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The use of hydrophobic amino acids in protecting spray dried trehalose formulations against moisture-induced changes

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Abstract

Trehalose is commonly used as a protein stabilizer in spray dried protein formulations delivered via the pulmonary route. Spray dried trehalose formulations are highly hygroscopic, which makes them prone to deliquescence and recrystallization when exposed to moisture, leading to impairment in aerosolization performance. The main aim of this study was to investigate and compare the effect of hydrophobic amino acids (i.e. L-leucine and L-isoleucine) in enhancing aerosolization performance and in mitigating moisture-induced changes in spray dried trehalose formulations. Trehalose was spray dried with 20–60% w/w of amino acid (i.e. L-leucine or L-isoleucine). The spray dried formulations were stored at 25 °C/50% RH for 28 days. Solid state characterization and in vitro aerosolization performance studies were performed on the spray dried formulations before and after storage. The addition of 20–60% w/w of amino acid (i.e. L-leucine or L-isoleucine) improved the emitted fractions of spray dried trehalose formulations from a dry powder inhaler. However, ≥ 40% w/w of L-leucine/L-isoleucine was needed to prevent recrystallization of trehalose in the formulations when exposed to 25 °C/50% RH for 28 days. X-ray photoelectron spectroscopy (XPS) demonstrated that samples with 40–60% w/w L-isoleucine had more amino acid on the surfaces of the particles compared to their L-leucine counterparts. This may explain the greater ability of the L-isoleucine (40–60% w/w) samples to cope with elevated humidity compared to L-leucine samples of the same concentrations, as observed in the dynamic vapour sorption (DVS) studies. In conclusion, this study demonstrated that both L-leucine and L-isoleucine were effective in enhancing aerosolization performance and mitigating moisture-induced reduction in aerosolization performance in spray dried trehalose formulations.

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Appendix A. Supplementary material

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L-isoleucine proved to be superior to L-leucine in terms of its moisture protectant effect when incorporated at the same concentration in the formulations.

Keywords

Aerosolization; Hygroscopic; L-leucine; L-isoleucine; Moisture protection; Recrystallization; Spray drying; Trehalose

1. Introduction

In the recent years, there has been a huge interest in the pharmaceutical sector in developing protein therapeutics. This is reflected by the fact that in 2017, protein therapeutics constituted 30% of all newly approved drugs by the Food and Drug Administration (FDA) in the United States of America [1]. Protein drugs are commonly administered via the parenteral routes which include the subcutaneous, intramuscular and intravenous routes [2]. These routes of administration are not patient friendly and pose a risk of needle stick injury to the patient/administrator [2]. The pulmonary route offers a viable and attractive alternative route for administering protein drugs, as it does not have the many shortcomings associated with injections. Examples of inhaled protein therapeutics approved by the FDA are dornase alfa (marketed as Pulmozyme [3] as a mucolytic agent in cystic fibrosis patients) and insulin (marketed as Afrezza [4] for the treatment of diabetes). The successful development of orally inhaled insulin demonstrated that an ideal orally inhaled protein formulation should be cost effective, convenient to use, have high patient acceptance and high dosing flexibility [5].

Oral inhalation formulation products that are available on the market include nebulizers, metered dose inhalers (MDIs) and dry powder inhalers (DPIs) [6]. There is a higher preference for DPIs over nebulizers and MDIs. The shortcomings of formulating protein drugs for nebulization include stability issues with liquid protein formulations [7], potential of shear induced denaturation during administration by means of the nebulizer [8], longer administration time than DPIs and MDIs and the requirement to clean the nebulizer after each use. On the other hand, the drawbacks of pressurised MDIs are the high tendency for protein denaturation when formulated with propellants [9] and also poor patient compliance due to the need for hand-breath coordination [6].

A drying step is often required in the production of dry powders for inhalation. Spray drying, which involves atomizing a solution into hot air to evaporate off the solvent to produce powder particles [10], is commonly used to prepare dry powders for inhalation. This drying process is able to engineer the particle size to a range suitable for inhalation (i.e. between 1 and 5 μm) [11]. Spray drying a protein solution will subject the protein to various stresses (i.e. mechanical, thermal and interfacial stresses), which may potentially lead to the physical and chemical degradation of the protein [7,12]. Thus, the inclusion of stabilizers (sugars are routinely used) into the protein formulation is necessary to preserve the integrity and ultimately, maintaining the therapeutic effectiveness of the protein during production and storage. It has been proposed that sugars form rigid scaffolds that kinetically immobilize proteins (i.e vitrification theory). Sugars are also able to form tight hydrogen bond

interactions with proteins, which reduces local mobility of the protein and thus preserves the protein's native conformation [7,12].

Sucrose and trehalose are the two sugars commonly used to stabilize proteins in the solid state [13]. Both sugars are disaccharides that have flexible backbones allowing them to participate in more hydrogen bonding and thus tighter interactions with proteins [7,12]. As they are small oligosaccharides, they have high miscibility with proteins (compared to sugars of larger size) which is essential in forming strong protein-sugar interactions [7,12]. Moreover, both sugars are non-reducing, indicating that they will not partake in Maillard reactions with proteins [14].

Trehalose is a more desirable protein stabilizer than sucrose for spray drying, as amorphous trehalose has a higher glass transition temperature (T_g) (i.e. 119 °C) than amorphous sucrose [15,16]. The low T g of amorphous sucrose (i.e. 74 °C) [15] presents a challenge in spray drying, as the outlet temperature of the spray dryer may be very close to sucrose's T_g, causing sucrose to change from a glass to a rubbery state, which typically results in a sticky mass being retained in the spray dryer and no powder collection [16]. Trehalose is used as an excipient in licensed protein formulations [7]. However, it has not been used in any licensed pulmonary dry powder formulations. Research in the literature demonstrates the use of trehalose as a promising excipient for dry powder protein formulations for the pulmonary route [17–24].

Spray dried trehalose is amorphous and highly hygroscopic [25] and thus poses a formulation challenge. Exposure of amorphous trehalose to moisture may result in the deliquescence and recrystallization of trehalose which ultimately may impair both the stability and aerosolization performance of the formulation. L-leucine (amino acid) which has been routinely used as a dispersion enhancer for spray dried powders [26–32], has been recently shown to be able to mitigate moisture-induced impairment in aerosolization performance [33,34]. Li et al. demonstrated that the inclusion of L-leucine in the spray dried powder was effective in maintaining the aerosolization performances of disodium cromoglycate and salbutamol sulfate powders on storage in elevated humidity conditions [33,34]. It was proposed that L-leucine preferentially enriched the surface of the particles during the drying process [33–36]. Due to L-leucine's hydrophobic nature, it acts as a barrier to moisture and slows down ingress of moisture into the particles. Yu et al. compared the ability of other hydrophobic amino acids (i.e. L-isoleucine, L-valine and L-methionine) to prevent moisture induced reduction in aerosolization performance in spray dried disodium cromoglycate [37]. They found that, of the three hydrophobic amino acids investigated, L-isoleucine offered the greatest moisture protection. L-isoleucine is an isomer of L-leucine, which means that they have the same chemical formula but a different atomic arrangement in the molecule. Both amino acids demonstrate good safety profiles when they were administered orally to animals [38,39].

L-leucine has been previously shown to enhance the aerosolization performance of spray dried formulations containing trehalose [29,35]. However, these studies have not explored the ability of L-leucine to protect trehalose against moisture-induced impairment of aerosolization performance from a dry powder inhaler. No previous studies have spray dried

L-isoleucine with trehalose. This is the first study that compares the effects of L-leucine and L-isoleucine in offering protection against moisture induced reduction in aerosolization performance for highly hygroscopic spray dried materials.

The main aim of this study was to investigate and compare the effect of hydrophobic amino acids (i.e. L-leucine and L-isoleucine) in enhancing aerosolization performance and in mitigating moisture-induced changes in spray dried trehalose formulations. This study involved preparing spray dried formulations comprising different ratios of trehalose and amino acid (i.e. L-leucine or L-isoleucine). The physical characteristics and aerosolization properties of the spray dried formulations were investigated initially and after storage for 28 days under conditions of 25 °C/50% RH. Finally, the effect of the two amino acids on the critical quality attributes of the spray dried formulations were compared.

2. Materials & methods

2.1. Materials

D(+)-trehalose dihydrate was purchased from AppliChem (Darmstadt, Germany). L-leucine (Brazil, Sigma-Aldrich) and L-isoleucine (China, Sigma-Aldrich) were used as received. Salbutamol sulfate was manufactured by Cambrex Profarmaco (Paullo, Italy). Low resistance RS01 monodose inhaler (Plastiape, Osnago, Italy) and no. 3 hydroxypropyl methylcellulose (HPMC) capsules (Qualicaps, Madrid, Spain) were used as received.

1-heptanesulfonic acid sodium salt (Fisher Scientific, Loughborough, United Kingdom), potassium phosphate monobasic (Sigma-Aldrich, Germany), acetonitrile (Sigma-Aldrich, Dorset, UK) and ultrapure Milli-Q water (obtained using a Synergy UV Millipore system, Merck, Darmstadt, Germany) were used to prepare the mobile phase for the high-performance liquid chromatography (HPLC) analyses. Deionized water was obtained from a Millipore Elix 3 system (Millipore, Saint-Quentin, France).

2.2. Spray drying

Feed solutions with a total solid content of 15 mg/ml (1.5% w/v) were prepared by dissolving salbutamol sulfate (at a level of 2% w/w of the total solute content) and varying mass ratios of D (+)-trehalose dihydrate and amino acid (i.e. L-leucine or L-isoleucine) in deionized water. Salbutamol sulfate was added into the formulations to enable quantification of the powders using HPLC coupled with a UV detector, since trehalose and the amino acids are not easily assayed by UV-HPLC. In terms of the trehalose/L-leucine or L-isoleucine content of the spray dried solutions (which together comprise 98% of the total solute content), the following formulation composition ratios were used: trehalose:L-leucine/L-isoleucine 100:0 (100% Tre), trehalose:L-leucine 80:20 (20% Leu), trehalose:L-leucine 60:40 (40% Leu), trehalose:L-leucine 40:60 (60% Leu), trehalose:L-isoleucine 80:20 (20% Iso), trehalose:L-isoleucine 60:40 (40% Iso) and trehalose:L-isoleucine 40:60 (60% Iso). Samples 100% Leu (trehalose:L-leucine 0:100) and 100% Iso (trehalose:L-isoleucine 0:100) were spray dried from solutions with a total solid content of 15 mg/ml without the inclusion of salbutamol sulfate. The solutions were spray dried using a Büchi Mini Spray-Dryer B-290 (Büchi Labortechnik AG, Flawil, Switzerland) which was equipped with a 0.7-mm two-fluid

nozzle. The spray drying parameters used were: feed rate of 10% (3.0 ml/min), atomizer setting 60 mm (742 L/h), aspirator setting of 90% (35 m^3/h), inlet temperature of 140 °C, which resulted in outlet temperatures of 69–72 °C. The spray dried powders were transferred into capped vials and immediately stored in a desiccator containing calcium sulfate at 4 °C, until further analyses.

2.3. Powder X-ray diffractometry (PXRD)

The spray dried samples were analyzed by PXRD with a Rigaku MiniFlex II desktop X-ray diffractometer (Rigaku, Japan) using Cu Kα radiation with λ = 1.54 Å (30 kV and 15 mA) and a dispersion slit of 1.25°. The samples were gently pressed into flat silicon sample holders using a spatula and scanned from 3° to 40° 2θ with a scanning rate of 0.05°/s.

2.4. Fourier-transform infrared (FT-IR) spectroscopy

Infrared spectroscopy measurements were conducted using a PerkinElmer Spectrum 1 FT-IR Spectrometer equipped with a UATR and a ZnSe crystal accessory. The final spectrum was the mean of 16 scans with a spectral range from 650 to 4000 cm^{-1}. The ATR spectra were evaluated using Spectrum version 5.0.1. software.

2.5. Modulated differential scanning calorimetry (mDSC)

DSC thermograms were recorded using a DSC Q200 (TA instruments, Elstree, UK). Samples (3–5 mg) were crimped in aluminum pans with pierced lids, heated up to 150 °C at a heating rate of 5 °C/min, with ± 0.796 modulation amplitude and a 60 s modulation period. The measurement cell was purged with dry nitrogen gas at a flow rate of 60 ml/min during the measurements. The DSC was calibrated with indium and tin. The samples were analyzed on the day of preparation and the filled DSC pans were prepared just prior to the DSC measurement to minimize waiting time. The glass transition temperature (T_g), were determined using the Universal Analysis® (version 4.7A) software. The T_g was defined as the midpoint change in heat capacity of the sample. Each sample was measured in triplicate.

2.6. Thermogravimetric analysis (TGA)

The residual moisture content of the samples was determined using TGA Q50 (TA Instruments, Elstree, UK). 5–10 mg of the samples were loaded onto aluminium pans and heated to 150 °C with a heating rate of 10 °C/min. Measurements were performed in triplicate.

2.7. Dynamic vapour sorption (DVS) analysis

The water vapour sorption–desorption isotherms of the spray dried samples were obtained at 25 °C using a DVS Advantage-1 instrument (Surface Measurements Systems Limited, London, UK). The samples were subjected to an initial equilibration under continuous nitrogen flow (to remove residual moisture) to determine the initial dry mass. Then, the relative humidity of the chamber was increased from of 0 to 90% RH, with 10% RH increments for the sorption cycles. Finally, the samples were subjected to a desorption cycle (i.e. decreasing relative humidity from 90 to 0% RH, with 10% RH decrements). Changes in

the relative humidity occurred when the mass of the samples reached equilibrium (i.e. weight change over 10 min (dm/dt) ≤ 0.002 mg/min).

2.8. X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectroscopy was performed under ultra-high vacuum condition (< 5×10^{-10} mbar) on a VG Scientific ESCAlab Mk II system equipped with a hemispherical analyzer using Al Kα X-rays (1486.6 eV). The emitted photoelectrons were collected at a take-off angle of 90° from the samples surface. The analyzer pass energy was set to 100 eV for survey scans and 40 eV for high-resolution core scans, yielding an overall resolution of 1.5 eV. Photoemission peak positions were corrected to C 1s at a binding energy of 248.8 eV.

2.9. Particle size analysis

The particle size distributions of the samples were determined using a Mastersizer 2000 laser diffraction instrument equipped with a Scirocco 2000 dry powder dispersion accessory (Malvern Instruments Ltd., Worcestershire, UK). A vibration feed rate of 50% and dispersive air pressure of 2.0 bar were used. D10, D50, D90 (i.e. particle diameters at 10, 50 and 90%, respectively, of the cumulative volume distribution) and span (determined by the following equation: (D90-D10)/D50)) were computed using the Malvern 2000 software. The measurements were performed in triplicate.

2.10. Scanning electron microscopy (SEM)

Scanning electron microscopy images of the powders were recorded using a Zeiss Ultra Plus (Oberkochen, Germany) scanning electron microscope. A working voltage of 15 kV was used. The powders were mounted on aluminium stubs with double-sided carbon tape and sub-sequently were coated with gold/palladium before imaging.

2.11. In vitro aerosolization studies

Aerodynamic particle size measurements were performed using the Next Generation Impactor (NGI) (Copley Scientific Limited, Nottingham, UK), coupled with two HCP pumps (Copley Scientific Limited, Nottingham, UK). The critical flow controller, TPK 2000 (Copley Scientific Limited, Nottingham, UK) was used to adjust the flow rate to 100 L/min and critical sonic flow (i.e. P3/P2 < 0.5) was achieved for every measurement. The aspiration time was adjusted to 2.4 s to allow the flow of 4 L of air. The NGI cups were lined with filter papers wetted with 1 ml of deionized water to minimize particle bounce. A low resistance RS01 monodose inhaler was used. 20 ± 1 mg of the spray dried powders were filled into no. 3 HPMC capsules. One capsule was used for each test and the in vitro aerosolization measurements were carried out in triplicate for each formulation. The deposited powders were dissolved in water and quantified using HPLC. The Copley Inhaler Testing Data Analysis Software (CITDAS, Copley Scientific Limited, Nottingham, UK) was used to evaluate the emitted fractions, the fine particle fractions (FPFs) of ≤3 μm and ≤5 μm, mass median aerodynamic diameters (MMADs) and geometric standard deviations (GSDs).

The HPLC method for the quantification of salbutamol sulfate was adapted from the British Pharmacopeia [40]. A Breeze™ HPLC system (Waters, Milford, USA) equipped with

Waters 1525 binary pump with an in-built degasser, Waters 717 plus autosampler and Waters 2487 dual λ absorbance detector was used. Isocratic chromatographic separation was carried out at room temperature using a 125 mm × 4.6 mm (5 μm particle size) Inertsil ODS3 column (GL Sciences, Eindhoven, Netherlands). The mobile phase comprised of 22% v/v acetonitrile and 78% v/v of an aqueous solution containing 0.264% w/v of 1-heptanesulfonic acid sodium salt and 0.25% w/v potassium phosphate monobasic adjusted to pH 3.7. The flow rate and injection volume used were 1.0 ml/min and 20 μL, respectively. Salbutamol UV detection was performed at 220 nm. The retention time for salbutamol sulfate was 4.0 min.

2.12. Storage

The Amebis Stability Testing and Monitoring System (Amebis Ltd., Ireland) [41] was employed to conduct storage stability studies at 50% RH, 25 °C for 28 days. Powders were stored in separate open glass vials (diameter of 20 mm and height of 35 mm) and then transferred to Amebis sample chambers containing an Amebis salt U036 (Amebis Ltd., Ireland). Each glass vial contained 150 mg of powder. The Amebis system wirelessly transmitted and logged (every 30 min intervals) the conditions in the chamber to the Amebis Control Software (Amebis Ltd., Ireland). This enabled remote monitoring of the conditions within the storage chambers, without the need to open the chamber for storage conditions monitoring.

2.13. Statistical analysis

The two-sample *t*-test (comparison of two data sets) and one-way ANOVA followed by posthoc Tukey's test (comparison ≥3 sets of data) were used to test the statistical significance of the samples when comparisons were made. Differences were found to be statistically significant when a confidence level of 95% (i.e. p value ≤0.050) was obtained. Minitab™ software (version 16) was used to perform the statistical analyses.

3. Results

3.1. Pxrd

Fig. 1a depicts the diffraction patterns of unprocessed trehalose and spray dried trehalose before and after storage. The PXRD pattern of the unprocessed trehalose corresponded to the diffractogram of the Cambridge Structural Database (CSD) reference for trehalose dihydrate (reference code: LEGFEK). 100% Tre was fully amorphous after spray drying (i.e. broad halo in the PXRD pattern). Storage of 100% Tre at 25 °C/50% RH for 28 days resulted in the crystallization of the sample to the dihydrate form.

Extra peaks (i.e. at 18.5°, 25.1°, 32.4° 2 theta for L-leucine; 17.7°, 19.1°, 19.8°, 22.3°, 22.9°, 32.6°, 33.6° 2 theta for L-isoleucine) were observed in the PXRD patterns of the spray dried amino acids when compared to those of the unprocessed amino acids. Differences in diffraction patterns of spray dried amino acids have been previously reported [34,37] and suggest that spray drying altered the crystalline form of the amino acids [34]. The PXRD patterns of 20% Leu, 40% Leu and 60% Leu, before and after storage are illustrated in Fig. 2a and b. All the L-leucine-containing formulations were crystalline after spray drying and

exhibited similar Bragg peaks as spray dried neat L-leucine. This suggests that whilst the L-leucine crystallized during spray drying, trehalose remained amorphous.. 20% w/w L-leucine was not adequate to prevent moisture-induced crystallization of trehalose when exposed to 50% RH for 28 days. The presence of 40 – 60% w/w of L-leucine in the spray dried formulations inhibited trehalose crystallization during the storage period, with the PXRD patterns of 40% Leu and 60% Leu remaining unchanged relative to the freshly spray dried materials.

Fig. 2c and d show the diffractograms of 20% Iso, 40% Iso and 60% Iso, before and after storage. The L-isoleucine component crystallized (to different degrees to the same form as spray dried neat L-isoleucine) during spray drying in all the L-isoleucine containing formulations. The diffraction pattern of 20% Iso after spray drying (Fig. 2c) has a broad halo background with small L-isoleucine peaks at 2θ positions of 6.4°, 19.0° and 24.9°. Storing 20% Iso for 28 days at 50% RH resulted in the growth of L-isoleucine peaks and the appearance of Bragg peaks corresponding to trehalose dihydrate also, suggesting crystallization of both L-isoleucine and trehalose. In comparison to its L-leucine counterpart, 20% Iso had a lower level of crystallinity after spray drying and had undergone a greater extent of crystallization on storage (indicated by the more pronounced Bragg peaks). The PXRD patterns of 40% Iso and 60% Iso appear to be unaltered after storage, indicating that trehalose remained amorphous over the storage period.

3.2. Infrared spectroscopy

The infrared spectra of unprocessed trehalose (dihydrate form), L-leucine and L-isoleucine are shown in Fig. S1 (supplementary information). The conversion of trehalose dihydrate (unprocessed) to the amorphous form (spray dried; Fig. 3) resulted in peak shifts, broadening of peaks and disappearance of peaks in the infrared spectrum. Differences in the infrared spectra between L-leucine and L-isoleucine, and their spray dried counterparts were also observed and are high-lighted in Fig. S2 (supplementary information). Spray drying L-leucine resulted in the change in relative intensities of the doublet peaks at 917 and 924 cm^{-1}, vibrational peak shifts (i.e. from 1511 to 1513 cm^{-1} and from 1576 to 1579 cm^{-1}) and disappearance of peaks at 1504 to 1556 cm^{-1}. On the other hand, subjecting L-isoleucine to spray drying resulted in the disappearance of the peak at 1383 cm^{-1} and the shifting of the peak at 1575 to 1579 cm^{-1}. This further supports the fact that spray drying changed the solid state form of L-isoleucine, as demonstrated in the PXRD section.

Fig. 3a and b depict the infrared spectra of spray dried L-leucine and L-isoleucine-containing formulations (wavenumber regions with spectral changes are highlighted). Increasing L-leucine content from 0 to 60% w/w in the spray dried formulations resulted in shifting of peaks as follows: 985 to 992 cm^{-1}; 1026 to 1030 cm^{-1}; 1042 to 1046 cm^{-1}; 1076 to 1079 cm^{-1}; 1102 to 1105 cm^{-1}. Similar spectral changes were observed for the spray dried formulations containing L-isoleucine when the L-isoleucine content was increased from 0 to 60% w/w in the spray dried formulations: peak shifts from 985 to 991 cm^{-1}, from 1026 to 1032 cm^{-1}, from 1042 to 1045 $cm^{-1}$from 1076 to 1080 cm^{-1}, and from 1102 to 1107 cm^{-1}. Spray dried L-leucine and L-isoleucine do not exhibit peaks at wavenumber positions for which peak shifts occurred. Those peaks exhibiting shifts in wavenumber were

most likely characteristic of trehalose and they are attributed to the functional group C-O. Shifts to lower wavenumbers in the peaks attributed to the carbonyl stretching [42] of the spray dried amino acids (i.e. 1513 cm^{-1} and 1579 cm^{-1} for leucine; 1511 cm^{-1} and 1579 cm^{-1} for isoleucine) were observed. The peak shifts could be attributed to molecular interactions between trehalose and the amino acids [42] and future studies to understand this further are warranted.

3.3. DSC

Differential scanning calorimetry was utilized to study the effect of the addition of different amounts of L-leucine or L-isoleucine on the glass transition of the formulations. The glass transition temperatures (T_gs) of all the trehalose-containing formulations (determined without any drying step in the DSC runs) are listed in Table 1. A single T_g was observed for all the samples. The T_g of the 100% Tre sample was 122.44 ± 0.31 °C and is similar to that reported in the literature [15,25]. A reduction in the T_g to 113.09 ± 0.14 °C was observed when 20% w/w L-isoleucine was added to the formulation. All the other trehalose-containing formulations (except sample 20% Iso) displayed similar glass transition temperatures to the 100% Tre sample. The reduction in the T_g of 20% Iso could be attributed to the presence of water that acts as plasticizer to lower the glass transition temperature of the formulation. In order to determine whether water plays a role in lowering the T_g of 20% Iso, the sample was subjected to drying at 100 °C for 15 min (to remove loosely bound water from the sample) before the T_g was reassessed. The T_g of dried 20% Iso was 119.98 ± 0.11 °C, which is similar to the other formulations, confirming that water was responsible for the T_g reduction.

3.4. Residual moisture analysis

Thermogravimetric analyses were performed on the samples before and after storage, to determine the moisture content of the samples. Table 2 shows the moisture content of the spray dried formulations before and after 28 days of storage at 25 °C/50% RH. The 100% Tre sample had a moisture content of 3.41 ± 0.13% w/w just after spray drying (day 0). The inclusion of 20% w/w L-leucine to the trehalose formulation did not affect the moisture content of the powder after spray drying. In contrast, the addition of 20% w/w L-isoleucine to the spray dried formulation resulted in a sample with higher moisture content (i.e. 4.28 ± 0.10%). This higher moisture content of sample 20% Iso when compared to other formulations explains the sample's glass transition reduction noted above. This could be due to the isoleucine being partially amorphous (as demonstrated by the PXRD results). Amorphous materials are more hygroscopic than their crystalline counterparts. Reduction in the moisture content was observed when the amino acid content was increased from 20 to 60% w/w.

All the L-leucine-containing samples gained moisture upon exposure to 50% RH for 28 days. Samples containing 60% w/w L-leucine or L-isoleucine gained the least amount of moisture when compared to formulations with lower amino acid contents.

3.5. DVS

The behavior of the samples upon exposure to different relative humidities at 25 °C was investigated by subjecting the samples to an initial sorption cycle, followed by an immediate desorption cycle. The sorption and desorption isotherms of L-leucine- and L-isoleucine-containing formulations are depicted in Fig. 4. PXRD analyses were performed on the samples at the end of the desorption cycles and the corresponding PXRD patterns are shown in Figs. S3 and S4 (supplementary information). 100% Tre sample absorbed moisture gradually when the relative humidity was increased from 0% RH up to 50% RH. As the relative humidity increased to 60% RH, there was a small drop in the mass of the sample. The mass of the sample plateaued off with a further increase in relative humidity. The point of inflection, which is also known as the critical relative humidity (i.e. 50% RH) indicates the maximum relative humidity that the sample can withstand. Increasing the humidity beyond the critical relative humidity resulted in crystallization of the sample to a stable form and expulsion of excess absorbed water (with a decrease in the sample's mass). The reduction in the relative humidity to which the sample was exposed (indicated by the corresponding desorption isotherm) did not alter the mass of the sample since the material has crystallized. The sample at the end of the desorption cycle was trehalose dihydrate, according to PXRD analysis. The mass of the 100% Leu and 100% Iso samples remained unaltered throughout the DVS cycles, suggesting that the samples were non-hygroscopic. The solid-state form of the 100% Leu and 100% Iso samples post-DVS analyses were the same as before the DVS analyses (Figs. S3 and S4 (supplementary information)).

All the spray dried formulations containing trehalose and L-leucine exhibited similar trends as was observed for the 100% Tre sample for both the sorption and desorption cycles. The trehalose component in all the L-leucine-containing formulations crystallized to the dihydrate form (Fig. S3 (supplementary information)). The key parameters of the DVS isotherms for all the spray dried formulations are presented in Table 3. Addition of 20% w/w L-leucine to the spray dried formulation had no effect on the critical relative humidity but decreased the maximum moisture uptake of the formulation by 2.3% w/w. Increasing the L-leucine content to 40 and 60% w/w, increased the critical relative humidity of the formulations to 60 and 70% RH respectively. The maximum moisture uptake remained unaltered when the L-leucine content was increased from 20 to 40% w/w. However, further increasing it to 60% w/w resulted in the decrease of the maximum moisture uptake to 7.2% w/w.

The trehalose formulations with 20 and 40% w/w L-isoleucine demonstrated similar sorption and desorption characteristics as the 100% Tre sample, and the trehalose constituent in the samples crystallized at the end of the desorption cycles (Fig. S4, supplementary information). The addition of 20% w/w L-isoleucine to the trehalose formulation did not offer any extra moisture protection benefit. However, increasing the L-isoleucine content to 40% w/w resulted in the sample being able to withstand higher relative humidity, (i.e. increase in the critical relative humidity and maximum moisture uptake, relative to those of sample 20% Iso). Increasing L-isoleucine to 60% w/w produced a sample that had the highest moisture uptake and trehalose did not recrystallize, even when exposed to 90% RH.

The DVS profiles of the formulation containing 20% w/w L-isoleucine were very similar to those of the 20% Leu sample, displaying similar DVS parameters, as shown in Table 3. 40% Iso and 60% Iso samples were more moisture resistant than their corresponding L-leucine formulations, as suggested by their higher critical relative humidities and maximum moisture uptakes. The mass change at the end of the desorption cycle for the 20% Iso and 40% Iso samples were similar to those demonstrated by their L-leucine counterparts.

3.6. XPS

X-ray photoelectron spectroscopy (XPS) was used to determine the surface chemical composition of the spray dried formulations, as it has been shown to have a sampling depth of 1–10 nm [33]. Table 4 summarizes the atomic elemental compositions on the surface of the particles of various formulations and the estimates of the surface molar fractions of the components of the formulations (for relative comparisons of the various formulations). 100% Leu and 100% Iso samples have similar carbon, oxygen and nitrogen contents, as expected, as L-leucine and L-isoleucine have the same molecular formula. Relative to the pure amino acids, 100% Tre has a higher oxygen content, lower carbon content and no nitrogen atoms. The surface atomic elemental compositions of the particles were plotted to enable easy comparison of the formulations (Fig. 5a–c). The surface content of nitrogen was used to estimate the amino acids content on the surface of the particles. Formulations with 20% w/w L-leucine/L-isoleucine have similar trehalose to amino acid surface molar fractions. However, increasing the amino acid contents to 40–60% w/w resulted in the observation of lower amino acid content on the surface for the L-leucine-containing formulations compared to their L-isoleucine counterparts. This is reflected by the lower nitrogen and carbon contents and higher oxygen contents on the surface for formulations comprising L-leucine, when compared to those of L-isoleucine.

3.7. Particle size

Table 5 shows the D10, D50, D90 and span of the spray dried formulations. The 100% Tre sample has D10, D50 and D90 of 1.00 ± 0.01 μm, 2.04 ± 0.03 μm and 3.98 ± 0.17 μm respectively, and a span of 1.46 ± 0.06. Adding 20% w/w of L-leucine to the formulation did not have any impact on the particle size of the resulting spray dried powder (*t*-test; p^{D90} = 0.205; p^{span} = 0.378). However, further increasing the L-leucine content to 40–60% w/w, increased the D50 and D90 values, and widened the range of the particle size distribution (ANOVA; *p* values for D50, D90 and span were all <0.0001). A similar trend in the particle size was observed when different amounts of L-isoleucine were added to the formulation, instead of L-leucine (ANOVA; *p* values for D50, D90 and span were all <0.0001).

3.8. SEM

Scanning electron microscopy was utilized to understand the morphology of the spray dried formulations before and after storage. Fig. 6 illustrates the SEM images of the spray dried formulations upon spray drying. The SEM images of unprocessed trehalose, L-leucine and L-isoleucine are shown in Fig. S5 (supplementary information) for comparison purpose. The SEM image of the 100% Tre sample shows fused particles. This could be due to the hygroscopic nature of the sample, resulting in the particles absorbing moisture quickly and

fusing during the SEM sample preparation process before imaging. Spray drying neat L-leucine or L-isoleucine resulted in irregular shaped particles.

Particles in samples with 20% w/w L-leucine or L-isoleucine have rough surfaces and were spherical in shape. The particles have diameters ranging from submicron to about 5 μm. Increasing the L-leucine or L-isoleucine content to 40–60% w/w resulted in the production of heterogeneously shaped particles with a wider size distribution. The 40% Leu and 60% Leu samples consist of small shrivelled raisin-like particles (submicron to about 3 μm) and large elongated shell-like particles (> 3 μm). Some of the larger shell-like particles were found to encase smaller shrivelled particles.. 40% Iso and 60% Iso particles have slightly different morphologies compared to their L-leucine counterparts. The shell-like particles were more common in the L-isoleucine samples and they were rounder in shape compared to those of samples comprising L-leucine. Moreover, the encasement of the small particles was not that common in the L-isoleucine (40 – 60% w/w) containing samples compared to those of formulations with L-leucine.

The SEM images of the various spray dried formulations after 28 days of storage at 25 °C/50% RH, are depicted in Fig. 7. The 100% Tre sample had undergone deliquescence and lost its structural integrity on storage, resulting in the formation of irregularly shaped large masses. Formation of solid bridges was observed in the 20% Leu and 20% Iso samples after 28 days of storage. However, samples containing 40–60% w/w of L-leucine or L-isoleucine appear unaltered after exposure to 50% RH for 28 days.

3.9. In-vitro aerosolization studies

The aerosolization performance of the trehalose containing formulations were assessed upon preparation and after 28 days of storage at 25 °C/50% RH. Fig. 8a and b depict the mass deposition profiles of spray dried formulations with L-leucine and L-isoleucine, respectively on day 0. Table 6 summarizes the key aerosolization parameters of the various formulations before and after storage at 50% RH. Fig. 8 demonstrates that the 100% Tre sample has the lowest emitted fraction (and thus lowest FPFs ≤3 μm and ≤5 μm (recovered)) when compared to other samples. It can be seen from Table 6 that the FPFs ≤3 μm and ≤5 μm (emitted), MMAD and GSD of the 100% Tre sample (day 0) are similar to those of other trehalose-containing formulations (day 0), except 60% Iso sample. This is not surprising, as these aerosolization parameters are not affected by the amount of powder left in the capsule and device. Whilst the FPFs ≤3 μm and ≤5 μm (emitted) are computed using the mass of powder deposited in the adapter, throat and various stages, the MMAD and GSD only consider the mass of powder deposited in the various stages. An aerosolization study was not performed on the 100% Tre sample stored for 28 days, as the stored sample was a deliquesced mass, as reflected by the SEM image (Fig. 7).

Samples containing 20 to 60% w/w L-leucine have different abilities to cope with moisture exposure during storage. Comparisons of the mass deposition profiles of samples 20% Leu, 40% Leu and 60% Leu before and after storage are shown in Fig. 9a, b and c, respectively. Sample 20% Leu experienced a significant reduction in aerosolization performance after storage, as reflected by the increased deposition in stage 1 of the NGI (Fig. 9a), reduction in FPF ≤ 5 μm (emitted) and increased MMAD compared to freshly prepared sample (*t*-test;

p^{stage1} = 0.001; $p^{FPF \leq 5\mu m\ (emitted)}$ = 0.034; p^{MMAD} = 0.001). Moisture-induced impairment in aerosolization performance was also observed for sample 40% Leu after storage compared to when tested initially, as indicated by the change in MMAD (*t*-test; $p^{FPF \leq 5\mu m\ (emitted)}$ = 0.083; p^{MMAD} = 0.010).. There were negligible changes in the aerosolization parameters for sample 60% Leu after storage compared to when it was first prepared and tested (*t*-test; $p^{FPF \leq 5\mu m\ (emitted)}$ = 0.181; p^{MMAD} = 0.579), suggesting that moisture had a negligible effect on the aerosolization performance of the sample.

The aerosolization performances of freshly spray dried samples 20% Iso and 40% Iso are similar (i.e. they have similar MMAD (*t*-test; p = 0.576) and GSD (*t*-test; p = 0.220) values). Increasing the L-isoleucine concentration from 40% w/w to 60% w/w in freshly spray dried formulations resulted in a reduction in the aerosolization performance (i.e. drop in FPF ≤ 5 μm (emitted) (*t*-test; p = 0.035) and increase in MMAD (*t*-test; p = 0.020)). The mass deposition profiles of samples 20% Iso, 40% Iso and 60% Iso, before and after storage, can be found in Fig. 9a, b and c, respectively. Storing sample 20% Iso at 25 °C/50% RH had little impact on the aerosolization parameters (*t*-test; $p^{FPF \leq 5\mu m(emitted)}$ = 0.965;p^{MMAD} = 0.034) of the sample (Table 6). The exposure of samples 40% Iso and 60% Iso to 50% RH during storage resulted in unexpected improvements in the aerosolization performances of the samples. Fig. 9b and c demonstrate that storing samples 40% Iso and 60% Iso resulted in increased deposition in stages 3 and higher, which is reflected in the increases in FPF ≤ 5 μm (emitted) (*t*-test; $p^{40\%\ Iso}$ < 0.0001; $p^{60\%\ Iso}$ = 0.028) and reductions in MMADs compared to freshly prepared samples (*t*-test; $p^{40\%\ Iso}$ = 0.010; $p^{60\%\ Iso}$ = 0.013).

Whilst freshly spray dried samples 20% Leu and 40% Leu possess aerosolization performances that are comparable to their L-isoleucine counterparts, sample 60% Leu (day 0) has better aerosolization properties than its L-isoleucine counterpart. However, samples spray dried with L-isoleucine have greater resistance to moisture-induced impairment in aerosolization performance compared to samples spray dried with leucine.

4. Discussion

The inclusion of L-leucine/L-isoleucine (regardless of concentration) in the spray dried formulations improved the aerosolization performance of trehalose, as reflected by the improvement in the emitted fractions determined in the in-vitro deposition studies. Increasing the amount of amino acids in the spray dried formulations resulted in a change in particle morphology from small spherical particles to larger hollow particles with thin shells, as evidenced by the SEM images and particle size results. The amino acids were present in the crystalline form, while trehalose appeared to be amorphous in all the spray dried formulations. The effect of increasing L-leucine concentration in spray dried trehalose formulations on the morphology of the microparticles was studied by Feng et. al. [35], and their results are similar to those observed in the current study. During the process of spray drying a solution containing trehalose alone, evaporation of water from an atomized droplet will result in a receding surface. The shrinkage of the atomized droplets due to solvent evaporation will lead to increasing trehalose concentration on the surface, and precipitation of trehalose into the amorphous form will occur when the trehalose concentration reaches its solubility limit. The inclusion of ≥40 %w/w L-leucine or L-isoleucine resulted in the

formation of large hollow particles with thin shells. Feng et. al. proposed that the reason for this observation was due to the different solubilities of the two components (trehalose versus amino acid) [35]. The aqueous solubilities of trehalose, L-leucine and L-isoleucine are 68.9 g/100 ml (20 °C) [43], 2.4 g/100 ml (25 °C) and 4.1 g/100 ml (25 °C) [44], respectively. Relative to trehalose, in the drying process, the amino acid will reach saturation at the surface of the atomized droplets earlier than trehalose, and thus will crystallize on the surface of the drying droplets (as demonstrated by the results from the PXRD and XPS measurements). As further water evaporation occurs, the droplets are not able to shrink inward due to the formation of solid outer layers that are enriched with L-leucine or L-isoleucine. Thus, trehalose solidification occurs mainly to the interior of the thin shells of the particles, resulting in the formation of large hollow particles.

It was determined that 20% w/w amino acid was insufficient to protect trehalose from moisture-induced recrystallization when exposed to 50% RH for 28 days. This observation was consistent with the DVS results, whereby it was demonstrated that these samples experienced recrystallization at 50% RH. The incorporation of amino acids at a level of 40 – 60% w/w was determined to be sufficient to prevent recrystallization of trehalose upon exposure to elevated humidity. This could be due to the greater amount of the hydrophobic amino acids on the surface of the particles (compared to those containing 20% w/w amino acid), resulting in repulsion of water and slowing down moisture entry into the particles, thus offering trehalose greater protection against moisture. This hypothesis is supported by the XPS results obtained in this study.

The fresh 60% Iso sample exhibited the poorest aerosolization performance compared to other freshly prepared trehalose formulations with L-leucine or L-isoleucine. Improvement in the aerosolization performance of 60% Iso was observed when the sample was exposed to 50% RH for 28 days. This unexpected improvement in the aerosolization performance upon exposure to 50% RH for 28 days was also noticed for 40% Iso (but not in the other L-leucine or L-isoleucine-containing samples). Particles in fresh samples of 40% Iso and 60% Iso were observed to have a high tendency to stick to foreign surfaces or one another, and the particles were not easily separated. Particles that are highly cohesive exhibit poor aerosolization properties. The stored samples did not present such phenomena. A plausible explanation for this could be due to the differences in the electrostatic charge of the samples after storage. Moisture has been shown to reduce electrostatic charge and the latter influences the aerosolization performance of particles [45]. Thus, moisture adsorption onto the surface of the particles which is composed predominantly of hydrophobic amino acid may result in a reduction in electrostatic charge, while the particles' interior is protected from moisture sorption.

The 40% Iso and 60% Iso samples appeared to be able to withstand higher relative humidity when compared to their L-leucine counterparts. This is demonstrated by the DVS results, as the former samples have higher critical relative humidities and levels of moisture uptake. The reason for this observation could be due to the presence of greater amounts of the hydrophobic amino acid on the surface of the particles containing L-isoleucine compared to those samples containing L-leucine, as indicated by the XPS results above.

There are two theories that have been proposed in the literature to explain surface enrichment of molecules in spray dried particles. The first theory describes how the occurrence of surface enrichment of the molecules is governed by the Peclet number of the solute molecules. The Peclet number (*Pe*) is defined by Equation (1) below [46], where, for a droplet being dried, K is the surface evaporation rate and D is the diffusion rate of the dissolved solute. If, on spray drying, the evaporation rate at the droplet surface is faster than the rate at which the dissolved component diffuses towards the centre, that component will form a shell around the droplet as solvent continues to evaporate. The non-displacement of L-leucine and L-isoleucine into the droplet centre (due to crystallization), and their accumulation on the surface of the spray dried particles, can be considered characteristic of a system where the ratio of time for solute diffusion from the droplet surface to its centre to time for droplet drying is greater than 1, i.e. $Pe > 1$.

$$Pe = \frac{K}{8D} \quad (1)$$

However, the evaporation rate of the spray dried formulations containing L-leucine and L-isoleucine should be similar, as they were exposed to the same drying conditions. L-leucine and L-isoleucine have the same molecular weight, and thus it can be assumed that the diffusion rates of both amino acids would be very similar. Hence, the Peclet number for L-leucine and L-isoleucine would also be very similar and it is highly unlikely that the differences observed between formulations containing L-leucine and L-isoleucine are related to differences in Peclet numbers of the amino acids.

The other theory describing surface enrichment during spray drying is related to crystallization of the component that reaches saturation earlier on the surface of the droplets, which has been described above [35]. However, while both amino acids have a lower solubility than trehalose, L-leucine (2.4 g/100 ml (25 °C)) has a lower solubility than L-isoleucine (4.1 g/100 ml (25 °C)) [44]. Thus, the phenomena of surface enrichment with amino acid would be expected to be more pronounced for formulations containing L-leucine, which contradicts the findings (i.e greater surface enrichment for spray dried formulation with L-isoleucine than formulations with L-leucine) observed in this study. It is possible that the discrepancy relates to differences in the crystallization of the two amino acids at the particle surface as they dry. Crystallization consists of the subprocesses of nucleation and crystal growth [47,48]. The degree of supersaturation before nucleation occurred (which affects the nucleation rate) and crystal growth rate could be different for the two amino acids and may have contributed to L-isoleucine crystallizing faster than L-leucine in the drying droplets. Future studies to understand the crystallization kinetics of L-leucine and L-isoleucine and also other factors that may contribute to the observed differences are warranted to provide further insight.

It is demonstrated in this study that the inclusion of ≥40 %w/w of L-leucine or L-isoleucine was able to prevent recrystallization of trehalose and maintained or improved the aerosolization performance of the formulations when exposed to 50 %RH. The feasibility of harnessing the potential of the combination of trehalose and the amino acid (i.e. L-leucine or

L-isoleucine) in protecting spray dried protein dry powder against moisture induced denaturation upon storage will be investigated in future studies.

5. Conclusions

The amino acids investigated, L-leucine and L-isoleucine were able to enhance the aerosolization performance of trehalose when included into the spray dried formulations. A low amount of the amino acid(s) (i.e. 20% w/w in this study) was not sufficient to mitigate moisture-induced recrystallization of trehalose when exposed to 25 °C/50% RH. 40% w/w or more of the amino acid(s) was needed in the formulation in order to protect the particles against moisture, and thus prevent moisture-induced recrystallization and reduction in aerosolization performance. Samples spray dried with ≥40% w/w L-isoleucine were more resistant to moisture-induced recrystallization than their L-leucine counterparts, when exposed to elevated humidity conditions. Overall, this study demonstrated that both L-leucine and L-isoleucine were effective in enhancing aerosolization performance and mitigating moisture-induced reduction in aerosolization performance in spray dried trehalose formulations. However, for the same concentrations incorporated in the formulations, L-isoleucine appears to have a superior moisture protectant effect compared to L-leucine. Further studies are required to investigate whether this apparent superior effect of L-isoleucine relative to L-leucine is generally applicable in other co-spray dried formulations, and to elucidate any differences in crystallization behaviour between the two amino acids, which may explain differing effectiveness in spray dried formulations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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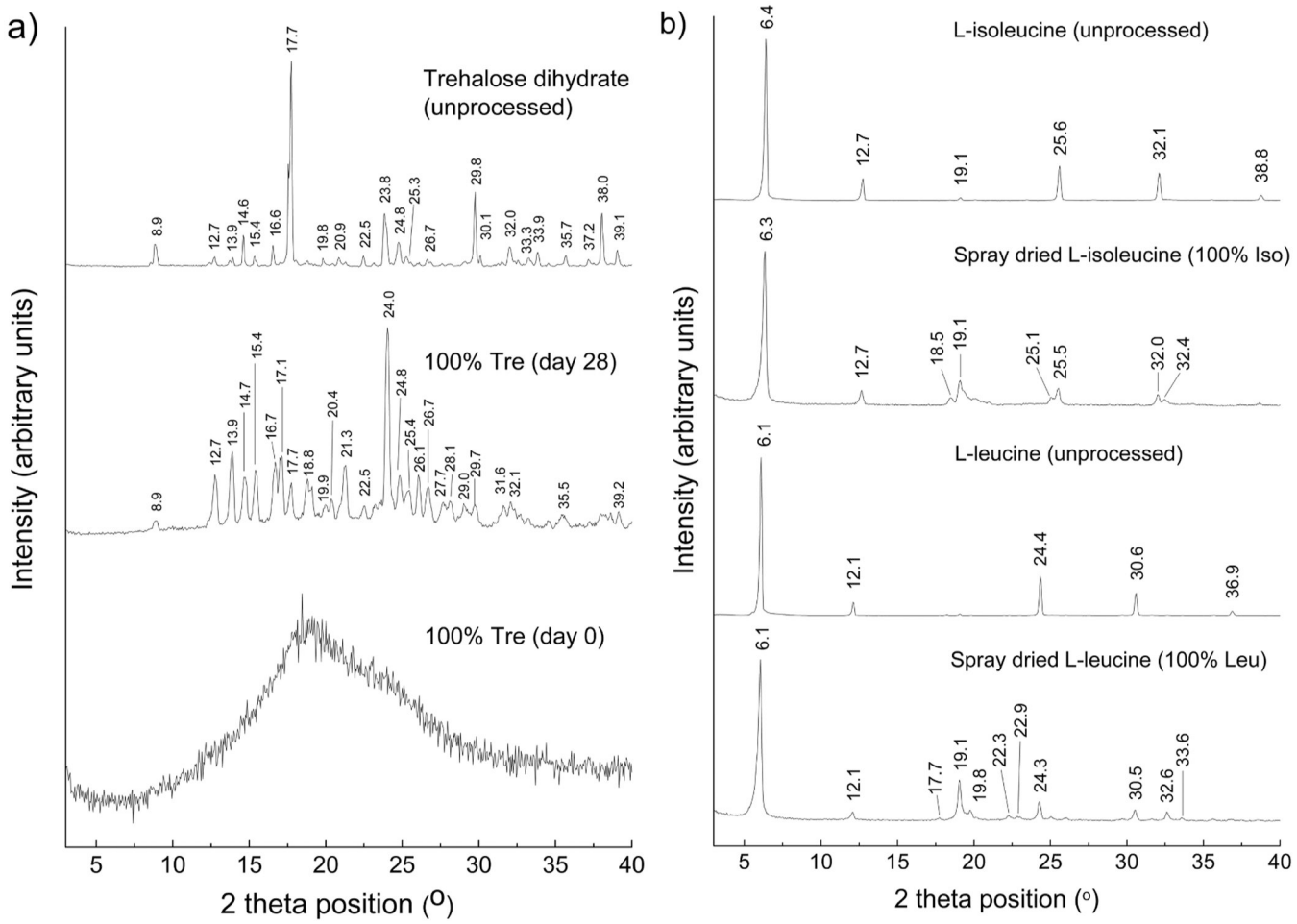

Fig. 1.

PXRD patterns of (a) unprocessed trehalose dihydrate, spray dried trehalose before and after storage at 50% RH/25 °C for 28 days and (b) unprocessed and spray dried L-leucine and L-isoleucine.

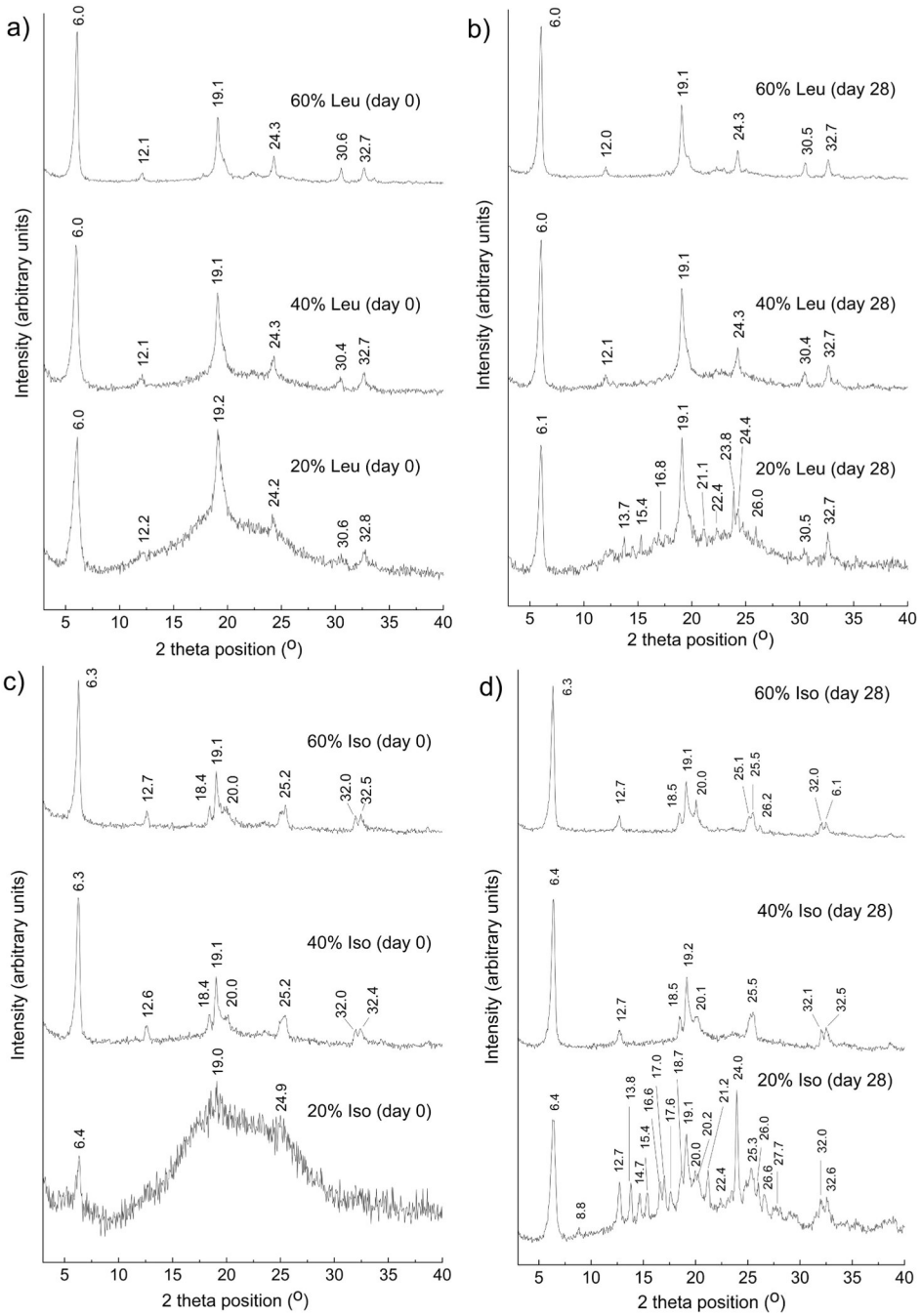

Fig. 2.

PXRD patterns of spray dried formulations containing different amounts of L-leucine (on (a) day 0 and (b) day 28) and L-isoleucine (on (c) day 0 and (d) day 28). Samples were stored at 50% RH/25 °C for 28 days.

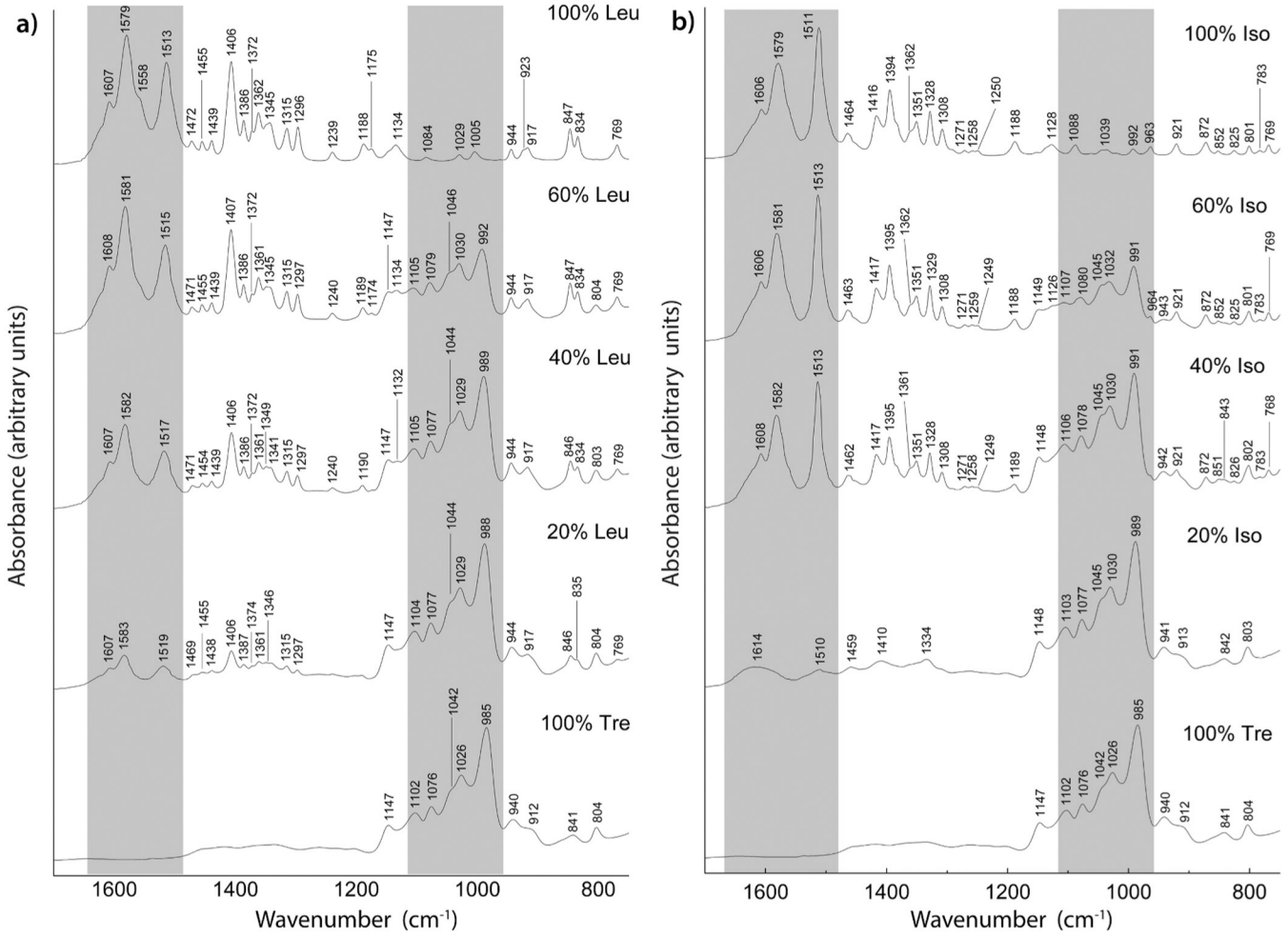

Fig. 3.

Infrared spectra of (a) L-leucine- and (b) L-isoleucine-containing spray dried formulations.

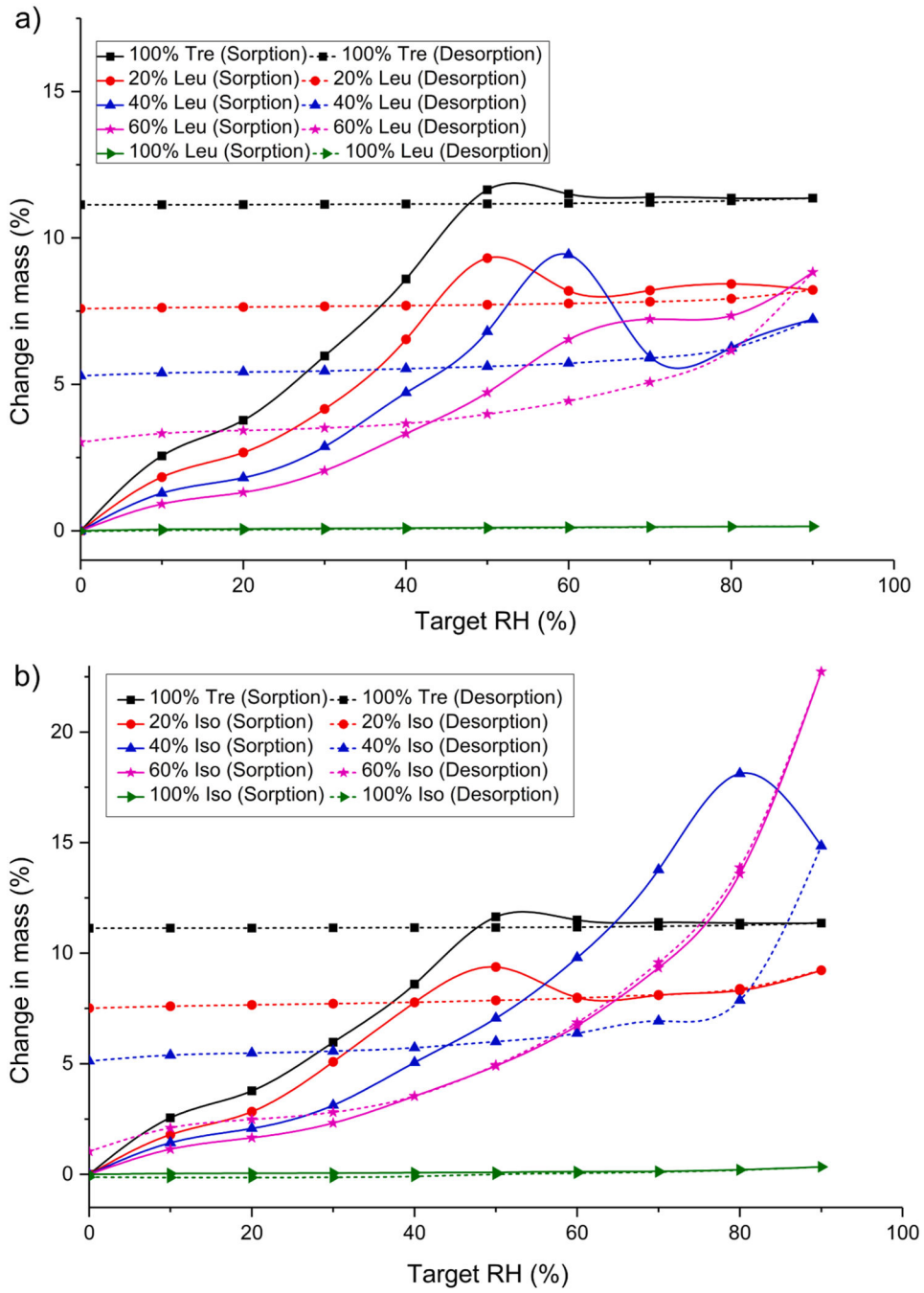

Fig. 4.

Vapour sorption and desorption isotherms of spray dried trehalose formulations containing (a) L-leucine and (b) L-isoleucine. The vapour sorption and desorption isotherms of spray dried trehalose (100% Tre), spray dried L-leucine (100% Leu) and spray dried L-isoleucine (100% Iso) were included for comparison purpose.

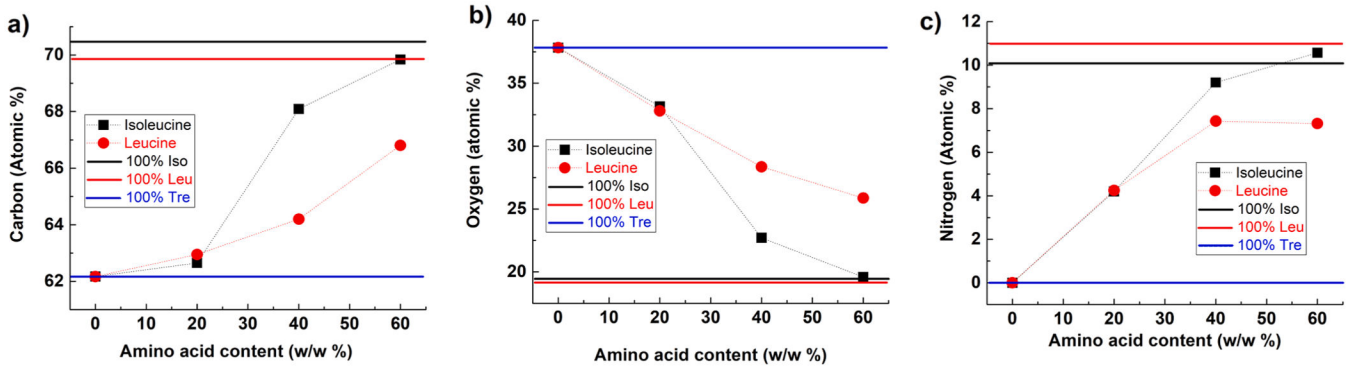

Fig. 5.

Plots of (a) carbon, (b) oxygen and (c) nitrogen surface chemical composition (%) as a function of the amount of amino acids in the spray dried formulations.

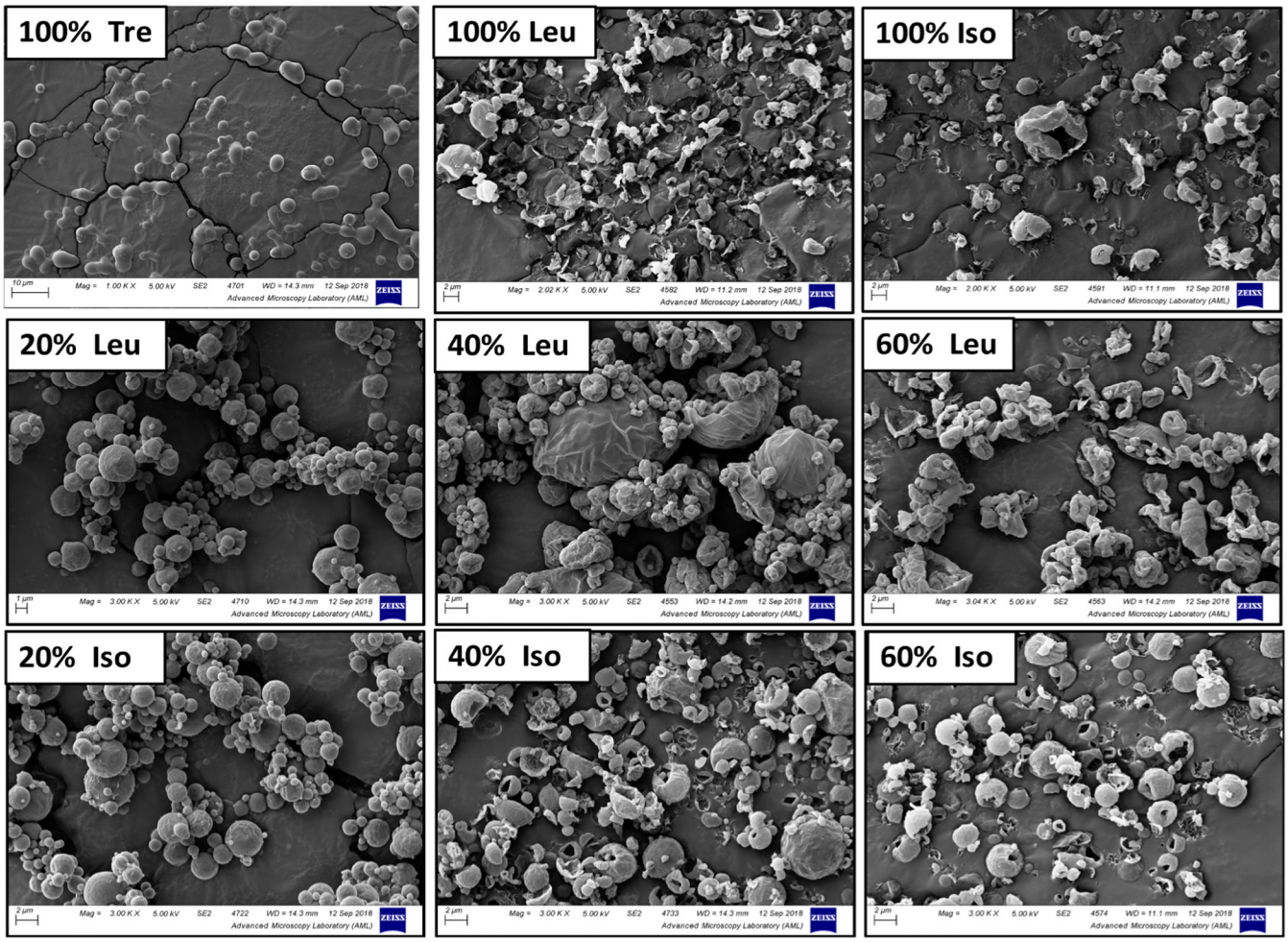

Fig. 6.

SEM images of the spray dried formulations on day 0. Images for samples 100% Tre and 20% Leu have scale bars equivalent to the length of 10 μm and 1 μm, respectively. The images for all the other sample have scale bars equivalent to the length of 2 μm.

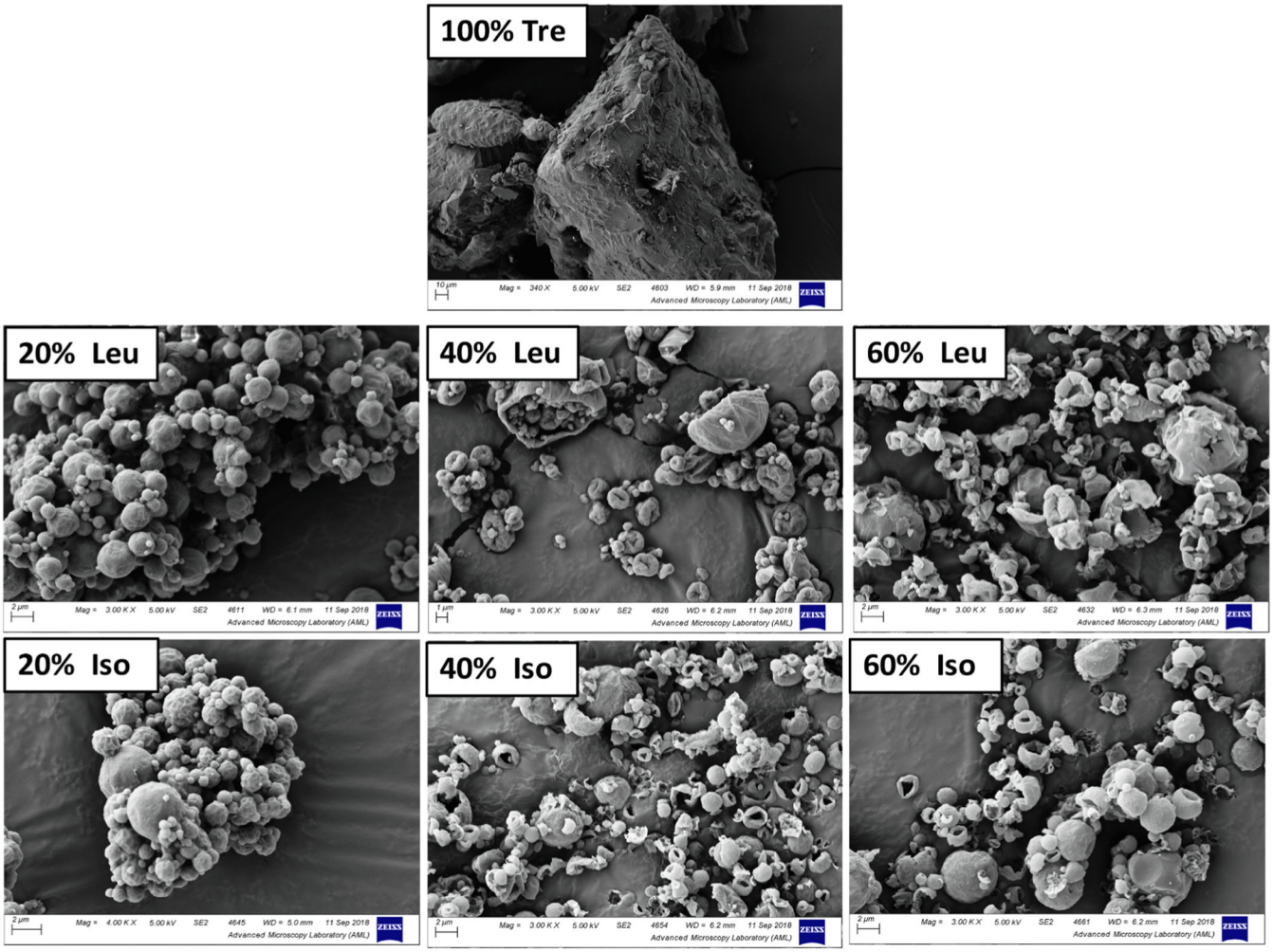

Fig. 7.

SEM images of the spray dried formulations stored at 50% RH/25 °C for 28 days. Images for samples 100% Tre and 40% Leu have scale bars equivalent to the length of 10 μm and 1 μm, respectively. The images for all the other sample have scale bars equivalent to the length of 2 μm.

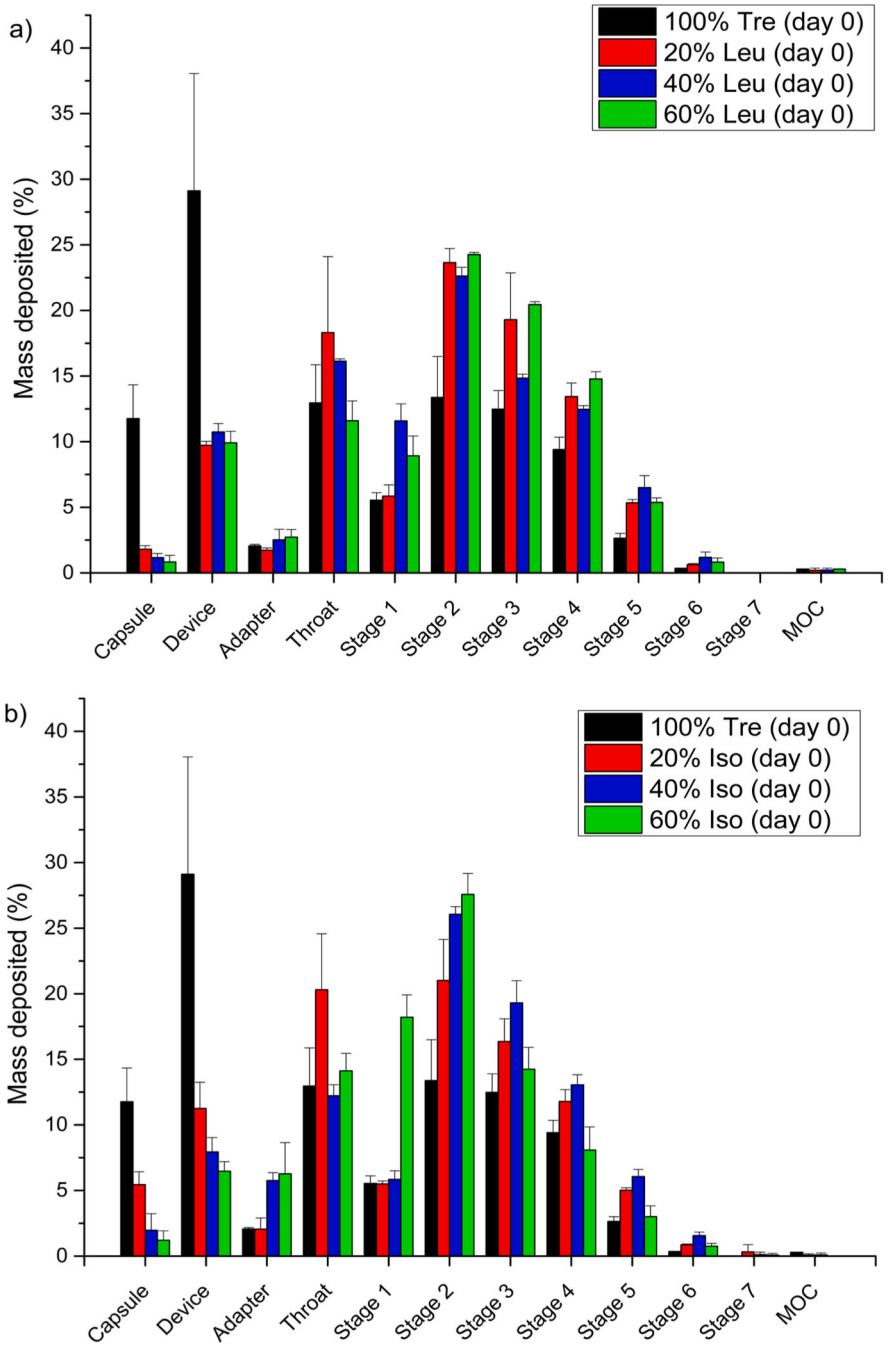

Fig. 8.

NGI mass deposition profiles of spray dried formulations containing (a) L-leucine and (b) L-isoleucine on day 0 (n = 3).

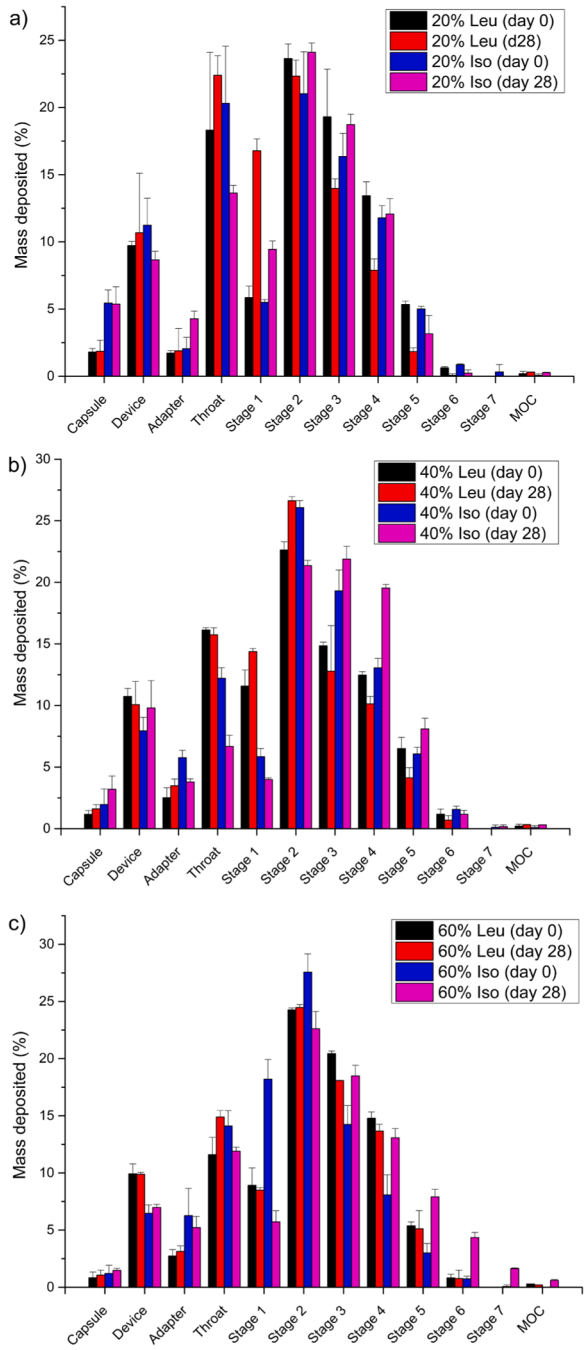

Fig. 9.

NGI mass deposition profiles of spray dried formulations containing (a) 20% w/w, (b) 40% w/w and (c) 60% w/w amino acids before and after storage at 50% RH/25 °C for 28 days (n = 3).

Table 1

Glass transition temperatures (T_gs) of the freshly prepared spray dried formulations. The samples were not subjected to any drying before determination of the T_gs.

	Glass transition temperature (°C)
100% Tre	122.44 ± 0.31
20% Leu	122.98 ± 0.13
40% Leu	120.77 ± 0.76
60% Leu	124.53 ± 7.26
20% Iso	113.09 ± 0.14
40% Iso	120.07 ± 0.20
60% Iso	120.15 ± 0.67

Table 2

Residual moisture contents (determined using TGA) of the spray dried formulations before and after storage at 50% RH, 25 °C.

	Moisture content on day 0 (%)	Moisture content on day 28 (%)
100% Tre	3.41 ± 0.13	7.06 ± 0.35 *
20% Leu	3.55 ± 0.24	7.96 ± 0.09 *
40% Leu	3.05 ± 0.17	5.30 ± 0.06
60% Leu	2.43 ± 0.09	3.34 ± 0.18
20% Iso	4.28 ± 0.10	7.32 ± 0.40 *
40% Iso	3.35 ± 0.06	6.95 ± 0.38
60% Iso	1.57 ± 0.09	2.82 ± 0.12

* Recrystallization of trehalose was observed.

Table 3

Key parameters obtained from the dynamic vapour sorption and desorption isotherms of the spray dried formulations.

	Critical relative humidity (%)	Maximum moisture uptake (%)	Mass change at the end of desorption cycle (%)
100% Tre	50	11.6	11.4
20% Leu	50	9.3	7.6
40% Leu	60	9.4	5.3
60% Leu	70	7.2	3.0
100% Leu	Not applicable	0.2	Negligible
20% Iso	50	9.4	7.5
40% Iso	80	18.1	5.1
60% Iso	90 *	22.7	1.0
100% Iso	Not applicable	0.3	Negligible

* Trehalose remained amorphous at the end of the desorption cycle.

Table 4

The atomic elemental compositions and estimated surface molar fractions of trehalose and amino acid for the spray dried formulations.

Formulation	Atomic elemental composition (%)			Estimated surface molar fraction* (%)		
	C	O	N	Trehalose	L-leucine	L-isoleucine
100% Tre	62.20	37.80	0.00	100	-	-
20% Leu	62.95	32.80	4.25	62	38	-
40% Leu	64.20	28.36	7.43	32	68	-
60% Leu	66.81	25.87	7.32	33	67	-
100% Leu	68.86	19.15	10.99	-	100	-
20% Iso	62.70	33.20	4.20	58	-	42
40% Iso	68.10	22.70	9.20	9	-	91
60% Iso	69.80	19.60	10.60	0	-	100
100% Iso	70.47	19.45	10.08	-	-	100

*Determined from the nitrogen (N) content of the sample.

Table 5

Particle size distributions of the freshly prepared spray dried formulations.

	d10 (μm)	d50 (μm)	d90 (μm)	Span
100% Tre	1.00 ± 0.01	2.04 ± 0.03	3.98 ± 0.17	1.46 ± 0.06
20% Leu	1.07 ± 0.03	2.18 ± 0.06	4.17 ± 0.09	1.42 ± 0.02
40% Leu	1.12 ± 0.01	2.68 ± 0.13	6.32 ± 0.34	1.94 ± 0.03
60% Leu	1.13 ± 0.01	2.51 ± 0.01	6.34 ± 0.13	2.08 ± 0.05
100% Leu	1.17 ± 0.01	2.44 ± 0.03	6.09 ± 0.17	2.01 ± 0.06
20% Iso	0.96 ± 0.01	2.01 ± 0.05	3.83 ± 0.12	1.43 ± 0.02
40% Iso	1.18 ± 0.01	2.39 ± 0.01	5.21 ± 0.15	1.68 ± 0.06
60% Iso	1.42 ± 0.04	3.36 ± 0.10	7.71 ± 0.26	1.87 ± 0.02
100% Iso	1.40 ± 0.02	3.01 ± 0.05	6.90 ± 0.10	1.83 ± 0.01

Table 6

Key parameters describing the aerosolization performances of the spray dried formulations before and after storage at 25 °C/50% RH (n = 3).

	Emitted fraction (% w/w)	FPF ≤ 3 μm (recovered) (%)	FPF ≤ 3 μm (emitted) (%)	FPF ≤ 5 μm (recovered) (%)	FPF ≤ 5 μm (emitted) (%)	MMAD (μm)	GSD
100% Tre (Day 0)	59.12 ± 8.42	21.40 ± 2.20	36.36 ± 2.01	34.90 ± 4.99	59.10 ± 2.69	3.06 ± 0.08	1.83 ± 0.08
20% Leu (Day 0)	88.47 ± 0.56	33.08 ± 3.74	37.40 ± 4.19	56.80 ± 5.67	64.21 ± 6.36	3.09 ± 0.07	1.66 ± 0.03
20% Leu (Day 28)	87.46 ± 5.00	19.40 ± 1.54	22.17 ± 0.64	39.16 ± 2.41	44.77 ± 0.30	4.14 ± 0.11	1.90 ± 0.02
40% Leu (Day 0)	88.09 ± 0.59	30.69 ± 2.01	34.84 ± 2.20	51.27 ± 2.87	58.2 ± 3.13	3.37 ± 0.13	2.05 ± 0.20
40% Leu (Day 28)	88.32 ± 1.58	24.00 ± 1.05	27.16 ± 0.75	46.51 ± 2.61	52.64 ± 2.07	3.90 ± 0.09	2.14 ± 0.24
60% Leu (Day 0)	89.24 ± 1.32	35.51 ± 1.17	39.80 ± 1.63	59.49 ± 1.28	66.68 ± 2.05	3.13 ± 0.08	1.77 ± 0.04
60% Leu (Day 28)	88.63 ± 0.36	33.09 ± 1.85	37.33 ± 2.17	56.50 ± 1.71	63.75 ± 2.08	3.18 ± 0.11	1.72 ± 0.01
20% Iso (Day 0)	83.30 ± 2.39	29.48 ± 1.85	35.37 ± 1.35	50.23 ± 4.84	60.23 ± 4.28	3.08 ± 0.07	1.68 ± 0.05
20% Iso (Day 28)	85.97 ± 0.65	28.56 ± 2.44	33.20 ± 2.60	51.90 ± 1.73	60.36 ± 1.59	3.38 ± 0.12	1.73 ± 0.02
40% Iso (Day 0)	90.09 ± 0.30	34.34 ± 0.80	38.11 ± 0.76	60.03 ± 0.53	66.63 ± 0.63	3.11 ± 0.08	1.63 ± 0.02
40% Iso (Day 28)	86.99 ± 1.79	44.98 ± 1.60	51.70 ± 1.00	67.90 ± 1.53	78.06 ± 0.80	2.62 ± 0.04	1.72 ± 0.02
60% Iso (Day 0)	92.33 ± 1.32	21.43 ± 4.14	23.19 ± 4.23	44.84 ± 5.96	48.53 ± 5.97	4.19 ± 0.26	1.95 ± 0.05
60% Iso (Day 28)	91.54 ± 0.41	40.72 ± 1.03	44.48 ± 1.01	63.28 ± 1.12	69.13 ± 0.95	2.76 ± 0.11	1.78 ± 0.04