initial η at $\dot{\gamma} = 10^{-1}$ s^{-1} without WPI (or WPM particles) decreased significantly, especially for the quercetin- stabilized system (one order of magnitude lower at 50 °C compared to at 25 °C). This decrease was possibly due to the re-arrangement of the polyphenol crystals at the interface. In the presence of WPI a slight decrease in the initial η was observed, suggesting disruption of flocculated particles, again mainly for the quercetin-stabilized system. The final η value at $\dot{\gamma} = 10^2$ s^{-1} decreased slightly for all the systems as the temperature increased from 25 to 50 °C. However, the WPM particle systems showed a very high initial η (~ 10^5 mPa s at 10^2 s^{-1}) compared to the systems with or without WPI (10^3 - 10^4 mPa s). These higher η values were presumably due to some flocculation in the continuous oil phase as already first noted in our previous work [19], where the WPM particles seemed to form aggregates with polyphenol crystals in the bulk oil phase, at the interface and also possibly between some water droplets, which may have partly explained the observed increase in resistance to coalescence under quiescent conditions [19]. Therefore, as expected for flocculated systems, shear thinning behavior was observed, as explained above [4, 39-40]. No significant effect of temperature increase from 25 to 50 °C was observed on the emulsions containing WPM particles.

Hysteresis was observed in all the above emulsions, but was more pronounced in those containing WPM particles, again probably due to more flocculated nature of the latter systems. Interestingly, η at $\dot{\gamma}$ = 10^{-1} s^{-1} of the WPM particle systems at 25 °C (10^3 - 10^4 mPa s) returned to values close to the initial values observed in the systems without (or with) WPI at the same initial shear rate. This suggests that flocculating effects of the WPM particles were broken by the application of the shear cycle. Hysteresis was less pronounced in the emulsions with or without WPI, presumably because these were less flocculated in the first place compared to the WPM particle systems. As the temperature increased (Figure 3 ($a_2 - a_3$) and ($b_2 - b_3$)), η decreased slightly, probably mainly due to the decrease of η of the continuous phase as observed in Figure 1. At 50 °C both curcumin- and quercetin- stabilized systems with or without WPI reached η values close to 10^2 mPa s at all shear rates (10^2 to 10^{-1} s^{-1}), indicating convergence to the same sort of state of droplet and/or protein aggregation.

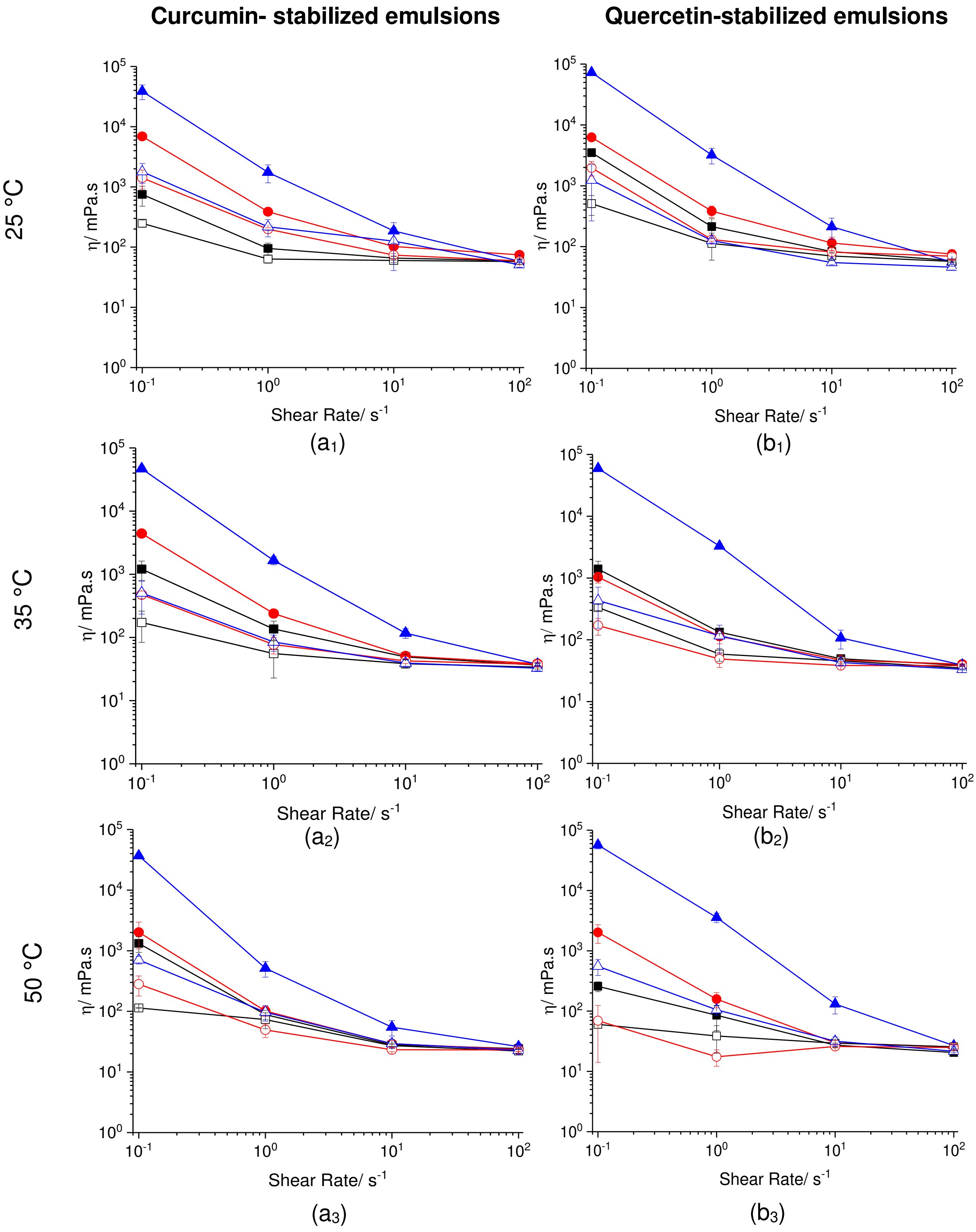


Figure 3. Viscosity (η) against shear rate ($\dot{\gamma}$) curves of 10 wt.% W/O emulsions stabilized by 0.14 wt.% curcumin (a) or quercetin (b) crystals dispersed in oil + water (■, □) or 2.0 wt.% WPI (●, ○) or 2.0 wt.% WPM particles (▲, ∆) as an aqueous phase (pH 3.0) at different temperatures 25 ($_1$), 35 ($_2$) and 50 ($_3$) °C. Viscosity values are

shown for ramping up (closed symbols) at shear rates from $10^{-1} – 10^{2}$ s^{-1} and ramping down (open symbols) at shear rates from $10^{2} – 10^{-1}$ s^{-1}.

Figure 4 shows the CLSM images of the same fresh 10 wt.% W/O Pickering emulsions stabilized by 0.14 wt.% curcumin or quercetin with or without 2.0 wt.% WPI or WPM particles. Figures 4 (a_1) and (b_1) show that the water droplets were surrounded by a dense layer of curcumin or quercetin crystals, respectively, confirming the preferential location of the polyphenol crystals at the W/O interface. The green brightness in the images was due to the auto-fluorescence of the polyphenol particles. Furthermore, some droplets appeared to be not completely spherical, which is another good indication of Pickering stabilization [41]. Images of the emulsions including 2.0 wt.% WPI plus curcumin or quercetin are shown in Figures 4 (a_2) and (b_2), respectively, demonstrating that again the water droplets were surrounded by a dense layer of polyphenol particles (green). 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 water droplets, as expected. Thus, both polyphenol crystals and WPI appeared to be in close proximity at the interface.

Images of fresh emulsions including 2.0 wt.% WPM particles plus curcumin or quercetin are shown in Figure 4 (a_3) and (b_3), respectively. As already discussed above, it is seen that in both systems there was an increased tendency for the whole system to aggregate in the oil phase. WPM particles seemed to be aggregated at the interface of the individual droplets and between interfaces of adjacent droplets, *i.e.*, causing flocculation of the water droplets. The polyphenol crystals seemed to be mixed in with these aggregates. In other words, there was increased tendency for microgels and polyphenol crystals to become shared between droplets prior to controlled shearing [19].

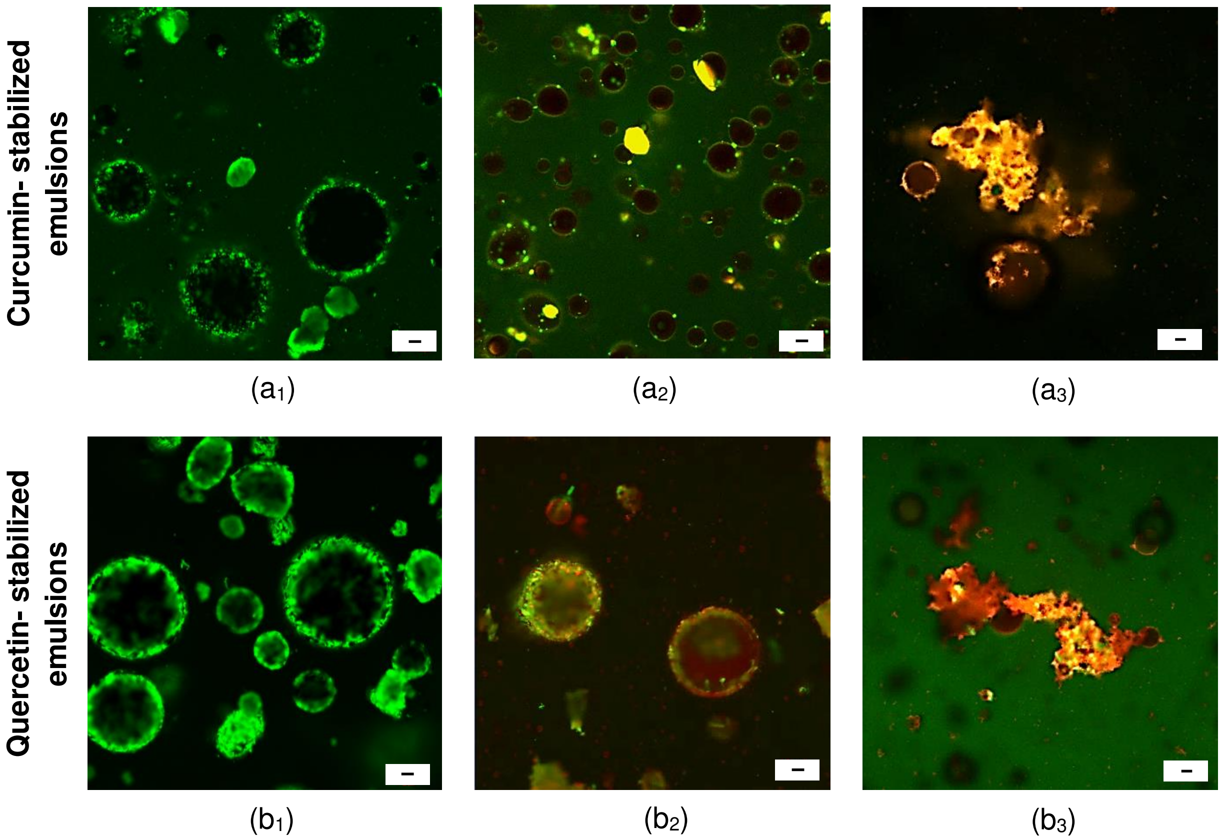


Figure 4. Confocal images of 10 wt.% W/O Pickering emulsions stabilized by 0.14 wt.% curcumin (a) and quercetin (b) crystals in the oil phase + water ($_1$), 2.0 wt.% WPI ($_2$) or 2.0 wt.% WPM particles ($_3$) in the aqueous phase at pH 3.0 for fresh samples. The green brightness in the images is caused by the auto-fluorescence of curcumin (488 nm excitation) or quercetin (405 nm excitation) crystals. The red brightness is due to the WPM particles stained by Rhodamine B (568 nm excitation). The scale bar represents 20 µm.

The PSDs of the systems were measured before and after the shear cycle at the different temperatures. Tables 1 and 2, show the $D_{3,2}$ and $D_{4,3}$ results for the curcumin- and quercetin- stabilized systems, respectively. It was seen that the size of the water droplets before shearing at 25 °C increased in the order of water < WPI < WPM particles, possibly indicating the presence of a thicker adsorbed particle-protein or particle-particle layer at the interface in the case of WPI and WPM particles, respectively. However, it should be remembered that the light scattering technique used cannot distinguish between water droplets, polyphenol crystals, WPM particles or their aggregates as scattering centers [42]. After shearing, the mean size ($D_{3,2}$ and $D_{4,3}$) for both curcumin- and quercetin- stabilized emulsions without WPI or WPM increased significantly in size ($p < 0.05$, Table 1 and 2), at all the temperatures, showing that these systems were unstable to shear and that coalescence occurred. In contrast, $D_{3,2}$ and $D_{4,3}$ for the samples containing WPI showed a small increase ($p < 0.05$, Table 1 and 2) due to shearing at 25 °C, *i.e.* the systems were more stable. However, as the temperature increased, $D_{3,2}$ and $D_{4,3}$ did not change ($p > 0.05$, Table 1 and 2). Furthermore, the samples containing WPM particles showed an approximate halving of the $D_{4,3}$ values after shearing ($p < 0.05$, Table 1 and 2) at all temperatures, again indicating a flocculated system disrupted by shear, whilst $D_{3,2}$ remained the same ($p > 0.05$, Table 1 and 2) before and after the shearing, suggesting that no coalescence was occurred.

From the above results, it can be concluded that the curcumin- and quercetin-stabilized emulsions containing WPI or WPM particles were more stable to shear and elevated temperatures than without these two forms of protein. Thus, interfacial complex formation between the oppositely charged polyphenol crystals in the oil phase + biopolymer or biopolymer microgel particles in the aqueous phase, not only improves the storage stability under quiescent conditions (as discussed in our previous work [19, 43]) but also enhances stability to shear and temperature.

Table 1. $D_{3,2}$ and $D_{4,3}$ values (μm) before and after shearing of 10 wt.% W/O emulsions stabilized by 0.14 wt.% curcumin crystals & water, 2.0 wt.% WPI or 2.0 wt.% WPM particles as an aqueous phase (pH 3.0) at different temperatures; 25, 35 and 50 °C. Samples with the same letter differ significantly ($p < 0.05$) according to Tukey's test for each component in the aqueous phase before and after shearing.

	Before Shearing		**After Shearing**					
	25 °C		**25 °C**		**35 °C**		**50 °C**	
	$D_{3,2}$	$D_{4,3}$	$D_{3,2}$	$D_{4,3}$	$D_{3,2}$	$D_{4,3}$	$D_{3,2}$	$D_{4,3}$
Water	$7.4 \pm 1.6^{a,b,c}$	$23.8 \pm 1.2^{d,e,f}$	21.5 ± 2.8^{a}	59.5 ± 3.2^{d}	24.2 ± 0.8^{b}	59.6 ± 3.1^{e}	27.2 ± 2.3^{c}	60.3 ± 5.7^{f}
WPI	18.5 ± 1.1^{g}	$51.5 \pm 0.9^{h,i}$	16.2 ± 0.9^{g}	47.7 ± 4.6^{h}	19.0 ± 1.2	42.4 ± 1.7^{i}	19.2 ± 1.8	50.4 ± 9.0
WPM particles	20.8 ± 0.5	$110.0 \pm 8.5^{j,k,l}$	18.0 ± 5.7	49.6 ± 6.1^{j}	18.0 ± 4.8	44.6 ± 10.0^{k}	20.6 ± 3.6	49.3 ± 2.4^{l}

Table 2. $D_{3,2}$ and $D_{4,3}$ values (μm) before and after shearing of 10 wt.% W/O emulsions stabilized by 0.14 wt.% quercetin crystals & water, 2.0 wt.% WPI or 2.0 wt.% WPM particles as an aqueous phase (pH 3.0) at different temperatures; 25, 35 and 50 °C. Samples with the same letter differ significantly ($p < 0.05$) according to Tukey's test for each component in the aqueous phase before and after shearing.

	Before Shearing		**After Shearing**					
	25 °C		**25 °C**		**35 °C**		**50 °C**	
	$D_{3,2}$	$D_{4,3}$	$D_{3,2}$	$D_{4,3}$	$D_{3,2}$	$D_{4,3}$	$D_{3,2}$	$D_{4,3}$
Water	$11.7 \pm 2.7^{a,b,c}$	$41.0 \pm 5.4^{d,e,f}$	18.9 ± 0.6^{a}	60.1 ± 1.4^{d}	23.6 ± 1.3^{b}	73.1 ± 5.4^{e}	32.7 ± 13.4^{c}	66.2 ± 6.9^{f}
WPI	$11.4 \pm 2.0^{g,h,i}$	$42.1 \pm 5.7^{k,l}$	14.1 ± 0.1^{g}	42.9 ± 0.4	18.2 ± 0.6^{h}	49.3 ± 1.4^{k}	18.6 ± 3.1^{i}	54.3 ± 13.4^{l}
WPM particles	18.5 ± 3.0^{m}	$92.7 \pm 21.6^{n,o,p}$	15.4 ± 0.8^{m}	49.8 ± 3.0^{n}	16.4 ± 1.3	51.0 ± 2.3^{o}	22.7 ± 5.2	42.9 ± 8.4^{p}

3.3. Stability of 10 wt.% W/O emulsions stabilized by curcumin or quercetin crystals + wt.% different concentrations of WPM particles

The curcumin and quercetin systems containing WPM particles were chosen for further testing since these had the highest viscosity values at high shear rates and different temperatures and no coalescence was observed. Emulsions stabilized by 0.14 wt.% curcumin or quercetin crystals + different WPM particle concentrations (0.1, 0.5, 1.0 and 2.0 wt.%) in the aqueous phase were tested. Figure 5 shows the results of η against WPM particle concentration at $\dot{\gamma}$ = 0.1 s^{-1}. It is seen that η of the emulsions stabilized by curcumin crystals were slightly lower than those stabilized by quercetin, possibly due to the smaller size of curcumin crystals compared to quercetin [12]. In addition, η increased as the concentration of WPM particles increased for both curcumin and quercetin emulsions confirming the tendency for the whole system to become more aggregated as more WPM particles were added.

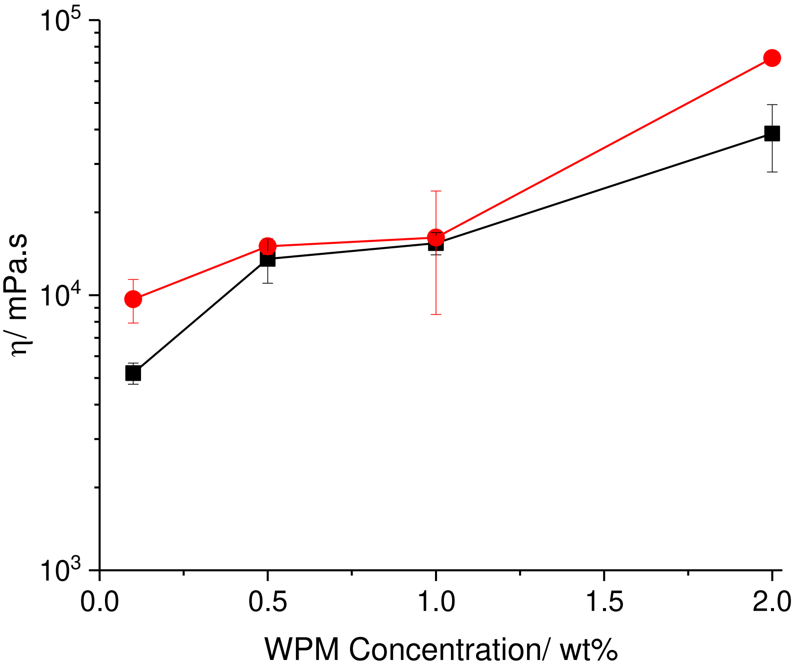


Figure 5. Viscosity (η) against WPM particle concentration at 0.1 s^{-1} shear rate of 10 wt.% W/O emulsions stabilized by 0.14 wt.% curcumin (■) or quercetin (●) crystals

dispersed in oil & WPM particles as an aqueous phase (pH 3.0) at different concentrations; 0.1, 0.5, 1.0 and 2.0 wt.%. The temperature was kept constant at 25 °C.

Tables 3 and 4 show the $D_{3,2}$ and $D_{4,3}$ values of the W/O emulsions stabilized by curcumin or quercetin crystals, respectively with different concentrations of WPM particles added in the dispersed phase, before and after shearing at 25 °C. All the emulsions had very similar $D_{3,2}$ values ($p > 0.05$, Table 3 and 4) before shearing but the $D_{4,3}$ values increased ($p < 0.05$, Table 3 and 4) as the concentration of WPM particles increased, again suggesting increased flocculation of the system as more WPM particles were added. After shearing, both the curcumin- and quercetin-stabilized emulsions + 0.1 wt.% WPM particles showed a significant increase in both the $D_{3,2}$ and $D_{4,3}$ values ($p < 0.05$, statistical data are not shown), possibly indicating some coalescence, but at the higher WPM particle concentrations (0.5, 1.0 and 2.0 wt.%) only $D_{4,3}$ decreased significantly ($p < 0.05$, statistical data are not shown), indicating greater stability with increased wt.% WPM particles.

Table 3. $D_{3,2}$ and $D_{4,3}$ values (μm) before and after shearing of 10 wt.% W/O emulsions stabilized by 0.14 wt.% curcumin crystals & WPM particles as an aqueous phase (pH 3.0) at different concentrations; 0.1, 0.5, 1.0 and 2.0 wt.%. Samples with the same letter differ significantly ($p < 0.05$) according to Tukey's test for each WPM particle concentration.

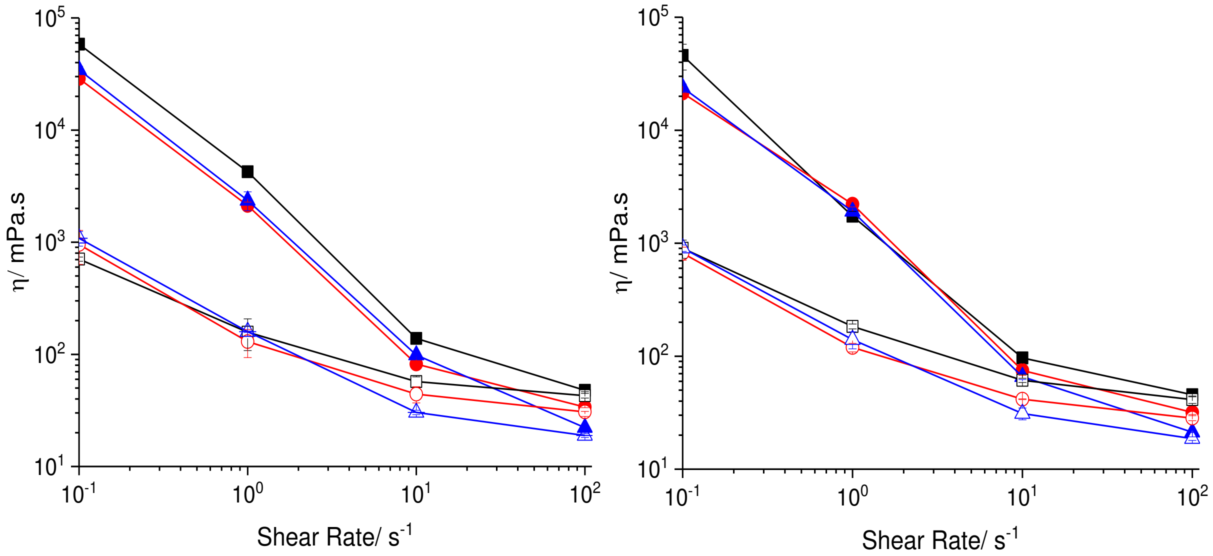
WPM particles concentration/ wt.%	**Before Shearing**		**After Shearing**	
	$D_{3,2}$	$D_{4,3}$	$D_{3,2}$	$D_{4,3}$
0.1	$22.0 \pm 1.7^{a,b}$	54.3 ± 0.8^{c}	$41.9 \pm 5.4^{d,e,f}$	61.1 ± 15.3^{g}
0.5	19.2 ± 1.4^{a}	83.5 ± 2.6^{c}	23.1 ± 3.0^{d}	70.9 ± 8.2^{e}
1.0	19.2 ± 3.2^{b}	92.7 ± 3.9^{c}	19.2 ± 0.3^{e}	$78.6 \pm 2.3^{g,f}$
2	20.8 ± 0.5	110.0 ± 8.5^{c}	18.0 ± 5.7^{f}	$49.6 \pm 6.1^{e,f}$

Table 4. $D_{3,2}$ and $D_{4,3}$ values (μm) before and after shearing of 10 wt.% W/O emulsions stabilized by 0.14 wt.% quercetin crystals & WPM particles as an aqueous phase (pH 3.0) at different concentrations; 0.1, 0.5, 1.0 and 2.0 wt.%. Samples with the same letter differ significantly ($p < 0.05$) according to Tukey's test for each WPM particle concentration.

WPM particles concentration/ wt.%	Before Shearing		After Shearing	
	$D_{3,2}$	$D_{4,3}$	$D_{3,2}$	$D_{4,3}$
0.1	$20.3 \pm 1.1^{a,b,c}$	65.8 ± 6.9^{d}	$38.1 \pm 13.6^{g,h,i}$	77.3 ± 1.3^{j}
0.5	18.5 ± 1.4^{a}	70.4 ± 7.3^{e}	25.3 ± 7.5^{g}	65.9 ± 23.8^{k}
1.0	17.5 ± 1.0^{b}	73.5 ± 6.2^{f}	17.3 ± 0.0^{h}	68.1 ± 5.0^{l}
2.0	18.5 ± 3.0^{c}	$92.7 \pm 21.6^{d,e,f}$	15.4 ± 0.8^{i}	$49.8 \pm 3.0^{j,k,l}$

3.4. Stability of 20 wt.% W/O emulsions stabilized by curcumin or quercetin crystals + 2.0 wt.% WPM particles

The shear stability of emulsions containing a higher water:oil ratio (20 wt.% water) was tested for systems stabilized by 0.14 wt.% curcumin or quercetin crystals + 2.0 wt.% WPM particles as Pickering stabilizers, since this WPM particle concentration appeared to impart enhanced stability for 10 wt.% W/O emulsions, (Figure 5 and Tables 3 and 4) [19]. Figure 6 shows η against $\dot{\gamma}$ at different temperatures (25, 35 and 50 °C). The emulsions were taken through the same shear cycle as described earlier. As at 10 wt.% water, all the systems showed shear thinning behavior at all temperatures. No significant differences were identified in the initial η (at $\dot{\gamma}$ = 10^{-1} s^{-1}) at any temperature. In addition, the initial η value (at all temperatures) was in the same range (10^4 – 10^5 mPa s) as that of the emulsions containing 10 wt.% water (Figure 3). At high shear rates (10^2 s^{-1}), a slight decrease of η was observed as the temperature increased for both the curcumin- and quercetin- stabilized systems. Hysteresis was observed for all the systems, with the final η in the same range (~ 10^3 mPa s) at all temperatures and similar to the value for the emulsions containing 10 wt.% water (Figure 3).



(a) (b)

Figure 6. Viscosity (η) against shear rate ($\dot{\gamma}$) curves of 20 wt.% W/O emulsions stabilized by curcumin (a) or quercetin (b) crystals dispersed in oil & 2.0 wt.% WPM particles as an aqueous phase (pH 3.0) at different temperatures; 25 (■, □), 35 (●, ○) and 50 °C (▲, △). Viscosity values are shown for ramping up (closed symbols) at shear rates from 0.1 - 100 s^{-1} and ramping down (open symbols) at shear rates from 10^2 – 10^{-1} s^{-1}.

Table 5 shows the size of the water droplets before and after shearing at different temperatures. The mean particle sizes in emulsions containing 20 wt.% water were smaller in size (~ 15 μm, Table 5) compared to those with 10 wt.% water (~ 20 μm, Tables 1 and 2). This is possibly due polyphenol crystal adsorption at the interface dominating over crystal aggregation in the bulk oil as the area of interface is increased. Also the $D_{4,3}$ value was lower in the emulsions with 20 wt.% (65 – 75 μm, Table 5) than those with 10 wt.% water (108 – 110 μm, Table 1 and 2), suggesting less aggregation in the continuous oil phase due to limited amount of unabsorbed WPM particles. After shearing, both $D_{3,2}$ and $D_{4,3}$ decreased slightly for both crystal types at all temperatures, due to the disruption of the aggregates, reaching mean sizes similar to those containing 10 wt.% water. In summary, the 20 wt.% W/O systems including 2 wt.% WPM particles were at least, if not more stable, than the 10 wt.% water emulsions.

Table 5. $D_{3,2}$ and $D_{4,3}$ values (μm) before and after shearing of 20 wt.% W/O emulsions stabilized by 0.14 wt.% curcumin or quercetin crystals & 2.0 wt.% WPM particles as an aqueous phase (pH 3.0) at different temperatures; 25, 35 and 50 °C. Samples with the same letter differ significantly ($p < 0.05$) according to Tukey's test for each polyphenol crystal before and after shearing.

	Before Shearing		**After Shearing**					
	25 °C		**25** °C		**35** °C		**50** °C	
	$D_{3,2}$	$D_{4,3}$	$D_{3,2}$	$D_{4,3}$	$D_{3,2}$	$D_{4,3}$	$D_{3,2}$	$D_{4,3}$
Curcumin	16.0 ± 1.0	$77.7 \pm 11.7^{a,b,c}$	12.9 ± 2.9	50.5 ± 7.9^{a}	15.5 ± 1.6	54.6 ± 1.6^{b}	13.6 ± 0.3	54.9 ± 0.4^{c}
Quercetin	14.2 ± 0.3^{d}	$65.9 \pm 6.9^{e,f,g}$	12.3 ± 1.4^{d}	52.4 ± 6.8^{e}	13.5 ± 2.4	54.1 ± 6.5^{f}	14.0 ± 1.9	50.6 ± 4.1^{g}

4. Conclusions

In this study, the viscosity (η) and mean particle size of W/O emulsions stabilized by curcumin or quercetin crystals dispersed in the oil phase, with or without WPI or WPM particles present into the aqueous phase at pH 3.0, were measured at different shear rates and temperatures. All the emulsions were shear thinning. The emulsions stabilized by the polyphenol crystals alone without the addition of WPI or WPM particles in the aqueous phase exhibited coalescence after shearing, with the size of the water droplets increasing significantly. More pronounced destabilization was observed at higher temperatures. The emulsions containing WPI in the dispersed phase were stable over shear and temperature without a significant increase in mean particle size. The emulsions with WPM particles showed a decrease in the $D_{4,3}$ values on shearing, indicating disruption of flocculated droplets, polyphenol crystals and WPM particles in the continuous oil phase. Aggregation appeared to be enhanced by increasing WPM particle concentration, but at ≥ 0.5 wt.% WPM particles no droplet coalescence occurred and the systems were significantly stable even at 20 wt.% water content. In summary, co-adsorbing biopolymers or biopolymer-based microgels to polyphenol crystal stabilized W/O emulsions appear as a promising new technique to improve the process stability of water droplets and can have applications in designing new formulations in food industries.

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Notes

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