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Protein content of amaranth and quinoa starch plays a key role in their ability as Pickering emulsifiers

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Abstract

Growing concerns about the safety of using synthetic surfactants to stabilize food emulsions have inspired a trend towards the use of natural ingredients like starch as alternative food stabilizers in what are called Pickering emulsions. The hydrophilicity of commercially available starches, however, necessitates further chemical treatment to increase their hydrophobicity and emulsifying ability. Here we demonstrate an alkaline isolation method to extract amaranth and quinoa starch from flour while retaining a high protein content, which gives these materials an emulsifying ability comparable to octenyl succinylated starches. We highlight the key role played by protein by showing that a serial reduction of the protein content leads to a parallel reduction in emulsifying ability, and that pH affects this ability. Our method of retaining proteins naturally present in amaranth and quinoa not only bolsters the nutritional profile of the food but also takes advantage of the proteins' native hydrophobicity for improved emulsification.

Key words: Pickering emulsion; protein content; amaranth and quinoa starch; solid surfactants; alkaline extraction method; octenyl succinic anhydride

1. Introduction

The use of emulsifiers to form stable emulsions and foams has afforded many incredible applications for the food, pharmaceutical, cosmetic, and drug industries (Berton-Carabin & Schroen, 2015; Dickinson, 2010). The creamy texture of a common food like mayonnaise, for example, can be attributed not to its oil content or water content alone, but to how emulsifiers are able to structure the oil and water together as an oil-in-water emulsion. Typical emulsifiers used today are small, amphiphilic molecules (~1 nm) that orient themselves at the oil/water interface to impart stability (see Fig. 1a). They can be categorized as either synthetic surfactants (e.g., polysorbates, monoacylglycerols) or biopolymers (e.g., proteins like casein or soy, and carbohydrates like gum arabic or carrageenan) (Berton-Carabin et al., 2015; McClements, 2016). Concerns over the biocompatibility, biodegradability, and carcinogenicity of synthetic surfactants, however, have led to a growing trend towards the use of natural emulsifiers in "clean label" food products. In the food and pharmaceutical industries, in particular, there has been increasing interest in what are called Pickering emulsifiers. These are large, solid particles (~10 nm–10μm) that possess the ability to stabilize emulsions due to their moderate hydrophobicity and larger size (see Fig. 1b) (Bon, 2015; Aveyard, Binks, & Clint, 2003; Timgren, Rayner, Sjoo, & Dejmek, 2011; Yang, et al., 2017). Plant-based solid particles like starch granules are especially good candidates for this application because they are cheap, widely available, biodegradable, non-allergenic, and GRAS (Timgren et al., 2011; Xiao, Li, & Huang, 2016; Zhu, 2019).

The problem with starch granules as Pickering emulsifiers is that in their commercial, purified, native form, they are very hydrophilic, making it difficult for them to adsorb onto the oil/water interface and thus making them poor emulsifiers in general (Aveyard et al., 2003). To

resolve this issue, chemical treatment with octenyl succinic anhydride (OSA) has been typically employed to increase their hydrophobicity and thus improve emulsifying ability (Zhu, 2019). It is in this OSA-modified form that starches in general are used in food emulsions, although the high amount needed relative to the oil content (~0.4-1:1 w/w) still presents a disadvantage compared to small-molecule surfactants (~0.05:1 w/w) (McClements, 2016). Another problem is that it is not yet clearly understood why certain kinds of native starches (e.g., quinoa, rice, barley) appear to have at least some ability to form emulsions while some others do not (e.g., maize, waxy maize, amaranth) (Timgren, Rayner, Dejmek, Marku, & Sjoo, 2013; Marefati, Wiege, Haase, Matos, & Rayner, 2017). Variations in source grain, isolation method, native granule hydrophobicity, granule particle size, and shape have all been hypothesized to play a role. However, since all these factors can confound each other when comparing starches of different sizes and shapes extracted from a myriad of botanical sources using different methods, it is difficult to ascertain exactly what role each factor plays (Marefati et al., 2017).

Recent papers have highlighted the important role that protein content may play in starch's hydrophobicity and emulsifying ability. For example, dry heating quinoa, rice, barley, and wheat starches at 100–160 ºC has been shown to improve their emulsifying ability and oil-binding ability. Heating is believed to lipophilize the residual proteins on the surface of the starch granules, changing their character from hydrophilic to hydrophobic and thus improving their emulsifying ability (Timgren et al., 2013; Seguchi, 1984; Baldwin, 2001). The protein contents were not quantified, however, and dry heating other kinds of starches (e.g., maize and waxy maize) did not produce the same improvement in emulsifying ability, making it difficult to make clear conclusions. In another recent paper, Marefati et al. (2017) have shown that quinoa starch with 0.69% protein were able to form emulsions, while amaranth starch with a lower 0.11%

protein content could not form emulsions, leading them to conclude that a higher protein content may be responsible for improved emulsification properties (Marefati et al., 2017; Marefati, Matos, Wiege, & Rayner, 2018). Differences in particle size and shape between quinoa and amaranth starches, however, again presented a confounding factor that prevented a direct comparison. A direct relationship between protein content and starch's emulsifying ability has therefore not yet been established.

In this study we seek to establish a direct relationship between the protein contents of two kinds of starches, amaranth and quinoa, and their respective Pickering emulsifying abilities. We chose these two pseudo-cereals because of their small granule size and their naturally high protein contents (with excellent amino acid profiles), and because they have not been as well studied as other cereals (Janssen, Pauly, Rombouts, Jansens, & Delcour, 2017; Bressani & Garcia-Vela, 1990; Gurbuz, Kauntola, Diaz, & Jouppila, 2018). We speculate that their proteins are adsorbed on the granule surface, serve to increase their hydrophobicity, and thus improve their emulsifying abilities (Figs. 1c and 1d). We first isolated amaranth and quinoa starches from flour using a NaOH-based method that retained a high protein content (> 2%), measured their respective emulsifying capabilities, then serially reduced the protein contents down to ~1% by NaOH extraction, and again studied their respective emulsifications in terms of emulsion index (EI), droplet size, rheology, surface charge, and 4-week stability. Our aim was to highlight the key role that proteins may play in the emulsification properties of starches, so we showed, in addition, how these emulsions were sensitive to changes in pH. Having established that a high protein content was key to starch's ability to form Pickering emulsions, we argued that the isolation method presented here can produce naturally high-protein starches that can successfully be used to make Pickering emulsions without any further chemical treatment.

2. Materials and Methods

2.1 Materials

Amaranth and quinoa starches were isolated from commercially available flours (see Section *2.2* for the isolation method). Corn oil was purchased from Healthy Brand Oil Corporation (Long Island City, NY, USA). Sodium hydroxide (NaOH, 95–100%) beads were purchased from Fisher Scientific (NJ, USA). ACS-grade hydrochloric acid (HCl, 36.5–38%) was purchased from VWR Chemicals (PA, USA). Denatured ethanol (<92%) was purchased from Thermo Fisher Scientific (MI, USA). Citric acid monohydrate (100.4%), sodium carbonate (100%), sodium bicarbonate (100.3%), and sodium phosphate dibasic were purchased from Fisher Scientific (NJ, USA). Monobasic sodium phosphate was purchased from VWR Life Science (Ohio, USA). Trisodium citrate dihydrate (>99%) was purchased from Sigma-Aldrich (MO, USA). All prepared emulsions were stored in Kimble KIMAX disposable culture tubes (15 mL) (NJ, USA).

2.2 Isolation of amaranth and quinoa starches from flours

Amaranth and quinoa starches were isolated from commercially available flours using an alkaline isolation method. First, 100 g of flour was dispersed in a 500-mL 0.15% NaOH solution and mixed using an overhead stirrer at ambient temperature for 1 h. The slurry was then filtered for 10 min using a laboratory test sieve vibrator (Derrick Mfg. Co., Buffalo, NY, USA) with a 270-mesh sieve (53-µm pore size). The remaining residue that did not pass through the sieve was collected, and then dispersed in 100 mL of a 0.15% NaOH solution, which was then stirred for a further 10 min, filtered again through the 270-mesh sieve, and washed with another 100 mL of the 0.15% NaOH solution. The filtrates from both filtrations were combined and centrifuged at 3000 g for 20 min. The supernatant was discarded, and the top yellow-brownish layer of protein

was removed using a spatula. The white starch layer was then re-suspended in deionized water, adjusted to pH 6.0 ± 0.1 using a 1 N HCl solution, and centrifuged at 3000 g for 20 min, again removing the top yellow-brown layer. It was then freeze-dried for 24 h and ground ultra-fine using a conical burr grinder. The starches isolated using this method had protein contents of 2.43% (amaranth) and 2.70% (quinoa). For the purposes of this paper, we labelled these as "high-protein starches," to differentiate them from lower-protein starches to be produced in the next section (see Section *2.3*). By comparison, these high-protein starches (2.4-2.7%) have protein contents much higher than those of commercially available starches (typically ~0.05%-0.6%) (Baldwin, 2001). The starch extraction yields from the amaranth and quinoa flours were approximately 12% and 30%, respectively.

2.3 Protein extraction from starch

Protein extraction was done to further reduce the protein contents of the high-protein amaranth and quinoa starches isolated from Section *2.2* by using a modified alkaline extraction method (Lim, Kyonggi-do, Shin, & Lim, 1999). 8 g of starch was dispersed in 30 mL of a 0.20% NaOH solution and mixed at 80 rpm for 1h using a vertical rotating mixer (BT Lab Systems, MO, USA). The slurry was then centrifuged at 3000 g for 10 minutes, removing the top yellow-brown protein layer. The white starch was then re-suspended in deionized water, adjusted to pH 6.0 ± 0.1 using a 1 N HCl solution, and centrifuged, followed by removal of the top yellow-brown layer. The white starch was then washed further sequentially with ethanol then deionized water, each time centrifuging at 3000 g for 10 min and removing any remaining yellow-brown top layer. The sample was then freeze-dried for 24 h. This reduced the protein contents of the amaranth and quinoa starches down to 0.87% and 1.4%, respectively. For the purposes of this paper, we

labelled these "mid-protein starches," to differentiate them from the high-protein starches produced previously in Section *2.2*.

To even further reduce the protein content, the starches isolated from Section *2.2* were mixed with a 0.20% NaOH solution for 4 h instead of 1 h, replacing the NaOH solution every hour with a fresh solution, and performing the centrifugation, removal of the top protein layer, pH adjustment, washing, and freeze-drying steps in the same way as above. This further reduced the protein contents of the amaranth and quinoa starches down to 0.67% and 1.17%, respectively. For the purposes of this paper, we labelled these "low-protein starches," to differentiate them from the mid-protein and high-protein starches produced previously.

2.4 Crude protein and crude fat analysis

Amaranth and quinoa flours, together with the high-protein starches isolated from them in Section *2.2* and the protein-reduced starches in Section *2.3* (mid-protein and low-protein) were tested in triplicate for crude protein using AOAC 992.23 (combustion method) and a nitrogen-to-protein conversion factor of 6.25, and tested for crude fat using AOAC 2003.05 (Randall-modified Soxhlet extraction), both on a % dry basis.

2.5 Scanning electron microscopy (SEM)

The amaranth and quinoa commercial flours were mounted on an SEM stub with conductive carbon tape. They were then sputter-coated with gold then imaged using a JCM-6000 Benchtop SEM (JEOL Ltd., Japan) at an accelerating voltage of 15 kV using a secondary electron detector at x1000 and x5000 magnifications.

160 The high-protein amaranth and quinoa starches isolated in Section *2.2*, together with the
161 low-protein starches from Section *2.3*, were both mounted on a stub and sputter-coated with
162 indium then imaged using a Zeis Gemini 500 Field Emission SEM.

163 *2.6 Particle size of starch granules*

164 The high-protein amaranth and quinoa starches isolated from flour in Section *2.2* were
165 imaged using a JCM-6000 Benchtop SEM at x5000 magnification. The granule size and
166 distribution were measured using ImageJ 1.51w software for over 200 granules. The surface
167 mean diameter (d_{32}), volume mean diameter (d_{43}), and polydispersity index (PDI) were
168 calculated based on the following equations, respectively (Li, Li, Sun, & Yang, 2013):

169 $$d_{32} = \frac{\sum di^3}{\sum di^2} \quad (1)$$

170 $$d_{43} = \frac{\sum di^4}{\sum di^3} \quad (2)$$

171 $$\mathrm{PDI} = \frac{d_{43}}{\sum di/N} \quad (3)$$

172 where d_i is the diameter of the particle to be measured, and N is the total number of particles.

173 *2.7 Preparation of emulsions*

174 Pickering emulsions were prepared using the high-protein, mid-protein, and low-protein
175 amaranth and quinoa starches produced in Sections *2.2* and *2.3*. These emulsions were prepared
176 with 30% v/v corn oil/water using a pH 7 buffer and a starch concentration of 0.15 g/mL oil.
177 These were then homogenized at 11,000 rpm for 4 min using a high-speed homogenizer (IKA
178 T25 digital Ultra Turrax, Germany) with S25N-18G dispersing tool. Six 10 mL replicates at each

protein level were prepared in total—3 for measuring the EI and the 4-week stability, and another 3 for measuring both the emulsion droplet size and rheology. They were stored in 15 mL culture tubes, covered with a cap, sealed with parafilm, and stored at ambient temperature.

Pickering emulsions were also prepared using the high-protein amaranth and quinoa starches using different aqueous pH buffers (pH 3, 5.7, 7.0, 7.5, 8.5, 9.5, 10), again using 30% v/v corn oil/buffer, and a starch concentration of 0.15 g/mL oil. These were then homogenized at 11,000 rpm for 4 min. Six 10 mL replicates at each pH level were prepared in total—3 for measuring the EI and the 4-week stability, and another 3 for measuring both emulsion droplet size and rheology. They were also stored in culture tubes, covered with a cap, sealed with parafilm, and stored at ambient temperature.

The buffers used above were prepared as follows: The pH 3 and pH 5.7 buffers were prepared using 0.1M citric acid monohydrate and 0.1M trisodium citrate dihydrate at 82:18 and 18:82 volume ratios, respectively. The pH 7, 7.5, and 8 buffers were prepared using 0.2M sodium phosphate dibasic and 0.2M monobasic sodium phosphate at 61:39, 84:16, and 94.6:5.4 volume ratios, respectively. The pH 9.2 and pH 10.0 buffers were prepared using 0.1M sodium carbonate and 0.1M sodium bicarbonate at 10:90 and 55:45 volume ratios, respectively.

2.8 Emulsion droplet size by optical microscopy

The Pickering emulsions prepared in Section *2.7* were placed on a microscope cover slip 1 day after preparation and imaged using a Leica Model DMIL LED Inverted Phase Contrast Microscope at 10x or 4x magnification, depending on their size. The emulsion droplet size was measured using ImageJ 1.51w software for over 200 droplets. The surface mean diameter (d_{32}),

volume mean diameter (d_{43}), and polydispersity index (PDI) of the droplets were calculated using equations 1-3 from Section *2.6*.

2.9 Emulsion index and 4-week stability

To monitor emulsifying ability and emulsion stability over a 4-week period, the emulsion indices (EI) of the Pickering emulsions prepared in Section *2.7* were measured 1d, 7d, 2 weeks, and 4 weeks after preparation using the following equation:

$$Emulsion\ index = \frac{V_E}{V_T} \qquad (4)$$

where V_E is the volume of the emulsion (upper cream layer) and V_T is the total volume of the whole sample (including all layers or phases) (Saari, Heravifar, Rayner, Wahlgren, & Sjoo, 2016). Three replicates were prepared.

2.10 Rheology

Two days after the preparation of the Pickering emulsions in Section *2.7*, oscillation frequency sweep and flow sweep experiments were performed using an AR 1000-N Rheometer (TA Instruments, USA) with a 40-mm-diameter parallel plate, a 1000-µm gap height, and a 2-min equilibration time between runs (Song, Pei, Qiao, Ma, Ren, & Zhao, 2015). An oscillation frequency sweep was first performed at 25 ºC using a frequency range of 0.01–10 Hz and a strain of 0.1%. Then, a flow sweep was performed at 25 ºC using a shear rate range of 0.02–100 s^{-1}. The viscosity, storage modulus G', loss modulus G", and tan δ were plotted logarithmically and investigated.

2.11 Zeta potential

The Zeta potential values of the emulsions prepared in Section *2.7* were measured 4 weeks after preparation by diluting them by a factor of 0.1 and determining their Zeta potential (mV) using a Malvern Zetasizer Nano Series. This was performed after the 4-week stability study.

3. Results and Discussion

3.1 Crude protein and crude fat analysis of flours and starches

The crude protein and crude fat analytical results are shown in Figures 2a and 2b. The commercial flours used in this study had crude protein contents of 17.37% (amaranth) and 14.93% (quinoa), and crude fat contents of 7.1% (amaranth) and 6.1% (quinoa), on a dry basis. Using the NaOH isolation procedure outlined in Section *2.2*, high-protein starches obtained from these flours featured protein contents of 2.43% (high-protein amaranth) and 2.70% (high-protein quinoa), and fat contents of 2.2% (amaranth) and 0.6% (quinoa). The protein contents were further reduced using the NaOH method outlined in Section *2.3*, producing mid-protein starches (0.87% protein and 0.5% fat for amaranth, and 1.40% protein and 0.4% fat for quinoa), and low-protein starches (0.67% protein and 0.2% fat for amaranth, and 1.20% protein and 0.3% fat for quinoa).

Proteins extracted from amaranth and quinoa were not fractioned in this study, but the available literature states that pseudo-cereal protein fractions (i.e., amaranth and quinoa proteins) are predominantly composed of albumins and globulins, which are particularly high in glutamic acid, aspartic acid, lysine, and arginine (Janssen et al., 2017). Different cultivars have been shown to have different protein compositions, but in general, protein fractions from amaranth

and quinoa grains using the Osborne fractionation scheme are composed mostly of water-soluble albumins and globulins (~40–77%), while the remaining fraction is composed of alkaline-soluble glutelins and alcohol-soluble prolamines (Bressani et al., 1990; Osborne, 1907; Janssen et al., 2017; Fairbanks, Burgener, Robison, Anderson, & Ballon, 1990). It is important to note that the protein extractions performed in Sections *2.2* and *2.3* (alkaline, water, and alcohol) did not totally extract all the proteins, so residual proteins remained on the starch granules, which is supported by the crude protein results (Gurbuz et al., 2018). In addition, amaranth and quinoa proteins (albumins, globulins, glutelins, and prolamins) are all globular proteins, which means that they take a longer time than random-coil proteins like casein to unfold and adsorb onto the oil/water interface (Joshi, Adhikari, Aldred, Panozzo, Kasapis, & Barrow, 2012).

3.2 Particle size of high-protein amaranth and quinoa starch granules

The high-protein amaranth starch isolated from flour in Section *2.2* featured a surface mean diameter, volume mean diameter, and a polydispersity index of 1.1μm (d_{32}), 1.2 μm (d_{43}), and 1.08, respectively. The high-protein quinoa starch had a slightly larger size with 1.4 μm (d_{32}), 1.5 μm (d_{43}), and 1.12 (PDI). These values agree with the literature (Timgren et al., 2013; Xia, Li, Liao, Zhang, Zheng, & Kan, 2015).

3.3 SEM of flours and starches

The SEM images of amaranth flour, high-protein amaranth starch, and low-protein amaranth starch are shown in Figures 2c, 2d, and 2e, respectively. Amaranth flour (Fig. 2c) is composed of aggregates of starch granules attached to each other by protein and fat. High-protein amaranth starch (Fig. 2d) is composed of well-separated polygonal granules 1.2 μm in diameter with sharp edges, with some small residual particles on the surfaces (red arrows), which

could perhaps be residual protein or fat. Low-protein amaranth starch (Fig. 2e) appears the same, but with fewer small residual particles. SEM images of quinoa flour, high-protein quinoa starch, and low-protein quinoa starch are shown in Figures 2f, 2g, and 2h, respectively. Like amaranth flour, quinoa flour (Fig. 2f) is composed of aggregates of starch granules attached together with protein and fat. High-protein quinoa starch (Fig. 2g) is composed of well-separated polygonal granules 1.5 μm in diameter with rounded edges, again with some small residual particles on the surface (red arrows), which could be residual protein or fat. Low-protein quinoa starch (Fig. 2h) appears the same, but with fewer residual particles.

3.4 Emulsification properties

3.4.1 Effect of protein content on emulsion droplet size, emulsion index, and 4-week stability

While conventional, small-molecule surfactants can stabilize emulsions because they are amphiphilic (their hydrophobic tails orient themselves towards the oil phase and their hydrophilic heads orient towards the aqueous phase), solid Pickering emulsifiers can stabilize emulsions because they are moderately hydrophobic over their entire surface (i.e., they are not amphiphilic). The more hydrophobic they are over their entire surface (e.g., higher degree of OSA substitution), the more easily they will adsorb onto the oil/water interface, promoting the formation of smaller droplets, and also the more deeply they will embed into the oil phase (higher Θ), thus making them more stable over time (i.e., higher desorption energy, harder to remove). If our contention that residual protein content adds hydrophobicity to starch like OSA, then a higher starch native protein content will be expected to likewise enhance starch's emulsifying ability, and reducing the protein content will conversely reduce this ability (We

provide a more thorough discussion of conventional vs. Pickering emulsifiers in Sections S1 and S2 under Supplementary Information).

We see exactly this trend in the results: High-protein amaranth starch (Fig. 3a) and quinoa starch (Fig. 3b) formed emulsions with small droplet sizes (27.9, 32.1μm) and high EIs (0.78, 0.62). As we reduced the protein content, the droplet sizes increased (91.3, 126.7 μm) and the EIs decreased (0.58, 0.32) (for tabular data, see Supplementary Tables S1 and S2 under Supplementary Information). We believe that reducing the protein content reduces starch's hydrophobicity, which in turn reduces starch's ability to adhere to the oil/water interface, thus promoting the formation of larger emulsion droplets (Timgren et al., 2013). In addition, larger emulsions have a smaller effective volume than smaller emulsions. The reason being that the effective volume fraction is $(1+\delta_x/r)^3$ times that of the actual volume fraction, where δ_x is the thickness of the adsorbed starch layer and r is the droplet radius (Chanamai & McClements, 2000). Thus, it follows that larger emulsions formed with low-protein, less hydrophobic starch will have lower emulsion volume, i.e., a lower EI, which is supported by Figs. 3a and 3b. Of course, larger emulsions are also more prone to creaming according to Stokes' law (McClements, 2016), thus further reducing the EI. The inverse of this relationship—increasing hydrophobicity leads to the formation of smaller emulsions and a higher EI—is supported by literature on OSA-treated starch (Timgren et al., 2013).

The larger emulsion droplets produced by lowering the starch protein content may also have been due to some of the low-protein granules (more hydrophilic) failing to adsorb onto the oil/water interface and settling down (Figs. 3a and 3b, see bottom of tubes). As a result, the effective concentration of starch that participates in the emulsification is lowered, which further contributes to the lower EI.

The effectiveness of amaranth and quinoa starches as Pickering emulsifiers appears to depend significantly on their protein content (Figs. 3c and 3d). In particular, high-protein amaranth and quinoa starches formed 27.9μm and 32.1μm droplets, respectively, which appear comparable to, if not better than, traditional OSA-treated starches, which typically form larger droplets in the 38–48 μm range at similar conditions (Marefati et al., 2017; Saari et al., 2016). Of course, that protein generally plays a role in emulsification is not new; it has been shown by literature outside starch research. For example, the proteinaceous fractions of natural carbohydrate-protein conjugates like gum arabic or sugar beet pectin are believed to be the one responsible for making them bind to the oil/water interface. Their direct removal reduces their emulsification ability (Ozturk & McClements, 2016; Sweedman, Tizzotti, Schafer, & Gilbert, 2013; Evans, Ratcliffe, & Williams, 2013; Zhang, Wu, Lan, & Yang, 2014; Randall, Phillips, & Williams, 1988). Of course, the high amount of natural carbohydrate-protein conjugates required relative to the oil content (1:1) to form emulsions pose a disadvantage (Ozturk et al., 2016; Evans et al., 2013).

The role that the crude fat content of starch plays in its emulsifying ability has been hypothesized in the literature, but the verdict remains unclear (Tang, 2007). To shed some light on this matter, we compared the emulsifying ability of amaranth starch with high fat content versus low fat content while keeping the protein content relatively constant, and found that the crude fat content appears to have no significant effect on emulsifying ability (see Section S3 and Figure S1 under Supplementary Information).

Amaranth starch outperformed quinoa starch in terms of forming smaller droplet sizes and larger EIs, despite the fact that in this study our amaranth starch had a lower protein content than quinoa at each protein level (at “low,” “mid,” “high”), which may be due to other factors. If

we cut a vertical line across Fig. 3c or Fig. 3d to interpolate the droplet size at a particular protein level, it appears that for the same level of protein, amaranth worked as a better emulsifier than quinoa. This may be due to several factors. The main factor may be size: amaranth starch (1.2μm) is smaller than quinoa (1.5μm), which makes it easier for it to adsorb onto the oil/water interface, and should allow it to theoretically form smaller droplets (Destribats, Ravaine, Heroguez, Leal-Calderon, & Schmitt, 2010; Berton-Carabin et al., 2015). In general, a particle can form a Pickering emulsion with a droplet size one order of magnitude greater than the particle size, and smaller particles generally form smaller emulsions (Berton-Carabin et al., 2015; Xiao et al, 2016; Timgren et al., 2013). Another factor could be the fact that amaranth granules have sharper edges than quinoa (Fig. 2e vs. Fig. 2h), which has been theorized to help adsorption at the oil/water interface (Tcholakova, Denkov, & Lips, 2008).

Geometrically (approximating a granule as a sphere), as a particle increases in size, its cross-sectional area (the area it can use to cover and stabilize an emulsion droplet) increases by the square of the radius only, while its volume or mass increases by the cube of the radius. It follows that smaller granules have a higher total cross-sectional area and can thus sterically cover more emulsion droplet surface area than larger granules. Thus, given the same amount of oil and the same amount of starch, smaller starch granules can afford to stabilize smaller droplets (with a larger total surface area) while larger granules can only afford to stabilize larger droplets (with a lower total surface area). This may explain why amaranth granules can form emulsions that are smaller than those formed by quinoa granules. Theoretical models elsewhere have confirmed this relationship (Destribats et al., 2010).

To check the stability of these emulsions, we also measured their EIs over 4 weeks. We found that all emulsions prepared using high-, mid-, and low-protein starches had a 10–20%

355 reduction in EI (Figs 3e and 3f). High-protein and mid-protein amaranth starches retained the
356 highest EIs after 4 weeks, while mid-protein and low-protein quinoa starches featured the lowest
357 EIs. This may be due to both gravitational separation and coalescence. Stokes' law predicts that
358 the gravitational phase separation rate of an emulsion increases by the square of the emulsion
359 droplet size (Berton-Carabin et al., 2015; Joshi, et al., 2012). Thus, the larger quinoa-stabilized
360 emulsion droplets should undergo phase separation (creaming) faster than smaller amaranth-
361 stabilized emulsions, which explains why quinoa has smaller EIs over 4 weeks. Other factors that
362 may have also affected the stability of these emulsions over time were their viscosity and Zeta
363 potential, which will be discussed in Sections *3.5* and *3.6*.

364 3.4.2 *Effect of pH on emulsion droplet size, emulsion index, and 4-week stability*

365 To highlight the role that protein plays in starch's emulsifying ability, we also
366 investigated the effect of pH on the emulsions formed by high-protein amaranth and quinoa
367 starches. As we increased the pH from 3 to 10, the emulsion droplet sizes appeared to increase
368 slightly for both amaranth- (Fig. 4a top) and quinoa-stabilized emulsions (Fig. 4b top). As
369 expected, this slight increase in droplet sizes led to a reduction of the EIs for both amaranth- (Fig.
370 4a bottom) and quinoa-stabilized emulsions (Fig. 4b bottom). These relationships are
371 summarized in Figs. 4c and 4d (see also Supplementary Tables S3 and S4).

372 Emulsions formed using small-molecule surfactants and proteins are generally affected
373 by pH due to the deprotonation or protonation of certain functional groups, which can change the
374 surface charge and affect the stabilizing mechanism of repulsion between droplets. Pickering
375 emulsions prepared using OSA-modified starch granules may show some slight variation when
376 the pH is changed—Song et al. (2015), for example, saw a slight decrease in the EI of OSA-
377 modified starch-stabilized emulsions when the pH was increased. However, in general, starch-

stabilized Pickering emulsions are known to be otherwise resistant to pH variations because of the lack of sensitive functional groups, and because their main stabilizing mechanism is steric hindrance rather than electrostatic repulsion (McClements, 2016). In contrast, our results show that Pickering emulsions stabilized by high-protein starches are actually affected by pH. This suggests that the ability of starch granules to form Pickering emulsions depends heavily on their protein content, because we can expect the protein to change starch's surface charge when pH is altered.

The literature shows that the proteins from amaranth starch granules have an isoelectric point (pI) at approximately pH 4–6 (Bolontrade & Scilingo, 2013). More specifically, the different protein fractions composing amaranth proteins have pIs at different pHs—7.5 (albumins); 5.6, 9.2, 5.2-5.8 (globulins); and 5.7-6.3 (glutelins), with the pIs for prolamins still unknown. Similarly, the proteins on quinoa starch granules have a pI at the following pHs: 5.0–6.5 (globulins), with the pIs for albumins, glutelins, and prolamins still unknown (Janssen et al., 2017). Thus, in general, at a pH of 4–6.5, both amaranth proteins and quinoa proteins will display a relatively neutral charge, and as we increase the pH farther away from the isoelectric point up to pH 10, we expect a more negative charge (Joshi, et al., 2012). Amaranth and quinoa protein extracts generally have been found to display poor solubility at low pH values of 3.0–5.0 (2–35% solubility), and good solubility at higher pHs of 5.0–11.0 (50–90% solubility) (Janssen et al., 2017). Lower solubility at low pHs indicates that these proteins are more hydrophobic under these conditions. Thus, we argue that at low pH, the relatively neutral charge of the residual proteins on the surface of both amaranth and quinoa starches makes them more hydrophobic and thus more effective as emulsifiers, which is why they form smaller emulsion droplets and higher emulsion indices at low pH. On the other hand, at higher pHs, the negative

charge of these proteins makes these starches less hydrophobic and thus less effective emulsifiers, resulting in larger emulsion droplets and lower EIs. (See Section *3.6.2* for supporting Zeta potential data showing that the surface charge indeed becomes more negative at higher pH.) Improved emulsifying ability at low pH could also be due to the extensive uncoiling of amaranth proteins that can be induced by low pH, which can further improve the emulsifying ability (Janssen et al., 2017). Furthermore, studies have shown that at very low pH (near pH 2), amaranth proteins may actually become denatured, dissociated, or partially hydrolyzed into smaller fragments by an endogenous peptidase, which will allow it to diffuse faster into an air/water interface to make a more flexible or viscoelastic film, thus forming more stable foams at low pH than at high pH (Janssen et al., 2017; Bolontrade et al., 2013).

To check whether pH also affected emulsion stability, we measured the EI over a 4-week period (Figs 4e and 4f; see also Supplementary Tables S3 and S4). We found that all the Pickering emulsions showed a 10–20% decrease in their EI after this period. And as expected, emulsions prepared at pH 3 retained higher EIs than those prepared at pH 10 after 4 weeks. Emulsions stabilized with amaranth starch (Fig. 4e) also appeared, again, to outperform those stabilized with quinoa starch (Fig. 4f), having higher EIs at all pH levels tested. Other factors that may have also affected the stability of these emulsions over time were their viscosity and Zeta potential, which will be discussed in the following sections.

3.5 Rheology

3.5.1 Effect of protein content on viscosity and elasticity

As the protein content of starch granules were increased, we saw in Fig. 3c that the droplet size of the Pickering emulsions decreased. Our rheology results demonstrate that a higher starch protein content (and smaller emulsion droplet size) also led to a higher viscosity for both

amaranth-stabilized emulsions (Fig. 5a) and quinoa-stabilized emulsions (Fig. 5b). Smaller emulsions had a higher viscosity than larger emulsions across almost the entire range of shear rates tested (0.02–100 s^{-1}). Several papers have shown the same relationship using surfactant-stabilized oil/water emulsions, citing several possible reasons for the trend (Pal, 1996; Chanamai et al., 2000): First, smaller droplets have a smaller mean distance of separation between them, leading to greater hydrodynamic interaction and collision frequency, and thus a higher viscosity. Second, as the droplet size decreases, the ratio between the thickness of the adsorbed starch layer and the droplet size increases, which leads to a higher effective volume fraction or a higher effective dispersed phase concentration. As we noted in the previous section, the effective volume fraction is $(1+\delta_x/r)^3$ times that of the actual volume fraction, where δ_x is the thickness of the adsorbed layer and r is the droplet radius (Chanamai et al., 2000). Thus, at smaller droplet sizes (lower r), the effective volume fraction of the dispersed phase is higher, leading to a higher viscosity. A recent modeling study on the rheology of Pickering emulsions has also shown that a smaller droplet size leads to higher viscosity (Pal, 2018). Lastly, smaller droplets tend to be more monodisperse, and this also leads to higher viscosity (Pal, 1996). This higher viscosity can further help explain why emulsions stabilized by high-protein starches retained the highest EI over 4 weeks (Figs. 3e and 3f), as compared to those stabilized by low-protein starches, because a higher viscosity delayed phase separation. Notice also that across all protein levels (high, mid, low), amaranth-stabilized emulsions (Fig. 5a) showed higher viscosity than quinoa-stabilized emulsions (Fig. 5b), which again may be due to the smaller size of amaranth granules and thus the smaller emulsion droplets that they tend to form, and thus the greater 4-week stability.

Both amaranth- and quinoa-stabilized emulsions also exhibited shear-thinning behavior. As the shear stress was increased, the randomly distributed emulsions may have begun to align

447 themselves with the flow into strings or layers, which reduced resistance to the flow of the fluid,
448 thus decreasing viscosity (McClements, 2016). We note here that any excess amount of granules
449 in the continuous phase may have also formed a 3D network to stabilize these emulsions, and in
450 addition could have increased the viscosity of the aqueous phase and thus also delayed phase
451 separation according to Stokes' law (Aveyard et al., 2003; Binks & Lumsdon, 2000).

452 In addition, we also found that these emulsions exhibited viscoelastic behavior, measured
453 by **tan δ** (tan δ=*G*"/*G*'), where *G*" is the loss modulus or a measure of energy lost due to viscous
454 dissipation in the material, and *G*' is the storage modulus, or a measure of the energy stored in
455 the material. For both amaranth-stabilized (Fig. 5c) and quinoa-stabilized emulsions (Fig. 5d),
456 low-protein starches formed less elastic Pickering emulsions, generally with tan δ >1, while mid-
457 and high-protein starches produced more elastic Pickering emulsions with tan δ <1.

458 This gel-like behavior may be attributed to inter-droplet network formation that resists
459 flow (Song et al., 2015). In general, Pickering emulsions show gel-like elasticity in frequency
460 sweep tests, which is ascribed to the rigid interface created by the solid particles, which leads to
461 surface elasticity (Xiao et al., 2016). Compressing a Pickering emulsion causes the solid
462 interface to be slightly deformed, but strong adhesion between the solid particles produces a kind
463 of scaffold that can cause it to revert back, thus exhibiting elasticity (Xiao et al., 2016). And
464 because a higher protein content leads to the formation of smaller emulsion droplets, and smaller
465 emulsion droplets in turn have a more packed structure with more granules rubbing against each
466 other in a scaffold, it follows that a higher protein content leads to more elastic Pickering
467 emulsions.

468 3.5.2 *Effect of pH on viscosity and elasticity*

As the pH was increased, we saw that emulsion viscosity decreased (Fig. 5e for amaranth, and Fig. 5f for quinoa) across the shear rates tested. We expect the protein on the starch granule surface to become more negative as the pH goes up, and this can reduce the emulsion viscosity in two ways: first, a more negative charge makes the starch more hydrophilic overall, and thus reduces its emulsifying ability, causing it to form bigger emulsion droplets with lower effective volume and thus lower viscosity. Second, a more negative charge at high pH also causes the droplets to repel each other more, which allows them to slide across each other more easily when shear is applied, which translates again to a lower viscosity. Thus, both amaranth and quinoa starches had better emulsifying abilities at low pH and the resultant higher viscosity at low pH further helped the emulsions retain a high EI over a 4-week period. Amaranth, in particular, formed more viscous emulsions than quinoa across all pH levels tested, which again may be due to the smaller emulsions it formed.

These Pickering emulsions also exhibited, in addition, viscoelastic behavior due to the solid granule scaffolds created between droplets. In general, these Pickering emulsions showed more elasticity at low pH than at high pH (Fig. 5g for amaranth and Fig. 5h for quinoa), likely because the smaller emulsion droplets formed at low pH allowed more starch granules to rub against each other when shear was applied, which deformed the droplet and, when the shear was removed, caused the granules to be pulled back into the oil/water interface, thus exhibiting better elasticity.

3.6 Zeta potential

3.6.1 Effect of protein content on Zeta potential

Zeta potential gives the net surface charge of an emulsion, and the higher the magnitude of this charge, the higher the repulsion between emulsion droplets and therefore the more stable they are over time (Joshi et al., 2012). As the protein content of starch was increased, we found

that the Zeta potential of the Pickering emulsions formed also became more negative (measured after the 4-week stability study) (Fig. 6a). A higher protein content thus not only makes the starch more hydrophobic to form more stable, smaller emulsions, it also lends these emulsions at the same time a more negative net surface charge due to the presence of more COO^- groups that can help further stabilize the droplets by electrostatic repulsion. This supports our previous results showing that emulsions stabilized by high-protein starches have better 4-week stability (higher EIs) than those stabilized by low-protein starches (Figs. 3e for amaranth and 3f for quinoa).

3.6.2 Effect of pH on Zeta potential

As the pH was increased from 3 to 10, the Zeta potential of the Pickering emulsions prepared using high-protein amaranth and quinoa starches became more negative (measured after the 4-week stability study) (Fig. 6b). This trend confirms our expectation that as we increase the pH farther away from the isoelectric point (pH 4–6) of the residual proteins on the starch, the charge of these residual proteins should become more negative. This more negative charge at high pH (at the same protein content and the same amount of hydrophobic moieties) makes starch more hydrophilic overall (i.e., less hydrophobic), and reduces its emulsifying ability. This helps explain why we found that high-protein starches had better emulsifying abilities at lower pH than at high pH (Figs. 4a for amaranth and 4b for quinoa) and why they are also more stable at lower pH over a 4-week period (Figs. 4e for amaranth and 4f for quinoa).

4. Conclusions

In the growing trend towards the use of natural, food-grade emulsifiers in food, we hope that the alkaline isolation method presented here can be used to produce high-protein amaranth

514 and quinoa starches that take advantage of their protein's native hydrophobicity for improved
515 emulsification without further chemical treatment. High-protein starches appear to have
516 emulsifying abilities comparable to, if not better than, that of OSA-treated starches in the
517 literature. Amaranth starch, in particular, exhibits a better emulsifying ability than quinoa starch
518 at all the protein and pH levels tested, which may be due to its smaller particle size or more
519 angular shape. We highlighted the main function that the protein contents of amaranth and
520 quinoa starches play in their emulsifying abilities by performing a serial reduction of the protein
521 content—from high-protein (> 2%) to low-protein levels (~1%)—and showing that this directly
522 reduced their respective abilities to form emulsions. High-protein starches, being more
523 hydrophobic than low-protein starches, formed smaller emulsion droplets with higher EIs, higher
524 viscosity and elasticity, and better 4-week stability. Reduction of starch's crude fat content, on
525 the other hand, did not have a significant effect. In addition, decreasing the pH from pH 10 to pH
526 3 improved these starches' emulsifying abilities, a trend that can be attributed to the less negative
527 charge of proteins at lower pH, making these starches more hydrophobic, and thus resulting in
528 smaller emulsion droplets with higher EIs, higher viscosity and elasticity, and better 4-week
529 stability. This variation of emulsifying ability with pH further underscores the key role that
530 protein content plays in starch's emulsifying ability.

531

532 **Conflict of interest**

533 None.

534 **Acknowledgments**

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Figure Captions

Figure 1. Schematic diagrams of (a) amphiphilic, small-molecule surfactants at the oil/water interface, (b) solid starch granules adsorbed at the oil/water interface at a contact angle θ, (c) proteins on the starch surface increasing its hydrophobicity and thus helping it adsorb deeper into the oil phase, increasing θ, and (d) how reducing the protein content of starch is expected to reduce its emulsifying ability. Note: Protein and fat are not drawn to scale; they have been enlarged to illustrate their influence on emulsification.

Figure 2. (a) Crude protein and (b) crude fat contents of amaranth and quinoa flours and their corresponding high-, mid-, and low-protein starches after isolation and further protein reduction. Error bars refer to standard deviation (SD). SEM images of (c) commercial amaranth flour, (d) isolated high-protein amaranth starch, and (e) low-protein amaranth starch at 1000x, 20,000x, and 20,000x magnifications, respectively. SEM images of (f) commercial quinoa flour, (g) isolated high-protein quinoa starch, (h) and low-protein quinoa starch at 1000x, 20,000x, and 20,000x magnifications, respectively.

Figure 3. Effect of starch's protein content on emulsifying ability. Digital camera and optical microscope images of emulsions prepared at pH 7 using (a) amaranth and (b) quinoa starches with different protein contents ("low", "mid", and "high"), taken 1 day after preparation. Scale bar is 50 μm. Relationship of starch protein content *vs.* (c) emulsion droplet size and (d) EI of emulsions prepared using amaranth and quinoa starches at pH 7 and 1d after preparation. Four-week stability of Pickering emulsions prepared using (e) amaranth and (f) quinoa starches with different protein levels. Error bars refer to standard deviation (SD).

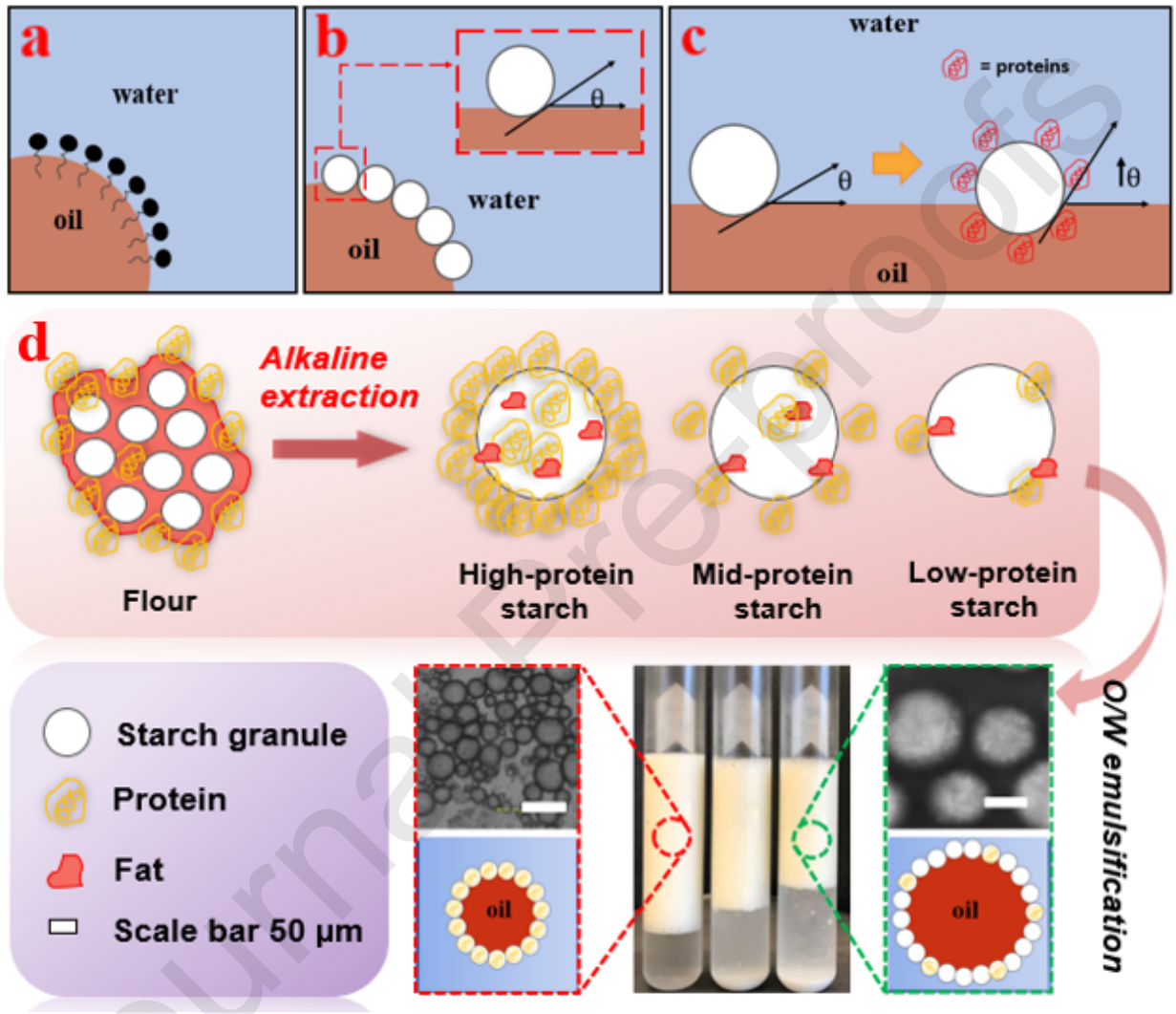
Figure 4. Effect of pH on starch's emulsifying ability. Digital camera and optical microscope images of emulsions prepared with high-protein (a) amaranth and (b) quinoa starches at different pHs (3.0, 5.7, 7.0, 7.5, 8.5, 9.2, 10) 1d after preparation. Scale bar is 50 μm. Relationship of the pH vs. (c) the emulsion droplet size and (d) the EI of emulsions prepared using high-protein amaranth and quinoa starches, 1 day after preparation. Four-week stability of Pickering emulsions prepared using high-protein (e) amaranth and (f) quinoa starches at pH 3, pH 7, and pH 10. Error bars refer to standard deviation (SD).

Figure 5. Effect of starch's protein content and the pH on the rheology of starch-stabilized Pickering emulsions. Viscosity vs. shear rate graphs of (a) amaranth- and (b) quinoa-stabilized Pickering emulsions at different protein levels using a log-log scale, measured 2 days after preparation at pH 7. Tan δ vs. frequency graphs of (c) amaranth- and (d) quinoa-stabilized Pickering emulsions at different protein levels using a log-log scale, also measured 2 days after preparation at pH 7. Viscosity vs. shear rate graphs of (e) amaranth- and (f) quinoa-stabilized Pickering emulsions at pH 3.0, 7.0, and 10.0, using a log-log scale and measured 2 days after preparation. Tan δ vs. frequency graphs of (g) amaranth- and (h) quinoa-stabilized Pickering emulsions at pH 3.0, 7.0, and 10.0, using a log-log scale and also measured 2 days after preparation.

Figure 6. Effect of (a) starch's protein content and (b) pH on the Zeta potential of starch-stabilized Pickering emulsions. For (a), emulsions were prepared at pH 7. All Zeta potential

values were measured 4 weeks after preparation, after a 4-week stability study. Error bars refer to standard deviation (SD).

Figure 1



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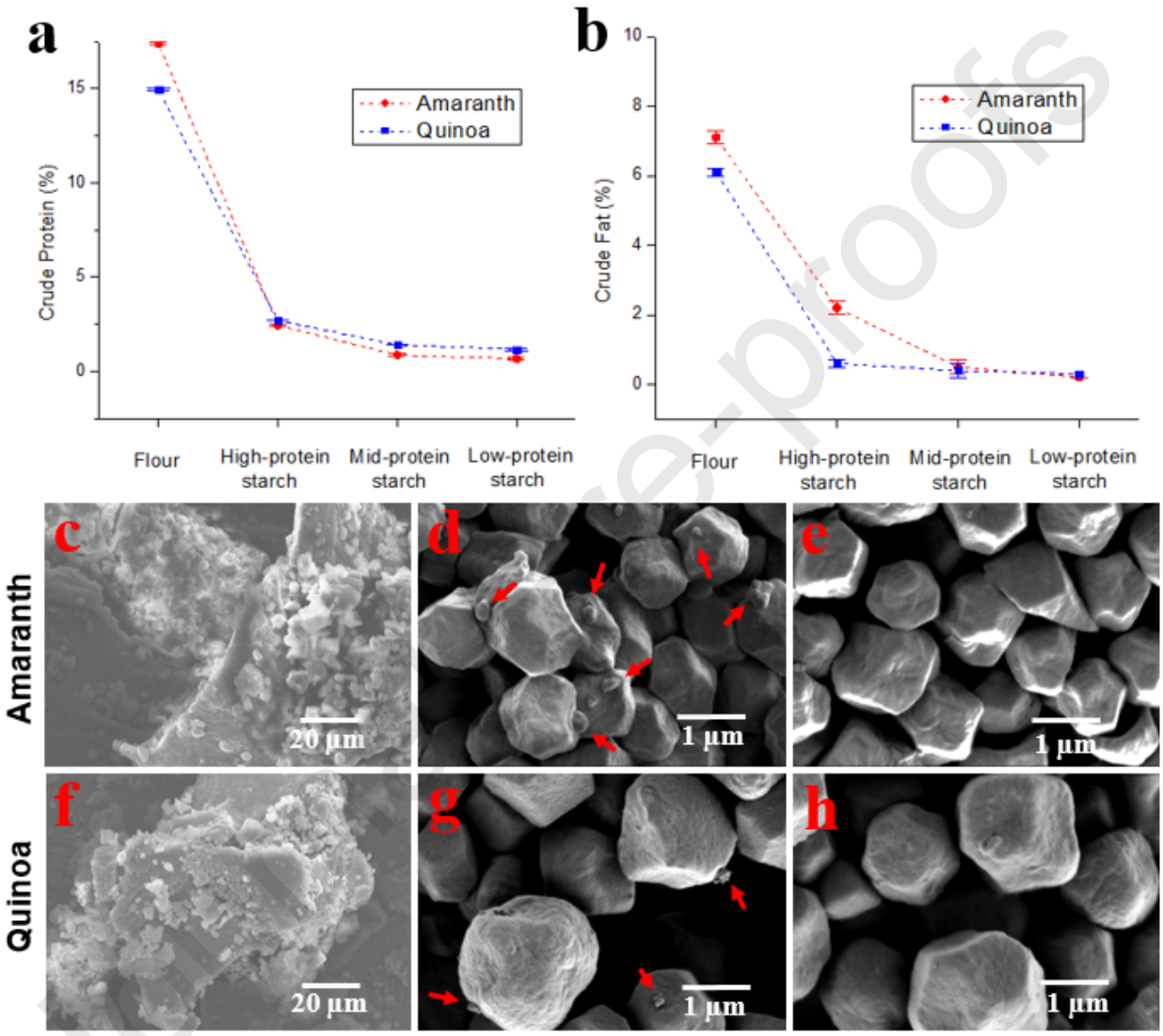
687 **Figure 2**



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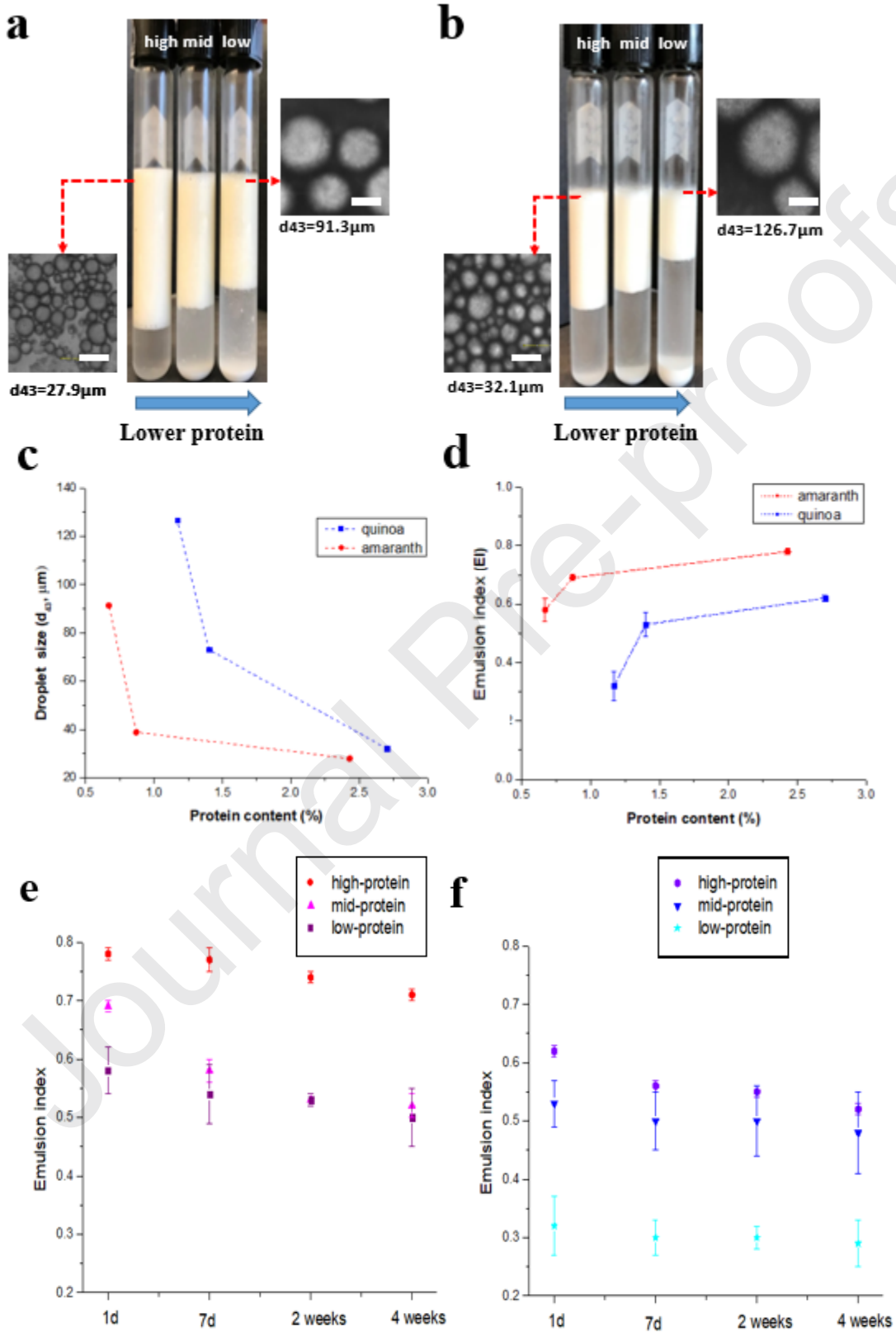

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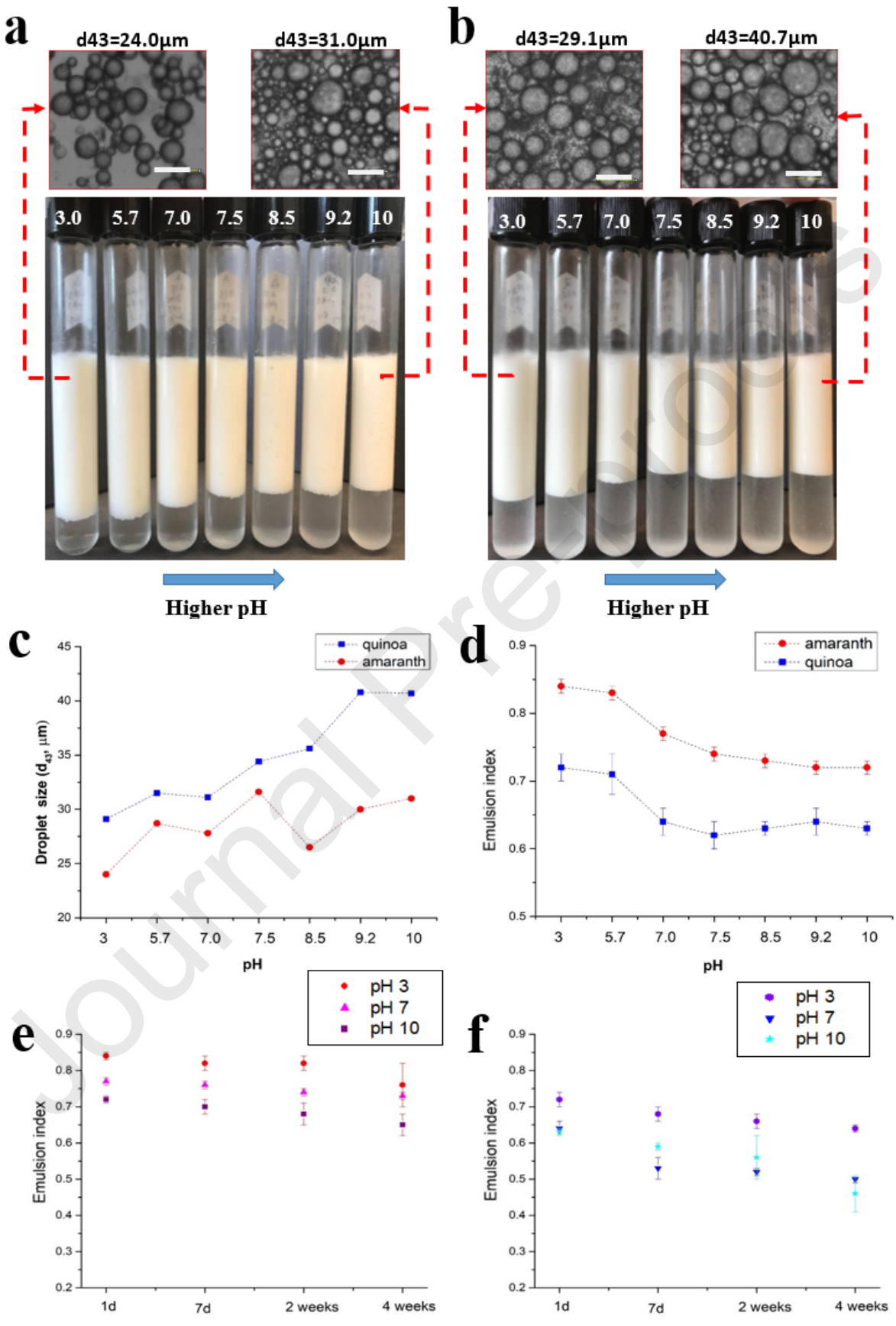

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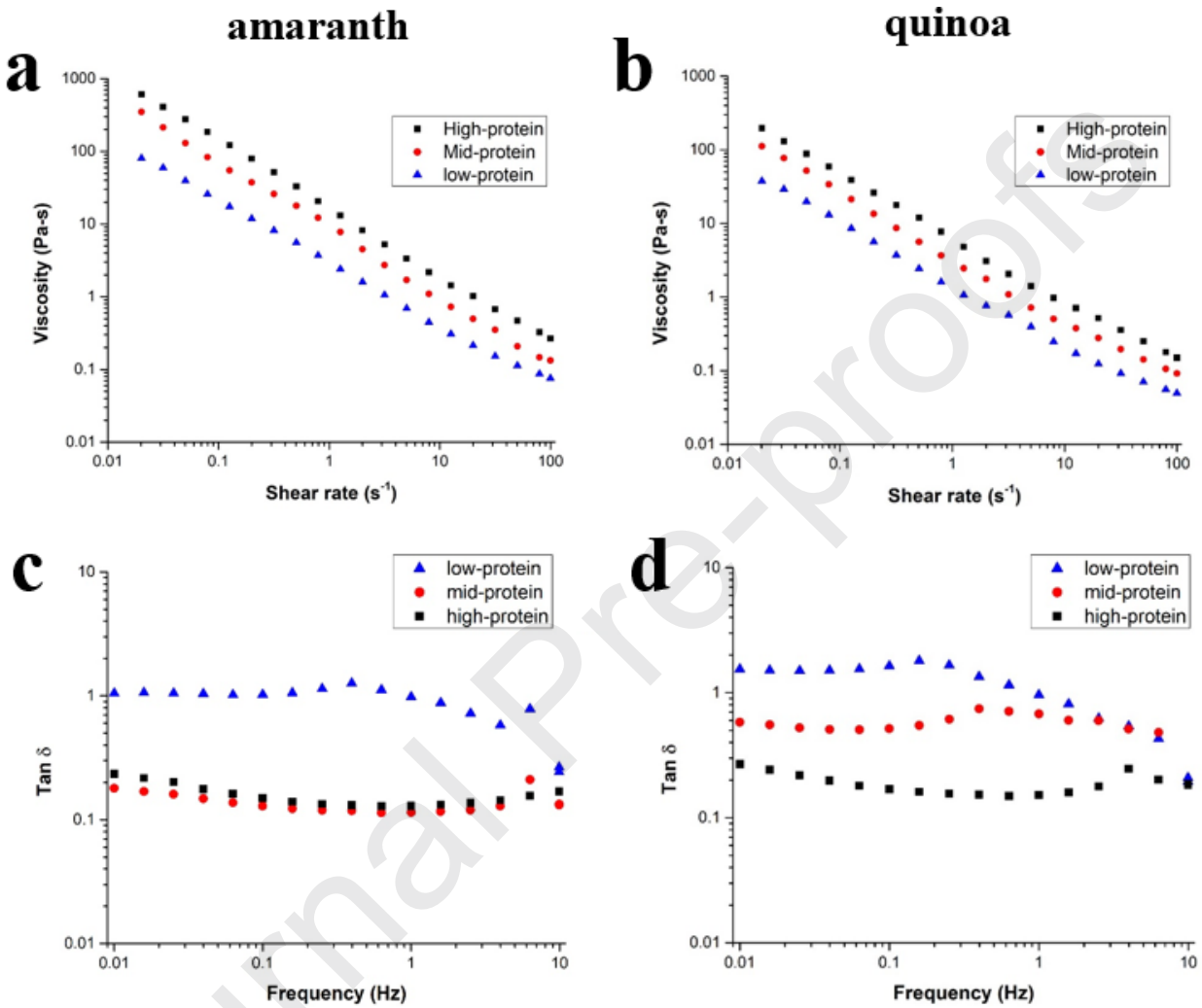

699 **Figure 5 (part 1 of 2)**

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706 **Figure 5 (part 2 of 2)**

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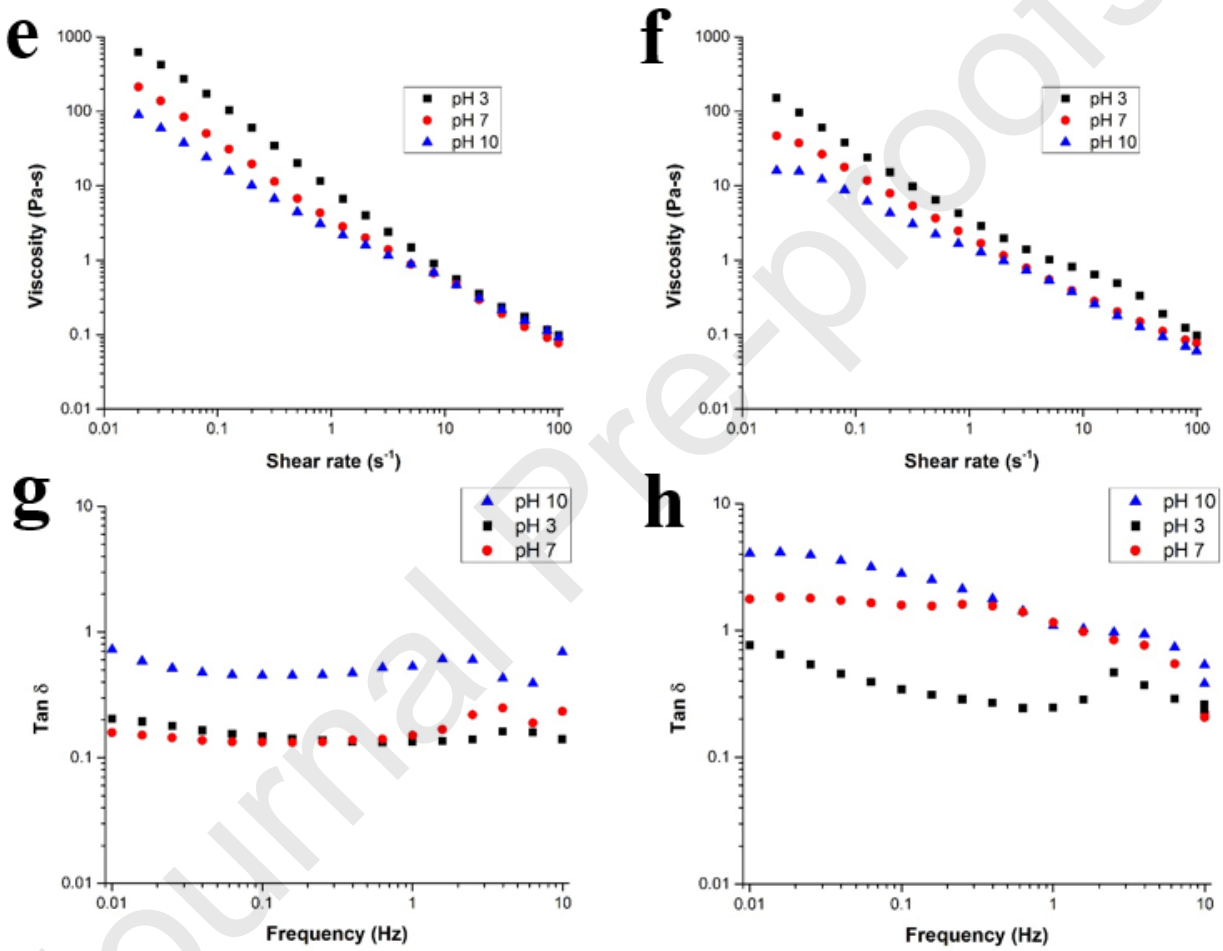

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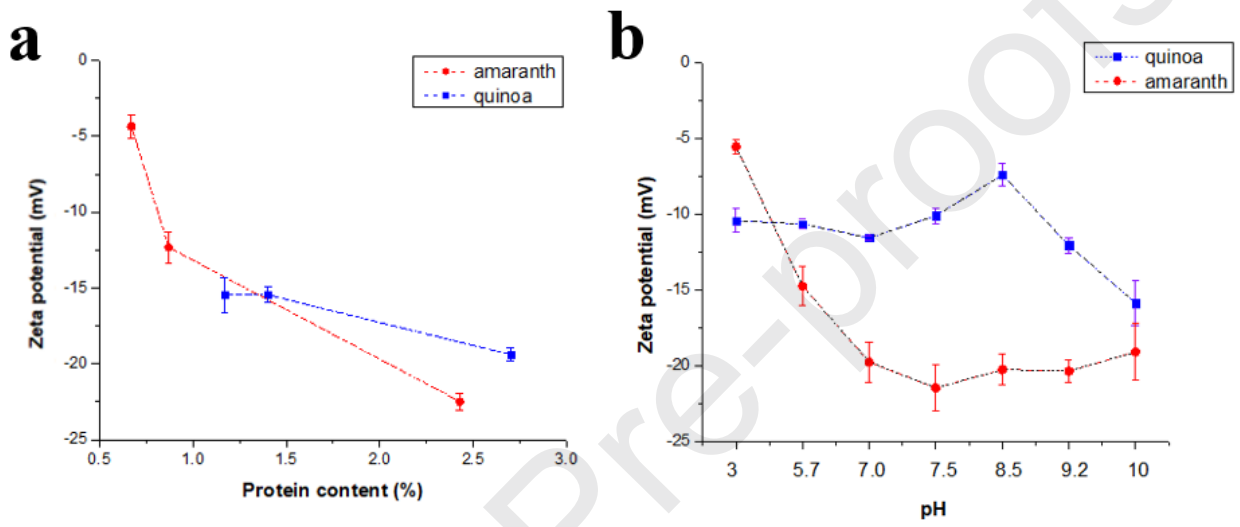
715 **Figure 6**



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718 **Highlights**

719 We demonstrate an alkaline method to isolate high-protein starch from flour.

720 High-protein starches are good Pickering emulsifiers, even w/o octenyl succinylation.

721 Higher protein content makes smaller droplets w/ greater emulsion index & viscosity.

722 Reducing protein content reduces emulsifying ability, but reducing fat has no effect.

723 Starch's emulsifying ability is affected by pH, w/c highlights protein's key role.

724