Development and characterization of the freeze-thaw and oxidative stability of edible rice bran wax-gelatin biphasic gels

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ABSTRACT

Edible biphasic gels with high lipid fractions (>50%) were developed and characterized for their freeze-thaw and lipid oxidation stability. Gels consisted of gelatin in aqueous buffer (hydrogel; HG), and rice bran wax (RBW) in high-oleic soybean oil (oleogel; OG). Freeze-thaw stability was studied by rheology, liquid loss measurement, and microstructural characterization of the gels before and after one freeze-thaw cycle. Biphasic gels were stored for 6 months under accelerated oxidation conditions to assess oxidative stability through peroxide value (PV) analysis. Biphasic gels led to superior systems compared to control OG and HG. The storage modulus (G') increased with increasing OG. Yield stress (σ *) for biphasic gels were greater than for OG alone, and σ * increased as the proportion of HG increased. Micrographs of biphasic gels showed OG-in-HG matrix for all gels, including those of >50% OG. After one freeze-thaw cycle, biphasic gels had less total liquid loss by approximately 50% compared to the controls and showed an increase in G'. No samples were rancid after the storage period, as demonstrated by the low PV (<3 meq/kg). Changing the ratio of OG:HG for this biphasic gel demonstrates the potential to design semi-solid fat replacers of desired properties with good overall physico-chemical stability.

1. Introduction

In the past decade, consumers have become more aware of the impact of diet on health and wellness, therefore increasing demand for healthier food products (Hwang, 2020). One of the macronutrients in the spotlight is fat. There is increasing evidence that associates the consumption of long chain saturated fatty acids with an increased risk for cardiovascular disease, whereas increased consumption of mono- or polyunsaturated fatty acids decreases this risk (Briggs, Petersen, & Kris-Etherton, 2017; Ding & Rexrode, 2020; Kim, Je, & Giovannucci, 2021; Kris-Etherton & Krauss, 2020; Smith, Lunt, Smith, & Walzem, 2020). Consumers are more aware of healthy and unhealthy fats due to press coverage on nutritional studies and governmental dietary regulations to limit saturated fat consumption (Hwang, 2020; Martins, Vicente, Pastrana, & Cerqueira, 2020; Zulim Botega, Marangoni, Smith, & Goff, 2013). According to the 2020–2025 Dietary Guidelines for Americans, consumers are recommended to limit daily caloric intake from saturated fats to less than 10% of the total calories (Dietary Guidelines for Americans 2020–2025, 2020). However, saturated fats play an important role in providing desirable organoleptic properties in food products, such as mouthfeel, creaminess, flavor, spreadability, and overall performance (Martins et al., 2020; Patel, 2015). Saturated fats are different from liquid oils because they are solids at room temperature, due to triacylglycerols (TAGs) with saturated fatty acids assembling into small crystals bound by non-covalent bonds (Rogers, 2009). Simply replacing saturated fats with unsaturated fats will therefore lead to changes in the quality of the final food product due to differences in their physicochemical characteristics.

Over the years, several saturated fat mimetics have been developed, all taking different approaches to structuring oil. An alternative to saturated fats is oleogels (OG), which are gels that have an edible lipophilic solvent as the continuous phase (Patel & Dewettinck, 2016; A. Singh, Auzanneau, & Rogers, 2017).

OGs have been demonstrated to be successful substitutes for shortening (Mert & Demirkesen, 2016), peanut butter (Tanti, Barbut, & Marangoni, 2016), margarine (Hwang, Singh, & Lee, 2016) and comminuted meat products among others. For example, Wolfer, Acevedo, Prusa, Sebranek, and Tarté (2018) incorporated rice bran wax (RBW)-soybean oil OGs into frankfurter-type sausages and observed that the 10% RBW OG incorporated sausages had fat globules averaging 559.6 μm², which was similar to the pork fat control sausage. Similar microstructures were observed with RBW-soybean oil OGs in

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chicken-based bologna sausages (Tarté, Paulus, Acevedo, Prusa, & Lee, 2020). Nevertheless, OGs are easily deformed, and thus cannot be used as saturated fat substitutes for food products that need particulate definition of saturated fats. For instance, OGs would not be suitable for sausages like salami, which have distinct particles of fat (0.1–0.5 cm²) throughout the product (Romano, Masì, & Cavella, 2018), because they cannot be passed through a grinder without losing structural integrity.

For food products that need saturated fat mimetics with greater mechanical strength, an alternative approach needs to be taken. One such approach is the use of biphasic gels, also known as hybrid gels or bigels (Martins et al., 2019). The biphasic gels are structured by synergistic interactions between two semi-solid phases, a hydrogel (HG) and an OG, leading to an unique thermodynamic and viscoelastic behavior (Martins et al., 2019; Shakeel, Lupi, Gabriele, Baldino, & De Cindio, 2018). If the continuous phase is the OG, the biphasic gel can be classified as an oleocollloid (Pușcaș, Mureșan, Socaciu, & Muste, 2020). On the other hand, HG-dominant biphasic gels have also been coined as particle filled composite gels and bigels (Gravelle, Barbut, & Marangoni, 2015; Shakeel et al., 2018). Because these gels have a unique duality of a hydrophilic and lipophilic phase, they have the potential to serve as carriers for both hydrophilic and lipophilic compounds (Martins et al., 2019; Shakeel et al., 2019). Different biphasic gels have been explored for drug delivery and development of functional foods (Rehman & Zulfakar, 2014; Bollom, Clark, & Acevedo, 2019; Martín-Illana, Notario-Pérez, Cazorla-Luna, Ruiz-Caro, & Veiga, 2019; Paul et al., 2018; Rehman, Mohd Amin, & Zulfakar, 2014; Saffold & Acevedo, 2021; Zheng, Mao, Cui, Liu, & Gao, 2020; Zhuang, Gaudino, Clark, & Acevedo, 2021). Despite these recent studies exploring biphasic gels, few studies have reported the freezing-thaw and storage stability of biphasic gels consisting of a high OG fraction. Understanding the storage stability of biphasic gels can be useful for assessing potential commercialization of biphasic gels for the food industry, as ingredients with better storage stability can be transported frozen or along the cold chain (Mercier, Villeneuve, Mondor, & Uysal, 2017). In addition, some processing conditions require chilling of raw ingredients near 0 °C to keep the ingredient from heating up, such as when processing meat through a grinder.

In this study, edible RBW-gelatin biphasic gels with different ratios of OG to HG (40:60, 50:50, 60:40, 70:30) were developed and characterized to evaluate differences in their physical properties, mechanical strength, thermal behavior, and oxidative stability during storage at 22 °C for 6 months. Additionally, the freeze-thaw stability of the biphasic gels was assessed upon exposure to a freeze-thaw cycle and storage.

2. Materials and methods

2.1. Materials

The raw materials for the biphasic gels were 250 bloom pork skin gelatin (Nitta Gelatin NA, Inc, Morrisville, NC, USA), high oleic soybean oil (HOOSO)(Plenish® brand, Corteva Agriscience, Johnston, IA, USA), and rice bran wax (RBW) (Koster Keunen, Inc., Watertown, CT, USA). All salts and reagents were obtained from Fisher Scientific (USA), unless otherwise specified below. Potassium sorbate was obtained from Bean brand, Corteva Agriscience, Johnston, IA, USA), and fluo- rescein isothiocyanate dye (FITC) from Acros organics (Czech Republic).

2.2. Preparation of gels

2.2.1. Buffer preparation

All biphasic gels and control hydrogels (HGs) were prepared with a buffer solution. To prepare 1 L of preservative solution, 4.6 g of trisodium citrate dihydrate, 5.1 g of citric acid monohydrate, 1 g of sodium benzoate, and 1 g of potassium sorbate were dissolved with deionized (DI) water. The pH was adjusted with 5 mol/L NaOH to pH 4.7 to yield a final buffer solution of 40 mmol/L with 0.1 g/100g preservatives.

2.2.2. Sample preparation

All gels were prepared according to Saffold and Acevedo (2021) with 7.5 g/100g RBW and 7 g/100g gelatin with varying ratios of OG:HG. Predetermined amounts of RBW, gelatin, preservative solution, and HOSO were weighed (Table S1). For the HG, gelatin was added to the preservative solution, then heated with stirring until the internal temperature reached 85 °C. Separately, RBW was melted in HOSO by heating to 85 °C with constant stirring, and later held between 85 and 95 °C for 10 min to erase the crystal memory. The hot OG mixture was cooled to 85 °C and homogenized (Ultra-Turrax T25, IKA-Werke, Germany) with the hot HG mixture for 20 s at 24,000 rpm and 30 s at 13,000 rpm/min. After homogenizing, the mixture was poured into plastic containers and then placed at 4 °C (cooling rate: 1.7 °C/min) overnight. All samples were prepared in triplicate and analyzed within 4 days of preparation. Controls included 7 g/100g gelatin HG and 7.5 g/100g RBW OG. Throughout this work, ratios of OG and HG by weight are expressed as OG:HG.

2.2.3. Freeze-thawed study

Gels were prepared as described in section 2.2.2. Gels were cut into 2 cm diameter x 3.2 mm-thick discs using a customized gel cutter. The discs were frozen to −20 °C for 24 h, thawed at 22 °C for 5 h before analysis.

2.2.4. Oxidative stability study

For the long-term storage study a pro-oxidant was added to the HOSO to promote lipid oxidation using a method adapted from (Gutierrez, Boylston, & Clark, 2018). Briefly, in a ceramic mortar and pestle, approximately 5 g CuSO₄·SH₂O crystals were ground to a fine powder and mixed with HOSO to a final concentration of 1 mg Cu²⁺/kg oil. The oil was stirred for 18 h in the dark at 20 °C before storing at 4 °C for a maximum of 3 d. This oil was used in place of the standard HOSO to prepare the biphasic gels as explained in section 2.2.2. Two controls, an OG sample and a 40:60 sample without prooxidant, were prepared in addition to the treatment samples. A 40:60 biphasic gel without prooxidants (std) was prepared as a control, and this ratio was selected because it has the highest amount of water, which could potentially accelerate lipid oxidation (Karel, 1980). This sample also served to observe the effect of prooxidant, if any, on the OG theoretically most susceptible to oxidation. All samples were prepared in triplicate and incubated at 22 ± 1 °C for 6 months. The temperature was set to 22 °C to ensure no physical changes (i.e., melting) occurred to the samples during storage.

2.3. Differential scanning calorimetry (DSC)

A Discovery DSC (TA Instruments, New Castle, DE, USA) was used for the thermal analysis of all samples. Approximately 2–3 mg of a gel sample were weighed directly in a Tzero pan. The pans were sealed with a Tzero hermetic lid and subsequently analyzed. The samples were equilibrated to 0 °C, heated to 90 °C, and cooled back to 0 °C, with the heating and cooling rate set to 10 °C/min. TRIOS software (version 4.1.1.33073, TA Instruments) was used to analyze the peak temperatures (ΔTcr = chain relaxation, ΔTrefl = crystallization, ΔTgel = sol-gel transition, and ΔTsol = gel-sol transition) and enthalpies (ΔHcr = chain relaxation, ΔHrefl = crystallization, ΔHgel = sol-gel transition, ΔHsol = gel-sol transition) of the thermal curves.

2.4. Rheology

Amplitude sweeps were performed according to Bollom et al. (2019) with minor modifications. All gels except the control OG were cut into 2
cm × 3.2 mm discs. A Discovery HR-2 Hybrid Rheometer with Peltier unit (TA Instruments, New Castle, DE, USA) with a 20 mm parallel plate XHatch geometry was used for rheological analysis. All sweeps were run from 0.001% to 150% strain, at an angular frequency of 10 rad/s and temperature of 20 °C. The elastic modulus (G’) and loss modulus (G”) were determined from the linear viscoelastic region (LVR). The yield stress (σ∗) was calculated as a 10% decrease from the LVR (Acevedo, Block, & Marangoni, 2012). A minimum of 6 replicates of each sample were run.

2.5. Water and oil loss determination

The method of Zhang, Yang, and Acevedo (2020), with modifications, was used to determine the water and oil loss of samples before and after being subjected to a freeze-thaw cycle. New 2 cm × 3.2 mm discs were weighed after thawing, and subsequently placed between two pre-weighed filter papers (Whatman #1, 90 mm diameter) and pressed with a 200 g weight for 30 min at 20 °C. The final weight of the disc and filter papers were measured. To determine oil loss, the filter papers were dried at 60 °C for 30 min and then set at room temperature for 30 min for equilibration before measuring the final weight. The water loss (WL) and oil loss (OL) were calculated according to equations (1) and (2), respectively.

\[
WL(\%) = \frac{(G_0 - G_1) - (F_1 - F_2)}{G_0} \times 100
\]

\[
OL(\%) = \frac{(F_1 - F_0)}{G_0} \times 100
\]

where G₀ and G₁ are the initial and final mass of the gel disc, respectively; and F₀ and F₁ are the initial and final mass of the filter papers. Three measurements were taken for each replicate, with a total of three replicates per sample. Visual observations of the gel discs before and after freezing were also done.

2.6. Confocal laser scanning microscopy (CLSM)

FITC (5–6 mg) and 0.01g/100g of Nile Red dissolved in HOSO were added to the HG and OG, respectively, before sample preparation. All samples were cut into discs (2 cm diameter x 3.2 mm thick) and kept cold with ice before placing on a round glass slide. Images were obtained using a SP5 X MP Confocal Microscope (Leica Microsystems Inc., Buffalo Grove, IL, USA), magnified with a 40× objective. To visualize each of the phases, two laser lines with excitation wavelengths of 495 nm and 552 nm for FITC and Nile Red, respectively, were used. For each replicate, 5 images (1024 × 1024 pixels) were obtained. Images were overlaid using the Leica LAS AF Lite software ver. 2.6.0 (Leica Microsystems Inc., Buffalo Grove, IL, USA).

2.7. Lipid oxidation analysis

2.7.1. Peroxide value (PV)

Total lipid was extracted according to a modified method by Bligh and Dyer (1959). Twelve mL of hexane and 4 mL of MeOH were added to 5 g of gel and stirred until the gel structure was broken down. Vials were sealed, vortexed for 1 min, then stored at 4 °C overnight to allow layers to separate. The top layer was then transferred to a new glass vial, and the solvent was evaporated at 20 °C under N₂ for 3 h. The final extract was weighed and stored at −20 °C until analysis.

The spectrophotometric method was adapted from Shantha and Decker (1994). Briefly, 10 mg/mL iron (II) chloride and 0.3 g/mL ammonium thiocyanate solutions were prepared and stored in a colored glass bottle at 4 °C until use. The standard solutions for the calibration curve of the [Fe(SCN)(H₂O)₃]²⁺ complex were prepared using 0, 2, 4, 8, 12, and 16 μg/mL Fe²⁺ standards from a 10 μg Fe⁵⁺/mL stock solution. Approximately 0.1 g of lipid extract was dissolved in 6 mL of 3:1 v/v butanol/n-hexane solvent solution in a test tube, and vortexed for 10 s. A 20 μl aliquot of 0.3 g/L NH₄SCN solution and 20 μL of 1 mg/mL Fe²⁺ solution were added to the test tube and vortexed for 10 s. The test tube was left to equilibrate at room temperature for 10 min. Afterwards, 2 mL of the solution were transferred to a quartz cuvette and the absorbance was measured at 500 nm. The blank was the 3:1 v/v butanol/n-hexane solvent and was used to obtain the corrected absorbance. The peroxide value (meq O₂/kg oil) was calculated using equation (3).

\[
PV = \frac{m \times 21.6}{55.845 \times Abs^2}
\]

where m = mass of sample (g), 21.6 = slope, 55.845 = atomic weight of Fe, Abs = corrected absorbance, and 2 = correction factor. Measurements were taken every 2–3 weeks.

2.8. Statistical analysis

Data were expressed as mean ± standard deviation when applicable. ANOVA and Tukey tests were used to test for significant differences between means. Significance level was established at P < 0.05. Statistical analyses were performed using RStudio (version 1.1.383) software, with packages dplyr (version 1.0.2), lme4 (version 1.1–23), nlme (version 3.1–142), emmeans (version 1.5.0), car (version 3.0–10), multcompView (version 0.1–8), and multcomp (version 1.4–15). All experiments were replicated three times, unless noted otherwise.

3. Results and discussion

3.1. Visual observations

3.1.1. Non-frozen-thawed gels

The gelatin HG and RBW OG controls were translucent and semi-opaque, respectively (Fig. 1). The OG was yellow in color which can be attributed to the large proportion of oil in the system. In contrast, all biphasic gels, regardless of the ratio of OG:HG, were semi-solid and uniformly white or off-white.

Tactile observations of biphasic gels with a high HG ratio displayed characteristics similar to the gelatin HG – they were more resilient and flexible. Since the biphasic gels were firm, they were able to be cut into cylinders without significant smearing or fracturing (Fig. 1). Biphasic gels with more OG proportion, however, displayed physical characteristics more similar to those of RBW OG, such as being more plastic or less resistance to deformation.

3.1.2. Freeze-thawed gels

After one freeze-thaw cycle, the most noticeable change occurred in
the control HG sample, in which the matrix appeared more opaque than before the freeze-thaw treatment (Fig. 1). After freeze-thawing, the physical structure of the control gel seemed to be affected as the disk appeared flattened and more opaque when compared to the initial sample. This is likely the result of ice crystals puncturing the gelatin network during freezing, causing fracturing of the HG and loss of structural integrity once it was thawed.

The biphasic gels and control OGs did not appear to change after freeze-thawing as they preserved their visual appearance. Surprisingly, despite the presence of the different water phase ratios, biphasic gels did not show signs of phase separation or macroscopic structural damage after freeze-thawing.

3.2. DSC

DSC thermograms obtained for the biphasic gels are shown in Fig. 2. The change in enthalpy, and peak temperature are summarized in Table S2.

During heating, an endothermic peak corresponding to the melting of gelatin was observed around 35 °C ($T_{\text{gel}}$) which falls within the determined range in previous studies (Fig. 2A). For instance, pork gelatin has been observed to have a broad gel-sol transition peak ranging from 25 to 37 °C depending on the pH, gelatin gel strength (bloom), presence of salts, and concentration (Gornall & Terentjev, 2008; Osorio, Bilbao, Bustos, & Alvarez, 2007).

An exothermic transition occurred around 20 °C in the control HG and the biphasic gel samples. The change in enthalpy was similar to that of the gel-sol transition, and therefore it is hypothesized that upon heating, the polypeptides have enough kinetic energy to rearrange or relax to a more stable configuration ($T_{\text{rel}}$), releasing energy in the process. As expected, a sharp endothermic peak corresponding to the melting ($T_{m}$) of RBW from the OG phase was observed to be near 66 °C (Fig. 2A). No statistically significant differences in $T_{\text{gel}}$ and $T_{m}$ were observed among the prepared biphasic gels regardless of the OE:HG ratio used.

The crystallization temperatures for RBW were around 61 °C (Fig. 2B). These temperatures are in agreement with previous studies on RBW OGs (Dassanayake, Kodali, Ueno, & Sato, 2011). No significant differences were observed in $T_{\text{cry}}$ across all samples; nevertheless, a trend for $T_{\text{cry}}$ to slightly increase as the proportion of OG increased in the biphasic gels was detected. Saffold and Acevedo (2021) observed a similar trend and attributed the increase to larger OG droplet sizes, which leads to an increase in impurities within the droplets. These impurities can act as nucleation sites to promote faster crystallization, thereby raising crystallization temperature (Palanuwech & Coupland, 2003). Not surprisingly, $\Delta H_{\text{cry}}$ values significantly decreased with the reduction of the OG phase in biphasic gels, which is attributed to a dilution effect of RBW in the gel’s composition.

The gelation peaks for gelatin were difficult to detect, likely due to the rapid cooling during the DSC run, which may have hindered occurrence of this event during the run. In the biphasic gels, very broad peaks were observed, with $T_{gel}$ between 22 and 24 °C, which is in agreement with current literature (Gornall & Terentjev, 2008) (Fig. 2, Table S2). From the heating and cooling therograms, the absence of the formation of new peaks and consistent peaks corresponding to the HG and OG demonstrate that the biphasic gels are comprised of two independent semi-solid phases.

3.3. Rheology

3.3.1. Effect of OG:HG ratio

The storage modulus ($G'$), loss modulus ($G''$) and yield stress ($\sigma^*$) values of the controls (100:0, 0:100) and biphasic gels (40:60, 50:50, 60:40, 70:30) obtained from the LVR in amplitude sweeps are shown in Fig. 3. All gels, regardless of gelators concentration and OG:HG ratio showed $G' > G''$, indicating a more solid-like than viscous behavior.

The control OG showed the highest $G'$ and $G''$ of 1100 kPa and 276 kPa, respectively, but the lowest $\sigma^*$ value of 95 Pa (Fig. 3A–C). This suggests that OGs have a high mechanical strength, but easily yield upon application of stress beyond 95 Pa. They are irreversibly deformed at a low stress despite their rigid structure (Mezger, 2006). Wolfer et al. (2018) described OGs with 10% RBW as forming rigid gels, as was observed in this study. Saffold and Acevedo (2021) also observed similar rheological behavior with RBW OGs, in which OGs exhibited $G'$ of 1700 kPa at high frequencies (near 100 rad/s) and could not withstand higher frequencies without $G'$ and $G''$ crossing over. In contrast, gelatin alone forms flexible gels (Van Vlierbergh, Schacht, & Dubrueil, 2011) that do not irreversibly deform as readily as OGs. Under standard conditions, the 7% gelatin HGs exhibited a $\sigma^*$ approximately four times higher than that of the control OGs.

The difference in $\sigma^*$ is likely due to the different structuring methods of the two gels. For the OG studied here, RBW crystals, which are mainly composed of wax esters and other long chain hydrocarbons, must assemble to form a crystal network and entrap the oil (Marangoni & Garti, 2011). Most of the interaction between wax esters and the entrapped oil are weak, non-covalent interactions (van der Waals forces), which do not require much energy to disrupt (Deman & Beers, 1987). Gelatin, however, forms a 3D network through partial renaturation of gelatin molecules from the coil to helical form, with each of the gelatin molecules interacting with each other and neighboring water

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Fig. 2. Thermograms of heating (A) and cooling (B) control gels (100:0, 0:100) and biphasic gels (40:60, 50:50, 60:40, 70:30). Annotations indicate phase transitions for gelatin and RBW. All ratios are expressed as OG:HG.
molecules via hydrogen bonding and electrostatic interactions (Gornall & Terentjev, 2008; Klein & Poverenov, 2020). Gelatin-based HGs have stronger intermolecular interactions than the RBW-based OGs, accounting for the difference in σ* observed.

The biphasic gels are composed of varying ratios of OG and HG, so it was presumed that they would exhibit intermediate characteristics from each of the component gels. As the proportion of OG increased, G′ and G″ increased, indicating a stronger gel (Fig. 3). This is expected because OGs are more rigid than HGS and agrees with observations by Bolllom et al. (2019), Saffold and Acevedo (2021) and Lupi et al. (2016). In contrast, as the proportion of HG increased, the σ* of the biphasic gel increased. The σ* was the highest in the 40:60 formulation, with σ* = 406 Pa, while the 60:40 and 70:30 gels had approximately half that value (Fig. 3). The characteristic of a biphasic gel is therefore greatly dictated by the proportion of HG and OG, with the OG fraction enhancing G′ and HG fraction enhancing σ*.

As a result of combining both gels, the overall mechanical properties of biphasic gels are significantly enhanced compared to their independent components, as shown by the increase in both rheological parameters, G′ and σ*. This enhancement can be beneficial when formulating semi-solid fat replacers requiring structure and resilience to processing conditions.

3.3.2. Effect of freezing and thawing

The rheological properties of the freeze-thawed samples showed similar overall trends to those of the unfrozen samples, in which the control HG had the highest G′ and G″, whereas the control HG had the highest σ*.

Comparing these attributes before and after the freeze-thaw process, the control OG significantly decreased in G′ and G″ after freeze-thawing by 47% and 55%, respectively and σ* remained unaffected (P > 0.05). In contrast, the control HG did not show changes in G′ and G″, while σ* significantly decreased by 16% (Fig. 3).

After freeze-thawing, biphasic gels containing a higher OG proportion displayed greater G′ and G″, while gels with more HG displayed greater σ* (Fig. 3). Overall, regardless of the OG:HG ratio, the freeze-thaw treatment resulted in an increase of G′ and G″ and a decrease of σ* with respect to non-treated samples. Nevertheless, the effect of the freeze-thaw treatment on G′ and G″ was more pronounced in gels with more OG. Statistically significant differences in G′ and G″ were not observed for 40:60 and 50:50 gels, but were seen for 60:40 and 70:30 gels (Fig. 3). The 60:40 and 70:30 gels significantly increased in G′ by approximately 14% and 38%, respectively, after the freeze-thaw cycle, indicating a significant improvement of their mechanical strength. Yield stress was observed to significantly decrease only for 40:60 and 60:40 gels, by 18% and 20%, respectively (Fig. 3). These results suggest that the effect of freeze-thawing on structural weakening is, therefore, less significant in biphasic gels than in control HGs, in particular in biphasic gels with a higher OG fraction.

There are several factors that may contribute to these changes in rheological properties of the gels. Gelatin gels are known to have poor freeze-thaw stability due to formation of ice crystals upon freezing and disruption of the 3D protein network (Zhu, Ramaswamy, & Le Bail, 2005). This is shown by the 25% decrease in G′ and 16% decrease in σ* after freeze-thawing of the control HG, suggesting that the gel has weakened in structure and thus yields at a lower deformation stress. The variation in the degree of protein network disruption likely accounts for the large standard deviation in the freeze-thawed gels (Fig. 3). On the other hand, the OG decreased in G′ after freeze-thawing, which may be due to the re-crystallization of HOSO during freezing, causing some RBW crystals to rearrange. HOSO is reported to have a solidification range from −0.5 to −17 °C (Liu, 1997; Shahidi, 2005), and the storage temperatures in the current study (−20 °C) were lower than that temperature range. Upon thawing, the internal structure may have shifted due to the loss of crystallized HOSO, leading to a weakened gel.

In the biphasic gels, a similar phenomenon is expected to occur, but to a smaller extent due to the presence of both gel components. The OG portion in these samples may have prevented some ice crystals from penetrating the gelatin network. These changes effectively enhanced the freeze-thaw stability of the biphasic gels. Another factor that may have affected the rheological parameters is the loss of water and oil from the samples due to the freeze-thaw process, which is discussed in section 3.4.2.

3.4. Water and oil loss determination

3.4.1. Effect of OG:HG ratio

The average total liquid loss for all biphasic gels were equal to or less than that of the control HG (0:100) and OG (100:0), which poses as evidence the ability of the biphasic systems to entrap the liquid more efficiently than the individual monophasic gels (Table 1). The control OG and HG lost the most total liquid (Table 1). There was no statistically significant difference (P > 0.05) in oil and total liquid lost among all biphasic gels, indicating that most of the difference in liquid loss was from water. As expected, the higher the proportion of OG or HG in the system, the higher the amount of oil or water lost from the samples, respectively. To analyze the matrix capability to retain water or oil within the network, and given that biphasic gels have different initial amounts of water and oil based on the OG:HG ratio, water and oil loss values were normalized based on the total amount of water and oil in the gels, respectively (Table 1).

Based on the normalized water loss values, biphasic gels with relatively low HG proportion (50:50, 60:40, and 70:30) showed 3–4% less ability to retain water than the control HG (Table 1). One hypothesis for this slight increase in water loss with a large OG fraction is the wider spread of HG throughout the biphasic matrix. As will be discussed in section 3.5, all biphasic gels appeared to have an HG dominant matrix. With increasing OG fraction, there is decreasing HG, yet for the matrix to be HG dominant suggests the HG is more spread out. This may have reduced the entrapment of water in the gelatin matrix, leading to greater water lost.
Table 1

Mean values (± standard deviation) for water loss (%), oil loss (%), and total liquid loss (%) of oleogel and hydrogel control and biphasic gel samples under standard conditions (not freeze-thawed; SC) and after one freeze-thaw (FT) cycle.

### SC-Biphasic Gel

<table>
<thead>
<tr>
<th>OG:HG</th>
<th>Water loss (%)</th>
<th>Normalized* water loss (%)</th>
<th>Oil loss (%)</th>
<th>Normalized* oil loss (%)</th>
<th>Total liquid loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:100</td>
<td>13.28 ± 2.65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.28 ± 2.85&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>13.28 ± 2.65&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>40:60</td>
<td>7.85 ± 0.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.81 ± 0.55&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.03 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.10 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.88 ± 0.29&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>50:50</td>
<td>7.63 ± 0.55&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>17.75 ± 1.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>7.63 ± 0.55&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>60:40</td>
<td>5.96 ± 0.53&lt;sup&gt;c&lt;/sup&gt;</td>
<td>18.06 ± 1.66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.31 ± 0.12&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.59 ± 0.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.27 ± 0.48&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>70:30</td>
<td>4.23 ± 0.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.40 ± 2.92&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.20 ± 0.83&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.92 ± 1.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.43 ± 0.34&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>100:0</td>
<td>ND</td>
<td>ND</td>
<td>16.83 ± 3.97&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>18.20 ± 4.30&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>16.83 ± 3.97&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

### FT-Biphasic Gel

<table>
<thead>
<tr>
<th>OG:HG</th>
<th>Water loss (%)</th>
<th>Normalized* water loss (%)</th>
<th>Oil loss (%)</th>
<th>Normalized* oil loss (%)</th>
<th>Total liquid loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:100</td>
<td>16.70 ± 1.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.95 ± 1.08&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>16.70 ± 1.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>40:60</td>
<td>8.25 ± 0.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.56 ± 0.90&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>8.25 ± 0.53&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>50:50</td>
<td>6.63 ± 0.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.43 ± 1.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>6.63 ± 0.49&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>60:40</td>
<td>6.27 ± 0.56&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.01 ± 1.10&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.05 ± 0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.10 ± 0.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.33 ± 0.34&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>70:30</td>
<td>4.61 ± 0.70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.04 ± 3.04&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.62 ± 0.58&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.19 ± 0.92&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.23 ± 0.43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>100:0</td>
<td>ND</td>
<td>ND</td>
<td>7.45 ± 1.50&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>8.06 ± 1.62&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.45 ± 1.50&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*<sup>A,B,C</sup> Within the same condition (standard or freeze-thaw), means within the same columns with different superscripts are significantly different (P < 0.05).

*<sup>a,b</sup> Within the same OG-HG but different conditions, means within the same columns with different superscripts are significantly different (P < 0.05).

ND = not detected (x < 0.01 g).

All ratios are expressed as OG:HG. N = 9.

* Normalized values are adjusted by taking the raw water or oil loss values over the expected initial mass of water or oil based on the OG:HG ratio.

Although there was no statistically significant difference in normalized oil loss among biphasic gels, there was a general, non-linear trend that gels containing more OG lost more oil (Table 1). The absence and minimal oil loss in the 40:60, 50:50, and 60:40 biphasic gels may be due to better oil retention because of more gelatin available for non-covalent interactions with HOSO. Another possible explanation is the HG mesh physically limiting oil movement, which is discussed further in section 3.5. As mentioned previously, relative to the control OG and HG, all biphasic gels showed significantly lower total liquid loss, illustrating the improved liquid binding capacity of biphasic systems.

### 3.4.2 Effect of freezing and thawing

As discussed previously, control OG discs and biphasic gel discs did not have discernible differences in appearance before and after freeze-thawing (Fig. 1). Control HG discs did change in appearance after freeze-thawing, turning from a clear, transparent gel to an opaque, shriveled gel (Fig. 1). As mentioned in section 3.3.2, the shrunken appearance of the HG is likely caused by the disruption of the gelatin network from ice crystals and water loss.

Water loss in the control HG was significantly higher (P < 0.05) after freeze-thawing, which was expected since this gel contained only gelatin HG (Tables 1 and 2).

Interestingly, oil loss for the control OG decreased by almost 50% after freeze-thawing. From the amplitude sweeps of the OG, all rheological attributes were observed to decrease (section 3.3), suggesting a weakened gel structure; therefore, an increase in oil loss was expected. This contradictory result may be due to modifications in the RBW crystal arrangement during freezing. As explained in section 3.3.2, OGs were frozen at –20 °C and it is possible that HOSO crystalized and shifted the arrangement of the RBW crystals such that the crystal structure became more porous, thus less oil was lost in the observed time frame. Blake, Co, and Marangoni (2014) observed that wax-based OGs retained more of the fast-leaking oil when porosity increased, since there is more “void-space” for the oil to fill. A more porous structure, however, can lead to a decrease in mechanical strength of the gel structure (de Vries, Lopez, Gomez, van der Linden, & Scholten, 2017).

No significant differences in water loss were observed for 40:60, 60:40, and 70:30 gels after freeze-thawing relative to the non-freeze-thawed gels (Table 1). Only the 50:50 gel showed a significant but slight decrease in water loss of around 3% (P < 0.05). It is hypothesized the decrease in water loss only for the 50:50 gel may be due to the equal ratio of OG and HG synergistically acting to retain each other. The gelatin contains hydrophobic amino acids that may be interacting with the RBW and HOSO, while the RBW crystal matrix may be containing some of the free water through hydrophobic interactions within the gel. It was noted in section 3.3.2 that the 50:50 gel did not show any significant changes in rheological properties after freeze-thaw. This observation in reduced water loss also suggests the 50:50 gel’s relative stability against the freeze-thaw process.

Only the 70:30 gels showed a significant increase in oil loss by approximately 2 times after freeze-thawing (P < 0.05). From preliminary work, it was found that 70:30 was the highest ratio of OG that could be used to form a uniform and stable biphasic gel, suggesting that beyond this OG:HG ratio, there may not have been an adequate concentration of gelatin to favorably interact with the HOSO and RBW through hydrophobic amino acids. If the 70:30 ratio is the threshold of OG for the system in this study, then an increase in oil loss may be due to this gel’s greater sensitivity to gelatin structure loss after freeze-thawing compared to the other gels with a higher HG ratio. Given that not all of the oil in gelatin gels are tightly bound to the protein (Lloyd & Moran, 1933), the frozen water expands as the ice crystals grow. Due to the higher ratio of OG than HG, some ice crystals may have penetrated and shifted parts of the RBW crystal network. Upon thawing, ice contracts back to water, and the shifts in the RBW crystal network may have loosened some of the HOSO held within the network, leading to increased oil loss.

Despite the increase in oil loss, freeze-thawed 70:30 gels significantly increased in G’ and G” (section 3.3.2). One explanation is that the loss of oil effectively increased the concentration of RBW crystals in the biphasic gel, since losing oil means a reduction in the solvent. Localized increase in RBW crystals may account for the apparent increase in G’ and G” after the freeze thaw process, as higher concentrations of RBW have been observed to increase gel strength (Dassanayake et al., 2011). A significant increase in oil loss may not have been observed for the other gels due to an abundance of the remaining gelatin network to physically hinder flow of any free HOSO. Further studies on the dynamic changes of the gel’s internal structure during freezing and thawing are needed to confirm the causes of these observed differences.
3.5. CLSM

3.5.1. Effect of OG:HG ratio

For all samples, the distribution of the HG did not change notably across ratios, and appeared to be the continuous phase surrounding the OG droplets and globules throughout the matrix (Fig. 4). This suggests that all biphasic gels were OG-in-HG type systems, even if the fraction of OG was greater than the HG. Park, Jimenez-Flores, and Maleky (2020) developed various biphasic systems with RBW OG and whey protein HG, but observed an inverse microstructure with the HG being surrounded by OG when OG was >50% of the total gel composition. Whey protein is mostly comprised of β-lactoglobulin, which contains mainly hydrophobic side chains to construct the globular structure (Dissanayake & Vasilevic, 2009). In contrast, gelatin is more hydrophilic and exists as long coils or helices (Alihosseini, 2016; Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011). Additionally, the re-natured structure of the two proteins after thermal and high shear treatment also differ. Gelatin is known to form a partially ordered helical structure after cooling from a sol form (Gornall & Terentjev, 2008); whereas whey protein, which consists of mostly β-lactoglobulin and α-lactalbumin, form a compact network of aggregate particles (Boye, Kalab, Alli, & Yung Ma, 2000). These structural differences in the protein may have allowed the whey protein to stabilize a HG-in-OG state, whereas the gelatin stabilized an OG-in-HG state.

The size and shape of the OG globules changed as the ratio of OG increased. As the proportion of OG increased from 40:60 to 70:30, the dispersed OG globules appeared to increase in size and distort away from a spherical structure (Fig. 4). In addition to the role of HG described in section 3.3, the smaller OG droplets may factor into the improved ε° of biphasic gels with less OG. Singh et al. (2014) noted that smaller droplet sizes enables tighter packing of the droplets, leading to increased stability of the entire system. The 70:30 gel had the largest OG globules, and this may be caused by more collisions of OG droplets and a resulting increase in partial coalescence. Partial coalescence is thought to build a crystal network by linking many fat droplets together, thus increasing the mechanical strength (Palanuwech & Coupland, 2003). As described in section 3.3, 70:30 gels were observed to have a higher G’ than 40:60 gels, presumably due to increased association between OG droplets.

For all biphasic gels, there appears to be minimal or no space between the OG droplets and the HG continuous phase, suggesting that the two phases are physically close to each other. Saffold and Acevedo (2021), who investigated a similar system at different ratios and concentration of gelators, stated that the two phases were independent from each other due to the lack of major peak shifts and formation of new peaks by FTIR, suggesting no new covalent bonds were formed; however, there is the possibility that non-covalent interactions facilitate the formation of a stable biphasic system, in particular during the sol stage when neither phases have solidified.

Non-covalent bonds such as hydrogen bonds, are reflected on FTIR spectra as peak shifts, which can vary in magnitude depending on the extent the X-H bond is disrupted (Behera & Das, 2018; Ngo, Liu, Chen, Kaya, & Zimudzi, 2020). Broad O-H and N-H peaks and overlapping peaks make it challenging to discern peak shifts as any changes would be covered up by other peaks. It is hypothesized that electrostatic effects, such as H-bonding and hydrophobic interactions, are facilitating biphasic gel formation during the sol stages of gel preparation. Considering that gelatin is a protein with hydrophobic amino acid groups, such as glycine, alanine, and lysine (Kariduraganavar, Kittur, & Kamble, 2014), the heating and high shear process may expose some of these amino acids to the OG for favorable hydrophobic interactions. This added stability may be extending the time the two phases remain emulsified, long enough for the gelators to set and form a network. Once the two phases are gelled, they physically prevent each other from separating, creating a kinetically stable system (Zheng et al., 2020).

At a high OG ratio, most, if not all, possible sites of interaction between the gelatin and the OG droplets are saturated; thus the OG droplets more readily coalesce to minimize the interfacial tension. At a low OG ratio, such as 40:60, there are sufficient interaction sites between the gelatin and OG droplet, such that the gelatin acts as a surfactant to effectively reduce interfacial tension and prevent phase separation.

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Fig. 4. Representative micrographs of all the formulated biphasic gels. The hydrogel is dyed with FITC and fluoresces green, while the oleogel is dyed with Nile Red and fluoresces red. All ratios are expressed as OG:HG. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
3.5.2. Effect of freezing and thawing

Freezing and thawing of the biphasic gels caused an increase in air cell size likely due to fracturing of the gelatin matrix, as shown by the presence of more dark areas in the CLSM micrographs (Fig. 4). Furthermore, the OG globules increased in size for all freeze-thawed gels. This effect is most noticeable in the 70:30 gel, in which relatively larger OG globules can be observed in the periphery of the micrograph (Fig. 4). These changes likely arose from ice crystals forming in the HG. During freezing, water freezes into ice, which takes up more volume than the aqueous state. Furthermore, the ice crystals can penetrate the 3D matrix of the gelatin and HG-OG interfaces. Upon thawing, the water reverts back to its denser liquid state, leaving new or expanded air cells in the gel matrix. The disrupted gelatin network is less effective at holding the OG in place, thus some free oil can migrate into air cells or coalesce with other oil droplets. Compared to the 70:30 gel, the 40:60, 50:50, and 60:40 gels may have had a high enough proportion of HG to physically restrict bulk oil movement, thus prevented the formation of very large oil globules. This is also supported by the lower oil loss in the 40:60, 50:50, and 60:40 freeze-thawed gels than 70:30 gels (section 3.4). Increasing the concentration of gelatin in the HG and increasing the freezing rate may improve the freeze-thaw stability of the biphasic gels by limiting ice crystal growth and enhancing protein-lipid interactions (Cao, Chen, Cui, & Foster, 2003; Wee, Yusoff, Lin, & Xu, 2017).

3.6. Peroxide value

The changes in PV as a function of storage time for the OG and biphasic gels are shown in Fig. 5. Visual inspection of the samples showed no phase separation in any of the biphasic gels during storage time, and the OG did not show any physical changes nor changes in color perceived by the naked eye (Fig. 5, inset).

Despite the addition of prooxidant, PV of all samples did not exceed 3 meq/kg (Fig. 5), which is considerably lower than the generally accepted threshold of 10 meq/kg for rancid oil (Kirk, Sawyer, & Egan, 1991). All PV for all samples fluctuated between 0.17 and 2.62 meq/kg. Relative to 40:60NP, biphasic gels and control OG containing prooxidants overall did not show significant differences in PV from day 0 to day 175; only slight fluctuations but with no practical impact were observed in samples with high HG proportion. The PV for all samples was expected to initially increase and then decrease as time progressed because most of the lipid hydroperoxides and free radicals would be expected to initially increase and then decrease as time progressed because most of the lipid hydroperoxides and free radicals would be expected to initially increase and then decrease as time progressed because most of the lipid hydroperoxides and free radicals would eventually be quenched through the formation of secondary oxidation products (Domínguez et al., 2019); however, gels did not show such a trend and maintained PVs well below 10 meq/kg, demonstrating the oxidation stability of systems where lipid structuring strategies were used under storage at 22 ºC. This is in agreement with da Silva et al. (2018) and Hwang, Phaner, Winkler-Moser, and Liu (2018).

OGs have been observed to have slow lipid oxidation rates, presumably due to the action of gelators structuring the oil and enforcing kinetic stability, preventing rapid migration of free radicals to susceptible double bonds (da Silva et al., 2018; Hwang, 2020; Lim, Jeong, Oh, & Lee, 2017; Tian & Acevedo, 2018; Zhuang et al., 2021). The gelatin in the HG likely prevented water mobility in the system by directly H-bonding to water molecules and indirectly entrapping water molecules in the 3D gelatin matrix, thereby limiting the movement of reactive radical species (Barden & Decker, 2016; Cengiz, Schroen, & Bertoni-Carabin, 2019; Chaijan, Cheong, & Panpipat, 2021; Karel, 1980). It should be noted that in this study, despite the biphasic gels containing 1 mg Cu²⁺/kg oil as prooxidant, the PV remained relatively low, thus demonstrating the oxidative stability of the biphasic system without the need for antioxidants.

4. Conclusion

This study demonstrated the oxidative stability after 6 months of storage and freeze-thaw stability of RBW-gelatin biphasic gels containing a high proportion of OG (>50%). Biphasic gels have the advantage of displaying superior structural and mechanical properties when compared to both control OG and HG, as demonstrated by their improved σt and reduced water and oil loss after freeze-thawing. Based on observations of the biphasic gel’s microstructure (OG-in-HG), freeze-thaw stability was enhanced presumably by physically limiting oil migration, thus creating more stable gels than the mono-component gels. Lipid oxidation of the biphasic gels was stable and minimal over a long storage period despite the presence of water from the HG. For future research, replacement of semi-solid fat with 60:40 and 70:30 biphasic gels in food products that need particulate fat appearance and mouthfeel, such as coarse-ground sausages and burger patties, may elucidate the performance of these gels in a food matrix. Biphasic gels may therefore be suitable semi-solid fat replacers for products requiring low temperature processing or a long shelf life.

CRediT authorship contribution statement

Karin Cho: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft.
Rodrigo Tartè: Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Reviewing & editing.
Nuria C. Acevedo: Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Reviewing & editing.

Declarations of competing interest

None.

Data availability

Data will be made available on request.

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