Heat induced denaturation, aggregation and gelation of almond proteins in skim and full fat almond milk

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ABSTRACT

The effect of thermal treatment (45-95 °C for 30 minutes) on the structure of almond milk proteins was assessed, as the unfolding and association of these proteins in response to heat is not well understood. Above 55 °C, protein surface hydrophobicity and particle size increased and alpha helical structure decreased, reducing the stability of skim or full fat milk. Fractal protein clusters were observed at 65-75 °C and weakly flocculated gels with a continuous protein network occurred at 85-95 °C, resulting in gels with high water holding capacity and a strength similar to dairy gels. The presence of almond fat increased gel strength but led to a more heterogenous microstructure, which may be improved by homogenisation. Elasticity could also be increased with protein concentration. This study improves our understanding of the heat stability of almond milk proteins and indicates their potential as a gelling ingredient for vegan and vegetarian products.

Keywords: Almond milk proteins, thermal treatment, partial protein denaturation, stability, microstructure, rheology

1. Introduction

Consumer interest in plant-based dairy and meat substitutes, including plant-based milk, yoghurt and cheese has grown over the last decade (Day, 2013), leading to an increased need to understand the functionality of plant proteins, including nut proteins such as almond. Almond kernel is rich in fats (44-61%) and proteins (16-26%) but low in carbohydrates (2-8%) (Yada, Lapsley, & Huang, 2011). The lipids and proteins in almonds are present in the form of oil bodies (or oleosomes) and protein bodies. Almond oil bodies are essentially oil droplets that are surrounded and stabilized by a monolayer of phospholipid in which proteins, called oleosins, are embedded (Beisson, Ferte, Bruley, Vouloury, Verger, & Arondel, 2001). The protein bodies house storage proteins, along with several hydrolytic enzymes, lectins and phytins (Weber & Neumann, 1980). Almond milk, which is a colloidal suspension made by grinding almond kernels with water, contains both oleosomes and water-soluble almond proteins (Gallier, Gordon, & Singh, 2012). The protein content in commercial almond milk is typically much lower than in dairy milk (~0.7% vs ~3%), although nutritional composition is a function of the ratio of almonds and water used in formulation (Vanga & Raghavan, 2018). Due to the small amount of almonds used, additional emulsifiers and stabilizers such as gums and celluloses are required to stabilize these low protein emulsions (Sethi, Tyagi, & Anurag, 2016). Almonds and almond milk also have broader potential as ingredients in the production of plant-based foods, although their high cost compared to starch based foods and their potential for allergenicity mean these ingredients may not be suitable for all applications (Costa, Mafra, Carrapatoso, & Oliveira, 2012).

The properties of almond protein extracts, including almond milk, depend on the method of extraction applied and the proteins extracted. These preparations are typically processed minimally, resulting in a complex suspension. The storage protein amandin constitutes 65-70% of the total water extractable proteins (Wolf & Sathe, 1998) and is the most studied almond protein to date. Despite being water soluble, amandin is classified as a globulin, similar to the 11-12S group of globulins (known as legumins), which share a hexameric structure, a similar sedimentation coefficient,

75 molecular weight (~ 275 - 450 kDa) and the presence of acidic and basic polypeptides with molecular
76 masses ~ 40 kDa and ~ 20 kDa linked through disulphide bonds (Kshirsagar, Fajer, Sharma, Roux, &
77 Sathe, 2011). A range of other almond proteins are also highly soluble in water, salt and buffer
78 solutions with pH ranging from 6 to 11; these proteins exhibit minimal solubility between pH 3 to 5
79 (Sathe, 1992; Wolf et al., 1998).

80 Heat stability is critical to the functionality of almond and other plant proteins and is relevant to
81 beverage pasteurisation, Ultra High Temperature (UHT), extended shelf life (ESL) and other thermal
82 processing. Heat also assists in the development of food structure in products such as yoghurts and
83 cheeses, where gelation is desirable and heat induced denaturation or aggregation can be used either
84 alone or in combination with other processes, including chemical or enzymatic modification, to
85 modify the native protein structure and obtain favourable sensory and textural attributes for optimal
86 functionality (Nicolai & Durand, 2013).

87 A range of denaturation temperatures have been observed for almond proteins, depending on the type
88 of extract studied. Denaturation was observed at ~ 98 °C for almond milk (Bernat, Chafer, Rodriguez-
89 Garcia, Chiralt, & Gonzalez-Martinez, 2015), whilst temperatures of 87 °C and 92 °C were recorded
90 by differential scanning calorimetry for two different almond protein isolates (each containing more
91 than 90% of almond proteins on a dry basis extracted under different conditions) (Amirshaghghi,
92 Rezaei, & Rezaei, 2017). In contrast, the denaturation temperature of proteins in fractionated protein
93 isolates ranged from ~ 46 °C (glutenin fraction) to ~ 63 °C (albumin fraction) and ~ 73 °C (globulin
94 fraction) or 79 °C (gliadin fraction) (Zhang, Zhang, Feng, & Shi, 2017). Isolated fractions also
95 denature between 54-62 °C (Li, Chu, Lu, Wang, & Ma, 2018), reflecting the type and concentration
96 of proteins present in these preparations. The combination of proteins in almond milk or almond
97 protein isolates, together with other ingredients, such as fats and carbohydrates, may also serve to
98 protect against thermal denaturation (Yusuf, 2003).

Less is known about the effect of heat treatment on the structure and properties of almond proteins in almond milk, including the thermal induced aggregation and stability of these proteins and changes in colloidal structure on heating. Moderate temperatures of 65-75 °C in combination with high pressures of 300 MPa during ultra-high pressure homogenisation or high temperature of 85 °C in combination with lower pressures of 172 MPa have been shown to produce almond beverages with higher physical stability than conventional heat treatments such as pasteurisation (90 °C, 90 s) and UHT processing (142 °C, 6 s) (Bernat et al., 2015; Valencia-Flores, Hernandez-Herrero, Guamis, & Ferragut, 2013). Almond proteins are also known to form gels (Albillos, Menhart, & Fu, 2009; Sathe & Sze, 1997; Sharma, Su, Joshi, Roux, & Sathe, 2010). There is little information on the gelation temperatures and properties of these gels, including the microstructural arrangement of protein, fat and water following gel formation or the resulting rheological properties. Yet these properties are of primary interest in the optimisation of almond based ingredients and food products, as they are some of the key determinants of product functionality and consumer perception.

The objective of this study was to characterise the proteins present in almond milk and to assess the effect of heat treatment on the thermal denaturation, aggregation, physical stability and gelation characteristics of skim and full fat almond milk preparations. We hypothesised that moderate heat treatment at 55-75 °C would denature almond proteins, inducing partial aggregation, whilst higher temperatures of 85-95 °C would induce more extensive denaturation and gelation, resulting in a continuous protein network in which fat droplets are integrated. These gelled structures could potentially act as a nutritious base for vegan or vegetarian products.

2. Materials and Methods

2.1 Materials and reagents

Raw whole **Australian** almonds (variety Non-pareil) were purchased from Woolworths Supermarket, Melbourne, Victoria. Purified water of resistivity > 18.2 MΩ.cm (at 25 °C), produced with a Milli-Q Direct-Q (Type 3) water purifier (Merck Millipore Corporation, Bedford, MA) was used for all

experiments. All chemicals were of analytical grade, purchased from Sigma-Aldrich (St. Louis, Missouri, USA) unless otherwise stated.

2.2 Sample preparation

Raw almonds were soaked in water in a ratio of 1:3 (w/w) for 18-20 hours in a beaker covered with cling wrap at 4 °C. After soaking was complete, this water was discarded, and the almonds were blotted dry to remove any surface water. The almonds were then deskinning by hand and mixed with water at a ratio of 1:4 by weight. This mixture was then ground using a homogeniser (Bellini Supercook Kitchen Master, GSM International Ltd, South Australia, Australia) at 20,000 rpm for 10 minutes, followed by stirring at 200 rpm for 30 minutes. The resulting slurry was filtered through two layers of muslin cloth and a 180-micron sieve to obtain full fat almond milk (FFAM).

To obtain skim almond milk (SAM), the FFAM was centrifuged twice at 15000 g for 30 minutes at 25 °C and the fat manually removed from the top of the preparation, yielding skim almond milk. Next the SAM was filtered through a 3 kDa centrifugal filter at 3000 g for 50 minutes to attain partial removal of the natural sugars. This filtered and concentrated protein solution containing 7.2% (w/w) protein was diluted to 0.18 mg/mL protein for surface hydrophobicity and circular dichroism measurements, to avoid light scattering due to presence of other non-protein components or the formation of precipitates. The SAM was further concentrated using the 3 kDa centrifugal filter to obtain concentrated skim almond milk (CSAM) with a protein content of 10.8% (w/w), to be used in select rheological measurements.

Sodium azide (0.02% (w/v), Chem-Supply, Gillman, SA) was added to all milk samples to prevent bacterial growth. The milk was stored at 4 °C and used within 3 days of production. Almond milk was extracted on three independent occasions.

2.3 Chemical composition and physical properties

The yield of FFAM was estimated as the percentage of milk obtained per unit of raw materials (dry almonds and water) on a w/w basis. The yield of SAM was calculated as percentage of the skim fraction obtained from the full fat milk (w/w basis). The moisture content and total solids were estimated by placing samples in an oven at $\sim 105^{\circ}\text{C}$ until a constant weight was obtained (Method 1990.20, (AOAC, 1995)). The protein content was quantified by nitrogen analysis following the Dumas combustion method (Method 992.23, (AOAC, 1995)) using Leco TruMac CNS Macro Analyser (LECO Corporation, Michigan, USA), where a nitrogen to protein conversion factor of 5.18 was used (Yada et al., 2011). The total fat was estimated using the Babcock method for cream (Method 995.18, (AOAC, 1995)). The pH was measured using a SevenCompact pH meter (Mettler Toledo Ltd., Victoria, Australia), while refractive index was measured using a DR-A1 Digital Abbe refractometer (ATAGO, Tokyo, Japan).

2.4 Thermal treatment

The full fat, skim and the diluted almond milk protein solutions were heat treated at the following temperatures: 45°C , 55°C , 65°C , 75°C , 85°C and 95°C . The change of temperature with time was recorded using an immersed Center 309 Data Logger thermometer. A 1 mL sample of each milk preparation was heated in a 1.7 mL microcentrifuge tube in a dry block heater. The sample was heated to the set temperature, a process that took 3.5 minutes for samples heated to $45-75^{\circ}\text{C}$ and 4 minutes for samples heated to $85-95^{\circ}\text{C}$. Each sample was then incubated for a further 30 minutes at the set temperature, followed by immediate cooling using running tap water. All samples were stored at 4°C until further analysis. Thermal treatment was performed for each of the three batches of almond milk ($n = 3$).

2.5 Fundamental characterisation of almond milk proteins

2.5.1 Surface hydrophobicity

The hydrophobicity index was determined by the method of Alizadeh-Pasdar and Li-Chan (2000), with slight modifications. A stock solution of 8 mmol/L 1-anilino-8-naphthalene sulfonate (ANS) was prepared in dimethylformamide. Protein solutions were prepared by serial dilution at final concentrations ranging between 0.036–0.18 mg/mL. A 5 μ L volume of ANS stock solution was added to each 1 mL of protein solution and the mixture vortexed for 10 s. The fluorescence intensity (FI) of the mixed solution was then measured at 470 nm after excitation at 390 nm, using a BMG FLUOstar OPTIMA Microplate Reader (BMG LABTECH Pty. Ltd., Mornington, Victoria, Australia) in fluorescence mode, with a constant excitation and emission slit width of 5 nm. The instrument gain settings were set such that fully denatured almond protein solution incubated at 105 °C for 30 minutes gave a fluorescence intensity approximately 90% of the maximum fluorescence intensity. A line of best fit was fitted to the fluorescence intensity of the dilution series for each protein sample by regression analysis and this line used to determine the protein hydrophobicity index. The grand average of hydropathy (GRAVY) value of amandin was calculated through the Expasy ProtParam tool (<http://expasy.org/tools/protparam.html>) using the amino acid sequence published by Jin et al. (2009).

2.5.2 Circular dichroism spectroscopy

Far-UV circular dichroism (CD) spectra of 0.18 mg/mL protein solutions were acquired between 190–260 nm using an AVIV CD-spectrometer (Biomedical Inc., New Jersey, USA) with a path length of 1 mm. The spectral resolution was 1 nm, data collection interval 1 s and bandwidth 1 nm.

2.5.3 SDS-PAGE electrophoresis

The milk samples were centrifuged at 2000 g for 10 minutes and the supernatants obtained were analysed under non-reducing conditions using the method of Laemmli (1970) with a pre-cast Bolt 4–12% gradient Bis-Tris Plus gel and apparatus from Bio-Rad laboratories. Each supernatant (15 μ L)

was mixed with 5 μ L of 4X Bolt LDS sample buffer (Thermo Fisher Scientific). Samples of $\sim 10 \mu$ g protein /well were loaded and the gels run using an SDS running buffer and voltage of 110V until the tracking dye reached the bottom of the gel. The gel was stained with 2.5g/L Coomassie brilliant blue (R-250) in 7.5% (v/v) acetic acid and 40% (v/v) methanol solution and destained in a 7.5% (v/v) acetic acid solution. Bio Rad markers were run in parallel with the samples (Precision Plus protein standards, All Blue, Bio-Rad, Richmond, CA, USA). The intensity of protein bands was measured using Image J (National Institute of Health, Maryland, U.S.A).

2.6 Effect of thermal treatment on almond milk

2.6.1 Gel formation and water holding capacity

Gel formation was assessed by inverting the sample after cooling to room temperature (Sharma et al., 2010). If the gel remained stable after gently tapping the sample tube 10 times, a gel was considered to have been formed. These gels were then centrifuged at 2000 g for 5 minutes and the released water drained. The water holding capacity (WHC) was calculated as follows:

$$WHC = \frac{W_2}{W_1} \times 100, \quad (1)$$

where, W_1 and W_2 correspond to the weight of the gel (g) before and after centrifugation, respectively.

2.6.2 Physical stability

The physical stability of the samples was measured using a LUMiFuge analytical centrifuge (LUM. GmbH, Berlin, Germany). Stability tests for FFAM and SAM samples were carried out at 2300g at 20 °C for 2 hours and 4.25 hours respectively, with measurements taken every 30 seconds.

The normalized transmission was calculated as a measure of the percentage of light transmission across the length of the sample tube. The integral transmission or the percentage of light transmitted

per unit time was also calculated, as a measure of the creaming, flocculation or sedimentation rate (Detloff, Sobisch, & Lerche, 2006).

2.6.3 Particle size analysis

A Malvern Mastersizer 3000 (Malvern Instruments Ltd., UK) in Can Hydro EV mode was used to assess the particle size. Samples were added dropwise to de-ionised water at 500 rpm until an obscuration rate of 2% for SAM samples and 8% for FFAM samples, allowing a volume-based particle size distribution to be obtained.

A refractive index of 1.464 was used for FFAM (Gallier et al., 2012) and a refractive index of 1.345 $\pm$ 0.001 was measured for SAM. In both cases, the samples had an absorbance of 0.001 AU.

2.6.4 Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) was used to observe the changes in microstructure of almond milk samples using the method described by Ong, Dagastine, Kentish, and Gras (2011). Nile Red and Fast green FCF were used to stain fat and proteins, respectively. For milk samples, 10 μ L of FCF solution was added to 480 μ L of milk followed by the addition of 10 μ L of Nile red solution. The mixture was vortexed and 50 μ L of this stained milk was then mixed with 200 μ L of agarose gel (0.5%). An aliquot of 5 μ L of this sample was transferred to a cavity slide (ProSciTech, Thuringowa, Queensland, Australia) and covered with a 0.17 mm thick glass coverslip (ProSciTech), so that the sample was flush with the coverslip. The coverslip was then fixed with nail polish. For gel samples, a thin section of the gel was cut using a surgical blade and placed on a slide. The section was stained with 0.02% Fast green FCF and 0.02% Nile red solution. Samples were then observed after 10 minutes using an inverted confocal microscope (Leica Microsystems, Heidelberg, Germany). For image analysis, single optical sections (512 $\times$ 512 pixels) were acquired for each sample using a $\times$ 63 magnification objective and dual-channel images were obtained using 488 nm and 633 nm laser excitation to visualise the protein and fat, respectively. A representative image is presented for each process variable.

2.6.5 Rheological analysis

The gelation of samples was assessed by temperature sweep tests conducted with a Discovery HR-2 Hybrid rheometer (TA Instruments, New Castle, USA) using a serrated parallel plate test geometry (40 mm diameter) with a geometry gap of 1000 μm . The temperature was controlled with a water thermo-circulator and a Peltier temperature control unit. The samples were heated from 25 $^{\circ}\text{C}$ to 95 $^{\circ}\text{C}$ at a rate of 3 $^{\circ}\text{C}/\text{minute}$ at a constant frequency (1 Hz) and strain (0.01%) and then cooled to 25 $^{\circ}\text{C}$. A two-piece solvent trap cover was used to prevent water evaporation. The temperature where the storage modulus G' crossed the loss modulus G'' was taken as the gelation temperature.

2.6.6 Statistical analyses

All properties were measured three times in replicate measurements and the average values compared between three independent batches of almond milk ($n=3 \pm$ the standard deviation). Wherever applicable, means were compared by one-way analysis of variance (ANOVA) using Minitab 18 software with a significance of 95%. For certain analyses, a representative graph or image is presented for the sake of clarity and ease of understanding.

3. Results and Discussion

3.1 Properties of almond milk formulations

Full fat almond milk (FFAM) was obtained by grinding almonds to obtain a particle slurry and the water-soluble extract filtered, using a process similar to industrial practice (Callewaert, Festjens, & Neiryneck, 2014). An off-white turbid solution consisting mainly of oleosomes (almond oil bodies surrounded by oleosins and phospholipids) and storage proteins was obtained, with a yield (defined in Section 2.3) of $84 \pm 2\%$ similar to previous studies (85-88% c.f. 84-86% here) (Dhakal, Giusti, & Balasubramaniam, 2016). Skim almond milk (SAM) was obtained after defatting the FFAM, providing a yield of $74 \pm 1\%$ for the two-step process.

The concentration of protein, fat and total solids content of the FFAM produced here (**Table 1**) is higher than in commercial almond milk but within the range reported in the literature, which varies due to differences in the extraction procedure including the ratio of almonds and water, the temperature and duration of soaking or blanching or roasting, the method of disintegration and the pore size of filters applied (Dhakal et al., 2016). The protein concentration in FFAM is similar to that of bovine milk (**Table 1**; ~ 3.5%), although the fat content, is significantly higher than in bovine milk ($9.5 \pm 0.5\%$ c.f. ~3-4% in bovine). The total solids content was also similar to that in fortified milk used for yoghurt formation (i.e. a total solids here of $15.0 \pm 0.3\%$ c.f. 15-16 g/100 g in a fortified yoghurt (Narayana & Gupta, 2016)). Each of these properties are relevant to gelation. The SAM retained a similar level of protein but had significantly lower fat content than the FFAM and ~ 60% lower total solids (**Table 1**). Both almond milk preparations had a pH similar to previous reports (**Table 1**).

Amandin is the major protein present in the both almond milk preparations in this study, representing ~60% of total protein present, as analysed by SDS- PAGE densitometry. This observation is consistent with prior studies (Wolf et al., 1998). The non-reduced amandin protein appeared at 60 kDa (**Figure 1A**), consistent with the expected molecular weight of 60.8 kDa calculated from the known protein sequence (Jin et al., 2009) and the molecular weight of 62-65.3 kDa, determined previously by non-reducing SDS-PAGE (Sathe, 1992). In the presence of reducing agent, several bands were present, including those at 39-42 kDa and 20-22 kDa corresponding to the known major acidic and basic polypeptides of amandin respectively, which are linked by disulphide bonds (**Figure S1A, Supplementary Information**) (Sathe, 1992; Wolf et al., 1998).

Several other proteins were observed following non-reducing SDS-PAGE analysis, between 11 kDa and 57 kDa (**Figure 1A**), collectively contributing 40% to the total protein present; their position on both reducing and non-reducing gels being consistent with minor proteins previously observed in other water based almond extracts (Li et al., 2018; Sathe, 1992). Many of these proteins have been

identified and characterised as potential allergens in literature (Che, Zhang, Lyu, Nadeau, & McHugh, 2018; Costa et al., 2012). The proteins present within the gel were grouped into three regions of interest based on band position for quantification by densitometry: 46-66 kDa containing the major component amandin, 35-38 kDa containing two other proteins and 25-28 kDa containing two other minor proteins. These latter two groups contain 15% and 10% of the protein in these preparations.

3.2 Effect of heating on the fundamental properties of almond milk proteins

The fundamental properties of SAM proteins, including hydrophobicity, secondary structure and size, as separated on an SDS-PAGE gel, were assessed using SAM and its diluted preparations. These same properties were then assessed following heating for 30 minutes at selected temperatures ranging from 45 -95 °C in order to assess the effect of heat treatment on these properties. The upper range of the temperature range selected is the highest temperature reported for potential denaturation of an almond protein isolate, as measured through differential scanning calorimetry (Amirshaghghi et al., 2017); the duration of 30 minutes was shown in preliminary experiments to induce changes across the temperature range.

Initially the proteins present in SAM were hydrophilic, as the fluorescence of the diluted preparation (0.18 mg/mL) assessed with hydrophobic probe ANS was low (**Figure 1B**) consistent with the high theoretical hydrophilicity of the amino acid sequence of amandin relative to other nut proteins. Specifically, the GRAVY value of Prunin-1, a major component of amandin, as calculated through the ProtParam tool (ExPASy, SIB Bioinformatics Resource Portal) using the amino acid sequence reported by Jin et al. (2009) is -1.125, where a more negative score indicates a higher hydrophilicity. In contrast, the 11S globulins of other nut proteins (cashew, pecan, pistachio and walnut; Pubmed references 12110836, 21718052, 15940148 and 27936684) range from -0.522 to -0.792. Previous studies have also found almond proteins to be hydrophilic, although the method applied and reference standards have varied, preventing direct comparison (Amirshaghghi et al., 2017; Li et al., 2018).

311 The ~~snape~~ of the CD spectrum (**Figure 1C**) resembled that obtained previously for both deaerated
 312 almond kernel powder and native purified amandin (Albillos et al., 2009; Li et al., 2018); with a
 313 strong positive peak at 193 nm and two minima at 208 nm and 222 nm, indicative of α -helical content.
 314 In addition, a signal at 216 nm was also present, typical of a β -sheet structure.

315 The hydrophobicity of the skim milk proteins increased significantly with incubation at temperatures
 316 greater than 55 °C ($p < 0.05$, **Figure 1B**), with a 2.45-fold increase in hydrophobicity occurring
 317 between 65 °C and 75 °C. This increase in surface hydrophobicity indicates the protein molecules are
 318 unfolding, exposing internal hydrophobic regions that are known to occur, for example, within the
 319 protein structure of amandin (Nakai, 1983). This increased hydrophobicity is also consistent with the
 320 thermal denaturation of almond protein extracts between 46-96 °C, as assessed by differential
 321 scanning calorimetry (Amirshaghghi et al., 2017; Li et al., 2018; Zhang et al., 2017).

322 Changes in secondary structure were observed by CD after heat treatment at temperatures > 65 °C.
 323 This heat induced change was most notable at 208 nm, a measure of alpha helical structure, where
 324 the ellipticity increased significantly ($p < 0.05$) at temperatures ≥ 75 °C (**Figure 1C and D**), indicating
 325 a decrease in alpha helical content, consistent with protein denaturation. The concentration of almond
 326 proteins employed here for analysis (0.18 mg/mL) was sufficiently low to prevent significant light
 327 scatter, as assessed by visual observation and **high-tension voltage during** CD measurements. Heating
 328 induced partial aggregation of the major almond proteins present in undiluted SAM (Figure 1D),
 329 which appeared less able to run through an SDS-PAGE gel after heat treatment at temperatures higher
 330 than 75 °C (**Figure 1E and Figure S1B, Supplementary Information**), with no protein observed
 331 within the gel at 95 °C. The greatest reduction in protein band intensity within the SDS PAGE gel
 332 occurred between 75 °C and 85 °C (**Figure 1E**). This temperature is slightly higher than the
 333 temperature at which the major changes in ANS fluorescence and secondary structure occurred (75
 334 °C), likely as these two methods are more sensitive to perturbations to the structure of amandin and
 335 other proteins that begin to occur at these more moderate temperatures.

Collectively these experiments using ANS fluorescence, circular dichroism and SDS-PAGE analysis, show that heat treatment at moderate temperatures (55 -75 °C) induces changes in the hydrophobicity and secondary structure of almond milk proteins, as the proteins unfolding become more hydrophobic, have less secondary structure and become less mobile within an SDS-PAGE gel. Heat treatment at higher temperatures (85-95 °C) induced more significant changes in each of these properties, consistent with more extensive denaturation of almond milk proteins.

3.3 Effect of heating on the functional properties of almond milk proteins

3.3.1 Heat stability and gelation of almond milk

The effect of heat treatment on the thermal stability and gelation of almond milk was investigated next. The ability of SAM and FFAM preparations to form self-supporting gels and the water holding capacity of these gels were assessed. The thermal stability of these preparations was also determined by sedimentation.

Visible aggregates were present along the wall of the tube at temperatures ≥ 75 °C and both SAM and FFAM preparations gelled at temperatures higher than 85 °C, after heating for 30 minutes followed by cooling to room temperature (**Figure 2A, Figure S2, Supplementary Information**).

Gel formation has previously been observed for purified amandin or partially purified almond protein isolates but not almond milk, although the protein concentrations tested were similar to those in the almond milk tested here. The heat treatment within these studies varied, as shown in **Table 2**, with differing temperature, time, heating rate and effective heat transfer area. The conditions reported to induce gelation potentially reflect both these different conditions but also differences in composition due to different extraction methods, buffers and pH. These experiments confirm, however, that proteins present in almond milk or more purified protein extracts can form a gel at high temperatures of 85 -100 °C, owing to extensive protein denaturation. Purified reduced basic polypeptides of amandin have also been shown to have a denaturation temperature of 62.5°C (Albillos et al., 2009).

360 The water holding capacity (WHC) of the gels formed by SAM in this study, as measured by
 361 centrifugation, was $72 \pm 2\%$; lower than dairy gels formed from 10% (w/v) whey protein ($\sim 100\%$
 362 WHC) but within the range of 10-15% (w/v) soy-whey protein mixtures (60-90% WHC) (Jose,
 363 Pouvreau, & Martin, 2016; Picone, Takeuchi, & Cunha, 2011). A higher WHC of $97.0 \pm 0.8\%$ was
 364 measured for FFAM gels formed under the same conditions, consistent with lipids increasing the gel
 365 strength and acting as filler particles in the matrix, reinforcing the protein gel network (Alzagat &
 366 Alli, 2002). Non-protein components are known to influence the gelation of almond proteins, as
 367 observed here. The presence of both fat and carbohydrate can also reduce the concentration of almond
 368 protein required for gelation. Almond protein concentrates ($\sim 90\%$ protein, 3% carbohydrates, wet
 369 basis), for example, were observed to gel at a minimum protein concentration of 22.5%, whereas
 370 almond flour (30% protein, 48% fat, 10% carbohydrate, wet basis) gelled at a lower minimum protein
 371 concentration of 4.2% (Yusuf, 2003). There are no prior reports of water holding capacity of these
 372 prior gels, however, to allow further comparison.

373 The thermal stability of almond milk after heat treatment was assessed by sedimentation analysis
 374 using a LUMifuge, where changes in the sample transmission are quantified as the proteins, fat and
 375 water separate during centrifugation. The SAM preparations were initially opaque due to light scatter
 376 (**Figure 2A**). Following centrifugation of the room temperature samples, there was a thin opaque
 377 layer at the surface and the sample was partially transparent (**Figure 2B**). Visual observation of the
 378 SAM preparations after centrifugation indicated flocculation at the meniscus, after moderate heat
 379 treatment at 55 °C, indicating protein instability with samples appearing clearer after sedimentation.
 380 In contrast, temperatures of 65 °C or higher induced sedimentation at the bottom of the tube. The
 381 thickness of this layer increased with higher temperatures. This sedimentation may be attributed to
 382 the greater extent of protein denaturation that occurs at higher temperatures, as seen in Section 3.2.
 383 Denaturation could affect the amandin protein and a range of polypeptides present in the sample
 384 (Albillos et al., 2009). This visual observation was also supported by changes in the integral
 385 transmission, a measure of the percentage of light transmitted across the LUMifuge tube, which

changes as a function of time (**Figure 2C**). While little change was observed in integral transmission for the unheated sample or samples heated to 45 °C, heating reduced the initial integral transmission of samples heated to 55 °C or higher (**Figure 2C**). Temperatures of 65 °C and above also lead to a much faster rate of change in integral transmission, indicating greater aggregation and lower protein stability. The rapid collapse of the gels formed by samples treated at 85 °C and 95 °C within the first minute of centrifugation also indicates a low gel strength.

FFAM behaved differently during sedimentation due to the presence of the fat. Centrifugation resulted in a thick opaque layer at the surface of the LUMifuge tube, due to creaming and the sample transparency increased for all samples (**Figure S2, Supplementary Information**). No sedimentation was observed at the bottom of the tube, in contrast to SAM and the almond proteins **unfolded and aggregated** by heat treatment at higher temperatures may associate with the **oleosomes** in these samples. Moderate heat treatments of 55 - 75 °C increased the separation rate, showing reduced stability in these samples, as occurred in SAM. The full fat gels formed at 85 °C and 95 °C were stable and did not collapse on centrifugation, indicating that the fat also provides greater strength to these gels.

The protein concentration (~3.5%) examined here, a further important variable in gelation, is similar to bovine milk and the total solids content resembles the concentration used to fortify milk for yoghurt production (see section 3.1). Previous studies have not observed almond milk gelation for full fat preparations when the protein concentration was lower (~ 1.4% c.f. ~3.5% here) and the same heat treatment was applied (85 °C for 30 minutes); there were no visual signs of gelation, although viscosity increased and phase separation was observed (Bernat et al., 2015), consistent with changes in protein structure and aggregation. These differences illustrate how temperature, fat and protein concentration each influence colloidal stability and can be used to induce or prevent almond milk gelation.

5.3.2 Effect of heating on particle size and microstructure

The size of particles in almond milk and extent of aggregation at each temperature was assessed through volume-based particle size distributions and confocal laser scanning microscopy (CLSM).

The microstructure of almond gels following gel formation was also assessed by CLSM.

The volume-based particle size distributions of SAM shown in **Figure 3A** exhibited a broad monomodal pattern with particles ranging from 40 nm to 8 μ m with a peak corresponding to particles with a diameter of $\sim$ 400 nm. CLSM also revealed a broad distribution of particle sizes (**Figure 3C**). The majority of the particles observed are much larger than the reported Stokes radius of amandin (6.6 nm) (Sathe, Wolf, Roux, Teuber, Venkatachalam, & Sze-Tao, 2002), **noting that this method sizing is unable to detect single particles less than 10 nm in size.** This data suggests that **a portion of the** almond proteins present have associated as globular clusters prior to heating. Protein clusters could be observed, stained green (**Figure 3C**). Fat stained red, was also observed to adhere to the surface of some particles (**Figure 3C**), potentially influencing particle association and size.

In the case of FFAM, the particle size distribution at 20 $^{\circ}$ C contained three peaks prior to heating, with the first peak at $\sim$ 405 nm, the second peak at $\sim$ 4 $\pm$ 1 μ m and the third peak at $\sim$ 100 $\pm$ 1 μ m (**Figure 3B**). The confocal micrographs indicate that the smaller $\sim$ 405 nm particles, stained in green, appear to be predominantly protein and resemble the particles seen in SAM. The larger $\sim$ 4 μ m particles, stained red, are predominantly fat, corresponding to almond oleosomes (**Figure 3D**), with an appearance similar to fat observed in previous microscopy studies of FFAM (Gallier et al., 2012). A significant mass of protein appears to adsorb at the oil body interface in the full fat samples (**Inset Figure 3D**). The third peak may be due to a small volume of particles from milling, such as soluble fibre or carbohydrate that may remain in the milk, these particles were not easily visible by confocal and may not be stained by the protein and fat specific dyes employed here. Interestingly, previous full fat almond protein preparations extracted under similar conditions contained particles with a different particle size distribution and appearance (Dhakal et al., 2016; Gallier et al., 2012),

suggesting that the extent of wet milling during extraction impacts on these properties and that these properties may be tuneable.

Heat treatment altered the appearance and size of proteins in the SAM samples. Low temperatures of 45 °C did not significantly change the particle size distribution (**Figure 3A**), while moderate temperatures of 55-75 °C led to the formation of two peaks, indicating an increase in the extent of aggregation. The proteins formed larger discrete clumps at 55 °C, lost their spherical globular shape and begin to form fractal like clusters at 65 °C and formed larger fractal aggregates at 75 °C (**Figure 3C**). The appearance of these larger aggregates at 75 °C corresponded with a much broader second peak in the particle size distribution at ~ 40 µm. These larger structures may influence the texture of gelled yoghurt-like almond products, as particles of this size are typically detected in sensory analysis, particularly when non-spherical in shape (Engelen, de Wijk, van der Bilt, Prinz, Janssen, & Bosman, 2005), potentially making it more difficult to achieve a smooth texture.

At higher temperatures of 85-95 °C, SAM formed a weakly flocculated particulate gel structure (**Figure 3C**). The protein network was highly porous, with asymmetric pores ranging from less than 1 µm to more than 10 µm in diameter that were filled with aqueous solution, consistent with the high-water holding capacity of these gels. Little fat could be observed in these low-fat preparations and the fat was likely incorporated within the protein network as it was not visible within the pores of the structure. The microstructure was similar for gels formed at higher temperatures of 85-95 °C, despite subtle differences measured in the hydrophobicity and aggregation of these samples by SDS-PAGE (**Figure 1**). The formation of these gels also prevented measurement of particle size distributions for these samples.

Heat treatment also changed the appearance and size of the structures present in FFAM. Subtle differences were noticed until 75 °C, where the second peak in the particle size distribution appeared to shift from $\sim 4 \pm 1$ µm to 18 ± 1 µm, with larger particles also present at $\sim 100 \pm 1$ µm. These changes coincided with the observation of larger aggregates (**Figure 3D**). In addition, a significant

decrease in the peak intensity at 405 nm occurred due to a reduction in the number of native globular clusters of water-soluble almond protein. These results indicate that incubation at a temperature of 75 °C significantly reduced the stability of water-soluble proteins, potentially due to hydrophobic association between these proteins and also between proteins and oleosins, resulting in large aggregates. Higher temperatures of 85-95 °C lead to gel formation, where the protein formed an extensive protein network, more continuous than the network formed at 75 °C. Some of the almond fat was incorporated into the protein network, while other fat droplets appeared loosely associated with the network or contained within large pores within the network. Interestingly, the fat droplets associated with the network appeared similar in size to the fat droplets within the unheated milk preparation, indicating that there was little coalescence of fat and that oleosins were comparatively much more stable at higher temperatures than the water-soluble almond proteins. Overall, the distribution of fat appeared quite heterogeneous, this is particularly evident in the images collected at 95 °C, where there were pockets more than 50 µm across containing numerous fat droplets. The networks of these full fat gels appear to have greater porosity and larger pore sizes than the skim milk gels, despite the higher solids content, potentially due to network disruption by the presence of the almond fat. Homogenisation of the fat present in these samples may reduce this variation and heterogeneity and could also be used alter the properties of the gels formed following thermal treatment.

The differences in particle size and gel microstructure observed by CLSM, as a function of temperature and between skim and full fat almond preparations, are expected to impact on the functional properties of these preparations, including the rheological properties.

3.3.3 Rheological analysis

The critical gelation temperature (T_G), as indicated by the crossover of G' over G'' , was ~ 87.5 °C for SAM containing 3.6% protein, with the G' value being ~ 0.2 Pa (**Figure 4**). The formation of a gel at ~85 °C is consistent with the temperature observed by sample inversion (**Figure 2A**) or confocal

microscopy (**Figure 5C and D**) and the extensive denaturation observed at 85-95 °C in Section 3.2, initiating gelation.

The elastic modulus was initially very low at the point of gelation for SAM but increased sharply, reaching a final value of 11.4 ± 0.8 Pa at a temperature of 95 °C. Interestingly, the gelation temperature T_G and gel strength measured here are close to those measured for heat induced dairy micellar casein gels at a similar protein concentration and pH (~4% and pH 6.2; $T_G \sim 88$ °C and gel strength ranging from ~ 0.1 to 10 Pa (Kharlamova, Nicolai, & Chassenieux, 2019)). Given this similarity, it would be interesting to further explore the potential interaction between micellar casein and almond proteins when used in combination to form a heat induced gel, as such networks may allow partial replacement of milk proteins and the formation of mixed protein products with complementary nutritional profiles. Further, this gelling ability of almond milk might also reduce the need for addition of **comparatively cheaper** starch based gelling agents that are often added to structure **low protein** vegan gelled products. **Thus, high protein, clean label products can potentially be obtained, although, at the expense of increased product cost.**

At a higher protein concentration of 10.8%, T_G was reduced to ~ 83 °C with a $G' \sim 0.4$ Pa, similar to the concentration dependence observed previously with other proteins, although the sensitivity to protein concentration is known to differ between protein systems (Kaspchak et al., 2017; Kharlamova et al., 2019). The change in elastic modulus as a function of temperature was also more rapid for 10.8% almond protein suspension, with samples reaching a final G' of 280 ± 70 Pa at a temperature of 95 °C ~ 25 times higher than the elastic modulus measured for 3.6% almond protein. The gel strength of these almond protein gels at 90 °C (29 ± 14 Pa) is also quite similar to soy proteins at a similar protein concentration (~ 40 Pa) but higher than the strength measured for pea proteins (~15 Pa) (Silva, Cochereau, Schmitt, Chassenieux, & Nicolai, 2019).

The gelation temperature and moduli were higher but also more variable for FFAM samples (**Figure S3, Supplementary Information**) and a range of crossover temperatures (69-74 °C) and moduli

(1200–2000 Pa) were obtained. The higher moduli of these samples are consistent with their stability when centrifuged (section 3.3). The variability measured may arise from the more heterogeneous microstructure of these gels, where the fat was observed to be unevenly distributed, as can be seen in the CLSM micrographs of full fat milk gels (**Figure 3D**); this property may be modified by the homogenisation of the FFAM samples prior to gelation. The temperature of gelation measured by rheology is slightly lower than observed by inversion (i.e. 69–74 °C c.f 85 °C, section 3.3), illustrating ability of rheological analysis to detect the formation of a protein network observed by CLSM at 75 °C, which is less continuous than at high temperatures of 85 °C (**Figure 3D**).

4. Conclusion

This study provides a better understanding of the heat sensitivity of almond milk proteins and reveals temperatures that are critical to almond milk processing for a variety of applications, including heat treatment to induce denaturation prior to almond yoghurt formation or heat-induced gelation to form products like almond tofu/cheese. Heating at moderate temperatures between 55–75 °C induced denaturation and partial aggregation. Higher temperatures of 85–95 °C induced more extensive denaturation and gelation and formed a self-supporting weakly flocculated particulate gel structure. The critical gelation temperature of skim almond milk, measured by rheology, was also ~ 87.5 °C. The continuous protein network formed by this gelled almond milk featured asymmetric pores and small amounts of integrated fat. These gels had high (~70%) water holding capacity and a gel strength similar to dairy gels (11.4 ± 0.8 Pa), although particles with size ≥ 30 μm at 75 °C may influence texture and result in ‘chalkiness’; the gels also collapsed rapidly on centrifugation. In contrast, full fat almond milk samples formed stronger gels with a higher but more variable gel modulus, likely due to the greater porosity of the gel network and more heterogeneous distribution of fat. The gel strengths observed indicate almond proteins can act as a base for development of vegan gelled products potentially without starch thickeners. Higher protein concentrations (10.8%) reduced the temperature of skim milk gelation to ~ 83 °C and increased the elastic modulus ~ 25-fold, suggesting that concentrating skim almond milk, together with homogenisation of almond fat in high fat milk

preparations may offer a route to optimise and tailor the texture of minimally processed, neat treated almond milk products. Almond proteins could also be combined with other plant or dairy proteins to fully or partially replace dairy proteins.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

- Albillos, S. M., Menhart, N., & Fu, T. J. (2009). Structural stability of Amandin, a major allergen from almond (*Prunus dulcis*), and its acidic and basic polypeptides. *Journal of Agricultural and Food Chemistry*, 57(11), 4698-4705. <https://doi.org/10.1021/jf803977z>.
- Alizadeh-Pasdar, N., & Li-Chan, E. C. Y. (2000). Comparison of protein surface hydrophobicity measured at various pH values using three different fluorescent probes. *Journal of Agricultural and Food Chemistry*, 48(2), 328-334. <https://doi.org/10.1021/jf990393p>.
- Alzagat, A. A., & Alli, I. (2002). Protein-lipid interactions in food systems: a review. *International Journal of Food Sciences and Nutrition*, 53(3), 249-260. <https://doi.org/10.1080/09637480220132850>.
- Amirshaghghi, Z., Rezaei, K., & Rezaei, M. H. (2017). Characterization and functional properties of protein isolates from wild almond. *Journal of Food Measurement and Characterization*, 11(4), 1725-1733. <https://doi.org/10.1007/s11694-017-9553-y>.
- AOAC. (1995). *Official methods of analysis of AOAC International*. Gaithersburg, MD, USA.

- Beisson, F., Ferte, N., Bruely, S., Vouloutoury, K., Verger, K., & Arondelet, V. (2001). Oil-bodies as substrates for lipolytic enzymes. *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids*, 1531(1-2), 47-58. [https://doi.org/10.1016/S1388-1981\(01\)00086-5](https://doi.org/10.1016/S1388-1981(01)00086-5).
- Bernat, N., Chafer, M., Rodriguez-Garcia, J., Chiralt, A., & Gonzalez-Martinez, C. (2015). Effect of high pressure homogenisation and heat treatment on physical properties and stability of almond and hazelnut milks. *LWT-Food Science and Technology*, 62(1), 488-496. <https://doi.org/10.1016/j.lwt.2014.10.045>.
- Callewaert, D., Festjens, G., & Neiryneck, N. (2014). Almond drinks and methods for their production. In E. P. Office (Ed.), *Google Patents* (Vol. EP2476317B1). European Union: Alpro Comm Va.
- Che, H., Zhang, Y., Lyu, S.-C., Nadeau, K. C., & McHugh, T. (2018). Identification of almond (*Prunus dulcis*) vicilin as a food allergen. *Journal of Agricultural and Food Chemistry*, 67(1), 425-432. <https://doi.org/10.1021/acs.jafc.8b05290>.
- Costa, J., Mafra, I., Carrapatoso, I., & Oliveira, M. B. (2012). Almond allergens: molecular characterization, detection, and clinical relevance. *Journal of Agricultural and Food Chemistry*, 60(6), 1337-1349. <https://doi.org/10.1021/jf2044923>.
- Day, L. (2013). Proteins from land plants—potential resources for human nutrition and food security. *Trends in Food Science & Technology*, 32(1), 25-42. <https://doi.org/10.1016/j.tifs.2013.05.005>.
- Detloff, T., Sobisch, T., & Lerche, D. (2006). Particle size distribution by space or time dependent extinction profiles obtained by analytical centrifugation. *Particle & Particle Systems Characterization*, 23(2), 184-187. <https://doi.org/10.1002/ppsc.200601028>.
- Dhakal, S., Giusti, M. M., & Balasubramaniam, V. M. (2016). Effect of high pressure processing on dispersive and aggregative properties of almond milk. *Journal of the Science of Food and Agriculture*, 96(11), 3821-3830. <https://doi.org/10.1002/jsfa.7576>.

- Engelen, L., de Wijk, R. A., van der Bilt, A., Prinz, J. F., Janssen, A. M., & Bosman, F. (2005).
Relating particles and texture perception. *Physiology & Behavior*, 86(1-2), 111-117.
<https://doi.org/10.1016/j.physbeh.2005.06.022>.
- Gallier, S., Gordon, K. C., & Singh, H. (2012). Chemical and structural characterisation of almond
oil bodies and bovine milk fat globules. *Food Chemistry*, 132(4), 1996-2006.
<https://doi.org/10.1016/j.foodchem.2011.12.038>.
- Jin, T., Albillos, S. M., Guo, F., Howard, A., Fu, T. J., Kothary, M. H., & Zhang, Y. Z. (2009). Crystal
structure of prunin-1, a major component of the almond (*Prunus dulcis*) allergen amandin.
Journal of Agricultural and Food Chemistry, 57(18), 8643-8651.
<https://doi.org/10.1021/jf9017355>.
- Jose, J., Pouvreau, L., & Martin, A. H. (2016). Mixing whey and soy proteins: Consequences for the
gel mechanical response and water holding. *Food Hydrocolloids*, 60, 216-224.
<https://doi.org/10.1016/j.foodhyd.2016.03.031>.
- Kaspchak, E., Oliveira, M. A. S., Simas, F. F., Franco, C. R. C., Silveira, J. L. M., Mafra, M. R., &
Igarashi-Mafra, L. (2017). Determination of heat-set gelation capacity of a quinoa protein
isolate (*Chenopodium quinoa*) by dynamic oscillatory rheological analysis. *Food Chemistry*,
232, 263-271. <https://doi.org/10.1016/j.foodchem.2017.04.014>.
- Kharlamova, A., Nicolai, T., & Chassenieux, C. (2019). Heat-induced gelation of mixtures of casein
micelles with whey protein aggregates. *Food Hydrocolloids*, 92, 198-207.
<https://doi.org/10.1016/j.foodhyd.2019.01.048>.
- Kshirsagar, H. H., Fajer, P., Sharma, G. M., Roux, K. H., & Sathe, S. K. (2011). Biochemical and
spectroscopic characterization of almond and cashew nut seed 11S legumins, amandin and
anacardein. *Journal of Agricultural and Food Chemistry*, 59(1), 386-393.
<https://doi.org/10.1021/jf1030899>.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of
bacteriophage T4. *Nature*, 227(5259), 680-685. <https://doi.org/10.1038/227680a0>.

- 612 Li, S. G., Chu, S., Lu, J. K., Wang, P., & Ma, M. H. (2018). Molecular and structural properties of
 613 three major protein components from almond kernel. *Journal of Food Processing and*
 614 *Preservation*, 42(3), e13536. <https://doi.org/10.1111/jfpp.13536>.
- 615 Nakai, S. (1983). Structure-Function-Relationships of Food Proteins with an Emphasis on the
 616 Importance of Protein Hydrophobicity. *Journal of Agricultural and Food Chemistry*, 31(4),
 617 676-683. <https://doi.org/10.1021/jf00118a001>.
- 618 Narayana, M. N. K. N., & Gupta, V. K. (2016). Quality of Plain Set Yoghurt as affected by Levels of
 619 Ultrafiltration Concentration of Milk and Inoculum of Yoghurt Culture. *Turkish Journal of*
 620 *Agriculture-Food Science and Technology*, 4(6), 508-514.
 621 <https://doi.org/10.24925/turjaf.v4i6.508-514.685>.
- 622 Nicolai, T., & Durand, D. (2013). Controlled food protein aggregation for new functionality. *Current*
 623 *Opinion in Colloid & Interface Science*, 18(4), 249-256.
 624 <https://doi.org/10.1016/j.cocis.2013.03.001>.
- 625 Ong, L., Dagastine, R. R., Kentish, S. E., & Gras, S. L. (2011). Microstructure of milk gel and cheese
 626 curd observed using cryo scanning electron microscopy and confocal microscopy. *LWT-Food*
 627 *Science and Technology*, 44(5), 1291-1302. <https://doi.org/10.1016/j.lwt.2010.12.026>.
- 628 Picone, C. S. F., Takeuchi, K. P., & Cunha, R. L. (2011). Heat-Induced Whey Protein Gels: Effects
 629 of pH and the Addition of Sodium Caseinate. *Food Biophysics*, 6(1), 77-83.
 630 <https://doi.org/10.1007/s11483-010-9177-9>.
- 631 Sathe, S. K. (1992). Solubilization, electrophoretic characterization and in vitro digestibility of
 632 almond (*Prunus amygdalus*) proteins 1, 2. *Journal of Food Biochemistry*, 16(4), 249-264.
- 633 Sathe, S. K., & Sze, K. W. C. (1997). Thermal aggregation of almond protein isolate. *Food Chemistry*,
 634 59(1), 95-99. [https://doi.org/10.1016/S0308-8146\(96\)00180-X](https://doi.org/10.1016/S0308-8146(96)00180-X).
- 635 Sathe, S. K., Wolf, W. J., Roux, K. H., Teuber, S. S., Venkatachalam, M., & Sze-Tao, K. W. (2002).
 636 Biochemical characterization of amandin, the major storage protein in almond (*Prunus dulcis*

- 637 L.). *Journal of Agricultural and Food Chemistry*, 50(15), 4333-4341.
 638 <https://doi.org/10.1021/jf020007v>.
- 639 Sethi, S., Tyagi, S. K., & Anurag, R. K. (2016). Plant-based milk alternatives an emerging segment
 640 of functional beverages: a review. *Journal of Food science and Technology*, 53(9), 3408-
 641 3423. <https://doi.org/10.1007/s13197-016-2328-3>.
- 642 Sharma, G. M., Su, M., Joshi, A. U., Roux, K. H., & Sathe, S. K. (2010). Functional properties of
 643 select edible oilseed proteins. *Journal of Agricultural and Food Chemistry*, 58(9), 5457-5464.
 644 <https://doi.org/10.1021/jf1002446>.
- 645 Silva, J. V. C., Cochereau, R., Schmitt, C., Chassenieux, C., & Nicolai, T. (2019). Heat-induced
 646 gelation of mixtures of micellar caseins and plant proteins in aqueous solution. *Food Research*
 647 *International*, 116, 1135-1143. <https://doi.org/10.1016/j.foodres.2018.09.058>.
- 648 Valencia-Flores, D. C., Hernandez-Herrero, M., Guamis, B., & Ferragut, V. (2013). Comparing the
 649 effects of ultra-high-pressure homogenization and conventional thermal treatments on the
 650 microbiological, physical, and chemical quality of almond beverages. *Journal of Food*
 651 *Science*, 78(2), E199-205. <https://doi.org/10.1111/1750-3841.12029>.
- 652 Vanga, S. K., & Raghavan, V. (2018). How well do plant based alternatives fare nutritionally
 653 compared to cow's milk? *Journal of Food Science and Technology*, 55(1), 10-20.
 654 <https://doi.org/10.1007/s13197-017-2915-y>.
- 655 Weber, E., & Neumann, D. (1980). Protein Bodies, Storage Organelles in Plant Seeds. *Biochemie und*
 656 *Physiologie der Pflanzen*, 175(4), 279-306. [https://doi.org/10.1016/S0015-3796\(80\)80070-9](https://doi.org/10.1016/S0015-3796(80)80070-9).
- 657 Wolf, W. J., & Sathe, S. K. (1998). Ultracentrifugal and polyacrylamide gel electrophoretic studies
 658 of extractability and stability of almond meal proteins. *Journal of the Science of Food and*
 659 *Agriculture*, 78(4), 511-521. [https://doi.org/10.1002/\(Sici\)10970010\(199812\)78:4<511::Aid-](https://doi.org/10.1002/(Sici)10970010(199812)78:4<511::Aid-Jsfa148>3.0.Co;2-X)
 660 [Jsfa148>3.0.Co;2-X](https://doi.org/10.1002/(Sici)10970010(199812)78:4<511::Aid-Jsfa148>3.0.Co;2-X).

- Yada, S., Lapsley, K., & Huang, G. W. (2011). A review of composition studies of cultivated almonds: Macronutrients and micronutrients. *Journal of Food Composition and Analysis*, 24(4-5), 469-480. <https://doi.org/10.1016/j.jfca.2011.01.007>.
- Yusuf, A. A. (2003). Physico-chemical properties of almond seed protein concentrate. *Italian Journal of Food Science*, 15(4), 531-539.
- Zhang, Q. A., Zhang, X. Y., Feng, Y. L., & Shi, F. F. (2017). Compositions and physicochemical properties of sweet almond isolate proteins. *Scientia Agricultura Sinica*, 50(13), 2564-2575.

FIGURE CAPTIONS

Figure 1. (A) Non reducing SDS-PAGE analysis of proteins in skim almond milk (Lane 2) on a 4-12% gradient gel. Molecular weight ladder is included in Lane 1. Numbers on the right side of the image indicate molecular weights of respective bands. Based on the appearance of the bands on the gel, proteins were grouped into 3 regions of interest: 46-66 kDa, 35-38 kDa and 25-28 kDa. **(B)** Changes in the hydrophobicity index as a function of incubation at different temperatures for 30 minutes. **(C)** Circular dichroism spectra of skim milk stored at 20 °C(—) or heated to 75 °C(--) for 30 minutes. **(D)** Change in ellipticity at 208 nm as a function of incubation at different temperatures for 30 minutes, derived from CD spectra. **(E)** Heat induced changes in intensities of regions of interest detected on an SDS-PAGE gel as a function of incubation at different temperatures for 30 minutes, measured by densitometry and Image J. The error bars in A, C and E represent the standard deviation (n=3) of the mean for three replicate heating experiments.

Figure 2. Heat stability and gelation of skim almond milk: visual observation of the occurrence of gel formation (the point when milk ceases to flow upon tube inversion) in skim almond milk **(A)**. Images of LUMifuge tubes following sedimentation **(B)** and change in the integral transmission over time as a function of temperature for unheated and heat-treated skim almond milk **(C)** during

centrifugation in LUMiuge at 2500 g for 4 hours. Each data series is a representative of three replicates, performed on three independent milk samples.

Figure 3. Effect of heating on the particle size and microstructure of skim almond milk (SAM) and full fat almond milk (FFAM). Particle size distribution of SAM samples (A) and FFAM samples (B). Particle size distributions could not be collected for samples heated at 85 °C or 90 °C, which formed gels. Error bars in the particle size distribution represent the standard deviation of the mean (n=3). Two-dimensional confocal laser scanning microscopy images for SAM samples (C) and FFAM samples (D) before and after heat treatment for 30 minutes. Treatment temperatures are indicated at the top of every image. The unheated SAM and FFAM control samples (20 °C), both contain an inset, where a digitally zoomed image, with a scale bar of 2 μ m is shown. For heated samples, all measurements were performed after samples were cooled to 20 °C. Fat globules stained with Nile Red appear red and protein particles stained with Fast Green FCF appear green. The scalebar is 25 μ m in length for all confocal images other than the insets.

Figure 4. Temperature dependence of the gelation of skim fat almond milk (3.6% protein) and concentrated skim almond milk (10.8% protein) upon heating from 25 °C to 95 °C at 3 °C/minute at a shear strain of 0.1% with a frequency of 1 Hz. The G' and G'' are shown as a function of temperature. The graph is representative of three replicates at each concentration performed on two independent milk samples. The inset on the right highlights the significant changes occurring in the temperature range of 80 to 95 °C.

Credit author statement

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Heat induced denaturation, aggregation and gelation of almond proteins in skim and full fat almond milk

Highlights

Almond milk stability decreased with increasing temperature.

Moderate temperatures between 55-75 °C induced protein denaturation and partial aggregation.

Almond milk with ~ 3.5 % protein gelled at 87.5 °C forming a protein network.

Full fat gels were stronger but the fat distribution was heterogeneous.

Almond proteins can act as a base for the development of vegan gelled products.