O/W emulsions stabilised by solid lipid particles

Zafeiri, Ioanna; Smith, Paul; Norton, Ian T.; Spyropoulos, Fotis

DOI:
10.1016/j.colsurfa.2020.124916

Document Version
Peer reviewed version

Citation for published version (Harvard):
Zafeiri, I, Smith, P, Norton, IT & Spyropoulos, F 2020, 'O/W emulsions stabilised by solid lipid particles: understanding how the particles' Pickering functionality can be retained post their dehydration and subsequent rehydration', *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, vol. 599, 124916. https://doi.org/10.1016/j.colsurfa.2020.124916

O/W emulsions stabilised by solid lipid particles: Understanding how the particles' Pickering functionality can be retained post their dehydration and subsequent rehydration

Ioanna Zafeiri [a,*], Paul Smith [b], Ian T. Norton [a], Fotis Spyropoulos [a]

[a]School of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

[b]Cargill, R&D Centre Europe, Havenstraat 84, B-1800 Vilvoorde, Belgium

*Corresponding author.
Email address: I.Zafeiri@bham.ac.uk

Abstract

Pickering particles have been extensively shown to hold an immense potential as emulsion stabilisers. Lipid-based particles in particular, are increasingly studied as edible Pickering structures. The aim of this work was to investigate whether specific formulation parameters that are key for the fabrication of lipid particles with a Pickering functionality can also impact upon the ability of these structures to withstand drying and subsequent rehydration events, without loss to their original capacity to provide stable o/w emulsions. The formulation parameters studied here included the type of the lipid source, type and concentration of the surface active species and type and concentration of cryoprotectants used. Freeze-drying was used as the process for the dehydration of the fabricated lipid particles, while two methods for the subsequent rehydration of these structures were tested. It is demonstrated that lipid particles fabricated using sodium caseinate during the melt-emulsification step can maintain their original Pickering functionality even following lyophilisation; i.e., once reconstituted, they provide stable o/w emulsions with the same droplet sizes as those produced by their precursors (lipid particles that haven't undergone the freezing and desiccation stages). In contrast, a typical small molecular weight surfactant (Tween 80) does not exhibit the same functionality as the protein, and lipid particle agglomeration following drying and reconstitution was unavoidable, even in the presence of traditional drying aids (cryoprotectants) in the formulation. Parameters relating to the drying and rehydration stages (drying kinetics, final moisture content and storage conditions) were also studied, but their impact upon the Pickering functionality of the reconstituted lipid particles was deemed secondary to the effects brought upon by changes to formulation elements.

Overall, the present work advances the current limited understanding on formulation approaches that enable the conservation of the Pickering functionality of the lipid particles following their drying and rehydration, in particular offering insight into the contribution of the specific emulsifier employed during particle fabrication.

Keywords:

Freeze-drying, solid lipid particles, protein, Pickering emulsions, cryoprotectants, emulsion stability

1. Introduction

Stabilisation of emulsions by particles of colloidal dimensions (Pickering stabilisation) that have been constructed from food grade sources has latterly been an energetic field of research, with a number of industrial applications, including several in the food and pharmaceutical sectors. Proteins, biopolymers and lipids are the most commonly investigated edible building blocks for these particulate stabilisers (Pichot, Duffus, Zafeiri, Spyropoulos, & Norton, 2014; Dickinson, 2016). With respect to lipid-based particles in particular, considerable share of research effort has been directed towards improving the physical and chemical stability and Pickering functionality of these solid lipid nanoparticles (SLN) within their host environment. The latter predominantly tends to be of an aqueous nature as the majority of published research has been conducted on water-continuous systems containing lipid particles (Gupta & Rousseau, 2012; Pawlik, Kurukji, Norton, & Spyropoulos, 2016).

Despite this, current literature somewhat lacks studies focusing on the removal/isolation of these lipid structures from their aqueous surrounding (dehydration) and whether subsequent reintroduction of water (rehydration) can easily result in the recovery of their original Pickering functionality. Understanding the dehydration/rehydration behaviour of these particulates will not only allow for greater flexibility in their use as food ingredients, but also prolong their shelf-life by halting microbial growth and ultimately assisting in their storage, distribution, and handling (Marefati, Rayner, Timgren, Dejmek, & Sjöö, 2013). However, the retention of particle performance following dehydration and later rehydration of such delicate structures is extremely problematic (Mehnert & Mäder, 2001). The challenges involved are a direct result of the dehydration process itself (e.g. high temperatures) and the close proximity between the (dehydrated) particles, brought up by the drastic reduction in the amount of solvent/water in the system. Unfavourable processing conditions (during dehydration) and uncontrolled particle-particle contacts (post-dehydration), can encourage/promote physical and chemical modification(s) to the particles, which then, upon rehydration, fail to recover their original microstructure and/or (Pickering) performance (Abdelwahed, Degobert, Stainmesse, & Fessi, 2006; Morais, *et al.*, 2016).

Over the last couple of decades, freeze-drying (lyophilisation) has been used by industry for the convenient production of solid forms or the stabilisation and preservation of perishable materials (Liu, Zhao, & Feng, 2008; Kasper, Winter, & Friess, 2013). Specifically in the case of SLN, lyophilisation has emerged as the preferred route for dehydration. Literature suggests that lyophilisation of SLN can improve their storage stability by inhibiting chemical degradation (e.g. hydrolysis) and Ostwald ripening (Mehnert, *et al*., 2001; Mishra, Dhote, Bhatnagar, & Mishra, 2012). Lyophilisation has also been suggested to improve the tolerance of these structures to temperature changes that may occur during transportation, as well as enhance their incorporation into tablets, pellets or capsules (Mehnert, *et al.*, 2001). Freeze-drying operation is based on the principle of lowering the system's temperature to subzero values, where the solvent or suspending medium (in this case water) crystallises, and subsequently allowing the frozen aqueous component to be removed by sublimation (direct transition from a solid to a vapour phase) and desorption under vacuum (Abdelwahed,

Degobert, Stainmesse, *et al.*, 2006; Ratti, 2013). Lyophilisation consists of three stages, namely freezing, primary drying and secondary drying, and therefore a plethora of processing parameters need to be controlled/optimised so that the resulting dehydrated product meets specific quality standards; (e.g. high yield, residual water content, structure and activity) (Liu, Zhao, & Feng, 2008).

In addition to processing elements, formulation aspects associated with the original "hydrated" structure must be tuned so that the lyophilisate can withstand the mechanical stresses involved during the freezing and desiccation steps; damage that is mainly associated with ice formation. A large body of literature reports on numerous compounds screened for the protective capacity (cryoprotectants) that they can provide to various structures (e.g. yeast cells, lactic acid bacteria, etc.) undergoing lyophilisation; polysaccharides, disaccharides, amino acids, polyols and honey are amongst such agents that have been reported (de Valdez, de Giori, de Ruiz Holgado, & Oliver, 1983; Champagne, Gardner, Brochu, & Beaulieu, 1991; Abadias, Benabarre, Teixidó, Usall, & Viñas, 2001; Soltanizadeh, *et al*., 2014). With regards to freeze-drying of nanoparticles, sugars such as sucrose, mannitol, trehalose and glucose are the most commonly investigated cryoprotective agents, with their effect (in the case of emulsified systems) being accentuated when added in concentrations from 5-20% (Morais, *et al.*, 2016). Two main mechanisms have been suggested in order to explain the manner by which these compounds impart stabilisation upon freeze-drying. The first one proposes that cryoprotectants form a glassy matrix that encloses the lyophilisate (vitrification theory), thus maintaining a sufficient distance from its close particulate neighbours (hindering aggregation) and also abating any mechanical damage caused by ice crystal growth (Abdelwahed, Degobert, Stainmesse, *et al.*, 2006). The second mechanism is relevant to systems containing surface active agents and postulates that hydroxyl groups present in the molecular structure of specific cryoprotectants can substitute water species during the course of drying. Using the example of lecithin, it has been proposed (Zhang, Liu, Qian, and Chen (2008)) that such replacement compensates for water loss during drying and essentially acts similarly to a hydrated layer preventing lecithin layers from coming close enough and fusing (which would result in a size increase).

Further to process control and incorporation of cryoprotective species, there is also scope to explore the role that formulation components used in the fabrication of hydrated SLN (such as emulsifiers/surfactants) can have in terms of supporting the recovery of particle functionality following transition to a dehydrated and consequently to a rehydrated state yet again. In contrast to formulation species with functionalities that only emerge during dehydration (e.g. as in the case of cryoprotectants), formulation components referred to here already possess a very specific microstructural function within SLN systems in the hydrated state (Zafeiri, Norton, Smith, Norton, & Spyropoulos, 2017), but could then, during dehydration and rehydration events, also provide additional (in the case of lyophilisation "cryoprotective-like") advantages enabling the particulate species to revive their original (pre-dehydration) performance. To the best knowledge of the authors, such dual formulation functionality has not been previously reported for SLN undergoing lyophilisation. However, comparisons can be drawn from a limited number of studies in the area of emulsions, where formulation design has been analogously employed to enable the subjection of oil droplets (as opposed to particles) dispersed within an aqueous continuous phase to a lyophilisation/spray-drying process with

minimal impact on their original microstructure upon rehydration (Adelmann, Binks, & Mezzenga, 2012; Marefati, *et al*., 2013; Hu, Marway, Kasem, Pelton, & Cranston, 2016). Two of these studies focus on food-grade particle-stabilised Pickering emulsions and highlight the vital role of the nature and properties of the stabilising material in the recovery of emulsion functionality following a dehydration stage. In more detail, Marefati, *et al.* (2013) investigated the manufacture of oil-filled powders from the dehydration of quinoa starch (OSA-chemically modified) stabilised Pickering oil-in-water emulsions. The authors found that partial gelatinised starch granules were able to preserve the original emulsion template throughout a freeze-drying process, with only minor levels of aggregation observed in the reconstituted emulsions. In that vein, complexation of tannic acid (added after emulsification) with cellulose derivatives (methyl cellulose or hydroxyethyl cellulose) already stabilising oil-in-water emulsions, was also shown to promote condensation of the cellulosic "shell" around the oil droplets, allowing the generation of solid dry emulsions that were then easily redispersed in water (Hu, *et al.*, 2016).

The present study focuses on lipid-based nanoparticulate structures and investigates how formulation parameters (e.g. lipid source and type and concentration of surface active species), previously demonstrated to be crucial for particle fabrication (Zafeiri, Norton, *et al.*, 2017) and subsequent Pickering functionality (Zafeiri, Smith, Norton, & Spyropoulos, 2017) can impact on the ability of these systems to withstand a harsh dehydration process and regain their microstructure and Pickering performance upon reconstitution. Edible lipid particles from two discrete lipid sources (either a pure monoacid triglyceride (tristearin) or a model wax (cetyl palmitate)), in the presence of two different types of amphiphilic species (either Tween 80 (surfactant) or sodium caseinate (protein)), were initially subjected to lyophilisation over different timescales and then rehydrated following two reconstitution methods of varied energy input. Lipid particle microstructure characteristics such as size and size distribution, interfacial behaviour, as well as their ability to stabilise oil-in-water emulsions (Pickering functionality) were studied before and after lyophilisation/rehydration. Dried powders were reconstituted either immediately after particle formation or following a storage period (under cold or ambient temperature conditions) of up to one month. The use of typical cryoprotectants was also studied to determine whether particle microstructure along with Pickering functionality can be maintained following freeze-drying. Retention of the lipid particles' microstructure and Pickering performance were linked to both formulation (type of lipid and type/concentration of surface active component) and processing (drying time, storage time, reconstitution method) parameters.

2. Materials & Methods

2.1. Materials

Microcrystalline glyceryl tristearate (Dynasan® 118) (tristearin hereafter) and cetyl palmitate were gifted from IOI Oleo (IOI Oleochemicals GmbH, Hamburg, Germany) and Gattefossé (France) respectively. Surface active components polyoxyethylene sorbitan monooleate (Tween 80) and casein sodium salt from bovine milk were purchased from Sigma-Aldrich

(Sigma-Aldrich, UK). High molecular weight hydrocolloids (hydroxypropyl)methyl cellulose (HMPC) (M_{HPMC}≅86 kDa) and low-methoxylated citrus pectin DE (LM-pectin, GENU® pectin type LM-104 AS) were purchased from Sigma-Aldrich (Sigma-Aldrich, UK) and CP Kelco (Copenhagen, Denmark) respectively. The disaccharide cryoprotectants D-(+)-trehalose dihydrate ($C_{12}H_{26}O_{13}$; molecular weight:378.33 g/mol; ≥98.5% purity) and sucrose ($C_{12}H_{22}O_{11}$;molecular weight:342.30 g/mol; ≥99.5% purity) were supplied by Fisher Scientific and Sigma respectively. A range of commercially available maltodextrins were also tested as drying aids during the dehydration process. To this end, maltodextrins of increasing dextrose equivalent values, i.e. decreasing molecular weights, were screened. Maltodextrin (DE 4-7) was provided by Sigma-Aldrich (Sigma-Aldrich, UK) and corn maltodextrin (DE 17.9, C*Dry MD 01915) was gifted by Cargill (Haubourdin, France). Commercial sunflower oil was used for all emulsions. Double distilled water from Milli-Q systems (Millipore, Watford, UK) was used throughout.

2.2. Methods

2.2.1. Particles preparation

Solid lipid micro- and nano- particles were produced via a melt-emulsification method, as described elsewhere (Zafeiri, Norton, *et al.*, 2017) . Firstly, an o/w emulsion is prepared at a temperature above the melting point of the lipid in the presence of surface active agents (Tween 80 or sodium caseinate) and using ultrasonication (2 minutes at 95% amplitude). The emulsion is then cooled resulting in the formation of discrete lipid particles. Final dispersions contained 2.5% (wt/wt) lipid with varying concentrations of surface active components. The protein was used at its native state (pH ~7.01).

Solid wax particles were also fabricated using 2% (wt/wt) (hydroxypropyl)methyl cellulose (HMPC) and low methoxyl pectin (LMP) as alternative to sodium caseinate high molecular weight species. Samples were subjected to lyophilisation and then characterised for their mean size.

2.2.2. Lyophilisation of lipid particles

Lyophilisation was undertaken using a bench top freeze dryer, Scanvac model 110-4 (Copenhagen, Denmark). Particulate dispersions were first cooled to -20 ºC overnight before being transferred to the freeze dryer. The freeze-drying process was carried out at -110 ºC under constant vacuum (0.100 hPa) for 48 hours. Post-lyophilisation, powders were collected, weighed and rehydrated at room temperature using the same volume of water (deionised) removed by the drying process. Particles were redispersed, via agitation supplied by vortex mixer for 30 seconds, followed by probe ultrasound treatment (Sonics & Materials, Inc., CT, USA) operating at 95% amplitude for approximately 20 seconds.

2.2.3. Effect of cryoprotectants

Solid lipid particle dispersions (2.5 and 5% (wt/wt)) formed in the presence of Tween 80 (0.8 and 2% (wt/wt)) were mixed with 2.5, 5 and 10% (wt/wt) aqueous cryoprotectant solutions (sucrose, trehalose, maltodextrins) in a weight ratio of 1:1 prior to freezing. Despite the fact

that particles were originally fabricated with a higher lipid and emulsifier content, the sizes and size distributions of the obtained particles were similar to the ones produced based on 2.5% Therefore, the total resulting lipid content was 1.25 and 2.5% wt/wt respectively, while typical concentration of the cryoprotectants in the final dispersion was 1.25, 2.5 or 5% wt/wt. Mixtures were subsequently lyophilised for 72 hours and the obtained powders were rehydrated as described in section 2.2.2.

2.2.4. Lyophilised powders – Storage conditions

A one-month stability study was conducted on the powders under different storage conditions. Freeze-dried samples were kept either at room temperature in an air-tight desiccator filled using silica gel as the desiccant material, or at 4 ºC (refrigerated) for fixed storage times (t=7, 30 days). At each time point, samples were reconstituted with distilled water and analysed for their physicochemical properties.

Powder water activity (a_w) was measured at ambient conditions, using a dew point hygrometer (AquaLab Series 4TE, Decagon Devices, Inc). Measurements were performed straight after powder collection to minimise moisture uptake. 1 g of lipid-based powder was used in each case, with the presented mean values showing at least duplicates.

2.2.5. Emulsion preparation

Re-suspended samples were subsequently used as the aqueous phase for following oil-in-water emulsions. O/W emulsions (sunflower oil 20% and 80% aqueous dispersions containing lipid particles and Tween 80 or sodium caseinate) were produced using a high shear mixer (Silverson L5M, UK) at 9,000 rpm for 2 minutes or via ultrasonication at 95% amplitude for 30 seconds. Emulsions were additionally fabricated using stored (30 days) reconstituted lyophilised lipid particles. Both sets of emulsions were analysed for droplet size.

2.2.6. Characterisation of powders and emulsions

2.2.6.1. Particle and droplet size analysis

Particle size distributions for both fresh and reconstituted samples were determined via static light scattering (SLS) using a Mastersizer 2000 (Malvern Instruments, UK). Means show the result of at least duplicated results. For redispersed systems, the refractive indices (RI) of 1.49 and 1.44 were used for tristearin and cetyl palmitate respectively.

Sunflower oil-in-water emulsions were also analysed using laser diffraction immediately after production (RI=1.47). Average droplet sizes were reported as volume weighted means, $D_{4,3}$.

2.2.6.2. Interfacial Tension measurements

Static sunflower oil/water interfacial tensions were determined using a K100 Krüss Tensiometer (Krüss GmbH, Germany) equipped with a Wilhelmy plate. The interfacial tension of freshly prepared and dried/resuspended lipid particle systems was measured at 20 ºC. To perform the measurement, ~50 mL of sunflower oil was carefully pipetted onto the surface of the aqueous phase (~25 mL). All measurements were conducted at least in duplicate, and average values along with standard deviations were calculated.

3. Results & Discussion

3.1. Drying and rehydration of solid lipid particles

Lipid particles were produced following the method described in previous work (Zafeiri, Norton, *et al*., 2017), in the presence of either Tween 80 or sodium caseinate, and from two different lipid sources; either a triglyceride (glyceryl tristearate) or a wax (cetyl palmitate). In the same study it was demonstrated that the size of the fabricated particles is affected by the type and the concentration of the employed surface active species, as well as the physicochemical properties of the lipid material used. Particle manufacturing was followed by lyophilisation and subsequent rehydration of the lyophilisates through the addition of distilled water matching the aqueous mass that was lost during the preceding drying process.

3.1.1. Effect of formulation parameters and reconstitution method

Reconstitution of the lyophilised lipid particles was carried out via two different methods: mixing with a magnetic stirrer for approximately 2 hours or vortexing for 30 seconds followed by sonication for another 20 seconds. The applied methods were selected to reflect two approaches with significantly different levels of shear (energy input) provided for reconstitution, and both these rehydration practices are routinely utilised in relevant literature (e.g. Abdelwahed *et al.*, 2006). Variation in the shear applied during reconstitution is also a useful measure of the extent of aggregation between particles that potentially occurs during drying, and whether this can be overcome upon rehydration (Ding & Pacek, 2008).

The size distributions obtained for non-freeze-dried and reconstituted cetyl palmitate particles formed originally in the presence of different concentrations (0.8 and 2 %wt) of surface active entities are shown in Fig. 1.

The data suggests that it is the type rather than the amount of the surface active component used in the formation of the original particles, which primarily dictates whether the wax entities will recover in terms of size upon reconstitution. In the case of wax particles produced using Tween 80, lyophilisation and resuspension using a low energy method (magnetic stirrer) results in large micron-sized aggregates, regardless of the amount of surface active component initially employed in the formulation (Fig. 1A and C). Assuming that upon drying particles are brought to close contact to give aggregated structures rather than individual fused wax bodies (particles), a high energy reconstitution method would revert such systems back to discrete particulate entities. In this study, mild reconstitution of dehydrated lipid particles, originally formed in the presence of Tween 80, gave rise to large wax bodies, and even rehydration under the provision of more extensive energy levels (ultrasound) was unable to fully reduce particle size to the pre freeze-drying levels. Although, following the more intensive reconstitution method, a population of smaller entities (~200 nm) is indeed apparent, a large cluster of micron-sized particles (of similar sizes to those rehydrated using a milder method) is still present. Tween 80 concentration appears to have a small effect on these bimodal distributions, with particles formed at a higher surfactant content giving a proportionally larger (in terms of volume) submicron size population once redispersed more thoroughly. Despite being highly effective (even at lower concentrations) in producing wax particles at sizes desired for

Pickering functionality, Tween 80 does not show any tendency to be as effective in maintaining these sizes after the drying and rehydration stages.

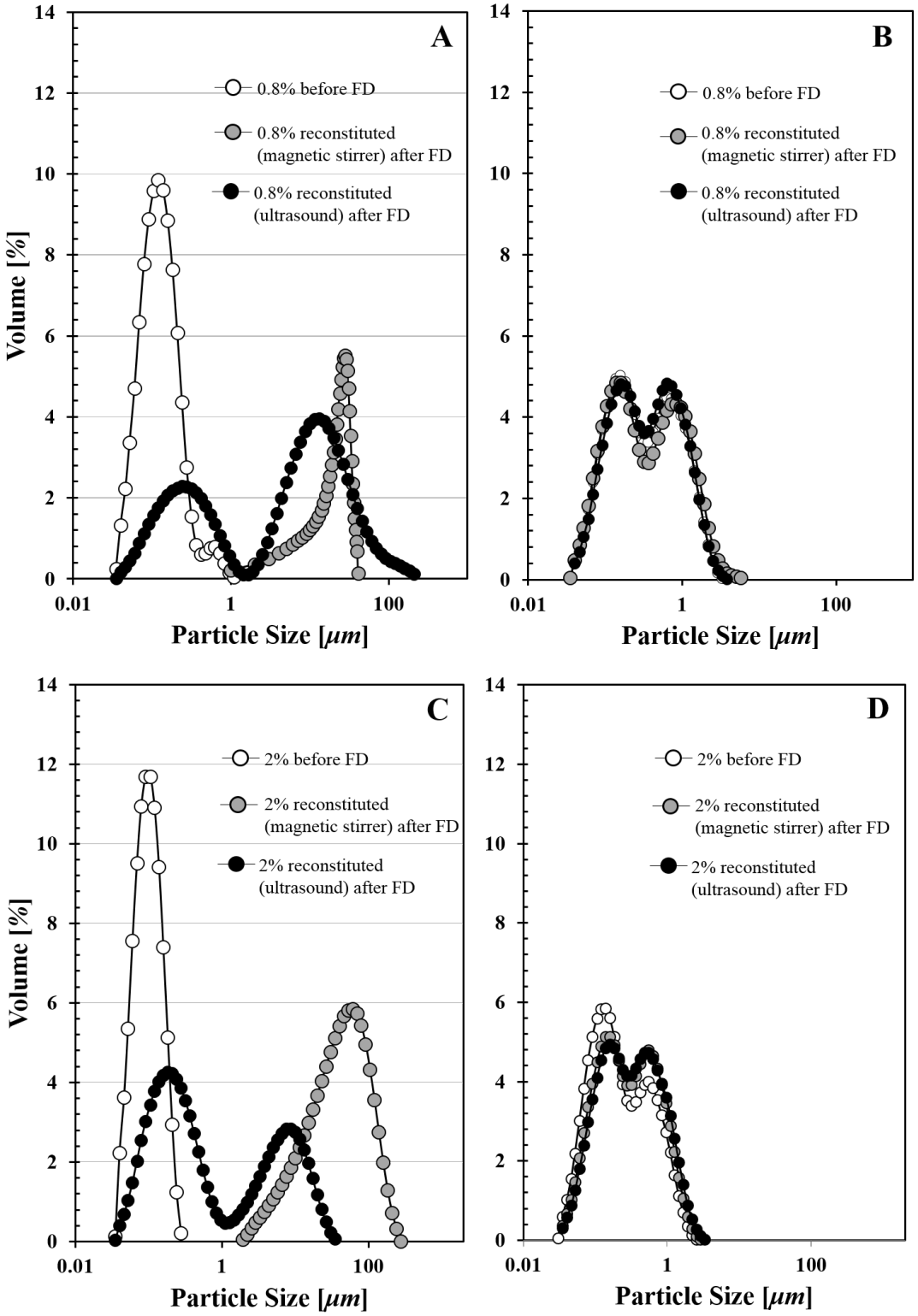

Fig. 1. Particle size distributions of solid cetyl palmitate (wax) particles in the presence of 0.8 and 2 %wt Tween 80 **(A, C)** or sodium caseinate **(B, D)**, before and after freeze-drying (FD) and for two different reconstitution methods.

On the other hand, sodium caseinate in addition to facilitating the production of small (although not at the same level as Tween 80) wax particles, also demonstrates a strong capacity to protect these from aggregation/fusion during the harsh drying conditions and thus significantly contributing in the conservation of their original size following rehydration. In fact, this behaviour appears to be independent of the resuspension method used or the amount of surface active agent (protein) present in the original formulation (Fig. 1B and D).

The protective behaviour of sodium caseinate was only observed at its native state (pH ~7) as any modification of pH to values closer to its isoelectric point (e.g. pH=5) before lyophilisation resulted in considerably bigger particle sizes (Fig. S1). This is probably due to the decrease in the protein's charge which, in turn, potentially also lessens electrostatic repulsion between particles.

The type of surface active species also appears to be more dominant than the type of lipid employed for particle manufacture, in terms of determining post freeze-drying reconstitution behaviour; lyophilisation and subsequent rehydration of solid tristearin particles gave very similar findings to those previously obtained for wax particles (Fig. 2). As in the case of wax particles, Tween 80 demonstrates the same deficiency in terms of maintaining the size of triglyceride particles post lyophilisation, with both the surfactant concentration and method of rehydration unable to assist (Fig. 2A and C). On the contrary, the presence of sodium caseinate at either concentration in the formulation proved to be key in preserving the original particle size upon reconstitution (Fig. 2B and D).

In view of this data, it is likely that the capacity of the surface active species initially used to fabricate the particles, and to also provide a certain level of microstructural recovery following the drying and subsequent rehydration events, is very much dependent on the type of interfacial component used. Furthermore, it is suggested that this is primarily dependent on the capacity of these species to form a significantly robust interfacial barrier arresting particle-particle interactions that are encouraged due to the low moisture environment resulting from drying. To an extent, the protective effect imparted to the particulate dispersion during the drying stage appears to be a function of the molecular size of the surface active component. A small molecular size surface active agent such as Tween 80, is not able to prevent contacts between the particulate entities and their subsequent increase in size as a result of particle coalescence/fusion. In contrast, particles that are coated prior to drying by a high molecular weight surface active component such as protein, retain their original dimensions upon the reintroduction of water. Evidence from previous work has shown that skim milk proteins, either alone or in combination with other components, have the potential to endow protection to labile systems subjected to freeze-drying. In particular, early studies have demonstrated the efficacy of proteins contained in milk in the preservation of lactic acid bacteria (Champagne, *et al.*, 1991) and the increase in viability of freeze-dried yeast cells and fungus spores (Berny & Hennebert, 1991), attributed to the formation of a protective coat.

Understanding how emulsifiers function in the systems in this study, can be facilitated by considering such effects in emulsion systems subjected to lyophilisation. There are various physicochemical processes that accompany ice formation and water removal during freeze-drying of oil-in-water emulsions that can lead to the destabilisation of these systems. The

amount of liquid water is lessened upon freezing, hence the emulsifier molecules adsorbed at droplet surfaces are not fully hydrated and this could favour interactions between the surfactant-coated oil droplets (Zhang, *et al.*, 2008). Additionally, the protective effect of the interfacial membranes around the oil droplets is dampened because of protruding ice crystals that can disrupt these barriers. Depending on their innate surface activity, emulsifiers can adsorb at the surface of newly formed ice crystals reducing their total content in the system. Consequently, it is likely that there is not sufficient material to provide surface coverage to emulsion droplets (McClements, 2004). In the course of both freezing and drying, it is possible that certain emulsifiers (e.g. proteins) are denatured and eventually lose their functionality (Farshchi, Ettelaie, & Holmes, 2013).

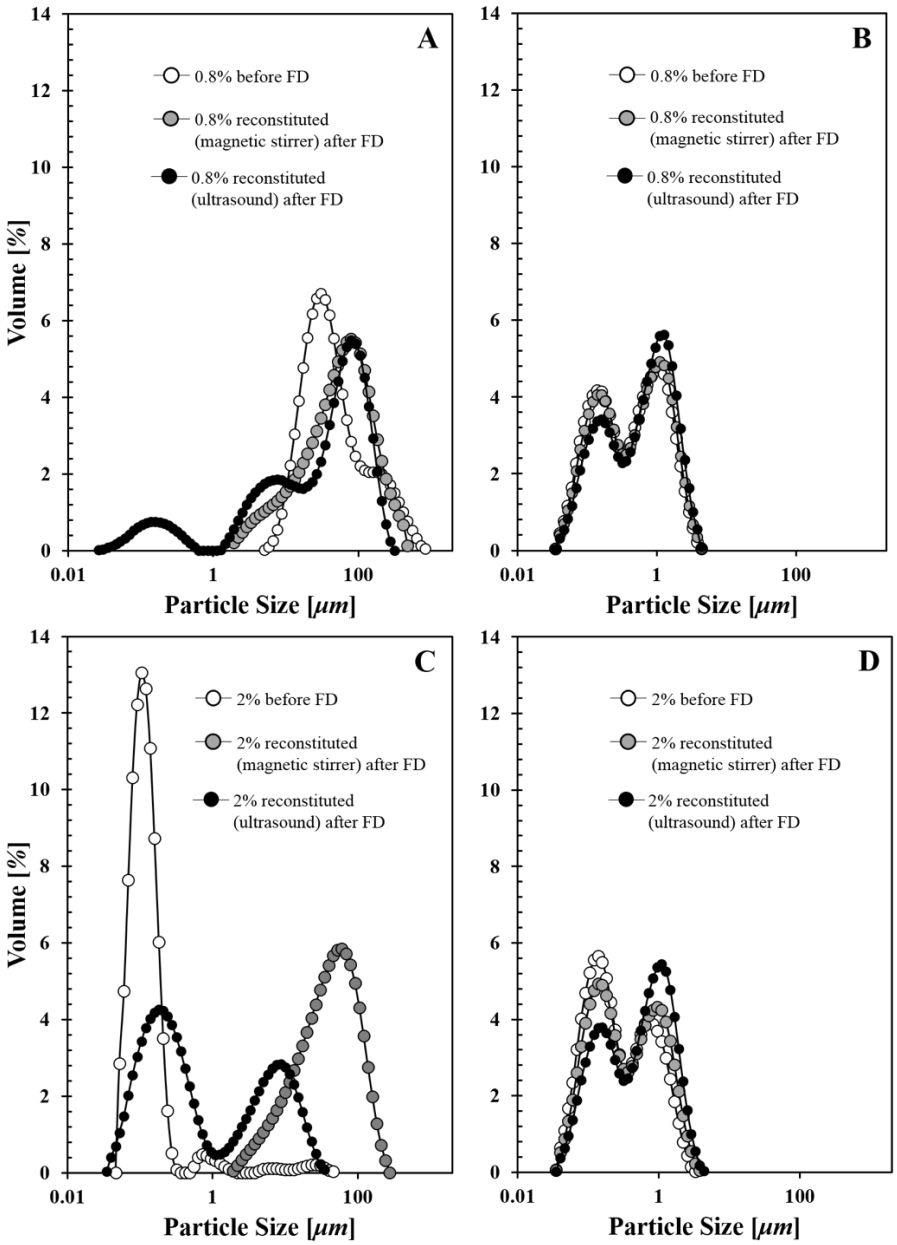

Fig. 2. Particle size distributions of solid tristearin (triglyceride) particles formed with 0.8 and 2%wt Tween 80 **(A, C)** and sodium caseinate **(B, D)** before and after freeze-drying (FD) and for two different reconstitution methods.

In this study, it is proposed that sodium caseinate provides a robust interfacial layer that limits the proximity between lipid particles during the lyophilisation stage and eventually, enables these to maintain their size and size distribution profiles. Similar observations have previously been reported when poly(vinyl alcohol) (PVA) or modified PVA (containing a long alkyl chain at the end of the molecule) were used as stabilisers of poly(ε-caprolactone) nanocapsules or liposomes (Takeuchi, *et al*., 1998; Abdelwahed, Degobert, Stainmesse, *et al*., 2006). These cryoprotectant-free systems were found to be able to undergo freeze-drying without any aggregation/fusion taking place during freezing. Takeuchi, *et al.* (1998) attributed the observed behaviour to the thick polymeric layer that is formed at the liposome's surface owing to the high molecular weight of the modified PVA together with its anchoring-like coating capacity.

It is also possible that advantages in terms of the recovery of particle performance could be arising from the presence of free (non-adsorbed) surface active species in the system. Abdelwahed, Degobert, Stainmesse, *et al.* (2006) highlighted the importance of the presence of free PVA in addition to the surface-adsorbed PVA, which possibly via inhibition of ice nucleation, protected the fragile nanocapsules throughout the freezing step.

Although the conservation of the size of dried and rehydrated lipid particles originally fabricated in the presence of NaCas was so far associated with surface active species positioned at the interface, the possibility that the observed enhanced performance also results from the presence of protein in the bulk phase, cannot be completely ruled out. This scenario was investigated by substituting sodium caseinate with two different water-soluble biopolymers, namely hydroxypropyl methyl cellulose (HMPC) and low methoxyl pectin (LMP). The underlying concept was to firstly assess a component that has a level of affinity for the interface but is even larger in molecular size than NaCas (i.e. HPMC). Secondly, this was taken a step further by considering another biopolymer of large molecular dimensions, that although has no apparent interfacial capacity (i.e. LMP), its presence in the bulk (aqueous) phase could still potentially provide a large barrier to particle aggregation upon lyophilisation. HPMC and LMP were used in the particle fabrication stage instead of NaCas, following exactly the same experimental protocol. The formed particles were subsequently freeze-dried and rehydrated and the yielded size and size distribution profiles of the reconstituted lipid particles are presented in Fig. 3.

The data in Fig. 3 shows that when high molecular weight entities were used for the preparation of particles, size distributions with a major peak at around 1 μm were obtained. What is more, the size of the formed particles was successfully maintained for both polymers after a lyophilisation cycle. As such, the data does suggest that water soluble macromolecular polymeric compounds act as a barrier preventing particles from fusing together during lyophilisation and thus, facilitate reconstitution back to their original sizes. Similar behaviour has been previously demonstrated for o/w emulsions in which different types of HPMC were used as both the emulsifier and the solid carrier (Christensen, Pedersen, & Kristensen, 2001). The authors produced redispersible dry emulsions of coconut oil that, upon reconstitution and up to a specific oil content (40%), could reform (in terms of droplet size and size distribution) the original o/w emulsion.

Although there is a clear impact in terms of conserving the size of dried and redispersed lipid entities, the investigated large molecular weight hydrocolloids, in contrast to surfactants and proteins, do not seem to provide particles within the desirable size ranges associated with Pickering functionality; particles at submicron sizes have been reported as necessary for the stabilisation of emulsion droplets of 0.5-10 μm (Dickinson, 2012). This is primarily due to their inherent lack of surface activity which results in only a minimal reduction to the oil/water interfacial tension compared to typical emulsifiers, e.g. protein (Fig. S2).

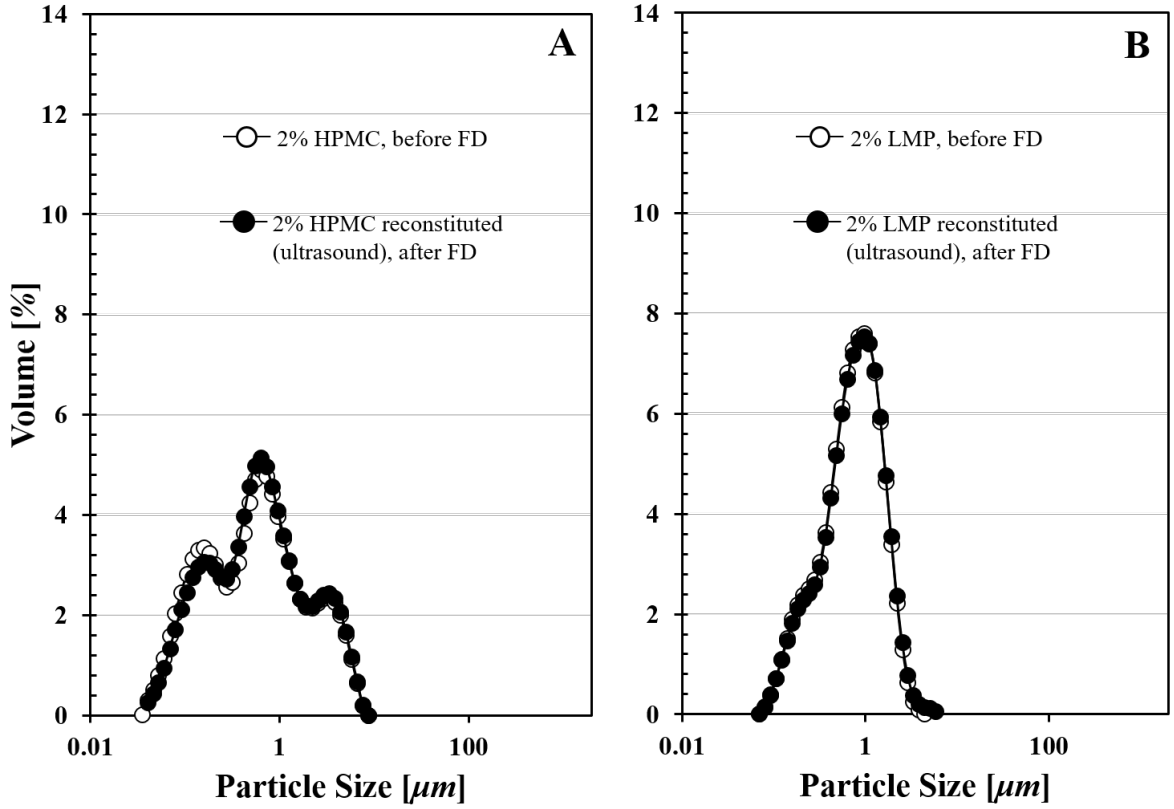

Fig. 3. Solid cetyl palmitate particles fabricated in the presence of 2 %wt HPMC **(A)** and 2 %wt LMP **(B)** before and after freeze-drying.

Nonetheless, the possibility that the observed profile is a result of the formation of an interfacial layer cannot be completely disregarded. The satisfactory drying and redispersion behaviour of the lipid particles when these are produced with either of the investigated biopolymers, could also be due to the polymers' impact at the interface or a combination of interfacial and bulk effects. Microscopy analysis from previous studies employing starch and cellulose have shown that the adsorption of these closely-packed entities surrounding emulsion droplets could confer a level of structural integrity to an o/w emulsion during the freeze-drying process (Marefati, *et al*., 2013; Hu, *et al*., 2016). Yet, hydrophobic modification (Marefati, *et al*., 2013) and use of an additional stabilising agent (Hu, *et al*., 2016) were shown to be essential for the stabilisation of the precursor emulsions.

As suggested in literature (Sheu & Rosenberg, 1995; Whitby, Scarborough, & Ngothai, 2017; Rezvankhah, Emam-Djomeh, & Askari, 2020), large molecular components such as

carbohydrates or proteins are commonly preferred to be added to the aqueous phase of emulsions since they encourage the formation of a protective film around droplets during the drying process. The said coating inhibits coalescence of oil drops occurring during drying or powder storage, which can lead to oil release onto the granule surface. This protective ability has been quantified using the dimensionless Peclet number as an aiding tool to predict localisation of the introduced solid carrier/solute in the system undergoing desiccation (Vehring, 2008). Therefore, polymers/proteins that have been associated with high Peclet numbers (>1) tend to accumulate at the droplet surface as water evaporates and can even form a solid shell/skin once a critical interfacial concentration is reached (Whitby, *et al*., 2017).

Because of their large dimensions, the lipid particles formed in the presence of either LMP or HPMC were not further investigated for their potential to stabilise o/w emulsions.

3.1.2. Effect of the presence of cryoprotective agent

In an attempt to improve the recovery performance of lipid particles originally formed in the presence of Tween 80, standard cryoprotective species used in other relevant studies were introduced to the formulations investigated here. Such species are typically sugars, which are added before freezing with a view to protect the lyophilised product of a colloidal suspension from freezing stresses and improve its stability upon storage (Abdelwahed, Degobert, Stainmesse, *et al.*, 2006). The non-reducing sugars sucrose (De Chasteigner, Cavé, Fessi, Devissaguet, & Puisieux, 1996; Lim & Kim, 2002; Abdelwahed, Degobert, & Fessi, 2006) and trehalose (Schwarz & Mehnert, 1997; Zimmermann, Müller, & Mäder, 2000; Dulieu & Bazile, 2005) are among the most common cryoprotectants used in literature for the freeze-drying of nanoparticles.

In the present study, these two sugar compounds were used at concentrations ranging from 1.25 to 5 wt% (of the final dispersion concentration). Maltodextrins derived from starch and with a variety of dextrose equivalent (DE) values, (varying from 5 to 18) were also used as alternatives to small molecular cryoprotectants, at 2.5 wt% concentration. These carbohydrates have also been shown to possess cryoprotective efficacy in studies concerning emulsions and bacteria undergoing lyophilisation (Reddy, Awasthi, Madhu, & Prapulla, 2009; Mun, McClements, & Surh, 2011). The protective action of maltodextrins has been identified to mostly take effect over the course of freezing and has been attributed to an increase in the volume fraction of unfrozen aqueous phase available to either keep the lipid droplets apart or hydrate the adsorbed emulsifier (Ogawa, Decker, & McClements, 2003; Mun, Cho, Decker, & McClements, 2008). They may also act to modify the number and properties of the formed ice crystals which can readily penetrate into the fat droplets and jeopardise their stability (Mun, *et al*., 2008). Different DE values correspond, among others, to maltodextrins of different glass transition temperatures (T_g'), which are expected to affect in a distinct way the resulting structure and physical properties of the lyophilised powders, for example their free flow behaviour or the ability to protect an encapsulated active ingredient (Michael J. Pikal & Shah, 1990; Wang, 2000).

These two different classes of cryoprotective species were introduced to aqueous dispersions of wax particles fabricated in the presence of Tween 80 and the resulting formulations were

lyophilised and rehydrated employing a high shear reconstitution method (i.e. ultrasonication) as described earlier. The resulting size distributions of rehydrated lipid particles as a function of the type and concentration of the cryoprotective agents used are presented in Fig. 4.

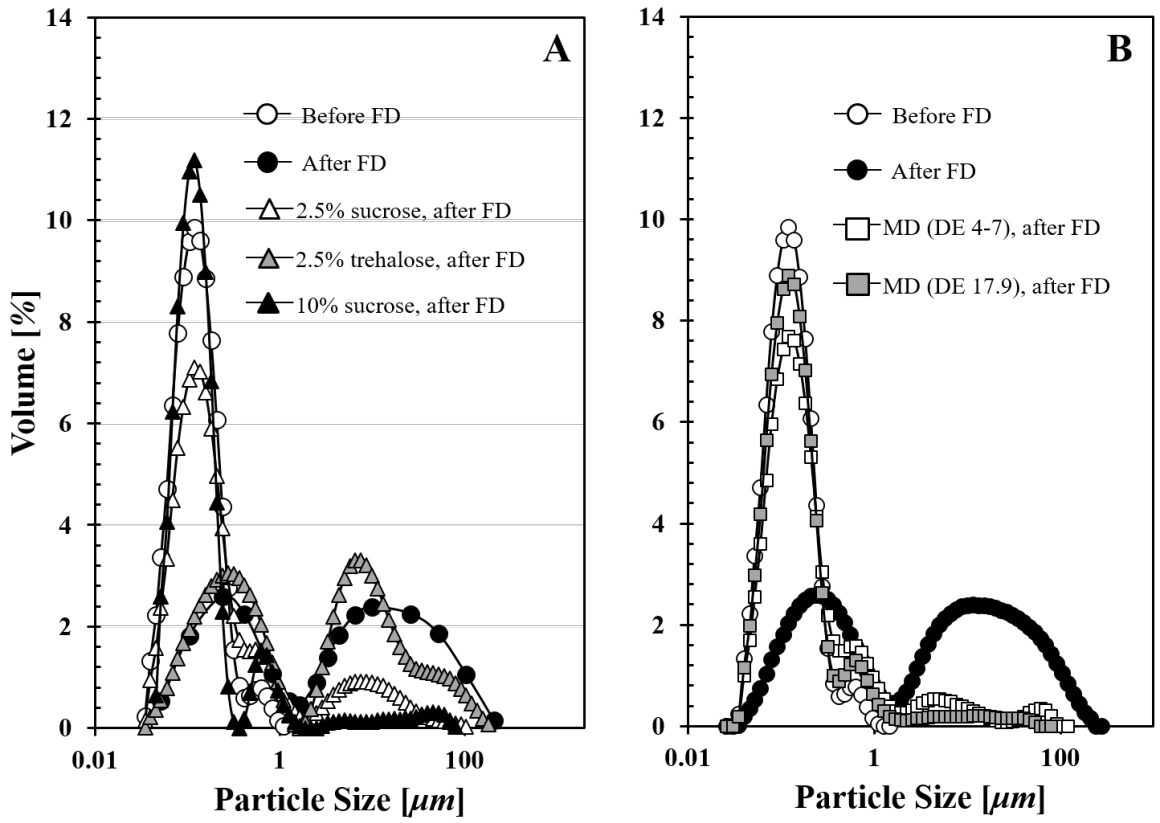

Fig. 4. Particle size distributions of 2.5 %wt solid cetyl palmitate particles produced with 0.8 % wt Tween 80 after reconstitution of the lyophilised samples in the presence of sucrose and trehalose as cryoprotectants at different concentrations **(A)**, as well as different DE maltodextrins (MD) at 2.5 %wt concentration (of the final dispersion) **(B)**.

The obtained data clearly suggest that only high concentrations of sugars provide freezing resistance to cetyl palmitate nanoparticles and thus mediate their recovery upon rehydration to sizes close to their original ones (Fig. 4A). The addition of cryoprotective species at relatively high concentrations has been identified in previous studies (Schwarz, *et al*., 1997; Hirsjarvi, Peltonen, Kainu, & Hirvonen, 2006; Lee, Kim, Kim, & Lee, 2009) as necessary to ensure the successful reconstitution of lyophilised products. This behaviour appears analogous to the effect of sugars on the stability of frozen dairy desserts (e.g. ice cream). Stability in these o/w emulsion-based systems is affected by sugar content due to the impact of the latter upon the size and growth of ice crystals (Goff, Caldwell, & Stanley, 1993). The cryoprotective effect of the non-reducing sugars is accentuated at higher concentrations as they confer increased viscosity to the aqueous medium on one hand, and immobilisation of the droplets within a glass matrix on the other (Zhang, *et al.*, 2008).

With regards to the role of maltodextrins, the observed reconstitution behaviour was similar to the high concentration sucrose formulations. The size of the redispersed wax particles was only minimally altered (from particles' original size), regardless of the DE value of the utilised maltodextrin (Fig. 4B). The discrepancies in the size distribution of the redispersed lipid particles formed with a different carrier phase (sucrose, trehalose, maltodextrin) could be ascribed to the ability of the latter compounds to form a glass. It is well known that glass transition temperature is a function of the water content of the sample and increases with a decrease in the moisture content (Avaltroni, Bouquerand, & Normand, 2004; Drake, *et al*., 2018). This effect, combined with the fact that sugars show glass transitions at lower temperatures (Augustin & Hemar, 2009) is likely to point to an easier and faster glass formation for the maltodextrin-containing systems. When maltodextrin is present, lipid particles are 'trapped' in a glassy matrix faster, preventing any further aggregation/fusion between them. This occurs at lower maltodextrin concentration (higher moisture content) as opposed to the ones needed for the case of sucrose or trehalose-including systems.

3.1.3. Effect of drying time on moisture removal and redispersion behaviour

An optimised freeze-drying process ensures that the duration of the freezing and drying stages (primary and secondary) are such that the requirements for specific final residual moisture contents are met and at the same time, time-consuming cycles that could lead to over-drying and unnecessary energy usage are avoided. Primary drying is usually the most extensive and multifactorial drying step (Patel, Doen, & Pikal, 2010); it allows ice sublimation to take place which, in turn, is dictated by several factors such as the shelf temperature and chamber pressure, the heat transfer coefficient of the containers and the geometrical characteristics of the product (e.g. thickness of frozen cake). Secondary drying involves water desorption which has normally faster kinetics at constant temperature and pressure (M. J. Pikal, Shah, Roy, & Putman, 1990). It has been suggested that secondary drying times longer than 3-6 hours have a negligible effect on the reduction of the moisture content (Tang & Pikal, 2004).

In the current study, it was postulated that different drying times would result in varying moisture contents and potentially affect the rehydration ability of the lyophilised powders, for instance by influencing particle proximity. Certainly, the moisture levels of the freeze-dried product depend on the interplay between formulation parameters and equipment characteristics as well as the specifics of the drying cycle *per se*.

Aqueous dispersions of wax particles fabricated in the presence of either Tween 80 or NaCas were dried over different time durations (i.e. 24, 40, 48 and 72 hours) and the resulting powders were weighed so that the amount of water that was removed is then calculated via a mass balance. The drying curves for the wax systems along with the water activity at each drying period are presented in Fig. 5.

The drying curves of cetyl palmitate-based particles exhibit two drying periods (Fig. 5A). During the initial 24 hours the amount of water that is removed increases rapidly for all four systems investigated. At the end of this 24 hours period, 54-65% of the initial water content is lost by sublimation. This provokes a sharp decay in the water activity values (α_w) over the

course of the same period (Fig. 5B). The time length of this desiccation stage is principally controlled by the preceding freezing step and more specifically, by the number and size of ice nuclei formed, which in turn define the product resistance to vapour flow and also the surface area that is available for desorption during secondary drying (Patel, Bhugra, & Pikal, 2009). The process of ice nucleation is affected by the freezing rate, vial surface area and certainly by solution properties, such as composition of the original product, water content and/or viscosity (Patel, *et al.*, 2009; Assegehegn, Brito-de la Fuente, Franco, & Gallegos, 2019). This high drying rate region is followed by a less pronounced increase in moisture removal (in the range of 23-30%) via sublimation over the following 24 hours. Between 40 and 48 hours, only a small percentage of water is still being removed. Accordingly, a decreasing trend in water activity is also observed, suggesting that free water content is significantly reduced and that the remaining moisture is closely bound within the powder (e.g. at 48 hours). Variation in the formulations appears to have a minimal effect on the amount of water that is sublimed following 48 hours of drying.

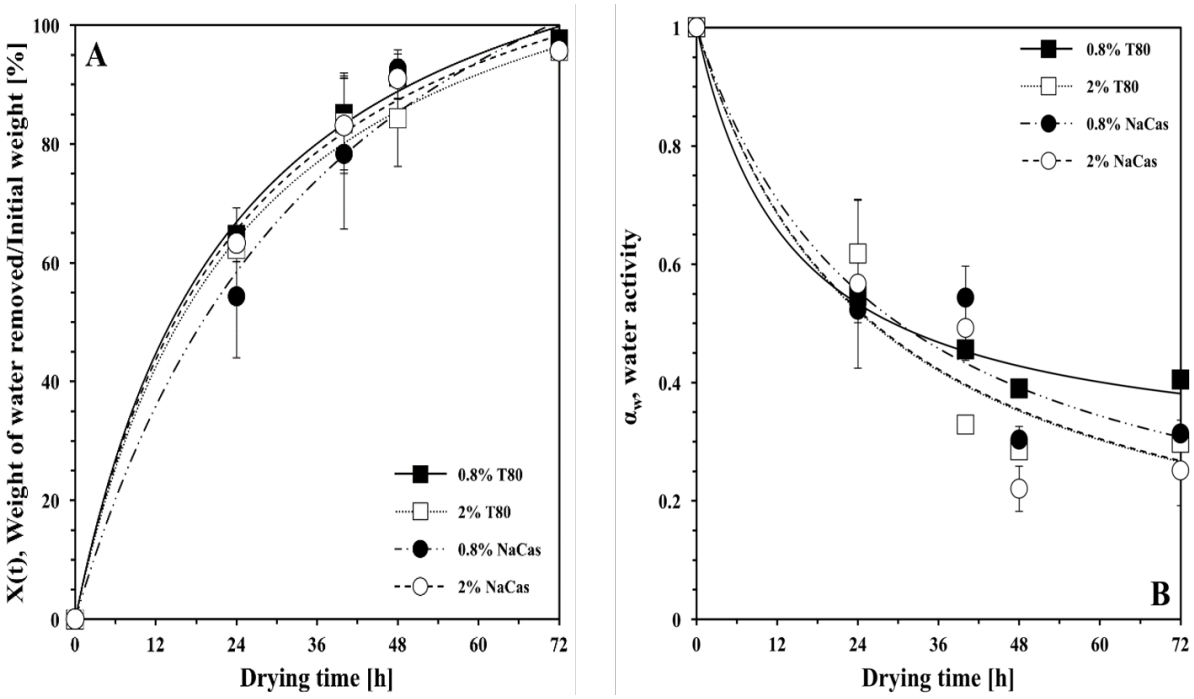

Fig. 5. A. Drying curves for different amounts of Tween 80 and NaCas for dried and resuspended cetyl palmitate particles formed in the presence of either surface active species. **B.** Water activity measurements of the dried powders. Measurements were carried out in duplicates with the mean values shown and the error bars representing ± 0.5 standard deviations.

During the last stages of the drying process (between 48 and 72 hours) and regardless of the type and amount of surface active species used, only negligible amounts of water are abstracted, which clearly indicates that no further benefit in terms of moisture removal can be realised by extending the drying cycle over the 72 hours period. This observation coincides with the very low water activity values obtained ($\alpha_w \cong 0.2$), which in fact indicate that a dry powder has already been acquired after 48 hours of drying.

Freeze-dried powders collected at different time intervals during the drying process were subsequently redispersed in water (via ultrasound) in order to evaluate the effect of the extend of drying and therefore the final moisture content on the size profile of the rehydrated wax particles. The size distributions of reconstituted cetyl palmitate particles fabricated in the presence of low amounts of surface active components are presented in Fig. 6.

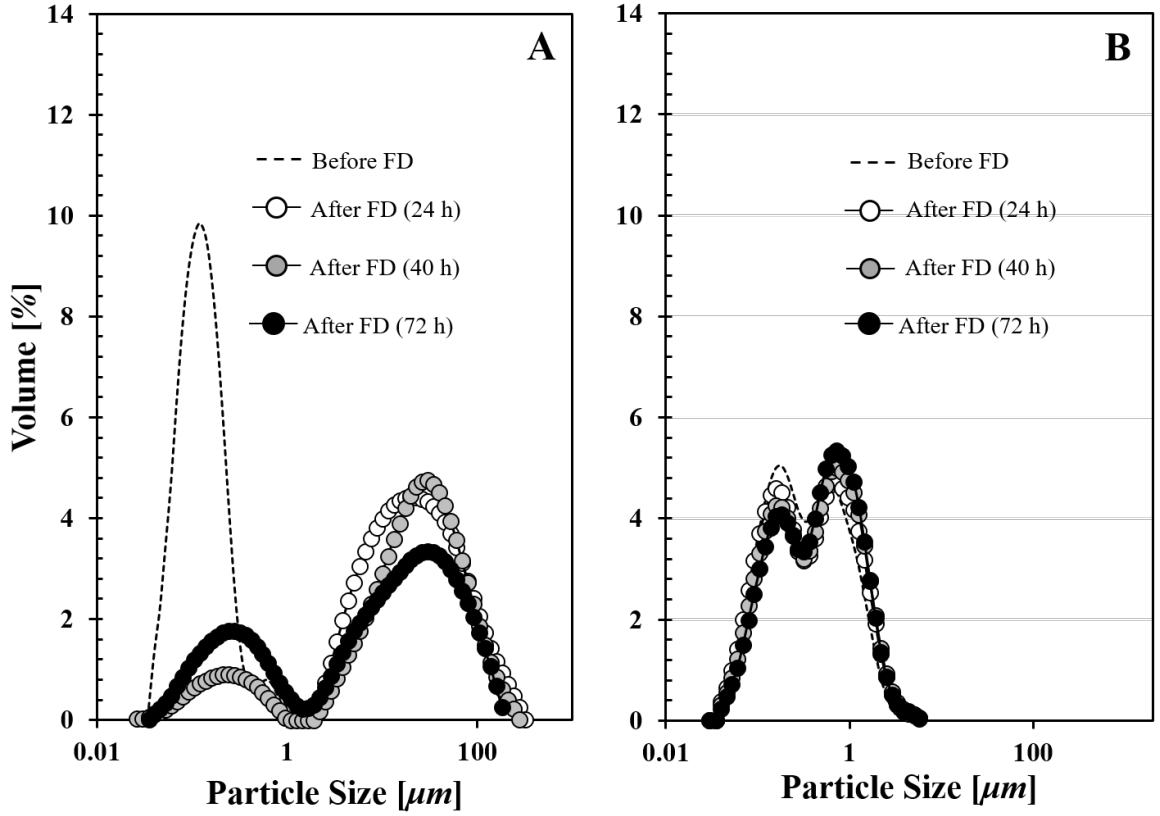

Fig. 6. Particle size distributions of dried and redispersed 2.5 %wt solid cetyl palmitate particles produced in the presence of 0.8% Tween 80 **(A)** and 0.8% NaCas **(B)** for different drying times. Original wax particles with the same amounts of surfactants serve as a reference.

Samples removed following 24 hours of freeze-drying were found to still contain significant amounts of ice, indicating that the freeze-drying cycle was still at its primary drying stage. This was also confirmed by the high values of water activity (α_w in the vicinity of 0.6 in most cases) as seen in Fig. 5B.

Increasing the drying time from 24 to 72 hours for wax particle systems made in the presence of Tween 80, gives a significant deviation (increase) in particle size that is not at all affected by the duration of the lyophilisation process (Fig. 6A). Antithetical to systems fabricated in the presence of the small molecular weight surfactant, incorporation of sodium caseinate in the formulations (at either of the 0.8 or 2 %wt concentrations) results in reconstituted wax particles of almost identical to the original size distributions (Fig. 6B). Therefore, it appears that the protein acts to retain the original particle dimensions, independently of the duration of the drying process and thus, regardless of the residual moisture content. It is proposed that a

protein-rich thick interfacial film forms around the wax particles, which upholds their integrity against the stresses developed during the drying process and prevents inter-particle aggregation/fusion. It has previously been reported that upon exposure of protein-sugar solutions to a convective drying environment, the protein film would convert into a glassy matrix that increases in thickness during the drying process (Adhikari, Howes, Bhandari, & Langrish, 2009).

3.1.4. Effect of storage conditions

A one-month stability study was performed on lyophilised wax particle powders to evaluate the effect of different storage times and temperatures (room or refrigerated temperature) on their capacity to be successfully reconstituted. Water activity of the dried particulates was also measured over the one month storage period. All dried formulations were rehydrated back to their original moisture content and the particle sizes in the reconstituted systems were measured. Data obtained from these measurements are presented in Fig. 7.

Visual observation did not reveal any significant differences between powders stored at different temperatures and/or over different time periods prior to rehydration. The general trend across all stored formulations was that they suffered an increase in their mean particle size, even after one week storage at any temperature, an effect which was significantly more pronounced for Tween 80-stabilised particles (Fig. 7A). Although the powders that were stored at 4 °C were exposed to much higher humidity levels, the particle size obtained after one week remains constant until the end of the one month storage. During storage, water activity values increase (i.e. water is less strongly bound) as the systems are extremely susceptible to moisture uptake, particularly when stored in a moisture-rich environment (i.e. refrigerator) (Fig. 7B). The effect of temperature was also crucial, as an increase in storage temperature was accompanied by a reduction in the mould-free shelf life.

For wax particles formed in the presence of Tween 80, the mean particle size of the resuspended powders stored at low temperatures for one month appeared to experience no variations. However, the span value increased by almost 80% (data not reported) which clearly points to particle aggregation. Given that this aggregation tendency was not observed when cetyl palmitate particles formed in the presence of Tween 80 and not having undergone freeze-drying were stored for the same time period at 20 °C, the behaviour could be due to the lyophilisation process and the different microstructures formed. The size of rehydrated wax particles produced with sodium caseinate remained unchanged after one month of storage at 4 °C. Nonetheless, room temperature conditions allowed an intense particle agglomeration, as seen by a doubling in the $D_{3,2}$ values (Fig. 7A).

The type of the surface active component or the presence of an additional solid carrier is likely to have an impact on the colloidal stability of the dried dispersion upon ageing. In an earlier study (Christensen, *et al.*, 2001), dry o/w emulsions formed in the presence of HPMC rehydrated back to their original form after 6 months of storage at 40 °C, with SEM micrographs confirming an unchanged outer structure morphology for the dried spherical

powder particles. However, the exact mechanism through which the dry powder reconstitutes to the original emulsion was not the focus of the former study.

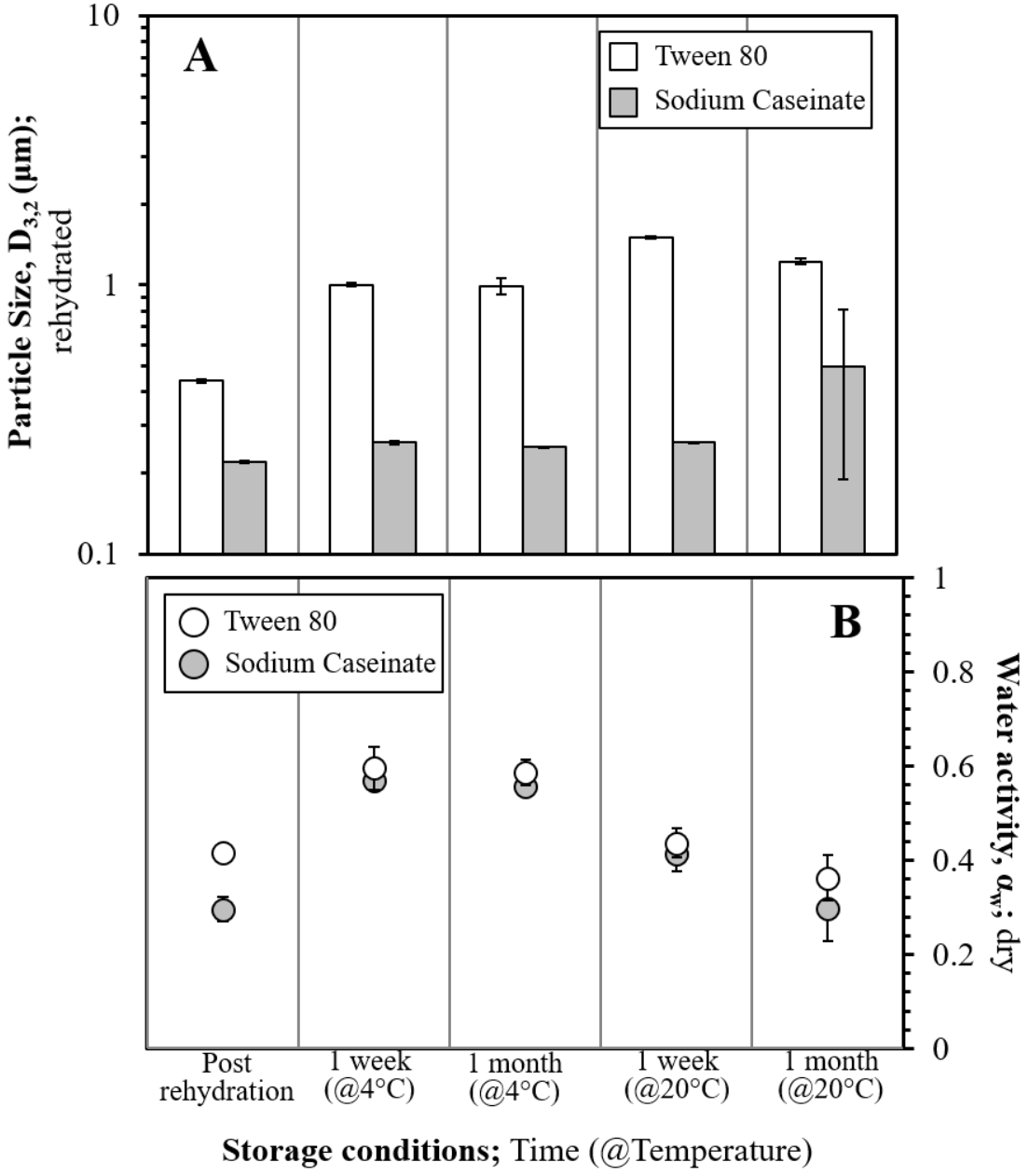

Fig. 7. Size properties **(A)** of redispersed cetyl palmitate particles formed with 0.8% Tween 80 or sodium caseinate under different storage times and conditions. Water activity of these systems measured in dry state **(B).**

Despite being in a dried state where Brownian motion is arrested, changes to the particles' surface properties could still be taking place. Tween 80 and sodium caseinate molecules may be desorbing from the interface over the course of freeze-drying and possibly the powder storage period (depending on the length and conditions of storage) in a similar way to interfacially adsorbed starch, as hypothesised in a recent study (Mu, Farshchi, Holmes, Chen,

& Ettelaie, 2019). Upon reconstitution, the free surface active compounds do not re-adsorb quickly enough (or barely adsorb at all) onto the particle surface resulting in coalescence or depletion flocculation events. These changes are less prominent in the rehydrated sodium caseinate-stabilised lipid particles (as seen in Fig. 7A), presumably due to the thicker interfacial layer present in this case, forming a barrier of protective capacity that is extended beyond lyophilisation and, during long periods of powder storage. Coarser emulsion droplets, following the storage and rehydration of dried powders were reported in the aforementioned study (Mu, *et al*., 2019) that specifically focused on the effect of storage temperature and humidity on the stability of freeze-dried emulsions stabilised by hydrophobically modified starch. The former work demonstrated the major impact of powder storage temperature on the droplet size and stability of the reconstituted emulsions, an effect that was ascribed to the limited diffusion of oil molecules at lower temperatures (e.g. 4 °C) which in turn, decelerated aggregation. In the present study, dried lipid particles are in crystalline form in both refrigerator and ambient temperatures and thus, desorption/re-adsorption behaviour of the surface active species used, is probably the most prevalent mechanism determining the rehydration of dried powders after prolonged storage.

3.2. Pickering functionality

3.2.1. Interfacial behaviour

In addition to size, the potential of particles to act as Pickering stabilisers of o/w emulsions is also dependent on their performance at the oil-water interface (Zafeiri, Norton, *et al*., 2017). Therefore, the aim was to study whether interfacial behaviour, analogously to size, can be maintained following drying and rehydration. The systems of focus here were reconstituted lipid particles that previously demonstrated a capacity to maintain their initial size post-lyophilisation (i.e. formed in the presence of sodium caseinate). The obtained dynamic interfacial tension profiles were compared to those produced for particles that hadn't undergone freeze-drying and subsequent reconstitution (Fig. 8).

Lipid particles formed in the presence of sodium caseinate (before FD) give interfacial tension profiles that are almost intermediate between those of particles formed in the absence of interfacially active entities and those of aqueous solutions of equivalent amounts of emulsifier alone. This has been previously ascribed to result from a proportion of the surface active component being entrapped/contained within the crystalline structure of the formed particles (Zafeiri, Norton, *et al*., 2017).

However, upon freeze-drying the interfacial tension profiles of the reconstituted particles seem to be more closely aligned to the pure emulsifier systems, an effect that is more pronounced for the resuspended tristearin particles (Fig. 8A & B) and for formulations with higher NaCas concentrations (Fig. 8B & D). The observed reduction in the interfacial tension of the reconstituted systems suggests that a proportion of the protein content, assumed to be associated with the wax particles prior to freeze-drying, has now been expelled. Several studies in literature concerning drug-loaded solid lipid nanoparticles have reported on the expulsion of the drug molecule to the surface of the crystalline particle as a result of a highly ordered

structure (Westesen, Bunjes, & Koch, 1997; zur Mühlen, Schwarz, & Mehnert, 1998; Dawoud & Nasr, 2016). This tendency could also be the case in the investigated lipid particulate systems, which crystallise in the most stable polymorphic forms allowing little space, particularly for a large protein molecule to fit in. Additionally, interfacial tension measurements for the NaCas-coated reconstituted lipid particles disclosed a profile that was nearly identical across the different drying times.

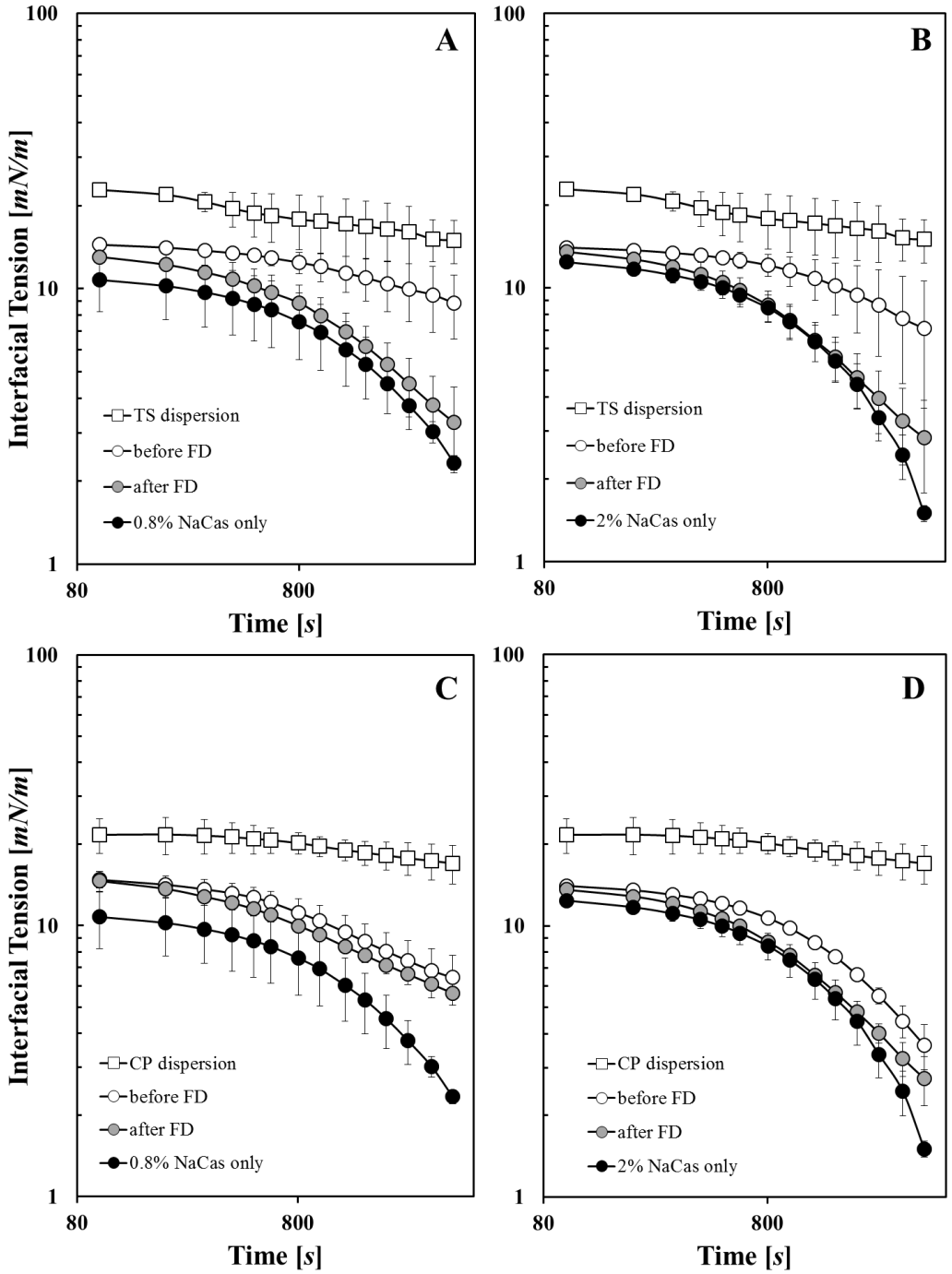

Fig. 8. Interfacial tension between sunflower oil and aqueous dispersions of solid tristearin **(A,B)** and cetyl palmitate particles **(C,D)** formed in the presence of 0.8 and 2 %wt NaCas before and after freeze-drying (FD). Surfactants in solution are presented on the graph as a reference. All formulations contain 2.5 %wt of lipid material.

Preliminary interfacial tension measurements conducted with lipid particles and Tween 80 (0.8 and 2 wt%) after lyophilisation, yielded a behaviour very similar to Tween only systems, or even in some cases exceptionally lower than that (Fig. 9).

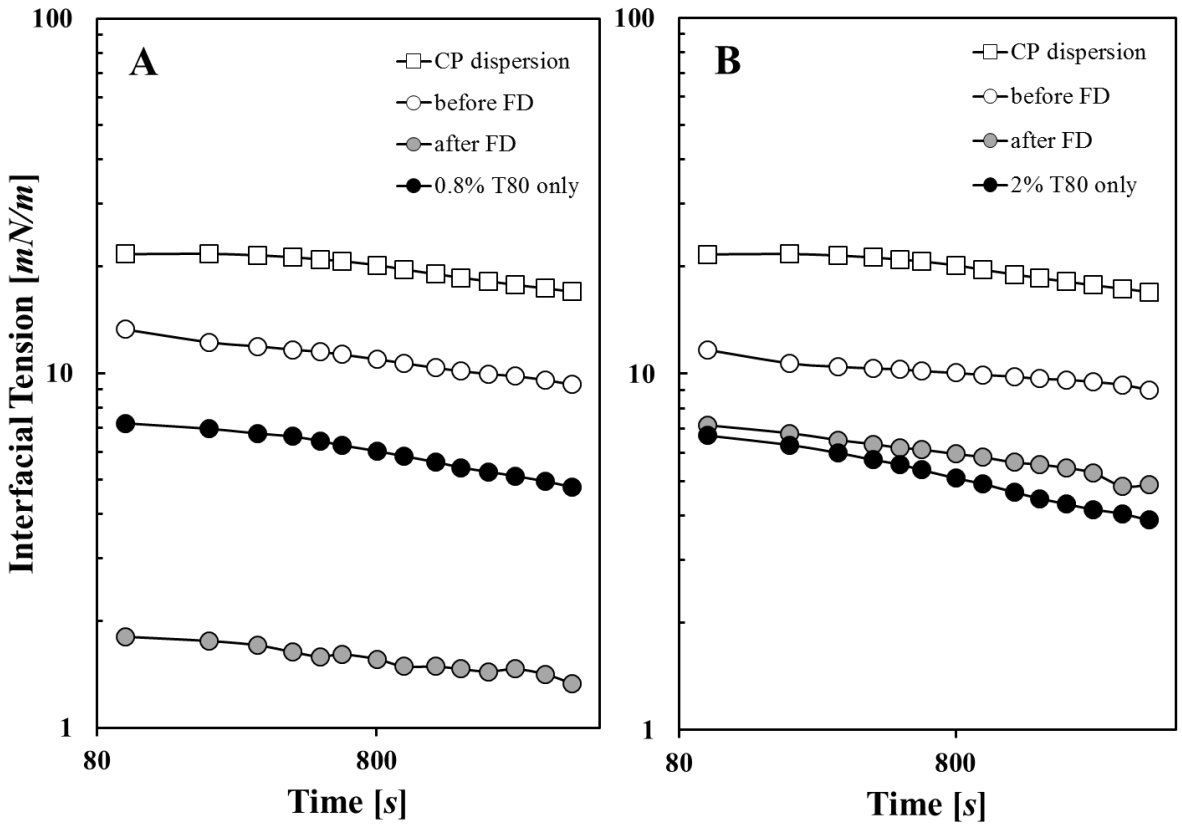

Fig. 9. Interfacial tension between sunflower oil and aqueous dispersions of solid cetyl palmitate particles formed in the presence of 0.8 **(A)** and 2 %wt **(B)** Tween 80 before and after freeze-drying (FD).

It appears that during freeze-drying part of Tween 80 is desorbed from the particles' surface as it is likely to be concentrated in the frozen state, due to the removal of water. This is in agreement with the hypothesis proposed earlier in section 3.1.4. Upon rehydration, Tween 80 is no longer associated with the particles, leading to irreversible aggregation as has also been discussed previously. In addition to Tween 80 being expelled from the particles (similarly to NaCas), an additional emulsifier content re-enters the bulk aqueous phase as a result of the decrease in overall interfacial area due to the increase in the size of the particles. Water crystallisation and ice growth in tandem could be responsible for the displacement of Tween 80/NaCas located at the surface of the particles, resulting in their desorption upon resuspension of the lyophilisate (De Chasteigner, *et al*., 1996).

3.2.2. Emulsion stabilisation

It was previously shown that key features linked to Pickering functionality such as size and interfacial behaviour can be controlled via process and formulation parameters (Zafeiri,

Norton, *et al.*, 2017). Additionally, in this work we have demonstrated the maintenance of these attributes after a secondary processing step (i.e. lyophilisation). Such characteristics enabled lipid-based particles fabricated in the presence of surface active species to act as Pickering stabilisers in o/w emulsion systems (Zafeiri, Smith, *et al.*, 2017). It was thus the ultimate objective of this study to investigate whether particles withstand a drying and desiccation environment and preserve their Pickering functionality.

To that end, lyophilised and reconstituted lipid particles formed with two different surface active species were used to produce 20% o/w emulsions. The effect of drying time on the ability of these systems to exhibit Pickering functionality was also investigated. In particular, it was explored whether duration of drying, therefore the moisture content of particles, had an effect on the capacity to stabilise emulsions. Droplet size distribution curves and mean droplet size values for o/w emulsions formed with cetyl palmitate particles originally fabricated in the presence of NaCas are presented in Fig. 10 and listed in Table 1.

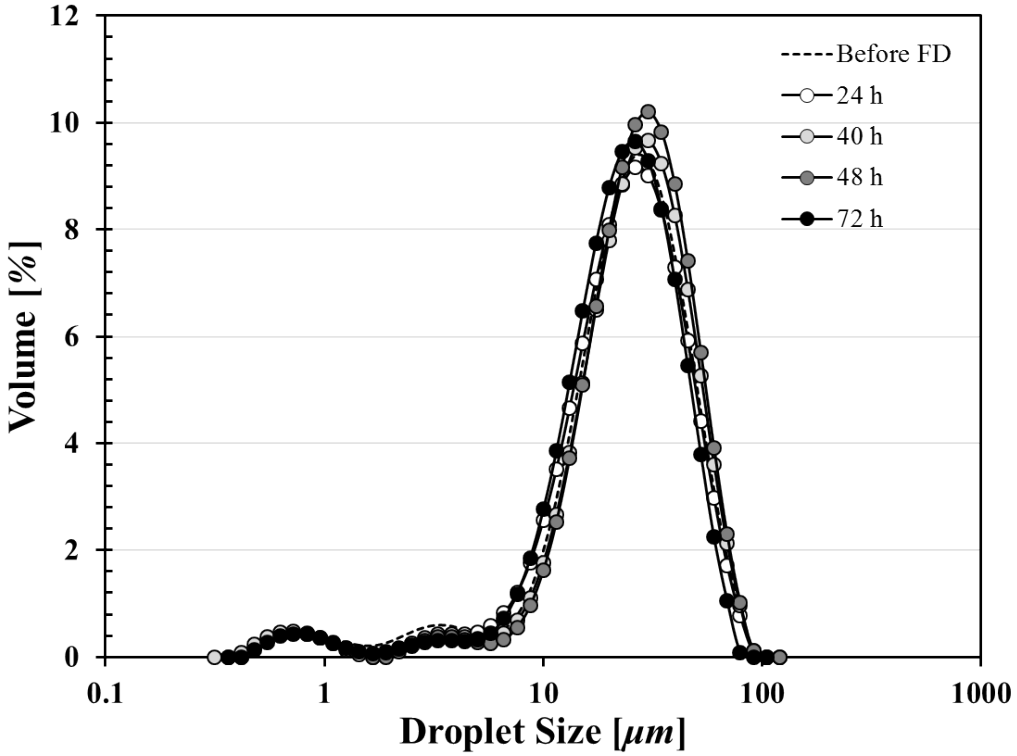

Fig. 10. Droplet size distribution curves for 20% sunflower oil emulsions formed with dried and reconstituted 2.5% wax particles and 0.8% NaCas for different drying times. The same emulsions produced with non-freeze-dried particles are also included for comparison.

As can be seen from Fig. 10, the capacity to stabilise emulsions seems to recover after a freeze-drying and rehydration cycle of lipid (wax) particles formed using NaCas as the surface-active species. This data suggests that maintaining key particle attributes such as size and a level of IFT performance appears to also correspond to a recovery of Pickering functionality. This capacity appeared also not to be influenced by either short or longer drying times.

Conversely, this was not the case with dried and rehydrated lipid particles formed with Tween 80 where all the resulting emulsions had larger average droplet size compared to the emulsions produced using non-dried particles (data not shown). This was an expected result as bigger sized particles such as the ones acquired after the lyophilisation of Tween 80-present lipid particle systems, would form and stabilise larger emulsion droplets.

Table 1. Mean droplet size and span values for 20 wt% sunflower oil emulsions formed with dried and rehydrated 2.5 wt% cetyl palmitate particles and different amounts of NaCas, for different drying times.

	0.8% NaCas			2% NaCas		
Drying time (h)	**$D_{3,2}$ (µm)**	**$D_{4,3}$ (µm)**	**Span**	**$D_{3,2}$ (µm)**	**$D_{4,3}$ (µm)**	**Span**
0	10.4±1.5	28.9±2.7	1.8±0.1	8.4±0.3	20.2±3.2	1.7±0.1
24	15.0±4.1	31.2±3.1	1.5±0.1	8.8±0.7	31.1±0.1	1.8±0.1
40	13.0±1.1	31.2±0.1	1.5±0.1	9.3±0.3	26.7±3.8	1.7±0.1
48	22.1±1.5	31.5±1.9	1.4±0.1	8.6±0.6	24.0±2.6	1.8±0.1
72	15.0±3.5	34.5±9.7	1.7±0.3	7.6±0.5	20.5±0.7	1.8±0.1

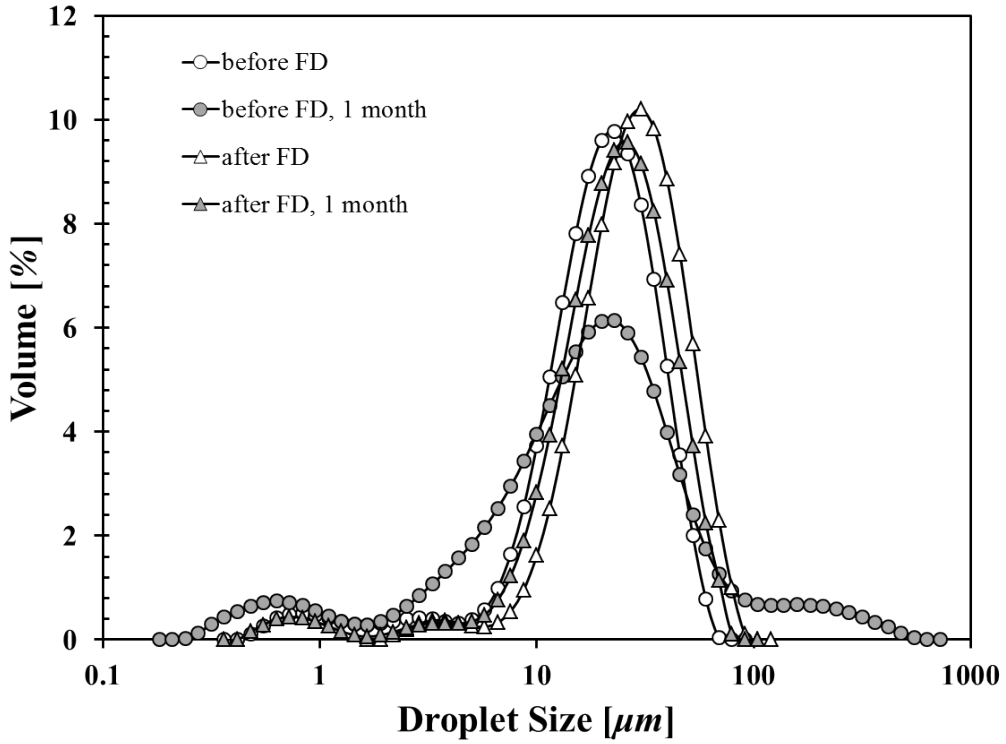

Fig. 11. 20 wt% o/w emulsions formed with dried and rehydrated 2.5 wt% wax particles and 0.8 wt% NaCas stored for different times at refrigeration temperatures. The same emulsions produced with non-freeze-dried particles are also included for comparison.

O/W emulsions were also produced after resuspending the powders that were stored for different time lengths. As shown previously, wax-based dried powders were more stable when stored at 4 ºC rather than at 20 ºC, hence the systems stored at the former temperature were chosen to stabilise sunflower-in-water emulsions. The resulting droplet sizes for a short and a longer duration storage time as a factor of the type of the surface-active component (here NaCas) are presented in Fig. 11.

In contrast to cetyl palmitate particles that were not subjected to a lyophilisation process, the emulsions formed with the dried and redispersed ones, appeared to be less sensitive to coalescence, with only minor changes in their droplet sizes after one month storage in the fridge. It seems that dried lipid particles not only maintain their Pickering functionality following a freeze-drying step, but also confer enhanced stability to coalescence when used as o/w emulsion stabilisers. These observations are speculative and require a more extensive study to elucidate the impact of a lyophilisation and rehydration process on the subsequent behaviour of particles as emulsion stabilisers.

4. Conclusions

Lyophilisation of solid lipid nanoparticles, a research topic heavily explored in literature, is examined in this work on the basis of formulation strategies that enable the retention of particles' original capacity to form stable Pickering emulsions after undergoing drying and rehydration. In contrast, studies so far, although scarce, have been mostly confined to obtaining dried powders from emulsions, in particular using particle-stabilised emulsions as the templates for drying processes (Mezzenga & Ulrich, 2010; Adelmann, *et al*., 2012; Marefati, *et al*., 2013; Hu, *et al.*, 2016; Whitby, *et al*., 2017).

The present study showed that the choice of surface active species during the initial fabrication of lipid particles is very crucial when it comes to drying or isolating the particulates from their aqueous environment. Sodium caseinate emerged as a "smart" drying aid which enabled particles to withstand the harsh freezing and desiccation conditions and retain their original size. This behaviour was found to be independent of the reconstitution method or concentration of sodium caseinate. It was postulated that the high molecular weight sodium caseinate provides a robust interfacial layer that protects lyophilised particulates from interactions and subsequent aggregation. A level of protection could additionally be provided by the presence of a large molecular mass entity in the bulk phase, demonstrated via the use of two biopolymers (HPMC and LMP). The above mentioned protective performance is in stark contrast to a low molecular mass surface active component (e.g. Tween 80) or the conventional cryoprotective agents – sucrose, trehalose and maltodextrins added prior to drying – which did not enable the conservation of the initial particle properties. Nevertheless, a 10 wt/wt% cryoprotectant concentration had a more drastic effect on the recovery of particle's original size. It would also be useful to investigate separately the freezing and drying processes and study the redispersion behaviour and stability of lipid particles after freeze-thawing, in parallel to past work on o/w emulsions (Magnusson, Rosén, & Nilsson, 2011; Marefati, *et al*., 2013). This would provide a

clear distinction between the effect of emulsifier surface layer formation during freezing and particle consolidation encouraged during the drying stage.

Retention of the lipid particles' microstructures was also observed for the rehydrated particles formed with NaCas upon different drying times (hence moisture contents) and storage of the dried powders under refrigeration temperatures for up to one month. The effect of powder storage on the (long-term) stability of rehydrated emulsions/dispersions is a topic that is seldom encountered in literature despite its significance in the development of formulations that can be truly reconstituted. Concurrently, colloidal crystalline structures fabricated in the presence of NaCas were shown, for the first time, to have the potential to undergo a drying and rehydration stage with a minimal loss on their Pickering functionality. These particulate structures could generate o/w emulsions with droplets of a size similar to emulsions formed with particles that had not undergone the additional processing step. This functionality could be achieved using only food-grade components and a facile-to-implement route that does not involve further ingredient modification.

Lipid-based Pickering particles and emulsions stabilised by such particles could be destined not only for food but also for cosmetics, pharmaceutical or agrochemical applications and, in particular, when drying is the intention. In the long run, it is envisaged that dried particulates could be manufactured by a pool of alternative templates such as natural lipids and waxes (e.g. carnauba, rice bran) together with thick interfacial layer forming surface active components, and the use of rapid drying techniques (e.g. microwave vacuum drying).

Acknowledgements
The authors gratefully acknowledge financial support from Engineering and Physical Sciences Research Council (EPSRC), Innovate UK and Cargill for completion of this work.

References

1. Abadias, M., Benabarre, A., Teixidó, N., Usall, J., & Viñas, I. (2001). Effect of freeze drying and protectants on viability of the biocontrol yeast Candida sake. *International Journal of Food Microbiology, 65*(3), 173-182.
2. Abdelwahed, W., Degobert, G., & Fessi, H. (2006). A pilot study of freeze drying of poly(epsilon-caprolactone) nanocapsules stabilized by poly(vinyl alcohol): Formulation and process optimization. *International Journal of Pharmaceutics, 309*(1–2), 178-188.
3. Abdelwahed, W., Degobert, G., Stainmesse, S., & Fessi, H. (2006). Freeze-drying of nanoparticles: Formulation, process and storage considerations. *Advanced Drug Delivery Reviews, 58*(15), 1688-1713.
4. Adelmann, H., Binks, B. P., & Mezzenga, R. (2012). Oil Powders and Gels from Particle-Stabilized Emulsions. *Langmuir, 28*(3), 1694-1697.
5. Adhikari, B., Howes, T., Bhandari, B. R., & Langrish, T. A. G. (2009). Effect of addition of proteins on the production of amorphous sucrose powder through spray drying. *Journal of Food Engineering, 94*(2), 144-153.
6. Assegehegn, G., Brito-de la Fuente, E., Franco, J. M., & Gallegos, C. (2019). The Importance of Understanding the Freezing Step and Its Impact on Freeze-Drying Process Performance. *Journal of Pharmaceutical Sciences, 108*(4), 1378-1395.
7. Augustin, M. A., & Hemar, Y. (2009). Nano- and micro-structured assemblies for encapsulation of food ingredients. *Chemical Society Reviews, 38*(4), 902-912.
8. Avaltroni, F., Bouquerand, P. E., & Normand, V. (2004). Maltodextrin molecular weight distribution influence on the glass transition temperature and viscosity in aqueous solutions. *Carbohydrate Polymers, 58*(3), 323-334.
9. Berny, J.-F., & Hennebert, G. L. (1991). Viability and stability of yeast cells and filamentous fungus spores during freeze-drying - Effects of protectants and cooling rates. *Mycologia, 83*(6), 805-815.
10. Champagne, C. P., Gardner, N., Brochu, E., & Beaulieu, Y. (1991). The freeze drying of lactic acid bacteria: a review. *Can. Inst. Sci. Technol. J., 24*, 118-128.
11. Christensen, K. L., Pedersen, G. P., & Kristensen, H. G. (2001). Preparation of redispersible dry emulsions by spray drying. *International Journal of Pharmaceutics, 212*(2), 187-194.
12. Dawoud, M. Z., & Nasr, M. (2016). Comparison of drug release from liquid crystalline monoolein dispersions and solid lipid nanoparticles using a flow cytometric technique. *Acta Pharmaceutica Sinica B, 6*(2), 163-169.
13. De Chasteigner, S., Cavé, G., Fessi, H., Devissaguet, J.-P., & Puisieux, F. (1996). Freeze-drying of itraconazole-loaded nanosphere suspensions: A feasibility study. *Drug Development Research, 38*, 116-124.
14. de Valdez, G. F., de Giori, G. S., de Ruiz Holgado, A. P., & Oliver, G. (1983). Comparative study of the efficiency of some additives in protecting lactic acid bacteria against freeze-drying. *Cryobiology, 20*(5), 560-566.
15. Dickinson, E. (2012). Use of nanoparticles and microparticles in the formation and stabilization of food emulsions. *Trends in Food Science & Technology, 24*(1), 4-12.
16. Ding, P., & Pacek, A. W. (2008). De-agglomeration of goethite nano-particles using ultrasonic comminution device. *Powder Technology, 187*, 1-10.
17. Drake, A. C., Lee, Y., Burgess, E. M., Karlsson, J. O. M., Eroglu, A., & Higgins, A. Z. (2018). Effect of water content on the glass transition temperature of mixtures of sugars, polymers, and penetrating cryoprotectants in physiological buffer. *PLOS ONE, 13*(1), e0190713.
18. Dulieu, C., & Bazile, D. (2005). Influence of lipid nanocapsules composition on their aptness to freeze-drying. *Pharmaceutical Research, 22*, 285-292.
19. Farshchi, A., Ettelaie, R., & Holmes, M. (2013). Influence of pH value and locust bean gum concentration on the stability of sodium caseinate-stabilized emulsions. *Food Hydrocolloids, 32*(2), 402-411.

20. Goff, H. D., Caldwell, K. B., & Stanley, D. W. (1993). The influence of polysaccharides on the glass transition in frozen sucrose solutions and ice cream. *Journal of Dairy Science, 76*, 1268-1277.
21. Gupta, R., & Rousseau, D. (2012). Surface-active solid lipid nanoparticles as Pickering stabilizers for oil-in-water emulsions. *Food & Function, 3*(3), 302-311.
22. Hirsjarvi, S., Peltonen, L., Kainu, L., & Hirvonen, J. (2006). Freeze-drying of low molecular weight poly(L-lactic acid) nanoparticles: effect of cryo- and lyoprotectants. *J Nanosci Nanotechnol, 6*(9-10), 3110-3117.
23. Hu, Z., Marway, H. S., Kasem, H., Pelton, R., & Cranston, E. D. (2016). Dried and Redispersible Cellulose Nanocrystal Pickering Emulsions. *ACS Macro Letters, 5*(2), 185-189.
24. Lee, M. K., Kim, M. Y., Kim, S., & Lee, J. (2009). Cryoprotectants for freeze drying of drug nano-suspensions: Effect of freezing rate. *Journal of Pharmaceutical Sciences, 98*(12), 4808-4817.
25. Lim, S.-J., & Kim, C.-K. (2002). Formulation parameters determining the physicochemical characteristics of solid lipid nanoparticles loaded with all-trans retinoic acid. *International Journal of Pharmaceutics, 243*(1–2), 135-146.
26. Liu, Y., Zhao, Y., & Feng, X. (2008). Exergy analysis for a freeze-drying process. *Applied Thermal Engineering, 28*(7), 675-690.
27. Magnusson, E., Rosén, C., & Nilsson, L. (2011). Freeze–thaw stability of mayonnaise type oil-in-water emulsions. *Food Hydrocolloids, 25*(4), 707-715.
28. Marefati, A., Rayner, M., Timgren, A., Dejmek, P., & Sjöö, M. (2013). Freezing and freeze-drying of Pickering emulsions stabilized by starch granules. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 436*, 512-520.
29. McClements, D. J. (2004). Protein-stabilized emulsions. *Current Opinion in Colloid & Interface Science, 9*(5), 305-313.
30. Mehnert, W., & Mäder, K. (2001). Solid lipid nanoparticles: Production, characterization and applications. *Advanced Drug Delivery Reviews, 47*(2–3), 165-196.
31. Mezzenga, R., & Ulrich, S. (2010). Spray-Dried Oil Powder with Ultrahigh Oil Content. *Langmuir, 26*(22), 16658-16661.
32. Mishra, D. K., Dhote, V., Bhatnagar, P., & Mishra, P. K. (2012). Engineering solid lipid nanoparticles for improved drug delivery: promises and challenges of translational research. *Drug Delivery and Translational Research, 2*(4), 238-253.
33. Morais, A. R. d. V., Alencar, É. d. N., Xavier Júnior, F. H., Oliveira, C. M. d., Marcelino, H. R., Barratt, G., Fessi, H., Egito, E. S. T. d., & Elaissari, A. (2016). Freeze-drying of emulsified systems: A review. *International Journal of Pharmaceutics, 503*(1–2), 102-114.
34. Mu, M., Farshchi, A., Holmes, M., Chen, J., & Ettelaie, R. (2019). Effect of storage temperature and relative humidity on long-term colloidal stability of reconstitutable emulsions stabilised by hydrophobically modified starch. *Food Hydrocolloids, 95*, 62-75.
35. Mun, S., Cho, Y., Decker, E. A., & McClements, D. J. (2008). Utilization of polysaccharide coatings to improve freeze–thaw and freeze–dry stability of protein-coated lipid droplets. *Journal of Food Engineering, 86*(4), 508-518.
36. Mun, S., McClements, D. J., & Surh, J. (2011). Influence of maltodextrin addition on the freeze-dry stability of β-lactoglobulin-based emulsions with controlled electrostatic and/or steric interactions. *Food Science and Biotechnology, 20*(4), 1143.
37. Ogawa, S., Decker, E. A., & McClements, D. J. (2003). Influence of Environmental Conditions on the Stability of Oil in Water Emulsions Containing Droplets Stabilized by Lecithin−Chitosan Membranes. *Journal of Agricultural and Food Chemistry, 51*(18), 5522-5527.
38. Patel, S. M., Bhugra, C., & Pikal, M. J. (2009). Reduced Pressure Ice Fog Technique for Controlled Ice Nucleation during Freeze-Drying. *AAPS PharmSciTech, 10*(4), 1406.
39. Patel, S. M., Doen, T., & Pikal, M. J. (2010). Determination of End Point of Primary Drying in Freeze-Drying Process Control. *AAPS PharmSciTech, 11*(1), 73-84.
40. Pawlik, A., Kurukji, D., Norton, I., & Spyropoulos, F. (2016). Food-grade Pickering emulsions stabilised with solid lipid particles. *Food & Function, 7*(6), 2712-2721.
41. Pikal, M. J., & Shah, S. (1990). The collapse temperature in freeze drying: Dependence on measurement methodology and rate of water removal from the glassy phase. *International Journal of Pharmaceutics, 62*(2), 165-186.

42. Pikal, M. J., Shah, S., Roy, M. L., & Putman, R. (1990). The secondary drying stage of freeze drying: drying kinetics as a function of temperature and chamber pressure. *International Journal of Pharmaceutics, 60*(3), 203-207.
43. Ratti, C. (2013). Freeze drying for food powder production. In B. Bhandari, N. Bansal, M. Zhang & P. Schuck (Eds.), *Handbook of food powders: Processes and properties* (pp. 57-84). Cambridge, UK: Woodhead Publishing Ltd.
44. Reddy, K. B. P. K., Awasthi, S. P., Madhu, A. N., & Prapulla, S. G. (2009). Role of Cryoprotectants on the Viability and Functional Properties of Probiotic Lactic Acid Bacteria during Freeze Drying. *Food Biotechnology, 23*(3), 243-265.
45. Rezvankhah, A., Emam-Djomeh, Z., & Askari, G. (2020). Encapsulation and delivery of bioactive compounds using spray and freeze-drying techniques: A review. *Drying Technology, 38*(1-2), 235-258.
46. Schwarz, C., & Mehnert, W. (1997). Freeze-drying of drug-free and drug-loaded solid lipid nanoparticles (SLN). *International Journal of Pharmaceutics, 157*(2), 171-179.
47. Sheu, T. Y., & Rosenberg, M. (1995). Microencapsulation by Spray Drying Ethyl Caprylate in Whey Protein and Carbohydrate Wall Systems. *Journal of Food Science, 60*(1), 98-103.
48. Soltanizadeh, N., Mirmoghtadaie, L., Nejati, F., Najafabadi, L. I., Heshmati, M. K., & Jafari, M. (2014). Solid-State Protein–Carbohydrate Interactions and Their Application in the Food Industry. *Comprehensive Reviews in Food Science and Food Safety, 13*(5), 860-870.
49. Takeuchi, H., Yamamoto, H., Toyoda, T., Toyobuku, H., Hino, T., & Kawashima, Y. (1998). Physical stability of size controlled small unilameller liposomes coated with a modified polyvinyl alcohol. *International Journal of Pharmaceutics, 164*(1–2), 103-111.
50. Tang, X., & Pikal, M. J. (2004). Design of Freeze-Drying Processes for Pharmaceuticals: Practical Advice. *Pharmaceutical Research, 21*(2), 191-200.
51. Vehring, R. (2008). Pharmaceutical particle engineering via spray drying. *Pharmaceutical Research, 25*(5), 999-1022.
52. Wang, W. (2000). Lyophilization and development of solid protein pharmaceuticals. *International Journal of Pharmaceutics, 203*(1), 1-60.
53. Westesen, K., Bunjes, H., & Koch, M. H. J. (1997). Physicochemical characterization of lipid nanoparticles and evaluation of their drug loading capacity and sustained release potential. *Journal of Controlled Release, 48*(2–3), 223-236.
54. Whitby, C. P., Scarborough, H., & Ngothai, Y. (2017). Drying oil-in-water Pickering emulsions to make redispersible powders. *Advanced Powder Technology, 28*(11), 2940-2946.
55. Zafeiri, I., Norton, J. E., Smith, P., Norton, I. T., & Spyropoulos, F. (2017). The role of surface active species in the fabrication and functionality of edible solid lipid particles. *Journal of Colloid and Interface Science, 500*, 228-240.
56. Zafeiri, I., Smith, P., Norton, I., & Spyropoulos, F. (2017). Fabrication, characterisation and stability of oil-in-water emulsions stabilised by solid lipid particles: The role of particle characteristics and emulsion microstructure upon Pickering functionality *Food & Function*.
57. Zhang, L., Liu, L., Qian, Y., & Chen, Y. (2008). The effects of cryoprotectants on the freeze-drying of ibuprofen-loaded solid lipid microparticles (SLM). *European Journal of Pharmaceutics and Biopharmaceutics, 69*(2), 750-759.
58. Zimmermann, E., Müller, R. H., & Mäder, K. (2000). Influence of different parameters on reconstitution of lyophilized SLN. *International Journal of Pharmaceutics, 196*(2), 211-213.
59. zur Mühlen, A., Schwarz, C., & Mehnert, W. (1998). Solid lipid nanoparticles (SLN) for controlled drug delivery – Drug release and release mechanism. *European Journal of Pharmaceutics and Biopharmaceutics, 45*(2), 149-155.

Supplementary Information:

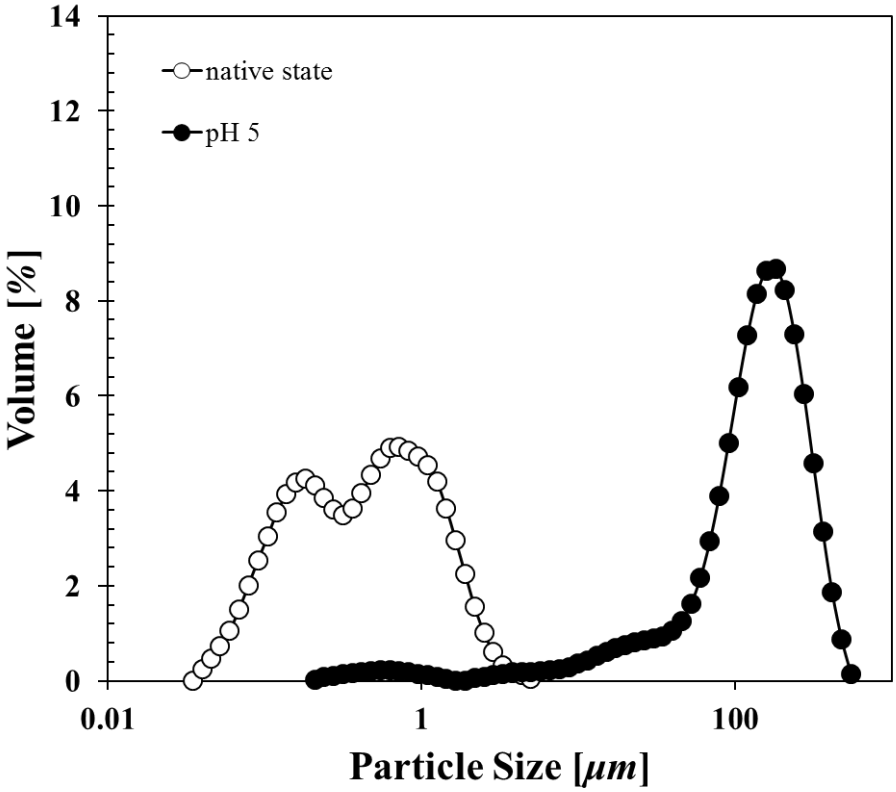

Fig. S1. Effect of protein's pH on lipid particle's redispersion behaviour. Size distributions refer to cetyl palmitate particles formed with 0.8% NaCas.

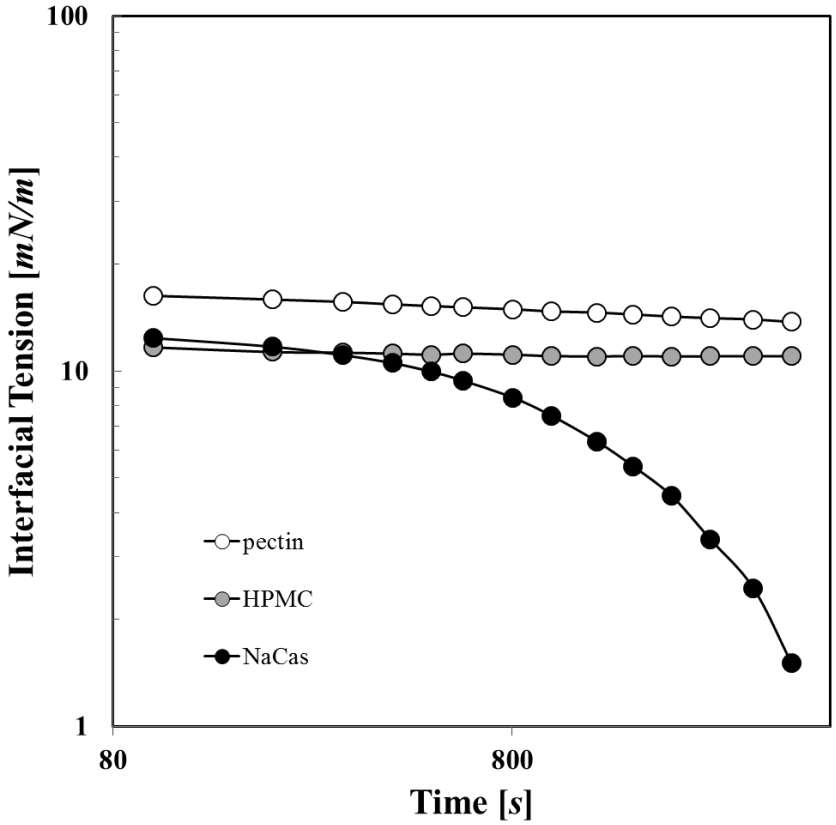

Fig. S2. Behaviour of 2% pectin, HPMC and NaCas (in solution) at the sunflower/water interface.