

Stabilization of liquid water-in-oil emulsions by modifying the interfacial interaction of glycerol monooleate with aqueous phase ingredients

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Abstract

Glycerol monooleate (GMO)-stabilized liquid water-in-vegetable oil (W/VO) emulsions are difficult to stabilize due to the desorption of GMO from the W-VO interface towards the oil phase. This work improved the stability of GMO-stabilized liquid 20 wt% water-in-canola oil (W/CO) emulsion by modifying the dispersed aqueous phase composition with hydrogen bond-forming agents. As a control, 20 wt% water-in-mineral oil (W/MO) emulsion was also utilized. Different concentrations of hydrogen bond-forming agents (citric acid (CA), ascorbic acid (AA), low methoxyl pectin (LMP)) with and without salts (sodium chloride (S) or calcium chloride (Ca)) was added to the aqueous phase before emulsification, which enhanced emulsifier binding to the water-oil interface. The emulsions were characterized by phase separation, stability against accelerated gravitation, microstructure and rheology. W/CO emulsion without any aqueous phase additive destabilized instantly, whereas W/MO emulsion stayed stable. The addition of hydrogen bond-forming agents and salts significantly improved emulsion stability. LMP, with many hydrogen bond-forming groups, was able to provide the highest emulsion stability after 7 days in both oils compared to AA, CA and their mixtures with S. Emulsions with both oils formed weak gels with viscous and elastic characteristics due to the formation of an extensive network of water droplet aggregates. Overall, the hydrogen bond-forming agents interacted with GMO at the interface, thereby improving their presence at the water droplet surface, allowing significantly improved stability of GMO-stabilized liquid W/CO emulsions. The knowledge developed in this research can be useful in applying GMO in stabilizing liquid water-in-oil emulsion without using any crystal network.

Stabilization of liquid water-in-oil emulsions by modifying the interfacial interaction of glycerol monooleate with aqueous phase ingredients

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ABSTRACT

Glycerol monooleate (GMO)-stabilized liquid water-in-vegetable oil (W/VO) emulsions are difficult to stabilize due to the desorption of GMO from the W-VO interface towards the oil phase. This work improved the stability of GMO-stabilized liquid 20 wt% water-in-canola oil (W/CO) emulsion by modifying the dispersed

aqueous phase composition with hydrogen bond-forming agents. As a control, 20 wt% water-in-mineral oil (W/MO) emulsion was also utilized. Different concentrations of hydrogen bond-forming agents (citric acid (CA), ascorbic acid (AA), low methoxyl pectin (LMP)) with and without salts (sodium chloride (S) or calcium chloride (Ca)) was added to the aqueous phase before emulsification, which enhanced emulsifier binding to the water-oil interface. The emulsions were characterized by phase separation, stability against accelerated gravitation, microstructure and rheology. W/CO emulsion without any aqueous phase additive destabilized instantly, whereas W/MO emulsion stayed stable. The addition of hydrogen bond-forming agents and salts significantly improved emulsion stability. LMP, with many hydrogen bond-forming groups, was able to provide the highest emulsion stability after 7 days in both oils compared to AA, CA and their mixtures with S. Emulsions with both oils formed weak gels with viscous and elastic characteristics due to the formation of an extensive network of water droplet aggregates. Overall, the hydrogen bond-forming agents interacted with GMO at the interface, thereby improving their presence at the water droplet surface, allowing significantly improved stability of GMO-stabilized liquid W/CO emulsions. The knowledge developed in this research can be useful in applying GMO in stabilizing liquid water-in-oil emulsion without using any crystal network.

Keywords: water-in-oil emulsion; glycerol monooleate; hydrogen bond-forming agents; emulsion stability; interfacial interaction

1. Introduction

Products containing water-in-oil (W/O) emulsions are widely used in the pharmaceutical, cosmetic, petroleum and food industries. In food, almost all water-in-vegetable oil (W/VO) emulsions (e.g., margarine) are used in a semi-solid state (Ushikubo & Cunha, 2014). The emulsion stabilization mechanism in margarin is mainly due to fat crystal-based Pickering and network formation (Ghosh & Rousseau, 2011). Emulsifiers are also critical in the formation of W/O emulsion. Most emulsifiers used to form W/O emulsions are non-ionic lipophilic molecules (e.g., polyglycerol polyricinoleate (PGPR), Spans, monoglycerides). The selection of emulsifiers is based on their hydrophilic-lipophilic balance (HLB), which must be low (3 – 6) for W/O emulsion. Various research groups have worked with different combinations of oil, emulsifiers and aqueous phase ingredients to obtain stable liquid W/O emulsions. Opawale and Burgess (1998) investigated concentrated 50 wt% water-in-light mineral oil emulsions with Span 20, 80, 83 and 85 (1 – 5 %w/v) in the oil and sodium chloride (0 – 1 M) in the aqueous phase. The addition of the salt (S) within an optimum range enhanced the stability of the emulsion. Scherze et al. (2006) investigated stabilization of 30 wt% water-in-medium-chain triglyceride (MCT) oil emulsion with PGPR (4 wt%) and soy lecithin (2.5 wt%) in the presence of 0.6 % S in the aqueous phase. For lecithin, S favoured water droplet coalescence; however, for PGPR, the incorporation of S was necessary to obtain a stable W/O emulsion (Scherze et al., 2006). Lindenstruth and Muller (2004) investigated 30 wt% W/VO emulsions prepared with a 1:1 mixture of olive oil and MCT with 20% soya lecithin as an emulsifier. It was proposed that the *cis* double bond of oleic acid needed more space at the W-O interface, which prevented efficient interfacial packing of lecithin (Lindenstruth & Muller, 2004). In another research, Márquez et al. (2010) prepared 20 – 40 wt% W/VO emulsion using sunflower oil with 0.2 – 1 wt% PGPR and an aqueous phase containing various concentrations of calcium chloride and other salts (calcium lactate or carbonate; sodium, magnesium or potassium chloride). The incorporation of any salt allowed emulsion stabilization with higher water content. The stabilization effect of calcium salts was associated with a reduced attractive force between the water droplets and a higher PGPR adsorption density at the droplet surface. Prichapan et al. (2017) prepared 20 wt% W/VO emulsions with rice bran oil and stabilized them with rice bran stearin and PGPR. Emulsions without rice bran stearin (a source of fat crystals) were highly unstable to phase separation after one day; however, the stability improved by increasing the PGPR concentration from 2 to 4 wt% due to the reduction in water droplet size. However, the most stable emulsion was obtained with an oil phase containing 45 wt% rice bran stearin, which indicates that incorporating fat crystals as a stabilizing factor was more important than increasing the PGPR concentration.

PGPR is one of the most commonly used emulsifiers to develop food-grade W/O emulsions for its ability to stabilize water droplets by a polymeric steric barrier (Goubran & Garti, 1988; Mettu et al., 2018). Although it is classified as GRAS (generally recognized as safe), in 1979, the Scientific Committee for Food of the

European Community determined an acceptable daily intake of 7.5 mg/kg body weight. In 1980, the Canadian Regulations reduced the maximum concentration of PGPR to 0.25% in chocolate (Wilson et al., 1998). However, in 2017, PGPR was limited just for chocolate products with a maximum of 0.5 wt% (Canada, 2017). For this reason, different groups investigated various combinations of PGPR with other ingredients to lower its concentration in W/VO emulsion (Ghosh & Rousseau, 2009; Rafanan & Rousseau, 2017). Nevertheless, there is an urgent need to completely replace PGPR from W/VO emulsions with a more natural small molecule emulsifier. Hence, one of the aims of the present study was to develop stable liquid food-grade W/VO emulsions by completely replacing PGPR with glycerol monooleate (GMO) without any stabilizing fat crystals. GMO is a widely studied low HLB (3 – 4) emulsifier that is non-toxic, biodegradable, and classified as GRAS. It is commonly used to prepare margarine-type semi-solid emulsions; however, the presence of saturated fat crystals is essential for emulsion stability (Ghosh & Rousseau, 2011). To our knowledge, no research so far has investigated stabilizing liquid W/VO emulsions without using PGPR and saturated fat crystals. The challenge here is that liquid W/VO emulsions prepared solely with GMO are impossible to stabilize due to its desorption from the water droplet interface towards VO. Ghosh et al. (2011) observed that GMO's desorption from the W-VO interface strongly relies on its stronger hydrogen bonding interactions with the VO triacylglycerol (TAG) fatty acid carboxylic groups than the weaker hydrogen bonding interaction with water hydroxyl groups. The authors also showed that, in the presence of a hydrocarbon oil (such as mineral oil), GMO could very well remain at the water droplet surface due to the lack of interaction with the oil, thereby improving emulsion stability (Ghosh et al., 2011). Therefore, we hypothesized that the presence of hydrogen bond-forming molecules in the dispersed aqueous phase would form strong hydrogen bonds with GMO at the W-O interface, thereby improving the stability of liquid W/VO emulsions. Three different hydrogen bond-forming agents (two small molecules with multiple carboxylic groups, ascorbic acid (AA), and citric acid (CA) and one biopolymer, low methoxyl pectin (LMP)) with or without the presence of salts (sodium chloride (S) or calcium chloride (Ca)) were investigated to improve the stability of water-in-canola oil (W/CO) emulsions. As a control, light mineral oil (MO, made of paraffin hydrocarbons) was also used to form water-in-mineral oil (W/MO) emulsions, and its influence on emulsion stability and rheology was compared to W/CO emulsions.

2. Materials and methods

2.1 Materials

Canola oil (CO) was purchased from a local grocery store and stored at 4 °C. Distilled monoglycerides DMG 0298 (90 – 95% monoglycerides, containing mainly 75 – 91% oleic, 2 – 17% linoleic, 3 – 6% stearic, and 3 – 5% palmitic) was donated by Palsgaard (Juelsminde, Denmark). L-ascorbic acid (AA) and Nile red were purchased from Sigma Aldrich (Oakville, ON, Canada). Citric acid anhydrous (CA), light mineral oil (MO, code O121-4), and hydrochloric acid (HCl) (1 N) were purchased from Fisher Scientific (Toronto, ON, Canada). Sodium chloride (S) and calcium chloride (Ca) were obtained from BDH (VWR International, USA). Low methoxyl pectin (LMP) amidated, Genu[®] pectin type LM-101 (degree of esterification 36% and degree of amidation 14%) was provided by CP Kelco (Lille Skensved, Denmark). Purified water by Milli-QTM (Millipore Corporation, MA, USA) was used for the dispersed phase.

2.2 Preparation of solutions

AA and CA aqueous solutions were prepared with 0.125 and 0.5 wt% acids with 0 and 0.125, wt% S at room temperature with stirring (400 rpm) and stored at 4 °C. LMP solutions at pH 3.0 with 1N HCl were prepared with 1.5 and 2 wt% LMP with 0 and 0.045 wt% Ca at 90 ± 0.5 °C, stirred at 200 rpm for 20 min. The final LMP solutions were cooled down to room temperature before W/O emulsions preparation. The concentrations of the ingredients were selected based on preliminary experiments with a wider range; however, only the ones showing significant effect at minimum concentrations are reported. The zeta potential of LMP, measured at pH 3.0 using a zeta potential analyzer (Litesizer500, Anton Paar, Montreal, QC, Canada), was -18.68 ± 0.2 mV. Control solutions with just S at 0.125, 0.5 and 1 wt% and Ca at 0.045 wt% were also prepared using similar methods. All the solutions were prepared the same day as emulsions. The oil phase

was prepared by dissolving 4 wt% GMO in canola oil (CO) or mineral oil (MO) under stirring (400 rpm) at 60°C for 25 min.

2.3 Preparation of W/O emulsions

The aqueous and oil solutions were warmed at 40 ± 2 °C before weighing. The oil phase (70 g, 80 wt%) was weighed in a glass beaker (100 mL), and the aqueous phase (17.5 g, 20 wt%) was added dropwise while stirring at 400 rpm. The same weight of each phase and similar size glass beakers were used for all emulsion batches. Coarse emulsification was done with a rotor-stator blender (Polytron, Brinkman, ON, Canada) at level 10 for 1 minute. The coarse emulsions were then further homogenized in a high-pressure homogenizer (Emulsiflex C3, Avestin Inc., Ottawa, ON, Canada) at 20,000 psi for three cycles. Preliminary experiments showed that three cycles were enough to reduce the droplet size, and after that, no further decrease was observed. The final emulsions were collected in 250 mL beakers and cooled down at room temperature while stirring with a magnetic stirrer (size 3.5 x 0.7 cm) at 290 rpm for 20 minutes.

2.4 Emulsion storage stability

Freshly-made emulsions (25 mL) were transferred to 40 mL clear glass vials (VWR, Edmonton, AB, Canada) and stored at room temperature for seven days. The images were taken with an iPhone 8 camera (12 MP). The images of the vials were used to measure the height of the emulsion layer on day 0 and day 7 with ImageJ (v1.5 2i – Fiji project) to calculate the fraction of emulsion height (Eq.1).

$$\text{Fraction of emulsion height} = \frac{\text{Emulsion height on day 7}}{\text{Total emulsion height on day 0}} \text{Eq. 1}$$

2.5 Microstructure analysis

2.5.1 Light microscopy

The emulsion microstructure was captured after seven days of storage. A drop of the emulsion was placed over a glass depression microscope slide with 1 – 1.2 mm thickness (VWR, Edmonton, AB, Canada) and covered with a coverslip (VWR, Edmonton, AB, Canada). A light microscope (Eclipse E400, Nikon Mississauga, ON, Canada) was used to observe the emulsion samples using a 10x and 100x objective lens.

2.5.2 Confocal microscopy

Emulsion microstructure was also captured with a confocal microscope (Nikon C2, Nikon Inc., Mississauga, ON, Canada). One drop of 0.1 wt% Nile red solution in mineral oil was mixed with nine drops of fresh emulsion, and a drop of this mixture was placed over a glass depression microscope slide covered with a coverslip and placed under the microscope. The sample was excited using a 543 nm laser, and the emission spectra were collected from 573 to 613 nm using a 40x and 60x objectives lens.

2.6 Determination of emulsion droplet size

The droplet size of the emulsions was determined by analyzing the images taken with the confocal microscope. It was essential to select multiple images of each sample so that the number of water droplets quantified was between 3,000 – 18,000. The image analysis methodology developed in Romero-Peña et al. (2020) was used to quantify the water droplet size using ImageJ software (v1.5 2i – Fiji project). The image analysis data (area of each droplet) was exported to an Excel spreadsheet (Microsoft Office 365), and further calculation was done to obtain the volume-weighted means droplet diameter ($d_{4,3}$) using Eq. 2, where d is the diameter of each droplet obtained from the image analysis and n is the number of droplets of a particular size.

$$d_{4,3} = \frac{\sum_1^n d^4}{\sum_1^n d^3} \text{Eq. 2}$$

2.7 Accelerated stability analysis

The accelerated storage stability of fresh emulsions was analyzed using an analytical photocentrifuge LUMiSizer[®] LS650 (LUM GmbH, Berlin, Germany) using the methodology used by Erramreddy et al. (2017). An aliquot of emulsion (400 µL) was added with a syringe into a rectangular polycarbonate cuvette (8 mm x 2 mm) (LUM GmbH, Berlin, Germany) and centrifuged at 4000 rpm (2325xg). The equipment

transmits an 865 nm wavelength laser through the cuvettes every 60 s (1000 transmission profiles collected in 16 h 40 min) during the centrifugation. The light intensity transmitted through the emulsion depends on droplet movement under the centrifugal force. Data analysis and calculation of sedimentation velocity ($\mu\text{m/s}$) from the final transmission profile were processed using the SEPView® software 4.1 (LUM GmbH, Berlin, Germany).

2.8 Rheological properties of W/O emulsions

The apparent viscosity and viscoelasticity of fresh and 7 days old emulsions were measured using an AR-G2 rheometer (TA Instruments, Montreal, QC, Canada) at 25 °C with an acrylic parallel plate (60 mm diameter). For viscosity measurement, a shear rate from 0.01 to 100 s^{-1} and a loading gap of 1000 μm were used. The oscillatory mode was used at a strain ranging from 0.01 to 1000% to measure storage (G') and loss moduli (G'') at a constant frequency of 1 Hz. (6.18 rad/s). The apparent viscosity values of emulsions at a shear rate of 0.1 s^{-1} on day 0 and 7 were used to calculate the viscosity index (Eq. 3) to determine the change in viscosity with time.

$$\text{Viscosity index} = \frac{\text{Apparent viscosity of emulsion on day 7}}{\text{Apparent viscosity of fresh emulsion}} \text{Eq. 3}$$

2.9 Determination of interfacial tension

A force tensiometer (K20 by Krüss GmbH, Hamburg, Germany) with a Wilhelmy plate (20 x 10 mm) was used to determine oil/water interfacial tension at room temperature. The oil phase (65 mL) with 4 wt% GMO was added in a wide-mouth glass beaker (130 mL). The Wilhelmy plate was immersed entirely in the oil phase, set as the zero-immersion depth. The plate was then removed and cleaned with acetone (A18-4, Fisher, US) and flamed with a butane torch (Bernzomatic, OH, USA). The aqueous phase (22 mL) was added to another similar glass beaker and placed over the stage. The plate was placed very close to the liquid-air interface for surface detection. Then, the oil phase was gently added to the aqueous solution with a pipette (VWR, Edmonton, AB, Canada) to fill the zero-immersion depth. The interfacial tension measurements were performed until a standard deviation of 0.1 mN/m could be reached between the readings.

2.10 Statistics

All emulsions were prepared in triplicate, and each of them was analyzed on day 0 and day 7, and the average and standard deviation were reported. The statistical analysis was performed using IBM SPSS Statistics 20, in which Tukey's test was performed to compare the average with a significance level of 0.05.

3. Results and discussion

3.1 Emulsion storage stability after seven days

Phase separation in the W/O emulsions was visually observed and recorded (Figure 1). After 7 days, a clear oil phase was separated at the top in most emulsions due to water droplet sedimentation. Higher emulsion height or lower oil phase separation indicates better emulsion stability. In Figure 1, the control emulsions were indicated with no-additive (S0). The emulsions with only salts are indicated as S0.125 (0.125% sodium chloride) and Ca0.045 (0.045% calcium chloride). For the emulsions without any additive (S0), CO emulsions showed larger phase separation (lowest stability) compared to MO emulsions (Figure 1A and 1B). The addition of S0.125 showed a significant increase in emulsions height compared to S0 in both bulk oils. The emulsions with S0.125 and AA or CA demonstrated higher emulsion height and lower oil separation for both CO and MO than those with only AA or CA. The addition of Ca also increased emulsion height compared to S0 in both oils (Figure 1C). The emulsions with only LMP reported a higher emulsion height than those with LMP and Ca, indicating Ca was not that critical for storage stability when LMP was present. Besides, LMP emulsions (Figure 1C) seem to be more stable with lower phase separation for both CO and MO than the emulsions formed with S-AA and S-CA (Figure 1A, B). For most emulsions, lower phase separation was observed with MO than CO, indicating higher emulsion stability with the MO, which followed the observation by Ghosh et al. (2011).

3.2 Quantification of emulsion kinetic stability against phase separation

The emulsion layer height was measured from Figure 1 and presented as a fraction of the total emulsion height (Eq. 1) (Figure 2). A higher value of emulsion height fraction signifies emulsions with higher stability against phase separation. CO-emulsions height showed a significant increase from S0 (0.16 ± 0.01) to S0.125 (0.54 ± 0.02) (Figure 2A). Similarly, in MO emulsions, the height significantly increased from S0 to S0.125 (0.39 ± 0.01 to 0.72 ± 0.01). For both CO and MO emulsions, only AA and CA (both 0.125% and 0.5%) did not improve the emulsion height further than the S0.125, which indicates that the organic acids added to the aqueous phase were not as effective as salt in improving emulsion stability. Interestingly, when both salt and organic acids were present, the CO-emulsions height significantly increased compared to only salt-added emulsions. In contrast, for MO-emulsion, no significant difference was observed when both salt and organic acids were present compared to only salt (Figure 2A). For both the CO and MO emulsions, S0.125 with either AA or CA showed no significant difference in emulsion height fraction considering the same oil phase (Figure 2A).

With the addition of LMP (both 1.5 and 2%) (Figure 2B), a significant improvement in both CO and MO emulsion stability was observed. It is possible that the acidic aqueous phase (pH 3) induced structural opening in the pectin (Schmidt et al., 2015), which could have resulted in interfacial interaction with GMO polar head groups, thereby stabilizing GMO at the interface and increasing emulsion stability. The emulsion height reached the highest in MO-emulsions with LMP, although the values in CO-emulsions with LMP were not significantly different. However, as soon as Ca was added to the LMP-containing CO-emulsions, the height decreased to 0.67 ± 0.02 and 0.61 ± 0.02 for LMP1.5 and LMP2, respectively. It could be possible that Ca formed ionic linkages between the carboxylic groups from two LMP chains, which decreased LMP interaction with GMO at the interface leading to an increase in emulsion destabilization (Flutto, 2003). In MO-emulsions, 1.5 % LMP with Ca demonstrated reduced emulsions height (0.66 ± 0.02). Conversely, 2 % LMP with Ca did not significantly diminish emulsions height (0.75 ± 0.02) than only LMP-added MO-emulsions. It might suggest that for LMP2-Ca, excess unreacted LMP was available to stabilize the interfacial GMO leading to better emulsion stability. Between CO and MO emulsions, it can be said that the effectiveness of LMP in improving liquid W/O emulsion stability was hindered by Ca addition, more extensively in CO than MO.

3.3 Emulsion microstructure

All emulsions formed water droplets dispersed in the continuous oil phase as a free and aggregated network. The control CO emulsions without any additive (S0) showed a few larger droplets with some aggregation as most droplets were destabilized phase-separated (Figure 3A). The incorporation of S0.125 showed the initial appearance of extensive water droplet network formation (dark irregular-shape matter in the image) (Figure 3B). Such droplet aggregation also appeared with AA or CA addition into the aqueous phase (Figure 3C, D and G, H). When S0.125% was incorporated with AA (Figure 3E, F) and CA (Figure 3I, J), a more extensive water droplet network structure was identified, where free droplets were hard to locate in the continuous phase. It can be said that the water droplet network's extension was responsible for their higher emulsion height (Figure 1 and 2). Similar water droplet aggregation was also observed for W/O emulsions stabilized with Span 80 and lecithin emulsifiers (Ushikubo & Cunha, 2014).

MO-emulsions without any aqueous phase additive (S0) showed water droplets well distributed in the continuous phase (Figure 4A). Similar to CO emulsion, the microstructure of MO-emulsions with S0.125 (Figure 4B), AA (Figure 4C, D) or CA (Figure 4G, H) also demonstrated a water droplet network formation. The addition of S0.125% with AA or CA in MO-emulsions developed an extensive water droplets network structure similar to CO-emulsions.

The microstructure of emulsions formed with LMP and Ca are presented in Figure 5. CO-emulsions without Ca (S0, Figure 5 I-A) and with Ca (Figure 5 I-B) showed dispersed water droplets with some degree of droplet coalescence. The incorporation of LMP formed an extensive water droplets network throughout the emulsion, making it challenging to capture the emulsion's clear images (Figure 5 I-C, I-D). However, the

mixture of LMP and Ca illustrated a different structure related to gel-formation due to the interaction between calcium ions and the carboxyl groups in the pectin molecules (Yulianti & Othman, 2018). The structure formed by LMP-Ca in the aqueous phase can coexist with the water droplet network (Figure 5 I-E, I-F). All MO-emulsions, in the presence of LMP (with or without Ca) (Figure 5 II-B, II-A), also formed an extensive water droplets network compared to the emulsions without LMP (Figure 5 II-C, II-D). Comparing the CO and MO-emulsions with LMP, it can be said that the presence of MO led to a finer droplet network that was better able to provide emulsion stability against phase separation. Overall, CO and MO-emulsions with LMP (Figure 5) showed a more expanded water droplets network of smaller water droplets than the emulsions with S, AA, CA, or their mixture (Figures 3 and 4). Such droplet networks could be responsible for increasing emulsion stability against phase separation reported in Figures 1 and 2.

3.4 Average water droplet size

The water droplet size was calculated using image analysis. Only the water droplets outside the network structure were analyzed for this calculation. Thus, emulsions with LMP and Ca in CO and MO were not reported because the water droplets were aggregated, forming a network spanning the whole microstructure. It was assumed that the free water droplet size would represent the entire emulsions for comparison purposes. The control CO emulsions (S0) showed a high $d_{4,3}$ ($9.2 \pm 0.1 \mu\text{m}$), which was similar to CA0.5, followed by CA0.125 ($8.2 \pm 0.0 \mu\text{m}$) and S0.125 ($7.9 \pm 0.0 \mu\text{m}$) (Figure 6A). The emulsions formed with AA showed considerably lower $d_{4,3}$ ($6.0 \pm 0.1 \mu\text{m}$) at both concentrations. The addition of S to either AA or CA showed the smallest droplet size of all CO emulsions, which could be related to their higher emulsion height fraction reported in Figure 2. All MO emulsions reported a much smaller droplet size (about 7 times lower) than CO emulsions (Figure 6B). The MO emulsions prepared with S0 and S0.125 showed a non-significant difference in $d_{4,3}$ ($1.2 \pm 0.03 \mu\text{m}$). However, the incorporation of AA and CA and their mixture with S reported a significant reduction of droplet size ($< 0.72 \pm 0.01 \mu\text{m}$). The smaller droplet size obtained in MO emulsions compared to CO-emulsions can be a factor for their higher emulsion stability against phase separation (Figure 1 and 2).

3.5 Accelerated sedimentation velocity of emulsions

Water droplets' sedimentation velocity under accelerated gravitation (2325g) was used to identify how fast the emulsions would phase separate (Figure 7), which was used as an indicator of long-term stability. The CO emulsions with S0 and S0.125 had high sedimentation velocity under accelerated gravitation (on average, $9.8 \pm 0.2 \mu\text{m/s}$), which could be due to the larger water droplets and coalescence under centrifugal force (Figure 7A). There was a sharp decrease in sedimentation velocity with the addition of AA, CA, or their mixture with S, which can be associated with the combined presence of smaller water droplets and the droplet network formation.

The sedimentation velocity in MO emulsions significantly decreased with S0 ($6.8 \pm 0.2 \mu\text{m/s}$) and S0.125 ($7.3 \pm 0.1 \mu\text{m/s}$) compared to CO-emulsions, which could be due to the smaller droplet size of the former (Figure 7A). However, the sedimentation velocity of all other MO emulsions with AA, CA, S-AA, or S-CA demonstrated higher values than CO emulsions, despite their visually higher emulsion height, under earth gravitation after seven days (Figure 1 and 2). It can be said that the water droplet network provided better emulsion kinetic stability against earth gravity. However, accelerated gravitational force probably broke the network structure, leading to a faster separation of large water droplet aggregates in MO-emulsions. Erramreddy et al. (2017) reported a correlation between gel yield point and centrifugal separation. The minimum relative centrifugal force required for the creaming of oil droplets increased with gel strength. In the present case, the higher sedimentation velocity of MO-emulsions under accelerated gravitation could be due to their lower-yielding behaviour than CO emulsions, which will be discussed under the viscoelasticity section.

The addition of Ca significantly reduced CO-emulsion sedimentation velocity ($7.4 \pm 0.4 \mu\text{m/s}$) compared to S0, indicating higher stability against phase separation (Figure 7B). Emulsions with LMP (both concentrations) exhibited a further reduced sedimentation velocity. Similar behaviour was also observed for MO

emulsions, except for the one with Ca. Overall, CO-emulsions with AA, CA, their mixtures with S and LMP exhibited lower sedimentation velocity than MO-emulsions. Lower sedimentation velocity indicates that the presence of aqueous phase ingredients could help to improve long-term W/O emulsion stability.

3.5 Change in emulsion viscosity with time

The emulsion viscosity changes were calculated using the viscosity index, a ratio of apparent emulsion viscosities on day 7 to day 0 (Figure 8). A value of viscosity index less than one indicates that the viscosity decreased upon storage, which could be due to the water droplet networks breakdown and lowering of emulsion stability against phase separation. All CO-emulsions reported viscosity indices less than one, except the emulsions with S-CA, where they remained close to one (Figure 8A). Conversely, all MO-emulsions with additives in the aqueous phase reported a viscosity index above one, except the S-CA emulsions, which can strengthen the water droplet network structure with time, leading to higher stability of MO-emulsions.

With LMP, all CO-emulsions showed viscosity indexes lower than one, except by LMP2-Ca0.045, which had the highest viscosity index (1.48 ± 0.02), which could be associated with the strongest network formation after storage (Figure 7B). On the contrary, all MO emulsions with LMP demonstrated a viscosity index very close to one (Figure 7B). Overall, the MO bulk phase might allow more interaction of the aqueous phase additives with GMO at the interface due to the less interaction of GMO with MO hydrocarbon. This way, higher droplet stability and a more robust droplet network could be developed during storage, leading to better viscosity protection for MO emulsions than CO-emulsions.

3.6 Emulsion viscoelasticity

All the emulsions reported higher storage (G') than loss (G'') moduli in the low-strain regime (Figures 9 and 10). No emulsions showed true linear viscoelastic region (LVR) except fresh S0 (Figures 9 A1 day 0), indicating weak gels. All emulsions showed a steady decline in G' with an increase in strain, also indicating a weak gel structure.

After storage, all CO-emulsions reported lower range G' than the fresh emulsions (Figure 9, A2), implying that the initial structure on day 0 has diminished in strength, similar to the lower viscosity index reported in Figure 8. Most CO-emulsions showed on day 0 showed a peak in G'' at the crossover, which indicates relaxation in droplet network structure, leading to gel breakdown. However, such peak in G'' mostly disappeared after 7-days storage, except for S-CA (both concentrations), indicating loss of network structure with time. All MO-emulsions (Figure 9, B) exhibited a faster drop in G' with strain and lower crossover strain than CO-emulsions, which could explain their higher sedimentation velocity under accelerated gravitational force reported in Figure 7. After 7 days, most MO-emulsions, except S-CA, showed unchanged or some increase in G' (Figure 9, B1), which was also similar to the viscosity index data (Figure 8)

Unlike CO-emulsions with S and AA or CA, no peak in G'' was observed with Ca and LMP. After 7 days, all CO-emulsions showed mostly unchanged weak viscoelastic behaviour (Figure 10, A2). Similar to what was observed in Figure 9, in Figure 10, MO emulsion showed a faster drop in G' as a function of strain and a crossover at a lower strain compared to CO-emulsions. After storage, MO-emulsions with Ca and LMP1.5 demonstrated an increase in gel strength (Figure 10, B2). Overall, CO and MO emulsions' viscoelastic behaviour showed different levels of weak gel strength, with MO emulsions appearing weaker than the CO emulsions. The presence of S, AA, CA or their combination in CO-emulsion created gels that can withstand more shear force than the CO-emulsions with Ca and LMP.

3.7 Interfacial tension between oil and aqueous phases and average droplet size

Oil-aqueous phase interfacial tension was determined to understand the GMO's surface activity in the presence of various aqueous phase additives. CO without GMO against water without any additive reported an interfacial tension of 21.23 ± 0.05 mN/m, while for MO, GMO's interfacial tension was 46.03 ± 0.73 mN/m (data not shown). According to Tuntiwattapanapun et al. (2013), vegetable oils with water displayed interfacial tension between 20 – 30 mN/m. With MO-water, Xaxa (2014) reported an interfacial tension of

45.65 ± 0.57 mN/m. Both the values from the present work are within the range found in the literature. Although made up of non-polar TAG, commercial vegetable oils reduced the interfacial tension due to minor polar components, such as free fatty acids, monoacylglycerols, or diacylglycerols (Gaonkar, 1989).

Interfacial tension measurement relied on the emulsifier diffusion and adsorption to the W-O interface. A higher concentration of surface-active molecules should lower interfacial tension unless the critical micelle concentration (CMC) has reached, after which no further decrease in interfacial tension would be observed. Figure 11 presents the interfacial tension of GMO in CO and MO against the various aqueous phases. All GMO interfacial tension for the CO-aqueous phase showed a non-significant difference (1.2 to 1.7 mN/m), and they were lower than all MO-aqueous phase interfacial tension (1.4 to 2.5 mN/m), except for S0, S0.125 (Figure 11A), and Ca0.045 (Figure 11B). Ghosh and Rousseau (2012) reported the interfacial tension of 4 wt% GMO in CO-water (1.6 ± 0.1 mN/m), which is similar to our data (1.2 ± 0.05 mN/m). GMO in MO-S0 system displayed a lower interfacial tension, which can be associated with GMO's ability to pack much better at the interface against MO. Lower interfacial tension of GMO in MO-aqueous phase without any additives was also responsible for the much smaller water droplet size in W/MO emulsion (0.7 to 1.2 μm) than W/CO emulsions (4.6 to 9.2 μm) (Figure 6).

The addition of S, AA, CA or their mixtures did not significantly change W-CO interfacial tension. In the W-MO system, with the addition of sodium chloride (S0.125), a slight increase in interfacial tension was observed. Amar-Yuli and Garti (2005) reported that S creates a "salting-out" effect, reducing the emulsifier head group hydrophilicity (due to dehydration) and promoting aggregation. The salting-out outcome may be favourable in oils with excess surface-active molecules, as in vegetable oils. However, in MO, the increase of GMO interfacial aggregation might promote some bare patches at the interface, which may not favour a decrease in interfacial tension. Besides, Amar-Yuli et al. (2007) addressed that the hydration layer of GMO polar heads decreased in size due to dehydration when GMO competes with other molecules for binding water, leading to an increase in interfacial tension. MO-aqueous phase system also observed a significant increase in interfacial tension with AA and CA compared to the aqueous phase without any additive. Therefore, increased emulsion stability against phase separation with AA and CA was not due to interfacial tension (their droplet size was also similar), but rather water droplet aggregation and network formation.

GMO in CO-water with Ca and LMP showed similar interfacial tension to the aqueous phase without additive (S0) (Figure 11B). Lutz et al. (2009) investigated the interfacial tension of limonene-water with 0.5 wt% pectin. They attributed the enhancement of GMO orientation at the interface due to the interaction of pectin carboxylic groups. Interfacial tension of the MO-aqueous phase with LMP showed an increase compared to the MO-aqueous phase without any additive or with Ca (Figure 12B). It could be due to the presence of LMP at the O/W interface (due to the lack of surface active molecule in MO) preventing GMO's free adsorption at the interface. Many researchers have previously reported the surface activity of LMP. For example, Leroux et al. (2003) reported a decrease in interfacial tension between paraffin oil and 2 wt% pectin solution with a reduction in the degree of methylation. The addition of Ca^{2+} to LMP in the aqueous phase led to a steep rise in GMO's interfacial tension for both the CO and MO emulsions. Calcium ions can promote gelation in LMP molecules due to their ionic binding with pectin carboxylic groups (Capel et al., 2006). LMP formed weak gels in the aqueous phase at room temperature with Ca^{2+} , which might reduce GMO adsorption at the interface, leading to increased interfacial tension. Higher interfacial tension of LMP-Ca systems can also be related to their reduced emulsion stability (61 – 67%) compared to only LMP-added systems (72%) (Figure 2). Overall, the interfacial tension was influenced by the oil type and the interfacial interaction between the GMO head group and the aqueous phase additives. Interestingly, interfacial tension had less influence on emulsion stability against phase separation, which was mostly influenced by droplet aggregation and network formation.

3.8 Discussion

We have shown that incorporating various aqueous phase ingredients interacted with GMO differently at the W-O interface, leading to improved W/O emulsion stability against phase separation for both TAG (in CO) and paraffin hydrocarbon (in MO)-based oils. The lowest stability was observed for the emulsions without

any aqueous phase additives. Incorporation of either sodium chloride (S) or calcium chloride (Ca) showed significant improvement in emulsion sedimentation stability. Salt-induced improvement in W/O emulsion stability has been well studied, and many authors reported that the presence of salt in the aqueous phase is essential for efficient stabilization of W/O emulsions (Ganguly et al., 1992; Márquez et al., 2010; Opawale & Burgess, 1998; Scherze et al., 2006). It has been proposed that salt improves non-ionic emulsifier interfacial adsorption density, improving interfacial elasticity and emulsion stability (Aronson & Petko, 1993). Besides salts, we have also added organic acids such as ascorbic acid (AA) and citric acid (CA) into the aqueous phase, which also showed significant improvement in W/O emulsion stability compared to the emulsions without any additives. It has been proposed that an ideal emulsifier for W/O emulsion must form a strong hydrogen bond with water with its polar head group (Villamagna et al., 1995). However, in canola oil (CO), GMO form stronger hydrogen bond with $>C=O$ groups of TAG glycerol polar heads compared to the $-OH$ groups of water, leading to an eventual disruption of GMO molecules from the W-O interface towards the oil phase (Ghosh et al., 2011). The addition of AA or CA with multiple $>C=O$ groups in the aqueous phase provided stronger hydrogen bonds with interfacial GMO towards the aqueous phase, thereby providing improved stability to the W/O emulsions. Such interaction of GMO with ascorbic acid in water has previously been reported by Bitan-Cherbakovsky et al. (2009). Improvement in W/O emulsion stability in the presence of AA and CA was also observed for mineral oil (MO). Interestingly, a synergistic improvement in emulsion stability was observed when both S and AA or S and CA were added to the aqueous phase. Such synergistic effect in increasing W/O emulsion stability was not reported before. Such differences in emulsion stability can also be seen from their average droplet size. For CO-emulsions, the smallest droplet size was observed when both S and AA or S and CA were present in the aqueous phase. Comparing CO and MO, a much smaller average droplet size and higher sedimentation stability were observed for MO-emulsions compared to CO-emulsions. This could be due to a lack of interactions between GMO and MO, leading to a favourable presence of GMO at the W-O interface than when GMO was present in CO. Such difference in W/O emulsion stability based on the continuous oil phase has also been reported before (Bus et al., 1990; Ghosh & Rousseau, 2011). For example, Bus et al. (1990) showed that if the emulsifier does not form any hydrogen bond with the oil (as in the case of GMO in MO), they will be more available to form hydrogen bonds with water, and their stability will be higher compared to a hydrogen-bonding oil such as CO.

We have also investigated the presence of polysaccharide pectin (LMP) with numerous $>C=O$ groups and $-OH$ groups on the stability of W/O emulsions. Our initial hypothesis was that the presence of a large number of hydrogen bond-forming groups in the aqueous phase would provide extensive stability to GMO at the W-O interface. We found the highest emulsion stability in the presence of LMP in the aqueous phase. Moreover, the hydrogen bonding ability of LMP with GMO was able to overcome the destabilization effect of CO on interfacial GMO, so that when LMP was present, no significant difference in the stability of the W/CO and W/MO emulsions was observed. However, the presence of Ca and LMP led to lower emulsion stability, which was due to the interaction of Ca with LMP making it less available to hydrogen bond with interfacial GMO. The effect of pectin on improving W/O emulsion stability has recently been reported for W/CO emulsion-based low-fat tablespread by Romero-Peña and Ghosh (2021).

The microstructure of the emulsions revealed extensive aggregations of water droplets and the formation of a droplet network in both oils. It is known that water droplets in W/O emulsion generally have a smaller energy barrier against flocculation (Kent & Saunders, 2001), which could also facilitate droplet aggregation. It can be said that the extensive water droplet network was eventually responsible for the higher stability of the W/O emulsions against sedimentation. For example, water droplets containing LMP aggregated massively, more than any other additives, which could be related to the interaction between GMO and LMP and interfacial strengthening of water droplets surfaces leading to the highest emulsion stability (Romero-Peña & Ghosh, 2021).

From the viscosity index, it was observed that while the water droplet network structure was protected and indices remained [?] 1 for all MO-emulsions, for most of the CO-emulsions viscosity, dropped with storage time, indicating breakdown of droplet network with time. Viscoelastic behaviour of all emulsions showed weak gel-like properties. Interestingly, all MO-emulsions showed a faster drop in storage moduli with strain

and lower crossover strain than the CO-emulsions. A similar indication of weaker droplet network structure for MO-emulsions was also observed when the emulsions were subjected to accelerated gravitation, where all MO-emulsion showed faster sedimentation velocities due to the faster breakdown of water droplet network in MO than that in CO. However, with LMP in the aqueous phase, such a difference in sedimentation velocity was smaller, which could be due to the enhancement of the droplet network in the presence of LMP. More research is needed to further understand the mechanism behind the difference in water droplet network strength in these two different oils

The excess of polar molecules in CO reduced the interfacial tension to a minimum which did not change in the presence of any aqueous phase ingredients. However, this mechanism was not enough to stabilize the CO emulsions without additives (S0) due to the stronger hydrogen bonds between GMO and TAG glycerol polar head groups of CO, which pulled the GMO out of the interface towards the oil phase in an emulsified state (Bus et al., 1990; Ghosh et al., 2011). It can be said that the interfacial tension determined using a platinum plate in a static setup was not able to simulate the dynamics in play at the water droplet interface in the presence of various aqueous phase additives and the oil phase. When GMO was present in MO, interfacial tension was the lowest without any aqueous phase additive, further supporting the hypothesis of lack of hydrogen bonding of GMO in MO leading to their free movement towards the interface. Interestingly, although the interfacial tension of GMO in MO was higher than GMO in CO in the presence of various aqueous phase additives, the droplet size of the final emulsions was significantly smaller for MO-emulsions than CO-emulsions. This apparent discrepancy could be explained by partial droplet destabilization in CO due to GMO's disruption towards the oil phase. Finally, LMP molecules demonstrated better stability of GMO and similar interfacial tension in both oils due to its numerous $>C=O$ groups that could form hydrogen bonds with interfacial GMO. Although the interfacial tension in the presence of LMP was not significantly different compared to AA, CA or their mixture with S, LMP provided a stronger interface which allowed higher stability of the droplets in an aggregated state (Ford & Furmidge, 1966), thereby improving emulsion stability and rheology. However, adding Ca ions with LMP led to the formation of weak gel in the aqueous phase, which hindered GMO adsorption at the O-W interface and reduced emulsion stability.

4. Conclusions

In this work, different concentrations of hydrogen bond-forming agents (citric acid (CA), ascorbic acid (AA), low methoxyl pectin (LMP)) with and without salts (sodium chloride (S) or calcium chloride (Ca)) was added into the aqueous phase to investigate the stability of the liquid W/CO and W/MO emulsions with a small molecule emulsifier, GMO. W/CO emulsion without additives destabilized instantly, whereas W/MO emulsion stayed partially stable due to extensive hydrogen bonds between GMO and CO TAG glycerol polar heads, leading to the desorption of interfacial GMO towards the CO phase. In MO, GMO favoured interfacial adsorption due to the lack of polar groups, leading to emulsion stability. The addition of hydrogen bond-forming agents and salts in the aqueous phase significantly improved both the CO- and MO-emulsion stability. LMP, with a large number of hydrogen bond-forming groups, was able to provide the highest emulsion stability after 7 days in both oils compared to AA, CA and their mixtures with S. However, the presence of Ca with LMP led to gelation in the aqueous phase which prevented interfacial interaction of LMP with GMO leading to a lowering of emulsion stability. Emulsions with both oils formed weak gels due to the formation of an extensive network of water droplet aggregates. Water droplets were smaller in MO-emulsions than CO-emulsions, leading to a more extensive droplet network in the former which provided higher stability against phase separation and preservation of viscosity after 7 days storage. Interestingly, the droplet network in MO-emulsions broke down at a lower crossover stain than the CO-emulsions, indicating weaker links between the droplets. Overall, the aqueous phase hydrogen bond-forming agents interacted with GMO at the interface allowing improved stability of GMO-stabilized liquid W/CO emulsions. This important finding could facilitate the development of highly stable food-grade low-saturated fat tablespreads. The knowledge developed could also improve our understanding of stability mechanisms in W/O emulsions for other industrial and cosmetics applications.

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Conflict of interest statement

The authors declare no conflict of interest.

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