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Effects of tannic acid on proteins and fat in cream

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Abstracts

The addition of polyphenols to ice cream mix has been shown to cause a decrease in melting rate of that ice cream, although the mechanisms of this effect are not well understood. To better understand this phenomenon, the objective of this study was to understand the effects of a polyphenol, tannic acid (TA), on dairy cream. TA was added to cream at 0.75%, 1.5% and 3% (wt/wt) concentrations. An increase in complex viscosity was seen as TA% increased. The effect of pH on protein aggregation was minimal since the pH values of the resulting TA-cream samples were generally above the isoelectric point of milk proteins. Microscope images and particle size distributions confirmed the presence of fat globule clusters in these samples, creating a network at 3% TA. Using sodium dodecyl sulfate (SDS) and ethylenediaminetetraacetic acid (EDTA) to disperse both fat and protein, average particle size and microscope images suggested that milk protein-TA interactions helped create the clusters. No evidence of partial coalescence was observed. These findings can help optimize the complex effect of polyphenols in the dairy matrix to create higher quality products.

Keywords Cream, Tannic acid, Gelation, Protein aggregation

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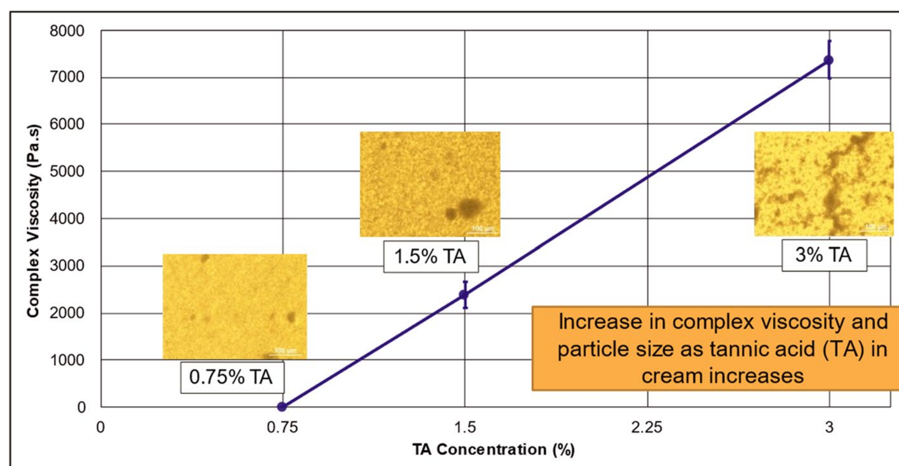
Graphical Abstract



Introduction

Polyphenols are known to interact with the components of various dairy systems, such as ice cream. Many studies have added polyphenol sources to ice cream in order to influence color (Kavaz et al. 2016; Yuksel 2015), nutritive value (Goraya & Bajwa 2015), and/or sensory characteristics (Çakmakçı et al. 2016; Salem et al. 2014). Many of these studies have also investigated various physicochemical attributes such as change in rheology of the ice cream and mix (Karaman & Kayacier 2012; Sun-Waterhouse et al. 2013; Tsevdou et al. 2019) and a decrease in the melting rate (Gabbi et al. 2018; Hwang et al. 2009; Karaman et al. 2014; Yazdi et al. 2020). However, these studies have used impure polyphenol sources that contain additional water or fiber, which can confound the effect of polyphenols on the system.

Others have capitalized on the ability of polyphenols to decrease the melting rate of ice cream. Strawberry extract, with naturally present polyphenols, was used to create a "no melt" ice cream (Ota 2011). This product is more resistant to melting and shape deformation for extended amounts of time at elevated temperatures (Bilbao-Sainz et al. 2019). Press releases share that the accidental ideation of this ice cream was sparked by observing dairy cream solidifying in the presence of a strawberry extract. This led our group to also add polyphenol extracts to cream, and a solidified, gelled cream product was created. When these samples were put under a microscope, an extensive network of particles was observed. These observations informed the hypothesis that polyphenols are affecting the two major components in the cream, the fat and/or protein.

Polyphenols can complex with various proteins, including both classes of milk proteins, casein and whey. These reactions are mostly facilitated by noncovalent hydrophobic or hydrogen bonding (Yildirim-Elikoglu & Erdem 2018). The proline content of casein (Arts et al. 2002) and the pH-dependent configurations of whey proteins (Riihimäki et al. 2008) are some of the primary drivers of these interactions. Proline-rich salivary proteins and TA also react through three stages of binding described by Charlton et al. (2002). The first stage includes the binding of a single, soluble peptide molecule with multiple polyphenols at the available sites. The second stage requires the presence of another polyphenol-coated peptide and a weak intermolecular interaction to create a new, insoluble complex. The last stage is the spontaneous aggregation of these insoluble complexes to create larger aggregates. The formation of these large aggregates was also observed by Han et al. (2011) in β-casein and tannic acid samples.

Protein–polyphenol complexes can have utility in colloidal systems that contain fat globules. Tea catechins have been found to complex with proline-rich sodium caseinate at the soybean/water interface (Sabouri et al. 2015). Sunflower protein–polyphenol complexes were also found at the sunflower oil–water interface. It was hypothesized that both protein–polyphenol and polyphenol-polyphenol interactions were synthesized through hydrogen bonding, which created bridges between complexes and improved emulsion stability (Karefyllakis et al. 2017). These polyphenol-protein interactions at the interface can also cause the clustering of fat globules. Rashidinejad et al. (2016) showed that green tea catechins interacted with milk fat globule membrane domains. In a

whey protein isolate (WPI)-stabilized sunflower oil emulsion, the fat globule clusters apparently increased in size with the increase in polyphenol-rich grape seed extract (Fuhrmann et al. 2019). Some have proposed that these fat globule clusters are a result of the partial coalescence of fat globules, something that is typically observed in ice cream (Goff & Hartel 2013).

Partial coalescence or fat destabilization occurs when two or more fat droplets collide but do not reach full coalescence due to the presence of internal crystalline fat or interfacial particles on the droplet surface (Thiel et al. 2016). Hwang et al. (2009) found that the addition of grape wine lees, a wine-making waste product high in polyphenols, to ice cream increased the fat destabilization index and fat globule particle size. Naeem et al. (2019) also observed a significant increase in fat destabilization for ice cream fortified with polyphenol-rich golden berry juice. Pundhir et al. (2018) hypothesized this correlation caused a destabilized fat structure that may "hold melted water", which led to a slower melting rate for ice cream with added polyphenol-rich fine wine lees. Some hypothesize that this increase in partial coalescence may be a contributing factor to the observed decrease in melting rate for these fortified ice cream systems (Bilbao-Sainz et al. 2019). The displacement of proteins at the oil–water interface can be a precursor of fat destabilization. Cui et al. (2014) found that the addition of rutin, a flavonoid, could create protein/polyphenol complexes, decrease the interfacial tension, and competitively displace native soy protein at the soybean oil /water interface. While many articles confirm a decrease in the melting rate of ice cream in the presence of polyphenol sources, the mechanism behind the polyphenol interactions is still unknown (Gabbi et al. 2018; Hwang et al. 2009; Karaman et al. 2014; Yazdi et al. 2020).

The objective of this study is to investigate the mechanisms that drive gelation in cream due to the presence of a polyphenol, tannic acid (TA). The hypothesis is that this resulting gelation is facilitated through either partial coalescence or protein-mediated fat aggregation, or both. Since polyphenols can also interact with many carbohydrates and hydrocolloids that are present in more complex systems, like an ice cream mix (Mateus et al. 2004), a simple cream and polyphenol system was utilized to reduce potential confounding variables. TA, a hydrolysable tannin, was chosen due to its purity, easy procurement, and known interactions with cream.

Materials and methods

Material

Pasteurized dairy cream (~37% fat) was supplied by Sassy Cow Creamery (Columbus, WI, USA). Tannic acid (TA) (98% purity) was supplied by Cayman Chemical (Ann Arbor, MI, USA). Sodium dodecyl sulfate (SDS) and HCl was supplied by Sigma Aldrich, Inc. (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA) was supplied by IBI Scientific (Peosta, IA, USA). Citric Acid was supplied by Tate and Lyle (London, UK). Deionized (D.I.) water was used throughout all experiments.

Methods

Sample preparation

TA was dissolved into D.I. water to create 7.5%, 15%, and 30% (wt/wt) solutions. TA solutions were then loaded into a syringe. Cream was stirred with paddle attachment at 180 rpm and the appropriate amount of TA solution was injected evenly. The samples were mixed for an additional 20 s to ensure homogeneity. The final TA concentrations of the samples were 0.75%, 1.5%, 3% (wt/wt) based on addition of the 7.5%, 15% and 30% TA solutions, respectively. The same amount of fluid was added to ensure similar dilutions regardless of TA level. The samples were stored at 4 °C for 24 h prior to analysis. Each sample was made in triplicate.

Rheology

A DHR-2 rheometer (TA instruments, Delaware, USA) with parallel plate geometry (dia. 40 mm) was used to measure the complex viscosity of the cream/TA samples. Plain cream (0% TA) was not measured due to its inability to produce consistent, sinusoidal torque and displacement waveforms. This sample may have been out of the range of this geometry. The 0.75% TA sample, which was a viscous liquid, was deposited on the plate using a 1 mL syringe. Small disks of the thicker, gelled, 1.5% and 3% (wt/wt) TA samples were cut from a cylindrical mold and placed in the center of the Peltier plate for analysis. The samples were measured at 4 °C using a recirculating chiller (Thermos Cube, Solid State Cooling System, Wappingers Falls, NY, USA) connected to the bottom plate. The geometry was moved vertically to reach the geometry gap (1 mm) and equilibrated at 4 °C for 90 s. An amplitude sweep test to confirm the linear viscoelastic region (LVR) was performed at a strain range of 0.01–3% and 1 Hz frequency. After the strain (0.019%) was properly adjusted for each sample, they were oscillated for 60 s and the complex viscosity was measured in triplicate.

pH

pH was measured with a FiveEasy Plus pH/mV meter with InLab® Viscous Pro-ISM probe (Mettler Toledo, Hampton, Schwerzenbach, Switzerland). The electrode was calibrated at pH four and seven. Samples were held at ~25 °C for 1 h before measuring pH in triplicate. To test the effect of pH on cream, solutions of citric acid and HCl at pH 3 and 3.5 were made and then added to cream

at the same concentration as the tannic acid solutions. After the samples were stored at 4 °C for 24 h, their pH was measured using the parameters above. Each sample was made and measured in triplicate.

Particle size distribution

Particle size distributions of cream/TA samples (1/10 dilution) were measured in triplicate using laser light scattering (Malvern Mastersizer 2000, Malvern Instruments Ltd., Worcestershire, UK). A refractive index of milkfat, 1.46, was used for these measurements. D.I. water was used as the dispersant with a refractive index of 1.33. Diluted samples were added dropwise into the attached Hydro 2000S liquid sampler until a laser obscuration value of 13–15% was reached. Mean particle size distributions and $d_{4,3}$, the weight-average mean diameters of the particles, were calculated by the Mastersizer.

Optical light microscopy

Cream/TA samples were lightly mixed with D.I. water to create a 1:10 dilution. One drop of diluted sample was placed on a glass slide and covered with a cover slip. Samples were imaged at 200 × magnification with a Nikon Eclipse FN1 optical microscope (Nikon Instruments Inc., Melville, NY, USA) and a Nikon Digital Sight DS-U3 camera control unit attached (ver. 1). NIS-Elements D Imaging Software (ver. 4) was used to capture images.

SDS and EDTA treatments

The preparation method as described in Méndez-Velasco and Goff (2012) was used to differentiate between the presence of partial coalescence or protein-mediated aggregation in these systems. A SDS solution (4% wt/wt) was prepared using D.I. water, and a Na_2H_2EDTA solution (3.7% wt/wt) was prepared using 0.1 M phosphate buffer (pH 6.8) as a solvent. The cream/TA samples were diluted at 1:1 ratio with each solution separately, then compared with a sample diluted with only D.I. water. Treated samples were mixed lightly for 2 min, then stored at 4 °C for 2 h. Optical microscopy and particle size measurements were conducted in triplicate for each treated sample.

Statistical analysis

Analysis of variance (one-way ANOVA) and Tukey's honest significant difference (HSD) test were used to compare means of the data taken. Analysis was performed using JMP® Pro version 15.0.0 (SAS Institute Inc., Cary, NC, USA). The level of significance was determined at $p < 0.05$.

Results

Characteristics of adding TA to cream

Rheology

Visually, the addition of TA solutions to cream caused an increase in viscosity, leading to gelation at higher TA addition levels. At 0.75%, the sample resembled a slightly thickened cream. The 1.5% and 3% (wt/wt) samples were significantly more viscous and had gelled. These gels were sufficiently solid that, when inverted, no sample fell out of the cup. The 3% sample had a cuttable texture, which differed from the other samples. Although the viscosity change was almost instantaneous upon TA addition, samples were analyzed quantitatively after 24 h at 4 °C.

Complex viscosity is the total resistance to flow of a viscous liquid (Dimitreli & Thomareis 2008). Figure 1 shows that as the TA% increased, the complex viscosity increased, which correlates with the visual observations. Note that no reading was possible for pure cream since it was too thin (low viscosity) for this analysis. Only the

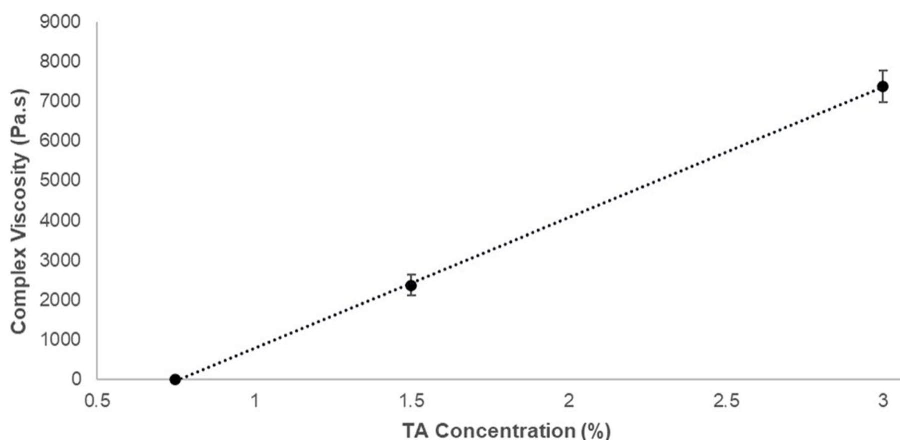


Fig. 1 Effect of tannic acid (TA) concentration on the complex viscosity of cream. The error bars represent standard error between sample and measurement replicates. ($n = 9$, 3 experimental replicates with 3 analytical replicates for each sample) (R value = 0.9998)

3% TA sample was significantly different from the other samples ($P < 0.05$).

Other studies have observed a similar increase in viscosity of ice cream mixes as a phenolic source was introduced. However, since a pure polyphenol was not used in many of those studies, the increase in viscosity might have been attributed to pectin and/or fiber in the dried or pureed fruit source (Bilbao-Sainz et al. 2019; Erkaya et al. 2012; Ürkek et al. 2019). In some cases, a decrease in viscosity was observed because of the additional water content of the fruit purees added (Karaman et al. 2014; Topdaş et al. 2017). Since this study used a pure polyphenol source without any other confounding components, it is possible to attribute this viscosity change to the presence of the polyphenols and their impact on the fat and protein found in cream. However, the role of pH change upon TA addition must be understood, especially with the possibility of protein precipitation at or around an isoelectric point of dairy proteins.

The role of pH on cream/TA samples

To understand the mechanism behind the gelation of these samples, the role of pH cannot be overlooked. The pH of the regular dairy cream was 6.67. At the isoelectric points (pI) of casein (pH 4.6) and whey proteins (pH ~ 5.5), protein coagulation occurs as the surface charges are reduced (Abugoch 2009). The pH of the TA solutions (prior to their addition to cream) were around 3.39–3.43. Table 1 lists the pH of the final cream/TA samples at each concentration. While the TA solutions were acidic, the resulting cream samples at 0.75% and 1.5% (wt/wt) TA remained above the pI for both proteins. The 3% (wt/wt) cream/TA sample had a pH below the pI of whey, which might suggest that whey protein coagulation had some potential impact on the sample's structure at this high TA concentration. However, since 1.5% and 3% samples both created a gelled product, a pH below the isoelectric point was not required for gelation. Diaz et al. (2020) also studied the role of pH while investigating the effect of polyphenol-rich berry juices on WPI solutions. For their control, they created imitation juices that contained comparable pH and sugar contents with no polyphenols. The berry juice caused more whey protein aggregates than the imitation juice, indicating the importance of polyphenols on the system beyond the pH they impart. Harbourne et al. (2011) observed that while tannic acid did not significantly change the pH of the system, it did cause significantly faster gelation times in an acid milk gel system. This was attributed to the effect that tannic acid has on proteins. These results suggest that, while pH does affect the overall environment of the sample, it was not the main driver of the viscosity and texture changes observed at these TA concentrations.

Table 1 The effect of tannic acid addition on the pH of resulting cream samples, as well as the addition of HCl and citric acid solutions at pH 3 and 3.5

Cream Sample	pH (Average ± SE)
0.75% TA	6.08 ± 0.05^{b}
1.5% TA	5.75 ± 0.03^{c}
3.0% TA	5.18 ± 0.03^{d}
pH 3 HCl solution	6.70 ± 0.02^{a}
pH 3.5 HCl solution	6.75 ± 0.02^{a}
pH 3 Citric acid solution	3.78 ± 0.02^{f}
pH 3.5 Citric acid solution	4.21 ± 0.06^{e}

Different subscript letters represent statistically different results ($p < 0.05$) ($n = 9$, 3 experimental replicates with 3 analytical replicates for each sample)

To test the effects of pH versus polyphenols on cream, solutions of HCl or citric acid were added (Table 1). The pH values of both acid solutions (pH 3 and 3.5) were chosen to mimic the pH range of the TA solutions. Viscosity data was assessed visually for these systems. The objective was to observe if the TA gelled cream product could be replicated using a solution at a similar pH (without any polyphenols present) at a constant weight added and preparation. When both HCl solutions were added to cream, there was little to no effect on the pH and the resulting viscosity did not change (similar to pure cream viscosity). There was a greater decrease in pH and increase in viscosity for the citric acid samples. Despite this sharper pH decrease, these samples were still semi-fluid, with a loose texture and did not create an invertible gel like the 1.5% and 3% (wt/wt) TA samples. Further, the citric acid samples did not cause an instantaneous viscosity change, as observed with TA addition, the effect required longer time. Lastly, another visual test was conducted that included adding citric acid into cream until a final pH of 5.20 was reached (similar to the final pH of 3% TA sample), and still a gelled product was not created despite pH below pI of some whey proteins. These experiments highlight that there are factors beyond pH that caused gelation of the cream/TA system at 1.5 and 3% (wt/wt) addition. While the role of pH cannot be completely disregarded, especially for the 3% TA sample, these results document that pH is not the primary mechanism in the viscosity changes observed in Fig. 1.

Microstructure of Cream/TA samples

The fat globule particle size of these samples was analyzed to gain deeper insight into the structure of the cream/TA systems. Figure 2 shows how the particle size of cream changed with the addition of TA. Regular cream had a

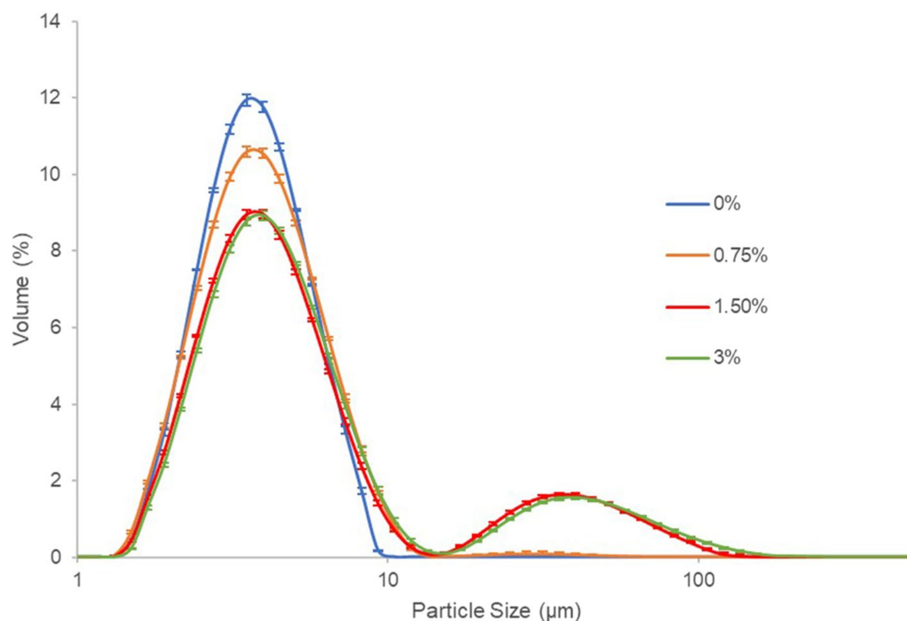


Fig. 2 Particle size distribution of cream at increasing concentrations of tannic acid, diluted 1:1 with D.I. water. The error bars represent standard error between sample and measurement replicates. ($n=9$, 3 experimental replicates with 3 analytical replicates for each sample)

singular peak around 5 µm, representative of the original emulsion droplets. As the concentration of polyphenols increased, a bimodal distribution was created with a secondary peak formed near the 50 µm range. The intensity of the first peak decreased with added TA because of the depletion of individual globules to create larger aggregates (Note, aggregation and clustering are used interchangeably in this work when describing a grouping of fat globules held together by various interactions).

To better understand this shift to larger particles, microscope images were taken of each sample (Fig. 3). The samples with added TA showed visible aggregates of fat globules (Fig. 3B-D), although some individual fat globules remained, but with decreasing amount as TA increased. These aggregates began to form a gel network at 1.5% and 3% (wt/wt) TA. Average particle size and microscope images together suggest that, as TA levels increased, the presence and size of the fat globule clusters increased.

To validate the importance of milkfat in this observed cream gelation and fat clustering, two tests were conducted. First, TA was added to skim milk at the same TA concentrations (0.75%,1.5%, 3% wt/wt), using the same procedure. No visual viscosity change was observed. Second, diluted samples similar to those shown in Fig. 3 were heated on the microscope (data shown later). Upon warming to 55 °C, melted fat globules were clearly observed, along with coalescence of some droplets in the same proximity. Clearly, both protein and fat play important roles in the formation of these aggregates.

While these experiments highlight the appearance of fat clustering, they do not confirm the mechanism of aggregation. Due to the composition of the cream system, it might be expected that clustering is driven by either fat aggregation mediated by protein interactions or by partial coalescence of the fat globules, or some combination of the two mechanisms. These expectations are informed by the likely presence of protein–polyphenol complexes and solid fat in this system, as discussed above. Protein-TA complexes could potentially act as a bridge between fat globules, as they have an affinity to the fat globule interface and can connect with other protein–polyphenol complexes. Polyphenols can potentially impact the fat globule interface through displacing proteins and influencing other surface-active properties. Based on the partially-crystalline milkfat, these changes could allow the fat globules to be more susceptible to partial coalescence as a method of fat clustering. Further analysis is needed to help understand these complicated interactions.

Protein-mediated fat aggregation versus fat destabilization by partial coalescence

By utilizing SDS and EDTA solutions, the binding mechanism of this observed colloidal network was investigated, along the lines of the work of Mendez-Velasco and Goff (2011). SDS can change the wettability of fat

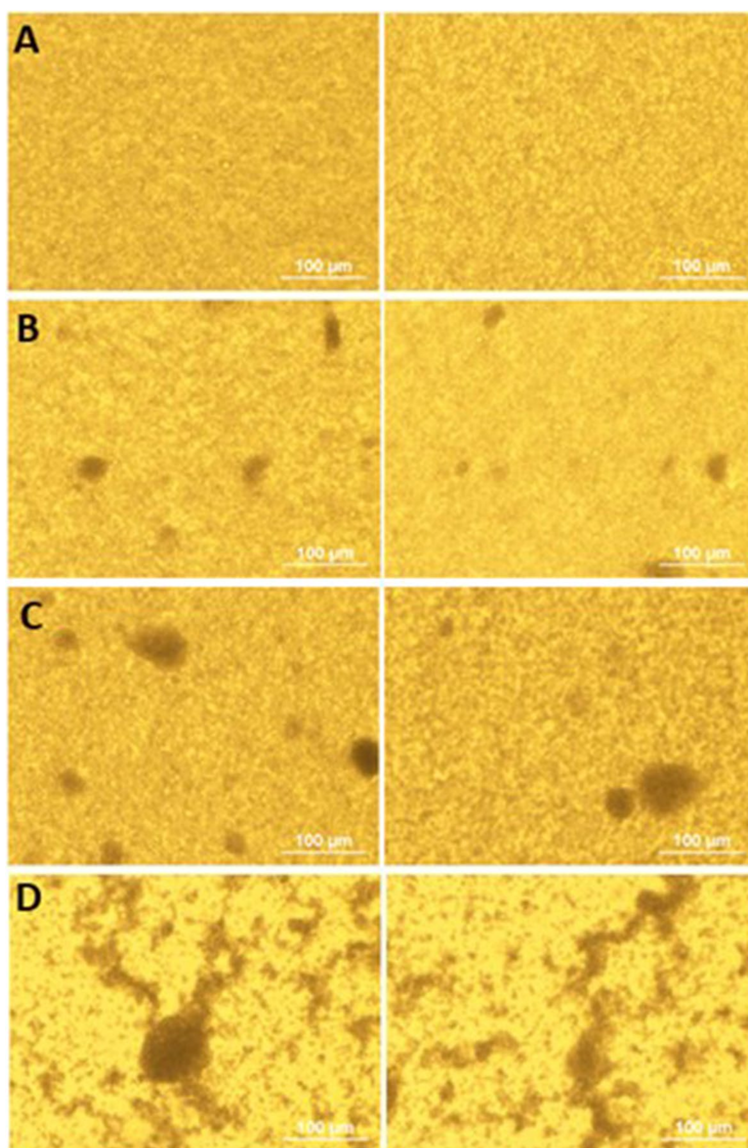


Fig. 3 Duplicate microscope images of cream samples with addition of (**A**) 0%, (**B**) 0.75%, (**C**) 1.5%, and (**D**) 3.0% (wt/wt) tannic acid. Samples diluted tenfold with D.I. water

crystals present at the interface of a partially-coalesced sample and cause the expulsion of fat crystals into the aqueous phase, thus redispersing the fat globules (Méndez-Velasco & Goff 2011). However, SDS is also used to break hydrophobic bonds between proteins (Hou et al. 2020), a critical point since polyphenols are thought to form aggregates with proteins through hydrophobic interactions (Yildirim-Elikoglu & Erdem 2018). EDTA, by sequestering solution calcium, removes colloidal calcium from the casein micelle, which causes the micelle to separate. By disrupting the micelles, casein-mediated fat aggregates can also be redispersed. Both treatments are necessary to distinguish between fat aggregation and partial coalescence.

The $d_{4,3}$ or volume-weighted mean is a measure of the average particle size (McClements 2015). Figure 4 shows $d_{4,3}$ of the fat globules in cream with increasing levels of TA, treated with water (control), SDS, and EDTA solutions to dilute samples for Mastersizer analysis. These values were analyzed via one-way ANOVA ($P<0.01$) and Tukey's HSD test. At 0.75% TA, only the SDS treatment was significantly different than the control (dilution with D.I. water). However, all treatments were significantly different at 1.5% and 3% (wt/wt) TA. At 1.5% TA,

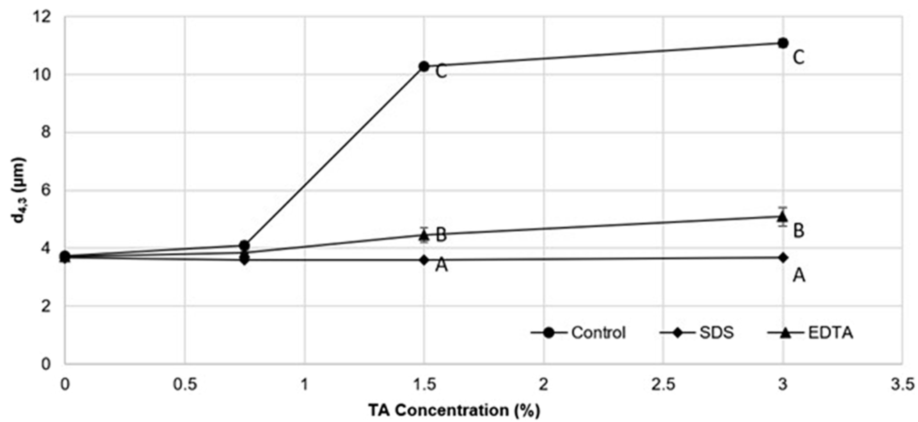


Fig. 4 Average fat globule diameter $d_{4,3}$ of cream with increasing levels of tannic acid (%) after 1:1 dilution with water (control), 4% SDS, and 3.7% EDTA. The error bars represent standard error between sample and measurement replicates ($n = 9$, 3 experimental replicates with 3 analytical replicates for each sample). Letters represent statistically different data points ($p < 0.05$)

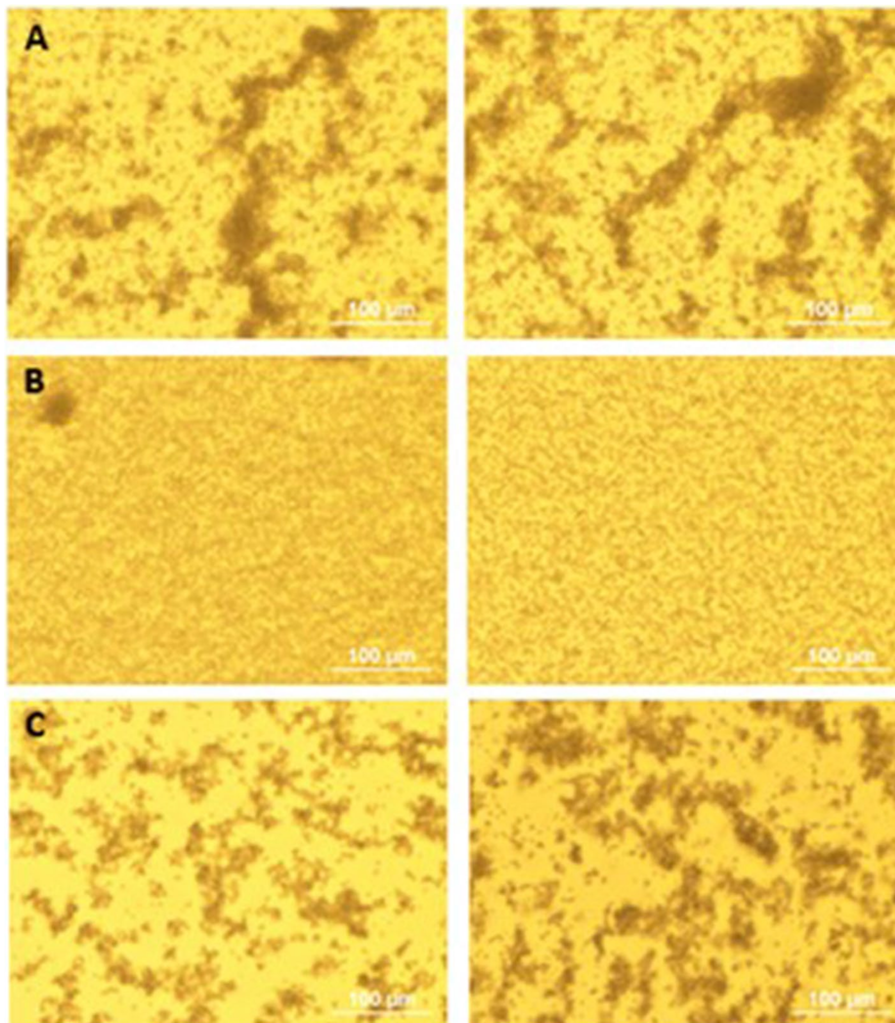


Fig. 5 Duplicate microscope images of 3% (wt/wt) tannic acid in cream after a 1:1 dilution with **A** water – control, **B** 4% (wt/wt) SDS and **C** 3.7% (wt/wt) EDTA

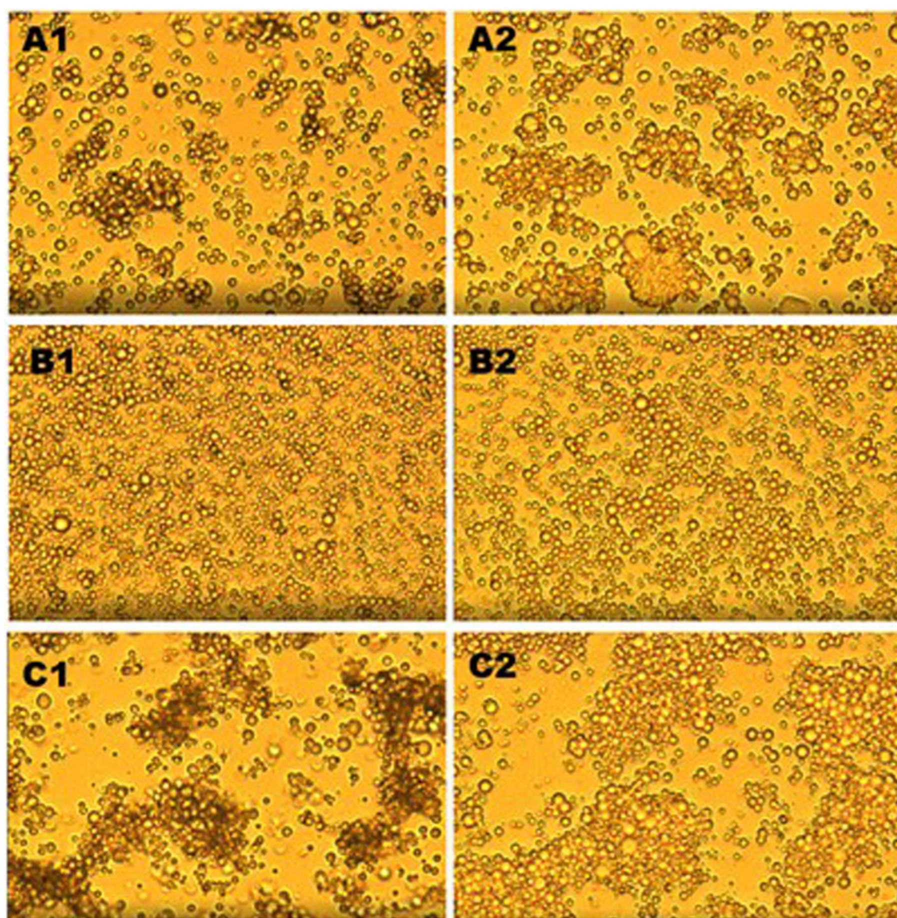


Fig. 6 Microscope images of 3% (wt/wt) tannic acid in cream after a 1:1 dilution with (A1) water – control, (B1) 4% (wt/wt) SDS and (C1) 3.7% (wt/wt) EDTA before and after heating at 55 °C (A2, B2, C2)

the control had a $d_{4,3}$ of 10.3 µm, and the EDTA and SDS samples were 4.5 µm and 3.6 µm, respectively. A similar trend can be seen at 3% TA for the control (10.3 µm), EDTA (5.1 µm), and SDS (3.7 µm) samples.

These trends are generally supported by the microscope images. Figure 5 depicts the water, SDS, and EDTA treated cream samples at 3% (wt/wt) TA. The SDS sample (Fig. 5B), shows an almost complete redispersion of the aggregated fat network found in the control (Fig. 5A), while the EDTA sample still has some fat clusters present (Fig. 5C). The $d_{4,3}$ value of the 3% EDTA sample is smaller than the clusters that appear in the microscope images. This may be attributed to a combination of the required mechanical agitation needed for particle size measurements and a weakened cream/TA matrix from EDTA treatment. Since image preparation needs only light mixing, there is less potential for disrupting a fragile network. To further confirm that these clusters contained fat, all treated samples (Fig. 5) were heated on a microscope stage and, once again, some coalescence of neighboring fat globules was observed (Fig. 6).

These treatments help us understand the components of the observed aggregate network. The redispersion of the SDS sample was attributed to the surfactant's ability to disrupt noncovalent bonds (within proteins and protein interactions with the fat globule membrane and polyphenols) and reverse partial coalescence. The EDTA treatment disrupted casein micelles and any casein micelle-TA complexes from the control. The statistically significant decrease in average particle size between the control and EDTA samples highlights the importance of casein micelles on the fat aggregation of this system. After the casein micelles were reversed in the EDTA sample, the remaining aggregates found in microscope images suggest that whey-TA complexes are also present in the system. The potential impact of partial coalescence

on these samples cannot be confirmed because protein interactions were not completely reversed during the EDTA treatment.

Conclusion

This work provides a basis to understand how TA interacts with pure cream to increase viscosity and promote gelation. The pH data revealed that the added acidity from TA was not the dominant driver of aggregation and increased viscosity in the samples. Average particle size and microscopic images confirmed that a stable network of protein and fat aggregates was created in the presence of TA. SDS and EDTA treatments document that both casein and whey proteins are involved in clustering of fat globules through protein aggregation. These protein mediated fat clusters could be attributed to the number of available binding sites on both the TA and proteins, the ratio of each present in the sample, and the ability to utilize these parameters to create bridges between globules by sharing TA binding sites between two or more proteins bound to the fat globule membrane (and vice versa) (Charlton et al. 2002; Siebert 1999; Wei et al. 2020). Based on these findings, partial coalescence did not seem to factor into formation of the clusters. However, due to its presence in cream, the contribution of crystalline fat to this system is a point of interest for future work.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43014-023-00166-9.

Additional file 1.

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Authors' contributions
CJW: conceptualization-equal; methodology-lead; project administration-supporting; validation-equal; writing-original draft-lead; writing review and editing-supporting. BWB: funding acquisition-lead; conceptualization-equal; methodology-supporting; project administration-lead; validation-supporting; writing-original draft-supporting; writing review and editing-supporting. RWH: funding acquisition-lead; conceptualization-lead; methodology-supporting; project administration-lead; supervision-lead; validation-lead; writing review and editing-lead. The author(s) read and approved the final manuscript.

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Availability of data and materials
The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate.
Not applicable.

Consent for publication
Not applicable.

Competing interests
No conflicts of interest.

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