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Crystallizing Amino Acids as Bulking Agents in Freeze-Drying

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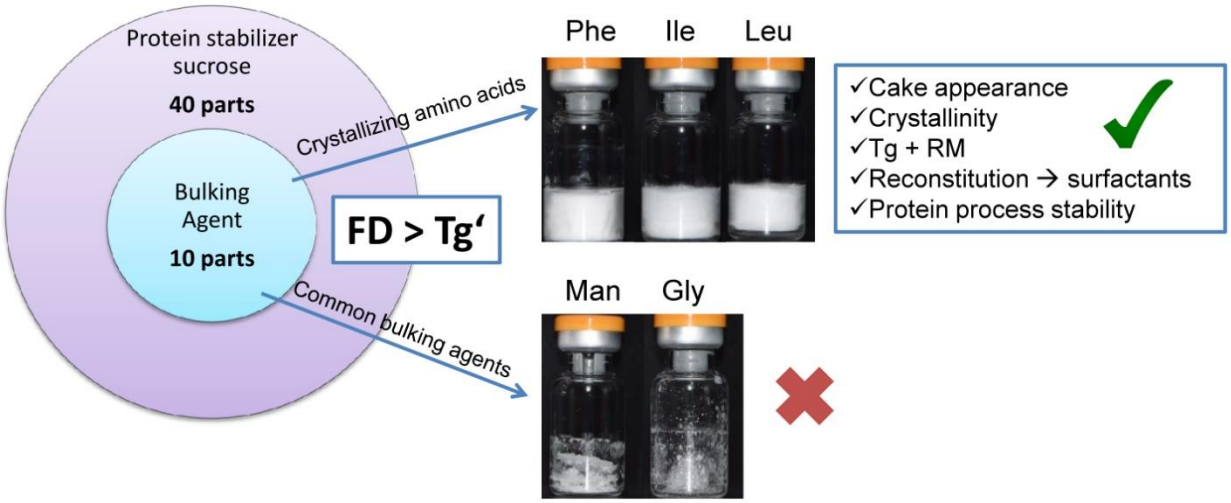
Graphical Abstract



Abstract

Bulking agents as mannitol (Man) and glycine (Gly) require high bulking agent to stabilizer ratios to ensure their crystallization during the freeze-drying process. The aim of this study was to investigate several amino acids (AA) as potential alternative bulking agents in low AA to sucrose (Suc) ratios. A fast freeze-drying process was performed above the collapse temperature (Tc) of the amorphous phase challenging the crystalline AA scaffold. Lyophilizates and liquid formulations were characterized by differential scanning calorimetry. Karl-Fischer titration and X-ray powder diffraction as well as macroscopic cake appearance and reconstitution times were evaluated. Stability of a monoclonal antibody was investigated by UV-Vis spectroscopy, light obscuration and size exclusion chromatography. Phenylalanine (Phe), leucine (Leu) and

isoleucine (Ile) crystallized upon freeze-drying at 5/45 and 10/40 AA/Suc ratios. 2.5/47.5 AA/Suc ratio showed less pronounced crystallization. Crystallization peaks were not suppressed by 2 or 50 mg/mL antibody in Leu and Ile lyophilizates. Reconstitution times could be improved by addition of surfactant. No signs of protein aggregation were detected after freeze-drying. Man and Gly yielded inacceptable cake appearance when dried with the fast freeze-drying cycle in such low bulking agent to Suc ratios. Leu, Ile and Phe can be used as alternate bulking agents at lower bulking agent to Suc ratios compared to Man or Gly. The selected AAs provided promising cake characteristics and good protein process stability.

Keywords

Freeze-drying, lyophilization, amino acid, leucine, isoleucine, phenylalanine, mannitol, bulking agent, crystallization, sucrose, protein stability, glass transition, collapse

Abbreviations

AA	Amino acid
AU	Absorbance unit
aFD	After freeze-drying
API	Active pharmaceutical ingredient
AUC	Area under the curve
bFD	Before freeze-drying
FD	Freeze-drying
Gly	Glycine
His	Histidine
HP-SEC	High performance size exclusion chromatography
HPW	Highly purified water
Ile	Isoleucine
Leu	Leucine
LO	Light obscuration
mAb	Monoclonal antibody
Man	Mannitol
DSC	Differential scanning calorimetry
Met	Methionine
Phe	Phenylalanine
PS 20 / 80	Polysorbate 20 / 80
RM	Residual Moisture
SD	Standard deviation
Suc	Sucrose
Tc	Collapse temperature
Te	Eutectic temperature
Tg	Glass transition temperature of the freeze-dried cake
Tg’	Glass transition temperature of the maximally freeze-concentrated solution
Tp	Product temperature
XRPD	X-Ray powder diffraction

1. Introduction

Freeze-drying of biopharmaceuticals is one approach to prolong their shelf life [1]. In order to achieve acceptable cake appearance, drying below the glass transition temperature of the freeze-concentrate (Tg') or at least below the collapse temperature (Tc) is recommended [2,3]. Operating at product temperatures (Tp) below Tg' and Tc may correspond to long drying times depending on the choice of excipients and the protein concentration [2–4]. In fact, high protein concentrations were reported to increase both, Tg' and specifically Tc, enabling faster drying at higher Tp [3]. Drying at even higher Tp would be favourable, especially at low protein concentration, but the resulting macrocollapse would result in rejected batches [5]. Collapse of freeze-dried matrices was already reported not to be detrimental for protein stability in some cases [6–10]. The use of crystalline bulking agents can solve this problem. Bulking agents are typically used to cover undesired cake appearance as "fillers" if the API dosage is low and they can also help to adjust tonicity [11–13]. Drying of crystalline bulking agents is not limited by Tg', but by their eutectic melting temperature (Te) which is markedly higher [14]. Since the crystalline excipients do not provide stabilization of the API like the amorphous cryo- and lyoprotectants, low concentrations are preferred. Unfortunately, the crystallisation is affected by the other formulation components such as the sugars used as cryo-and lyoprotectant, buffer components and the protein drug itself [12,15–17]. Mannitol (Man) as one of the common used bulking agents typically requires a 4/1 ratio of Man to sucrose (Suc) for complete crystallization [18,19]. Incomplete crystallization can lead to subsequent crystallization during storage which might induce instability reactions or lead to inadequate cake appearance due to collapse of the whole matrix. Man is also prone to crystallize in different polymorphs or pseudopolymorphs (α, ß, δ, hemihydrate) depending on process and formulation [20,21]. Highly concentrated protein formulations can facilitate incomplete crystallization despite high Tc of the formulation. Thus, the essential ratio of bulking agent to amorphous stabilizer needs to be established in order to ensure crystallinity of the bulking agent. A lower bulking agent concentration would hence be favoured in exchange for a higher stabilizer concentration without loss of cake appearance if dried above Tc in a fast feeze-drying cycle.

A few amino acids (AA) are known for their crystallization tendency upon freeze-drying. Table 1 gives an overview of the common AAs ordered by hydrophobicity. Their crystallization tendency upon freeze-drying was investigated in a few studies. The most comprehensive studies are based on glycine (Gly) [6,22–30]. Although it shows a higher crystallization tendency than Man [24], the essential Gly/Suc ratio to induce Gly crystallization is still approximately 5/2 [22].

Recently, a variety of AAs was compared to Suc regarding their potential to stabilize proteins [31]. Almost all AAs showed beneficial results upon two months storage of lyophilizates at 50 °C. Crystallinity of the products was not evaluated. Arginine, glutamine, glycine and histidine showed crystallization tendency with substantial impact of the counter-ion in case of arginine combined with citric acid [32–36].

Table 1. Physico-chemical characteristics of AAs and Man. They are sorted by their hydrophobicity. Man was included for comparison purposes. "Crystalline" refers to the crystallization of the AA or Man.

Amino Acid / Additive	Hydro-phobicity [37]	Sample morphology aFD of		pI [38]	pk_{a1} (COOH) at 25 °C [38–40]	pk_{a2} (NH_4^+) at 25 °C [38,41]	Solubility at 25 °C [g/100 g] [40,42]
		pure AA solution [43,44]	AA/Suc 2.5/47.5 solutions*** [8]				
Isoleucine	1.000	Crystalline**	Crystalline	6.05	2.32	9.76	4.12
Phenylalanine	0.951	Crystalline*	Amorphous	5.49	2.16	9.18	2.97
Valine	0.923	Crystalline*	-	6.00	2.29	9.74	8.86
Leucine	0.918	Crystalline**	Crystalline	6.01	2.33	9.74	2.19
Methionine	0.811	Crystalline**	Crystalline	5.74	2.13	9.28	3.38
Alanine	0.806	Crystalline*	-	6.11	2.35	9.87	16.51
Glycine	0.770	Crystalline*	Amorphous	6.06	2.35	9.78	24.99
Cysteine	0.721	Crystalline*	-	5.05	1.92	10.78	-
Proline	0.678	Crystalline*	-	6.30	2.95	10.65	162.3
Threonine	0.634	Crystalline*	-	5.60	2.09	9.10	-
Serine	0.601	Crystalline*	-	5.68	2.19	9.21	5.02
Histidine	0.548	Tg = 37 °C**	Amorphous	7.60	1.80	9.33	4.29
Glutamic acid	0.458	-	-	3.15	2.10	9.47	0.84
Glutamine	0.430	-	-	5.65	2.17	9.13	-
Aspartic acid	0.417	-	-	2.85	1.99	9.90	0.50
Lysine	0.263	Tg = 68 °C*	-	9.60	2.16	9.78	-
Arginine	0.000	Tg = 42 °C	Amorphous	10.76	1.82	8.99	-
Mannitol	-	Crystalline***	-	-	13.5	-	21.6

AA – Amino acid, aFD – after freeze-drying, total solid contents: * 0.24 M, **0.12 M, ***50 mg/mL

Phenylalanine is a promising candidate. Vacuum-drying time could be accelerated if Phe was included into Suc/rhG-CSF formulations due to Phe crystallization, without losing protein stability [45]. Phe, leucine (Leu), isoleucine (Ile) and methionine (Met) combined with trehalose and sucrose showed crystalline structures after "aggressive" freeze-drying [8]. A low AA/stabilizer ratio of only 2.5/47.5 was sufficient to detect crystallization peaks via XRPD. An acceptable macroscopic appearance and considerably lower residual moisture levels compared to other AAs were found. Consequently, in the present study, Phe, Leu, Ile and Met were evaluated as potential crystalline bulking agents that could be used at much lower concentrations compared

to the typical bulking agents Man and Gly. Additionally, the crystalline AA scaffolds should be mechanically stable enough to enable fast drying at high Tp above Tc of the amorphous phase. The cycle may require optimization including a potential annealing step to foster crystallization in terms of best balance between fast process and satisfying AA crystallization [26,46,47].

One of the drawbacks of the AAs with a higher crystallization tendency is their poor solubility in water due to their mainly hydrophobic character (Table 1). Their use in high concentrations is hence additionally limited. Furthermore, AAs can react sensitively on changes of the pH environment because of their dipolar character. Consequently, also the effect of pH on the bulking agent properties of the AAs was to be evaluated.

For this study, three AA/Suc ratios with increasing AA content were investigated: 2.5/47.5, 5/45 and 10/40 with Phe, Ile, Leu, and Met. The aim of this study was to establish alternative bulking agents that (1) crystallize in low bulking agent to Suc ratio and (2) crystallize at fast freeze-drying cycle conditions above the formulations' Tg'. It was further focused on the impact of pH, annealing step, chamber pressure and protein concentration on AA crystallization and other cake characteristics as macroscopic appearance, glass transition temperature (Tg) or reconstitution time.

2. Materials and Methods

2.1. Materials

Stock solutions of L-glycine (m/V) (VWR International, Ismaning, Germany),L-isoleucine (m/V) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), L-leucine (m/V) (Sigma-Aldrich Chemie GmbH), L-methionine (m/V) (Sigma-Aldrich Chemie GmbH), L-phenylalanine (m/V) (Sigma-Aldrich Chemie GmbH), D-mannitol (m/V) (VWR International), Poloxamer 188 (m/m) (Kolliphor® P188, BASF AG, Ludwigshafen am Rhein, Germany), polysorbate 20 (m/m) (Tween® 20, VWR International), polysorbate 80 (m/m) (Tween® 80, VWR International), and sucrose (m/V) (Sigma-Aldrich Chemie GmbH) were prepared in highly purified water (HPW) or in 10 mM L-histidine (Sigma-Aldrich Chemie GmbH) buffer pH 6.0 if protein was included in the formulation. Stock solutions of the amino acids of up to 35 mg/mL were used. The pH of aqueous solutions without histidine was adjusted with sodium hydroxide or hydrochloric acid. Different bulking agent/sucrose (AA/Suc or Man/Suc) weight ratios of equal total solid content (50 mg/mL) were investigated: 2.5/47.5, 5/45 and 10/40 [mg/mL]. Bulking agents were amino acids or mannitol. The formulations were filtered with 0.2 µm polyethersulfone membrane syringe filters (VWR International GmbH) prior to use.

A monoclonal IgG_1 antibody (MW ~ 150 kDa, $\varepsilon = 1.49$ mL mg^{-1} cm^{-1}, referred to as mAb) served as model protein. A 81 mg/mL (for 5/45 AA/Suc formulations) or 94 mg/mL (for 10/40 AA/Suc formulations) mAb stock solution in 10 mM L-histidine buffer pH 6.0 was prepared by tangential flow filtration in Minimate™ capsules (30 kDa Omega membrane, PALL Life Science, Port Washington, NY, USA). The protein was formulated at 2 mg/mL and 50 mg/mL leading to total solid contents of 52 mg/mL and 100 mg/mL, respectively.

2.2. Methods

2.2.1. Lyophilization

Lyophilization stoppers (Westar®, B2-TR coating, West Pharmaceutical Services Deutschland GmbH & Co. KG, Eschweiler, Germany) and ISO 2R Vials (Fiolax®, Schott AG, Mainz, Germany) were cleaned with HPW and dried for 8 hours at 100 °C and 40 °C, respectively. 1.2 mL sample per vial were investigated. The two outer rows of vials in the batch were not used for subsequent analysis: this is due the fact they are also heated by radiation from chamber

walls, beside the shelf, and, thus, additional and uncontrolled overheating may occur. Thermocouples were placed center bottom in different vials within the freeze-dryer.

The formulations were freeze-dried according to the protocols given in Table 2 to

Table 5. At first, the operating conditions were selected in such a way that the limit product temperature is trespassed in all the formulations investigated (Table 2; Table 3), and the influence of the annealing step in the freezing stage was investigated. Then, with the goal of improving cake appearance, the original cycle was modified in such a way that product temperature is lowered: chamber pressure was decreased to reach this goal, at first to 0.52 mbar (Table 4) and, then, to 0.4 mbar (Table 5). The two lab scale freeze-dryers of comparable processing, an FTS Lyostar 3 (SP Scientific, Stone Ridge, USA) (Cycle 3) and an Epsilon 2D-6 LSCplus (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) (Cycle 1, 2 and 4) were used. The end of primary drying was controlled by comparative pressure measurement between Pirani and MKS sensor (FTS) or set manually (Christ). After secondary drying, the vials were stoppered under nitrogen atmosphere at 800 mbar to prevent popping off of the stoppers, crimped with flip-off seals and stored at 2-8 °C.

Table 2. Freeze-drying cycle with annealing step ran on the Christ freeze-dryer (Cycle 1).

Step	Ramp [°C min^{-1}]	Shelf temperature [°C]	Pressure [mbar]	Hold Time [min]
Freezing 1	1	-50	-	60
Annealing	1	-20	-	120
Freezing 2	1	-50	-	120
Primary Drying	1	20	2.2	420
Secondary Drying	1	40	2.2	120

Table 3. Freeze-drying cycle without annealing step ran on the Christ freeze-dryer (Cycle 2).

Step	Ramp [°C min^{-1}]	Shelf temperature [°C]	Pressure [mbar]	Hold Time [min]
Freezing	1	-50	-	120
Primary Drying	1	20	2.2	420
Secondary Drying	1	40	2.2	120

Table 4. Freeze-drying cycle without annealing step and at reduced chamber pressure of 0.52 mbar ran on the FTS freeze-dryer (Cycle 3).

Step	Ramp [°C min^{-1}]	Shelf temperature [°C]	Pressure [mbar]	Hold Time [min]
Freezing	1	-50	-	120
Primary Drying	1	20	0.52	750
Secondary Drying	1	40	0.52	120

Table 5. Freeze-drying cycle without annealing step and at reduced chamber pressure of 0.40 mbar ran on the Christ freeze-dryer (Cycle 4).

Step	Ramp [°C min^{-1}]	Shelf temperature [°C]	Pressure [mbar]	Hold Time [min]
Freezing	1	-50	-	120
Primary Drying	1	0	0.40	1200
Secondary Drying	1	40	0.40	120

2.2.2. Macroscopic Appearance

Macroscopic appearance was evaluated visually and pictures were taken with a Nikon digital camera D5300 (Nikon GmbH, Düsseldorf, Germany) in a black photobox.

2.2.3. Digital Microscopy

Digital microscopy was performed with the digital microscope Keyence VHX-500F (Keyence Corporation, Osaka, Japan). The lyophilizates were extracted from the vial without destruction of the cake. A 50-fold magnification was recorded.

2.2.4. Differential Scanning Calorimetry (DSC)

Glass transition temperatures (Tg) of the lyophilizates were determined with a Mettler Toledo DSC 821e (Mettler-Toledo GmbH, Giessen, Germany). Samples were prepared under controlled humidity conditions (< 10% r. h.). 5 to 15 mg of crushed cake were weighted into aluminum crucibles and sealed hermetically. The modulated DSC scan was conducted from 25 °C to 100 °C or 140 °C at 2°C/min and modulated with an amplitude of ± 1°C every 120 s [48]. The Mettler StarE Software was used for data analysis.

Glass transition temperatures of maximally freeze-concentrated solutions (Tg') were evaluated with the same instrument and additionally with a DSC 204 Phoenix (Netzsch, Selb, Germany). 30 µL of the liquid formulations were investigated in hermetically sealed crucibles. Samples were cooled at 10 °C/min from 20 °C to -60 °C (Mettler) and -100 °C (Netzsch), respectively, and heated to 20 °C at the same rate.

2.2.5. Karl-Fischer Titration

Karl Fischer titration was used to determine the residual moisture (RM) of the freeze-dried cakes. It was performed with an Aqua 40.00 titrator (Analytik Jena AG, Halle, Germany) connected to a headspace oven set at constant temperature of 100 °C. For analysis, 5 to 20 mg of crushed cake were transferred to 2 R vials under controlled humidity conditions (< 10% r. h.).

2.2.6. X-Ray Powder Diffraction (XRPD)

Crystallinity of the excipients was determined by XRPD. An XRD 3000 TT diffractometer (Rich. Seifert & Co. GmbH &Co. KG, Ahrensburg, Germany) equipped with a copper anode (40 kV, 30 mA, λ = 0.154178 nm) and a scintillation detector at 1000 V was used. The freeze-dried samples were crushed and smoothened homogenously on copper sample holders of 0.2 mm height. Samples were analyzed in steps of 0.05° 2-Θ for 2 s/step from 5 to 45° 2-Θ. Reference peaks used for identification of the crystalline phases are listed in Table 6.

Table 6. Reference XRPD peaks of the excipients.

Excipient		Main peaks [° 2-θ]								References
Sucrose		11.8	12.7	13.1	18.9	20.9	24.8	25.2		[49–51]
Phenylalanine	hydrate	6.4	14.7	17.4	21.3					[8]
	hydrate	6.8	10.9	15.2	17.9	21.7	24.1	26.6	27.9	[52]
	hydrate	6.4	8.4	13.8	17.4	19.3	20.7	22.4	27.5	[53]
	anhydrate	5.7			17.1		22.8	28.6	34.5	[52]
	anhydrate	5.5			16.9	17.7	22.6	28.4	34.3	[53]
Isoleucine		6.5	13.0		25.7	32.4				[54]
		6.4	12.8	19.1	25.4	32.3	38.6			[8,55]
Leucine		6.1								[8]
		6.5		19.2	24.4	30.6				[54]
		6.4	12.3	19.1	24.4	30.7				[56]
Methionine		5.8								[8]
		6.1	23.6	35.2						[54]
Mannitol	α	9.3	13.7	17.2	18.7					[57–59]
	ß	10.4	14.6	16.8	18.8	20.3	23.4			
	δ	9.7				20.4	22.0	24.6	25.3	
	hemihydrate	9.6		17.9			23.1		25.7	

2.2.7. Reconstitution Time

Reconstitution times were determined by dissolving the lyophilizates with the required volume of HPW. The reconstitution volume was calculated based on formulation density and solid content. The time until complete dissolution of the cake was considered. The vials were gently rolled to ensure wetting.

2.2.8. Turbidity

Turbidity of the samples before freeze-drying and after reconstitution of the lyophilizates was measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at 350 nm. Each formulation was measured as triplicate with its corresponding placebo without protein as blank solution.

2.2.9. Light Obscuration (LO)

A PAMAS SVSS-35 particle counter with a HCB-LD-25/25 sensor (PAMAS – Partikelmess- und Analysesysteme GmbH, Rutesheim, Germany) was used to determine the number of subvisible particles before freeze-drying and after dissolution of the lyophilizates. The system was cleaned with HPW between the analyses. The rinsing volume was 0.2 mL, followed by four measurements of 0.2 ml according to USP 788 [60]. PAMAS PMA software was used to determine the number of particles ≥ 1 µm, ≥ 10 µm and ≥ 25 µm per mL.

2.2.10. High Performance Size Exclusion Chromatography (HP-SEC)

Size exclusion chromatography was performed with an Agilent 1100 series HPLC system equipped with an UV/Vis detector for detection at 280 nm (Agilent Technologies, Santa Clara, CA, USA). A TSKgel® G3000 SWXL column (dimension: 300 x 7.8 mm, TOSOH Bioscience GmbH, Stuttgart) and 100 mM sodium phosphate / 100 mM sodium sulfate buffer at pH 6.8 mobile phase with a flow rate of 0.5 mL/min was used. Prior to analysis, 50 mg/mL samples were diluted to 2 mg/mL. All samples were centrifuged (5 min, 4000 rpm, Sigma 1-15 microfuge, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). After blank subtraction the integrated peak intensity was compared before and after freeze-drying using the ChemStation software (Agilent Technologies, Santa Clara, CA, USA). Soluble aggregates and fragments were also referred to the AUC of monomer before freeze-drying.

3. Results and Discussion

3.1. Characterization of AA - Suc Formulations

In order to cover unwanted cake appearance, crystalline bulking agents are preferably used at low concentration. Several AAs crystallize already at a 2.5/47.5 AA/Suc ratio [8]. As proteins and amorphous excipients can suppress bulking agent crystallization, increased AA/Suc ratios (5/45 and 10/40 AA/Suc ratio) were also considered in this study. Phe, Leu, Ile, Gly, and Met were selected as crystallizing AAs based on literature [22,23,31,36,43,45]. Man/Suc 10/40 was also tested although a ratio of 40/01 is considered essential for complete Man crystallization [18,19].

3.1.1. DSC of Freeze-Concentrated AA/Suc Formulations

Formulations in the frozen state were characterized by DSC. Increasing AA/Suc ratios of Phe/Suc and Leu/Suc formulations resulted in increased Tg' values indicating that the individual Tg' value of the AA was higher than the one of pure Suc Table 7. A Tg' of -10.5 °C was reported for Phe [8]. Tg' values of Leu, Ile and Met were not found in literature and could not be measured since the AAs crystallized already during cooling. Ile/Suc mixtures showed Tg' values close to that of pure Suc. Ile was either already crystalline below -33 °C or the Tg' value of pure Ile is close to the one of Suc. Gly, Met and Man were investigated at the 10/40 ratio only. Tg' values were close to the results of Suc for Man/Suc (-34.5 °C) and for Met/Suc (-33.5 °C). Tg' values of Gly/Suc were lower than pure Suc samples in accordance with a Tg' of -51.5 °C recorded to Gly/Suc 5/2 reported by [22].

Table 7. Tg' values of AA/Suc and Man/Suc formulations. For n≥3: mean ± SD, for n=2: mean. n. d.: not determined.

AA/Suc ratio	Tg' [°C]					
	Phe/Suc	Ile/Suc	Leu/Suc	Gly/Suc	Met/Suc	Man/Suc
0/50	-32.9 ± 0.3					
2.5/47.5	-32.0 ± 0.2	-33.3 ± 0.3	-33.1 ± 0.2	n. d.	n. d.	n. d.
5/45	-31.0 ± 0.6	-32.9 ± 0.5	-32.5 ± 0.5	n. d.	n. d.	n. d.
10/40	-29.6 ± 0.6	-33.0 ± 0.6	-28.5 ± 0.8	-37.6 ± 0.8	-33.5	-34.5 ± 0.5

Exothermic crystallization peaks were detected in the heating scans of Phe/Suc 10/40 at -18.3 ± 0.2 °C, Ile/Suc 10/40 at -14.4 ± 1.7 °C, Leu/Suc 10/40 at -20.8 ± 0.2 °C and Met/Suc 10/40 at -17.1 °C. During cooling, peaks were detected for all AAs at approximately -30 °C. Thus, DSC analysis of the liquid samples pointed at pronounced crystallization tendencies of the selected AAs but also at the potential for incomplete crystallization after freezing.

3.1.2. Screening of AAs and the Impact of Annealing on the Lyophilizates

AA/Suc formulations were freeze-dried applying a fast freeze-drying cycle with or without annealing (Table 2, Table 3).

Macroscopic and Microscopic Appearance

Phe, Ile and Leu lyophilizates showed promising macroscopic appearance at 5/45 and 10/40 AA/Suc while the 2.5/47.5 cakes were shrunken (Figure 1). Cakes with Leu, Ile and Met were lifted up from the bottom probably due to the high chamber pressure of 2.2 mbar resulting in fast sublimation. The microscopic structure of Phe/Suc 10/40 and Leu/Suc 10/40 lyophilizates could not be differentiated by the selected freezing protocol and homogeneous, dense cakes were detected (Figure 2). The two typical bulking agents Man and Gly resulted in collapsed matrices except of the annealed 10/40 Man/Suc formulation. Microscopy confirmed a slightly improved cake appearance of Man/Suc 10/40, but still, many visible larger pores were evident. The bulking agent/Suc ratio was below the crystallization threshold of Gly and Man of Man/Suc ≥4/1 and Gly/Suc ≥2/5 [6,22,58]. Except for the annealed 10/40 Man/Suc formulation, annealing did not improve the macroscopic and microscopic appearance of the AA/Suc cakes indicating the crystallization of the AAs without the need of an additional annealing step.

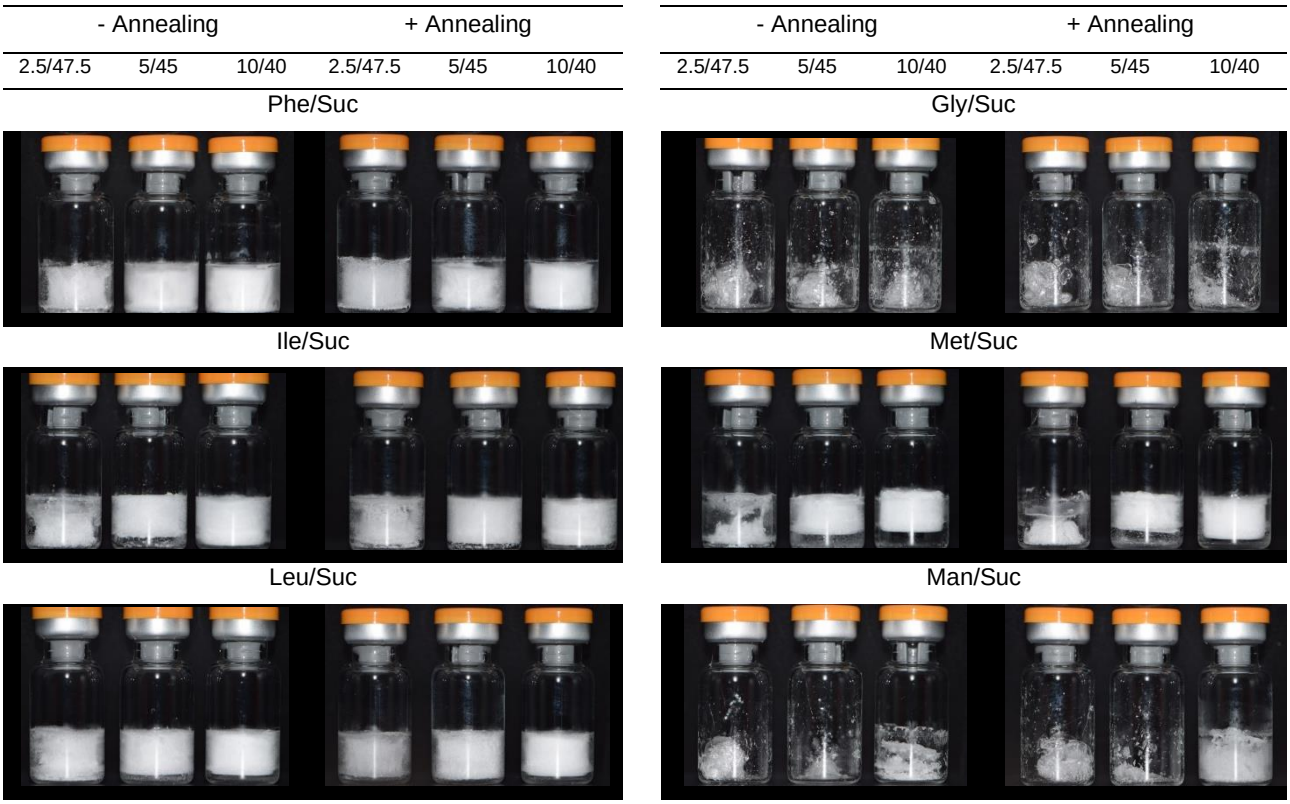


Figure 1. Macroscopic appearance of AA/Suc and Man/Suc lyophilizates freeze-dried with (Table 2) or without annealing (Table 3).

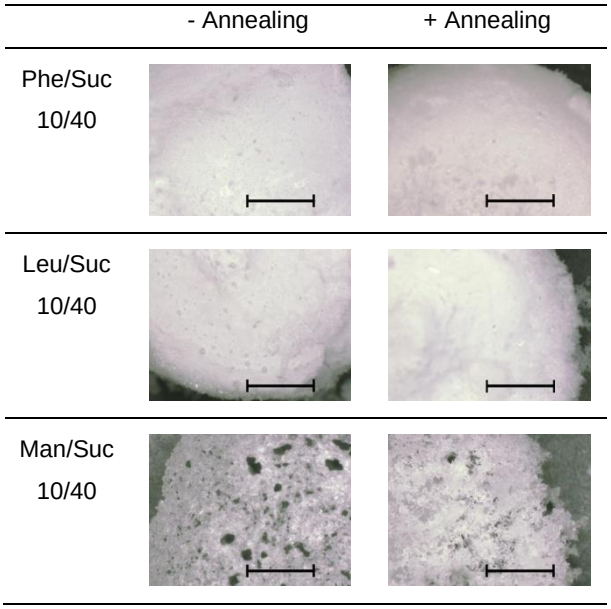


Figure 2. Microscopic Appearance of Phe/Suc 10/40, Leu/Suc 10/40 and Man/Suc 10/40 lyophilizates freeze-dried with (Table 2) and without annealing step (Table 3). The bar refers to a 2.0 mm length.

XRPD

AA based lyophilizates with an acceptable macroscopic appearance (Phe, Ile, Leu and Met), in our study the most important quality attribute, were further investigated by XRPD (Figure 3 A-D). Peaks that indicated AA crystallization were detected (Table 6). Man/Suc 10/40 showed crystalline Suc and Man peaks (Figure S1). Independent of the annealing step the higher the AA/Suc ratio the more pronounced was the peak pattern. Ile and Leu showed significant peaks also at the lowest AA/Suc ratio of 2.5/47.5 while detection of Phe peaks was more difficult and at 2.5/47.5 no peaks could be assigned.

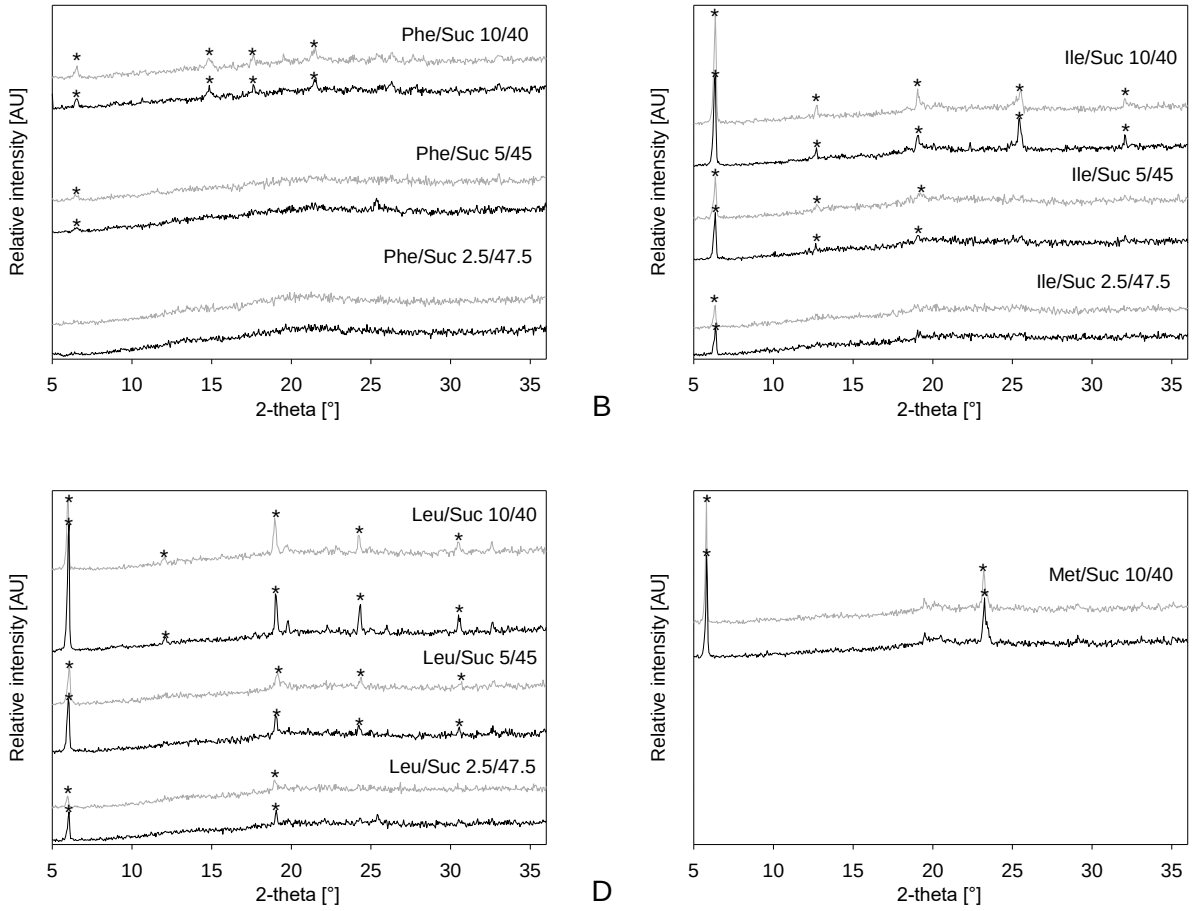


Figure 3. XRD diffractograms of A - Phe/Suc, B - Ile/Suc, C - Leu/Suc, D - Met/Suc lyophilizates freeze-dried with (Table 2, grey lines) or without annealing step (Table 3, black lines).

DSC of Freeze-Dried AA/Suc Systems

Tg values of the freeze-dried formulations were above 25 °C for all AA/Suc ratios and above 50 °C for the 10/40 AA/Suc ratio (Table 8). Annealing had no impact on the Tg values since the amorphous phase was not affected except from Ile/Suc 5/45. This formulation showed

a distinct increase in the Tg leading to the conclusion that annealing fostered Ile crystallization in this case. Higher AA/Suc ratios showed higher Tg values compared to the 2.5/47.5 AA/Suc ratios. Microcollapse of these formulations was a possible reason for insufficient drying behaviour leading to higher RM values and hence lower Tg values (Table 9).

Table 8. Tg values of freeze-dried AA/Suc formulations. The formulations were freeze-dried with (Table 2, + Annealing) or without annealing step (Table 3, - Annealing). For n=3: mean ± SD, for n=2: mean.

AA/Suc ratio		Tg [°C]	
		- Annealing	+ Annealing
Phe/Suc	10/40	67.4 ± 0.6	69.7 ± 1.1
	5/45	66.1 ± 2.4	67.2
	2.5/47.5	58.8	62.1
Ile/Suc	10/40	54.7	54.9
	5/45	36.3 ± 2.3	47.0
	2.5/47.5	29.7	28.2
Leu/Suc	10/40	50.5	51.4
	5/45	50.9	51.0
	2.5/47.5	38.9	43.4
Met/Suc	10/40	51.0	50.9
	5/45	55.4	55.4

Reconstitution Times

Reconstitution times are an important quality attribute of freeze-dried formulations although they are not directly coupled to API stability and a diversity of different reconstitution times has been reported [61–65]. A prolonged reconstituion time can be caused by excipients or by the API itself, e.g. at high protein concentration. Approaches for improvement are addition of wetting agents, adapted freezing procedures, heated reconstitution media or reconstitution with the 2-fold volume [62–68]. The reconstitution times differed significantly (Figure 4 A-B) whereas Phe/Suc lyophilizates dissolved in less than one minute. Most other lyophilizates required one to two minutes increasing with higher AA portion and annealed Leu/Suc 10/40 needed about four minutes for complete reconstitution. Compromised wetting behaviour was observed for Leu and Ile lyophilizates (Figure 4C). Dissolution was not hindered as long as the lyophilizate was submersed. As soon as the outer cake region dissolved and the cake became detached from the vial wall, floating occurred and reconstitution was retarded.

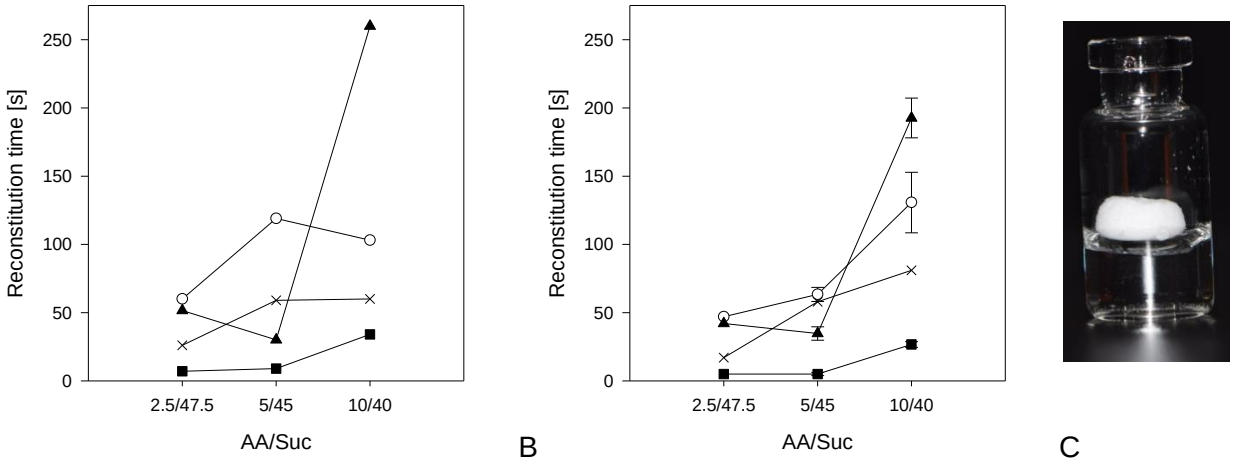


Figure 4. Reconstitution times AA/Suc formulations freeze-dried with (A, Table 2) or without annealing step (B, Table 3). Phe/Suc - black squares, Ile/Suc - white circle, Leu/Suc - black triangles and Met/Suc - black cross. For n=3: mean ± SD, for n=2: mean. C - Leu/Suc 10/40 lyophilizate during reconstitution after loss of contact with the vial wall.

The reconstitution times were in an acceptable range of less than five minutes. Nevertheless, different approaches were tested to improve the rehydration, in particular for Leu/Suc and Ile/Suc 10/40 lyophilizates (Figure 5 A-D). The hydrophobic character of the selected AAs implied poor wetting and dissolution behavior of the crystalline phases. However, reconstitution of Phe lyophilizates was clearly faster than the reconstitution of Ile and Leu lyophilizates. In order to improve solubility, the reconstitution medium was adjusted to the pK_a and pK_b values of the AAs (Table 1). The reconstitution times of Ile/Suc 10/40 decreased at pH 2.33 and 9.75 compared to the reconstitution at pH 6.0 while the ones of Leu/Suc did not change significantly (Figure 4 A). Both amino acids have comparable physical characteristics, such as chemical structure, pI and solubility. Nevertheless, their reconstitution behaviour differs. We explain this by the different crystal structure in combination with sucrose. The reason, why Ile showed a pH dependence of the reconstitution time but Leu did not, is unclear.

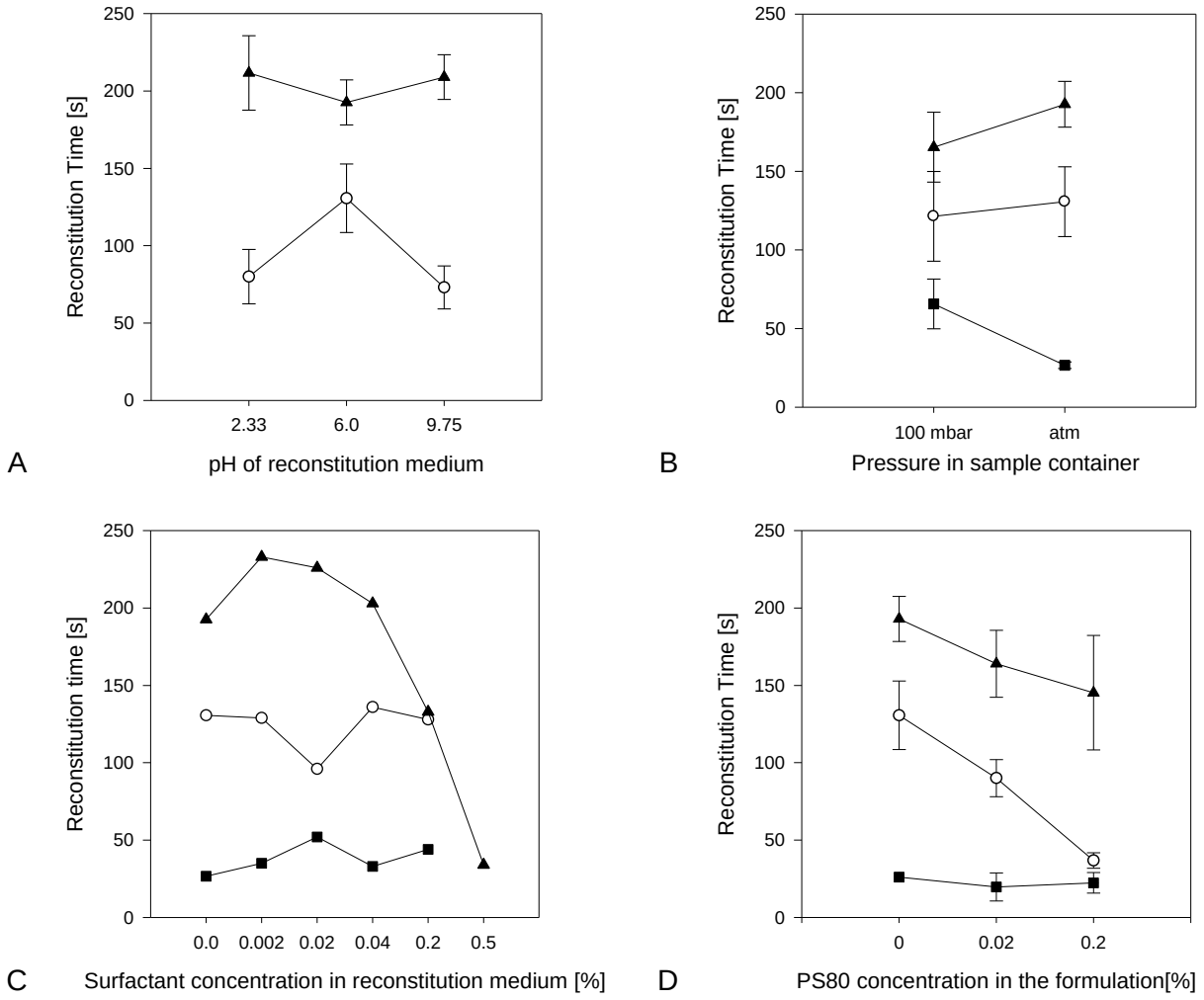


Figure 5. Reconstitution times of AA/Suc 10/40 lyophilizates freeze-dried without annealing step (Table 3). For n≥3: mean ± SD, for n=2: mean. A - pH adjustment of reconstitution medium. B - Pressure adjustment within the sample container. C – Addition of surfactant to reconstitution medium. D – Addition of surfactant to the formulation. Phe/Suc 10/40 - black squares, Ile/Suc 10/40 – white circles and Leu/Suc 10/40 – black triangles.

Werk et al. reported that reconstitution times decreased if a warmed reconstitution medium was utilized [67]. However, for the AA/Suc lyophilizates, a prewarmed reconstitution medium of 40 °C resulted in the same reconstitution times as water at ambient temperature. Bhatnagar showed that vacuum within the sample container could help to improve reconstitution times if complete disintegration of the cake was caused by the incoming liquid flow [62]. Ile and Leu lyophilizates showed slightly, but not significantly faster reconstitution when the sample container was stoppered at 100 mbar after freeze-drying instead of ventilation of the vial before reconstitution (Figure 4 B). Since the limiting factor was the wetting of the cake and the contact with water, neither warmer HPW and pH adjustment nor vacuum could increase the contact area with the lyophilizate and hence the rate determining floating of the cake was not prevented.

Surfactants are commonly used in freeze-dried formulations to cope with the interface related stress such as at the ice-freeze concentrate interface during freezing [69–72]. Surfactants at high concentration in the reconstitution medium act as wetting agents and decrease reconstitution times [64,65]. The reconstitution times of Leu/Suc 10/40 decreased only if surfactant concentrations of 0.2% and higher were used (Figure 4 C). Reconstitution of the other lyophilizates was not affected by the addition of surfactant because floating of the cake could not be prevented. As an alternative, 0.02% or 0.2% polysorbate 80 in the solutions to be lyophilized were evaluated (Figure 4 D). Reconstitution was facilitated, in particular for the Ile/Suc 10/40 formulation. Phe/Suc 10/40 and 5/45 lyophilizates did not benefit as they dissolved already fast without surfactant. Phe/Suc 5/45 and Leu/Suc 5/45 suffered shrinkage when 0.2% PS80 was included. In addition, in combination with Ile, 0.2% PS80 induced the formation of crystalline Suc at both AA/Suc ratios. Thus, the addition of 0.2% PS80 could improve the reconstitution time but it affected other important quality attributes negatively. As a compromise, lower polysorbate concentrations were added to the formulations for the following experiments. 0.04% PS20 was chosen in order to avoid shrinkage and Suc crystallization.

Thus, the screening of the AAs demonstrated that Phe, Ile and Leu resulted in lyophilizates with appropriate physical characteristics when combined with Suc in different ratios. Higher AA/Suc ratios of 5/45 and 10/40 were superior with respect to macroscopic appearance and crystallinity compared to the 2.5/47.5 AA/Suc ratio. Annealing did not improve these characteristics. Inclusion of polysorbate into the formulations could improve the reconstitution times of the poorly wetting Ile/Suc and Leu/Suc lyophilizates. Phe/Suc lyophilizates reconstituted faster in spite of comparable hydrophobicity (Table 1). Higher Tg values may correlate to a different lyophilizate structure enabling better wetting and hence reconstitution.

3.1.3. Impact of pH on Physical Characteristics of AA/Suc Lyophilizates

Solubility and crystallization of the amphoteric AAs strongly depend on pH. Indeed, the macroscopic appearance of lyophilizates based on AA/Suc mixtures was affected by the formulation pH especially at lower AA/Suc ratio (Table S 1). In general, pH conditions from pH 5 to pH 7 resulted in better cake appearances than more extreme acidic or basic conditions. Met/Suc and Gly/Suc lyophilizates did not improve macroscopic appearance under the applied pH conditions. Within the formulations with good cake structure, the crystallization tendencies were not affected, except of Phe/Suc 10/40 which showed a different peak pattern at a formulation pH equal to the pK_a of Phe despite comparable macroscopic appearance

(Figure S 2). The peaks could be assigned to the anhydrous form of Phe, whereas the common form of crystalline Phe after freeze-drying is the monohydrate [8]. Physical instabilities of these two Phe forms in lyophilizates have not yet been described in literature. Potentially, release of the hydrate upon storage might induce degradation reactions, however, only its formation occurred at low pH. The known Maillard reaction was also favoured at acidic pH condition [73,74] and was detected by browning of the acidic Phe/Suc formulations after few days of storage at ambient temperature. Leu/Suc 10/40 at a pH equal to pK_a did show additional peaks that could not be assigned to further typical Leu reference peaks. In contrast to Phe, no other Leu modification could be found in literature. The peaks were also not in accordance with Suc peaks. Thus, macroscopic appearance and AA crystallinity were affected only by extreme pH conditions but not in the pH region of interest. Tg values decreased at pH values equal to the pK_a, pK_b, pK_a+1 and pK_b-1, but were stable in the pH region of pH 5 – 7. (Table S 2). Reconstitution times were comparable over a broad pH range (pH 5 - 7) (Figure S 3). Only Leu/Suc 10/40 showed an improved reconstitution in the extreme basic or acidic region. A formulation pH of 6.0 resulted in the best characteristics regarding appearance, crystallinity, Tg values and reconstitution times for Phe/Suc, Ile/Suc and Leu/Suc lyophilizates.

3.1.4. Impact of Lower Tp on the Quality of AA:Suc Lyophilizates

As cake shrinkage and lifted cakes were detected during the screening experiments, the freeze-drying cycle was adapted to improve macroscopic appearance without losing AA crystallinity at still short process time. First, the chamber pressure was reduced from 2.2 mbar to 0.52 mbar (Table 4) resulting in a reduction of Tp from -10 °C to -20 °C during primary drying. Thus, the Tp was still markedly above the Tg' of Suc. Alternatively, the chamber pressure was set to 0.40 mbar and the shelf temperature set to 0 °C instead of 20 °C during primary drying (Table 5) resulting in a Tp of -27 °C, only slightly above Tg'.

At the reduced chamber pressures settings, lifting of the lyophilizates could be prevented whereas the cake appearance was not changed (Figure 6). Phe/Suc 5/45 dried at 0.52 mbar and Ile/Suc 5/45 dried at 0.40 mbar formed shrunken cakes. A decreased Tp seemed to hinder the arrangement of AA crystals (Figure 7). However, Phe/Suc 5/45 was not collapsed at a Tp of -27 °C despite of an amorphous XRD pattern since the Tp was close to the Tg' of the formulation. Thus, crystallization of the AAs was not completed after the freezing step, but was favoured at increased Tp during primary drying. The crystallization tendency depended on the

AA portion. Low AA/Suc ratio combined with low Tp during primary drying prevented AA crystallization.

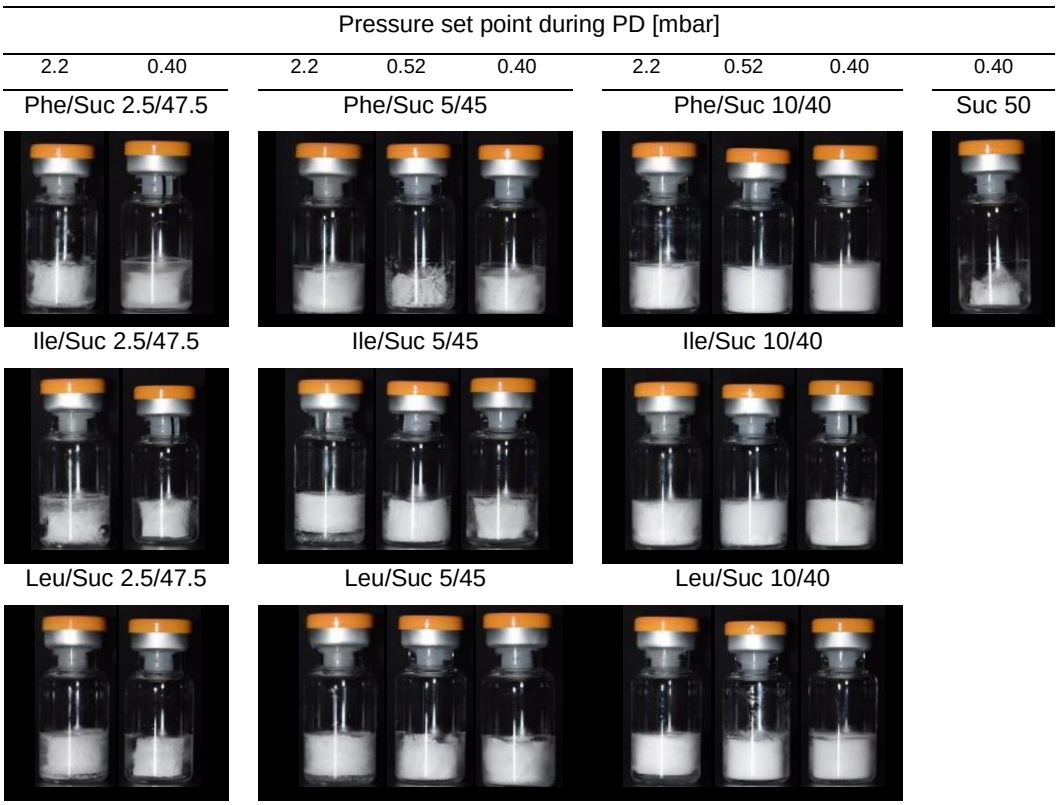


Figure 6. Macroscopic Appearance of AA/Suc and Suc lyophilizates dried at different chamber pressure settings (Table 3 - Table 5).

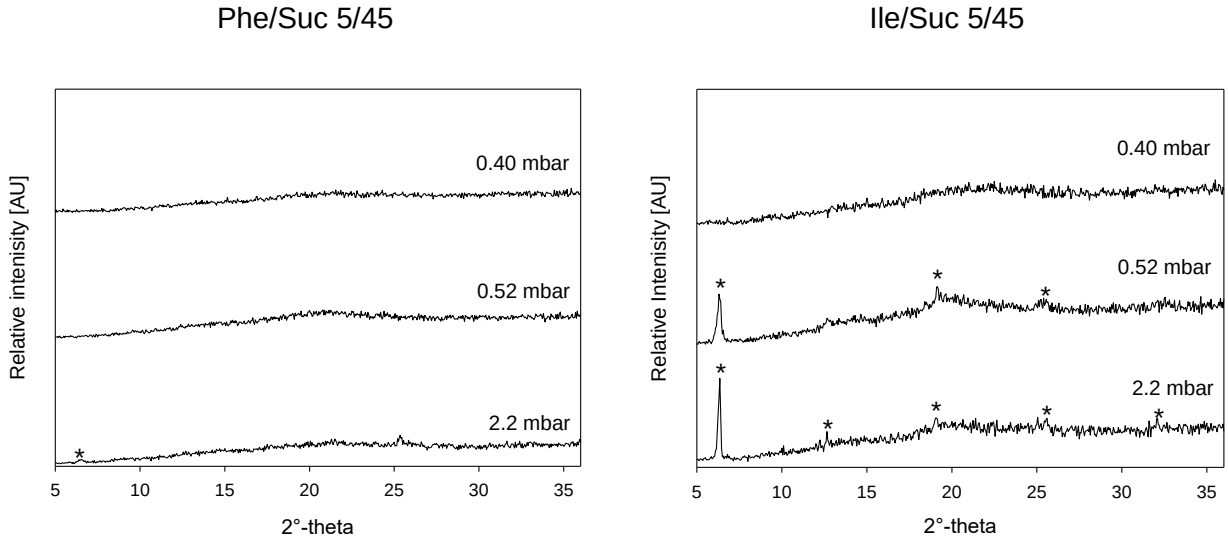


Figure 7. XRD diffractogram of Phe/Suc 5/45 and Ile/Suc 5/45 lyophilizates freeze-dried according to Table 3 – Table 5. The chamber pressure set points during PD are given.

Tg values correlated to the RM levels (Table 9). Fast freeze-drying cycle at the highest Tp of -10 °C led to the highest RM levels in Ile/Suc and Leu/Suc formulations. This cycle was implemented for Man/Suc 4/1 formulations with acceptable RM levels [21]. For Phe/Suc 10/40 RM below 0.5% resulted. For Ile/Suc and Leu/Suc freeze-dying at 0.52 mbar resulted in lower RM levels and hence higher Tg values. The lack of crystalline bulking agent in AA/Suc 2.5/47.5 resulted in collapse and as a consequence in higher RM and lower Tg values. A chamber pressure of 0.52 mbar resulted in the most suitable RM levels of 0.1 – 0.7% leading to Tg values above 55 °C.

Table 9. Tg values and residual moisture levels of AA/Suc freeze-dried at different chamber pressure settings according to Table 3 - Table 5. For n=3: mean ± SD, for n=2: mean. – not determined.

AA/Suc ratio		Tg [°C]			Residual Moisture [%]		
		2.2 mbar	0.52 mbar	0.40 mbar	2.2 mbar	0.52 mbar	0.40 mbar
Phe/Suc	10/40	67.4 ± 0.6	70.9	68.9 ± 2.0	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.1
	5/45	66.1 ± 2.4	39.9	62.1 ± 2.2	0.5 ± 0.1	0.7 ± 1.1	1.0 ± 0.3
	2.5/47.5	58.8	-	55.3 ± 3.6	-	-	1.3 ± 0.4
Ile/Suc	10/40	54.7	55.1	61.5 ± 5.0	1.7	0.6 ± 0.5	0.8 ± 0.2
	5/45	36.3 ± 2.3	56.4	52.6 ± 2.2	2.8 ± 0.02	0.3 ± 0.3	1.9 ± 0.3
	2.5/47.5	29.7	-	50.1 ± 2.4	-	-	1.5 ± 0.3
Leu/Suc	10/40	50.5	62.8	58.3 ± 5.9	1.6 ± 0.1	0.3 ± 0.2	0.7 ± 0.1
	5/45	50.9	55.3	65.9 ± 2.3	2.5 ± 0.05	0.3 ± 0.2	0.7 ± 0.2
	2.5/47.5	38.9	-	51.6 ± 6.7	-	-	1.3 ± 0.2
Suc	50	-	-	51. 1 ± 4.1	-	-	2.3 ± 0.4

Reconstitution times were measured for these cycles as well, but did not lead to significant differences between the freeze-drying cycles.

Thus, a lower chamber pressure of 0.52 mbar leading to a Tp of -20 °C prevented lifting of the cakes and led to acceptable cake appearances, Tg values and RM levels in most formulations. AA crystallinity was detected in all AA/Suc formulations processed with this cycle except of Phe/Suc 5/45. An even lower pressure set point of 0.40 mbar resulting in a Tp of -27 °C reduced crystallinity of the AAs, higher RM levels and lower Tg values. The low 2.5/47.5 AA/Suc ratio led to crystalline AAs but the ratio of crystalline to amorphous phase was not investigated quantitatively. The macroscopic appearance was also not acceptable in the 2.5/47.5 AA/Suc

ratios. As a consequence, protein runs were only performed with two AA/Suc ratios, 5/45 and 10/40, dried at 0.52 mbar.

3.2. Freeze-Drying of the Model mAb in AA/Suc Formulations

To test whether the AA/Suc formulations were able to stabilize proteins upon freeze-drying a model mAb was investigated at two different concentrations (2 mg/mL and 50 mg/mL). The concentrations reflect the limits of a broad range as physical characteristics such as glass transition temperature, drying behaviour, thus residual moisture, crystallization of the excipients as well as protein stability may be different at low or high protein concentration. Phe/Suc, Ile/Suc and Leu/Suc formulations were evaluated (AA/Suc 5/45 and 10/40). 0.04% Polysorbate 20 was included in the formulations for improvement of the reconstitution times. The resulting lyophilizates were characterized in terms of their physical properties. Furthermore, protein process stability was examined regarding aggregate formation. Freeze-drying was carried out at 0.52 mbar with a Tp of -20 °C during the 12 h primary drying step (Table 4).

3.2.1. Physical characterization

Lyophilizates with 50 mg/mL mAb did not suffer any cake defects independent of the chosen AA (Figure 8). This was due to the high total solid content of 100 mg/mL. The 10/40 AA/Suc ratio resulted in acceptable macroscopic appearance for all formulations and marked AA crystallinity. In contrast, for the 5/45 AA/Suc ratio and 2 mg/mL mAb, best cake appearance was given for Leu/Suc 5/45 followed by Ile/Suc 5/45 and Phe/Suc 5/45 which suffered shrinkage. The crystallization tendency of Phe was strongly affected by the protein while Ile and Leu crystallized at both mAb concentrations (Figure 9). The higher mAb concentration of 50 mg/mL suppressed Phe crystallization completely whereas Ile/Suc and Leu/Suc lyophilizates showed crystalline Ile and Leu, respectively. Thus, Ile and Leu had a stronger crystallization tendency in contrast to Phe and were less affected by crystallization suppressing excipients as protein or surfactant.

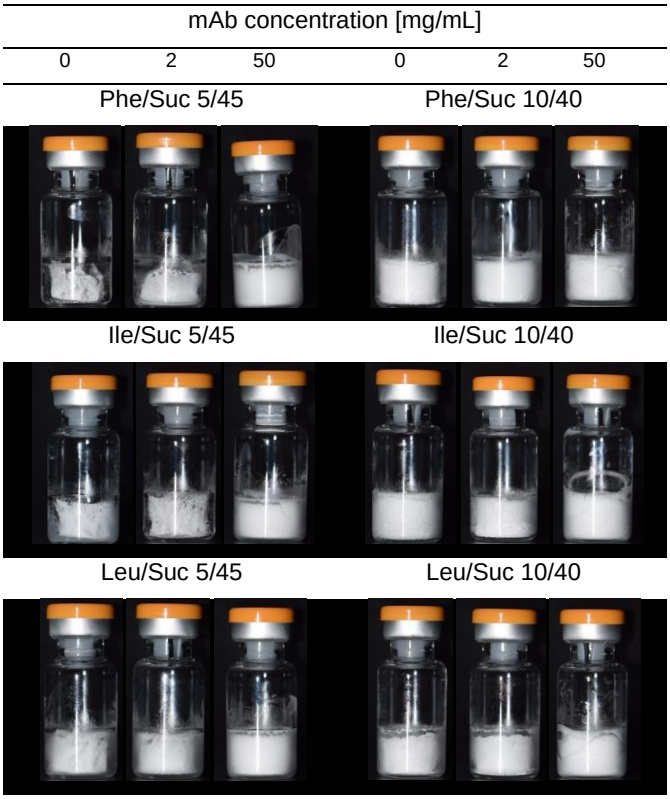


Figure 8. Macroscopic Appearance of AA/Suc lyophilizates at different protein concentrations (0 mg/mL, 2 mg/mL and 50 mg/mL) freeze-dried according to Table 4.

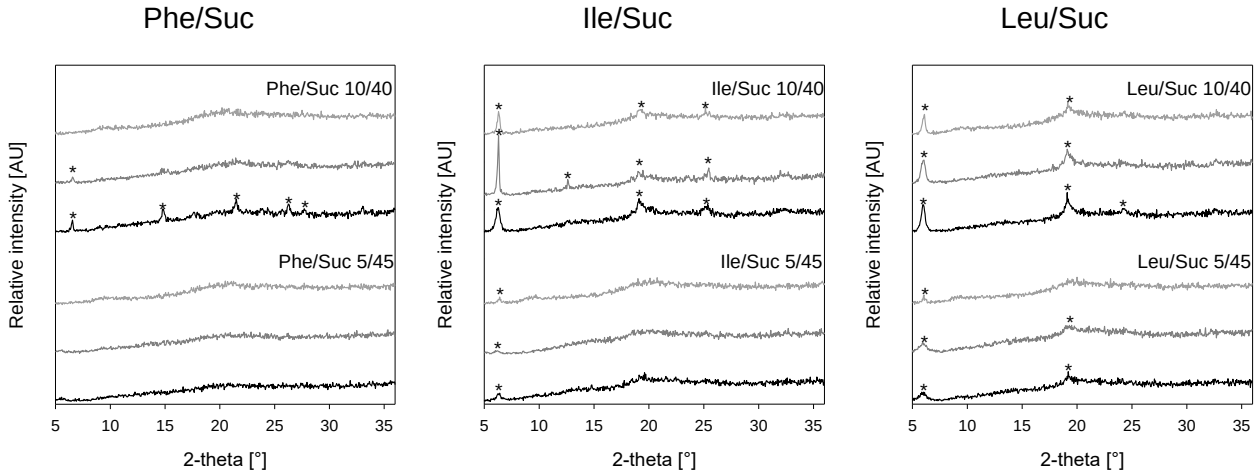


Figure 9. XRD diffractograms of Phe/Suc, Ile/Suc and Leu/Suc lyophilizates at different mAb concentrations (0 mg/mL – black, 2 mg/mL – grey, 50 mg/mL dark grey). * mark reference peaks according to Table 6.

Tg values and RM contents complemented macroscopic appearance and XRPD (Table 10). Inacceptable cake appearance was accompanied by high RM levels and low Tg values (Phe/Suc 5/45). The mAb concentration of 50 mg/mL led to better cake appearance, improved

drying behavior and hence low RM levels and higher Tg values. Similar results were achieved by Ile/Suc and Leu/Suc in this AA/Suc ratio of 5/45. The higher AA/Suc ratio of 10/40 resulted in consistently lower residual moisture levels coupled to higher Tg values.

Table 10. Impact of protein concentration on Tg values and RM levels of AA/Suc lyophilizates freeze-dried according to Table 4. n=3: mean ± SD, n=2: mean, -: not detected.

AA/Suc Ratio		Tg [°C]			RM [%]		
		0 mg/mL	2 mg/mL	50 mg/mL	0 mg/mL	2 mg/mL	50 mg/mLL
Phe/Suc	10/40	67.4 ± 2.8	59.0	75.0	0.1 ± 0.1	0.1	0.1
	5/45	39.1	43.6	88.0	2.3	2.4	0.1
Ile/Suc	10/40	67.4 ± 2.4	72.8	87.2	0.1 ± 0.2	0.3	0.04
	5/45	57.9 ± 4.6	52.5	-	0.7	0.7	0.1
Leu/Suc	10/40	65.1 ± 2.2	57.0	46.3	0.2 ± 0.2	0.1	0.0
	5/45	61.4	67.5	65.5	0.4	0.4	0.03

Reconstitution times of formulations with only 2 mg/mL protein concentration were below 30 s for AA/Suc 5/45 and below 100 s for AA/Suc 10/40 and comparable to protein free formulations independent of the AA. In contrast, 50 mg/mL protein induced a pronounced prolongation of the reconstitution as described in literature [62,64,66].

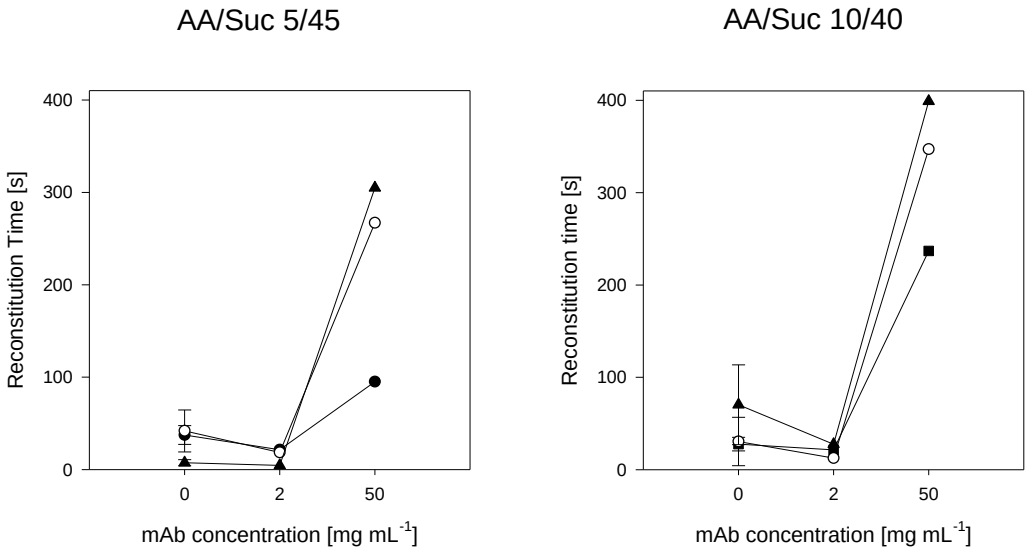


Figure 10. Impact of protein concentration on reconstitution times of AA/Suc 5/45 and 10/40 lyophilizates freeze-dried according to Table 4.Phe/Suc – black squares, Ile/Suc – white circles, Leu/Suc – black triangles.

3.2.2. Protein stability

Insoluble aggregates larger than 35 nm can be detected due to their scattering of light at 350 nm [75]. After freeze-drying and reconstitution the formulations were clear without indications of visible aggregate formation. Absorbance values before and after freeze-drying were comparably low at 0.06 AU for all formulations. Thus, no insoluble aggregates were formed during freeze-drying. Light obscuration was used to determine the amount of sub-visible particles. The particle numbers were below 25,000 particles per mL (≥ 1 µm), below 250 particles per mL (≥ 10 µm) and below 20 particles per mL (≥ 25 µm) and did not increase with freeze-drying. The amount of soluble aggregates before and after freeze-drying and reconstitution was below 1% for all samples without significant impact of freeze-drying at both protein concentrations (Figure 11).

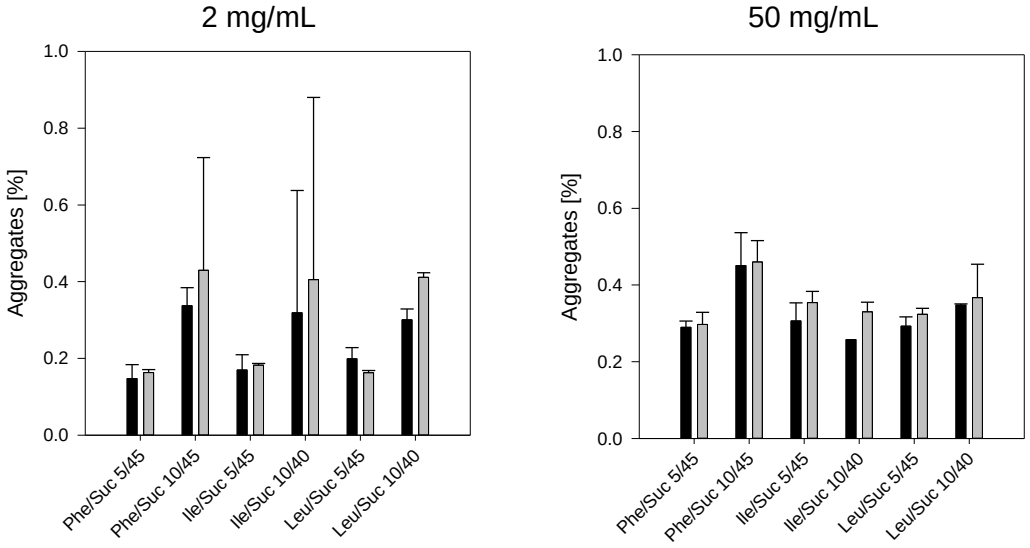


Figure 11. Soluble aggregates of mAb/AA/Suc formulations freeze-dried according to Table 4. n=3: mean ± SD. bFD - black bars, aFD - grey bars.

Thus, the analysis of mAb process stability did not show any significant protein aggregation. As only a small amount of bulking agent was used in comparison to other common crystalline bulking agents, sufficient amorphous Suc was present. Upon storage, the high fraction of amorphous Suc should provide adequate stabilization. It has to be ensured that Suc remains amorphous upon storage and that AA crystallization, if amorphous, is avoided. Partial crystallization and hence subsequent crystallization of the residual bulking agent have to be monitored in stability studies, but was beyond the scope of the study at hand.

4. Conclusions

The aim of this study was to investigate several AAs as alternatives to the typical bulking agents Man and Gly which require high bulking agent/Suc ratios. Low AA/Suc ratios were freeze-dried above Tg' to enable fast freeze-drying. The amino acids Phe, Ile and Leu showed better crystallization characteristics than the typical bulking agents Man and Gly at the essential bulking agent to Suc ratio freeze-dried above Tg'. At 2.5/47.5 AA/Suc acceptable cake appearance could not be achieved despite of crystalline Ile and Leu peaks. 5/45 and 10/40 AA/Suc offered a substantially improved lower bulking agent concentration at high stabilizer content. Annealing at -20 °C could not enhance crystallization in contrast to varied Tp during primary drying. Lower Tp decreased the crystallization tendencies of the AAs. Thus, fast freeze-drying at -20 °C for 12 hours primary drying time rendered the best physico-chemical characteristics of the AA/Suc lyophilizates. A pH range of pH 5 to pH 7 was suitable at the tested AA/Suc ratios while extreme pH conditions induced collapse. Reconstitution times were longer in Ile and Leu lyophilizates compared to Phe and Man due to poor wettability and floating of the cakes containing the hydrophobic AAs. Including surfactants in the formulation improved the reconstitution behaviour. The protein was protected by the high amount of sucrose. The crystallization tendency of Phe was reduced by protein in contrast to Ile and Leu.

Concluding, the three AAs Ile, Leu and Phe were found to be alternative bulking agents to Man and Gly enabling fast drying at high stabilizer content but low bulking agent concentration. In contrast to other crystallizing excipients, Ile and Leu crystallization was not suppressed by protein enabling also increased protein concentrations.

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