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Freeze-drying of protein pharmaceutical in vials with different character

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Abstract

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Freeze-drying of protein pharmaceuticals is a procedure frequently used to obtain stability of the active pharmaceutical ingredient during distribution and storage. It can be performed in pre-filled syringes, with a lubricous coating of silicone on the inside, to enable the piston moving. The coating changes the environment potentially affecting the features of the freeze-dried cake since the wetting behavior and adhesion to the inner wall is affected. This project aimed to investigate the effect of the siliconization of the cakes. Three different formulations were freeze-dried in non-siliconized (NS) and siliconized vials using different siliconization protocols. Analysis was done using differential scanning calorimetry (DSC), thermal gravimetric analysis (TGA), scanning electron microscopy (SEM), X-ray photoelectron spectroscopy (XPS) and an embedding method, intended to give information about the cake's shrinkage, cracking and pore-structure. The water content in the bottom of the cakes was consistently higher than in the top, a difference decreasing over time. Increased surface hydrophobicity lead to increased shrinkage of the cake's volume and a decrease in fogging. The bottom of the protein cake in the vial siliconized with a commercial silicone emulsion consisted of pores with regularly equal pore size and thick pore walls, a structure not seen in any other cake. All cakes in the silicone emulsion siliconized vials had lower water content than the cakes in the vials using the other siliconization method. The XPS-analysis showed that the cakes in the emulsion siliconized vials contained silicon, indicating an excess of silicone when siliconizing and/or an unstable coating.

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Popular scientific summary

Biopharmaceuticals are a type of medicine where the active pharmaceutical ingredient (the molecule having the desired pharmaceutical effect) is a biological macromolecule or a cellular component. Proteins are biological macromolecules used as the active pharmaceutical ingredient in several drugs on the market today; as for example insulin which is used to treat diabetes. In 2017, FDA (U.S. Food and Drug Administration) claimed that 29% of the total new molecule entities approved by the FDA were biopharmaceuticals, showing the importance of these macromolecules treating diseases now and in the future. The importance of biopharmaceuticals, such as proteins, in treating diseases is mostly because of their high specificity and therapeutic efficacy. They also have the potential to treat diseases which the most common class of pharmaceuticals, small molecule pharmaceuticals, are not able to treat.

However, there are problems arising with proteins due to their chemical and physical instability. Usually, a pharmaceutical is taken orally as a pill, which is considered to be a simple and secure way of administration. When a protein is taken orally the body is not able to distinguish if the protein is a pharmaceutical or if it is food. Therefore, the body digests it into amino acids, and hence the pharmaceutical effect is completely lost. This problem is solved by injecting the protein, which gets the protein into the blood stream without passing through the gastrointestinal tract. To inject the protein, it must be in solution, leading to the next problem, which is the instability of proteins in solution. When the protein is in solution it may lose its structure, and since the structure is important for the way of action, the protein also loses its effect as a drug.

This stability problem can be solved by freeze-drying the solution. During freeze-drying the solution is first frozen and the ice is removed by sublimation (in other words, the ice transforms into water vapor without becoming a liquid) to avoid high temperatures which could be detrimental for the protein stability. Sublimation happens when the ice is held under vacuum, leaving the protein with the additives as a porous and dry cake. In this way, the problem with the instability of the protein in the presence of water is solved, although the additives must be chosen with care to support the protein stability during drying. Freeze-drying of proteins is usually done in a ready-to-use glass syringe which can be surface treated with a silicone coating from the inside. The silicone coating works as a lubricant to enable easier movement of the piston during injection. This silicone coating changes the environment for the pharmaceutical when it is freeze-dried in the syringe compared to non-coated containers possibly affecting the attributes of the final pharmaceutical product.

In this project, a commercially available protein pharmaceutical was freeze-dried in vials with and without silicone coating to investigate the effect of the silicone coating in the final product. The protein used was a truncated human recombinant protein with a molecular weight of 16192.7 Da with mainly a β-sheet structure. The final product was analyzed visually and with several analytical techniques at different scales. The coating affected the freeze-dried cakes in some of the aspects that was examined, but it would have been interesting to examine more replicas. However, this could provide important information which could hopefully be used to improve the stability and the quality of protein pharmaceuticals freeze-dried in ready-to-use syringes.

Table of content

1. Introduction

Biopharmaceuticals (such as proteins) offer, compared to small molecules, advantages in high specificity and therapeutic efficacy[1,2]. According to FDA's center of drug evaluation, 29% relative to the total new molecule entities approved by the FDA in 2017 were biopharmaceuticals[3]. There are two major problems often discussed when it comes to protein pharmaceuticals, one is their instability in solution and the other is their low bioavailability when administered orally. To avoid the bioavailability problem, administration through injection is used and pre-filled syringes are then often the primary packaging of choice. Both liquid and solid forms of protein pharmaceuticals are available but proteins in solution tend to involve stability problems, such as denaturation, degradation, chemical destabilization and aggregation[4]. When these problems cannot be overcome by formulation improvement or storage at low temperature a different approach is needed. The liquid form is then dried into a solid form. Solid forms of protein pharmaceuticals can be manufactured by freeze-drying which allows the formulation to obtain stability during storage, distribution and delivery[1] to achieve acceptable shelf life.

Freeze-drying as a drying method is suitable since proteins are instable at high temperatures. The process of freeze-drying consists of three main segments freezing, primary drying and secondary drying. During freezing, most of the water is transformed into ice crystals by lowering the temperature while the solutes (excipients and protein) and some non-crystallizable water remain as a freeze concentrate[5]. The freeze concentrate is an amorphous glass with a glass transition temperature denoted as T_g'[6]. Primary drying is carried out under low pressure while the temperature is slowly ramped up providing the energy necessary to sublimate the ice. One important aspect during primary drying is to ensure that the temperature is lower than the collapse temperature, T_c, usually several degrees higher than T_g'. T_c is the temperature when collapse in the cake is visually seen and depends not only on the T_g' but also on how the structure develops during drying. These macroscopic structural changes such as deformation and collapse originate from a high energy input leading to temperatures higher than T_c in the drying material, allowing viscous flow, contributing to loss of the porous structure of the cake. Therefore, having knowledge about both T_g' and T_c while freeze-drying is important to obtain acceptable final products[1]. The secondary drying aims to remove the non-crystallized water entrapped in the freeze concentrate by desorption and it is carried out under low pressure and higher temperature than the primary drying to enable desorption of the unfrozen water. After drying the moisture remaining in the product is usually <1%.

When freeze-drying, the appearance of the final product is expected to be an elegant cake with a certain cake volume and a homogenous appearance since these aspects are considered to be critical when inspected by health authorities[7]. Two structural changes, that are not desirable to a great extent, observed in freeze-dried cakes are shrinkage and cracking. Where shrinkage is defined as detachment and contraction of the cake from the vial's inner wall and cracking as formation of fractures through the cake. These structural changes are caused by the buildup of drying tension (tensile stress) within the freeze-dried product when the solvent is removed. Then the cake's tensile stress is released by shrinkage, if possible, or otherwise by cracking[8–10] and it has been shown in several studies that these two phenomena are linked[5,8–10]. Ullrich et al.[9] concluded that shrinkage results from the allowance of plastic flow whereas cracking results from water desorption, and therefore, it is claimed that cracking and shrinkage depend of the amount of residual water at the end of primary drying[9]. Since shrinkage is defined as detachment, adhesion to the wall could affect the cake's ability to shrink. Ullrich et al.[10] investigated the contributions of the vials properties to the shrinkage ability of the cake, which evidenced that the degree of shrinkage was higher in siliconized (hydrophobic) vials than glass (hydrophilic) vials, showing that wetting of the wall has a significant impact. When freeze

drying in siliconized vials, the contraction occurs more uniformly, resulting in more uniform distribution of cake's volume and cracking within the cake[10].

Another quality attribute of freeze-dried products is the appearance of fogging, which can be described as a white haze on the wall of the vials after freeze drying. This feature is believed to occur due to the solution wetting the walls of the vial followed by adsorption of solutes. The solution creeps up the inner wall due to its wetting ability and when freeze-dried, the solutes remain adsorbed to the wall of the vials resulting in fogging.[11] To date, the only approach considered to eliminate fogging is the use of hydrophobic vials, such as siliconized vials, when freeze-drying[11,12].

Pre-filled syringes are ready-to-use devices which have the advantages of improved dose accuracy, reduced contamination risk and improved patient compliance. A lubricious coating enabling the piston to move easily inside the syringe is needed and an appropriate alternative is a silicone coating[13]. However, after siliconization the surface properties of the glass changes from hydrophilic to hydrophobic. The interactions between the protein, excipients and silicone can affect the final product. Several studies on protein formulations in these devices have shown that there is a risk of silicone migrating into the solution, increasing protein aggregation[14–16].

This study aims to evaluate how the use of siliconized glass vials affect the cake's attributes (such as fogging, cracking and shrinkage), the solid-state properties of the excipients and how freezing occurs, potentially affecting the cake's porous structure. To evaluate the effect of the siliconized vials, the study also aims to develop a cake embedding method to visualize the cake structure, based on the embedding method used by Lam and Patapoff 2011[17]. To this end, three different formulations of different complexity (sugars only, sugars with surfactant and buffer and sugars with surfactant, buffer and protein) have been freeze-dried in siliconized and non-siliconized vials.

2. Experimental

2.1 Chemicals and materials

The protein used was a commercially available protein pharmaceutical. Deconex® NS-20x was obtained from Fischer Scientific (Västra Frölunda, Sweden). Tween® 20 (polysorbate) and D(-)-Mannitol was obtained from Merck KGaA (Darmstadt, Germany). L-Histidine, Sucrose, Silicone oil DC 200 and toluene was obtained from Sigma-Aldrich (Steinheim, Germany). Sylgard® 184 silicone elastomer curing agent and base and Dow Corning® 365 and 35% Dimethicone NF Emulsion was obtained from Dow Corning (Midland, Michigan, United States).

The vials used were 6.00mL Fiolax® clear obtained from Pharmaceutical Systems SCHOTT (Müllheim, Germany). The stoppers used were 20mm Flurotec® freeze dry stopper Westar® RS obtained from Adelphi Healthcare Packaging (Haywards Heath, United Kingdom). The seals used where 20mm complete tear off aluminum seal silver from Adelphi Healthcare Packaging (Haywards Heath, United Kingdom).

2.2 Instruments

Contact angle measurements were carried out with a Dataphysics OCA-40 Micro. The differential scanning calorimeter used was a Mettler Toledo DSC 1 STARe System. Thermogravimetric analyzes were done in a Mettler Toledo thermogravimetric analyzer, TGA2. The freeze-drier used to dry the samples was a LyoLogplus, Christ GmbH, Germany. The SEM analyzes were done with a FEI-XL, 30 series and Philips XL30. The x-ray photoelectron spectrometer was a Kratos Analytical AXIS Ultra^{DLD} with a monochromatic Al x-ray source. The photos of the freeze-dried and embedded cakes were taken with a Canon EOS 550D.

2.3 Procedures

2.3.1 Preparation of non-siliconized and siliconized vials

Four different types of vials, with different character, were used when freeze-drying. The siliconization was done using two different methods. The vials siliconized by the first method are denoted 0.5% siliconized vials and 3.0% siliconized vials and the vials siliconized with the second method are denoted 3.5% siliconized vials. The vials that were not siliconized are denoted non-siliconized (NS) vials. To remove any impurities possibly remaining from manufacturing before siliconization, the vials were sonicated for 1h at 30°C in a 1% solution of deconex® NS-20x in Milli-Q, rinsed 5 times with Milli-Q and sonicated for 1h at 30°C in Milli-Q. The vials were then incubated in Milli-Q in room temperature overnight and let dry in room temperature. When dry, the vials were siliconized using two different methods and the first method was based on an article by Gerhardt et al.[18] and two articles by Funke et al.[16,19]. A 0.5% solution of silicone oil in toluene was incubated in half of the vials, while half of the vials were incubated with a 3.0% solution of silicone oil in toluene, both in room temperature overnight. The solution was removed, and the vials were rinsed three times with acetone and placed in an oven at 300°C for 15 minutes.

When siliconizing using the second method, the vials were cleaned and let dry as described previously. The 35% emulsion was diluted to 3.5% with Milli-Q water, and 1.5mL of the emulsion was added to the vials, swirled to wet all surfaces and poured out. The vials were dried in a neck down position on adsorbent tissue paper for 30 minutes and baked in an oven at 300°C for 15 minutes. The vials siliconized by this method are denoted 3.5% siliconized vials.

The non-siliconized (NS) vials was cleaned in the same way as the siliconized vials before use.

After siliconization, 2 mL Milli-Q was added to one of each vial to visually evaluate the stability of the coating.

2.3.2 Contact angle measurements

Hydrophobicity of the surfaces of glass vials and siliconized vials was evaluated with contact angle measurements using water droplets. A droplet of 5µL was placed on the surface one time in three different places and the contact angles were calculated using a line tangent from the intersection from the droplet and the surface to the curve of the droplet. When evaluating, a hydrophobic surface was expected to result in a larger contact angle.

Surface roughness was evaluated with a dynamic sessile drop method placing a droplet on the surface and with a constant rate of 0.5µL/s, increasing the volume of the droplet while monitoring the increase of the droplet's base diameter on the surface.

2.3.3 Freeze-drying process

Three different formulations were prepared consisting of i) 4% mannitol and 2% sucrose (sugar formulation), ii) 4% mannitol, 2% sucrose, 10mM histidine and 0.01% polysorbate 20 (placebo formulation) and iii) 4% mannitol, 2% sucrose, 10mM histidine, 0.01% polysorbate 20 and 5mg/mL protein (protein formulation). The formulation components were dissolved in Milli-Q, 2mL of the solution was pipetted into the vials and stoppers were placed in an open position. The filled vials were placed on the freeze-drier shelf and freeze-dried according to a previously developed process, Table 1, illustrated in Figure 1. After freeze-drying, the vials were flushed with nitrogen, closed and placed in a desiccator, and stored in room temperature. To evaluate the effect of the siliconization methods on the wetting and dissolution, 2mL Milli-Q was added to the freeze-dried cakes while recording the process with an iPhone camera in the slow-motion setting.

Table 1. Freeze-drying process in which the three different formulations were freeze-dried.

Segment	Shelf Temperature [°C]	Pressure [mbar]	Rate [°C/h]	Time [h]
Freezing	-45		-21.7	5
Primary drying	4	0.1	24.6	18.25
Secondary drying	20	0.001	16.0	5.08

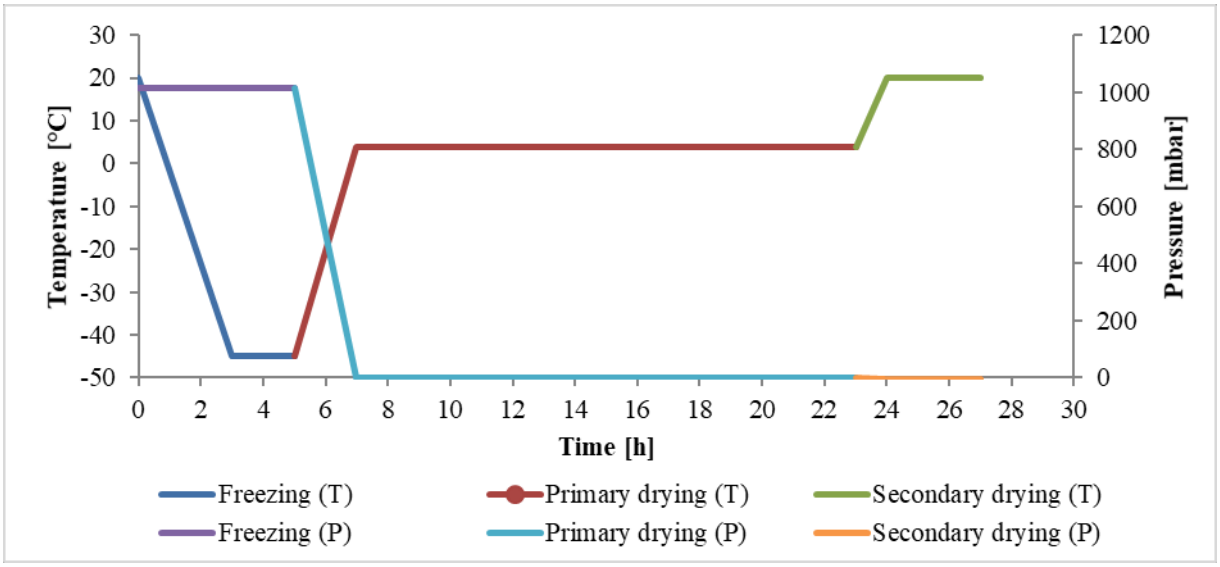

Figure 1. Diagram of the freeze-drying cycle with the temperature of the shelf [°C] and the chamber pressure [mbar] against time [h], showing the three main segments freezing, primary drying and secondary drying.

2.3.4 Differential Scanning Calorimetry

The DSC instrument was initially checked with iodine and zinc, for which the melting temperature, T_m, and the melting enthalpy, ΔH, were evaluated. Top and bottom layer of the freeze-dried cakes were separately analyzed in duplicates. Approximately 1.5mg of the sample was weight into a 40µL aluminum pan and sealed with a pinhole on the lid. The measurement was performed by heating the sample from 20°C to 180°C with a heating rate of 10°C/min under a nitrogen gas flow of 50mL/min, using an empty aluminum pan as reference. The total crystallinity of mannitol was calculated by integrating the melting peak and dividing the normalized integral value with the enthalpy of fusion for D-mannitol[20] (54.69kJ/mol at 437.3K) and adjusting for the mannitol content in the sample. The mean value of the crystallinity from the two measurements was calculated.

2.3.5 Thermal Gravimetric Analysis

Top and bottom layer of the freeze-dried cakes was separately analyzed one time each. 1-2mg of the sample was weight in a sample pan and measurement was performed by heating the sample from 25°C to 250°C with a heating rate of 20°C/min under a nitrogen gas flow of 50mL/min. The weight loss occurring before 120°C was considered to happen due to dehydration, giving the water content of each sample from this weight loss.

2.3.6 Embedding of freeze-dried cakes

To be able to evaluate the cracking and shrinking behavior, freezing procedure and pore structure of the freeze-dried cakes, the entire cake needs to be removed from the vial without breaking. Freeze-dried cakes are fragile, making the removal of the cakes from the vials without breaking them unlikely. Therefore, an embedding method, based the procedure in an article by Lam and Patapoff 2011[17], was used to enable removal of the whole cake from the vial. Sylgard® 184 base and curing agent were mixed in a 10:1 ratio and placed under vacuum to remove air bubbles. Figure 2 illustrates the setup for the embedding process where the vial was placed in a tilted position. The tilted position was evaluated to be the most suitable position for the vial, since when it was not tilted, a "lid" of silicone elastomer was created on top of the cake before the whole cake had been penetrated. Approximately 2.5mL of the silicone elastomer was dripped, under vacuum created with a syringe, onto the cakes and the silicone elastomer was left to cure in room temperature. When fully cured, the vial was broken and the embedded cakes were cut in the middle, creating two equally sized semicircles. The interface of each semicircle

was cut into thin slices by hand (approximately 1mm thick) and to 500µm thick slices by microtome before photographed while on a LED-screen. The shrinkage was evaluated by measuring the width of the cakes, on magnified images of the embedded cakes, and comparing it to the width of the vial with Equation 1,

$$Shrinkage\ [\%] = \left(1 - \frac{width_{cake}}{width_{vial}}\right) \times 100\%, \quad (1)$$

giving a measure of the shrinkage.

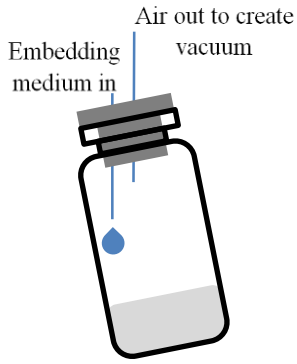

Figure 2. Illustration of the embedding procedure setup.

2.3.7 Scanning electron microscopy (SEM)

The SEM analysis was performer together with a skilled operator. The freeze-dried cakes consisting of sugar, placebo and protein formulation freeze-dried in NS, 0.5%, 3.0% and 3.5% siliconized vials were under low vacuum using scanning electron microscopy with an accelerating voltage of 5kV and secondary electron detector. One of each cake were examined in four different parts of the cake, at the bottom (in contact with the glass), right above the bottom, in the middle and at the top. The samples were attached to a double-sided carbon tapes mounted on aluminum holders.

2.3.8 X-ray photoelectron spectroscopy (XPS)

XPS-measurements and evaluation of the atomic composition was performed by an XPS-specialist. X-ray photo electron spectroscopy (XPS) is an analyzing technique based on the photoelectric effect and provides information about the elemental composition of solid surfaces. The technique is able to analyze with a dept of 2-10nm. The sample is irradiated with x-ray energy and emission of photoelectrons occur. The photoelectrons reaching the detector are only the ones emitted from the outermost surface layer and by analyzing the kinetic energy of these and calculating their binding energy, their origin in terms of element and orbital can be determined. XPS were performed on the freeze-dried cakes consisting of placebo and protein formulation freeze-dried in the vials siliconized with the 3.0% solution and the freeze-dried cakes from the placebo and protein formulation freeze-dried in 3.5% emulsion siliconized vials. Samples from each cake were analyzed by placing a small portion of each sample in a 40µL aluminum pan. The analysis was performed under high vacuum and the analysis area was below 1 mm^2, with most of the signal from an area of about 700×300µm. Each cake was analyzed in two places from four parts of the cake, where the samples was taken from the bottom of the cake (in contact with the vial), right above the bottom (not in contact), from the middle of the cake and at the top of the cake. The placebo and protein cakes freeze-dried in NS vials (not cleaned) had previously been analyzed and were analyzed in the top and the bottom of the cake.

Wide survey spectra were run to detect elements (C, O, N, S, Si) present in the surface layer. By quantification of detail spectra run for each element, the relative surface compositions expressed in atomic% were obtained and in addition, the high-resolution carbon spectra were curve-fitted to obtain information about chemical shifts in the carbon signals due to different functional groups. High resolved nitrogen spectra were also measured, and curve fitted. By analyzing the relative amounts of the elements, the surface composition of the samples can be estimated by assuming that the elemental composition of the surface is a linear combination of the elemental composition of the components[21]. A matrix equation, Equation 2, was created,

$$\boldsymbol{A\gamma} = \boldsymbol{f}, \qquad (2)$$

where **A** was the matrix containing the pure components elemental composition, **γ** was the vector of the different components relative coverage and **f** was the vector containing the samples surface elemental composition. Each component in the sample has different relations between C, O, N and S, including different relations between different types of C and N (determined by the atoms functional groups as previously mentioned). The matrix formula, Equation 2, thereby is expressed as

$$\begin{bmatrix} I^1_{comp.1} & \cdots & I^1_{comp.i} \\ \vdots & & \vdots \\ I^n_{comp.1} & \cdots & I^n_{comp.i} \end{bmatrix} \begin{bmatrix} \gamma_1 \\ \vdots \\ \gamma_i \end{bmatrix} = \begin{bmatrix} I^1_{sample} \\ \vdots \\ I^n_{sample} \end{bmatrix},$$

where $I^n_{comp.i}$ is the relative atomic concentration of element n in pure component I, γ^i is the relative coverage of component i on the surface and I^n_{sample} is the relative amount of element n in the composed sample.

Equation 2 was solved by applying the least-squares method creating an overdetermined system which was solved by using Equation 3,

$$\boldsymbol{\gamma} = (\boldsymbol{A^T A})^{\mathbf{1}} \boldsymbol{A^T f}. \qquad (3)$$

3. Results and discussion

3.1 Hydrophobicity of vials after siliconization

The contact angle measurements (mean value with error bars as max and min value in Figure 3) of the NS vials, after being washed as previously described, resulted in contact angles in a range of 20-25° while the contact angles of the siliconized vials were in a range of 58-64° (0.5% siliconized vial), 70-82° (3.0% siliconized vial) and 94-110° (3.5% siliconized vial). The conclusion from this result was that the hydrophobicity increased with increasing amount of silicone oil in the incubation solution and all three types of siliconized vials were used when freeze-drying to evaluate if different degree of hydrophobicity affects the cake appearance. During the siliconization with the 3.5% emulsion, the emulsion was stuck on the neck of the vials (illustration in Figure 4), which meant that the emulsion was not poured out completely. This could result in an excess of silicone in the vial. The dynamic sessile drop measurement showed smooth surfaces, with an even increase of the base diameter as the drop volume increased and an even decrease of the base diameter as the drop volume decreased.

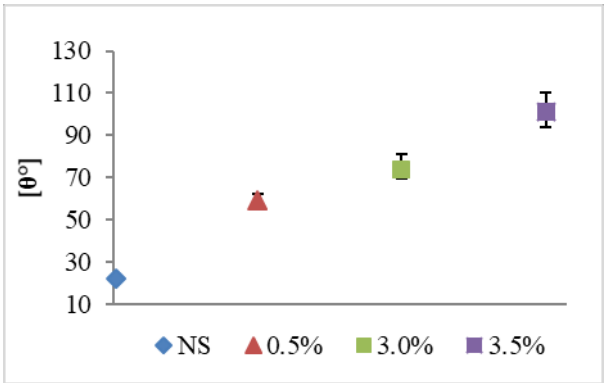

Figure 3. Contact angle measurements [θ°] for the different vials, measured as described in the Methods section. The diagram shows the mean value of three measurements with error bars showing max and min value.

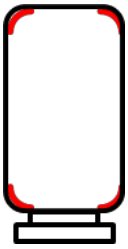

Figure 4. Illustration of how the silicone emulsion, marked in red, gets stuck on the curvature of the vial, possibly creating an excess of silicone.

3.2 Cake appearance (fogging, cracking and shrinkage)

The cake appearance was visually evaluated after the samples were photographed in two different angles, as seen in Figure 5 and Figure 6. The freeze-dried sugar cakes in the 3.0% and 3.5% siliconized vials (Figure 5c and d) were not as adhered to the vials inner wall as the cakes in the NS and 0.5% siliconized vials (Figure 5a and b). The same result was seen when the placebo formulation was freeze-dried in the same kind of vials (Figure 5e-h). The protein formulation showed a decrease in adhesion to the inner wall only when freeze-dried in 3.5% emulsion siliconized vials (Figure 5l). The height of the sugar cakes in 3.0% and 3.5% siliconized vials (Figure 5c and d) and placebo and protein cakes in 3.5% siliconized vials (Figure 5h and l), appears lower than the height of the other cakes. If this is due to the higher degree of wetting of the other cakes or if it is due to shrinkage (in height) is not possible to tell by visual inspection of these pictures. Both the adherence behavior and the change in height

indicates that the surface-active ingredients in the placebo and protein formulations tends to counteract the otherwise declining wettability of the inner wall when the hydrophobicity of the surface increases.

The peak on the surface seen in the upper part of the sugar cake in the 0.5% and 3.0% siliconized vials (Figure 6b and c) also existed, without any distinct connection, in sugar cakes in the other vials as well. Although, this peak originates from the freezing behavior when the solution freezes and the water expand as it crystallizes, the solution in the middle of the vial is pressed upwards, creating this peak in the middle of the cake.

The fogging behavior of the placebo cakes when freeze-dried in vials with increasing hydrophobicity showed a distinct decreasing degree of fogging as the hydrophobicity increased. This phenomenon was not observed as clearly in the protein formulation, but a distinct decrease in fogging was seen when the protein formulation was freeze-dried in the 3.5% emulsion siliconized vials (Figure 6l) compared to when freeze-dried in the other vials. The sugar formulation did not show any fogging in any of the vials (Figure 6a-d). It can be anticipated that the surface tension of the surfactant and protein containing formulations are lower than the sugar formulation, which would result in higher rising on the wall for the sugar formulation. All these observations of the fogging behavior indicate that poor wetting of the glass decreases fogging.

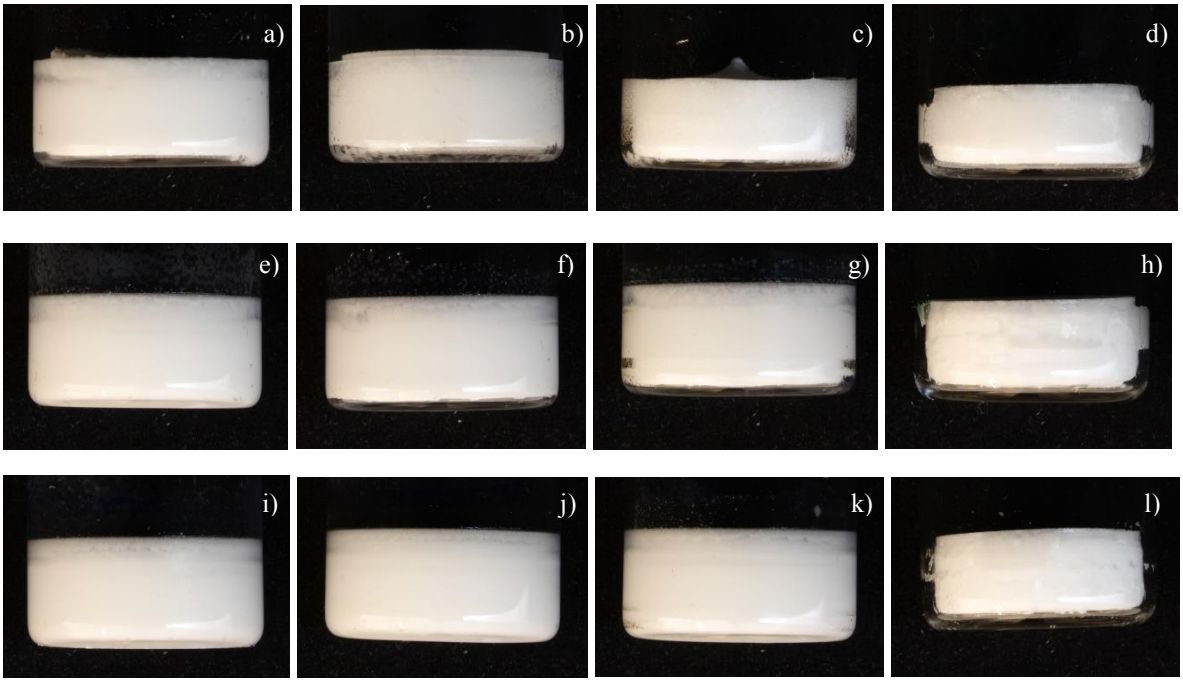

Figure 5. Images of the samples freeze-dried in the different vials. A) sugar formulation in NS vial, b) sugar formulation in 0.5% siliconized vial. c) sugar formulation in 3.0% siliconized vial, d) sugar formulation in 3.5% siliconized vial, e) placebo formulation in NS vial, f) placebo formulation in 0.5% siliconized vial, g) placebo formulation in 3.0% siliconized vial, h) placebo formulation in 3.5% siliconized vial, i) protein formulation in NS vial, j) protein formulation in 0.5% siliconized vial, k) protein formulation in 3.0% siliconized vial and l) protein formulation in 3.5% siliconized vial.

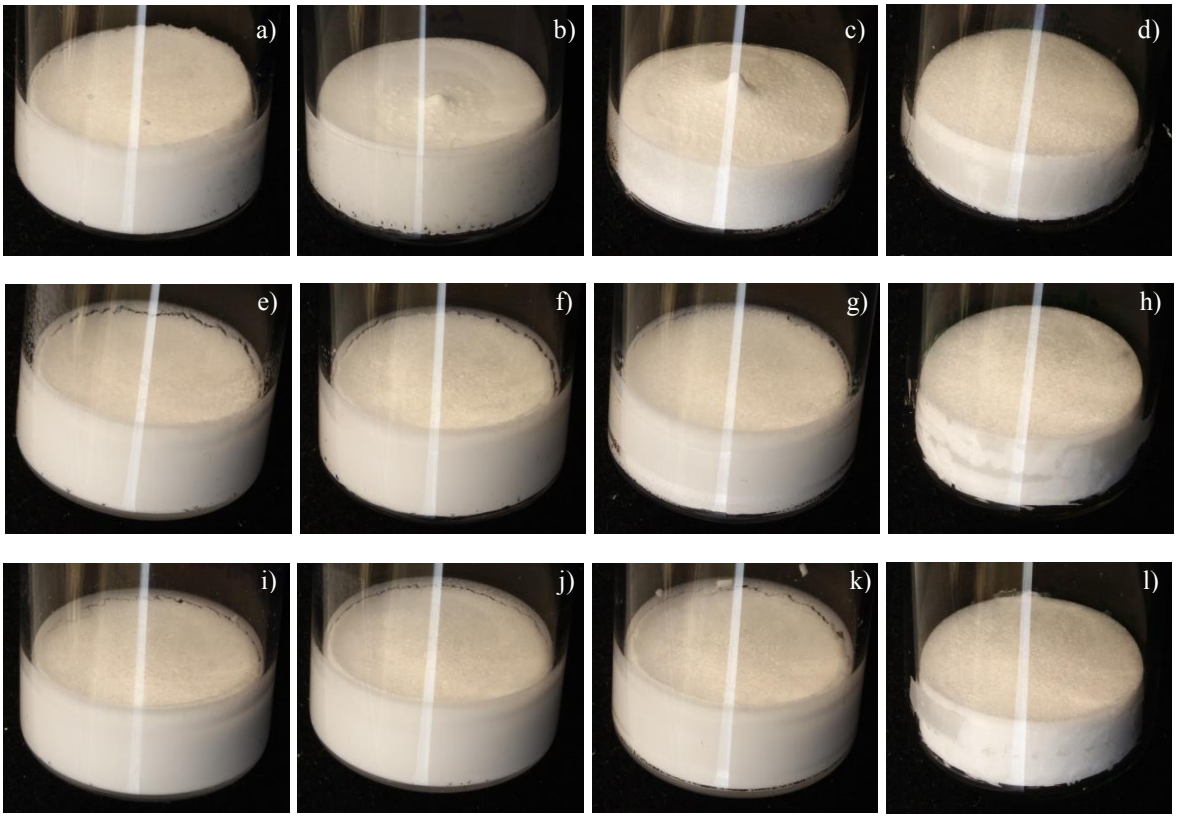

Figure 6. Images of the samples freeze-dried in the different vials. A) sugar formulation in NS vial, b) sugar formulation in 0.5% siliconized vial. c) sugar formulation in 3.0% siliconized vial, d) sugar formulation in 3.5% siliconized vial, e) placebo formulation in NS vial, f) placebo formulation in 0.5% siliconized vial, g) placebo formulation in 3.0% siliconized vial, h) placebo formulation in 3.5% siliconized vial, i) protein formulation in NS vial, j) protein formulation in 0.5% siliconized vial, k) protein formulation in 3.0% siliconized vial and l) protein formulation in 3.5% siliconized vial.

Figure 7 shows the embedded and cut cakes of all three formulations freeze-dried in NS, 3.0% and 3.5% siliconized vials. Some of the cuts resulted in parts of the cut being broken, and the cuts shown in Figure 7 were evaluated to be the most representative in order for the entire cake and pore structure to be visible. Some traces from the cutting process are seen in some of the cakes and appear as vertical lines through the whole cake, most clearly visible in the placebo cake freeze-dried in the 3.0% siliconized vial (Figure 7e). The embedded and cut cakes reveal a toplayer in the cakes, partly separated from the rest of a cake in the sugar cakes (Figure 7a, b, c), placebo cakes freeze-dried in NS and 3.0% siliconized vials (Figure 7d, e) and protein cakes freeze-dried in NS and 3.0% siliconized vials (Figure 7g, h). This toplayer was seen in all the formulations in all types of vials during the study even though it is not visible in these pictures. All cakes were before the embedding checked for visible cracking, and none of them contained visible degree of cracking. Therefore, the cracking of the placebo cake freeze-dried in the 3.5% siliconized vial (Figure 7f) was considered to occur during the embedding process since this high degree of cracking would have been seen during the visible inspection before the embedding. The placebo and protein formualtion cakes freeze-dried in NS vial shows a higher degree of cracking in the bottom of the cake (Figure 7d, g). Ullrich et al.[8–10] concluded that shrinkage and cracking was coupled and that if shrinkage is possible (due to less adhesion to the vials inner wall) it relieves the tension built up during the freeze-drying process. This could explain the cracking of the cakes freeze-dried in NS vials (Figure 7d, g) since the adhesion is higher in the NS vials than in the more hydrophobic vials.

The embedded cakes also show more wetting of the wall for the freeze-dried cakes in NS vials (Figure 7a, d, g) and placebo and protein cakes in 3.0% siliconized vials (Figure 7e, h), similar

to what is seen in Figure 5 and Figure 6. That the formulation wets the surface more was determined by the upwards creaping of the cake on the inner walls of the vial.

The cakes from the sugar formulation (Figure 7a-c) show pores which are longer in the 3.5% siliconized vial than the pores in the cakes freeze-dried in the NS and 3.0% siliconized vials. The pores in the sugar cake in the 3.5% siliconized vial reveals that the solution freezes from the sides, and to some extent from the bottom, since the remaining pores have this shape. The pores in the placebo cakes reveal a freezing process that gives ice crystals from the side in both NS and 3.0% siliconized vials (Figure 7d, e). The pores in the placebo cake from the 3.5% siliconized vial (Figure 7f) shows ice formation vertically upwards, and a smaller degree of pores from the side. The protein cakes shows small pores from the side and bottom in the cakes freeze-dried in NS and 3.0% siliconized vials (Figure 7g, h) and large pores from the side in the 3.5% siliconized vial (Figure 7i).

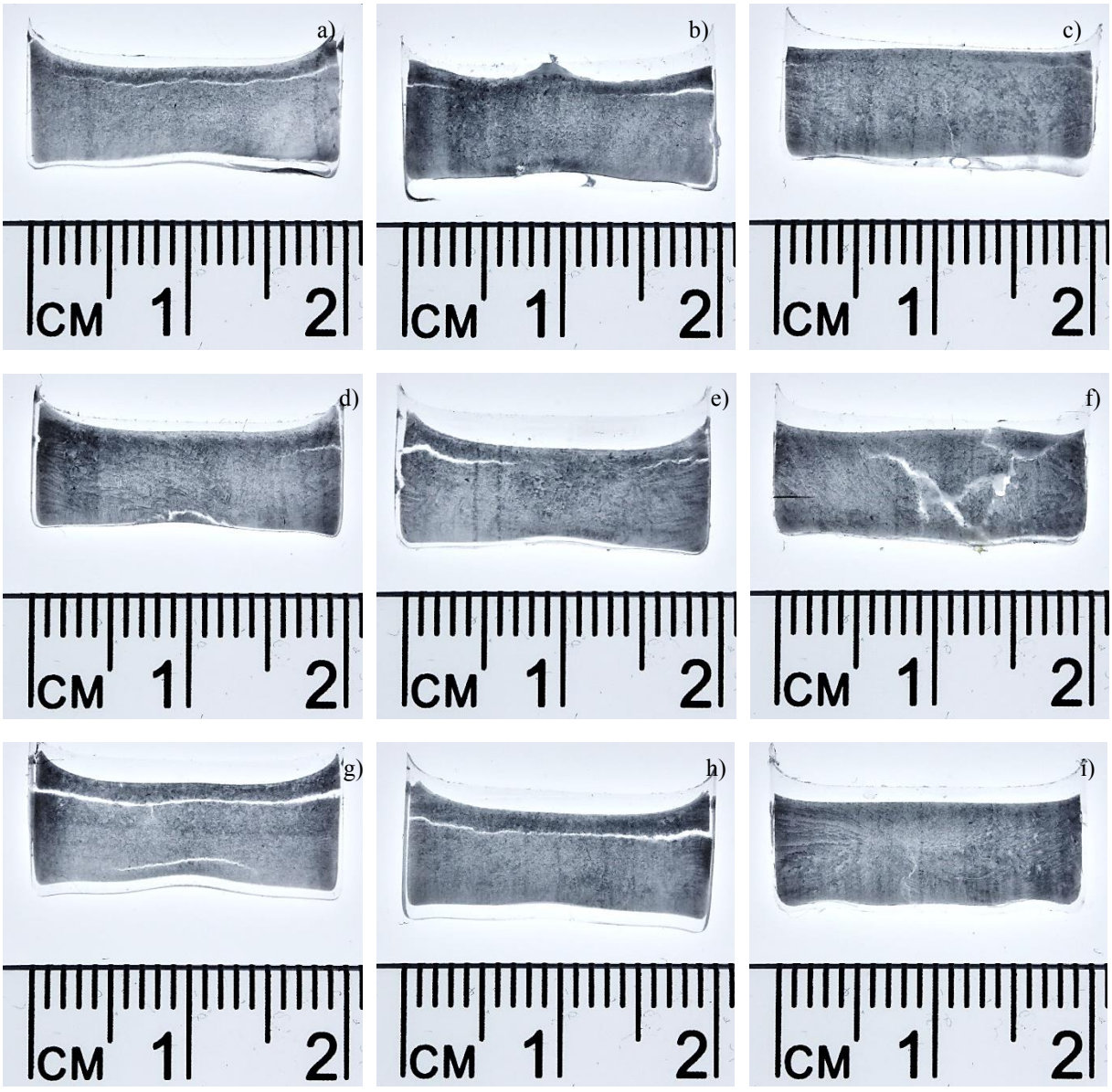

Figure 7. Pictures of embedded cakes. A) sugar formulation in NS vial, b) sugar formulation in 3.0% siliconized vial, c) sugar formulation in 3.5% siliconized vial, d) placebo formulation in NS vial, e) placebo formulation in 3.0% siliconized vial, f) placebo formulation in 3.5% siliconized vial, g) protein formulation in NS vial, h) protein formulation in 3.0% siliconized vial and i) protein formulation in 3.5% siliconized vial.

The shrinkage of the cakes was calculated with Equation 1, and the result is presented in Table 2. The shrinkage in width increased with increased hydrophobicity of the vials, with exception to the placebo cake freeze-dried in the 3.5% siliconized vial. As seen in Figure 7, this cake underwent significant macroscopic cracking during the embedding process which naturally increases the width of the cake, making the shrinkage percentage presented in Table 2 questionable for this sample.

Table 2. The shrinkage in width [%] of sugar, placebo and protein cakes freeze-dried in NS, 3.0% and 3.5% siliconized vials. The shrinkage was calculated by measuring the width of the cake and dividing it to the width of the vial. The shrinkage percentage of the placebo cake freeze-dried in 3.5% siliconized is placed in brackets due to cracking during the embedding process, probably increasing the width of the cake.

	Shrinkage [%]		
Vial	Sugar formulation	Placebo formulation	Protein formulation
NS	2.0	2.6	2.6
3.0% siliconized	2.0	2.9	2.8
3.5% siliconized	2.7	0.9	3.1

3.3 Dissolution of the cake

The vials' effect on the dissolution was investigated by adding 2mL of Milli-Q water to the freeze-dried cakes. The total time for most of the cake to dissolve was <1s and therefore the dissolution of the cakes was recorded with an iPhone camera in the slow-motion setting. No difference in the dissolution behaviour could be seen with this type of study, but the remaining solution presented a difference between the siliconization methods. The cakes freeze-dried in the 3.5% siliconized (siliconized with the emulsion) vials left a cloudy solution while the solution after dissolving in NS and the vials siliconized with silicone oil in toluene (both 0.5% and 3.0%) was clear, and this was observed for sugar, placebo and protein cakes (for pictures of the dissolved cakes see Appendix Figure S1-3). The cloudiness of the solution was investigated immediately and after one and two days showing that the solution in the 3.5% emulsion siliconized vials remained cloudy over time. This indicates that the silicone and/or the surfactant from the emulsion is present in the solution after dissolving the cake. Since the cloudiness could be seen in both the sugar, placebo and protein cakes, the surfactants and protein in the formulation do not show any essential effect on the release of silicone and/or surfactant from the siliconized surface. The fact that the cloudiness was seen when dissolving the sugar cake also indicates that the cloudiness is due to the whole emulsion being released, not only the silicone from the emulsion.

To investigate if the siliconized surface is stable in water solution, 2mL Milli-Q water was added to the siliconized vials. In the vials siliconized with 3.5% emulsion, no difference was seen after 8 hours but small white droplets were seen on the surface of the vials inner wall after one day (for picture see Appendix Figure S4). This fact, together with the dissolution tests, could indicate that the silicone coating is released from the glass to the surface of the cake during freeze-drying, and since some of the silicone coating has stuck on the surface of the cake it dissolves together with the rest of the cake when water is added.

Problems can arise when silicone oil droplets are present in the solution since studies have shown that proteins tend to adsorb to the droplets resulting in aggregation and tertiary structural changes of the protein[14,22,23]. However, is has also been shown that the presence of surfactants (like polysorbate) in the formulation reduces the aggregation[14,22,24]. The surfactants adhere to the silicone oil droplets, inhibiting the adhesion of the protein and thereby the protein remains stable. In this case, it was the silicone coating made by the emulsion which was possibly present in the solution after dissolving the sample, which could mean that the silicone oil droplets already contained surfactants on the surface. If the surfactants would inhibit the protein to adhere to the released silicone in was not investigated in the present study.

3.4 Solid state analysis of freeze-dried cakes

The solid form of the excipients is important since a stable cake structure depends on that there is some crystalline excipient present and their protein stabilizing effects depends on that there is some amorphous form present[25]. Information about the solid form of the excipients is also important since crystallization of amorphous forms or structural changes of different polymorphs in a closed system can result in water being released, exposing the protein to water, which plays a key role in the aggregation and stability of proteins in the solid-state[26]. The excipients were analyzed with DSC to obtain information about the solid form of the excipients. This method gives information about any changes in the material that are associated with a thermal response during a heating or cooling ramp, such as crystallization, melting, glass transition and loss of hydrate water.

The DSC analysis (Figure 8) shows an endothermal peak followed by an exothermal peak at around 50°C when the top of the cake was analyzed, indicating a crystallization which in literature has been attributed to crystallization of amorphous mannitol[27]. When the bottom part was analyzed (Figure 8b), an endothermal peak is seen at 70-80°C attributed to the dehydration of the hemihydrate (crystalline hydrate form, containing one water molecule per two molecules of mannitol) form of mannitol[28]. The TGA (Figure 8b) also shows that the amount of water in the cake decreases at this temperature as well, strengthening the contention that the endotherm in DSC is due to the dehydration of the hemihydrate form releasing the bound water. The TGA reveals that the water loss profile is different for the top and bottom parts of the cake. The water loss profile in the bottom shows a more distinct decrease in weight at about 80°C when the water was removed from the hemihydrate in contrast to the more extended removal of water in the top of the cake, where the existence of hemihydrate is undetectable when analyzed with DSC. At around 130°C in Figure 8b an endothermal peak immediately followed by an exothermal peak is seen, indicating a melting transition followed by crystallization. Studies have shown that this crystallization is the recrystallization of the δ-polymorph of mannitol to the α/β-polymorph[28,29]. At 160°C an endotherm is seen in all cases, attributed to the melting of α- and/or β-mannitol[28]. No difference was seen in the DSC-curves when the formulations were freeze-dried in vials with different hydrophobicity.

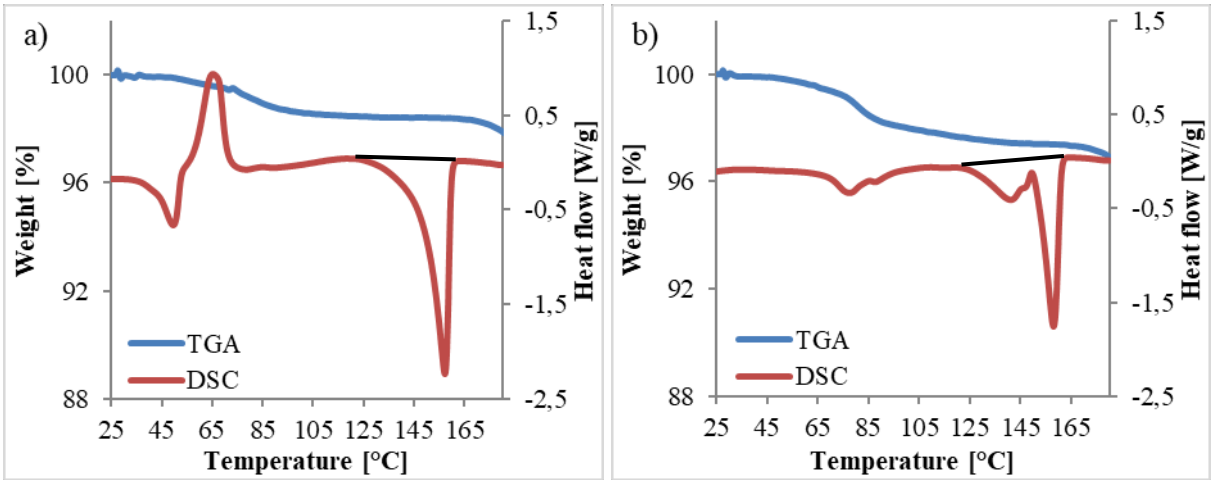

Figure 8. DSC- and TGA-curves of the protein cakes with the normalized heat flow [W/g] and weight [%] against the temperature of the sample [°C]. a) top of the cake, b) bottom of the cake.

The total crystallinity of mannitol was calculated as described in the Methods section, and the result (mean value from the two measurements) is summarized in Figure 9, and the part of the curve which was integrated is marked with a black line in Figure 8a and b. The amount of crystalline mannitol was lower in all the protein cakes than in the sugar and placebo cakes, showing that the protein to some extent inhibits the crystallization of mannitol. No effect on the amount of crystalline mannitol of the hydrophobicity of the vials was seen.

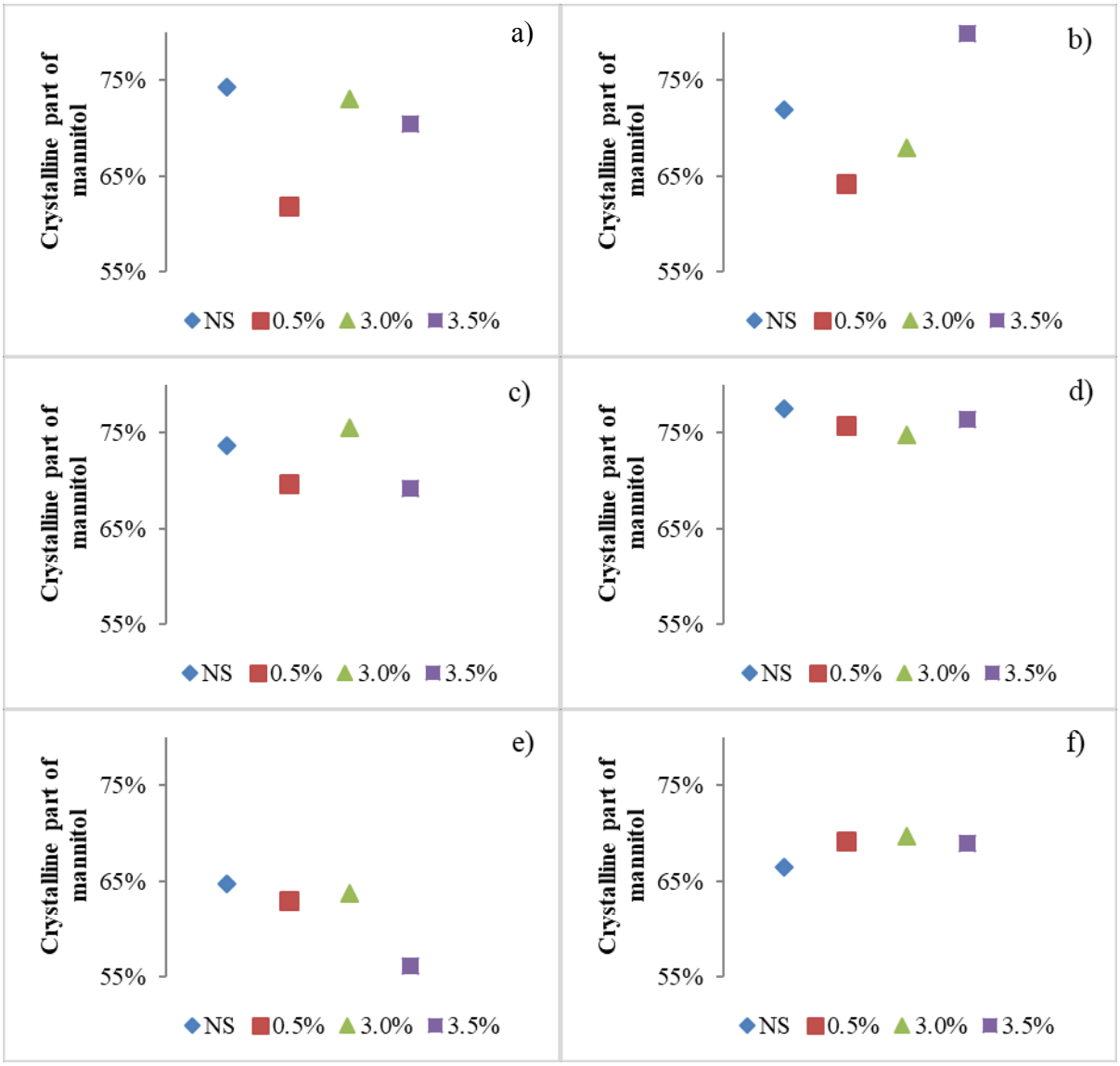

Figure 9. The percentage of the total amount of mannitol that is crystalline in each cake (shown as the mean value of two measurements), calculated from the integral of the melting peak in the DSC-curves, as described in the Methods section, a) top of sugar samples, b) bottom of sugar samples, c) top of placebo samples, d) bottom of placebo samples, e) top of the protein formulation samples and f) bottom of the protein formulation samples.

The water content in the cakes is presented in Figure 10 and was above 1% in all cases. The bottom of the cakes consistently has a higher water content than the top of the cakes. No effect of the hydrophobicity of the vial on the water content of the cake could be seen.

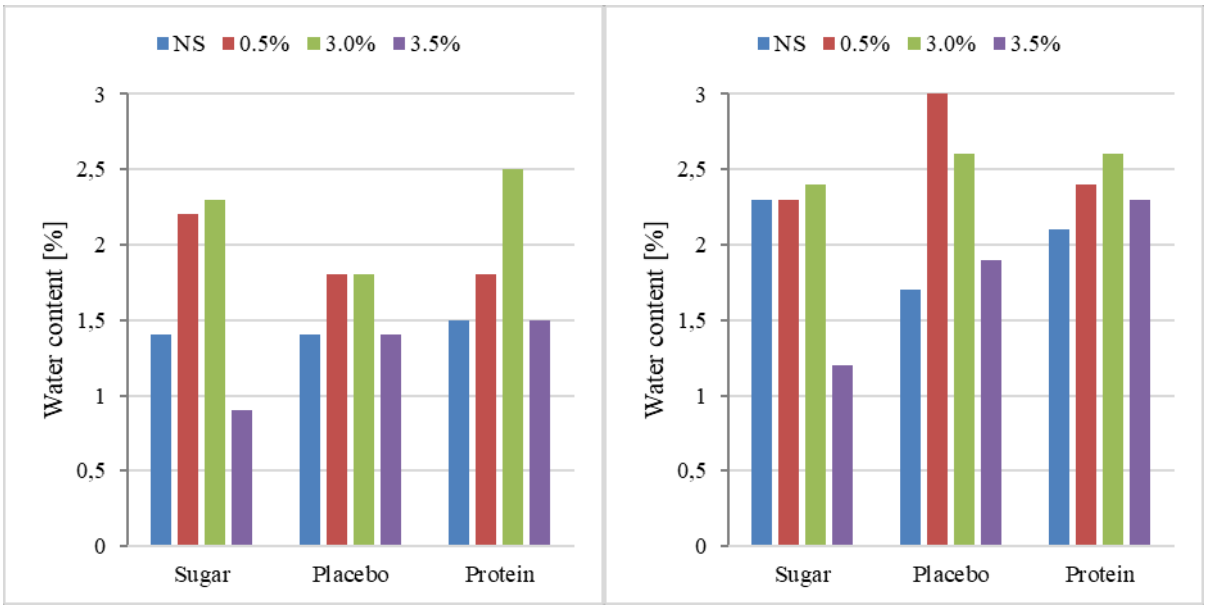

Figure 10. The water content [%] of top (left) and bottom (right) of the freeze-dried cakes, calculated from the weight loss occurring before the samples reached 120°C when analyzed using TGA.

An observation during the study showed that the crystallization peak at about 50°C was not present when one of the samples was analyzed after 3 weeks and therefore, the cakes freeze-dried in the NS, 3.0% and 3.5% siliconized vials were analyzed in duplicates with DSC after 1, 2 and 3 weeks of storage in room temperature in the desiccator. After 1, 2 and 3 weeks, the crystallization peak in the sugar cake from the NS vial was reduced to an almost invisible peak indicating that the crystallization had already taken place in room temperature. In the remaining samples there was no difference seen in the DSC-curve after 1, 2 and 3 weeks. The top and bottom of one of each cake freeze-dried in NS and 3.0% siliconized vials was analysed using TGA after 1, 2 and 3 weeks as well. The result is presented in Figure 11. Over time, the water in the sugar cakes seems to migrate from the bottom to the top, making the water content in the cake more evenly distributed. The placebo cakes also show that the water migrates from the bottom to the top, although the accuracy of the water content of 4.8% in the top of the placebo cake during week 2 is questionable. The water content in the protein cakes freeze-dried in the NS vial showed a constant water content in the top and the bottom over time, with a higher water content in the bottom of the cake. Besides the equally distributed water in the protein cake in the 3.0% siliconized vial in week 0, the water content in the protein cakes in the 3.0% siliconized vials also had a higher water content in the bottom over time. From this, the protein seems to have an impact of the water moving in the cake during storage. If the proteins' effect of the amount of crystalline mannitol (Figure 9), leading to a higher amount of amorphous mannitol when freeze-dried with the protein, and the proteins' effect of the mobility of the water over time has a connection was not investigated. Although, since the samples were not analysed in more than one sample each, that these trends need to be treated with caution.

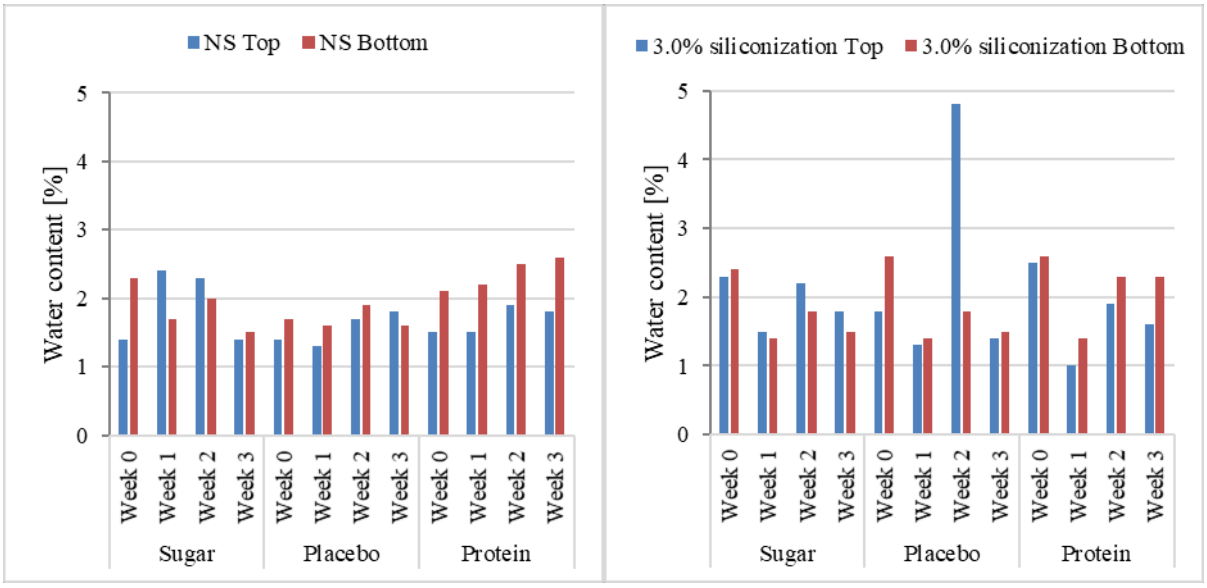

Figure 11. Water content of the sugar, placebo and protein cakes from the NS vial and 3.0% siliconized vial over a three-week period.

3.5 SEM characterization of the freeze-dried cakes

3.5.1 Sugar formulations

In the bottom of the vials, a thin film was created when all three formulations were freeze-dried. When this film from the sugar cakes was analysed by SEM, only the films from NS, 0.5% and 3.5% siliconized vials were analysed. The film from the 3.0% siliconized vials was not analysed since this was the first sample being analysed, when the film was not regarded as an attribute of the cakes. The film from NS and 0.5% siliconized vials showed a crystalline structure with pores positioned in lines (like the cracks seen in the top in Figure 14 and Figure 17), seen in Figure 12, in difference to the film from the 3.5% siliconized vials which shows a crystalline structure with significantly less pores. The film from the 3.5% siliconized vial also shows droplets on the surface, potentially being silicone emulsion released from the glass surface onto the cake. For pictures of the bottom films of the sugar cakes from NS, 0.5% and 3.5% siliconized vials with larger magnification, see Appendix Figure S5-7.

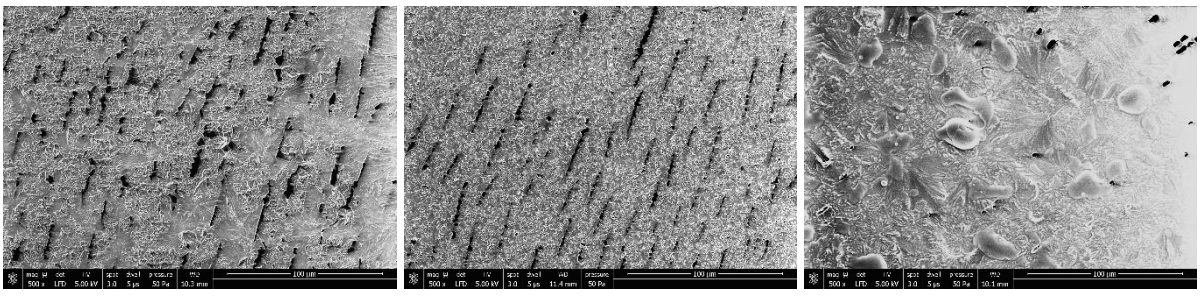

Figure 12. SEM images of the film created in the bottom of the vial of the sugar cakes freeze-dried in (from the left) NS, 0.5% and 3.5% siliconized vials. The images have a 500 x magnification.

In the SEM pictures (Figure 13) the bottom part of the sugar cakes from the NS, 0.5% and 3.0% siliconized vials shows a porous structure with crystals in a flower like shape. In the structure of the cake from the 3.0% siliconized vial, a droplet like structure is seen, which could indicate minor collapse in the cake, see Appendix Figure S8 for magnifications of the flower like crystal structure and the droplet like structure. When the added energy during drying is not dissipated as heat for sublimation it results in locally higher temperature which locally exceeds Tg'. This

leads to local collapse in the amorphous part of the cake since the material has the ability to flow. The pores in the sugar cake in the 3.0% siliconized vial appear to become larger than the pores in the NS, 0.5% and 3.5% siliconized vials. The sugar cake in the 3.5% siliconized vial shows parts of the cake where the droplet like structure is seen and at those places, a larger pore structure can be seen. The middle of the sugar cakes from the NS, 0.5%, 3.0% and 3.5% siliconized vials all showed similar pore structures with flower like crystals. The top of the sugar cakes freeze-dried in 0.5% and 3.0% siliconized vials shows an amorphous structure with a structure of thin lines, indicating cracking of a film in the top layer during the freeze-drying. The sugar cake freeze-dried in the 3.5% siliconized vials shows a top layer not completely cracked into the thin lines, Figure 14. The top of the sugar cake in the NS vial showed a similar structure with the cracking in thin lines, but consisted of a crystalline form seen in Figure 14, see Appendix Figure S9-12 for greater magnification of the cracks. The cracking of the top layer of the sugar cake thereby decreased when siliconization was done with the 3.5% emulsion, and the top layer was crystalline when the sugar formulation was freeze-dried in the NS (hydrophilic) vial.

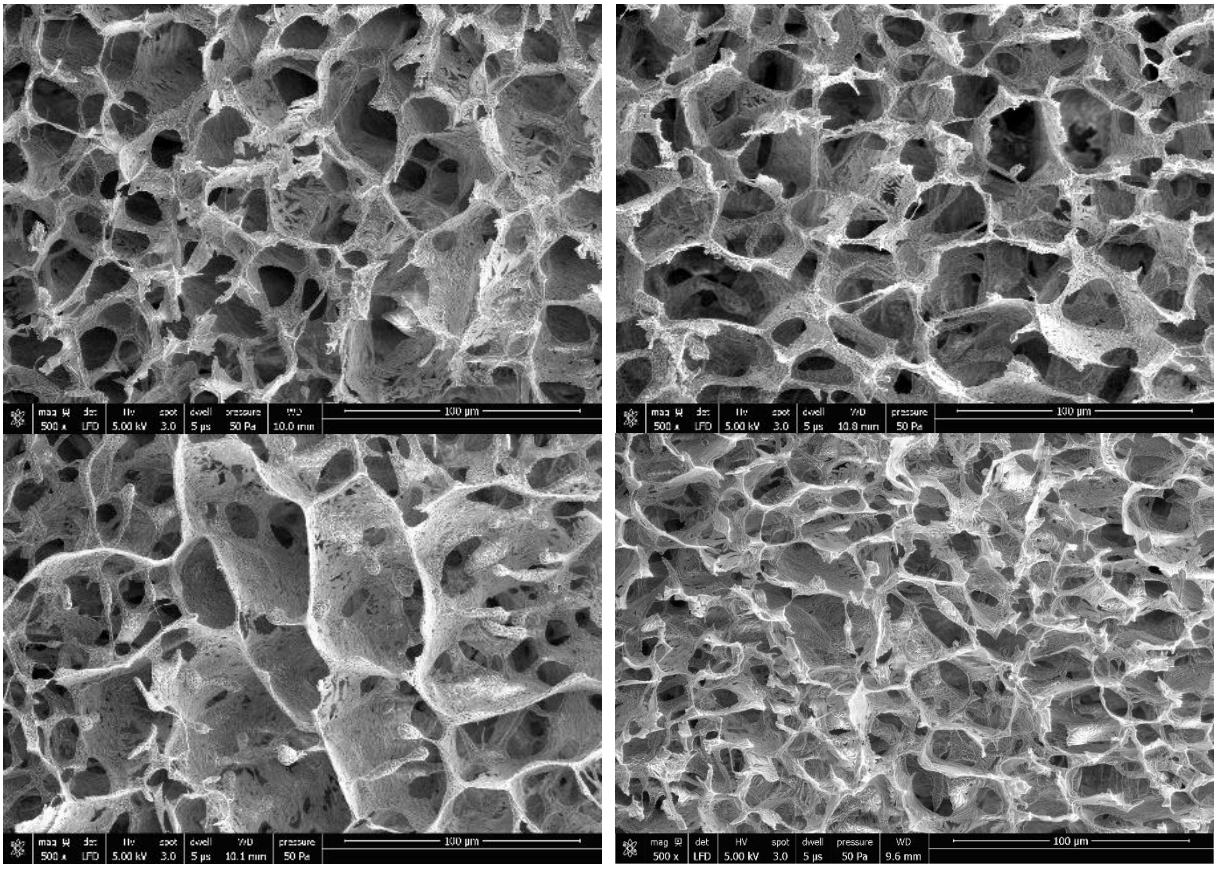

Figure 13. SEM images of the bottom (not in contact with the vial) of the sugar cakes freeze-dried in NS (top left), 0.5% (top right), 3.0% (bottom left) and 3.5% (bottom right) siliconized vials. The images have a 500x magnification.

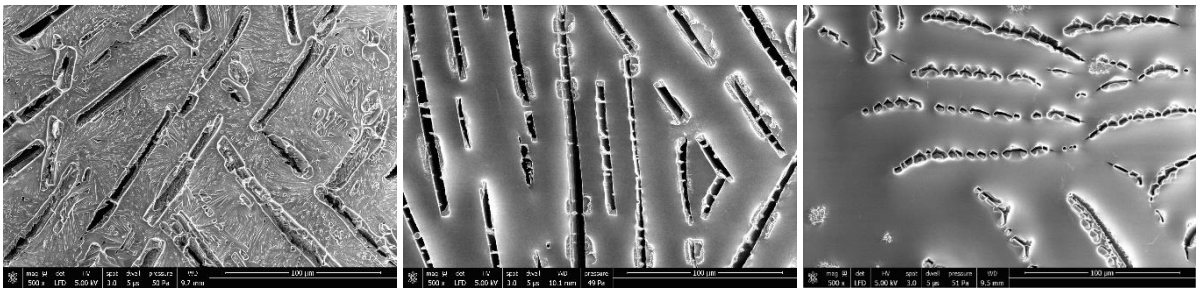

Figure 14. SEM image of the top layer of the sugar cakes freeze-dried in the NS (left) 0.5% siliconized vial (middle) and 3.5% siliconized vial (right) with a magnification of 500.

3.5.2 Placebo formulations

As described above, there was a thin film created in the bottom of the vial and the only films from placebo cakes analysed by SEM were the ones from the 0.5% and 3.5% siliconized vials, seen in Figure 15 (greater magnification in Appendix Figure S13 and S14), as the other films got stuck on the bottom of the vial or the film was not able to be separated from the rest of the cake. The film from the 3.5% siliconized vials shows larger pores and droplets which potentially could be silicone emulsion released from the coating, similar to the ones seen in the sugar cake from the 3.5% siliconized vial in Figure 12. The bottom of the placebo cakes (not in contact with the vials) showed a porous structure with a flower like crystal structure with no difference between the vials. Although, the middle of the cakes, Figure 16, revealed a droplet like structure (similar to the one in the sugar cake freeze-dried in 3.0% siliconized vial) in the placebo cakes in 3.0% and 3.5% siliconized vials. This droplet like structure was present in a higher degree in the 3.5% siliconized vial than in the 3.0% siliconized vial and was not found in the NS and 0.5% siliconized vials. The middle of the placebo cakes consisted of the flower like structure with longer crystals than the middle of the sugar cakes, see Appendix Figure S15 for greater magnification. The top of the placebo cakes all consists of an amorphous film with thin cracks and some crystal structures, like seen in Figure 17.

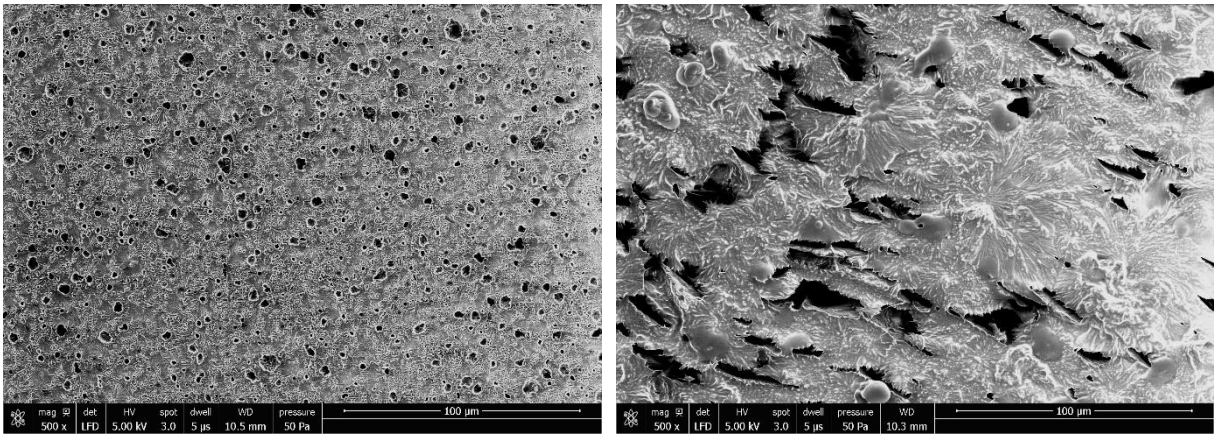

Figure 15. SEM images of the bottom film (in contact with the vial) of the placebo cakes freeze-dried in (from the left) 0.5% and 3.5% siliconized vials. The images have a 500x magnification.

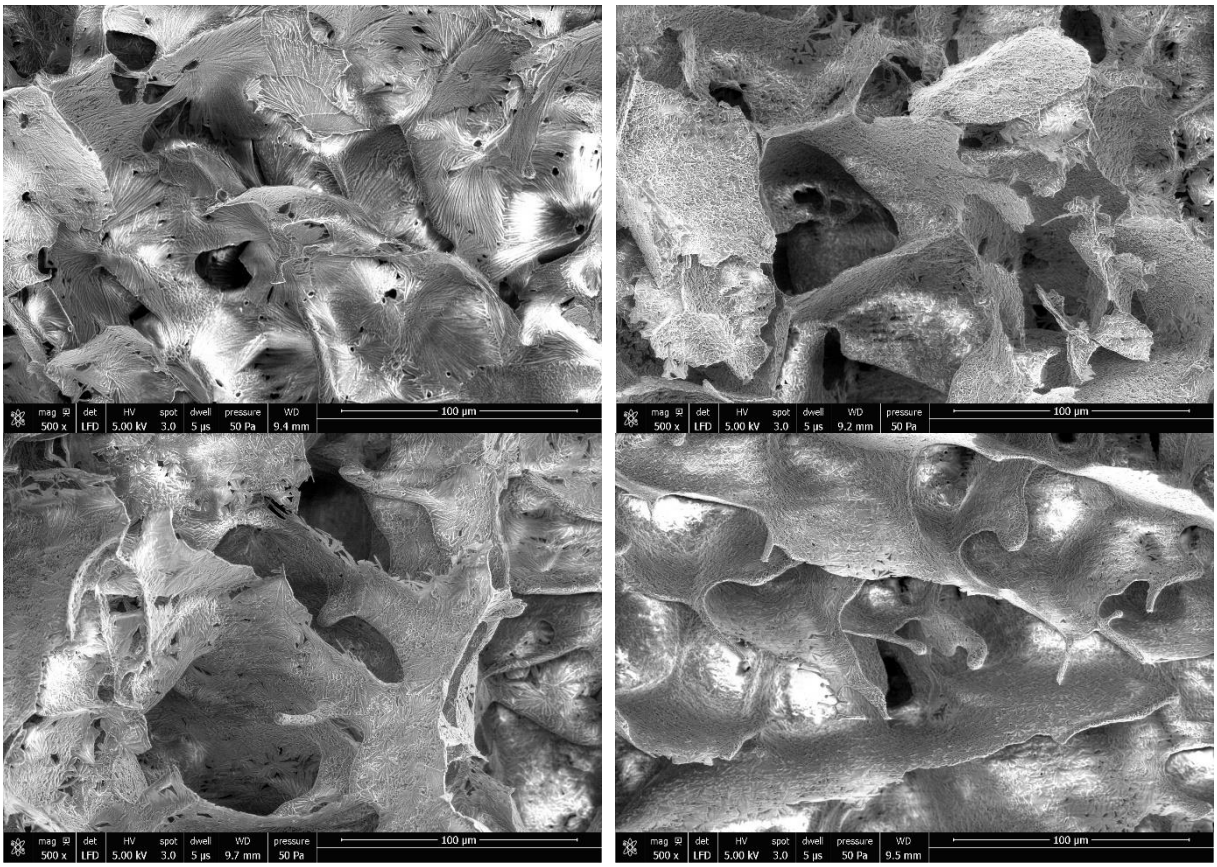

Figure 16. SEM images of the middle of the placebo cakes freeze-dried in (from the left) NS% and 0.5% siliconized vials in the top row and 3.0% and 3.5% in the second row. The images have a 500x magnification.

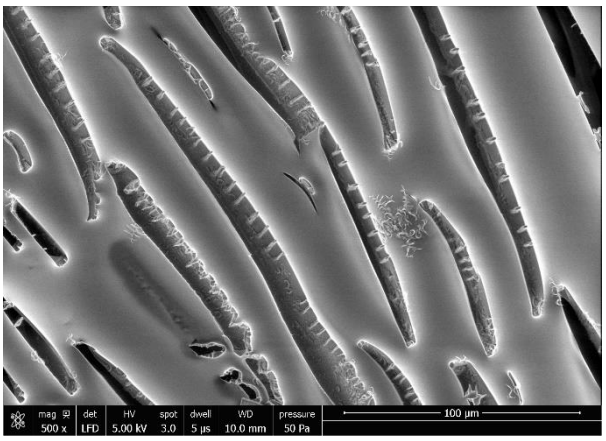

Figure 17. SEM images of the top layer of the placebo cakes freeze-dried in the 3.0% siliconized vial with a 500x magnification.

3.5.3 Protein formulations

When the protein cakes were analysed with SEM only the bottom films from the 3.0% and 3.5% siliconized vials was able to be analysed as the other films got stuck on the bottom of the vial or the film was not able to be separated from the rest of the cake. The structure of the films showed a similar structure with bigger pores compared to the pores in the sugar and placebo cakes from the NS and 0.5% siliconized vials. As in the other cakes from the 3.5% siliconized vials, there were droplets potentially being silicone emulsion released from the glass surface onto the cake. The bottom of the protein cakes (not in contact with the vial) freeze-dried in NS, 0.5% and 3.0% siliconized vials showed a similar structure as previously seen in both sugar and placebo cakes. Figure 18 shows that the structure of the protein cake in the 3.5% siliconized

vial differs from all the other samples. The pores appear to have a more regularly equal pore size and the walls of the pores appear to be thicker, for greater magnification of this structure see Appendix Figure S16. The middle part of the protein cakes shows similar pore structure with the flower like crystals in the NS, 0.5% and 3.5% siliconized vials while the 3.0% siliconized vial shows more amorphous parts. The top film of the cakes was, as in the placebo cakes, consisting of an amorphous structure with thin cracks.

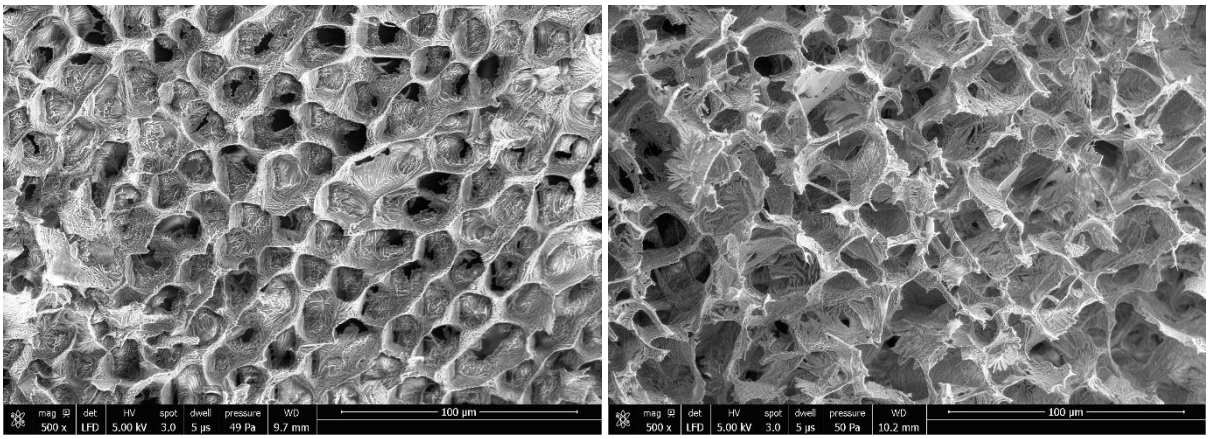

Figure 18. SEM image of the bottom (not in contact with the vial) of the protein cake freeze-dried in the 3.5% siliconized vial (left image) and in NS vial (right image) with a magnification of 500.

3.6 XPS

The result of the XPS-analysis is presented in Figure 19 (placebo) and Figure 20 (protein) and tabulated in Appendix Table S1-S6. These data were analyzed using Equation 2, and this analysis is complemented by inspecting the content of the different elements in reference samples and freeze-dried samples. A simplified way to estimate the content of a molecular species is to divide the content of a specific element in the sample with the content in the reference material, e.g. $N_{sample}/N_{protein}$. No data of the composition of pure polysorbate was able to be analyzed due to its volatility at high vacuum. Due to the low amount of polysorbate present and the lack of experimental data for its atomic composition, its contribution to the composition was ignored in the analysis by leaving out its elemental composition in the matrix **A**. From the calculation a least squares approximation of the surface composition in terms of molecular species is obtained. As seen in Figure 19 and Figure 20 the sum of the composition is close to 100%, but not exactly 100%. This indicates experimental deviations from the ideal patch model. Furthermore, when using a large number of components in the sample, and especially components with similar composition makes the estimation less certain. Therefore, the data from the patch model calculations are cross-checked with estimations based on content of heteroatoms (N and S) as well as information from the high-resolution spectra for N.

The original ratio between sucrose and mannitol is 1:2. despite this, the amount of sucrose in most cases turned out higher than the amount of mannitol. This may indicate that the amorphous sucrose is placed on the crystals of mannitol resulting in a higher amount of sucrose when analyzing, or the model used while calculating the composition does not manage to result in correct amounts.

Like described above, the patch model's calculations on the placebo cakes was cross-checked by assuming that the atomic amount of nitrogen in the samples comes from histidine. By relating the atomic amount of nitrogen in the sample to the atomic amount of nitrogen in pure histidine, an estimation of the histidine amount could be obtained. The difference between these cross-checked values (from the nitrogen data) and the values obtain from the model gave similar

amounts of histidine (2.7-5.5% for the nitrogen-based values and 1.0-6.0% for the model-based values). Both the model-based values and the cross-checked values showed that the amount of histidine was less in the top of the vial in both NS and 3.0% siliconized vials. In the 3.5% siliconized vials, the histidine distribution was even, with about 6.1-8.7% throughout the cake. The middle and near the bottom parts of the cakes from the 3.0% and 3.5% siliconized vials showed the same histidine amounts as the bottom of the cakes.

The total amount of protein in the protein formulation was about 7% but the XPS-analysis resulted in protein amounts ranging from 5-60%, in most cases between 20 and 30%. Proteins are surface active and accumulate at interfaces, which could explain the high amounts. The crystallization of mannitol could also contribute to high amounts of protein, since this leaves sucrose, potentially some mannitol, protein and histidine in an amorphous phase containing higher amount of protein than the original formulation. Although, the high amounts of protein and large range of values makes it fair to question the model's suitability for this purpose. Therefore, the high resolved nitrogen spectra were used to analyze if there is more or less protein or histidine in different parts of the cake, since the nitrogen in the sample either comes from histidine or protein. This was only possible for the NS and 3.0% siliconized vials, since no high resolved nitrogen spectra was performed for the cake in the 3.5% siliconized vial. The ratio between N1 and N2 for pure protein was about 92:8 and for histidine it was 26:74. Therefore, a higher amount of N1 indicates more protein, which was the case in the top and of the NS vial and top, middle and bottom of the 3.0% siliconized vial. The part of the cake right above the bottom (that was not in contact with the vial) in the 3.0% siliconized vial and the bottom of the cake in the NS vial showed a higher amount N2, indicating a higher amount of histidine in that part of the samples. The amount of S in the protein samples was also used to give an idea of the protein amount. These values where in the range of 5-26%, most of them in the range of 15-20% with no connection of protein amount in different parts of the cake and hydrophobicity of the vial. Since the total S content is very low, the protein content based on S only becomes somewhat uncertain.

The XPS-analysis showed no silicon in neither of the placebo and protein cakes freeze-dried in NS and 3.0% siliconized vials. The siliconization method using silicone oil in toluene thereby creates a hydrophobic surface without releasing silicone into or onto the freeze-dried cake. As seen in the SEM-pictures in Figure 15 the siliconization method using the emulsion (3.5% siliconized vials) released, what was thought to be, silicone emulsion onto the surface of the cakes. As previously mentioned, these cakes also showed a cloudiness when being dissolved, which also was thought to be release of silicone emulsion into the solution. The XPS-results of the silicon content in different parts of the cake is presented in Figure 19 and Figure 20 which reveals that the cakes freeze-dried in 3.5% siliconized vials (emulsion siliconization) contains silicone, where the bottom contains the highest amount and the middle contains the lowest amount. Potentially, the distribution of the silicone in the cake could indicate that the release of emulsion happens throughout the drying, including afterwards. The silicone possibly is released on the surface of the dried cake and over time, it is spread into the cake as well. If so, the silicone amount in different parts of the cake would even out over time. As previously mentioned, the amorphous excipients have an important role in the stabilization of the protein[25,26] and if the silicone contributes to crystallization of the amorphous parts stability problems can be thought to arise. Even though there are surfactants in the formulation, which aims to prevent the aggregation of the protein[14,22,24], the solid-state aggregation could still be of interest if the silicone destabilized the solid form of the excipients. Although, the effect of the silicone on the amorphous parts or the aggregation of the protein was not evaluated.

As mentioned before, the patch model used for calculating the amounts of the different components in some cases resulted in the total percentage adding up to >100%, and sometimes resulting in amounts <0% for some components. This makes these results questionable and there is place for development when it comes to the model. The model is based on that the different components are distributed as thick layers and if they in this case are distributed as thin layers, the model is not suitable. Still, the raw data was considered to give an idea of the distribution of some components (histidine in placebo cakes and protein in protein cakes) making it somehow interesting to present the results from the calculations as well.

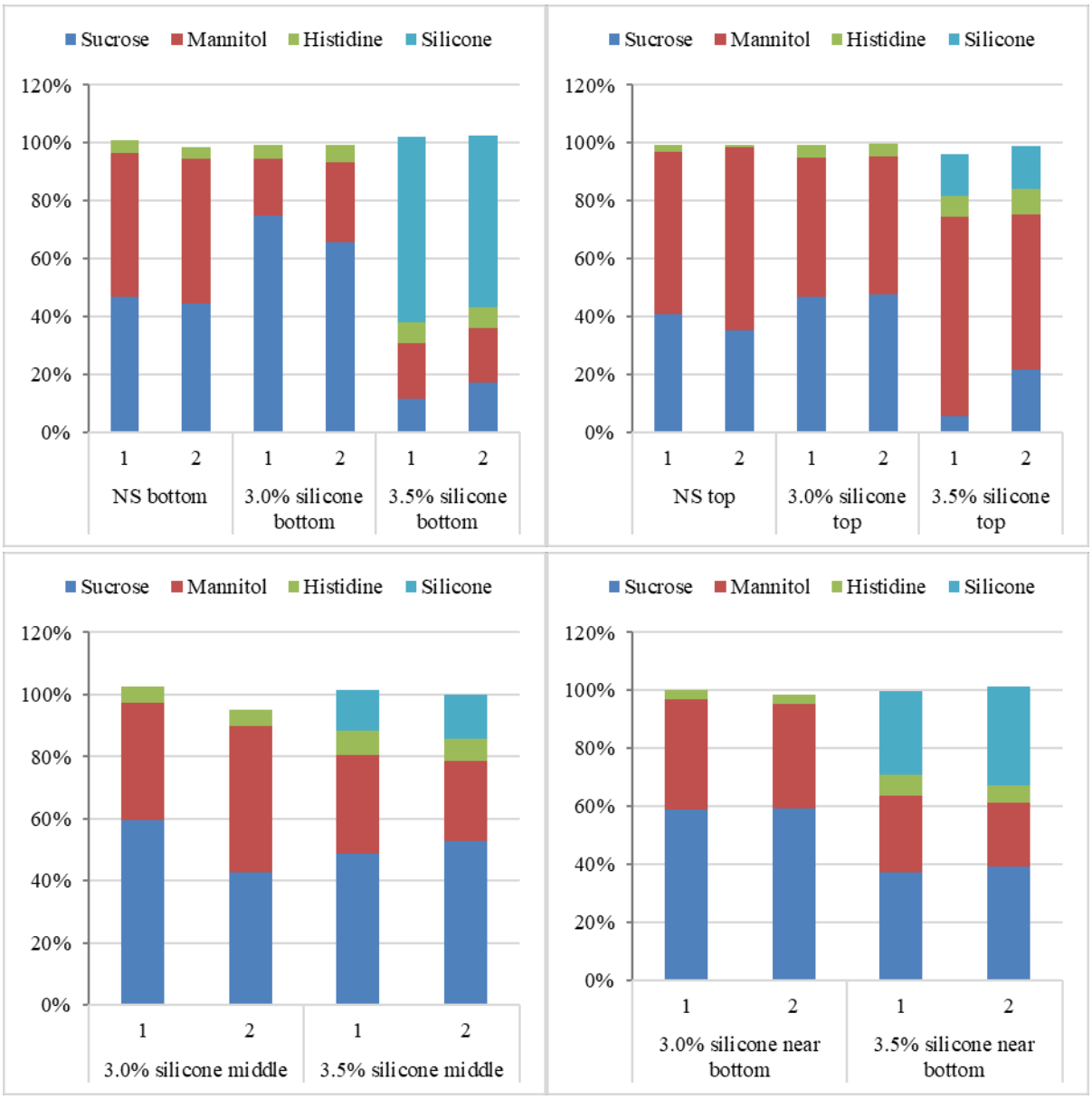

Figure 19. Component composition [%] in different parts of the placebo cakes freeze-dried in the NS, 3.0% and 3.5% siliconized vials.

Figure 20. Component composition [%] in different parts of the protein cakes freeze-dried in the NS, 3.0% and 3.5% siliconized vials.

4. Conclusion

4.1 Hydrophobicity of the vial and the attributes of the freeze-dried cake

When the three different formulations were freeze-dried in the four different vials, the wetting of the walls decreased with increased hydrophobicity of the vial, directly leading to a decrease in fogging with increasing hydrophobicity. The shrinkage in width of the cakes increased with increased hydrophobicity and since no cake showed a significant degree of cracking, no conclusion between hydrophobicity of the vial and cracking behavior could be drawn. Although, it was visually seen that the adhesion of the cake to the vials inner wall decreased with increased hydrophobicity.

4.2 Embedding method

The embedding method gave the possibility to evaluate the cake structure macroscopically, providing a clear relationship between the structure seen in the photos and the hydrophobicity of the vials, although more replicas are needed. Furthermore, the method needs to be further developed to obtain better contrast between the cake and the embedding medium. This could be obtained, for example, by adding a fluorescent dye to the formulation before freeze-drying and looking at the cakes through a fluorescence microscope. This assumes that the cakes with dye is compared to the cakes without to conclude that the dye does not affect the properties of the cake.

4.3 Hydrophobicity of the vial and the solid-state properties of the freeze-dried cake

The hydrophobicity of the vial did not affect the solid-state properties analyzed with TGA and DSC when it comes to crystallinity (including polymorph) of mannitol or water content. But differences in the structures of the cake when the cakes were analyzed with SEM was found. Most apparent was the change in pore structure of the protein cake that emerged when it was freeze-dried in the 3.5% siliconized vial compared to the pore structure seen in all the other samples. Another attribute was the larger pore structure and droplet like structure appearing when the placebo formulation was freeze-dried in the 3.0% and 3.5% siliconized vials and when the sugar formulation was freeze-dried in the 3.0% siliconized vials and to some extent in the 3.5% siliconized vial. These two facts lead to the conclusion that when the character of the vial changes from hydrophilic to hydrophobic, the structure of the freeze-dried cake changes. Although, it would have been interesting to analyze what the droplets in the structure consisted of, to evaluate if they appear due to local collapse. This can be done by SEM/EDX to conclude if this consist of sucrose which has a low collapse temperature.

4.4 Silicone release and XPS-analysis

The two different siliconization methods showed that the surface became more hydrophobic when it was siliconized with the emulsion, but this method did not create a stable surface since emulsion droplets on the surface of the cakes were seen in the SEM images. Silicone was also found when the cakes were analyzed by XPS in all parts of the cake. The largest amount of silicone was found in the bottom and the lowest amount in the middle of the cake. The release of the coating when using the emulsion for the siliconization procedure resulted in cloudy solutions when the cakes were dissolved. The cloudiness was not seen in the other cakes. Although, the XPS-data requires more work and the model used for the calculation of the distribution of the excipients in the cake needs to be further developed to fit for this purpose, hopefully leading to more reliable results.

The migration of silicone oil and silicone emulsion from the surfaces after siliconization also needs to be further examined together with the silicone sources' impact of the stability of the protein. Another aspect interesting to analyze is how uniform the silicone coating on the glass

vial gets with the different methods, since this could potentially affect the amount of silicone emulsion released onto the surface of the cake. The uniformity could also affect the wetting and adhesion to the wall since an irregular coating results in some parts of the surface being more hydrophobic than other parts. The amount of silicone emulsion used to siliconize the surface (and potentially the siliconization procedure using the emulsion) needs to be evaluated and developed to avoid the droplet formation on the surface when getting in contact with water in the siliconized vial and to avoid the cloudiness when dissolving the cake.

As an overall conclusion, this study showed that the attributes of the freeze-dried cake to some extent depends on the character of the surface of the container, giving interesting insights for the use of siliconized pre-filled syringes in the pharmaceutical industry.

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