

Degree project in Pharmaceutical Formulation – KLGM06

Spray-dried powders for inhalation

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POPULAR SUMMARY

Spray drying is a method that has been used widely in the pharmaceutical industry to produce powders containing therapeutic drugs. It is a method that takes a liquid formulation, sprays it into a chamber in the form of droplets, and dries it with high temperature air. Then the solvent evaporates from the droplets, producing solid particles in powder form. It is a cost-effective method which is easy and quick to use.

The powders produced with spray drying can be tailored for use in inhalation. These dry powders are made to cure lung specific diseases and need to have specific size and shape to have a therapeutic effect in the lungs. One specific characteristic required is that the aerodynamic size of the particles is below 5 μm . The aerodynamic diameter (AD) of the particles is the size of a sphere (density 1 g/cm^3) that moves at the same speed as the particles when an individual inhales.

To make dry inhalation powders with spray drying, two things need to be considered: the effect of the spray drying conditions and the effect of the formulation on the powder characteristics.

This study examined both objectives by using three sets of spray drying conditions on pure lactose and trehalose dihydrate powders (pre-study) and changing the additives in the formulation to study their effect on the particles (main study).

Bovine Serum Albumin, a standard protein, was used as the model drug in the formulation, and L-leucine, an amino acid, was added to the formulation in different concentrations to improve the aerosol performance of the powders.

The powders were analyzed with different methods to determine their size, shape, surface morphology and aerosol characteristics such as the aerodynamic diameter and their efficiency as an inhalation powder.

It was found that the powders produced had the ideal aerodynamic size to perform well as aerosol powders ($<5 \mu\text{m}$) and reach the deep part of the lungs. They were also irregularly shaped which typically improves aerosol performance. Additionally, it was discovered that L-leucine improves the powders ability to reach the lungs and have a therapeutic effect.

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ABSTRACT

Spray drying is a widely utilized method for producing protein pharmaceutical powders due to its cost-effectiveness, efficiency, ability to extend shelf life and tailor particle characteristics. These advantages make spray drying an optimal option to produce dry powders suitable for inhalation.

Dry powders are extensively used in treating lung diseases primarily due to their increased stability, their ability to preserve drug activity, and achieve high deposition efficiency in the lungs, compared to other delivery forms. Therefore, protein pharmaceutical powders can be produced with spray drying and tailored specifically for inhalation.

This project aimed to produce spray-dried protein powders for inhalation using specific spray drying conditions and excipients. The research was conducted in two parts: the pre-study and the main study. The pre-study identified the ideal spray drying conditions for inhalation powders containing only lactose or trehalose dihydrate. The main study focused on spray drying powders with Bovine Serum Albumin as the active pharmaceutical ingredient and trehalose dihydrate and L-leucine as excipients. These powders were analyzed to determine their size, morphology, and aerodynamic properties using laser diffraction, light microscopy, bulk density measurements, Scanning Electron Microscopy, Next Generation Impactor analysis and Aerodynamic Particle Sizing.

All spray-dried powders produced under different conditions were in the size range between 3.5-5 μm . In the pre-study pure powders containing lactose or trehalose dihydrate were smaller, smooth, and spherical. The chosen spray drying conditions had an inlet temperature of $T_{\text{in}} = 175\text{ }^{\circ}\text{C}$, an outlet temperature of $T_{\text{out}} = 73\text{ }^{\circ}\text{C}$, aspirator rate of 32 m^3/hr , and pump rate of 5 mL/min .

In the main study, complex powders containing BSA, and L-leucine were produced with a high yield, above 75%. They contained particles between 4-5 μm , with corrugated, and raisin-like shapes. Samples with L-leucine exhibited a flake-like surface, likely due to crystalline L-leucine on the surface. NGI analysis showed that all powders containing BSA had an aerodynamic diameter below 5 μm , suitable for efficient aerosolization. The sample with the highest amount of L-leucine (27% w/v) had the highest Fine Particle Fraction at 49.3%.

The findings suggest that spray drying was a suitable method to produce inhalable protein powders with high yield. The addition of BSA and L-leucine resulted in raisin-shaped particles, while higher L-leucine concentrations improved dispersibility and aerosolization performance.

Keywords: Spray drying, dry powders, protein, inhalation, aerodynamic diameter, trehalose, L-leucine, Bovine Serum Albumin, corrugated particles.

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LIST OF ABBREVIATIONS

AD: Aerodynamic Diameter

API: Active Pharmaceutical Ingredient

APS: Aerodynamic Particle Sizing

BSA: Bovine Serum Albumin

DPI: Dry Powder Inhaler

FDA: Food and Drug Administration

FPD: Fine Particle Dose

FPF: Fine Particle Fraction

MMAD: Mass Median Aerodynamic Diameter

NGI: Next Generation Impactor

SEM: Scanning Electron Microscopy

1 INTRODUCTION

1.1 Spray-dried protein pharmaceuticals

Spray drying is a technique vastly used in the food, pharmaceutical and chemical industry. It includes the atomization of the liquid feed and drying of the droplets in the drying chamber to obtain solid particles in the form of a stable powder (Figure 1) (Lechanteur & Evrard, 2020; Maa et al., 1997).

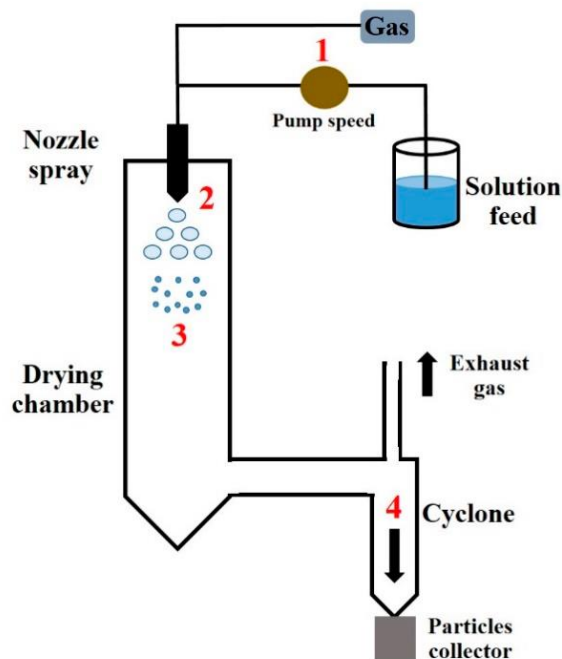


Figure 1: Spray drying process of liquid feed solution to eventually produce a powder (Lechanteur & Evrard, 2020).

Protein pharmaceuticals have been extensively produced with spray drying as it offers many benefits compared to other methods. This technique can tailor particle characteristics such as size and morphology, which can enhance their application efficiency. Spray drying provides stability to protein pharmaceuticals by preserving their activity to a greater degree than liquid formulations and thereby extending their shelf life. At the same time, the powder obtained has increased purity and the particles are more homogeneous in size. Additionally, it is more cost and time efficient, suitable for continuous operations and scalable for larger operations (Eedara et al., 2021; Pinto et al., 2021).

1.2 Dry powder inhalers

Dry powder inhalers (DPI) have become essential pharmaceutical products in treating lung-specific diseases. Dry powders deliver the active pharmaceutical ingredient (API) to the lungs with high deposition efficiency. For APIs that are biomolecules, dry powders provide a more stable form, extending shelf life and maintaining drug activity (Lechanteur & Evrard, 2020; Pinto et al., 2021).

Unlike other inhalers, DPIs do not require a propellant but rather rely on the patient's inhalation. They offer improved chemical stability and have a convenient and user-friendly design. DPIs typically deliver micronized drug particles in the form of a dry powder to the

lungs, often using excipients such like carrier particles. These carrier particles are often used to increase the flowability of the powder, to ensure dose accuracy and generally to improve the aerosolization performance (Lechanteur & Evrard, 2020; Pinto et al., 2018). However, it is required that the API particles adhere homogeneously to the carrier particles and dissociate upon inhalation. This requires a thorough understanding of the cohesive and adhesive forces, to achieve good aerosolization properties of the powder (Pinto et al., 2018).

Increased cohesive forces between drug particles may lead to aggregation, reducing powder dispersibility and limiting deposition in the lower airways, whereas strong adhesive forces between API and carrier particles will result in poor flowability of the powder (Adi et al., 2008; Maa et al., 1999).

Therefore, a new method involving spray-dried powders has emerged with the ability to tailor particle characteristics such as size and shape, enhancing drug dispersibility in the lungs (Pinto et al., 2018). These powders are produced by spray drying the excipients along with the API, under controlled conditions (Figure 2). Adjusting the spray drying conditions and the feed formulation allows for tailoring of the particle characteristics for inhalation (Lechanteur & Evrard, 2020; Maa et al., 1997; Pinto et al., 2018).

By altering spray drying conditions and using specific excipients, it is possible to modify the particle surface characteristics. Specifically, using shell-forming excipients may enhance the stability of the powder and improve aerosol performance. There are two mechanisms that contribute to the formation of a protective layer around the particles, due to the high Peclet number of the excipients, and their surface activity (Ordoubadi, 2024).

The Peclet number of the excipients, defined as the ratio of the solvent's evaporation rate to the excipient's diffusion rate, has an important role. When Pe is less than 1, the excipients diffuse into the particle at a rate equal to the solvent evaporation, resulting in uniform spherical particles. Whereas when Pe is greater than 1, the excipient will diffuse slower than the solvent evaporates, causing the excipient to accumulate on the surface and form corrugated particles. These corrugated particles have been studied and found to improve the aerosolization performance of DPI powders (Adi et al., 2008; Lechanteur & Evrard, 2020; Ordoubadi, 2024).

The second mechanism involves the surface activity of the excipients, which allows them to adsorb on the surface of the droplets. When the excipient is adsorbed on the droplet's surface, it forms a monolayer that later hardens into a shell, which will contribute to powder stability, decreased interparticle cohesion and improved dispersibility (Ordoubadi, 2024).

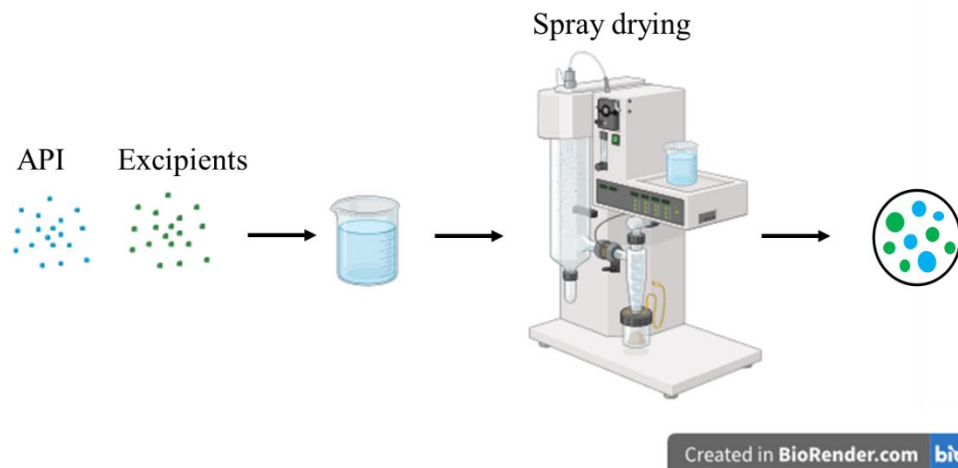


Figure 2: Spray drying drug particles and excipients for use in inhalation (Image made with BioRender).

1.3 Characteristics of inhalation powders

As previously mentioned, tailoring particle characteristics for inhalation is crucial to achieving efficient respiratory deposition. Particle size, shape and morphology significantly impact aerosolization performance. Specifically, for high-efficiency drug delivery to the deeper part of the lungs, inhalation particles need to have an aerodynamic size between 1-5 μm (Chang & Chan, 2022; Lechanteur & Evrard, 2020; Thalberg, 2022). The aerodynamic diameter (AD) is an important characteristic for respiratory drug delivery. The AD of a non-spherical particle is equivalent to that of a sphere with density equal to 1 g/cm^3 that has the same velocity in the aerosol stream (Shekunov et al., 2007).

The drug delivery efficiency is evaluated using two metrics, the Fine Particle Dose (FPD) and the Fine Particle Fraction (FPF). The FPD represents the amount of drug particles in the aerosol cloud with the ideal size range to reach the lungs, while the FPF indicates the efficiency of drug delivery and is the percentage of the particles in the range 1-5 μm . FPF is calculated by the FPD divided by the delivered dose (Lechanteur & Evrard, 2020; Thalberg, 2022).

Furthermore, particle shape and morphology influences aerosol behavior by affecting powder flowability, dispersibility and the ability to release successfully from the inhaler (Chang & Chan, 2022). It has been shown that non-spherical particles with elongated shape have a smaller aerodynamic size but lower flowability (Elsayed, 2019). Additionally, spherical particles often do not exhibit the best aerosolization performance (Pinto et al., 2018).

Studies also indicate that smooth surfaced particles tend to increase FPF, while other research suggests that corrugated carrier particles promoted a higher FPF (Telko & Hickey, 2005). Both findings may be correct, as aerosolization performance depends on various factors. Another study found that more corrugated particles resulted in higher FPF values due to reduced contact between particles, which decreased adhesive forces (Adi et al., 2008; Lechanteur & Evrard, 2020).

However, while corrugated surfaces seem to enhance aerosolization performance and drug deposition in the lungs, excessively corrugated particles may decrease FPF. Increased surface

roughness and high degree folding, may cause particles to stick together, leading to aggregation and poor aerosolization (Figure 3) (Lechanteur & Evrard, 2020).

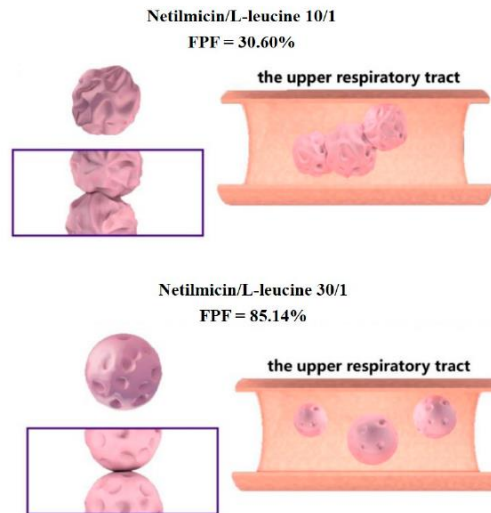


Figure 3: Differently corrugated particles: Top image portrays spray-dried particles (Netilmicin/L-leucine, ratio 10/1) with highly corrugated surface and FPF=30.6 %. The bottom image shows spray-dried particles (Netilmicin/L-leucine, ratio 30/1) with a less corrugated surface and a higher FPF (85.1 %) (Lechanteur & Evrard, 2020).

1.4 Spray-drying conditions

Powder characteristics can be tailored by changing the spray-drying conditions. During the process, temperature at the entrance and exit of the drying chamber, the drying air flow, and the spray gas flow are conditions that can be set depending on the required powder characteristics

The inlet temperature (T_{in}) affects the particle shape and moisture content (Lechanteur & Evrard, 2020). A lower inlet temperature leads to particles with more wrinkled surface and less hollow. Whereas higher inlet temperatures (more than 190 °C) lead to less wrinkled particles (Maa et al., 1997). For lower moisture content in the spray-dried powder, higher inlet temperatures are more efficient along with lower liquid feed rates. It has been reported that for less than 3% moisture content, inlet air temperatures should be higher than 140 °C (Ameri & Maa, 2007).

The outlet temperature (T_{out}) is a critical parameter as it is the temperature that the droplets will come into contact with. Lower outlet temperature results in more spherical particles with higher moisture content (Maa et al., 1997). This will produce stickier particles that may adhere to the drying chamber, leading to a decrease in production yield. A higher outlet temperature can be achieved by changing parameters such as the inlet temperature and feed flow rate and will result in deformed particles that deviate from the spherical shape (Ameri & Maa, 2007; Lechanteur & Evrard, 2020).

The feed flow rate affects the size of the particles and possibly their morphology. By increasing the rate, larger droplets are formed during atomization, due to more feed solution entering the system and therefore larger particles (Lechanteur & Evrard, 2020).

In addition, the nozzle pressure plays a major role in the size of the particles. When increasing the nozzle pressure, smaller droplets are formed, which are then dried and form smaller particles (Lechanteur & Evrard, 2020; Maa et al., 1999).

It should be noted that the room temperature and relative humidity also determine the powders characteristics and should be taken into account during spray drying (Maa et al., 1998). Additionally, storage humidity should be kept below 30% to avoid agglomeration (Wu et al., 2014), although depending on the type of powder, this may differ.

1.5 Formulation and excipients in spray drying

The active pharmaceutical ingredient (API) in the dry powder formulation will be represented by Bovine Serum Albumin (BSA), a model protein commonly used in experiments as a standard. The protein concentration is tailored based on the desired particle characteristics, and dosage wanted for the inhalation product. For higher protein concentrations, the particles are larger in size, whereas their surface becomes more wrinkled for lower protein concentrations (Lechanteur & Evrard, 2020; Maa et al., 1997).

When spray drying protein pharmaceuticals, it is critical to address certain issues to maintain the activity and structure of the protein. During spray drying, the high temperatures and stresses from atomization may affect the proteins activity and cause degradation. Additionally, spray drying often results in low production yields and should therefore be optimized for each protein pharmaceutical (Mutukuri et al., 2023; Sosnik & Seremeta, 2015; Wu et al., 2014).

One way of improving the stability and processability of the pharmaceutical, as well as its dispersibility, is by adding excipients to the formulation.

Carbohydrates are usually the main excipient used in protein pharmaceuticals due to their ability to stabilize the protein. More specifically, amorphous sugars such as lactose and trehalose have a high glass transition temperature (T_g) and can stabilize molecules at room temperature (Chang & Chan, 2022; Lechanteur & Evrard, 2020). The methods with which carbohydrates stabilize the protein vary, with the main theories being the formation of hydrogen bonds with the proteins, by replacing the bonds with water (water replacement theory) and the stabilization of proteins by forming an amorphous matrix and halting any structural changes or degradation (vitrification hypothesis) (Chang & Chan, 2022; Eedara et al., 2021; Pinto et al., 2021).

Lactose is a disaccharide that has been extensively used in powder inhalation formulations as a delivery carrier as it is Generally Recognized as Safe (GRAS). With a high glass transition temperature of 101 °C, lactose helps produce an amorphous dry powder characterized by weak stickiness and good flowability. However, it is important to note that lactose is a reducing sugar and can participate in the Maillard reaction by reacting with amine groups (Lechanteur & Evrard, 2020; Wu et al., 2014).

Similarly, trehalose is recognized as safe (GRAS) and has a higher T_g of 120 °C (Chang & Chan, 2022). The higher T_g aids in stabilizing proteins and extending the shelf life of the pharmaceuticals. Trehalose can form an amorphous matrix that protects the active pharmaceutical ingredient (API) during spray drying and storage. However, its sticky nature can lead to aggregation when used above a certain concentration (Maa et al., 1997). At the same

time, trehalose has an enhanced ability to form hydrogen bonds with proteins further stabilizing them through the water replacement theory (Pinto et al., 2021).

Another group of excipients highly used in protein pharmaceuticals are amino acids. These stabilize protein formulations and protect them from moisture during storage. The methods are generally unknown, but it has been suggested that they could form hydrogen bonds with the proteins (Pinto et al., 2021)

L-leucine is a hydrophobic amino acid widely used in protein pharmaceuticals, particularly in inhalation powders. It has been suggested that L-leucine diffuses towards the air-liquid interface to form an outer hydrophobic shell around the particles as it accumulates there in crystallized form, protecting them from moisture (Lechanteur & Evrard, 2020; Ordoubadi, 2024; Pinto et al., 2021) and causing the particles to corrugate and become more wrinkled (Juptner & Scherliess, 2019; Pinto et al., 2021). Additionally, it enhances powder flowability and dispersibility by decreasing surface cohesiveness (Eedara et al., 2021; Pinto et al., 2021). Increasing L-leucine concentrations improves powder dispersibility and resistance to high relative humidity. There are notable improvements in the FPF values observed for powders containing up to 20% w/w L-leucine (Chang & Chan, 2022).

At the same time, when combined with trehalose, higher concentrations of L-leucine (20-30%) result in more stable powder in humid environments, whereas a lower concentration of 10% improves aerosolization performance, enabling deposition in deeper part of the lungs (Lechanteur & Evrard, 2020).

1.6 Project layout

The project consisted of two parts: the pre-study and the main study. The pre-study focused on identifying the most suitable spray drying conditions for producing inhalable particles. The sugars used for this study were lactose and trehalose in aqueous solutions and the resulting powders were analyzed using light microscopy and laser diffraction to determine particle shape and size.

The main study examined the impact of spray drying conditions on formulations containing trehalose and L-leucine. After finalizing the ideal spray drying conditions for producing inhalation powders, the study aimed to produce protein powders with varying ratios of BSA, trehalose dihydrate and L-leucine. These powders were then analyzed for size, morphology and aerodynamic properties. Characterization techniques included light microscopy, laser diffraction, bulk density measurements, Scanning Electron Microscopy (SEM) and their aerosolization performance was analyzed using a Next Generation Impactor (NGI) and Aerodynamic Particle Sizing (APS).

2 MATERIALS AND METHODS

The experimental work for the master's thesis was conducted in the department of Process and Life Science Engineering, Division of Food and Pharma. It took place from the month of January 2024 until May 2024.

2.1 Materials

The materials used were made available by the Division of Food and Pharma and are presented in Table 1.

Table 1: Materials used for the production and characterization of dry powders for inhalation.

Materials	Supplier
Lactose (Lactohale LH200)	DFE pharma
Trehalose Dihydrate	Sigma-Aldrich
L-leucine	Sigma-Aldrich
Bovine serum albumin (BSA)	Sigma-Aldrich
Lamp oil	Petromax Alkan
Sorbitan Monolaurate (Span20)	Sigma-Aldrich
Dimethyl Sulfoxide (DMSO)	VWR Chemicals
Fluorescamine	Sigma-Aldrich
Brij/glycerol mixture	Made by Emmace Consulting AB

2.2 Preparation of powder formulations

2.2.1 Pre-study

In the first stage of this study, spray drying conditions were examined to produce powders with particle sizes appropriate for inhalation (below 5 μm). The spray dryer used was the Mini Spray Dryer B-290 (Büchi Labortechnik AG) with a two fluid nozzle (Figure 4).

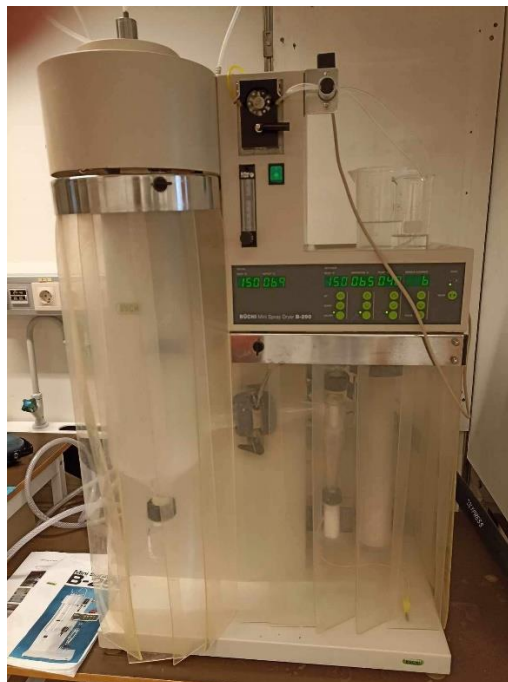


Figure 4: Mini Spray Dryer B-290 by Büchi Labortechnik AG, located in the department of Process and Life Science Engineering, Division of Food and Pharma.

The feed solution used contained lactose 10% w/v dissolved in distilled water. Three sets of spray drying conditions were used to produce the lactose powders (Table 2), which were then characterized using light microscopy and laser diffraction. Detailed conversion tables for the spray drier settings can be found in the Appendix section 7.1.

Table 2: Spray drying conditions of lactose formulations in the pre-study.

Batch	T _{in} °C	T _{out} °C	Aspirator	Pump
Lactose_01	150	70	65%	16%
Lactose_02	170	80	80%	20%
Lactose_03	150	80	80%	10%
<i>Aspirator 65% = 26 m³/hr, 80% = 32 m³/hr</i> <i>Pump 10% = 3 mL/min, 16% = 5 mL/min, 20% = 6 mL/min</i> <i>Nozzle = 6</i> <i>Spray gas flow (Q_{gas}, actual volume flow) = 45 mm = 0.831 m³/hr</i>				

Similar powders were made with trehalose dehydrate (7.2% w/v), instead of lactose, to determine the effect of the spray-drying conditions on the main sugar excipient of the final inhalation powder. Two powders were made with the following conditions, Trehalose_01 and Trehalose_02 (Table 3).

Table 3: Spray drying conditions of trehalose dihydrate formulations in the pre-study.

Batch	T _{in} °C	T _{out} °C	Aspirator	Pump
Trehalose_01	150	67	65%	16%
Trehalose_02	172	77	80%	20%

All powders were kept in a desiccator with RH% below 10%, to protect from moisture.

2.2.2 Main study

In the main study, three powders were firstly produced that contained trehalose dehydrate and leucine, with similar spray drying conditions used in the pre-study above. The powders contained 27% w/v leucine, with a total percentage of solids 7.4% w/v (Table 4). The feed solutions were made by initially dissolving trehalose dihydrate in distilled water. The addition of L-leucine was done under magnetic stirring, with simultaneous low heating to fully dissolve the hydrophobic amino acid. The pH was adjusted to 6.0-6.5, using HCl or NaOH 0.1 M solutions.

Table 4: Spray drying conditions of trehalose-leucine formulations (27% w/v leucine).

Batch	T _{in} °C	T _{out} °C	Aspirator	Pump
Trehalose_Leucine_01	150	65	65%	16%
Trehalose_Leucine_02	175	73	80%	20%
Trehalose_Leucine_03	150	80	80%	10%

Following this, the conditions selected to produce the final powders containing BSA, were the second combination: T_{in} = 175 °C, T_{out} = 73 °C, aspirator = 80%, pump = 20%.

The powders contained varied ratios of excipients. More specifically, all powders contained 10% w/v BSA, and varied amounts of L-leucine. The total solids percentage was kept stable at 7.4% w/v. The three formulations are presented in Table 5.

Table 5: Formulations of spray dried powders with BSA.

Batch	BSA	L-leucine	Trehalose dihydrate
Trehalose_BSA_01	10%	-	90%
Trehalose_Leucine_BSA_02	10%	27%	63%
Trehalose_Leucine_BSA_03	10%	10%	80%
Trehalose_Leucine_BSA_03_A	10%	10%	80%
Trehalose_Leucine_BSA_03_B	10%	10%	80%
<i>Samples Trehalose_Leucine_BSA_03_A and B are replicates of the third batch, to examine the reproducibility of the results.</i>			

The formulations were made similarly to the above method. Following the addition of leucine, BSA was added when the feed solution reached room temperature. Light magnetic stirring was used so as not to cause foaming. Finally, the pH was adjusted to 6.0-6.5. The production masters for these powders can be found in the Appendix section 7.2.

2.3 Characterization of powders

The dry powders characteristics were determined using laser diffraction, light microscopy, and Scanning Electron Microscopy, whereas their applicability as aerosol powders in inhalation devices was evaluated using a Next Generation Impactor (NGI) and an Aerodynamic Particle Sizer (APS).

2.3.1 Light microscopy

The particles morphology and shape were examined with light microscopy. The instrument used was Olympus System Microscope Model BX50, with ocular magnification x10.

The samples were prepared by dispersing 20 mg of powder in 2 mL of lamp oil and mixing with the pipette for 10 seconds. A drop of the suspension was added onto a glass slide and a cover slip was applied on top. The images were taken with the following objectives: x20 and x50.

Images were also taken of a scale, 1 mm long with 100 divisions of 0.01 mm (made by GRATICULES LTD.) to compare particle sizes.

2.3.2 Laser diffraction

The size distribution of the dry powders was measured using laser diffraction. The method uses a He-Ne laser to emit light, which then diffracts as it passes through a dispersed particulate sample. The resulting light scattering pattern is analyzed to estimate the size distribution of the particles using either the Mie theory or Fraunhofer diffraction theory. Both theories are used

under the assumption that the particles are smooth and spherical (Merkus, 2009; Shekunov et al., 2007)

Mie theory is applicable for particles of all sizes, and requires the optical properties of the samples, including the refractive index of the particles and the medium they are dispersed in. This theory calculates the intensity of the scattered light by considering light diffraction, absorption, and refraction as well as wide angle scattering, which is ideal for measuring particles smaller than 25 μm . However, if the refractive index of the powders is unknown or cannot be measured, the accuracy of the method is not as good (Merkus, 2009; Shekunov et al., 2007).

Fraunhofer diffraction theory relies solely on particle size and is accurate for particles larger than the wavelength of the light. This theory measures the particle diameter based on the intensity of diffracted light without considering the refractive index of the sample. However, for smaller particles, the absorption and refraction of light significantly influence the scattering pattern, making Mie theory more appropriate to apply in this case. Under certain conditions, measurements from Fraunhofer theory and Mie theory are equivalent (Merkus, 2009; Shekunov et al., 2007).

In this study, both theories were examined for powders with known refractive indices (pure lactose and trehalose dihydrate) and unknown (powders containing percentage of BSA, L-leucine). The instrument used was the Malvern Mastersizer 2000 (Figure 5). Samples were prepared by sonication, with the addition of a surfactant to assist in the dispersion of the powders in lamp oil.

The sample was prepared by weighing 50 mg of powder and dispersing it in 4 mL lamp oil and 1 mL Span20 (25% w/v solution). The powder was fully dispersed in the oil with a sonication probe (Branson Sonifier B-12 Ultrasonic Tip), for 2 mins at power level 7, in an ice bath.

Following this, the sample was added dropwise into the oil dispersion unit of the Mastersizer 2000, until the wanted obscuration (5, 10 or 15%). Refractive indexes used for the samples were 1.53 for the sugars and 1.43 for the lamp oil, with absorption 0.01.

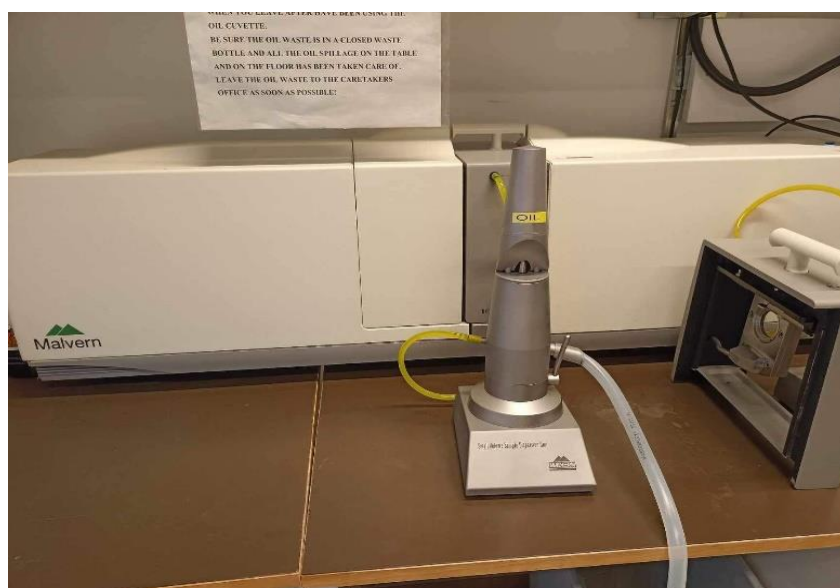


Figure 5: Malvern Mastersizer 2000 used for determining the size distribution of particles in powders.

2.3.3 Scanning Electron Microscopy (SEM)

The Scanning Electron Microscope was used to examine the particles shape and morphology and to observe differences found between the powder batches produced. SEM is a method widely used for the characterization of materials and relies on the production of electrons by the materials to form an image. By using an electron beam that passes through a microscope, the sample will produce secondary and backscattered electrons, as well as characteristic X-rays that will be used for imaging (Paredes, 2014).

The samples were prepared by applying a thin layer of each powder on a brass mount that was fitted with a thin layer of tape. Following this, the mounts were coated with gold/palladium (Au/Pd) to create a conductive layer that will enhance the secondary electron signal and provide improved imaging. For the coating, a Sputter Coater was used with argon as the operating gas, working pressure 0.05 mbar, sputtering current 15 mA and for sputtering time 180 seconds. This would result in a film thickness of 15 nm Au/Pd coated on the samples.

The SEM instrument used was the JEOL JSM-6700F scanning electron microscope. The accelerating voltage used during the SEM procedure was 10 kV, and the emission current 10 μ A. Images of samples were taken at magnifications x250, x1000 and x10000.

2.3.4 Bulk density measurement

Powders used for inhalation should ideally have a bulk density below 0.4 gr/cm³ for deposition in the deeper part of the lungs (Chaurasiya & Zhao, 2020).

The bulk density of the powders was determined using a cylinder with 5.2 cm³ volume. Each sample was packed into one cylinder, and any excess powder was scraped off with a spatula. The cylinder was weighed on an analytical scale, and the value was recorded before and after filling with powder. Each measurement was performed in triplicates.

2.3.5 Next Generation Impactor (NGI)

To simulate the use of the powders in an inhaler and to calculate the aerodynamic particle diameter (AD) and the Fine Particle Dose (FPD) and Fraction (FPF), a Next Generation Impactor was used by the manufacturer Copley (Figure 6.A).

The process included preparation of the capsules by weighing 20-30 mg of powder. For each NGI, 5 capsules were filled. The inhaler used was the RS01 monodose dry powder inhaler with high resistance (blue colour) by the company Plastiaple (Figure 6.B). In this specific model the capsule spins laterally in the inhaler.



Figure 6: A. Next Generation Impactor (NGI) by Copley (promosciences.com), B. RS01 monodose dry powder inhaler with high resistance by Plastiap (www.berryglobal.com).

The impactor was prepared by setting the inspiratory flow rate at 62 L/min with the use of a Trigger Box, which is necessary to produce an aerosol that will reach the dedicated area of the lungs, at the required dose. The pressure drop that is reached with this flow rate is 4 kPa. This was confirmed with a manometer.

After setting the flow rate and pressure, the plates were covered with a few drops of Brij/glycerol mixture and spread out evenly onto the surface with a KIMTECH wipe. 15 mL of distilled water was added to the pre-separator (P).

The Brij/glycerol mixture was provided by Emmace Consulting AB and was made by dissolving 15 gr of Brij in 100 ml Ethanol and then mixing 10 ml of this solution with 40 ml glycerol (85%).

The capsule is placed in the inhaler and pierced by pressing the blue side buttons. The RS01 inhaler was fitted to the throat (T) of the NGI and the start button was pressed on the Trigger Box. The successful inhalation of the powder was indicated by a rattling noise. Each capsule was used twice to ensure that all powder was deposited into the NGI system. All five capsules used were collected after the measurements and each batch was repeated twice.

After the experimental procedure, 15 mL of water was added to each disk, to dissolve the powder. These were set on a rocking platform for 15 mins at a speed of 60 rpm. At the same time, 15 mL was added to the throat and pre-separator of the system. Table 6 indicates the batches and the naming system used to store the samples from each batch.

Table 6: Dry powders used in NGI and their equivalent names.

Batch	Sample	NGI	Name
1	Trehalose_BSA_01	1	B1 N1
		2	B1 N2
2	Trehalose_Leucine_BSA_02	3	B2 N3
		4	B2 N4
3	Trehalose_Leucine_BSA_03	5	B3 N5
		6	B3 N6
4	Trehalose_Leucine_02	7	B4 N7
		8	B4 N8

Depending on which stage each sample came from, they were cataloged with a number or letter in Table 7.

Table 7: Numbering system for powders found in different NGI stages.

Stage 1	B N 1
Stage 2	B N 2
Stage 3	B N 3
Stage 4	B N 4
Stage 5	B N 5
Stage 6	B N 6
Stage 7	B N 7
Stage 8	B N 8
Capsules	B N C
Throat	B N T
Pre-separator	B N P
Capsules Blank (5 capsules dissolved in 15 ml water)	CB

2.3.5.1 Fluorescamine assay

To quantify the amount of BSA in each sample from the 11 NGI stages, a fluorescamine assay was used, based on the protocol by AAT Bioquest, Sunnyval, CA (Amplite® fluorescamine protein assay). Fluorescamine is a non-fluorescent heterocyclic dione that reacts with primary amines of amino acids and proteins and produces a fluorescent product. It has an excitation wavelength of 365 nm and an emission wavelength at 470 nm.

A fluorescence plate reader (FLUOROSKAN, by Thermo Scientific) was used to detect the fluorescence signal of each sample with a 96-well black microplate. The process for the fluorescence assay can be seen below in Figure 7.

For each batch of powder, a standard curve was made using serial dilution, from a stock solution of 1 mg/mL BSA or L-leucine or the equivalent BSA/leucine ratio for Batch 2 and 3 (Table 5).

Firstly, fluorescamine was dissolved in dimethyl sulfoxide (DMSO), at a concentration of 3 mg/ml. It was stored in an amber vial, to protect from light.

Each sample was added in a well in the microplate, at a volume of 75 μ L and mixed with 25 μ L Fluorescamine. The plate was protected from light and left to react for 10 minutes. The fluorescence was read at excitation wavelength 350 nm and emission wavelength 460 nm.

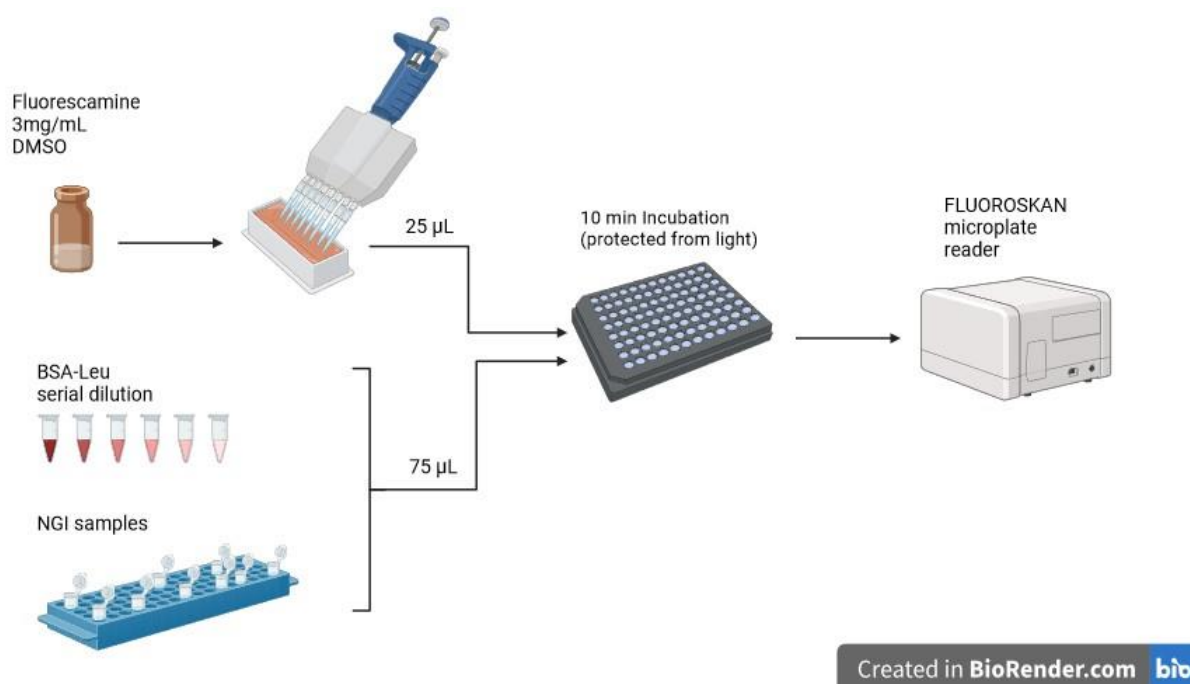


Figure 7: Fluorescamine assay, based on the protocol by AAT Bioquest, for the quantification of BSA in the NGI stages (Image made with BioRender).

2.3.6 Aerodynamic Particle Sizing (APS)

Another method used to measure the aerodynamic particle size was with the Aerodynamic Particle Sizer Model 3321, which was modified by Emmace Consulting AB with an additional 5L tube to achieve the needed volume, in combination with a Trigger Box (Figure 8).

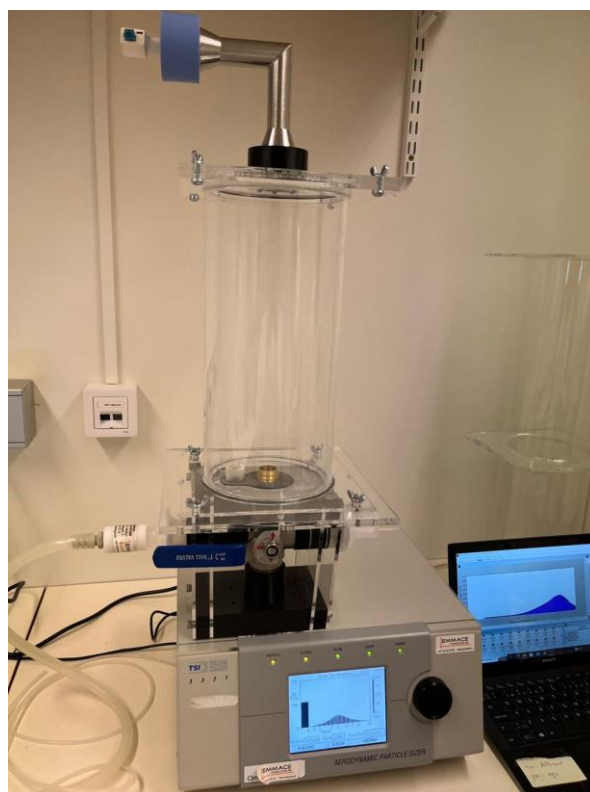


Figure 8: Aerodynamic particle size, fitted with a 5L tube, by Emmace Consulting AB.

The powders used in the analysis were Trehalose_BSA_01, Trehalose_Leucine_BSA_02, Trehalose_Leucine_BSA_03, Trehalose_Leucine_02 and Trehalose_02.

Samples were prepared by weighing 20-30 mg of powders in capsules and kept in a desiccator. The same inhaler used for the NGI was used here (RS01 high resistance by Plastiapex).

Each sample was sprayed into the device for 2 seconds, at a flowrate of 62 l/min and each measurement lasted for 30 seconds. The system was cleaned of any powder residues in between samples and all powders were measured in duplicates. The experiment was performed a second time for all samples, where each one was sprayed into the device for 1 second and with a flowrate of 120 l/min.

3 RESULTS

Spray-dried powders were produced successfully with the Mini Spray Dryer B-290. The powders produced in the pre-study and the main study were characterized based on their size, morphology, and aerodynamic properties.

For powders containing L-leucine and BSA the yield was calculated by weighing the spray dryer's collection vessel before and after the production process. The resulting yields can be seen in Table 8.

Table 8: Production yields of spray-dried powders.

Batch	Yield (%)
Trehalose_BSA_01	78.6
Trehalose_Leucine_BSA_02	85.0
Trehalose_Leucine_BSA_03	81.8
Trehalose_Leucine_BSA_03_A	82.2
Trehalose_Leucine_BSA_03_B	77.4

Typically, the spray drying yields on this scale reach values above 75%, which is considered a very successful process (Sosnik & Seremeta, 2015). In the main study the values for all spray drying processes were above 70%, which indicates that the spray drying of trehalose dihydrate powders under these conditions is highly efficient.

3.1 Pre-study

The results from the pre-study were used to determine the most efficient spray drying conditions for the sugars used.

3.1.1 Light microscopy

Light microscopy was used to initially view the shape and size of the spray-dried particles. Below, images are presented of the powders produced with different magnifications.

3.1.1.1 Spray-dried Lactose

Lactose formulations were spray dried under three different conditions (Table 2). All three powders had similar shape viewed under light microscopy. Powder Lactose_03 can be seen in Figure 9, at magnifications x20 and x50. The particles are spherical with polydisperse sizes, many particles have a dark ring inside which indicates the presence of vacuoles.

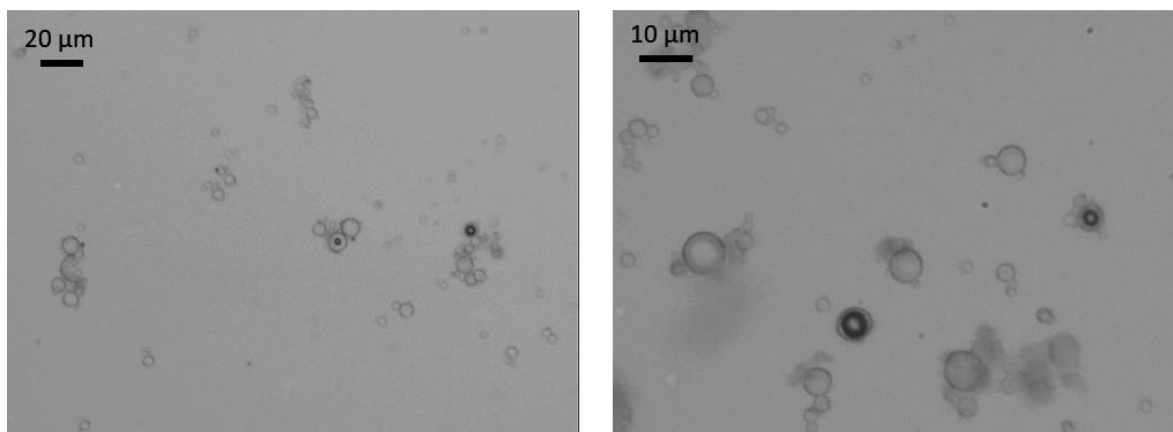


Figure 9: Light microscopy image of spray-dried Lactose_03 at magnification x20 (left) and x50 (right).

3.1.1.2 Spray-dried Trehalose dihydrate

Formulations containing trehalose dihydrate were spray dried under two different conditions presented in Table 3. Trehalose_01 and Trehalose_02 were examined under light microscopy and were similar in shape and size. The images taken at magnifications x20 and x50 for Trehalose_01 can be seen in Figure 10. Trehalose particles are spherical with sizes below 10 µm, with very few vacuoles present.

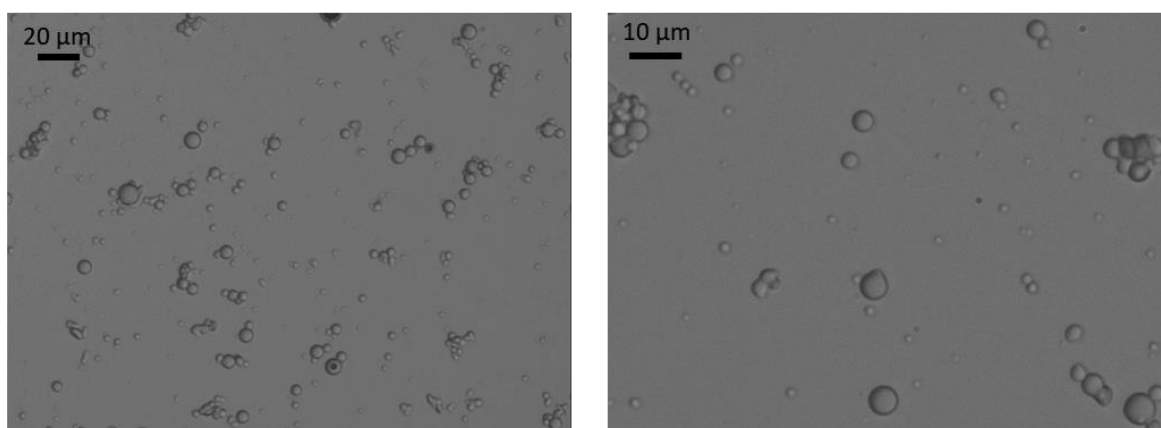


Figure 10: Light microscopy image of spray-dried Trehalose_01 at magnification x20 (left) and x50 (right).

3.1.2 Laser diffraction

3.1.2.1 Spray-dried Lactose

The three lactose powders produced had similar sizes around 4.5 µm, with Lactose_01 being slightly larger in size close to 5 µm with weighted residuals below 1%. Lactose_02 and Lactose_03 had smaller sizes with average particle size (d50) closer to 4.5 µm and weighted residuals 0.9%. The size distribution for Lactose_02 can be seen in Figure 11, using Mie theory, for obscuration 5%. Figure 12 depicts the particle size distribution of Lactose_02 using Fraunhofer theory, which calculated average particle size of 3.8 µm and weighted residuals below 1%.

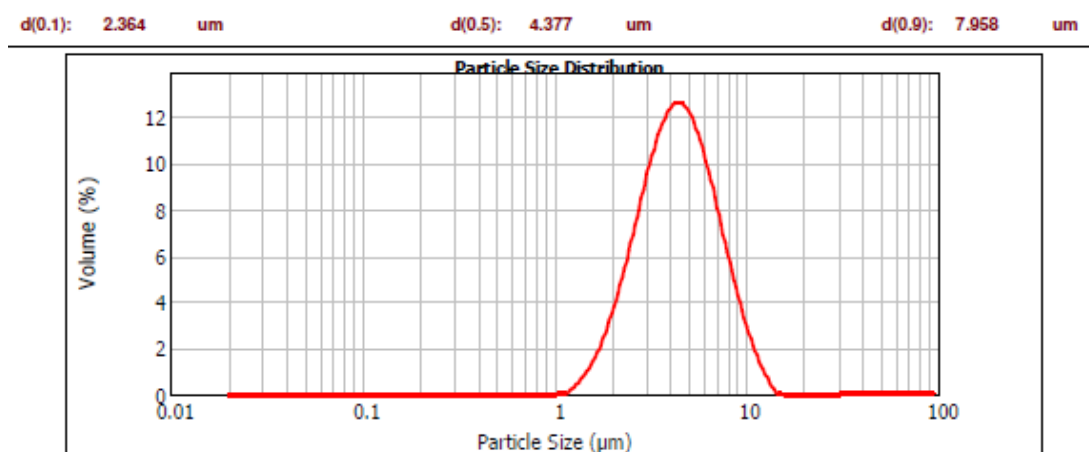


Figure 11: Particle size distribution of sample Lactose_02 with 5% obscuration (Mie theory).

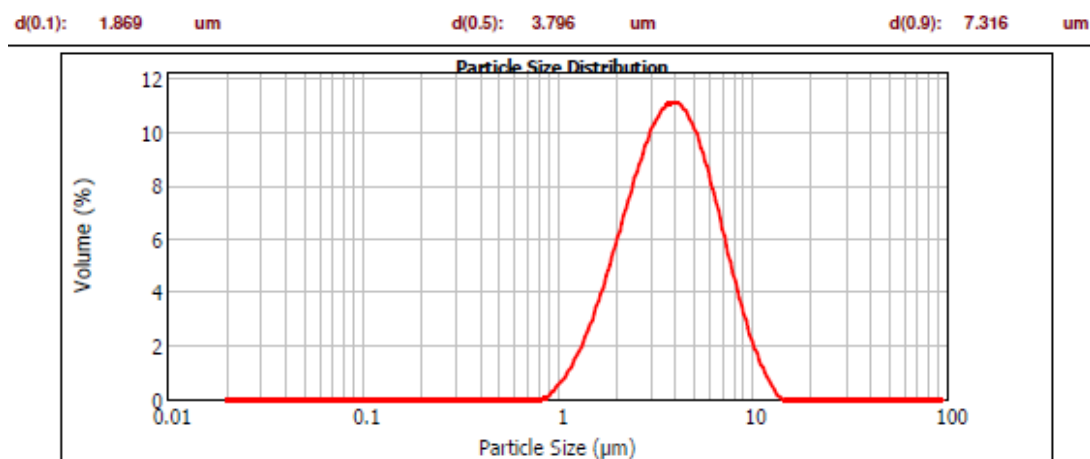


Figure 12: Particle size distribution of sample Lactose_02 with 5% obscuration (Fraunhofer theory).

3.1.2.2 Spray-dried Trehalose dihydrate

Spray-dried trehalose dihydrate samples (Trehalose_01 and Trehalose_02) were examined under light microscopy. For Trehalose_01 the average particle size d50 was 4.4 μm , with weighted residuals close to 1% for Mie theory and 3.8 μm with 0.4% weighted residuals for Fraunhofer theory. The size distribution can be seen in Figure 13 with Mie theory and Figure 14 with Fraunhofer theory. Similar size distributions were seen for sample Trehalose_02.

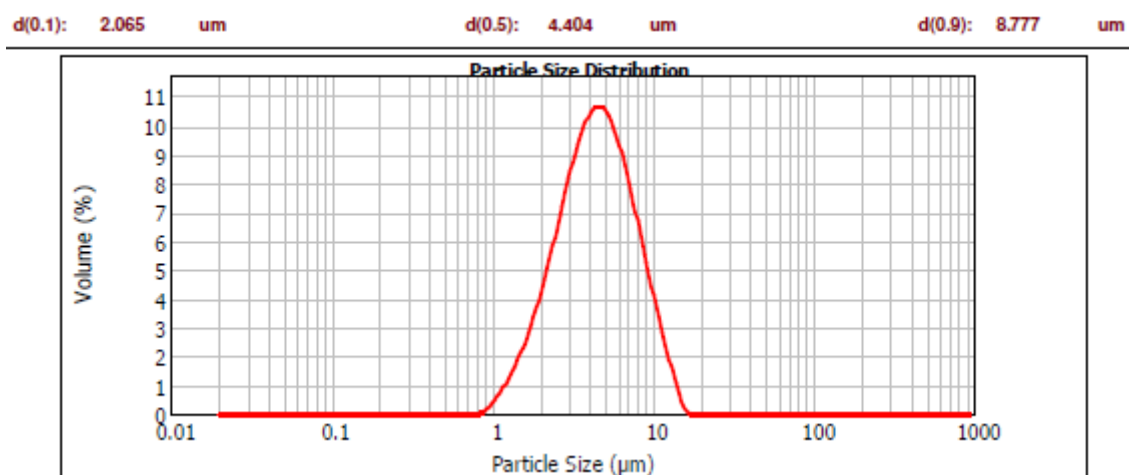


Figure 13: Particle size distribution of sample Trehalose_01 with 10% obscuration (Mie theory).

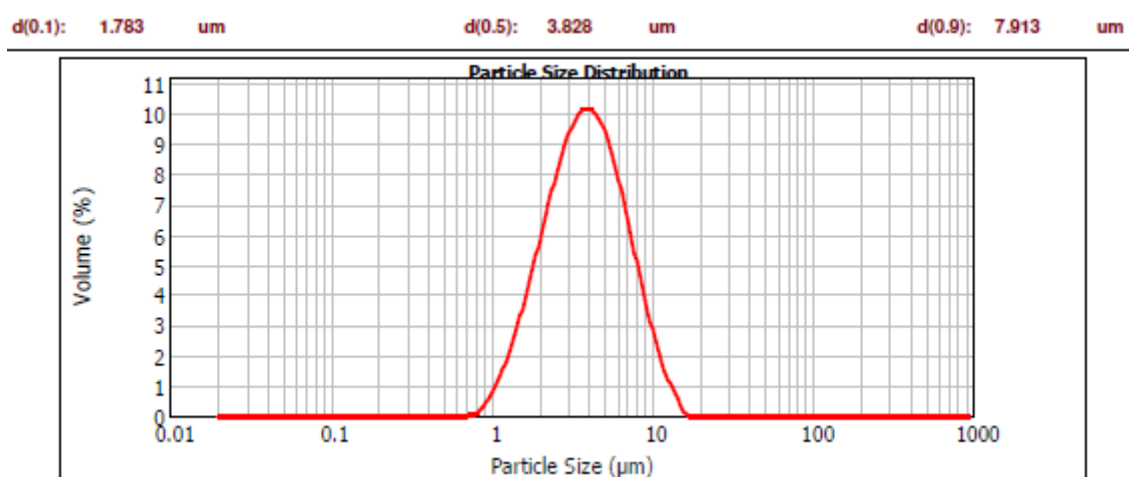


Figure 14: Particle size distribution of sample Trehalose_01 with 10% obscuration (Fraunhofer theory).

3.2 Main study

Based on the results in the pre-study, new formulations were made with trehalose dihydrate as the main excipient shown in Tables 4 and 5 in section 2.2.2. These spray-dried powders contained L-leucine and BSA, and were characterized by light microscopy, laser diffraction, SEM, bulk density, NGI, and APS.

3.2.1 Light microscopy

3.2.1.1 Spray-dried Trehalose and L-leucine

L-leucine containing powders were produced under different conditions presented in Table 4. Three powders were produced: Trehalose_Leucine_01, Trehalose_Leucine_02 and Trehalose_Leucine_03. Light microscopy images of the three powders, at magnifications x20 and x50, are shown in Figure 15. The particles for all three powders deviated from sphericity and had a raisin-like shape. There were multiple vacuoles present in the particles, distinguished by the darker shapes inside, which also deviated from sphericity and were irregularly shaped.

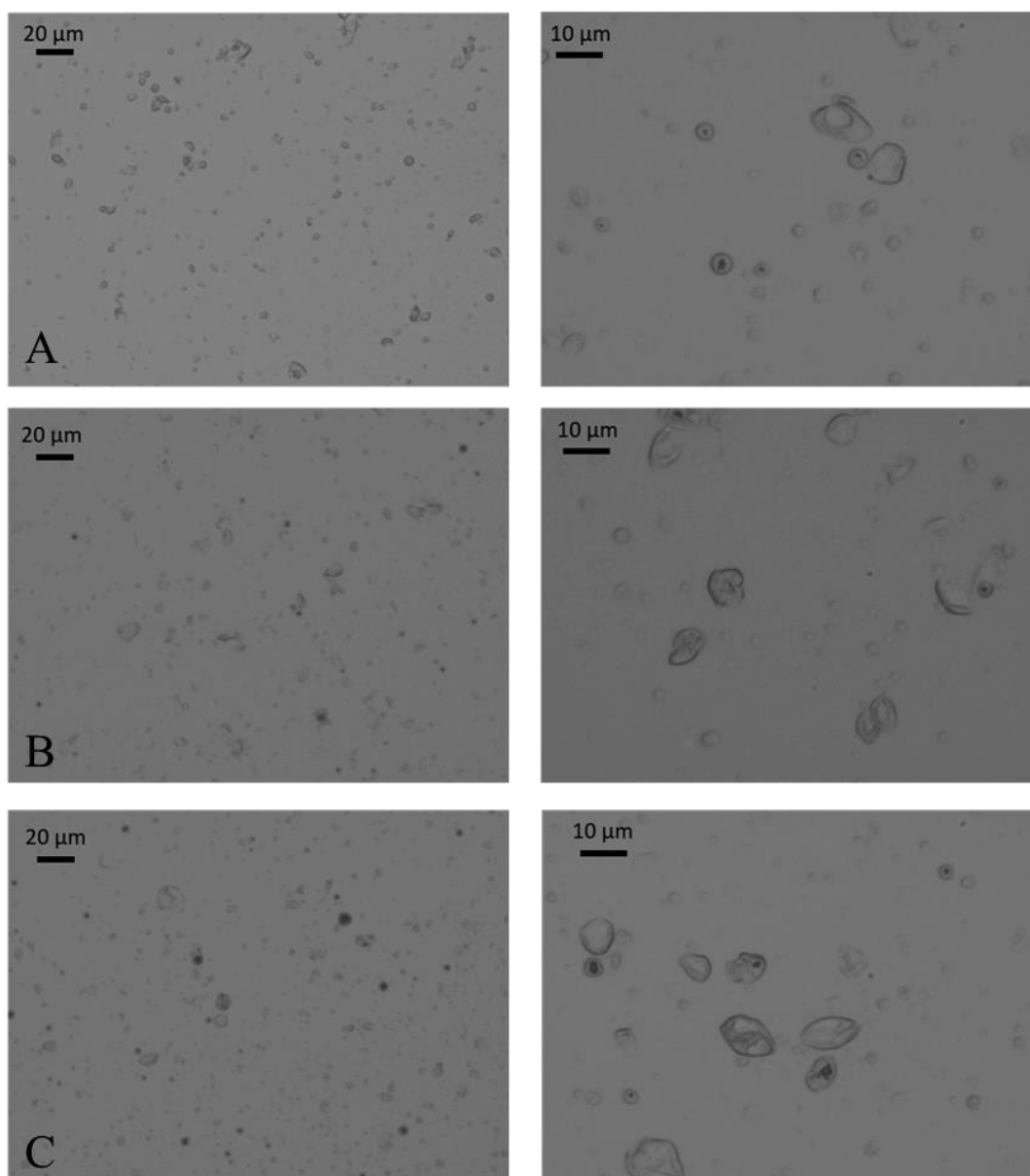


Figure 15: Light microscopy images of Trehalose_Leucine_01 (A), Trehalose_Leucine_02 (B) and Trehalose_Leucine_03 (C), at magnifications x20 (left column) and x50 (right column).

3.2.1.2 Spray dried Trehalose dihydrate and Bovine Serum Albumin

Following spray drying formulations containing leucine, the conditions chosen to produce BSA containing formulations were similar to batch Trehalose_Leucine_02 and more specifically $T_{in} = 150\text{ }^{\circ}\text{C}$, $T_{out} = 80\text{ }^{\circ}\text{C}$, aspirator = 80%, pump = 10%.

The first spray-dried powder containing the protein BSA can be seen in the light microscopy images in Figure 16. The percentage of protein in the formulation was 10% w/v. The particles were raisin-like with a range of sizes. They had a limited number of round vacuoles inside some particles, which can be seen as a smaller darker ring.

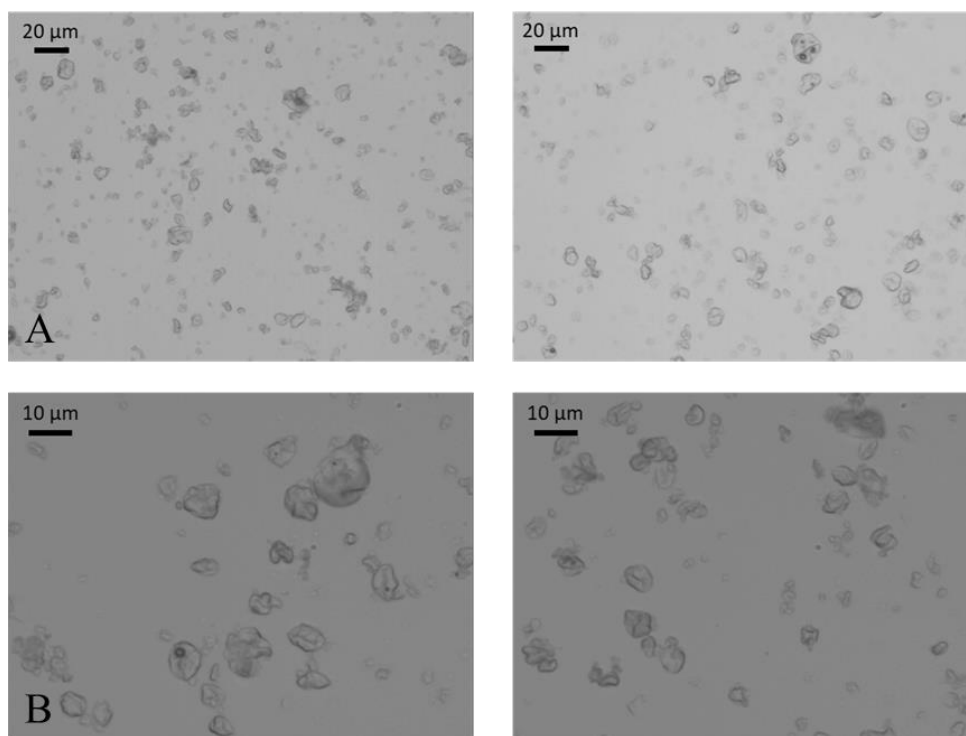


Figure 16: Light microscopy images of Trehalose_BSA_01 at magnifications x20 (top row A) and x50 (bottom row B).

3.2.1.3 Spray dried Trehalose dihydrate, L-leucine and Bovine Serum Albumin

Two formulations containing BSA were spray-dried under the same conditions but with different ratios of L-leucine and BSA. Light microscopy images of powders Trehalose_Leucine_BSA_02 and Trehalose_Leucine_BSA_03 can be seen below (Figure 17 and Figure 18). These particles deviated from sphericity, with raisin-like shapes and a larger number of vacuoles. The vacuoles in powder Trehalose_Leucine_BSA_02 were irregularly shaped, whereas in Trehalose_Leucine_BSA_03 they were rounder.

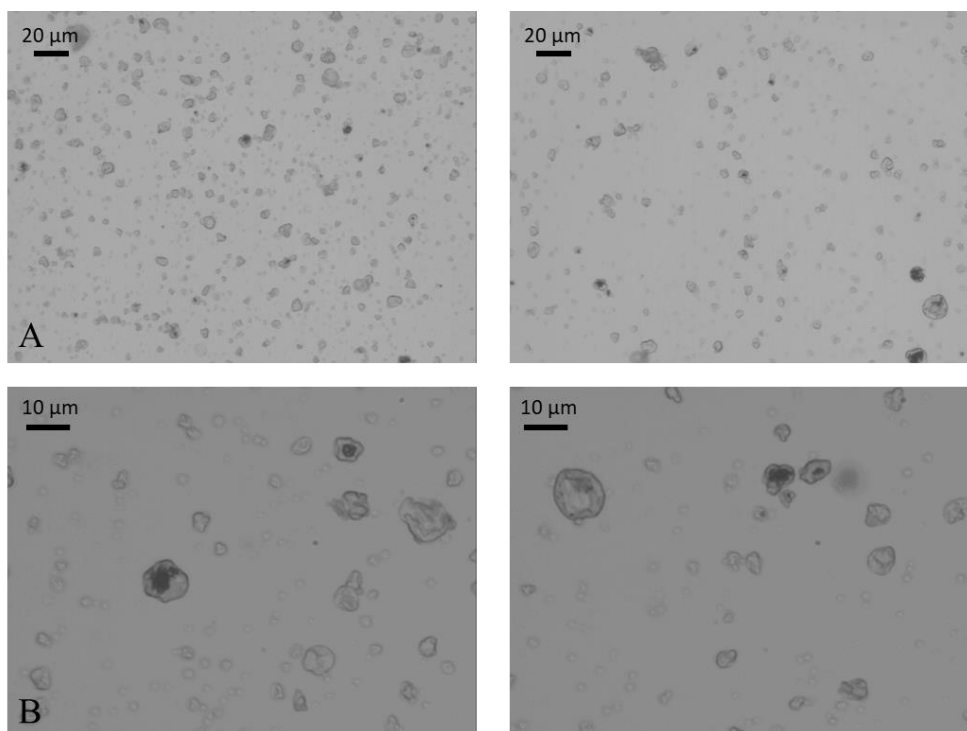


Figure 17: Light microscopy images of Trehalose_Leucine_BSA_02 at magnifications x20 (top row A) and x50 (bottom row B).

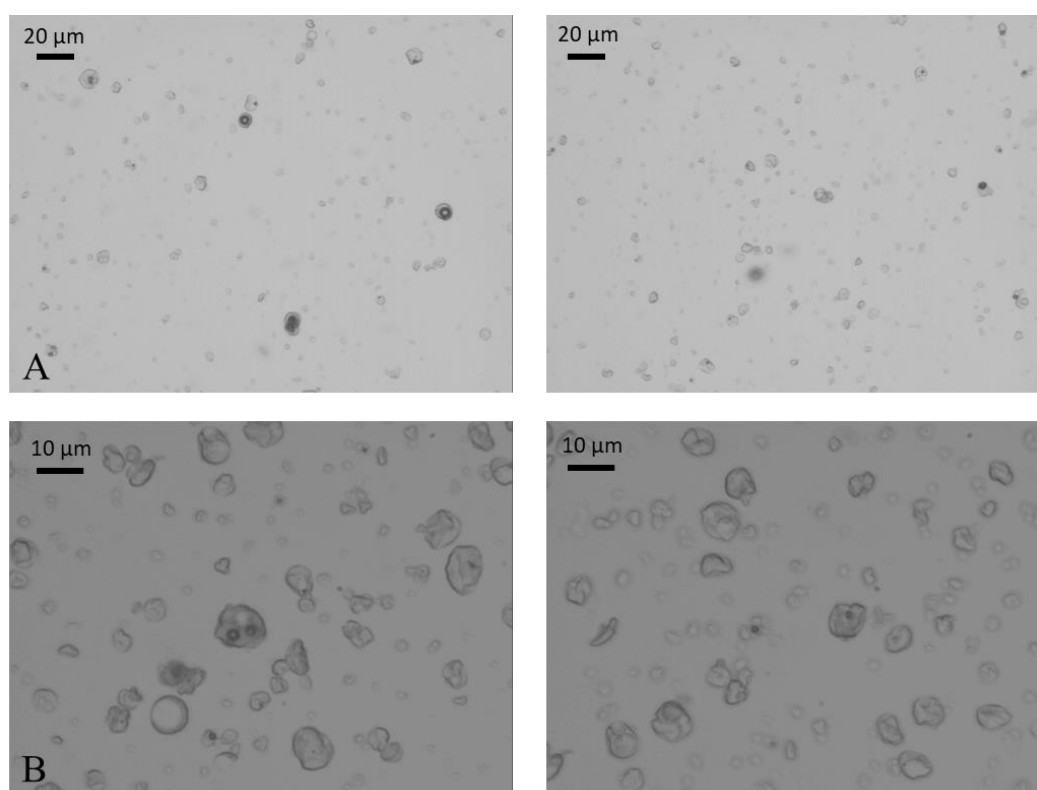


Figure 18: Light microscopy images of Trehalose_Leucine_BSA_03 at magnifications x20 (top row A) and x50 (bottom row B).

Light microscopy images of the replicates of batch 3 (Trehalose_Leucine_BSA_03_A and Trehalose_Leucine_BSA_03_B) are similar to that in Figure 18 (Appendix 7.3).

3.2.2 Laser diffraction

3.2.2.1 Spray-dried Trehalose and L-leucine

Three powders with the same formulation Trehalose dihydrate and L-leucine (27% w/v) were produced with different spray drying conditions. All three powders had similar size distribution with a d50 in the range of 5-6 μm with Mie theory and 4-5 μm with Fraunhofer theory. Figures 19 and 20 portray the typical size distribution found with these samples, with 5% obscuration.

For all three samples, the weighted residuals were below 1%, and more specifically between 0.5 - 0.9% for Mie theory and 0.3 - 0.7% for Fraunhofer theory.

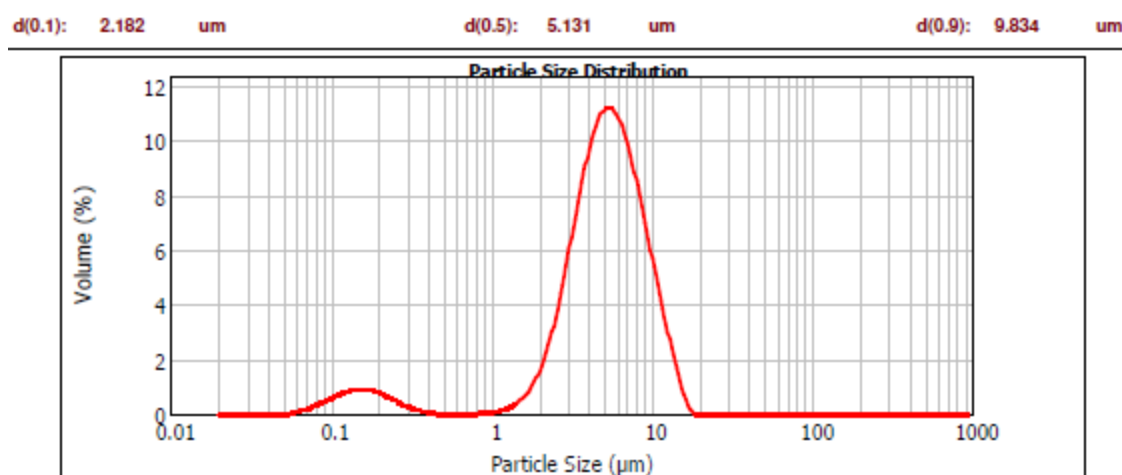


Figure 19: Particle size distribution of sample Trehalose_Leucine_02 with 5% obscuration (Mie theory).

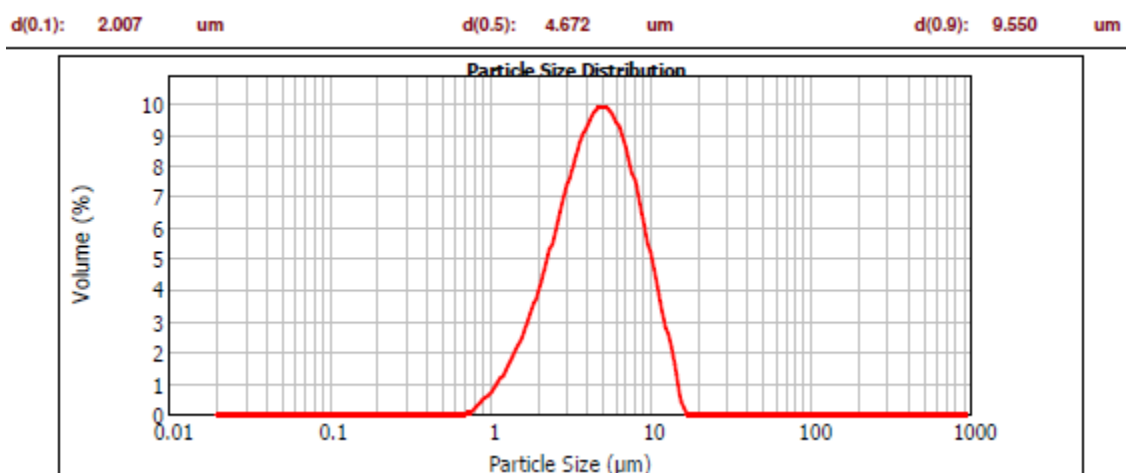


Figure 20: Particle size distribution of sample Trehalose_Leucine_02 with 5% obscuration (Fraunhofer theory).

3.2.2.2 Spray dried Trehalose dihydrate and Bovine Serum Albumin

Samples containing BSA were produced with the following conditions: $T_{in} = 175\text{ }^{\circ}\text{C}$, $T_{out} = 73\text{ }^{\circ}\text{C}$, aspirator = 80%, pump = 20%. For the first sample containing BSA (Trehalose_BSA_01) the average particle size (d_{50}) was $5.5\text{ }\mu\text{m}$ (Mie theory), with a size distribution presented in Figure 21. The weighted residuals were 0.8% for Mie theory and 0.4% for Fraunhofer theory (Figure 22) with a mean particle size of $4.7\text{ }\mu\text{m}$.

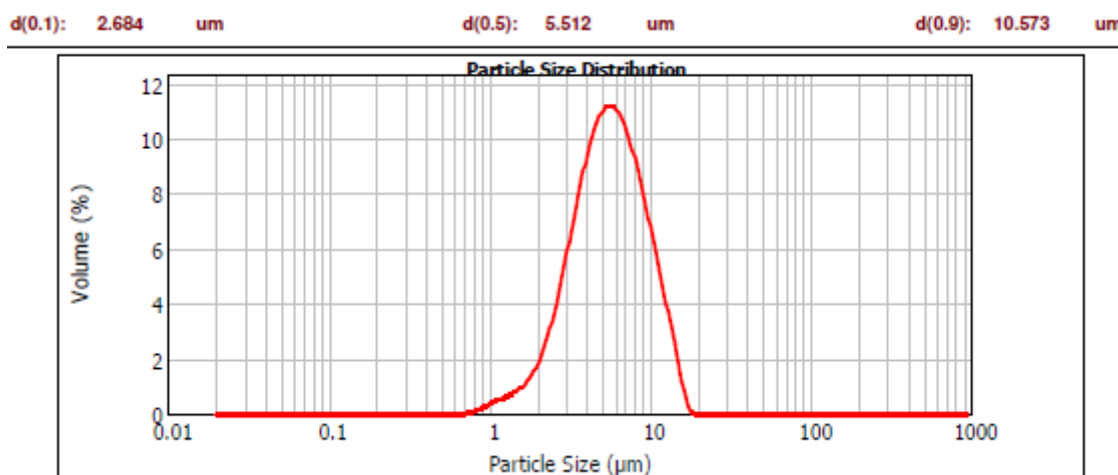


Figure 21: Particle size distribution of sample Trehalose_BSA_01 with 5% obscuration (Mie theory).

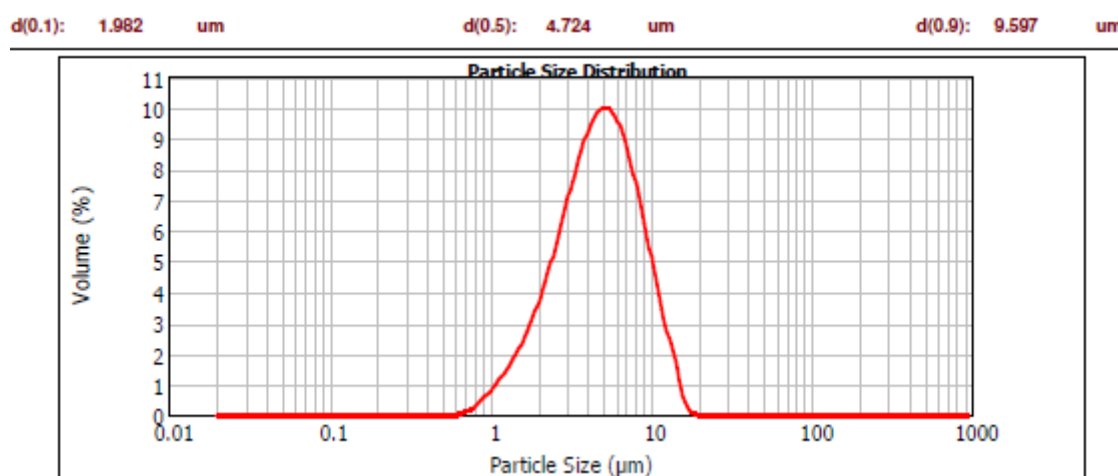


Figure 22: Particle size distribution of sample Trehalose_BSA_01 with 5% obscuration (Fraunhofer theory).

3.2.2.3 Spray dried Trehalose dihydrate, L-leucine and Bovine Serum Albumin

Two powders were spray-dried with different ratios of BSA and L-leucine, under the same conditions. Sample Trehalose_Leucine_BSA_02 had 27% w/v L-leucine and 10% w/v BSA, as presented in Table 5. The particle size distribution based on both Mie and Fraunhofer theories can be seen in Figure 23 and 24. The average particle size was $5.5\text{ }\mu\text{m}$ based on Mie theory, and $4.6\text{ }\mu\text{m}$ based on Fraunhofer theory. Weighted residuals were below 1% for both methods of measurement.

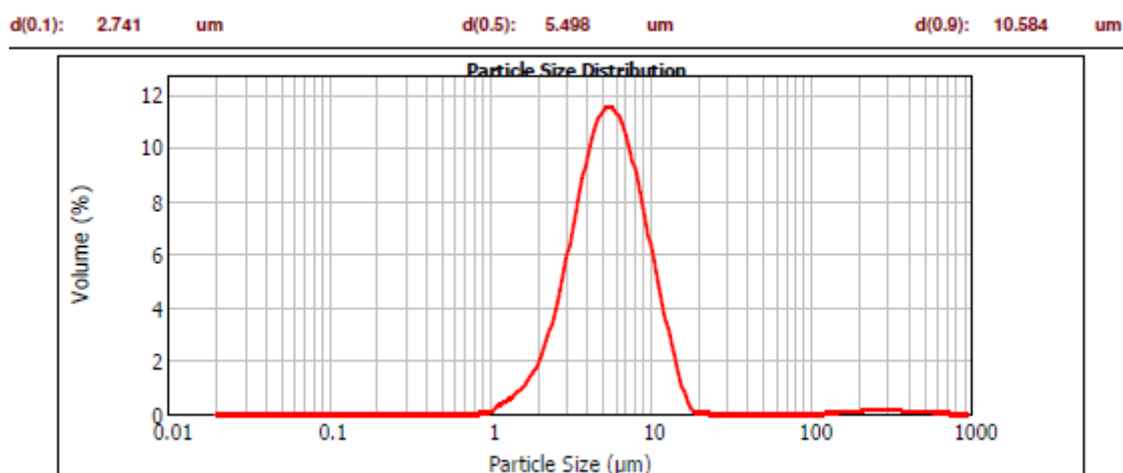


Figure 23: Particle size distribution of sample Trehalose_Leucine_BSA_02 with 5% obscuration (Mie theory).

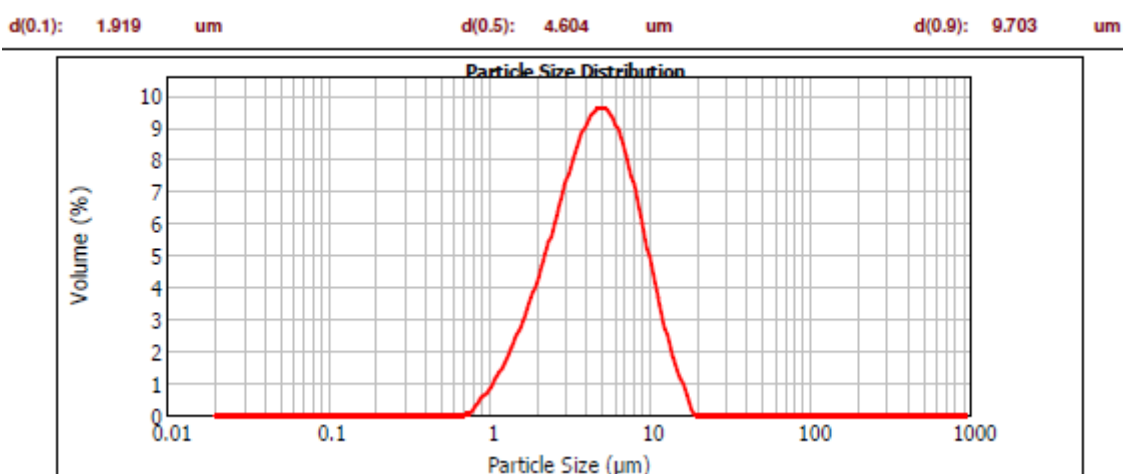


Figure 24: Particle size distribution of sample Trehalose_Leucine_BSA_02 with 5% obscuration (Fraunhofer theory).

Sample Trehalose_Leucine_BSA_03 contained 10% w/v L-leucine and BSA (1:1 ratio). The resulting size distribution of this powder is shown in the two figures below (Figure 25 and 26). The average particle size is 6.2 μm for Mie theory and 4.9 μm for Fraunhofer theory which in both cases are slightly larger than their counterparts in the previous sample Trehalose_Leucine_BSA_02. At the same time, weighted residuals were below 1%.

Similar results were found for repetition batches Trehalose_Leucine_BSA_03_A and Trehalose_Leucine_BSA_03_B.

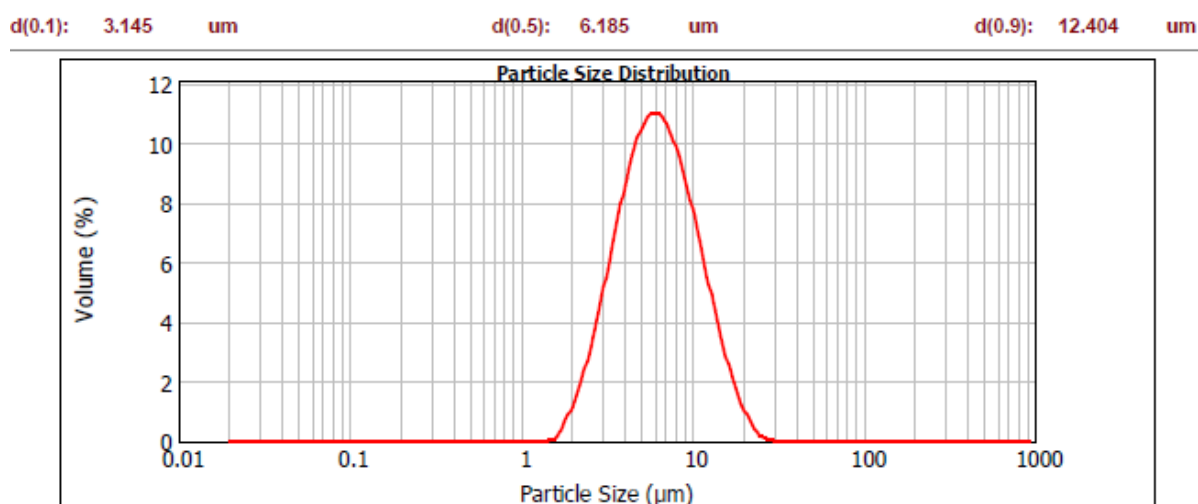


Figure 25: Particle size distribution of sample Trehalose_Leucine_BSA_03 with 5% obscuration (Mie theory).

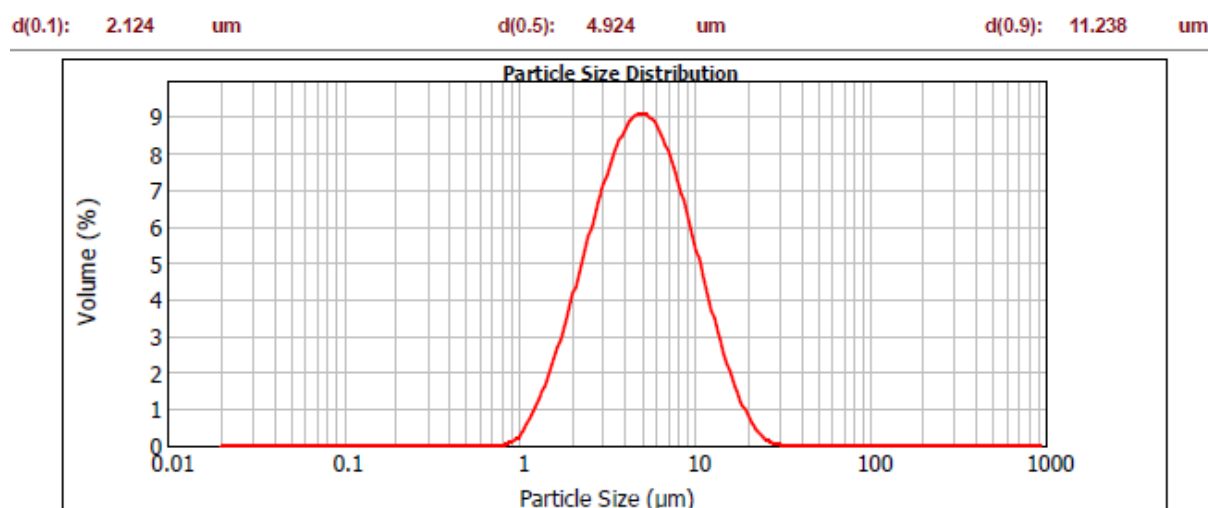


Figure 26: Particle size distribution of sample Trehalose_Leucine_BSA_03 with 5% obscuration (Fraunhofer theory).

3.2.3 Scanning electron microscopy

3.2.3.1 Spray-dried Trehalose and L-leucine

Samples Trehalose_02 and Trehalose_Leucine_02 which contained 27% w/v L-leucine, were examined with SEM, to compare with BSA containing samples that were produced under the same spray-drying conditions ($T_{in} = 175\text{ }^{\circ}\text{C}$, $T_{out} = 73\text{ }^{\circ}\text{C}$, aspirator = 80%, pump = 20%).

Images from these samples can be seen in Figures 27 and 28 at magnifications x250, x1000 and x10000. Trehalose_02 had spherical particles with smooth surfaces, whereas Trehalose_Leucine_02 had corrugated particles compared to just trehalose. They have a coffee bean-like shape, with deeper dents and thicker ridges, as well as shrivels on the surfaces (indicated with arrows in Figure 28). Smaller particles have smaller dents and thinner ridges; therefore, they are described as wrinkled.

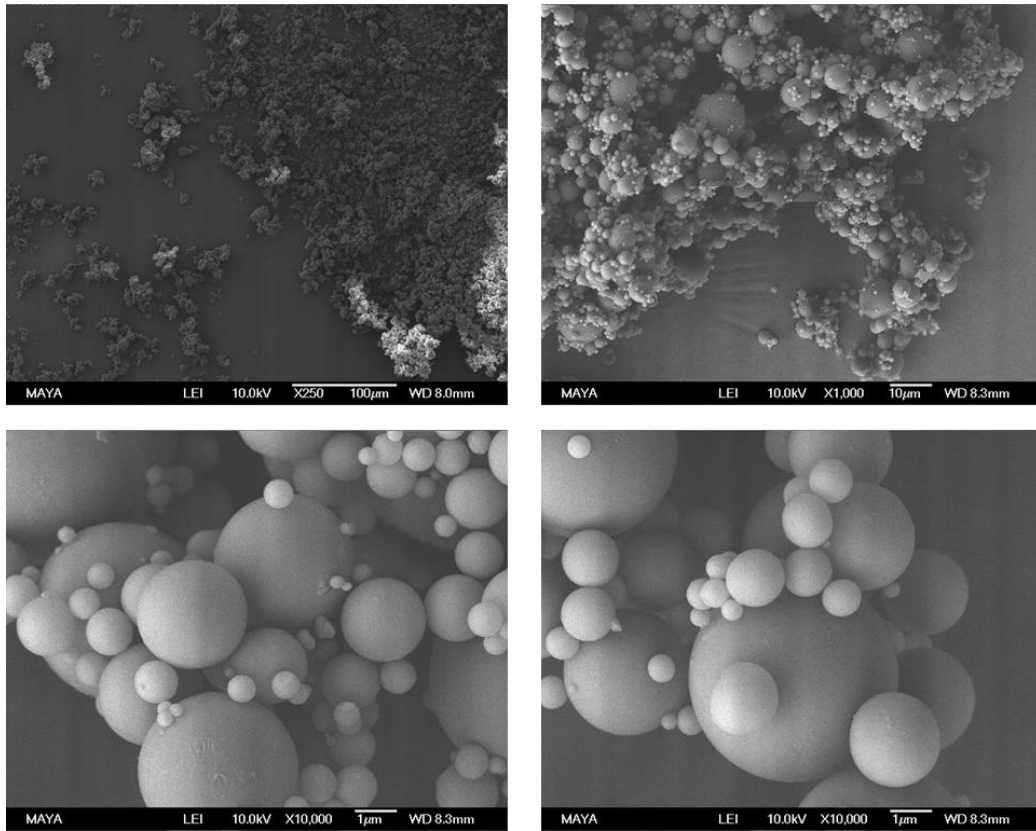


Figure 27: SEM images of Trehalose_02 at magnifications x250, x1000, x10000.

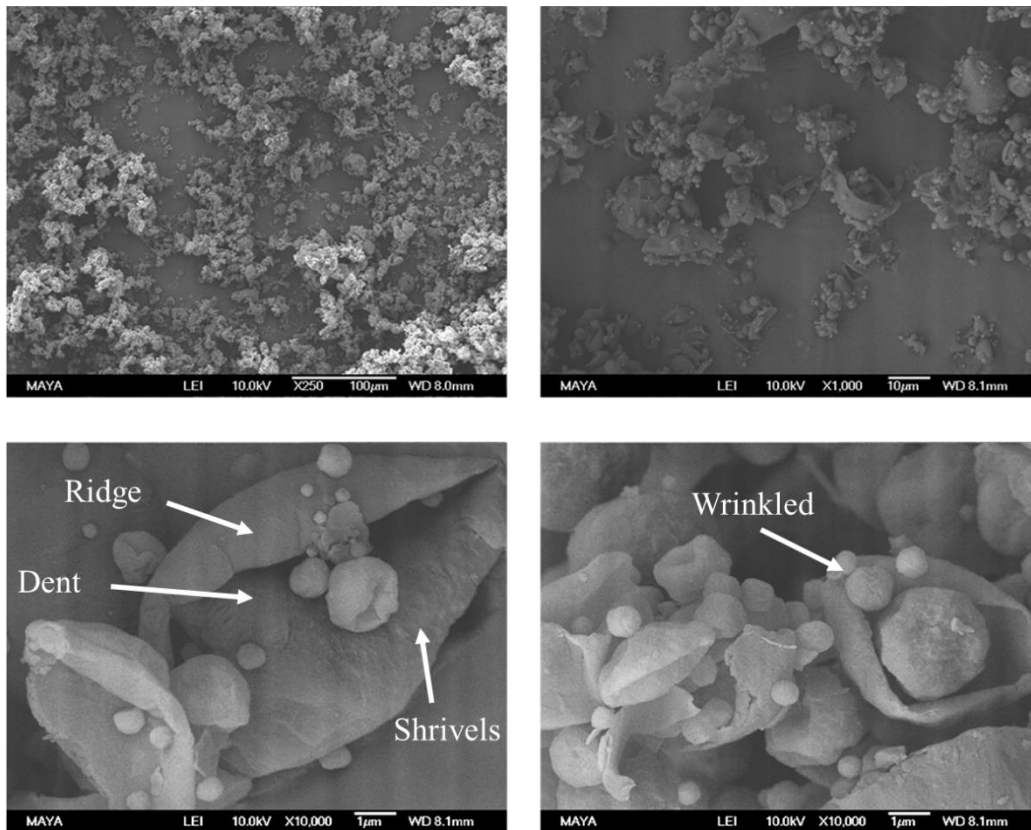


Figure 28: SEM images of Trehalose_Leucine_02 at magnifications x250, x1000, x10000.

3.2.3.2 Spray dried Trehalose dihydrate and Bovine Serum Albumin

The first powder containing BSA (Trehalose_BSA_01) with no leucine, was examined under SEM. Figure 29 presents the particles with magnifications x250, x1000 and x10000. The particles had a raisin-like shape, with dents and thick ridges on the larger particles. The smaller particles were heavily wrinkled with sharper ridges.

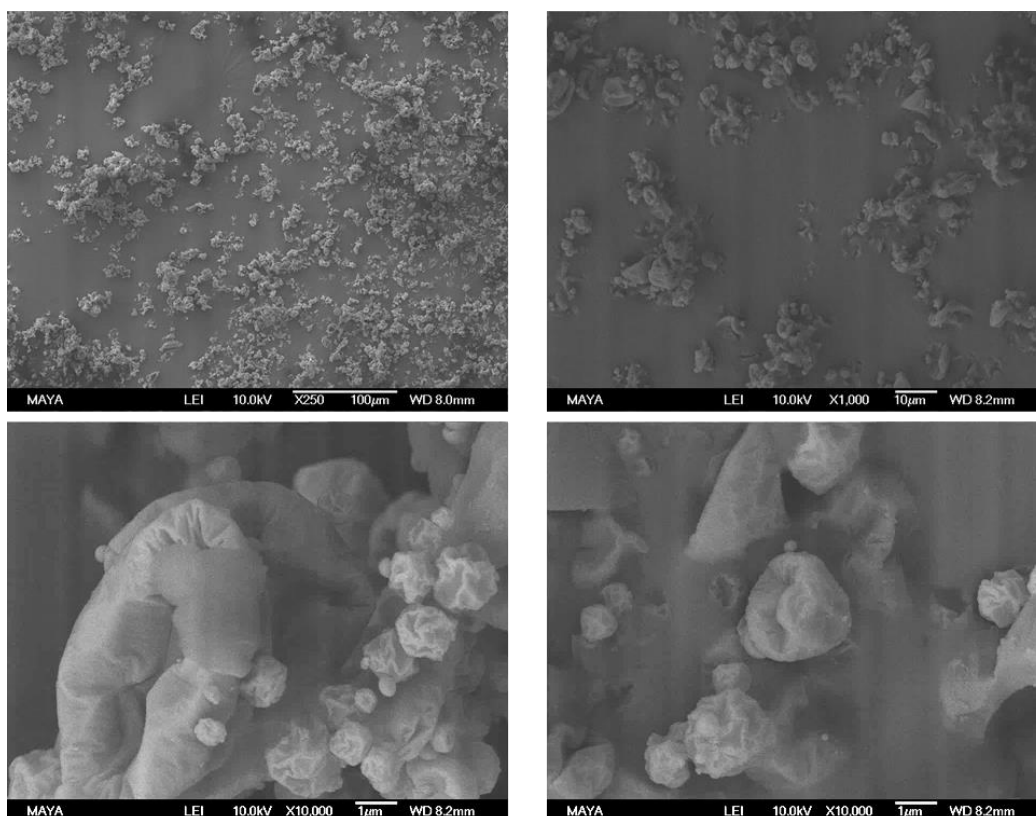


Figure 29: SEM images of Trehalose_BSA_01 at magnifications x250, x1000, x10000.

3.2.3.3 Spray dried Trehalose dihydrate, L-leucine and Bovine Serum Albumin

SEM images of powders containing both BSA and L-leucine, Trehalose_Leucine_BSA_02 and Trehalose_Leucine_BSA_03, at different ratios can be found below (Figure 30, Figure 31). Batch 03 was repeated twice and SEM images were captured of the first repetition batch Trehalose_Leucine_BSA_03_A (Figure 32). Both powders contained corrugated particles with dents, thick ridges and shrivels on their surface. Trehalose_Leucine_BSA_02 was more flake-like compared to Trehalose_Leucine_BSA_03. Both contained smaller wrinkled particles, with batch 03 having sharper ridges compared to batch 02.

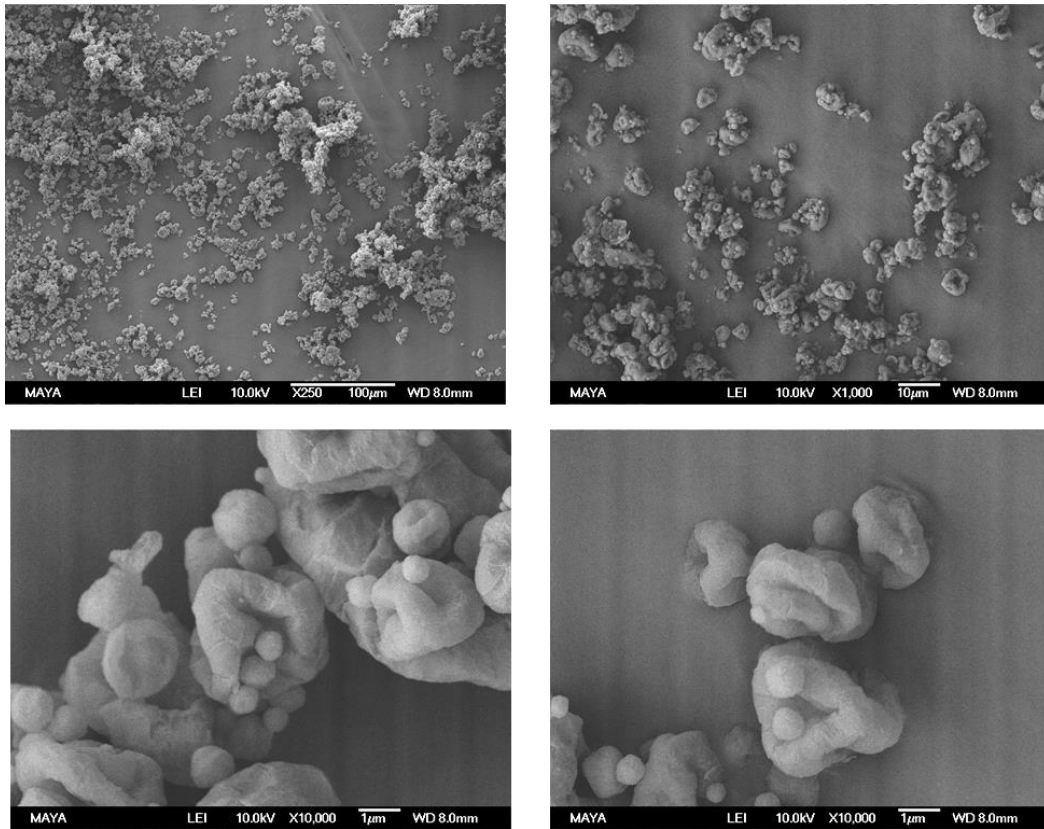


Figure 30: SEM images of Trehalose_Leucine_BSA_02 at magnifications x250, x1000, x10000.

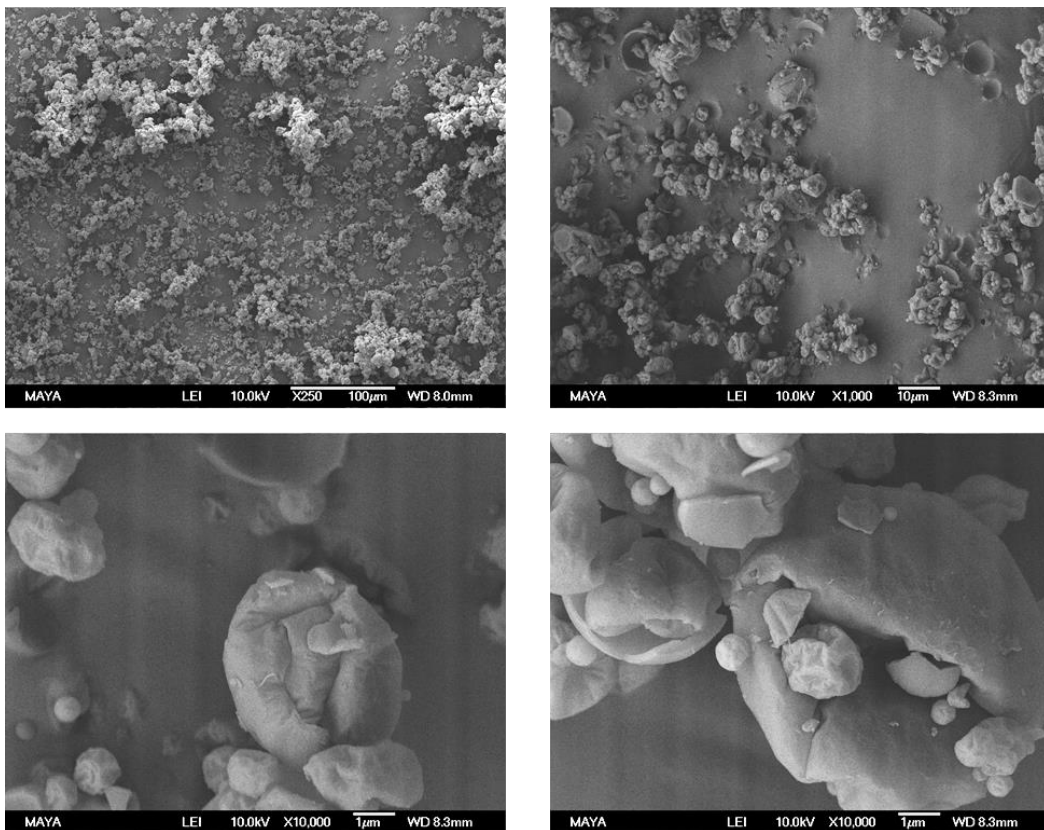


Figure 31: SEM images of Trehalose_Leucine_BSA_03 at magnifications x250, x1000, x10000.

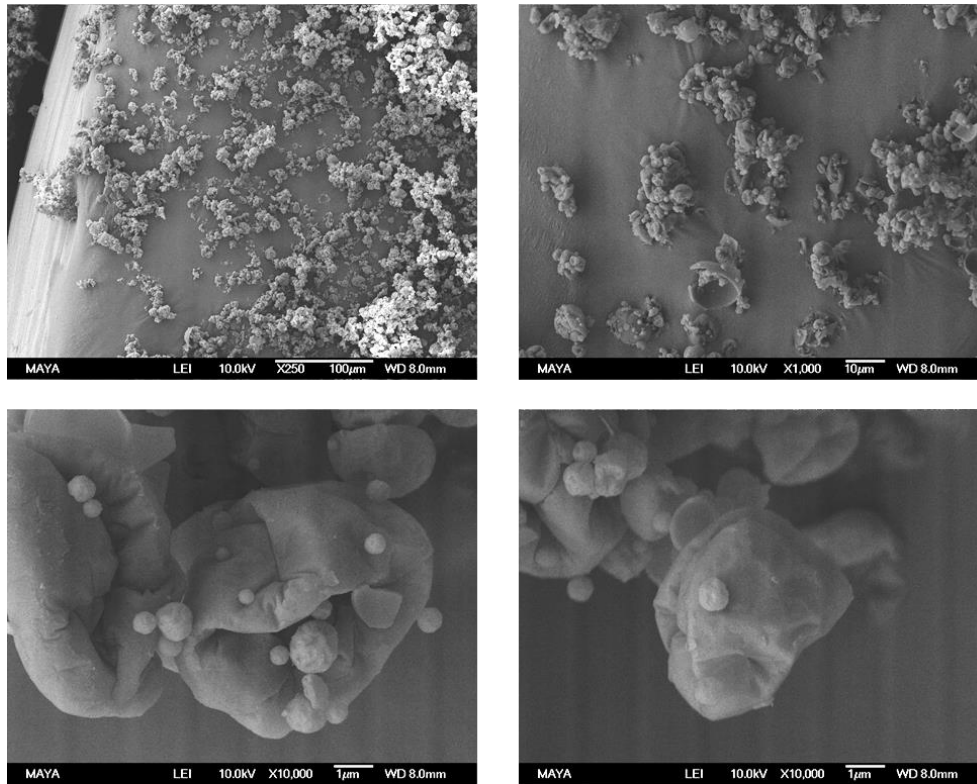


Figure 32: SEM images of Trehalose_Leucine_BSA_03_A at magnifications x250, x1000, x10000.

3.2.4 Bulk density measurements

Bulk density results can be seen in Table 9. Standard deviation was calculated for each sample to determine the variation of the measurements.

Table 9: Bulk density measurements of spray-dried powders for inhalation.

Samples	Weight (gr)	Bulk density (gr/cm ³)	Average Bulk density (gr/cm ³)	STDEV
Trehalose_BSA_01	1.564	0.301	0.306	0.005
	1.590	0.306		
	1.615	0.311		
Trehalose_Leucine_02	1.170	0.225	0.222	0.002
	1.152	0.222		
	1.145	0.220		
Trehalose_Leucine_BSA_02	2.001	0.385	0.377	0.008
	1.968	0.378		
	1.917	0.369		
Trehalose_Leucine_BSA_03	1.511	0.291	0.287	0.006
	1.502	0.289		
	1.457	0.280		
Trehalose_Leucine_BSA_03_A	1.548	0.298	0.291	0.007
	1.515	0.291		
	1.479	0.284		
Trehalose_Leucine_BSA_03_B	1.480	0.285	0.287	0.002
	1.498	0.288		
	1.493	0.287		
Trehalose_02	1.253	0.241	0.253	0.013
	1.303	0.251		
	1.385	0.266		

In the graph in Figure 33, a comparison can be made between the different spray-dried powders and their bulk densities.

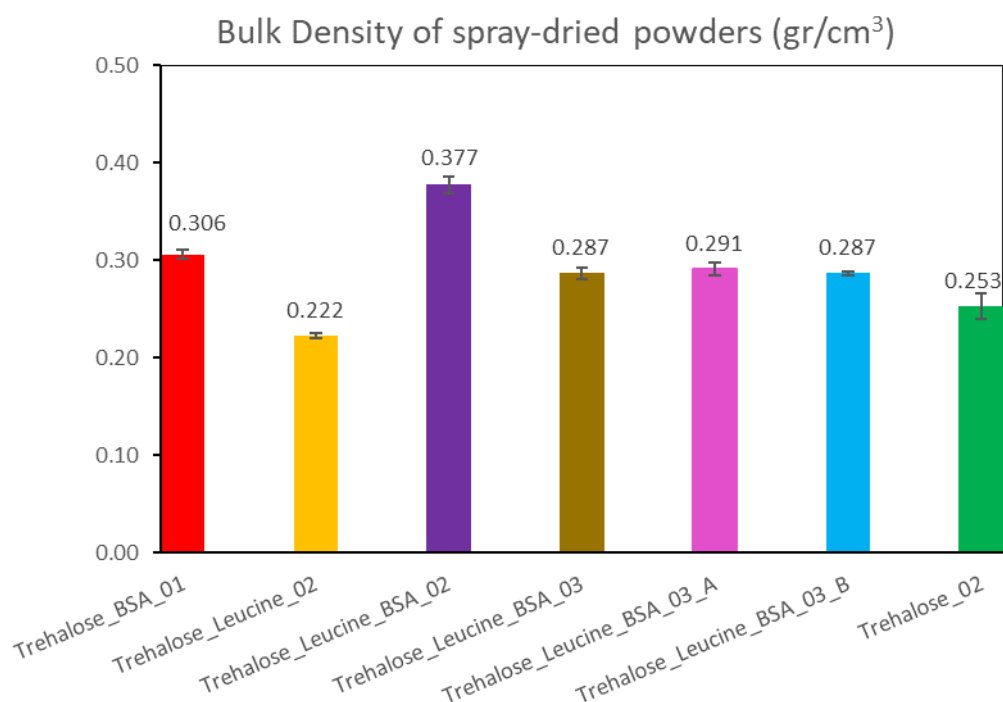


Figure 33: Graph depicting the bulk density values with the standard deviation of the spray-dried powders.

3.2.5 Next Generation Impactor (NGI)

The aerodynamic characteristics of the powders were determined with the Next Generation Impactor. Four powders were used with the RS01 inhaler (high resistance) and by performing the fluorescamine assay the BSA/L-leucine quantity was determined on each NGI stage. A summary of the results can be found in the Appendix section 7.4 and an example image of the pattern of powder on the NGI stages can be found in Figure 34.

During the calculations in the Fluorescamine assay, it was assumed that the fluorescence signals from BSA and L-leucine were of equal strength.

Also, it should be noted that there were variations between blank measurements of fluorescamine in water. This could be due to fluorescamine's sensitivity to light during the procedure. Despite adequate covering, the experiment should be conducted in a dark room with minimal light exposure. Additionally, the time between dissolving fluorescamine in DMSO and conducting the measurement in the fluorescence plate reader should be minimized to ensure accuracy. To avoid inconsistencies, the blank measurements and the standard curve were prepared separately for each powder and each individual NGI (Appendix 7.4.1).



Figure 34: NGI stages after using Trehalose_Leucine_BSA_02 with RS01 inhaler.

The Fine particle dose (FPD), Fine particle fraction (FPF), Mass median aerodynamic diameter (MMAD) and the delivered dose were calculated for the four powders Trehalose_BSA_01, Trehalose_Leucine_BSA_02, Trehalose_Leucine_BSA_03 and Trehalose_Leucine_02. The results are presented in the figures below (Figures 35, 36, 37, 38).

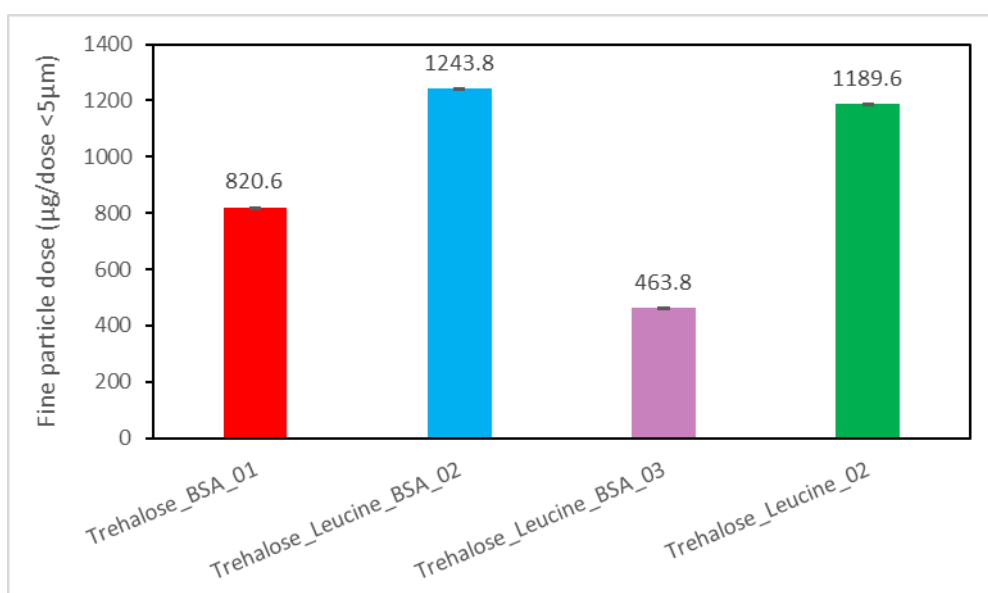


Figure 35: Fine particle dose (µg/dose < 5 µm) of four powders in NGI analysis.

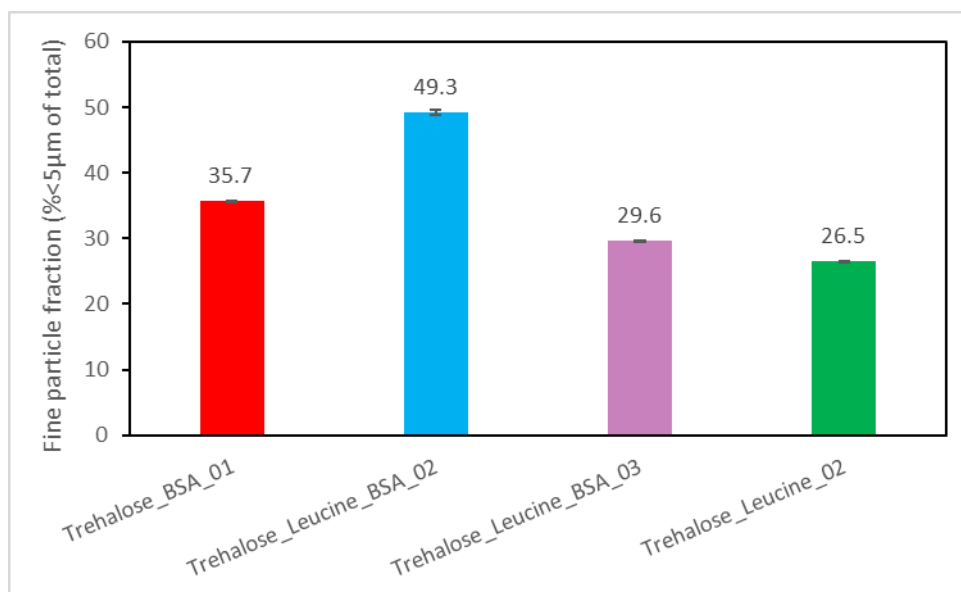


Figure 36: Fine particle fraction (% < 5 μm of total) of four powders in NGI analysis.

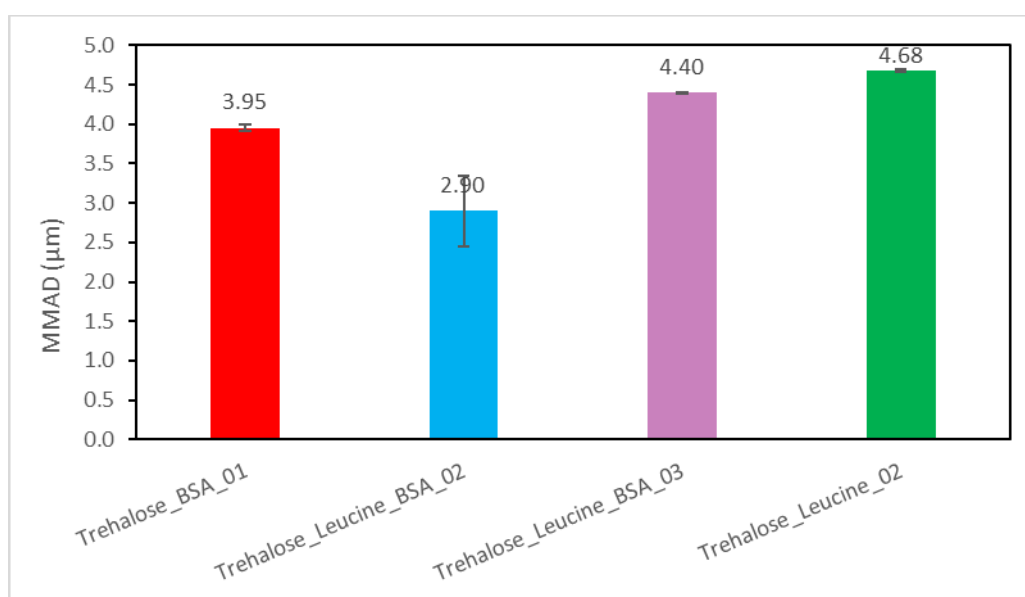


Figure 37: Mass median aerodynamic diameter (μm) of four powders in NGI analysis.

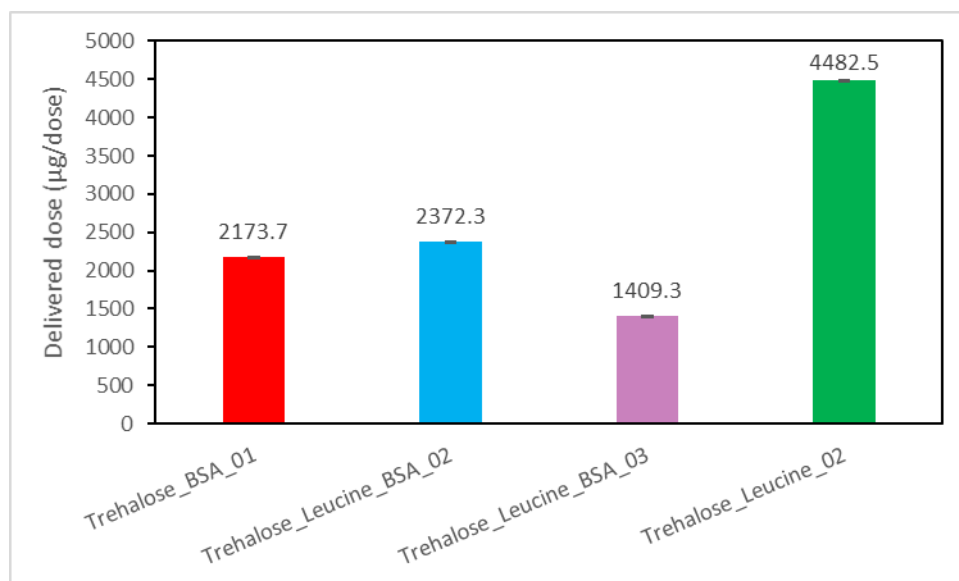


Figure 38: Delivered dose (µg/dose) of four powders in NGI analysis.

Finally, the aerodynamic size distribution for the four powders can be seen below (Figure 39).

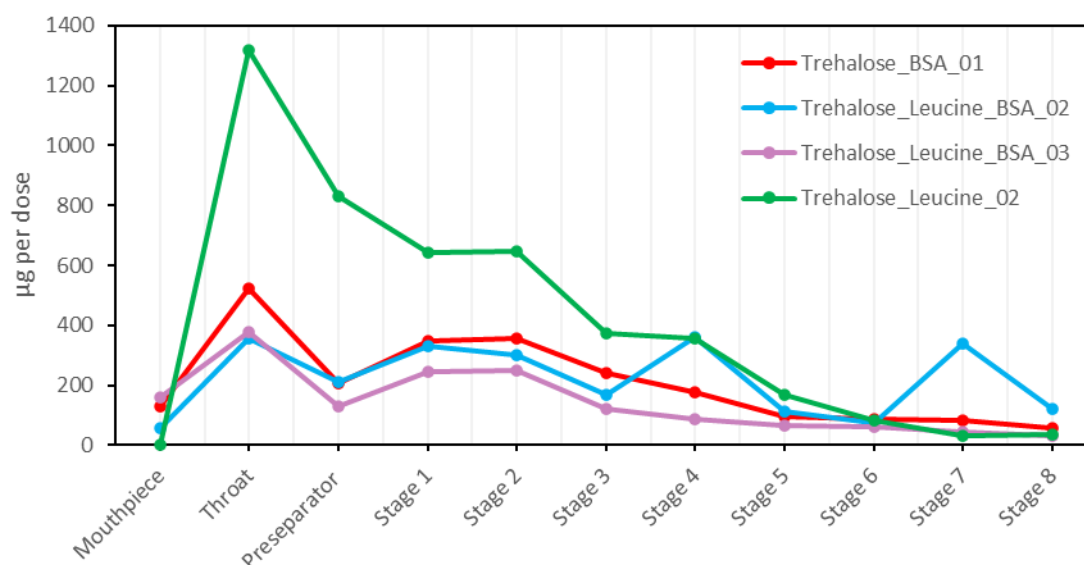


Figure 39: Aerodynamic size distribution (µg/dose) of four powders in NGI analysis.

3.2.6 Aerodynamic Particle Sizing (APS)

Aerodynamic particle sizing was an analysis used to examine the aerodynamic particle size of the inhalation powders. Figure 40 depicts the median aerodynamic diameter of powders containing BSA, L-leucine and Trehalose_02, which was spray dried under the same conditions as the rest.

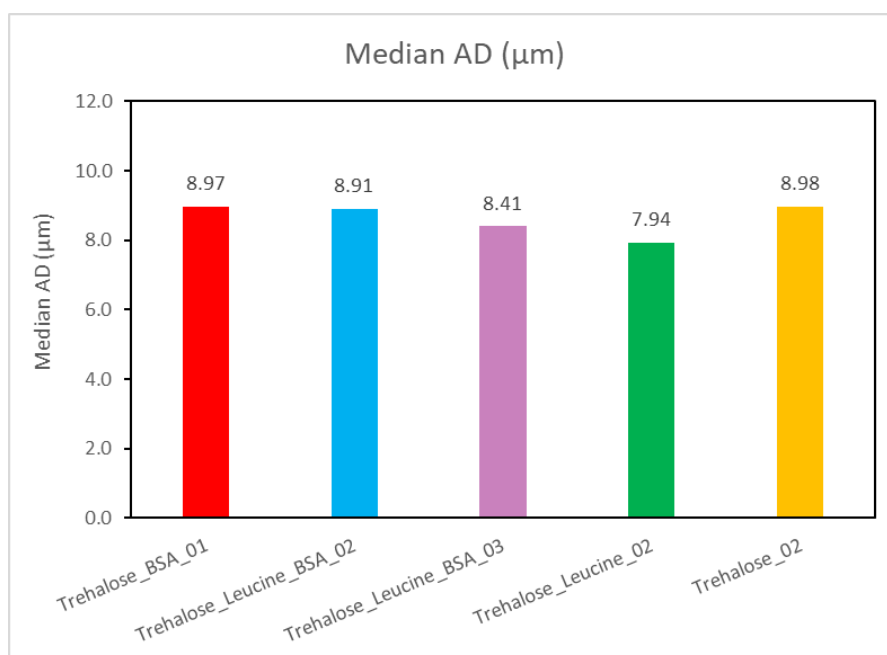


Figure 40: Median aerodynamic diameter of powders in APS analysis with 62 l/min flow rate.

Below is the normalized concentration distribution versus aerodynamic size of the particles of powder Trehalose_BSA_01. Samples were taken every 30 seconds, with a total of 6 samples collected (Figure 41). The normalized concentration is symbolized with $dM/d\log D_p$, where dM is the particle concentration and $d\log D_p$ is the midpoint particle diameter. The distribution was consistent across all samples analyzed in the APS system (Appendix 7.5).

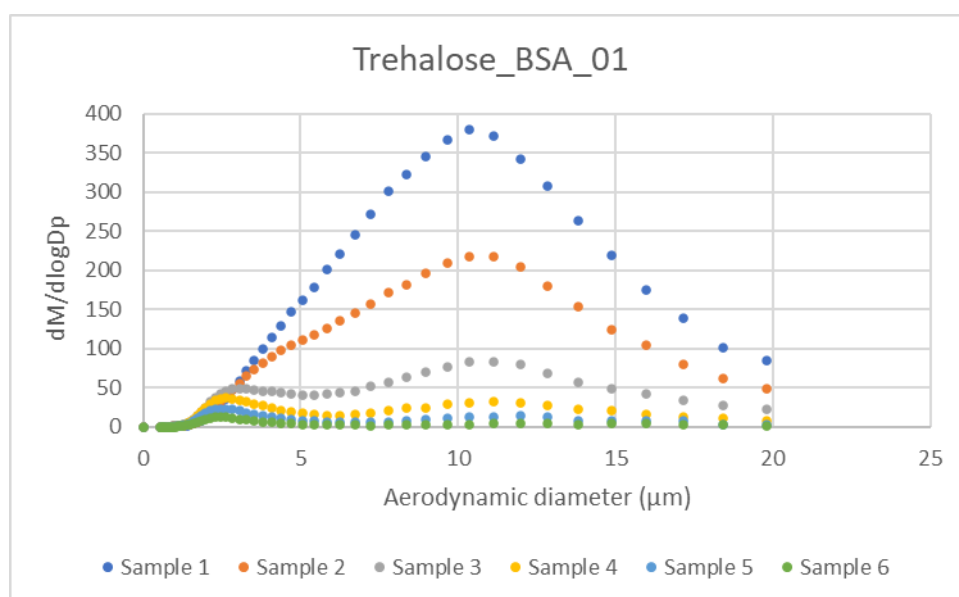


Figure 41: Normalized concentration vs aerodynamic particle size of powder Trehalose_BSA_01 sprayed with 62 l/min flow rate.

The total concentration of each powder over time in the APS system is seen in Figure 42.

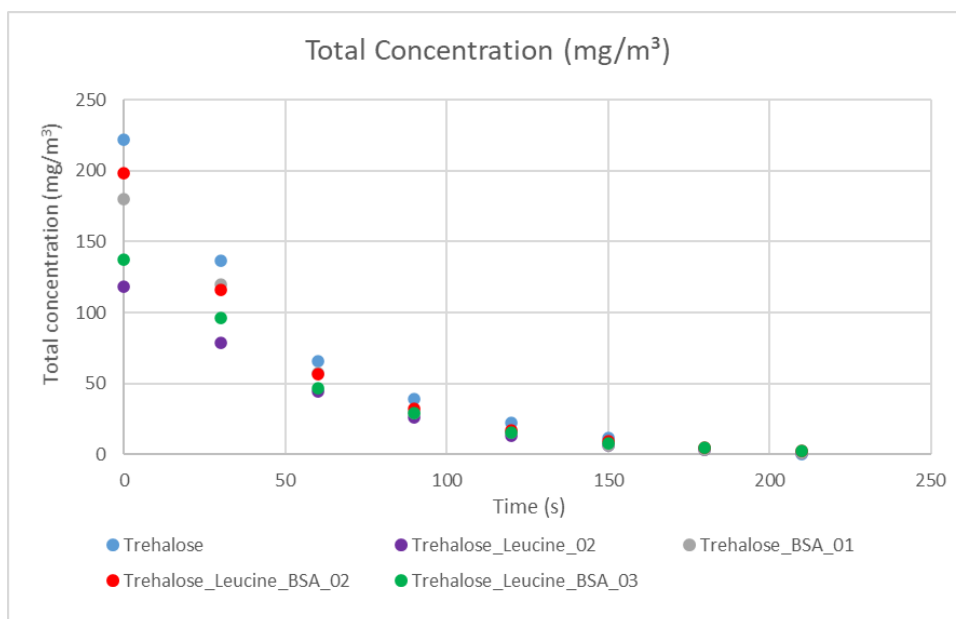


Figure 42: Total concentration of powders over time in the APS system for flow rate 62 l/min.

For comparison reasons, the samples were sprayed with a flow rate of 120 l/min. The median aerodynamic size of all powders was similar (Figure 43), as well as the normalized concentration distribution versus the aerodynamic size of the particles (Figure 44).

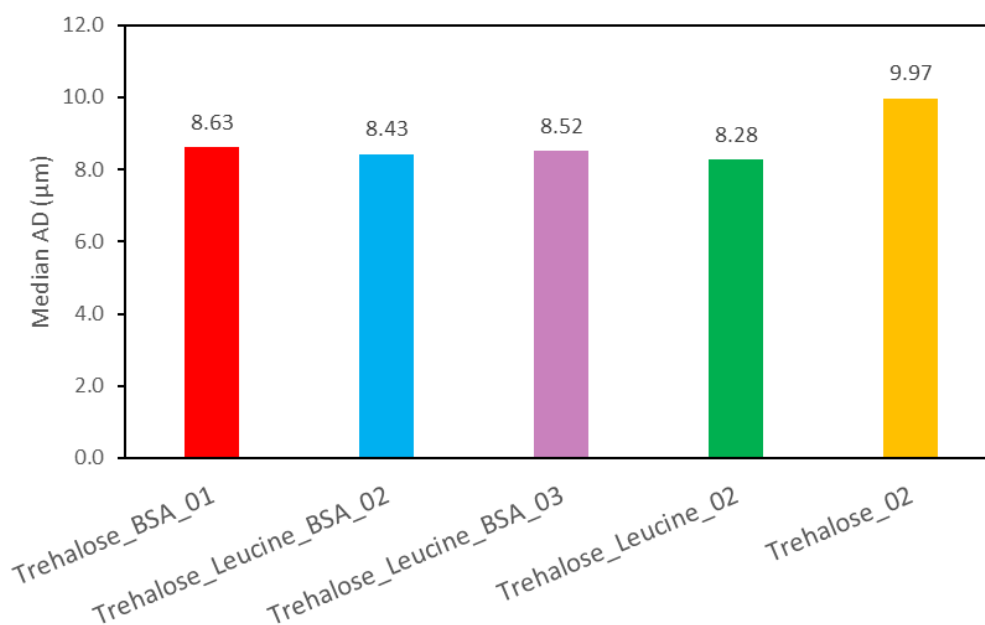


Figure 43: Median aerodynamic diameter of powders in APS analysis with 120 l/min flow rate.

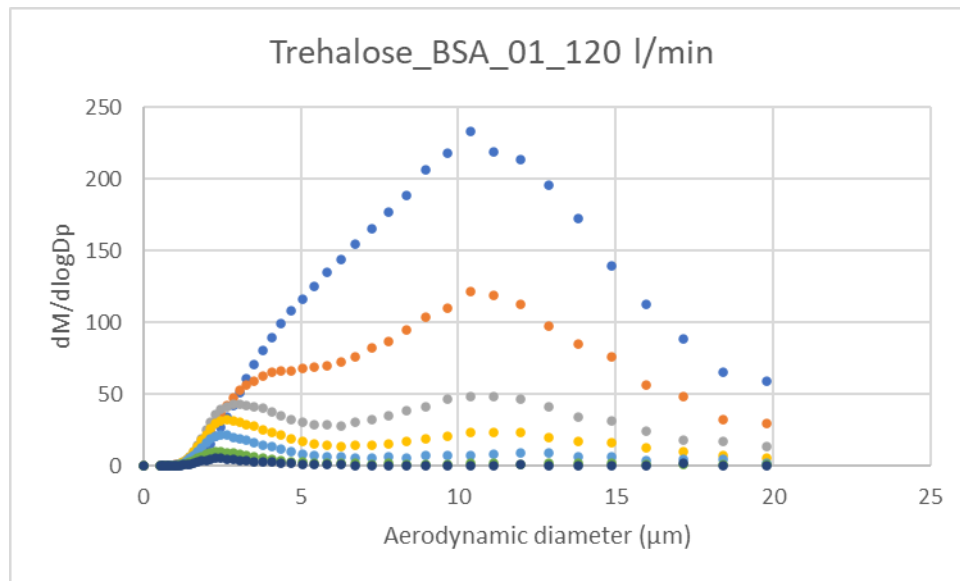


Figure 44: Normalized concentration vs aerodynamic particle size of powder Trehalose_BSA_01, sprayed with 120 l/min flow rate.

4 DISCUSSION

4.1 Pre-study

Lactose and trehalose formulations were spray dried under different conditions (Tables 3 and 4) to determine which were ideal to produce powders for inhalation. These were examined with light microscopy and laser diffraction.

All lactose powders (Lactose_01, 02, 03) had similar shape and size. From light microscopy they have a spherical shape, with many vacuoles (Figure 9). Lactose_01 had slightly larger size compared to Lactose_02 and Lactose_03, but all were below 5 μm and had weighted residuals below 1%.

Similarly, Trehalose_01 and Trehalose_02 powders contained spherical particles with a smaller number of round vacuoles (Figure 10), and an average particle size of 4.4 μm with weighted residuals below 1% (Mie theory).

It should be noted that when using Fraunhofer theory for size calculation in laser diffraction, the particle sizes for all powders appeared smaller compared to those calculated using Mie theory. Additionally, the Fraunhofer theory results exhibited lower weighted residuals, indicating a better fit.

Due to the similarities of the resulting powders, all three spray drying conditions seemed viable and were used to produce three trehalose-leucine powders in the main study.

4.2 Main study

4.2.1 Comparison of Trehalose Leucine powders

In the main study, trehalose was initially spray dried with L-leucine (27%) under three different settings (Table 5). Light microscopy images revealed that the powders Trehalose_Leucine_01, Trehalose_Leucine_02 and Trehalose_Leucine_03 contained spherical-like particles with irregular, cup-like shapes and vacuoles that deviated from sphericity (Figure 15). The irregular shaped vacuoles could be due to the crystallization of leucine. Furthermore, Trehalose_Leucine_03 particles deviated more from the spherical shape and had a higher number of vacuoles. This could be attributed to the combination of lower inlet and higher outlet temperatures, which led to more deformed and wrinkled particles (Ameri & Maa, 2007; Lechanteur & Evrard, 2020). Notably, all batches contained smaller spherical particles, many of which also had vacuoles.

Regarding the size distribution of the three batches, the average size was between 5-6 μm for Mie theory and between 4-5 μm for Fraunhofer theory, with weighted residuals below 1%. The size distribution curve with Mie theory (Figure 19) shows a noticeable smaller peak in the size ranges below 1 μm , which suggests the presence of smaller particles. This is also confirmed by light microscopy and indicates that the powders have a high degree of polydispersity. The size distribution curve with Fraunhofer theory (Figure 20) lacks the smaller peak, although there is a noticeable tilt to the curve for smaller particle sizes. This indicates a better fit when using Fraunhofer theory, which will therefore be used for the complex powders. On the other hand, Mie theory could have a degree of inaccuracy because the index of the complex powders is unknown.

4.2.2 Comparison of powders containing BSA

Following the main study, three powders containing BSA were produced using the same spray drying settings as Trehalose_02 and Trehalose_Leucine_02 (Tin = 175 °C, Tout = 73 °C, aspirator = 80%, pump = 20%). Based on the results in the pre-study, all three conditions appeared viable. However, based on the literature mentioned in the introduction, the second condition with a higher inlet temperature ensures lower moisture content in the final powder, which is crucial for the powder's stability (Ameri & Maa, 2007). The increased pump speed did not affect the particle size, as the sizes were similar for all trehalose-leucine samples.

The powders produced were Trehalose_BSA_01, Trehalose_Leucine_BSA_02, Trehalose_Leucine_BSA_03, with two replicates for the third powder (Trehalose_Leucine_BSA_03_A and Trehalose_Leucine_BSA_03_B). All powders contained 10% w/v BSA, and additionally Trehalose_Leucine_BSA_02 contained 27% w/v L-leucine and Trehalose_Leucine_BSA_03 had 10% w/v L-leucine.

Microscopy images of Trehalose_BSA_01 revealed wrinkled, raisin-like particles with a small number of circular vacuoles (Figure 16). Trehalose_Leucine_BSA_02 (Figure 17) exhibited more spherical-like wrinkled particles compared to Trehalose_BSA_01, with irregularly shaped vacuoles. The difference could be due to the crystalline structures of L-leucine. On the other hand, Trehalose_Leucine_BSA_03 (Figure 18) appeared more cup-like than Trehalose_Leucine_BSA_02, with round vacuoles. This could be related to the lower amount of L-leucine (10% w/v) in Trehalose_Leucine_BSA_03 compared to the second batch, which contains 27% w/v leucine. Additionally, all samples contained noticeably smaller wrinkled spherical particles.

For all three batches the average particle size was between 4.5 and 5 µm with weighted residuals below 1% (Fraunhofer theory), with similar size distributions.

4.2.3 Comparison of morphology (SEM images)

Six powders were analyzed using SEM imaging to examine their shape and surface morphology. All the powders were produced with the same spray drying conditions (settings 02).

- Trehalose_02 (Figure 27): The particles were spherical with smooth surfaces and sizes ranging from 1 µm to below 10 µm.
- Trehalose_Leucine_02 (Figure 28): The addition of L-leucine resulted in particles collapsing and becoming corrugated. They had a coffee bean-like shape, with dents and thick ridges. A fine graininess on the surface (shrivels) is likely due to the crystallization of L-leucine, making the surface more flake-like. Smaller particles were wrinkled (smaller dents and thinner ridges).
- Trehalose_BSA_01 (Figure 29): Containing only trehalose and BSA, the larger particles were corrugated with raisin-like shape. They had dents and thick ridges, but with smoother surfaces compared to powders with L-leucine. Smaller particles had sharper ridges and smooth wrinkles, appearing as if they have collapsed inward.
- Trehalose_Leucine_BSA_02 (Figure 30): Powders containing both protein and amino acid resembled both the previous two samples due to both BSA and L-leucine determining the shape. Trehalose_Leucine_BSA_02 had particles with dents, ridges

and shrivels on the ridges. The surface was more flake-like due to the higher amount of crystallized L-leucine.

- Trehalose_Leucine_BSA_03 (Figure 31): This batch was more coffee bean-shaped than batch 02, with larger dents. The surface graininess was less pronounced due to the lower amount of crystallized L-leucine.

For all powders, the sizes ranged from 1 to 10 μm . The larger sized particles were heavily corrugated with dents and thick ridges, whereas the smaller particles were heavily wrinkled. These results suggest that the presence of L-leucine leads to the formation of shrivels on the surface of the particles ridges, due its crystalline structure. At the same time larger particles, possibly due to slower drying times, developed larger and deeper grooves, resulting in raisin or coffee bean-like shapes. Whereas smaller particles are coated with protein at an early stage and due to the amorphous nature of BSA, these particles do not dry in a specific direction, but rather maintain their shape and become wrinkled.

4.2.4 Comparison of bulk density

The bulk density of the samples measured were below 0.40 g/cm^3 (Figure 33), with standard deviations below 0.01 g/cm^3 . Trehalose_Leucine_BSA_02 (27% leucine) had the highest bulk density at $0.377 \pm 0.008 \text{ g/cm}^3$, followed by Trehalose_BSA_01 and Trehalose_Leucine_BSA_03 (10% leucine). The last two powders could have lower bulk densities compared to Trehalose_Leucine_BSA_02, due to their open coffee bean-like shape.

The lowest bulk density was recorded for sample Trehalose_Leucine_02 (27% leucine) at $0.222 \pm 0.002 \text{ g/cm}^3$, which similarly could be due to the open coffee bean-like particle shapes.

As mentioned in section 2.3.4, powders ideal for deposition in the deeper part of the lungs have a bulk density smaller than 0.4 g/cm^3 (Chaurasiya & Zhao, 2020).

4.2.5 Comparison of NGI and APS

4.2.5.1 NGI analysis

From the NGI analysis of the powders containing BSA, Trehalose_Leucine_BSA_02 had the highest Fine Particle Dose (FPD) at $1243.8 \mu\text{g/dose}$, followed by Trehalose_BSA_01 and Trehalose_Leucine_BSA_03 (Figure 35). This suggests that Trehalose_Leucine_BSA_02, which contained 27% leucine and 10% BSA, had the largest amount of particles within the ideal size range (1-5 μm) for reaching the lungs.

Regarding the Fine Particle Fraction (FPF) values (Figure 36), sample Trehalose_Leucine_BSA_02 achieved the highest FPF at 49.3%, followed by Trehalose_BSA_01 (35.7%) and Trehalose_Leucine_BSA_03 (29.6%). Higher FPF values indicate more efficient drug delivery, as they represent the percentage of particles with the optimal size range to deposit in the lungs and have a therapeutic effect.

The Mass Median Aerodynamic Diameter (MMAD) for all samples was below 5 μm (Figure 37), which is necessary for deep lung deposition. Trehalose_Leucine_BSA_02 had the smallest MMAD at 2.90 μm , though with a high %RSD (Relative Standard Deviation). The other samples were in the range of 4-4.7 μm , with Trehalose_Leucine_02 exhibiting the largest

MMAD overall. Amongst samples containing BSA, Trehalose_Leucine_BSA_03 had the largest aerodynamic diameter.

It should be noted that SEM images showed particles in the 10 μm range, as well as smaller sized particles below 5 μm . This is consistent with Figure 39, where the distribution of the powders in the NGI stages was broad, indicating that the powders are polydisperse.

The highest delivered dose of BSA was achieved with the powder Trehalose_Leucine_BSA_02 (27% leucine), followed by Trehalose_BSA_01 and Trehalose_Leucine_BSA_03. In these calculations, the leucine amount was not included in the delivered dose of BSA. Since Trehalose_Leucine_02 contains only leucine (27%), the delivered dose was calculated specifically for leucine.

All NGI parameters taken together indicate that Trehalose_Leucine_BSA_02 is the most efficient powder for delivery of BSA to the deeper parts of the lungs, compared to Trehalose_BSA_01 and Trehalose_Leucine_BSA_03. Despite all three powders having an ideal aerodynamic size, the higher percentage of L-leucine (27% w/v) in Trehalose_Leucine_BSA_02 appears to enhance aerosol performance. This is further supported by literature, where studies have shown that L-leucine enhances flowability, dispersibility and FPF of the powder (Chang & Chan, 2022; Pinto et al., 2021). However, this was not observed with Trehalose_Leucine_BSA_03, which had a lower FPF compared to Trehalose_BSA_01. This could be due to the differences in the surface chemistry of the particles. Despite having similar morphological features and aerodynamic size, the surface interactions likely influenced the aerosolization performance.

4.2.5.2 APS analysis

The aerodynamic size of the powders was determined using APS analysis. The powders analyzed included the same samples as in the NGI analysis, as well as Trehalose_02. For all powders examined, the aerodynamic size ranged from 7.9 to 9.0 μm (Figure 40), which is above the ideal size for inhalation powders (less than 5 μm). The small differences between samples in this analysis is quite surprising.

The figure depicting the normalized concentration distribution versus the aerodynamic size of the particles was similar for all samples (Figure 41). There is a noticeable peak around 10 μm representing the size of most particles in the powder. For samples taken after 60 seconds (samples 3-6), an additional peak appears in the size ranges below 5 μm (3-4 μm), indicating the presence of smaller particles in the powder within the ideal inhalation size.

Similar results were found when the samples were sprayed with a flow rate of 120 l/min (Figures 43 and 44).

The unexpectedly large aerodynamic size calculated with APS could be attributed to moisture uptake by the powders due to high relative humidity in the laboratory, causing particle aggregation.

5 CONCLUSIONS

The project aimed to produce spray dried powders suitable for inhalation and to characterize their aerosolization performance. Using BSA as the model API, the necessary excipients were used to ensure the powders had ideal characteristics for inhalation, such as good flowability, dispersibility and an aerodynamic diameter below 5 μm .

The powders produced in the main study had a yield above 75%, indicating a highly successful spray drying process. Light microscopy images revealed wrinkled, raisin-like particles, with vacuoles. Powders containing a higher amount of L-leucine (27% w/v) had irregularly shaped vacuoles, compared to the rounder vacuoles in other samples without L-leucine or with lower concentration (10% w/v). This difference is likely due to the higher amount of crystalline structure present, which is formed by L-leucine.

The Fraunhofer theory was selected to compare particle size due to better fit, compared to Mie theory. The pure powders produced in the pre-study (lactose and trehalose dihydrate) were between 3.5 and 4 μm in size, smaller than the more complex powders containing BSA and L-leucine, which ranged from 4 to 5 μm . Notably, the powders had a high degree of polydispersity, indicated by the light microscopy images and the size distribution.

Comparing SEM images of the samples, powders containing pure lactose or trehalose dehydrate were spherical with smooth surfaces, as expected. In contrast, powders containing BSA and L-leucine, deviated from the spherical shape and smooth surface. The addition of BSA resulted in highly corrugated particles with thick ridges and dents on the surface. The addition of L-leucine led to the formation of particles with more flake-like surfaces due to the crystallization of L-leucine on the surface.

All powders had bulk density below 0.4 g/cm^3 , which is considered optimal for inhalation powders. Lower bulk densities amongst BSA-containing powders were observed for powders with open coffee bean-like shapes. These powders contained BSA and low concentrations of L-leucine.

The aerodynamic size of the particles was calculated using NGI and APS analysis. The results from both methods do not correlate due to insufficient deaggregation of the particles in the APS system, resulting in an inaccurately high MMAD of 8 μm .

NGI results showed that the samples had an MMAD below 5 μm , which is ideal for inhalation powders. Trehalose_Leucine_BSA_02 had the smallest MMAD and the highest FPF (49.3%) which suggests that the addition of 27% w/v L-leucine gives the best powder dispersibility.

Overall, the produced powders were suitable for inhalation as they had an ideal MMAD, a corrugated shape that increases aerosol performance and a good shelf life when stored in desiccators below 10% RH. The spray drying method used was time efficient, cost effective, reproducible, and robust.

Future studies should examine the effect of protein concentration on the size, shape and aerosol characteristics of the particles. Additionally, the effect of L-leucine concentration should be studied further, to determine the concentration range at which it enhances aerosolization performance of the powders. Regarding the analysis methods, further optimization is also needed for protein quantification, due to the unstable nature of fluorescamine. Similarly, the

APS analysis should be repeated to confirm the aerodynamic diameter of the particles, while ensuring there is no risk of moisture uptake during the experiment.

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7 APPENDIX

7.1 Conversion tables for spray drying parameters

- Peristaltic pump

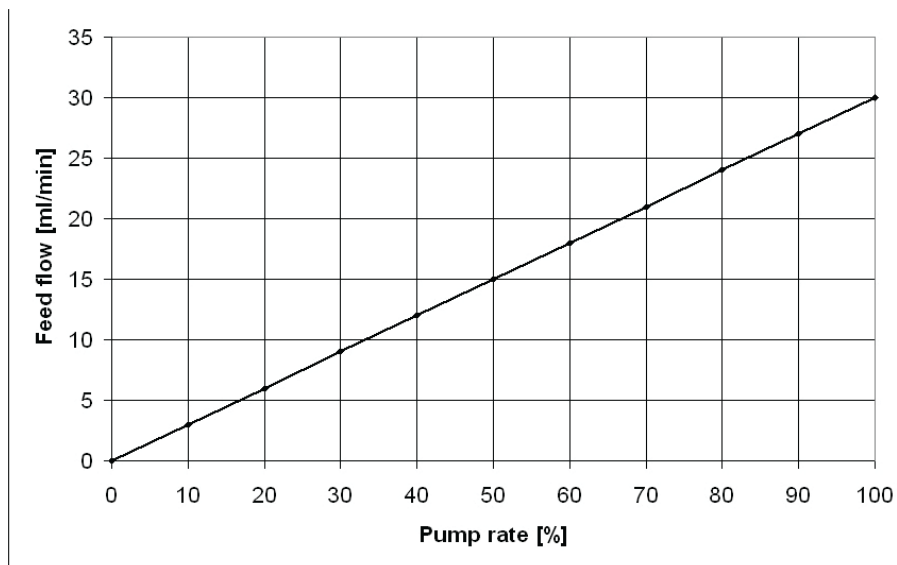


Figure A: Pump settings (%) vs actual feed flow (ml/min) (B-290 Mini Spray Dryer Operation Manual, 2018)

- Aspirator

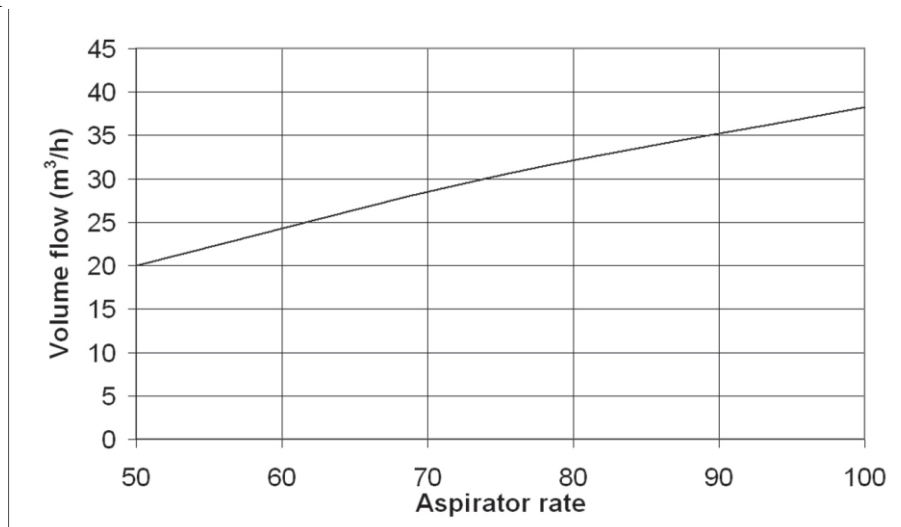


Figure B: Aspirator settings (%) vs actual volumetric flow (m³/h) (B-290 Mini Spray Dryer Operation Manual, 2018).

- Spray gas flow (rotameter)

Height (mm)	Liters/hour	Pressure drop (bar)	Actual volume flow (at standard temperature and pressure) in liters/hour
5	84		
10	138		
15	192		
20	246	0.15	283
25	301	0.18	355
30	357	0.23	439
35	414	0.3	538
40	473	0.41	667
45	536	0.55	831
50	601	0.75	1052
55	670	1.05	1374
60	742	1.35	1744
65	819	1.8	2293

Figure C: Indicated height (mm) and volume throughput (L/h) (B-290 Mini Spray Dryer Operation Manual, 2018).

7.2 Production masters of spray-dried powders

7.2.1 Trehalose_BSA_01

Production master for production of "Spray dried powders for inhalation";
Division of Food Technology

master-f version
1(6)

Production master		M-J-version nr
Spray dried powder for Inhalation		
Author	Trehalose-BSA (9:1)	
Ell. M. Kousoudi	12/03/24	

Special Information

Production of spray dried powder for inhalation with the purpose of using it in a dry powder inhaler (DPI). The spray dryer used is the BUCHI 290 (B-290).

Formulation Ingredients	Supplier	Lot/Batch
Trehalose Dihydrate	Sigma-Aldrich	V0330236 4 332
BSA (Bovine Serum Albumin)	Sigma-Aldrich	
Bovine Serum Albumin (BSA)	Sigma-Aldrich	0000287318

Production master for production of "Spray dried powders for inhalation";

master-f version

Batch number 01 Date of production 12/03/24

3(6)

Product name: Trehalose-BSA powder	Batch number 01
Date of production 12/03/24	
Signature	

Weighing of ingredients	Signature	Comments
Ingredient: Trehalose dihydrate		
Weigh the container (G270) and zero the balance.		
Weigh <u>4.5g</u> amount of Trehalose. Actual m = <u>4.5004g</u>		
Ingredient: BSA		
Weigh the container (G270) and zero the balance.		
Weigh <u>4.5g</u> amount of BSA. Actual m = <u>4.556g</u>		
Ingredient:		
Weigh the container and zero the balance.		
Weigh amount of . Actual m = .		

Production master for production of "Spray dried powders for inhalation";

master-f version

Batch number 01 Date of production 12/03/24

5(6)

Spray drying steps	Signature	Comments
Set up spray dryer as instructed in the manual and start with distilled water.		
Set spray drying conditions referred in the start of the production master:		
T _{in} = 70 °C		
Feed rate = 20%		
Aspirator rate = 30%		
Spray gas flow = 45 mm		
Nozzle cleaner = 6		
When conditions are steady, start spray drying the feed solution (V = 100 mL).		
Monitor the conditions and take note of inlet and outlet temperature:		
T _{in}	T _{out}	
172 °C	71 °C	
171 °C	75 °C	
171 °C	78 °C	
174 °C	71 °C	
175 °C	72 °C	
173 °C	77 °C	
172 °C	78 °C	
		End: 12:57

Production master for production of "Spray dried powders for inhalation";
Division of Food Technology

master-f version
2(6)

Spray drying conditions	Comments
Inlet Temperature (T _{in})	175 °C
Outlet Temperature (T _{out})	76 °C
Feed rate	20%
Aspirator rate	30%
Spray gas flow	45 mm
Nozzle cleaner	6
Room temperature	21.5 °C
Room relative humidity (%RH)	20.5%

Production master for production of "Spray dried powders for inhalation";

master-f version

Batch number 01 Date of production 12/03/24

4(6)

General comments:

Preparing feed solution	Signature	Comments
Dissolving ingredients for 7.4 %w/v total solids.		
Add 200 mL deionized water in beaker (volume size 200 mL)		
Add weighed powder.		
Mix until dissolved using magnetic stirring and heating (if necessary)		No heating
Add the rest of weighed ingredients and mix until dissolved.		No heating (Prep. 17:1)
Monitor pH 6.8		
General comments: BSA is not stable at pH 6.0-8.0. Most stable at pH 7.0		

Production master for production of "Spray dried powders for inhalation";

master-f version

Batch number 01 Date of production 12/03/24

6(6)

When feed solution is finished, switch to distilled water and start shut down procedure of spray dryer as described in manual.		
General comments: product produced: m = 5.8136 g		
Yield = 78.64%		

Storing of product	Signature	Comments
Store product in glass container.		
Place in desiccators for one week for equilibration (%RH less than 25-30% and room temperature)		Trehalose-BSA-01
General comments:		

7.2.2 Trehalose_Leucine_BSA_02

Production master for production of "Spray dried powders for inhalation";
Division of Food Technology

master-2 version
1(6)

Production master		Master-2 version nr
Spray dried powder for inhalation		
Author	Date	
Elli, M. Kauszuli	18/03/24	

Special information

Production of spray dried powder for inhalation with the purpose of using it in a dry powder inhaler (DPI). The spray dryer used is the BUCHI 290 (B-290).

Formulation Ingredients	Supplier	Lot/Batch
Trehalose Dihydrate	Sigma-Aldrich	N223083676 338
L-Leucine	Sigma-Aldrich	0000287228
Bovine Serum Albumin (BSA)	Sigma-Aldrich	0000287228

Production master for production of "Spray dried powders for inhalation";
Batch number 02 Date of production 18/03/24

master-2 version
3(6)

Product name: Trehalose-Leucine-BSA powder	Batch number 02
Date of production 18/03/24	
Signature <i>[Signature]</i>	

Weighting of ingredients	Signature	Comments
Ingredient: Trehalose dihydrate Weigh the container 0.280g and zero the balance. Weigh 18.5g amount of Trehalose. Actual m = 18.362g.	<i>[Signature]</i>	
Ingredient: Leucine Weigh the container 0.284g and zero the balance. Weigh 0.335g amount of Leucine. Actual m = 0.332g.	<i>[Signature]</i>	
Ingredient: BSA Weigh the container 0.283g and zero the balance. Weigh 2.35g amount of BSA. Actual m = 2.351g.	<i>[Signature]</i>	

Production master for production of "Spray dried powders for inhalation";
Batch number 02 Date of production 18/03/24

master-2 version
5(6)

Spray drying steps	Signature	Comments														
Set up spray dryer as instructed in the manual and start with distilled water.	<i>[Signature]</i>															
Set spray drying conditions referred in the start of the production master: T _{in} = 175 °C T _{out} = 70 °C Feed rate = 10 l/min Aspirator rate = 80 % Spray gas flow = 45 m/min Nozzle cleaner = 6	<i>[Signature]</i>															
When conditions are steady, start spray drying the feed solution (V = 100 mL).	<i>[Signature]</i>	Start: 14:06														
Monitor the conditions and take note of inlet and outlet temperature:																
<table border="1"> <thead> <tr> <th>T_{in}</th> <th>T_{out}</th> </tr> </thead> <tbody> <tr><td>173 °C</td><td>76 °C</td></tr> <tr><td>174 °C</td><td>77 °C</td></tr> <tr><td>174 °C</td><td>78 °C</td></tr> <tr><td>175 °C</td><td>78 °C</td></tr> <tr><td>178 °C</td><td>78 °C</td></tr> <tr><td>178 °C</td><td>78 °C</td></tr> </tbody> </table>	T _{in}	T _{out}	173 °C	76 °C	174 °C	77 °C	174 °C	78 °C	175 °C	78 °C	178 °C	78 °C	178 °C	78 °C	<i>[Signature]</i>	End: 14:30
T _{in}	T _{out}															
173 °C	76 °C															
174 °C	77 °C															
174 °C	78 °C															
175 °C	78 °C															
178 °C	78 °C															
178 °C	78 °C															

Production master for production of "Spray dried powders for inhalation";
Division of Food Technology

master-2 version
2(6)

Spray drying conditions	Comments
Inlet Temperature (T _{in})	175 °C Average: 174 °C
Outlet Temperature (T _{out})	70 °C Average: 77.5 °C
Feed rate	10 l/min 6 ml/min
Aspirator rate	80 % 38 m/min
Spray gas flow	45 m/min 0.832 m ³ /hr
Nozzle cleaner	6
Room temperature	21.5 °C
Room relative humidity (%RH)	90.5 %

Production master for production of "Spray dried powders for inhalation";
Batch number 02 Date of production 18/03/24

master-2 version
4(6)

General comments:		
Preparing feed solution	Signature	Comments
Dissolving ingredients for 2.1 %w/v total solids. Add 150 mL deionized water in beaker (volume size 500 mL) Add weighted powder. Mix until dissolved using magnetic stirring and heating (if necessary).	<i>[Signature]</i>	
Add the rest of weighted ingredients and mix until dissolved. Monitor pH: 6.43	<i>[Signature]</i>	
General comments:		

Production master for production of "Spray dried powders for inhalation";
Batch number 02 Date of production 18/03/24

master-2 version
6(6)

When feed solution is finished, switch to distilled water and start shut down procedure of spray dryer as described in manual.	Signature	Comments
General comments: positive product, m = 6.4265g. Yield = 85 %	<i>[Signature]</i>	

Storing of product	Signature	Comments
Store product in glass container. Place in desiccators for one week for equilibration (%RH less than 25-30% and room temperature)	<i>[Signature]</i>	Trehalose-BSA-Leucine-02
General comments:		

7.2.3 Trehalose_Leucine_BSA_03

Production master for production of "Spray dried powders for inhalation";
Division of Food Technology

master-3 version
116)

Production master		MS-version nr
Spray dried powder for Inhalation		Trehalose-Leucine-BSA - 8.1.1
Author	Date	
El. Martina Kossuth	2010/2/24	

Special information

Production of spray dried powder for inhalation with the purpose of using it in a dry powder inhaler (DPI). The spray dryer used is the BLCH 290 (B-290).

Formulation Ingredients	Supplier	Lot/Batch
Trehalose dihydrate	Sigma Aldrich	V0230826 338
Leucine	Sigma Aldrich	51890824V 000023272
Bovine Serum Albumin	Sigma Aldrich	0000287218

Production master for production of "Spray dried powders for inhalation";

master-3 version
116)

Batch number 03 Date of production 2010/2/24

Product name: Trehalose-Leucine-BSA 8.1.1	Batch number 03
Date of production 2010/2/24	
Signature	

Weighting of ingredients	Signature	Comments
Ingredient: Trehalose dihydrate		
Weigh the container 2.91g and zero the balance.		
Weigh 16.5g amount of solid. Actual m = 16.459g		
Ingredient: Leucine		
Weigh the container 0.986g and zero the balance.		
Weigh 1.35g amount of leucine. Actual m = 1.323g		
Ingredient: BSA		
Weigh the container 2.312g and zero the balance.		
Weigh 1.81g amount of BSA. Actual m = 1.853g		

Production master for production of "Spray dried powders for inhalation";
Division of Food Technology

master-3 version
116)

Spray drying conditions		Comments
Inlet Temperature (T _i)	85°C	Average: 12.5°C
Outlet Temperature (T _o)	36°C	Average: 20.45°C
Feed rate	20 l	6 ml/min
Aspirator rate	80 l	32 m/hr
Spray gas flow	45 m³/h	0.43 l m³/h
Nozzle cleaner	6	
Room temperature	25°C	
Room relative humidity (%RH)	34%	

Production master for production of "Spray dried powders for inhalation";

master-3 version
116)

Batch number 03 Date of production 2010/2/24

General comments:

Preparing feed solution	Signature	Comments
Dissolving ingredients for 2.4 %w/v total solids.		
Add 200 ml deionized water in beaker (volume size 250 ml)		
Add weighed powder.		
Mix until dissolved using magnetic stirring and heating (if necessary)		
Add the rest of weighed ingredients and mix until dissolved.		
Monitor pH: 6.62		
General comments:		

Production master for production of "Spray dried powders for inhalation";

master-3 version
116)

Batch number 03 Date of production 2010/2/24

Spray drying steps	Signature	Comments
Set up spray dryer as instructed in the manual and start with distilled water.		
Set spray drying conditions referred in the start of the production master:		
T _i = 85°C		
T _o = 36°C		
Feed rate = 20 l		
Aspirator rate = 80 l		
Spray gas flow = 45 m³/h		
Nozzle cleaner = 6		
When conditions are steady, start spray drying the feed solution (V = 100 mL)		
Monitor the conditions and take note of inlet and outlet temperature:		
T _i	T _o	
173°C	78°C	
176°C	73°C	
171°C	78°C	
178°C	80°C	
171°C	80°C	
171°C	72°C	
173°C	78°C	
174°C	78°C	
172°C	77°C	
174°C	78°C	
172°C	78°C	

Production master for production of "Spray dried powders for inhalation";

master-3 version
116)

Batch number 03 Date of production 2010/2/24

When feed solution is finished, switch to distilled water and start shut down procedure of spray dryer as described in manual.
General comments: When switching inlet temperature, had to change inlet temperature due to the cyclone had no position. The actual mass was: m = 6.02 g (Batch 8.1.1.1)

Storing of product	Signature	Comments
Store product in glass container.		
Place in desiccators for one week for equilibration (%RH less than 25-30% and room temperature).		
General comments:		

7.2.4 Trehalose_Leucine_BSA_03_A

Production master for production of "Spray dried powders for inhalation";
Division of Food Technology

master 4 version 1/16

Production master	
Spray dried powder for inhalation	Trehalose-Leucine-BSA-03-A (g/l)
Name: F. M. Karsouli	Date: 25/03/24

Special information

Production of spray dried powder for inhalation with the purpose of using it in a dry powder inhaler (DPI). The spray dryer used is the BUCHI 290 (B-290).

Formulation Ingredients	Supplier	Lot/Batch
Trehalose Dihydrate	Sigma-Aldrich	V02302X76-232
Leucine	Sigma-Aldrich	000028722
Bovine Serum Albumin (BSA)	Sigma-Aldrich	0000028722

Production master for production of "Spray dried powders for inhalation";
Division of Food Technology

master 4 version 2/16

Spray drying conditions		Comments
Inlet Temperature (T _i)	175 °C	Average: 175 °C
Outlet Temperature (T _o)	76 °C	Average: 73.3 °C
Feed rate	20 %	6 ml/min
Aspirator rate	80 %	32 m ³ /h
Spray gas flow	45 mm	0.351 m ³ /h
Nozzle cleaner	6	
Room temperature	20.5 °C	
Room relative humidity (%RH)	30 %	

Production master for production of "Spray dried powders for inhalation";

master 4 version 3/16

Batch number 03-A Date of production 25/03/24

Product name: Trehalose-Leucine-BSA-03-A	Batch number: 03-A
Date of production: 25/03/24	
Signature: [Signature]	

Weighting of ingredients	Signature	Comments
Ingredient: Trehalose Dihydrate		
Weigh the container 0.283g and zero the balance.		
Weigh 2.83g amount of Trehalose. Actual m = 2.885g	[Signature]	
Ingredient: Leucine		
Weigh the container 0.283g and zero the balance.		
Weigh 1.11g amount of Leucine. Actual m = 1.114g	[Signature]	
Ingredient: BSA		
Weigh the container 0.283g and zero the balance.		
Weigh 1.11g amount of BSA. Actual m = 1.111g	[Signature]	

Production master for production of "Spray dried powders for inhalation";

master 4 version 4/16

Batch number 03-A Date of production 25/03/24

General comments:	
Preparing feed solution	Signature: [Signature] Comments:
Dissolving ingredients for 1.9 %w/v total solids.	
Add 150 ml deionized water in beaker (volume size 250 ml)	[Signature]
Add weighted powder.	
Mix until dissolved using magnetic stirring and heating (if necessary)	[Signature] Heating for 5 min to dissolve leucine
Add the rest of weighted ingredients and mix until dissolved.	
Monitor pH: 6.62	[Signature]
General comments: 1.1-1.2 bar of foaming in beaker	

Production master for production of "Spray dried powders for inhalation";

master 4 version 5/16

Batch number 03-A Date of production 25/03/24

Spray drying steps	Signature	Comments
Set up spray dryer as instructed in the manual and start with distilled water.	[Signature]	
Set spray drying conditions referred in the start of the production master:		
T _i = 175 °C		
T _o = 76 °C		
Feed rate = 20 %		
Aspirator rate = 80 %		
Spray gas flow = 45 mm		
Nozzle cleaner = 6		
When conditions are steady, start spray drying the feed solution (V = 100 mL)	[Signature]	10.46 (Started)
Monitor the conditions and take note of inlet and outlet temperature:		
T _i	T _o	
175 °C	77 °C	
176 °C	76 °C	
174 °C	77 °C	
172 °C	75 °C	
176 °C	74 °C	
173 °C	76 °C	11.02 (End)

Production master for production of "Spray dried powders for inhalation";

master 4 version 6/16

Batch number 03-A Date of production 25/03/24

When feed solution is finished, switch to distilled water and start shut down procedure of spray dryer as described in manual.	
General comments: Dry mass = 6.0503g ⇒ yield = 88.2% Almost no residues in cyclone.	
Storing of product	Signature: [Signature] Comments:
Store product in glass container.	
Place in desiccators for one week for equilibration (%RH less than 25-30% and room temperature).	[Signature]
General comments:	

7.2.5 Trehalose_Leucine_BSA_03_B

Production master for production of "Spray dried powders for inhalation";
Division of Food Technology

master: 5 version
116)

Production master		Master: 5 version	
Spray dried powder for inhalation		Trehalose, Leucine, BSA, 03-B (4.1.1)	
Date:	25/03/24		
Signature:			

Special information

Production of spray dried powder for inhalation with the purpose of using it in a dry powder inhaler (DPI). The spray dryer used is the BUCHI 290 (B-290).

Formulation Ingredients	Supplier	Lot/Batch
Trehalose Dihydrate	Sigma-Aldrich	V02020656 332
L-Leucine	Sigma-Aldrich	0000203022
Bovine Serum Albumin (BSA)	Sigma-Aldrich	0000203010

Production master for production of "Spray dried powders for inhalation";
Division of Food Technology

master: 5 version
216)

Spray drying conditions		Comments
Inlet Temperature (T _{in})	175°C	Average: 173.8°C
Outlet Temperature (T _{out})	76°C	Average: 72.4°C
Feed rate	20%	6 ml/min
Aspirator rate	30%	38 m ³ /hr
Spray gas flow	45 m ³ /hr	0.831 m ³ /hr
Nozzle cleaner	6	
Room temperature	20.5°C	
Room relative humidity (%RH)	30%	

Production master for production of "Spray dried powders for inhalation";

master: 5 version
316)

Batch number: 03-B	Date of production: 25/03/24
Product name: Trehalose-Leucine BSA-03-B	
Batch number: 03-B	
Date of production: 25/03/24	
Signature: [Signature]	

Weighing of ingredients	Signature	Comments
Ingredient: Trehalose Dihydrate		
Weigh the container 0.00g and zero the balance.		
Weigh 2.267g amount of Trehalose (Actual m = 2.86g)	[Signature]	
Ingredient: Leucine		
Weigh the container 0.00g and zero the balance.		
Weigh 1.11g amount of Leucine (Actual m = 1.11g)	[Signature]	
Ingredient: BSA		
Weigh the container 0.00g and zero the balance.		
Weigh 1.11g amount of BSA (Actual m = 1.11g)	[Signature]	

Production master for production of "Spray dried powders for inhalation";

master: 5 version
416)

Batch number: 03-B	Date of production: 25/03/24
General comments:	

Preparing feed solution	Signature	Comments
Dissolving ingredients for 4.4 %w/v total solids.		
Add 450 mL deionized water in beaker (volume size 500 mL)	[Signature]	
Add weighed powder.	[Signature]	
Mix until dissolved using magnetic stirring and heating (if necessary)	[Signature]	Heating only to dissolve Leucine
Add the rest of weighted ingredients and mix until dissolved.	[Signature]	
Monitor pH: 6.65	[Signature]	
General comments:		

Production master for production of "Spray dried powders for inhalation";

master: 5 version
516)

Batch number: 03-B	Date of production: 25/03/24
--------------------	------------------------------

Spray drying steps	Signature	Comments
Set up spray dryer as instructed in the manual and start with distilled water.	[Signature]	
Set spray drying conditions referred in the start of the production master:		
T _{in} = 175°C		
T _{out} = 76°C		
Feed rate = 20%		
Aspirator rate = 30%		
Spray gas flow = 45 m ³ /hr		
Nozzle cleaner = 6		
When conditions are steady, start spray drying the feed solution (V = 100 mL)	[Signature]	Started 12:08
Monitor the conditions and take note of inlet and outlet temperature:		
T _{in}	T _{out}	
172°C	77°C	Approx. set to 20% for correct T _{in} and T _{out}
175°C	78°C	
176°C	78°C	
173°C	77°C	
174°C	77°C	[Signature] End 12:28

Production master for production of "Spray dried powders for inhalation";

master: 5 version
616)

Batch number: 03-B	Date of production: 25/03/24
--------------------	------------------------------

When feed solution is finished, switch to distilled water and start shut down procedure of spray dryer as described in manual.	[Signature]	
General comments:		
Mp _{product} = 5.7271g		
Yield = 0.7750 = 77.53%		
Approx. 50 mg of product (0.5-1.0g) kept in vials for analysis (this may explain lower yield)		
Storing of product		
Store product in glass container.	[Signature]	Trehalose-Leucine-BSA-03-B
Place in desiccators for one week for equilibration (%RH less than 25-30% and room temperature)	[Signature]	
General comments:		

7.3 Light Microscopy images

7.3.1 Trehalose_Leucine_BSA_03_A

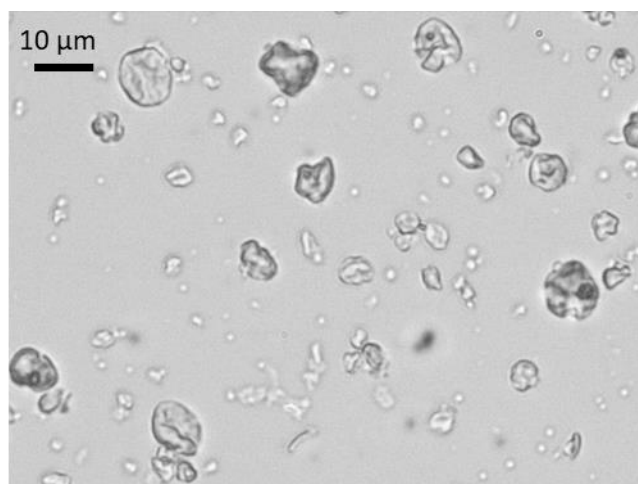
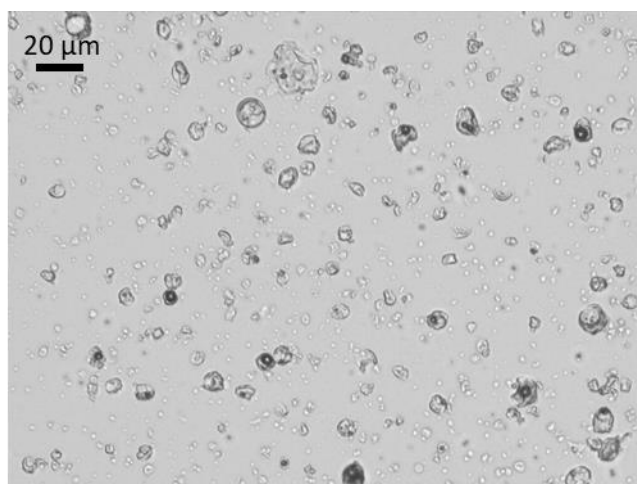


Figure D: Light microscopy images of Trehalose_Leucine_BSA_03_A at magnifications x20 (left) and x50 (right).

7.3.2 Trehalose_Leucine_BSA_03_B

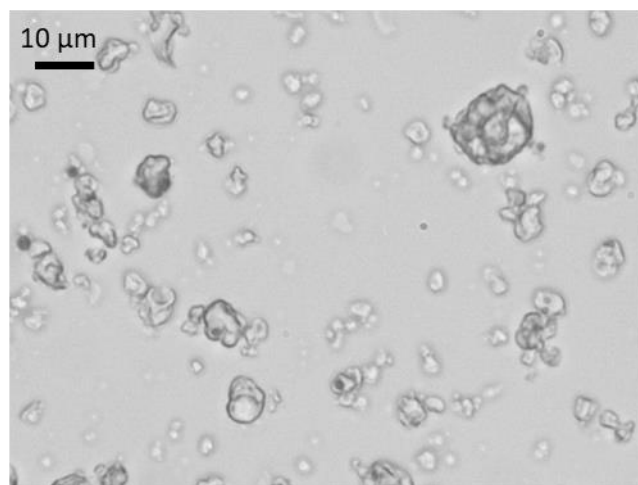
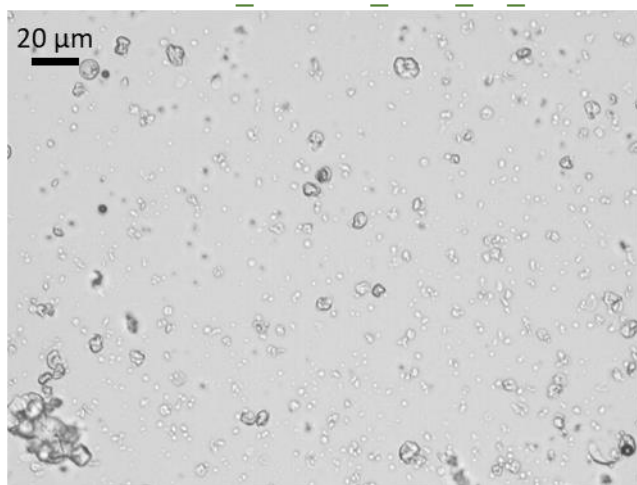


Figure E: Light microscopy images of Trehalose_Leucine_BSA_03_B at magnifications x20 (left) and x50 (right).

7.4 Summary of NGI results

Summary of Results		RUN 1	PS used	RUN 2	PS used	RUN 3	PS used	RUN 4	PS used
Comment		T B 10%		T L B 27:10		T L B 1:1		T L 27%	
Flow rate in impactor	l/min	62		62		62		62	
No of inhalers		2		2		2		2	
No of doses per inhaler & label claim		5		5		5		5	
		Mean	RSD%	Mean	RSD%	Mean	RSD%	Mean	RSD%
Total dose	µg/dose	2303.73	3.39	2430.15	21.85	1570.95	3.50	4482.54	6.76
Delivered dose	µg/dose	2173.65	4.87	2372.29	25.83	1409.35	12.32	4482.54	6.76
Throat	% of DD	23.99	0.85	15.53	26.63	26.52	14.58	29.41	2.09
MP	% of total	5.51	141.42	2.82	141.42	10.04	141.42	0.00	
Total Retention	µg/dose	130.08	141.42	57.85	141.42	161.61	141.42	0.00	
Total Retention	% of total	5.51	141.42	2.82	141.42	10.04	141.42	0.00	
Fine particle dose	µg/dose <5µm	820.63	3.52	1243.80	55.42	463.81	7.28	1189.57	6.78
Fine particle fraction	%<5µm of total	35.66	6.90	49.26	35.74	29.58	10.76	26.54	0.02
Fine particle fraction	%<5µm of DD	37.77	1.35	50.36	31.87	33.01	5.06	26.54	0.02
Fine particle fraction	%<3µm of DD	24.92	12.67	41.33	44.74	21.90	6.26	16.51	6.39
Fine particle fraction	%<1µm of DD	11.04	14.87	22.13	35.79	10.29	3.91	3.88	1.00
MMAD	µm	3.95	4.61	2.90	44.53	4.40	0.86	4.68	1.28
GSD		2.95	12.76	3.56	10.94	3.17	0.01	2.58	8.22
R-value		0.99	0.56	0.98	0.33	0.98	0.38	1.00	0.08

7.4.1 Standard curves for BSA/L-leucine ratios

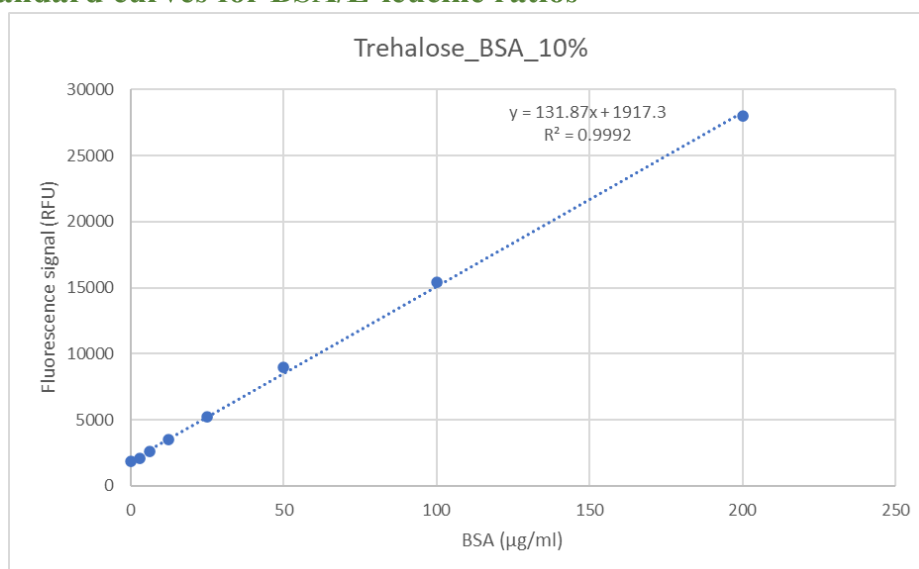


Figure F: Standard curve of BSA for sample Trehalose_BSA_01 in NGI analysis.

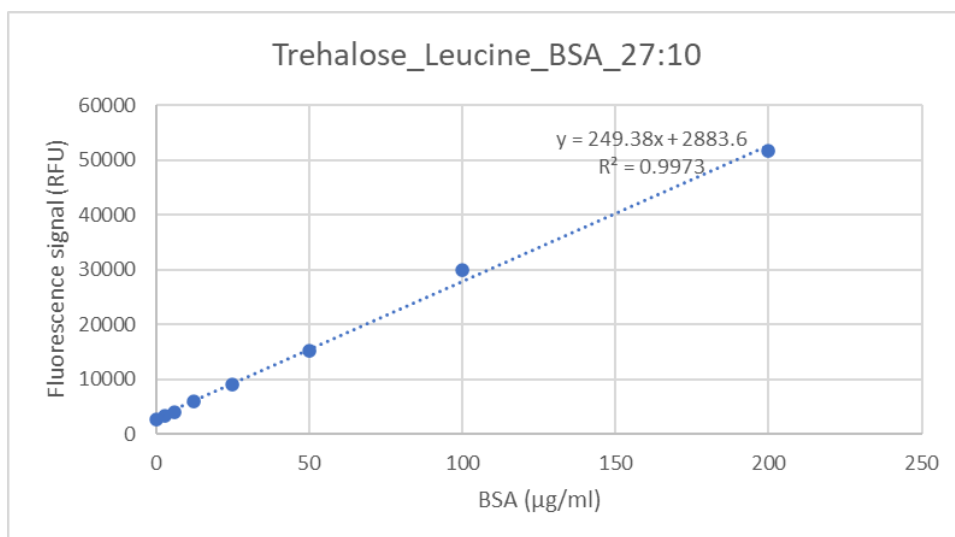


Figure G: Standard curve of BSA:L-leucine ratio 27:10 for sample Trehalose_BSA_02 in NGI analysis.

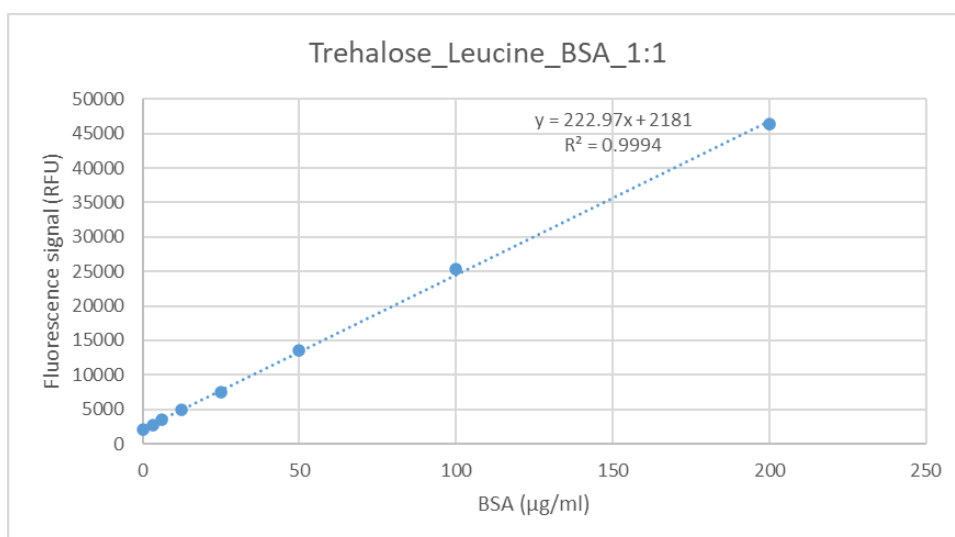


Figure H: Standard curve of BSA:L-leucine ratio 1:10 for sample Trehalose_BSA_03 in NGI analysis.

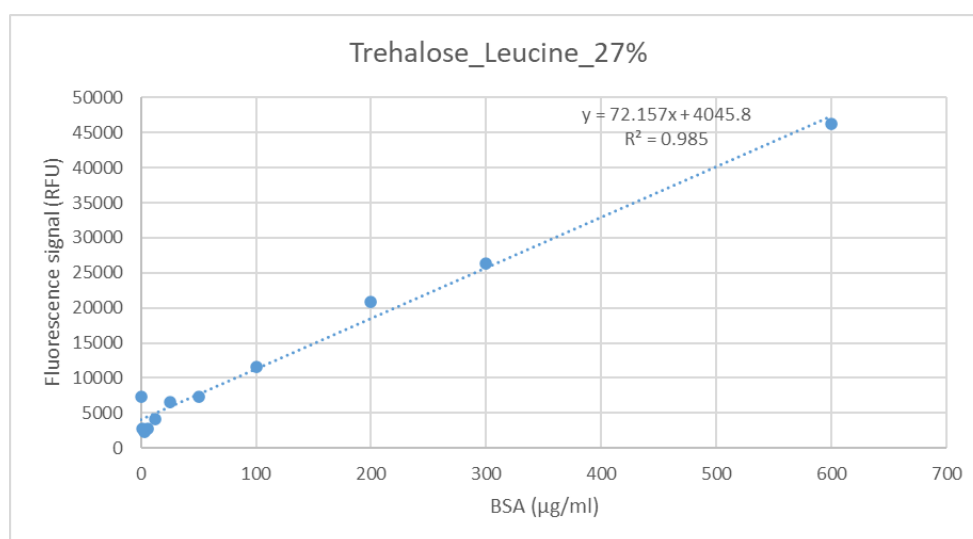


Figure I: Standard curve of L-leucine for sample Trehalose_BSA_02 in NGI analysis.

7.5 APS graphs: Normalized concentration vs aerodynamic particle size of powder

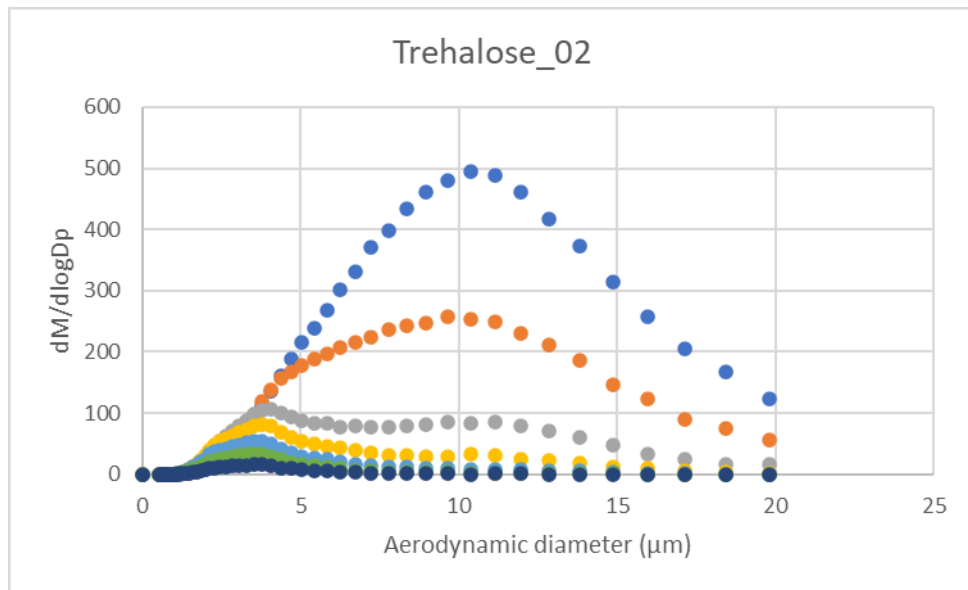


Figure J: Normalized concentration vs aerodynamic particle size of powder Trehalose_02.

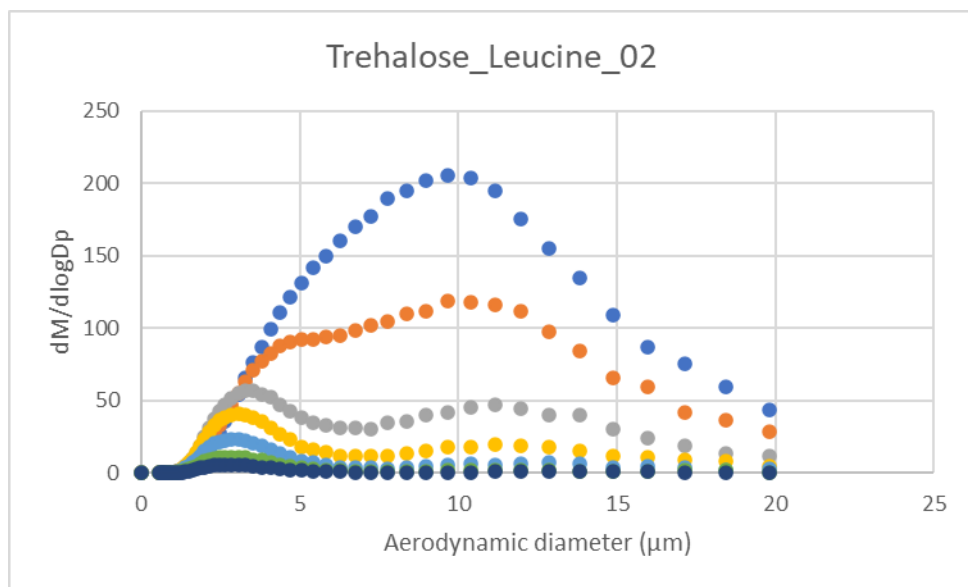


Figure K: Normalized concentration vs aerodynamic particle size of powder Trehalose_Leucine_02.

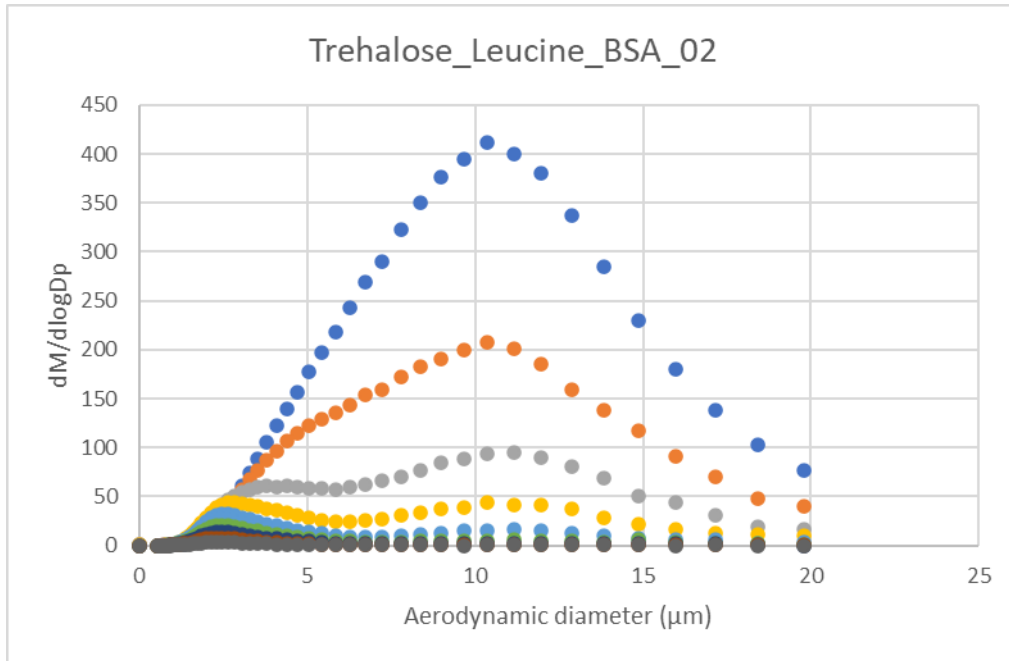


Figure L: Normalized concentration vs aerodynamic particle size of powder Trehalose_Leucine_BSA_02.

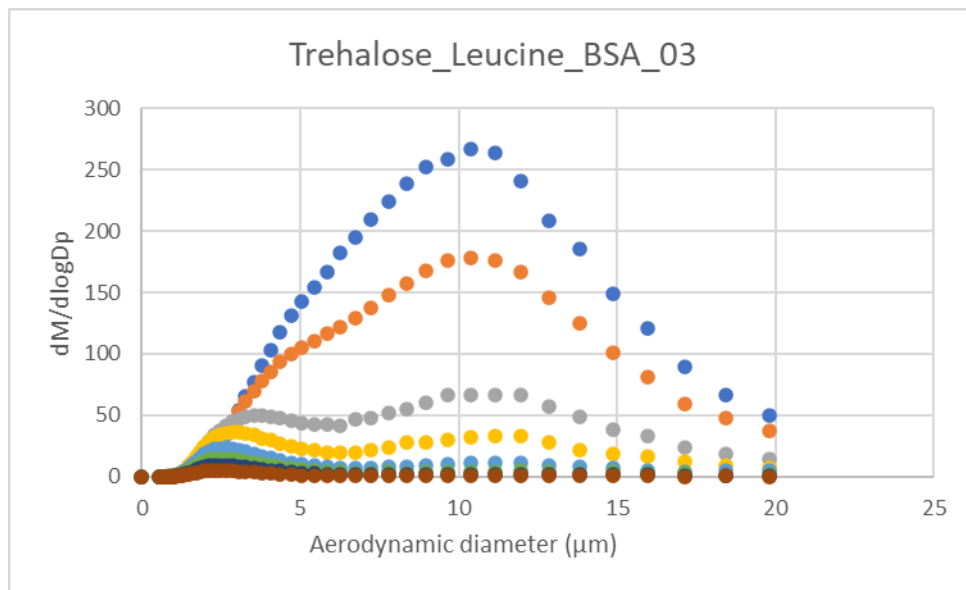


Figure M: Normalized concentration vs aerodynamic particle size of powder Trehalose_Leucine_BSA_03.