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Formation and structure of insoluble particles in reconstituted model infant formula powders

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ABSTRACT

Phase separation of insoluble particles, so called “white flecks”, is a physical defect in reconstituted milk obtained from spray-dried, fat-containing dairy powders. Compared with bulk powder particles, white flecks have quite different chemical and morphological characteristics. They consist mainly of proteins and fat, forming a dense network. Here, two concurrent formation mechanisms are suggested based on the structures revealed by confocal microscopy imaging. Fused spherical particles with free fat on the surface were the most abundant and likely formed during the manufacturing process. Sharp-edged particles containing shapes typical of lactose crystals formed during storage. Fat globule stability was thus determined to be critical in preventing white fleck formation. Stability was increased with (a) the presence of lecithin in the oil phase; (b) systems with high protein-to-fat ratio and (c) storage in dry conditions since humidity leads to lactose crystallisation and disruption of the fat globules in powders.

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1. Introduction

Milk powder production is based on pre-concentrating of milk by thermal evaporation followed by drying with a spray dryer or a rotary wheel atomiser. During spray drying, the concentrate is sprayed through an orifice with high pressure and exposed to a flow of hot air. Water is rapidly removed and solid particles are formed (Kelly & Fox, 2016). Reconstituted milk powder should ideally reflect the organoleptic, nutritional and colloidal properties of fresh milk. However, an issue that is often encountered upon reconstitution of IMF is the presence of lecithin in the oil phase; (b) systems with high protein-to-fat ratio and (c) storage in dry conditions since humidity leads to lactose crystallisation and disruption of the fat globules in powders.

In this work, WF is defined as the insoluble material that suspends atop the aqueous dispersion forming a thin layer (Písecký, 1997). Therefore, the WF is assigned to fat. They typically have diameters of a few hundred micrometres and are prone to adhesion onto the contacting surfaces of the container. Their presence is easily determined by pipetting reconstituted milk onto a black plate. Besides the unappealing appearance of WF in reconstituted milk, they also promote clogging of the orifices in bottle nipples, reducing usability of the IMF. WF are only observed in reconstituted fat-containing milk powders, while they are absent in the case of skim milk powder. Therefore, their formation in milk powders is ascribed to the presence of milk fat. In IMF, the role of other components, such as vegetable fat, protein or lactose in the formation of WF is still unclear, as well as the morphology and composition of the particles. Thorough characterisation of the particles is needed to understand the mechanisms of formation and the conditions to minimise their occurrence in the reconstituted IMF.

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Insoluble particles with various sizes may form with several mechanisms at different stages of the manufacturing process. Insolubility originates from heat instability of the system before or during drying and depends on the composition, pretreatment (homogenisation, pasteurisation, lecithination) characteristics of the concentrate (pH, solids content, viscosity), dryer parameters (nozzle design, inlet and outlet temperatures) and finally, the transport and storage conditions (Sadek et al., 2014; Schuck et al., 2016; Sharma, Jana, & Chavan, 2012). Lactose, native whey proteins and some inorganic salts are the only completely water soluble components in milk powder. Therefore, fat globules, casein micelles, denatured whey protein and their combinations have the ability to form insoluble material (McKenna, 2000).

Protein insolubility is initiated by the unfolding of whey proteins followed by aggregation with casein (Baldwin, 2010; Sharma et al., 2012; Straatsma, Van Houwelingen, Steenbergen, & De Jong, 1999) and in fat-containing systems this results in formation of protein-bridged fat globule clusters (Singh & Ye, 2010). Based on the previous research (De Ruyck, 1991; McKenna et al., 1999; Mol, 1975; Ohba, Takahashi, & Igarashi, 1989) the formation of insoluble material is hypothesised to originate from heat and shear applied during spray drying resulting in changes in fat-protein interactions. Also, emulsion quality before drying (Schmidmeier, O’Gorman, Drapala, & O’Mahony, 2017) and fat crystallisation (Regost, 2016) as well as whey protein denaturation (Joyce, Brodkorb, Kelly, & O’Mahony, 2017; McKenna et al., 1999) at different stages of drying may play a role. Lactose crystallisation during humid storage has been reported to result in disruption of fat globules, higher amount of free fat and rougher particles in model infant formula powders made with different protein-to-fat ratios (McCarthy et al., 2013). This could contribute to insoluble material formation during storage.

This study presents the isolation and characterisation of white flecks from a model IMF powder and a suggestion of their origin. WF morphology and composition was determined with confocal imaging, chemical and thermal analyses. Fat globule distribution, fat globule clustering and size in the native powders were characterised to elucidate what type of powder structure is related to the WF formation. The experimental results suggest at least two different formation mechanisms, the control of which may assist in minimising the formation of such insoluble particles.

2. Materials and methods

2.1. Infant formula powders

IMF powders were composed from fresh fat standardised milk, demineralised whey powder (Valio Demi™90, Lapinlahti, Finland), infant formula fat OP3 (Cargill, Izegem, Belgium) containing sunflower, rapeseed and coconut oil and lecithin, and minerals to meet the standard IMF for stages 1–3 according to Technical Regulation of the Customs Union “On Safety of Milk and Dairy Products” (TR TS 033/2013). Target values are reported in Table 1. IMF powders were prepared with an industrial scale drying process in controlled conditions in a series of 33 preparations at Valio Ltd, Lapinlahti, Finland. Vegetable oil was homogenised at 140/40 bar (Tetra Alex 350, Tetra Pak, Lund, Sweden) and the mixture was evaporated to average dry matter of 50% using mechanical vapour recompression (MVR) type evaporator (GEA, Skanderborg, Denmark) and spray dried (Tetra Magna, Tetra Pak, Lund, Sweden).

In this study, detailed microstructural analysis of two stage 1 IMF powders (Powder A and Powder B) from the test series is presented and correlations between composition, structure and white flecks formation are evaluated from the 33 test powders. Apart from the lecithin addition in Powder B, the composition of the powders was similar. Moisture content of powder A was lower (2.3% in powder A and 1.4% in powder B) but this was not significant for white fleck formation as described later in section 4. Results and discussion. Six of the test runs (including Powder A) had a second homogenisation with a high pressure pump in the feed line (pressures 80/40 bar). Three test runs (including Powder A) had a higher second stage homogenisation pressure (140/80 bar). The composition of the powders (averages ± standard deviations) is shown in Table 1. Eight powders were stage 1–2 powders without lecithin, 15 were stage 1–2 powders with lecithin, seven were stage 3 powders without lecithin and three were stage 3 powders with lecithin. Target levels of lecithin in the powder were 0.2–0.3%. Free calcium was determined from reconstituted solution (12%) using an ion selective electrode (ionised calcium micro volume electrode, Konelab™T-series) and an Arena 30 analyser (Thermo Scientific, Vantaa, Finland). The effect of the compositional variables on white fleck formation was evaluated by calculating the correlation coefficient between variables whereas values < 0.5 indicate negative correlation.

The powders were stored in closed containers in ambient conditions. To demonstrate the effect of increased humidity, the powders were kept for 3 weeks in evacuated desiccators in the presence of saturated NaCl and K2CO3 salt solutions (Merck KGaA, Darmstadt, Germany) for water activity (aw) of 0.08 and aw 0.43, respectively.

2.2. Extraction and quantification of white flecks

WF were extracted while trying to minimise the interference of any other insoluble material or material originating from the soluble fractions and dried during sample preparation. In particular, the extraction of SDP and WF has not been well established. SDP are extracted and quantified based on the fact that they remain on the walls of the test tube after pouring off the reconstituted milk or by filtration after 2 min of standing time (Niro, 2005; Piscecky, 1997; GEA). The WF number (WFN) test (ISO/IDF, 2009), unlike SDP, is based on the clogging of a 63-μm sieve that results in slower filtration. However, these tests do not distinguish between WF and SDP. For quantification of WF, the IDF method cannot be used to appropriately distinguish between samples with low number of WF.

Here, WF were determined by dissolving 30 g of powder in 250 mL water at 50 °C followed by stirring for 1 min. The solution was allowed to stand for 10 min and 2 mL was pipetted from the top onto a black plate. The visible WF were counted and a lower score 45 were assigned unacceptable for consumption. The particles were counted only when the total count was <45, otherwise the powder was assigned with the score 45.

2.3. Compositional analysis of white flecks

For compositional analysis, reconstituted solution was filtered through a 63 μm sieve, the insoluble material was washed with water at 50 °C two times and collected from the sieve. Protein, fat and dry matter contents were analysed in duplicate from the WF material extracted by sieving. Protein was determined with the Kjeldahl method (ISO/IDF, 2014) and fat with the Schmid-Bodzynski-Ratzlaff method (ISO/IDF, 2004).

2.3.1. SDS-PAGE

Protein composition was determined with SDS-PAGE ran in duplicate from reconstituted stage 1–3 bulk powders and
Table 1

Composition, particle size parameters and number of white flecks in of the bulk IMF powders in the series of 33 model powders and target compositions for stage 1–3 powders.\(^a\)

<table>
<thead>
<tr>
<th>Component</th>
<th>Stage 1–2 no lecithin</th>
<th>Stage 1–2 with lecithin</th>
<th>Target</th>
<th>Stage 3 no lecithin</th>
<th>Stage 3 with lecithin</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (%)</td>
<td>1.8 ± 0.3</td>
<td>1.8 ± 0.3</td>
<td>2</td>
<td>1.8 ± 0.1</td>
<td>1.6 ± 0.4</td>
<td>2</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>26.0 ± 1.2</td>
<td>26.4 ± 0.8</td>
<td>27</td>
<td>27.8 ± 0.3</td>
<td>28.4 ± 0.2</td>
<td>28.5</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>11.5 ± 0.5</td>
<td>11.3 ± 0.3</td>
<td>10.9</td>
<td>16.9 ± 0.3</td>
<td>16.6 ± 0.5</td>
<td>16.8</td>
</tr>
<tr>
<td>Fat:Protein</td>
<td>2.3 ± 0.2</td>
<td>2.3 ± 0.1</td>
<td>2.5</td>
<td>1.6 ± 0.0</td>
<td>1.7 ± 0.0</td>
<td>1.7</td>
</tr>
<tr>
<td>Lactose (%)</td>
<td>56.7 ± 1.3</td>
<td>56.2 ± 0.7</td>
<td>57</td>
<td>48.2 ± 0.4</td>
<td>47.9 ± 1.2</td>
<td>48</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>2.4 ± 0.2</td>
<td>2.4 ± 0.1</td>
<td>2.5</td>
<td>3.5 ± 0.1</td>
<td>3.7 ± 0.2</td>
<td>3.5</td>
</tr>
<tr>
<td>GOS (mg 100 g(^{-1}))</td>
<td>1.7 ± 0.1</td>
<td>1.8 ± 0.3</td>
<td>1.6</td>
<td>1.8 ± 0.0</td>
<td>1.6 ± 0.0</td>
<td>1.6</td>
</tr>
<tr>
<td>Ca (mg 100 g(^{-1}))</td>
<td>429 ± 66</td>
<td>401 ± 12</td>
<td>410</td>
<td>584 ± 14</td>
<td>700 ± 20</td>
<td>690</td>
</tr>
<tr>
<td>Free Ca (mg L(^{-1}))</td>
<td>63 ± 4</td>
<td>63 ± 12</td>
<td>58 ± 14</td>
<td>67 ± 2</td>
<td>32 ± 13</td>
<td>14 ± 8</td>
</tr>
<tr>
<td>Lecithin (%)</td>
<td>0</td>
<td>0.2 ± 0.0</td>
<td>0</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Emulsion particle size (µm)</td>
<td>16.7 ± 7.8</td>
<td>2.4 ± 1.2</td>
<td>4.5 ± 2.7</td>
<td>1.6 ± 0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat globule size (µm)</td>
<td>0.9 ± 0.2</td>
<td>0.9 ± 0.3</td>
<td>0.8 ± 0.1</td>
<td>0.7 ± 0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White flecks (pcs)</td>
<td>41 ± 8</td>
<td>18 ± 14</td>
<td>32 ± 13</td>
<td>14 ± 8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Values are averages ± standard deviation. Target is as per the Technical Regulation of the Customs Union "On Safety of Milk and Dairy Products" (TR TS 033/2013).

- Corresponding WF samples extracted from the stage 1–2 powders as described in section 2.2. WF sample (20 mg) was mixed with the sample buffer (1 mL containing 23% glycerol (95.5%, BDH Prolabo, Paris, France), 4% sodium dodecyl sulphate (SDS; Sigma–Aldrich, St. Louis, MO, USA), 10% 2-mercaptoethanol (Sigma–Aldrich) and 0.02% bromophenol blue (Merck KGaA, Darmstadt, Germany) in 0.1 M Tris–HCl (Trizma\(^®\)hydrochloride, 99%, Sigma–Aldrich) buffer, pH 6.8. The sample was centrifuged at 10,000 × g for 10 min. The middle phase (40 µL) was heated for 5 min at 100 °C and centrifuged at 13,500 × g for 30 s followed by deposition (7.5 µL) on the electrophoresis gel. Criterion\(^TM\)TXG\(^TM\) 12% Precast Gels and Precision Plus Protein\(^TM\) All blue standards (Bio-Rad Laboratories Inc., Hercules, CA, USA) were used. The liquid fractions were diluted 1:30 and mixed with the sample buffer (23.1 µL sample ± 7.7 µL sample buffer for stage 1–2 and 16.7 µL sample ± 5.6 µL sample buffer for stage 3 powder).

- The electrophoresis buffer contained 0.025 M Trizma base (Sigma–Aldrich), 0.192 M glycine (Sigma–Aldrich) and 0.1% SDS. Electrophoresis was run at 200 V and 100 mA for approximately 1 h using a PowerPac Basic Power Supply (Bio-Rad Laboratories Inc, Kaki Bukit, Singapore). The staining solution contained 0.1% Coomassie brilliant blue R-250 (Bio-Rad Laboratories Ltd., Watford, UK), 30% ethanol (Altia, Rajamäki, Finland) and 10% acetic acid (Merck KGaA). The gels were imaged with Gel Doc\(^TM\)EZ Imager (Bio-Rad Laboratories Inc., Hercules, CA, USA).

2.4. Microscopy

2.4.1. Optical microscopy

IMF powder samples and WF particles were imaged with a stereomicroscope (Zeiss Stemi 2000-C, Jena, Germany) to compare the morphologies of bulk powder and WF. The sample was spread on the microscopy slide. The WF sample was prepared by adding a drop of the reconstituted solution on the microscopy slide. Lactose crystals in powders were imaged with an optical microscope (Nikon Eclipse Ci-L, Nikon Instruments, Tokyo, Japan). The powder was dissolved in cold water and a drop was added on the microscopy slide, covered with a cover slip and imaged with 40 × magnification.

2.4.2. Confocal laser scanning microscopy

IMF powder samples and WF particles were imaged with confocal laser scanning microscopy (CLSM; Leica TCS SP2, Leica Microsystems GmbH, Heidelberg, Germany) to visualise fat globules in bulk powders and morphologies of the WF particles in detail. The powder sample for CLSM was spread onto the microscopy slide in an area outlined with double-sided tape. The staining solution (5 µL) containing 0.02% Nile Red (for microscopy, Sigma–Aldrich) in 1,2-propanediol was added and the sample was then covered with a cover slip. The WF sample was prepared by collecting individual particles on the microscopy slide from the black plate after reconstitution as described in section 2.2. The slide was subsequently washed with water at 50 °C to remove the possible residues of a soluble fraction.

- The WF particle sample was prepared by adding ~10 µL 0.05% Acidine Orange base (Sigma–Aldrich) and 2 µL Nile Red staining solution on top of a single particle on a microscope slide. The sample was imaged without a cover slip. A 63 × oil immersion objective or 20 × dry objective was used for imaging the powder samples and a 10 × dry objective for imaging the WF particles. Ar/Kr laser at 488 nm was used for excitation. For powder, the cross-section image provided the best visualisation of the fat globules in powders. For WF particles, the 3D projections were created from a stack of optical sections.

2.4.3. Scanning electron microscopy

Powder samples were also imaged with scanning electron microscopy (SEM; FE-SEM, JEOL JSM-7500FA, JEOL Ltd., Tokyo, Japan) to compare morphologies of bulk powders A and B. The powder sample for SEM was attached on the SEM stub with double-sided carbon tape, blown with air to remove loose particles and sputter-coated with gold and imaged at 2.00 kV.

2.5. Particle sizing

Emulsion particle sizes were determined by laser diffraction (Mastersizer 2000, Malvern Instruments, Malvern, UK) in distilled boiled water medium. Powders were reconstituted as described in section 2.2. The solution was stirred before adding to the dispersion unit. Each result is an average of four measurements. Particle size in dispersions without pretreatment originated from various components, namely, casein micelles, fat globules, fat globule clusters and insoluble material. Particle sizing, therefore, was taken only as an indication of both the insoluble material and fat globule clusters in powders. For WF particles, the 3D projections were created from a stack of optical sections.
pH 7 (adjusted with NaOH), was added directly to the dispersion unit. The measurement had a delay time of 40 s and each result is an average of five measurements.

2.6. Differential scanning calorimetry

Differential scanning calorimetry (DSC) was performed with DSC3+ instrument (Mettler Toledo AG, Schwerzenbach, Switzerland). One to five milligrams of sample was weighed in 40 μl aluminium pans and sealed. The given sample was heated from 20 °C to 80 °C to remove the thermal history, then cooled to −50 °C and heated again to 130 °C at 5 °C min⁻¹. Each sample was measured in duplicate. The second heating scans were used in the characterisation of the powders.

3. Results and discussion

3.1. Composition of white flecks

Compositional and morphological characterisation indicated that the nature of WF is completely different from the typical bulk powder particles. WF material was comprised of 75% fat and 22% protein based on dry matter of the extracted material. The composition of the WF was thus very different from that of the bulk powder (Table 1), namely, the former was highly enriched with fat. Both fat and protein and their interactions are expected to play a critical role in the formation of WF.

Protein profiling with SDS-PAGE showed that the WF material included both caseins and whey proteins (Fig. 1). The percentage ratios of caseins and whey proteins were estimated from the intensities of the bands shown in Table 2. The estimations for bulk powders are in line with the actual values although for stage 1–2 bulk powders, SDS-PAGE results in a slightly higher casein-to-whey ratio than the actual value (34:66). The result for stage 3 bulk powder is very close to the actual value (59:41). The slight differences compared with the actual values can result from loss of protein during sample preparation or effectiveness of staining. Percentage ratio of caseins to whey proteins in WF can be estimated to be 60:40. Casein was therefore enriched in the WF material compared with bulk stage 1–2 powder. Due to the interactions of the milk proteins with fat during the manufacturing process, the presence of both casein and whey protein in the fat-protein complex forming the insoluble material is expected.

Chemical composition of WF in whole milk powder (WMP) or IMF has been reported very scarcely. According to Ohba et al. (1989), the insoluble material from spray-dried WMP with fat (27%), protein (26%) and lactose (38%) contained fat (50%) and casein (40%) and the particles were spherical. Despite the fact that the percentage of fat in IMF powder is close to that of WMP, they display a different overall composition and, therefore, the formation mechanisms of the insoluble material are likely to be different for these materials. Compared with bovine milk, IMF contains less protein, completely different whey-casein (20:80 in bovine milk and 70:30 in IMF) and elevated calcium-to-protein ratio. In addition to this, unlike WMP, ingredients such as whey protein concentrate (WPC), lactose, vegetable fat and vitamins are used in the IMF powder production (Guo, 2014; Jiang & Guo, 2014). The higher protein content determined for WF from WMP compared with WF from IMF can be understood as the protein content of WMP is also higher than that of IMF. Recently, Regost (2016) reported the composition for WF in IMF powder. The WF material in IMF powder included 91.5% fat and 0.6% protein. The higher fat and lower protein content compared with our results indicate that WF can be formed by various different mechanisms. The composition of the fat blend is not known and could also be the origin of the different characteristics of the WF material. In the process by Regost (2016), fat crystallisation was probably the dominating cause for the WF material formation.

3.2. Morphology of bulk powders and white flecks

The morphologies of the bulk powder particles and WF were also completely different, as shown in the stereomicrograph of bulk powder and WF as well as the SEM images of Powders A and B (Fig. 2). Bulk powder particles consisted of agglomerates of spherical particles with a primary particle diameter ranging from 10 to 100 μm. In contrast, the WF appeared as fusions of large spherical particles with porous surfaces. Powder A and Powder B showed similar morphologies (SEM images): they consisted of agglomerated powder with smooth surfaces. From the observations, it is concluded that the underlying reason for WF formation cannot be found in the morphology of the bulk powder particles.

Morphologies and fat distribution of the individual WF particles were visualised in more detail with CLSM. CLSM images of WF particles isolated from the black plate after reconstitution of Powder A are shown in Fig. 3a–c. The images are 3D projections created from a stack of optical sections with red and green indicating protein and fat, respectively. Particles (a) and (b) had a clearly different structure from the powder particles (Fig. 3d): they did not appear as agglomerates of spherical powder particles with clearly distinct fat globules. They rather showed a more continuous network of protein and fat with some distinct areas enriched in fat on the surface. Particle (a) appeared as a fusion of spherical particles having a porous surface with spherical openings. In some locations, the surface was disrupted with fat overhangs projecting outward from the particle. Particle (b) had a more continuous mass of fat and a more sharp-edged shape. Also, angular shapes were observed as negative contrast on the particle. These angular shapes

<table>
<thead>
<tr>
<th>Samples</th>
<th>Caseins (%)</th>
<th>Whey proteins (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk powder Stage 1</td>
<td>42 ± 2</td>
<td>58 ± 2</td>
</tr>
<tr>
<td>Bulk powder Stage 2</td>
<td>44 ± 2</td>
<td>56 ± 2</td>
</tr>
<tr>
<td>Bulk powder Stage 3</td>
<td>63 ± 1</td>
<td>37 ± 1</td>
</tr>
<tr>
<td>WF 1</td>
<td>56 ± 0.2</td>
<td>45 ± 0.2</td>
</tr>
<tr>
<td>WF 2</td>
<td>61 ± 0.5</td>
<td>39 ± 0.4</td>
</tr>
</tbody>
</table>

Values are averages ± standard deviation.

Fig. 1. SDS-PAGE gel for stage 1 (lanes 2 and 3), stage 2 (lanes 4 and 5) and stage 3 (lanes 6 and 7) powders and WF materials (lanes 9 and 10) and (11 and 12).
indicated lactose crystallisation. Cross-section images of particle (b) between 20 μm steps in the vertical direction are presented in Fig. 3c, whereby arrows were drawn to point to the angular shapes that were observed on the surfaces. Based on the shapes of lactose crystals and their visualisation as negative contrast in CLSM images (McKenna, 2000), these features are proposed to originate from lactose crystals. The square shape on the right penetrates through the particle while the axe-shaped feature on the left was located on the surface of the particle. The presence of lactose crystals in the bulk powder was verified with optical microscopy after dissolving the bulk powder in cold water (Fig. 4).

The significant impact of storage on the fat structure was verified by CLSM imaging of powders stored for 3 weeks at different water activities, 0.08 and 0.43 (Fig. 3d and e). The powder stored in dry conditions \( (a_w = 0.08) \) showed a typical morphology that included intact fat globules. The powder stored at humid conditions \( (a_w = 0.43) \) showed abundant lactose crystallisation shown as angular and needle-like negative contrast on the surfaces and in the interior of the bulk powder particles. Compared with the small number of lactose crystals observed after room temperature storage, the powders stored in humid conditions contained abundant lactose crystals. Leakage of the fat is shown as bright green areas. It is evident that lactose crystallisation resulted in disruption of fat globules and leakage of fat from the powder as has also been shown previously (McCarthy et al., 2013). The angular shapes on insoluble material in Fig. 3b could be of this origin and suggest a possible mechanism of insoluble material formation during humid storage.

We hypothesise that the origins of the WF particles are associated with concurrent mechanisms whereby large, porous, fusions of spherical particles (Fig. 3a) formed during the manufacturing process. This type of WF particles was clearly the most abundant. On the other hand, the smaller, sharp-edged particles with shapes corresponding to lactose crystals (Fig. 3b) were formed during storage. Lactose crystals growing inside the particle may have contributed to the breaking of the fat globule structure, as shown in Fig. 3e, resulting in formation of the insoluble core, while the outer layer of the particle was dissolved. Lactose crystallisation resulting in breakdown of the fat globule structure during storage is proposed here as the reason for the formation of the structure in Fig. 3b.

3.3. Phase transitions and thermal behaviour of bulk powder and white flecks

Differential scanning calorimetry (DSC) was used to detect phase transitions in bulk powder and WF. Possible phase transitions in these materials include fat melting, lactose glass transition and instant crystallisation (Jouppila & Roos, 1994). DSC was therefore applied to detect fat melting as well as amorphous lactose. The phase transitions are best visualised in the derivative of the heat flow (see Fig. 5 for the derivative of heat flow normalised with sample mass for WF and bulk powder). Milk fat triglycerides melt in a wide range of temperatures, between \(-40\) and \(35\) °C (van Aken, ten Grotenhuis, van Langevelde, & Schenk, 1999). Small, broader peaks were detected below \(0\) °C, together with sharp, distinct peaks at around \(5\), \(15\) and \(35\) °C. These peaks were labelled 1, 2 and 3. The melting profile depends on the interaction of vegetable fat, milk fat and other components. As the fat melting peaks 1, 2 and 3 are better defined in the WF than bulk powder, it can be concluded that fat was enriched in the WF material compared with bulk powder. However, the DSC signal does not allow distinguishing between milk fat and vegetable fat. In addition to fat melting, phase transitions of lactose were detected in the DSC thermograms of the bulk powder (Fig. 5a). Lactose glass transition (\(T_g\)) occurred at around \(45\) °C while instant crystallisation (\(T_{cr}\)) took place at \(85\) °C for a powder stored in ambient conditions. These features indicated the presence of amorphous lactose in the bulk powder, which were absent in WF. Regost (2016) compared the extracted total fat from the powder and the WF material and observed that WF contained fat with a higher melting point than the bulk fat. It was proposed that WF formation originated from low emulsion quality and fat crystallisation to the form with a high
melting point. In the present work, no such effect was observed and therefore, fat crystallisation to a form with a higher melting point was not the root cause of the WF material formation.

3.4. Impact of particle size in the reconstituted emulsions on formation of white flecks

As the WF phenomena is known to be associated solely to fat-containing powders, the internal fat structure of the powder particles was further explored, aiming to determine if WF formation was related to the fat structure of the corresponding powders. CLSM images were compared for a powder that resulted in WF (Powder A) and one that did not develop WF (Powder B) upon reconstitution were compared. Representative images of the powders are shown in Fig. 6a and b with fat globules visualised in green. McKenna et al. (1999) reported that uneven size distribution and spreading of the fat globules was the origin of the poor solubility. In our powders, no major differences were observed in the fat globule structure of the two powders. Both powders displayed evenly spread fat globules within the powder particles, with no clear difference in their size, whether on the surface or the interior of the particle. Therefore, it can be concluded that, in the present case, the distribution of the fat globules within the particles was not the main reason for WF formation.

Particle size determinations of reconstituted powders in Fig. 6c and d showed a main population at 1 μm, assigned to fat globules. The population at 100 μm originated from insoluble materials that could be removed by sieving: the 10 μm population is assigned as fat coalescence or flocculation (Drapala, Auty, Mulvihill, & O’Mahony, 2017). Removal of particles with diameters greater than 3 μm by

Fig. 3. CLSM images showing 3D projections of the two different types of WF particles: fusion of spherical particles (a) and a sharp-edged particle (b). The cross section images of the sharp-edged particle between 20 μm steps in (c) reveal angular shapes shown by arrows. The impact of lactose crystallization on bulk powder particle during humid storage is shown in d and e. Fat is visualised in green in all images and protein in red in a–c. (For interpretation of the references to color/colour in this figure legend, the reader is referred to the Web version of this article.)
the SDS/EDTA pretreatment is an indication of dissociation of protein-bridged fat globule clusters: $d_{4,3}$ changed from 19 $\mu m$ to 1.3 $\mu m$ in Powder A, before and after the pretreatment, respectively. The particle size distribution of Powder B remained almost unchanged after the SDS/EDTA treatment ($d_{4,3}$ = 1 $\mu m$ before and after pretreatment) showing that there was little protein-bridged fat globule clustering in Powder B. The correlation of $d_{4,3}$ before the SDS/EDTA treatment with WF formation was evident in the series of 33 powders (correlation coefficient 0.656). Table 1 also shows that even though fat globule size was similar in all powders, less fat globule clustering (shown as the smaller emulsion particle size without the SDS/EDTA treatment) occurred when lecithin was included. This also reduced the WF formation.

3.5. Impact of bulk powder composition on formation of white flecks

The presence of lecithin in the oil blend significantly decreased the amount of white flecks in powders (correlation coefficient –0.557). The impact of fat percentage and fat to protein ratio became evident when the white flecks were plotted grouped by the lecithin content and samples with lower fat:protein ratio were distinguished (Fig. 7). Without lecithin in (a), the amount of white flecks could only be reduced for a low fat percentage or fat-to-protein ratio. With lecithin, in (b), the amount of white flecks was low unless the fat percentage was high, but even at high fat percent, the amount of white flecks was low if the fat-to-protein ratio was low. With the highest amount of lecithin, amount of white flecks remained low.

Powder A and Powder B had similar compositions, except that Powder B included lecithin and had a lower moisture content. Moisture and white flecks formation were not correlated in the 33 test powders (correlation coefficient 0.099). The presence of lecithin in the oil blend (emulsifier) and a low fat-to-protein ratio was determined to be critical to reduce the number of WF. This suggests that a better protection of the fat globules in the concentrate would reduce the number of WF. In model infant formula emulsions, lecithin has been shown not only to function as an emulsifier but also to increase heat stability due to formation of protein-phospholipid complexes (McSweeney, Healy, & Mulvihill, 2008). Whey protein hydrolysate conjugation with maltodextrin has shown to have a similar effect (Drapala, Auty, Mulvihill, & O’Mahony, 2016). Fig. 7 shows the WF count as a function of the fat percentage for powders with varying fat to protein ratio and lecithin content. Ohba et al. (1989) proposed that calcium phosphate aggregates the fat-protein complexes and thus insoluble material was formed in WMP. The amount of the insoluble material increased with fat content and decreased with addition of sodium polyphosphate (calcium chelator), with addition of emulsifying agents or with reduction of calcium content. Here, neither the calcium content nor the free calcium content in the powders varied, so lecithin emulsifier likely protected the fat globules from aggregation with calcium. In a recent discussion, Schmidmeier et al. (2017) have also indicated that emulsion stability was the most important reason for WF formation in the coarse particle fraction in fat-filled powders.
4. Conclusions

We found that white flecks (WF) have a quite different structure compared with that of powder particles. WF consist of fat (75%) and protein (22%) forming a continuous network that contains both casein and whey protein. Increased fat-protein clustering in the powder is associated with WF formation. WF have at least two structures types originating either from the fusion of spherical particles (with porous, partly broken surfaces and fat leaking out of the particle), or from sharp-edged morphologies containing lactose crystals. CLSM images of powder obtained after humid storage indicates that lactose crystals formed during storage. To the best of our knowledge, this is the first report to address the formation and characterisation of key morphological features of WF. The identification of the two different types of WF particles highlights that both, the drying process and the storage conditions, needs consideration to reduce WF formation. The stability of the fat globules can be achieved by addition of an emulsifier and by lowering the fat-to-protein ratio. Fat globule clustering should be avoided as well as humid storage. Humid storage promotes lactose

Fig. 6. Cross-section CLSM images of (a) Powder A that resulted in a high amount of WF upon reconstitution and (b) Powder B that resulted in no WF and corresponding particle size distributions without pretreatment (solid line) and after sieving and SDS/EDTA pretreatment (dashed line) for (c) Powder A and (d) Powder B. Removal of the tail in (c) after the pretreatment indicates fat globule clustering in the powder.

Fig. 7. WF count with increasing fat percentage of the powder with target lecithin contents (a) 0%, (b) 0.2% and (c) 0.3% in powders. High fat-to-protein ratios (stage 1–2 powders) are marked with (×) and low (stage 3 powders) with [■].
crystallisation in powders, which is known to cause remarkable changes in the powder structure, resulting in disruption of the fat globules and reconstituting behaviour.

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