

The influence of Arginine and counter-ions: Antibody stability during freeze-drying.

Original

The influence of Arginine and counter-ions: Antibody stability during freeze-drying / Seifert, Ivonne; Bregolin, Alessandro; Fissore, Davide; Friess, Wolfgang. - In: JOURNAL OF PHARMACEUTICAL SCIENCES. - ISSN 0022-3549. - STAMPA. - 110:(2021), pp. 2017-2027. [10.1016/j.xphs.2020.12.009]

Availability:

This version is available at: 11583/2894543 since: 2021-04-16T11:20:08Z

Publisher:

Elsevier

Published

DOI:10.1016/j.xphs.2020.12.009

Terms of use:

This article is made available under terms and conditions as specified in the corresponding bibliographic description in the repository

Publisher copyright

Elsevier postprint/Author's Accepted Manuscript

© 2021. This manuscript version is made available under the CC-BY-NC-ND 4.0 license
<http://creativecommons.org/licenses/by-nc-nd/4.0/>. The final authenticated version is available online at:
<http://dx.doi.org/10.1016/j.xphs.2020.12.009>

(Article begins on next page)

The Influence of Arginine and Counter-Ions: Antibody Stability during Freeze-Drying

Ivonne Seifert¹, Alessandro Bregolin², Davide Fissore², Wolfgang Friess¹

¹Department of Pharmacy, Pharmaceutical Technology and Biopharmaceutics, Ludwig-Maximilians-Universität München, Munich, Germany

²Politecnico di Torino, Dipartimento di Scienza Applicata e Tecnologia, Turin, Italy

Abstract

Amino acids, for example L-arginine, are used in lyophilisation as crystalline bulking, buffering, viscosity reducing or stabilising excipients. In this study, arginine was formulated with different counter ions (hydrochloride, citrate, lactobionate, phosphate, and succinate). A monoclonal antibody was investigated in sugar-free arginine formulations and mixtures with sucrose regarding to cake appearance and protein stability with respect to protein aggregation and fragmentation. Arginine hydrochloride formulations collapsed during the selected lyophilisation process, and partially crystallised during storage, but provided the best protein stability at low antibody concentration, followed by arginine succinate formulations. Arginine citrate/ phosphate/ lactobionate formulations resulted in amorphous elegant cakes, but lacked in protein stability. Increasing pH from 5.0 to 7.0 resulted in decreased protein stability. Addition of sucrose to the arginine formulations improved cake appearance and protein stability. Arginine phosphate with sucrose resulted in similar protein stability to the sucrose reference. At 2 mg/ml antibody, arginine hydrochloride (\pm sucrose) provided very good protein stabilising characteristics. Mixing sucrose with arginine hydrochloride/ lactobionate/ succinate were superior to pure sucrose. While 50 mg/ml antibody improved cake appearance, only arginine lactobionate provided sufficient protein stability next to sucrose. So, sugar-free arginine formulations were able to stabilise the antibody in lyophilisates in a comparable or better way than sucrose formulations depending on counter ion, specifically the hydrochloride and the lactobionate, and antibody concentration. Best protein stability was found for mixtures of arginine hydrochloride/ lactobionate/ succinate with sucrose.

1. Introduction

In order to prolong the shelf life of biopharmaceuticals, freeze drying can be used ¹. Sucrose is the most frequently utilised excipient due its cryo- and lyoprotecting effect. Its moderate T_g' of -32°C and relatively high T_g of 75°C make sucrose favourable with respect to both process and storage stability ². Furthermore, isotonic sucrose solutions result in amorphous and elegant cakes ³. Sucrose is a non-reducing sugar, but the glycosidic bond between the two building blocks, glucose and fructose, may be broken and the reducing monosaccharides can lead to protein glycation at accelerated or long term storage ⁴.

Besides saccharides like sucrose or trehalose, other bulk stabilising excipients are utilised with amino acids comprising an interesting group already used in commercially available products ⁵⁻⁸. A few sugar free lyophilisation formulations based on amino acids for biologicals are on the market, e.g. ATryn[®] (antithrombin alfa), with glycine and sodium citrate, Omnitrope[®] (somatropin), with glycine, and Metalyse[®] (tenecteplase), with arginine and phosphoric acid as main excipients ⁹. Various lyophilisates comprise both amino acid and sugar, the amino acid in most cases acting as buffer ⁹.

The amino acids differ in their physical and chemical behaviour after freeze drying. L-arginine, L-histidine, L-lysine, and L-citrulline form amorphous glasses, whereas glycine, L-phenylalanine, L-leucine, and L-isoleucine crystallise ^{6,7,10}. Crystallising amino acids are able to act as scaffolds to achieve elegant cake appearance ^{6,11}. Nevertheless, proteins lack in stability when freeze dried in crystalline excipients only ¹²⁻¹⁴. Glass forming amino acids can stabilise proteins by the same mechanisms as sugars, i.e. preferential exclusion, water replacement or void filling ^{5,15-17}.

Arginine has received specific attention as it can reduce protein-protein interaction and aggregation in liquid formulations and reconstituted lyophilisates ^{19,20}. Its stabilising effect derives from a weak binding affinity to the protein surface via hydrogen bond formation and ion-dipole interactions ²¹⁻²³. Arginine containing formulations have shown their protein stabilising efficacy in freeze dried products depending on the protein, further excipients and concentrations ²⁴. Arginine in combination with further amino acids was able to prevent changes in the secondary structure of anti-CD11a and

anti-IgE antibodies during lyophilisation²⁵. A mixture of the three amino acids arginine, glutamic acid, and isoleucine was suitable to stabilise b-domain-deleted recombinant FVIII (GreenGene™ F) during production and storage to overcome the need for albumin as stabiliser²⁶. In a potassium phosphate buffered solution pH 7.0, the addition of at least 2% (w/v) arginine preserved the stability of bovine liver catalase during lyophilisation²⁷.

The basic pH of 10.4 of an arginine base solution is not suitable for protein formulation and an acid or counter ion is essential for pH adjustment¹⁰. Furthermore, arginine base shows a very low T_g' of -42.7°C ²⁸. Different multivalent acids such as phosphoric acid, citric acid, or tartaric acid lead to an increase of T_g' ¹⁰. The counter ion also affects protein stability. After 6 months at 40°C antibody formulations containing sucrose and arginine citrate, phosphate or succinate showed more aggregates than those with sucrose and arginine hydrochloride^{29,30}. Schersch et al. showed a protein stabilizing effect of arginine phosphate for lyophilisates which collapsed during freeze-drying³¹.

Although, the stabilising potential of amino acids and especially of arginine containing formulations has been shown, a detailed investigation of the stabilising potential of arginine in combination with different counter ions and in the absence of additional excipients is still lacking^{29,30}. Therefore, we evaluated sugar-free based solely on arginine in combination with hydrochloride, citrate, lactobionate, phosphate, and succinate as counter ions and compared them to sucrose-based formulations. The formulations were studied with respect to their freeze drying properties, cake characteristics, and stabilisation of a monoclonal antibody (mAb) at 2 mg/ml in the pH range between 5.0 and pH 7.0. The arginine formulations were also combined with sucrose or high protein concentration of 50 mg/ml to evaluate the potential to improve the characteristics of the lyophilised product as well as mAb stability.

2. Materials and Methods

2.1 Materials

Stock solutions of sucrose (Merck KGaA, Darmstadt, Germany), L-arginine base (Sigma-Aldrich Chemie GmbH, Steinheim Germany), citric acid anhydrous (VWR International, Ismaning, Germany), succinic acid (Merck KGaA), and lactobionic acid (Acros Organics, Geel, Belgium) were prepared with highly purified water (HPW) or in 15 mM L-histidine buffer (Sigma-Aldrich Chemie GmbH) pH 5.0, 6.0, and 7.0. In addition, 1 M hydrochloric acid and conc. phosphoric acid (both VWR International) were used.

The pH of arginine base stock solution was titrated to pH 7.0, 6.0 or 5.0 with citric acid (ArgCitr), hydrochloric acid (ArgHCl), lactobionic acid (ArgLacto), phosphoric acid (ArgPhos), and succinic acid (ArgSucc). For sucrose formulations (Suc), sucrose was dissolved in the corresponding histidine buffer. Arginine formulations containing sucrose were prepared by adding sucrose to the titrated arginine formulations at pH 6.0. The following acronyms were used to describe these formulations: SucArgCitr, SucArgHCl, SucArgLacto, SucArgPhos, SucArgSucc. Suc based formulations contained 7% (w/w) sucrose in 15 mM histidine buffer, Arg based formulations 4% (w/w) arginine and mixed formulations 3.5% (w/w) sucrose and 2% (w/w) arginine.

A monoclonal IgG₁ antibody (MW ca. 148 kDa, $\epsilon = 1.37 \text{ mL mg}^{-1} \text{ cm}^{-1}$, referred to as mAb) in 15 mM histidine buffer pH 5.3 was used as model protein. Formulations contained 2 mg/ml mAb. Formulations with high mAb concentration contained 50 mg/ml mAb and were named ArgHCl-HC, ArgCitr-HC, ArgLacto-HC, ArgPhos-HC, ArgSucc-HC, Suc-HC. For HC formulations the mAb was dialysed against HPW with a 10-fold buffer exchange in Vivaspin® 20 Ultrafiltration Uni (30 kDa, Sartorius, Goettingen, Germany) and up-concentrated.

The formulations were filtrated with 0.2 μm polyethersulfone membrane syringe filters (VWR International) prior to filling.

2.2 Methods

2.2.1 Freeze-thaw

Freeze-thaw experiments were performed using a FTS LyoStar™ 3 freeze dryer (SP Scientific, Stone Ridge, NY, USA). DIN 2R Vials (Fiolax®, Schott AG, Mainz, Germany) were cleaned with highly purified water and dried at 60°C for 8 h. The vials were filled with 1.5 mL of sample solution and stoppered with lyophilisation stoppers (B2-TR coating, West Pharmaceuticals Services Deutschland GmbH & Co. KG, Eschweiler, Germany). Samples were cooled from 20°C to -50°C at a ramp of 1°C/min followed by an isothermal hold for 3 h, thawed back to 20°C at a ramp of 1°C/min followed by an isothermal hold of 3 h. This cycle was repeated three times. Thermocouples in selected vials confirmed complete ice crystallisation and thawing and that -50°C and 20 °C resp. were reached in the samples.

2.2.2 Lyophilisation

Lyophilisation stoppers (B2-TR coating, West) and DIN 2R Vials (Fiolax®, Schott) were cleaned with highly purified water and dried at 60°C for 8 h. The vials were filled with 1.5 mL and semi-stoppered subsequently. The two outer rows of vials in each batch were not used for analysis. The product temperature in different vials at different position over the whole shelf area was record with thermocouples. Formulations were freeze-dried according to the protocols shown in Table 1 using a FTS LyoStar™ 3 freeze dryer (SP Scientific). End of primary drying was controlled by comparative pressure measurement between Pirani and MKS sensor. The vials were stoppered after secondary drying under nitrogen atmosphere at 800 mbar and crimped with flip-off seals.

For accelerated stability studies, sucrose reference and sugar free arginine samples pH 5 to 7 were stored at 50°C for 6 months, mixtures with sucrose pH 6 and high concentration pH 6 samples were stored at 40°C for 6 months.

2.2.3 Cake appearance

Images of the lyophilised products were taken with a Nikon D5300 camera (Nikon GmbH, Düsseldorf, Germany) in front of a black background.

2.2.4 Differential scanning calorimetry (DSC)

For the analysis of the glass transition temperature of the maximally freeze-concentrated solution T_g' , 15 μ l sample were filled in aluminium 40 μ l crucibles (Mettler Toledo, Gießen, Germany), hermetically sealed and analysed with a DSC 821^e (Mettler Toledo). The samples were cooled to -60°C at 10 K/min, held at -60°C for 5 min and reheated to 20°C at 2 K/min. The T_g' was determined as the midpoint of the phase transition using the STAR^e software for data analysis.

The glass transition temperature (T_g) of the lyophilisates was determined with a DSC 821^e (Mettler Toledo) or a Polyma 314 (Netzsch, Selb, Germany). 5 to 10 mg of crushed cake were filled under controlled humidity conditions (less than 10% rel. humidity) into aluminium 40 μ l crucibles (Mettler Toledo) or concavous 40 μ l crucibles (Netzsch) and sealed hermetically. Samples containing sucrose or ArgHCl were heated to 80°C at 10°/min, cooled to 20°C at 10°/min and reheated to 120°C at 10°/min. Other samples were heated to 120°C at 10°/min, cooled to 20°C at 10°/min and reheated to 150°C at 10°/min. T_g was determined as the midpoint of the phase transition during the second heating using the STAR^e software (Mettler Toledo) or Proteus 7.1 software (Netzsch) for data analysis.

2.2.5 Karl-Fischer titration

The residual moisture (RM) was analysed by coulometric Karl-Fischer titration using the AQUA 40.00 titrator with a headspace module (Analytik Jena AG, Halle, Germany). For the measurement about 20 mg of the lyophilised sample was prepared in a dry atmosphere and heated in the headspace module up to 100°C. The evaporated water was transferred into the titration solution and the remaining moisture determined.

2.2.6 X-Ray powder diffraction (XRPD)

The crystallinity of the lyophilised samples was analysed with the X-Ray Diffractometer XRD 3000 TT (Rich. Seifert & Co. GmbH & Co. KG, Ahrensberg, Germany), equipped with Cu K α (40 kV, 30 mA, wavelength 154.17 pm). The powders were analysed from 5 – 45° 2 θ with steps of 0.05° 2 θ and 2 seconds per step.

2.2.7 Reconstitution time

Reconstitution time was determined by dissolving the lyophilisates with the required volume of HPW. The required volume was calculated based on the solid content of each formulation. The time span from adding the HPW until complete dissolution was determined visually and considered as reconstitution time. During reconstitution, the vials were gently rolled by hand.

2.2.8 Light obscuration

Light obscuration was used to characterise subvisible particles in the range of 1 to 200 μm . For this purpose, a PAMAS SVSS-C35 particle counter with an HCB-LD-25/25 sensor (Partikelmess- und Analysensysteme GmbH, Ruthesheim, Germany) was used. Samples were analysed 4 times with 0.2 ml each after rinsing the system with 0.2 μm filtrated HPW and a sample pre-rinse with 0.2 ml according to USP 788³². At least 3 samples were measured to evaluate the subvisible particle count per ml of particles $\geq 1 \mu\text{m}$.

2.2.9 Turbidity

To further assess protein aggregation the turbidity was measured by static light scattering at 90° ($\lambda = 860 \text{ nm}$) using a NEPHLA turbidimeter (Dr. Lange, Düsseldorf, Germany). The turbidity was recorded in formazine nephelometric units (FNU). 1.5 ml of each sample was analysed in triplicates.

2.2.10 High performance size exclusion chromatography (HP-SEC)

Size exclusion chromatography was performed with an Agilent 1100 series HPLC system with UV detection at 280 nm (Agilent Technologies, Santa Clara, CA, USA). A Waters AQUITY UPLC Protein BEH 200Å column (1.7 μm ; 4.6 x 150 mm; Waters GmbH, Eschborn, Germany) and 50 mM sodium phosphate with 400 mM sodium perchlorate buffer at pH 6.0 were used as stationary and mobile phase, respectively. The flow rate was set to 0.4 mL/min. Prior to analysis, samples were centrifuged (10 min, 7000 rpm, Force7, Denver Instruments, Bohemia, NY, USA). The area of the peaks as well as relative areas of monomer, aggregates and fragments were determined after blank subtraction using the ChemStation software (Agilent Technologies).

3. Results and Discussion

3.1 Evaluation of arginine formulations during freeze-thaw cycles

Already the freezing step during freeze drying can result in a loss in product quality and was therefore addressed separately as a first step by characterising the influence of freezing and thawing on mAb stability in the presence of arginine and different counter ions ³³.

Freezing and thawing of sucrose reference formulations (pH 6.0) resulted in formation of approx. 50,000 particles $\geq 1 \mu\text{m/ml}$ (Figure 1). The addition of 0.05% polysorbate 80 to sucrose formulations reduced particle formation to less than 10,000 particles $\geq 1 \mu\text{m/ml}$. After freezing and thawing, ArgPhos pH 5 resulted in the most pronounced particle formation with 1.2 million, followed by ArgCitr pH 5 and SucArgCitr with around 100,000, and ArgPhos pH 6 and pH 7 as well as SucArgSucc with around 80,000 particles $\geq 1 \mu\text{m/ml}$. The other samples were comparable or better than the sucrose reference. Overall, there was a trend towards less particles with higher pH.

3.2 Protein stability after freeze drying

Subsequently the stabilising effect of arginine both in pure amino acid based as well as in arginine and sucrose containing mAb formulations with different pH and counter-ions was evaluated. The formulations were studied with respect to their lyophilisation properties and cake characteristics by characterising T_g' of the frozen liquid as well as T_g , residual moisture, reconstitution times, and cake appearance. The colloidal stability of the mAb was assessed by characterising the formation of soluble aggregates as well as subvisible particles and turbidity. Chemical degradation of a mAb was assessed on the basis of fragmentation.

3.2.1 T_g' of arginine based formulations

Pure arginine base has a rather low T_g' of -42.7°C ²⁸. The T_g' of arginine salts strongly depends on the counterion and the salt form and does not only reflect the difference in pH value resulting from a different amount of acid added. Multivalent acids increase the T_g' of arginine until a 1:1 mixture is reached, whereas monovalent acids tend to decrease T_g' . For example, citric acid brings the T_g' up to around -25°C , whereas acetic acid shows a continuous decrease in T_g' to values below -50°C ^{10,24,34}.

Too low T_g' values can cause issues during conventional freeze drying causing poor cake appearance due to collapse³⁵.

As shown in Table 2, ArgHCl and ArgSucc exhibited T_g' values of approx. -46.5°C and -38.0°C , respectively, which was not affected by pH and was below the T_g' of sucrose of approx. -32.5°C . The other sugar free arginine formulations ArgCitr and ArgLacto showed substantially higher T_g' values. ArgPhos exhibited a pH dependence of T_g' with -30.6°C at pH 5 and -27.3°C at pH 7. As the strong pH dependent shift of T_g' was only seen for phosphoric acid but not for the other trivalent acid, citric acid, a correlation with nearing one of the pK_a values of phosphoric acid cannot be concluded. Furthermore, a dependency of T_g' with respect to different acids in combination with arginine or with increasing phosphoric acid concentration were reported without a correlation to the pK_a ^{10,34}. Addition of sucrose led to a shift towards the T_g' of pure sucrose. The T_g' for SucArgCitr, SucArgLacto, and SucArgPhos became decreased by approx. 3°C and we observed an increase for ArgHCl and ArgSucc by 8 and 5°C respectively. This shift can be explained by the Gordon-Taylor equation, which states a mixed T_g' consisting of the mass fraction and the T_g' values of the individual components³⁶. Nevertheless, the bell-shaped transition temperature profiles of arginine with organic acids is not explainable by Gordon-Taylor¹⁰. Interestingly, T_g' of ArgLacto was higher if compared to the pure arginine T_g' , although lactobionic acid belongs to the monovalent acids³⁴. T_g' of a pure lactobionic acid solution was $-30.2 \pm 0.3^\circ\text{C}$ leading to a mixed T_g' explainable by the Gordon-Taylor equation¹⁰. Increasing the mAb concentration from 2 mg/ml to 50 mg/ml mAb led to an increase of T_g' ^{35,37}. Suc-HC exhibited an increase of about 2°C , whereas the T_g' values for ArgHCl-HC and ArgSucc-HC was increased by almost 15°C .

Thus, most of the arginine formulations can be handled by a standard conventional lyophilisation process and are expected to result in pharmaceutically acceptable cakes. ArgHCl and ArgSucc must be seen critical due to their low T_g' values. T_g' could be increased by adding sucrose or using high mAb concentrations.

3.2.2 Powder characteristics of the lyophilisates

Subsequently, sucrose and arginine formulations pH 5 to 7 were freeze dried according to cycle 1. ArgCitr, ArgLacto, and ArgPhos resulted in pharmaceutically elegant cakes (Figure 3). The pure sucrose lyophilisates showed some defects. The pH value did not affect the macroscopic appearance. ArgHCl and ArgSucc were substantially collapsed and shrunken since the product temperature of -32°C during primary drying in cycle 1 was significantly above T_g' (Figure 2). Less defects could be observed when lyophilised at a lower pressure according to cycle 2 resulting in a product temperature of -34°C during primary drying (Figure 3). Secondary drying temperature was decreased to 30°C for freeze drying the mixed arginine sucrose formulations according to cycle 3 (Figure 3). The addition of sucrose did not influence cake appearance of ArgCitr, ArgLacto, and ArgPhos based lyophilisates and improved the appearance of ArgHCl and ArgSucc based cakes. 50 mg/ml mAb formulations freeze dried according to cycle 1 resulting in a product temperature of -36°C during primary drying were all elegant products due to the higher T_g' values and the increased total solid contents. Cake appearance of all the formulations did not change over 6 months storage at 40°C or 50°C .

Overall, ArgHCl and ArgSucc required low product temperatures during primary drying to gain elegant cakes. A mixture with sucrose or the addition of a high mAb concentration can improve slow primary drying times due to their low T_g' values and resulted in acceptable cakes. Other arginine formulations showed elegant cakes after freeze drying in a standard procedure.

The sucrose reference showed a T_g value of 65°C and a RM of 0.5% after lyophilisation³⁸. T_g did not significantly change upon storage at 50°C for up to 6 months while RM increased slightly to 0.9% (Figure 4). Overall, the Arg based lyophilisates showed very high T_g and low RM values after production. Similar to T_g' , the T_g of freeze dried arginine with multivalent counter ions increased with increasing pH³⁴, whereas ArgHCl and ArgLacto lyophilisates were not affected by pH. ArgCitr showed the highest T_g values of 90°C to 120°C (RM of 0.3%), followed by ArgPhos and ArgSucc. ArgHCl and ArgLacto lyophilisates had similar T_g values of 80°C independent of pH. After 6 months storage at 50°C , arginine based products increased in RM to between 1% and 2% and correspondingly the T_g

values decreased by up to 15°C^{20,39}. Even after storage, T_g was above storage temperature indicating potential protein stability through immobilisation in the amorphous matrix^{40,41}.

Mixtures of sucrose and arginine salts were freeze dried according to cycle 3 with a lower secondary drying temperature resulting in higher RM levels after production except for ArgLacto. The higher RM levels and the addition of sucrose led to a decrease in T_g to 55°C for SucArgHCl and between 72°C and slightly above 80°C for SucArgCitr, SucArgPhos, SucArgSucc and SucArgLacto. During 6 months storage at 40°C, the T_g slightly decreased by approx. 5°C.

Products with 50 mg/ml mAb resulted in similar RM and the higher protein concentrations led to even further increased T_g values⁴². An increase by 10°C up to 20°C was found for ArgPhos-HC, ArgLacto-HC, and Suc-HC, a drastic increase by 40°C for ArgSucc-HC. Hardly any change was found for ArgCitr-HC, which already exhibited an extremely high T_g , and ArgHCl-HC, which was unexpected as an increase was identified in literature²⁹.

Sugar-free Arg based formulations exhibited extremely high T_g values compared to the sucrose reference formulation pointing towards high protein stability in the immobilised amorphous phase^{40,41}. In combination with sucrose, T_g values were decreased slightly according to a mixed T_g based on the Gordon-Taylor equation¹⁰ but the values are still high overall.

All samples showed a T_g in DSC analysis and were fully amorphous according to XRPD except for ArgHCl stored for 3 or 6 months, independent of pH (Figure 5). The addition of sucrose as well as a high mAb concentration of 50 mg/ml suppressed the crystallisation of ArgHCl upon storage.

Reconstitution of all 2 mg/ml mAb samples was completed within 30 s at t_0 and after storage. The 50 mg/ml mAb samples based on ArgHCl showed the same fast reconstitution. For all other formulations, reconstitution took longer with up to 60 s for ArgCitr-HC, ArgLacto-HC, and ArgSucc-HC and 3 min for ArgPhos-HC, which increased further upon storage. Thus, arginine based lyophilisates dissolve which makes especially ArgHCl a promising alternative candidate for highly concentrated mAb sugar based lyophilisates with delayed dissolution times^{6,43}.

3.2.3 mAb stability during lyophilisation and upon storage

The mAb process and storage stability was analysed with respect to aggregation by SEC, turbidity and subvisible particle analysis by light obscuration in order to assess colloidal stability of the mAb. Furthermore, information on fragmentation was collected via the SEC measurements in order to evaluate chemical stability of the formulations.

The sucrose reference formulations pH 5, 6, and 7 resulted in low particle counts after lyophilisation with less than 30,000 particles $\geq 1 \mu\text{m/ml}$ and a turbidity of < 5 FNU (Table 3). Less than 2% HMWS were found for all pH values (Figure 6). After 6 months storage at 50°C, particle count and turbidity increased in a pH dependent manner. While pH 5 revealed no changes, pH 7 resulted in around 90,000 particles and 11.7 FNU turbidity. Additionally, large and rapidly sedimenting visible particles had formed. The HMWS level did not increase but the relative monomer recovery compared to t_0 decreased to 85 – 90% due to larger particle formation⁴⁴.

The sugar-free arginine based 2 mg/ml mAb products differed depending on counter ion and pH. Whereas the number of particles $\geq 1 \mu\text{m/ml}$ was similar or slightly higher for ArgCitr, ArgLacto and ArgPhos, the mAb showed substantially better process stability in ArgHCl and ArgSucc with less than 5,000 and $< 17,000$ particles, respectively. Turbidity and HMWS content were similar for all formulations and comparable to sucrose.

After 6 months storage at 50°C, the particle count of ArgCitr, ArgLacto, and ArgPhos was higher than 100,000 and turbidity reached values above 10 FNU. These formulations showed a slight pH dependency. ArgCitr and ArgLacto showed an increase in particle count with increasing pH, moving closer to the IEP of the mAb of around 8.0. This tendency is less pronounced for ArgPhos, which correspond to the freeze/thaw experiments where higher particle formation only occurred at pH 5. Overall similar trends were observed in HMWS content and relative monomer recovery. The HMWS content increased up to 4% in ArgLacto and up to 10% and 14% at pH 7 for ArgCitr and ArgPhos respectively. The relative monomer recovery decreased to 85% in ArgLacto, 80% in ArgCitr, and 75 – 82% in ArgPhos. The mAb stability after 6 months at 40°C in ArgSucc lyophilisates was superior at pH 5 with less than 50,000 particles $\geq 1 \mu\text{m/ml}$, turbidity below 10 FNU, approx. 3% HMWS and more

than 97% monomer recovery. At pH 6 and 7 the particle numbers were higher. ArgHCl formulations exhibited the best stability with the lowest particle numbers, turbidity values below 10 FNU, no increase in HMWS level and no monomer loss in SEC.

In general, mixtures of sucrose and arginine at pH 6 showed a better process stability. Only for SucArgHCl a slightly higher particle count compared to pure ArgHCl product was seen. After 6 months storage at 40°C, all formulations resulted in a less pronounced increase in particle count compared to the sugar free formulations. Most particles were found for SucArgLacto with around 55,000 particles, which was comparable to pure sucrose. The turbidity was similar or lower compared to both pure sucrose and the sugar-free products. The HMWS levels increased and the relative monomer recovery decreased in none of the formulations. Only a slight increase in HMWS formation from 2.9% to max. 4.1% upon storage at 40°C was observed in literature for sucrose mixtures with 50 mg/ml mAb³⁰.

Increasing the mAb concentration to 50 mg/ml resulted in similar or slightly lower particle levels. The turbidity values were significant higher with around 10 FNU compared to 2 mg/ml products, except for ArgHCl-HC which was low with 4.2 FNU. Aggregate levels of around 3% HMWS were found. After 6 months storage at 40°C, particle counts increased less compared to the corresponding 2 mg/ml samples. Again, ArgCitr-HC, ArgPhos-HC, and ArgSucc-HC were comparable to the sucrose reference Suc-HC reference and ArgLacto-HC and ArgHCl-HC were superior. The turbidity increased slightly in all formulations and the HMWS levels in Suc-HC and ArgLacto-HC went up to 4% and to 7 – 10% in all other arginine formulations including ArgHCl-HC. A decrease in relative monomer recovery was not observed. Thus, at high mAb concentration ArgHCl and ArgLacto were beneficial with ArgHCl forming the least amount of larger aggregates and ArgLacto the least soluble aggregates.

All formulations showed comparable stability against cleavage. Overall, the mAb did not undergo severe fragmentation in any formulation upon freeze drying and storage (Figure 6B).

Thus, despite providing the lowest T_g of all tested counter ions, the monovalent hydrochloric acid was the most suitable counter ion for arginine to stabilise the mAb during freeze-drying and storage, independent of the pH value and addition of sucrose. Overall it provided better protein stability compared to the sucrose reference formulations. This is in good agreement with previously

published data similarly concluding best protein stability for ArgHCl lyophilisates ³⁰. Multivalent counter ions resulted in reduced protein stability compared to ArgHCl, with the counter ion succinic acid being an exception at pH 5. The amorphous matrices of arginine with multivalent counter ions are different to ArgHCl matrices, indicated by high T_g values of up to 120 °C. Accordingly, they seem to act differently in terms of stabilising a mAb. The use of arginine lyophilisates in marketed products further supports the potential of sugar-free arginine formulations ⁹.

The addition of sucrose to arginine formulations resulted in good or better results for particle formation and protein stability compared to the sucrose reference, again similar to previous reports ^{29,30}. A high mAb concentration of 50 mg/ml resulted in overall low particle counts and ArgLacto-HC turned out to be an interesting formulation candidate next to ArgHCl-HC.

4. Conclusion

The aim of this study was to test sugar-free arginine based formulations as alternatives to sucrose or sucrose-containing arginine formulations for lyophilisation of biopharmaceuticals. By using a sugar-free formulation approach, potential glycation issues, which may occur in sucrose based formulations, can be circumvented in an elegant way ^{2,4}. We studied the freeze-drying performance of sugar-free arginine formulations with different counterions at different pH with 2 and 50 mg/ml of an exemplary mAb. The freeze-thaw stability of the mAb as well as the physicochemical characteristics and the mAb stability during freeze-drying and upon subsequent storage up to 6 months at 40°C or 50°C were studied. The sucrose references pH 5 to pH 7 showed good cake appearance and remained amorphous over storage. They were characterised by a T_g of 65°C ³⁸. The lyophilisates exhibited low particle counts and did not show an increase in HMWS but substantial subvisible and visible particle formation, which led to a loss in monomer recovery ⁴⁴. Stability of the antibody upon freezing and thawing in ArgHCl, ArgLacto or ArgSucc formulations was found to be comparable or better than in a sucrose formulation. Potential protein instabilities during the freezing step of the applied freeze-drying process for arginine formulations could be excluded for these formulations.

T_g' was increased compared to the sucrose formulation by using arginine in combination with citric, lactobionic, and phosphoric acid potentially enabling a more robust and faster lyophilisation process. The T_g' values of ArgHCl and ArgSucc at 2 mg/ml mAb were very low with -46°C and -38°C , respectively, which poses challenges in the opposite way. This challenge can be overcome by adding sucrose or using high mAb concentration leading to an increase in T_g' . By running an adapted freeze drying cycle for ArgHCl and ArgSucc, all formulations resulted in good cake appearance which did not change over storage. The T_g values of ArgHCl and sucrose lyophilisates were similar with approx. 65°C whereas the other arginine formulations showed even higher T_g values of up to 120°C . All formulations exhibited T_g values well above the intended storage temperature providing a good forecast for protein stabilisation through protein immobilisation in the amorphous matrix ^{40,41}. ArgHCl partially crystallised after 3 months at 50°C but crystallisation was suppressed in the sucrose mixtures or at 50 mg/ml mAb at 40°C storage temperature. The Arg based lyophilisates reconstituted fast. At 50 mg/ml mAb, reconstitution times were prolonged except for ArgHCl-HC taking only 30 sec for complete dissolution, which could be highly valuable asset for high concentration formulation or large fill volumes ⁴³. Both process and storage stability of the mAb was superior in ArgHCl and ArgSucc if compared to sucrose with respect to aggregation. Mixtures of arginine and sucrose improved both process and storage stability of the mAb compared to a sucrose formulation without arginine. At high mAb concentration ArgHCl-HC and ArgLacto-HC provided good process and storage stability with respect to aggregation and fragmentation.

In summary, this study presented sugar-free Arg based formulations as potential alternative to sucrose or sucrose-containing arginine lyophilisates for biopharmaceuticals, which were already reported in literature ^{29,30}. While multivalent acids as counter ions enhance T_g' and T_g to values above corresponding sucrose formulations and provide good process characteristics, they showed a lack in protein stabilisation. Arginine formulations in combination with the counter ions succinate and lactobionate were comparable in both cake appearance and protein stabilisation efficiency to the sucrose reference formulation. The monovalent hydrochloride rendered outstanding protein stability. The low T_g' values of ArgSucc and ArgHCl with low mAb concentration may pose challenges

in freeze drying and corrupt the cake appearance but this can be overcome by addition of sucrose and potentially other crystalline bulking agents. Further studies should address these drawbacks while maintaining protein stability. Overall, arginine based lyophilisates do have both advantages and disadvantages but should be used more frequently as alternatives to sugar based formulations.

References

1. Kasper JC, Winter G, Friess W 2013. Recent advances and further challenges in lyophilization. *Eur J Pharm Biopharm* 85(2):162-169.
2. Heljo P. 2013. Comparison of disaccharides and polyalcohols as stabilizers in freeze-dried protein formulations. ed.: University of Helsinki.
3. Franks F 1998. Freeze-drying of bioproducts: putting principles into practice. *Eur J Pharm Biopharm* 45(3):221-229.
4. O'Brien J 1996. Stability of Trehalose, Sucrose and Glucose to Nonenzymatic Browning in Model Systems. *J Food Sci* 61(4):679-682.
5. Forney-Stevens KM, Bogner RH, Pikal MJ 2016. Addition of Amino Acids to Further Stabilize Lyophilized Sucrose-Based Protein Formulations: I. Screening of 15 Amino Acids in Two Model Proteins. *J Pharm Sci* 105(2):697-704.
6. Horn J, Tolardo E, Fissore D, Friess W 2018. Crystallizing amino acids as bulking agents in freeze-drying. *Eur J Pharm Biopharm* 132:70-82.
7. Mattern M, Winter G, Kohnert U, Lee G 1999. Formulation of Proteins in Vacuum-Dried Glasses. II. Process and Storage Stability in Sugar-Free Amino Acid Systems. *Pharm Dev Technol* 4(2):199-208.
8. Mattern M, Winter G, Rudolph R, Lee G 1997. Formulation of proteins in vacuum-dried glasses. I: Improved vacuum-drying of sugars using crystallising amino acids. *Eur J Pharm Biopharm* 44(2):177-185.
9. Gervasi V, Dall Agnol R, Cullen S, McCoy T, Vucen S, Crean A 2018. Parenteral protein formulations: An overview of approved products within the European Union. *Eur J Pharm Biopharm* 131:8-24.
10. Izutsu K-I, Kadoya S, Yomota C, Kawanishi T, Yonemochi E, Terada K 2009. Freeze-Drying of Proteins in Glass Solids Formed by Basic Amino Acids and Dicarboxylic Acids. *Chem Pharm Bull* 57(1):43-48.
11. Varshney DB, Kumar S, Shalaev EY, Sundaramurthi P, Kang SW, Gatlin LA, Suryanarayanan R 2007. Glycine crystallization in frozen and freeze-dried systems: effect of pH and buffer concentration. *Pharm Res* 24(3):593-604.
12. Chang L, Shepherd D, Sun J, Ouellette D, Grant KL, Tang X, Pikal MJ 2005. Mechanism of protein stabilization by sugars during freeze-drying and storage: Native structure preservation, specific interaction, and/or immobilization in a glassy matrix? *J Pharm Sci* 94(7):1427-1444.
13. Hawe A. 2006. Studies on Stable Formulations for a Hydrophobic Cytokine. ed.: Ludwig-Maximilians-Universität München.
14. Meyer JD, Nayar R, Manning MC 2009. Impact of bulking agents on the stability of a lyophilized monoclonal antibody. *Eur J Pharm Sci* 38(1):29-38.
15. Chang BS, Randall CS 1992. Use of subambient thermal analysis to optimize protein lyophilization. *Cryobiology* 29(5):632-656.
16. Arakawa T, Timasheff SN 1983. Preferential interactions of proteins with solvent components in aqueous amino acid solutions. *Arch Biochem Biophys* 224(1):169-177.

17. Sane SU, Wong R, Hsu CC 2004. Raman Spectroscopic Characterization of Drying-Induced Structural Changes in a Therapeutic Antibody: Correlating Structural Changes with Long-Term Stability. *J Pharm Sci* 93(4):1005-1018.
18. Al-Hussein A, Gieseler H 2013. Investigation of Histidine Stabilizing Effects on LDH During Freeze-Drying. *J Pharm Sci* 102(3):813-826.
19. Tsumoto K, Umetsu M, Kumagai I, Ejima D, Philo JS, Arakawa T 2004. Role of Arginine in Protein Refolding, Solubilization, and Purification. *Biotechnol Prog* 20(5):1301-1308.
20. Hackl E, Darkwah J, Smith G, Ermolina I 2018. Effect of Arginine on the Aggregation of Protein in Freeze-Dried Formulations Containing Sugars and Polyol: 1—Formulation Development. *AAPS PharmSciTech* 19(2):896-911.
21. Schneider CP, Shukla D, Trout BL 2011. Arginine and the Hofmeister Series: the role of ion-ion interactions in protein aggregation suppression. *J Phys Chem B* 115(22):7447-7458.
22. Arakawa T, Tsumoto K, Kita Y, Chang B, Ejima D 2007. Biotechnology applications of amino acids in protein purification and formulations. *Amino Acids* 33(4):587-605.
23. Ohtake S, Kita Y, Arakawa T 2011. Interactions of formulation excipients with proteins in solution and in the dried state. *Adv Drug Del Rev* 63(13):1053-1073.
24. Stärtzel P 2018. Arginine as an Excipient for Protein Freeze-Drying: A Mini Review. *J Pharm Sci* 107(4):960-967.
25. Tian F, Middaugh CR, Offerdahl T, Munson E, Sane S, Rytting JH 2007. Spectroscopic evaluation of the stabilization of humanized monoclonal antibodies in amino acid formulations. *Int J Pharm* 335(1–2):20-31.
26. Paik SH, Kim YJ, Han SK, Kim JM, Huh JW, Park YI 2012. Mixture of three amino acids as stabilizers replacing albumin in lyophilization of new third generation recombinant factor VIII GreenGene F. *Biotechnol Prog* 28(6):1517-1525.
27. Lale SV, Goyal M, Bansal AK 2011. Development of lyophilization cycle and effect of excipients on the stability of catalase during lyophilization. *International Journal of Pharmaceutical Investigation* 1(4):214-221.
28. Izutsu KI, Yoshida H, Shibata H, Goda Y 2016. Amorphous-Amorphous Phase Separation of Freeze-Concentrated Protein and Amino Acid Excipients for Lyophilized Formulations. *Chem Pharm Bull* 64:1674-1680.
29. Stärtzel P, Gieseler H, Gieseler M, Abdul-Fattah AM, Adler M, Mahler HC, Goldbach P 2015. Freeze-Drying of L-Arginine/Sucrose-Based Protein Formulations, Part 2: Optimization of Formulation Design and Freeze-Drying Process Conditions for an L-Arginine Chloride-Based Protein Formulation System. *J Pharm Sci* 104(12):4241-4256.
30. Stärtzel P, Gieseler H, Gieseler M, Abdul-Fattah AM, Adler M, Mahler H-C, Goldbach P 2015. Freeze Drying of L-Arginine/Sucrose-Based Protein Formulations, Part I: Influence of Formulation and Arginine Counter Ion on the Critical Formulation Temperature, Product Performance and Protein Stability. *J Pharm Sci* 104(7):2345-2358.
31. Schersch K, Betz O, Garidel P, Muehlau S, Bassarab S, Winter G 2010. Systematic investigation of the effect of lyophilizate collapse on pharmaceutically relevant proteins I: Stability after freeze-drying. *J Pharm Sci* 99(5):2256-2278.
32. United States Pharmacopeial Convention. 2012. USP<788>Particulate Matter In Injections. In Rockville MUSPC, editor United States Pharmacopoeia (USP 36-NF 31), ed., Rockville, MD.
33. Kasper JC, Friess W 2011. The freezing step in lyophilization: physico-chemical fundamentals, freezing methods and consequences on process performance and quality attributes of biopharmaceuticals. *Eur J Pharm Biopharm* 78(2):248-263.
34. Izutsu K-I, Fujimaki Y, Kuwabara A, Aoyagi N 2005. Effect of counterions on the physical properties of L-arginine in frozen solutions and freeze-dried solids. *Int J Pharm* 301(1–2):161-169.
35. Depaz RA, Pansare S, Patel SM 2016. Freeze-Drying Above the Glass Transition Temperature in Amorphous Protein Formulations While Maintaining Product Quality and Improving Process Efficiency. *J Pharm Sci* 105(1):40-49.
36. Seo J-A, Oh J, Kim HK, Hwang Y-H, Yang YS, Kim SJ 2005. Study of Glass Transition Temperatures in Sugar Mixtures. *Journal of the Korean Physical Society* 46(3):606-609.

37. Garidel P, Presser I. 2019. Lyophilization of High-Concentration Protein Formulations. *Lyophilization of Pharmaceuticals and Biologicals*, ed. p 291-325.
38. te Booy MP, de Ruiter RA, de Meere AL 1992. Evaluation of the physical stability of freeze-dried sucrose-containing formulations by differential scanning calorimetry. *Pharm Res* 9(1):109-114.
39. Schersch K, Betz O, Garidel P, Muehlau S, Bassarab S, Winter G 2012. Systematic investigation of the effect of lyophilizate collapse on pharmaceutically relevant proteins, part 2: stability during storage at elevated temperatures. *J Pharm Sci* 101(7):2288-2306.
40. Grasmeyer N, Stankovic M, de Waard H, Frijlink HW, Hinrichs WL 2013. Unraveling protein stabilization mechanisms: vitrification and water replacement in a glass transition temperature controlled system. *Biochim Biophys Acta* 1834(4):763-769.
41. Mensink MA, Frijlink HW, van der Voort Maarschalk K, Hinrichs WL 2017. How sugars protect proteins in the solid state and during drying (review): Mechanisms of stabilization in relation to stress conditions. *Eur J Pharm Biopharm* 114:288-295.
42. Carpenter JF, Pikal MJ, Chang BS, Randolph TW 1997. Rational Design of Stable Lyophilized Protein Formulations: Some Practical Advice. *Pharm Res* 14(8):969-975.
43. Cao W, Krishnan S, Ricci MS, Shih LY, Liu D, Gu JH, Jameel F 2013. Rational design of lyophilized high concentration protein formulations-mitigating the challenge of slow reconstitution with multidisciplinary strategies. *Eur J Pharm Biopharm* 85(2):287-293.
44. Barnard JG, Singh S, Randolph TW, Carpenter JF 2011. Subvisible Particle Counting Provides a Sensitive Method of Detecting and Quantifying Aggregation of Monoclonal Antibody Caused by Freeze-Thawing: Insights Into the Roles of Particles in the Protein Aggregation Pathway. *J Pharm Sci* 100(2):492-503.

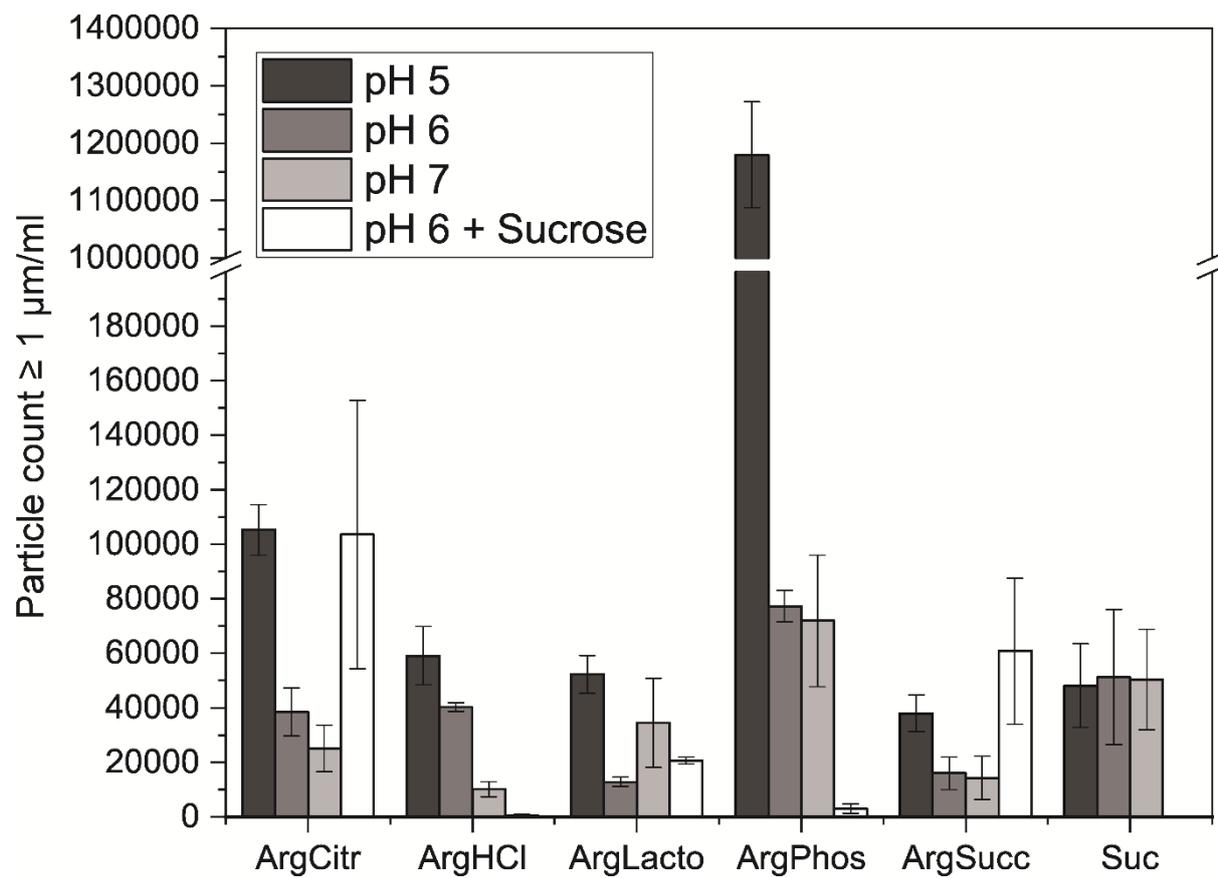


Fig. 1. Subvisible particle count after 3 freeze-thaw cycles.

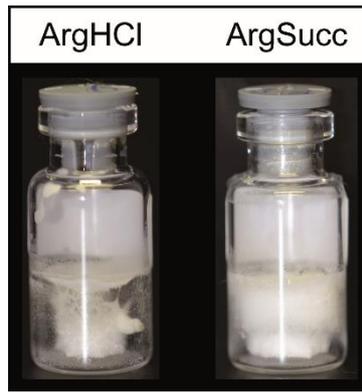


Fig. 2. Macroscopic appearance of ArgHCl and ArgSucc at pH 6 lyophilised according to cycle 1.

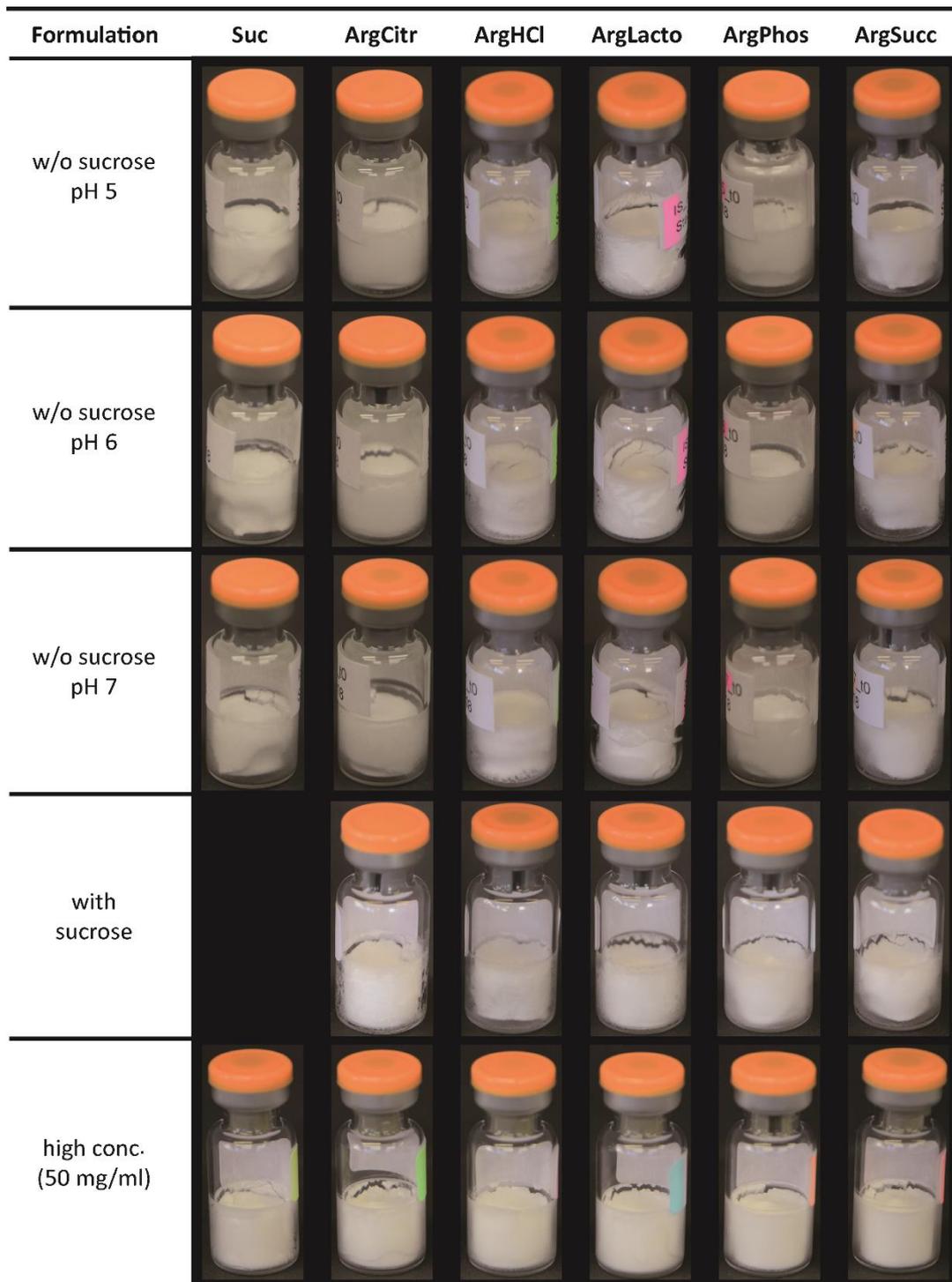


Fig. 3. Macroscopic appearance of sucrose and arginine lyophilisates pH 5.0, 6.0, and 7.0 that differ in the counter ion, the addition of sucrose, and mAb content. Freeze drying cycle 1 was used for Suc, ArgCitr, ArgLacto, and ArgPhos without sucrose, as well as all 50 mg/mL mAb lyophilisates; cycle 2 was used for ArgHCl and ArgSucc without sucrose; cycle 3 was used for all arginine-sucrose mixtures. The symbol p represents good cake appearance, o minor cake shrinkage/minor structural defects, and e major cake shrinkage/structural defects.

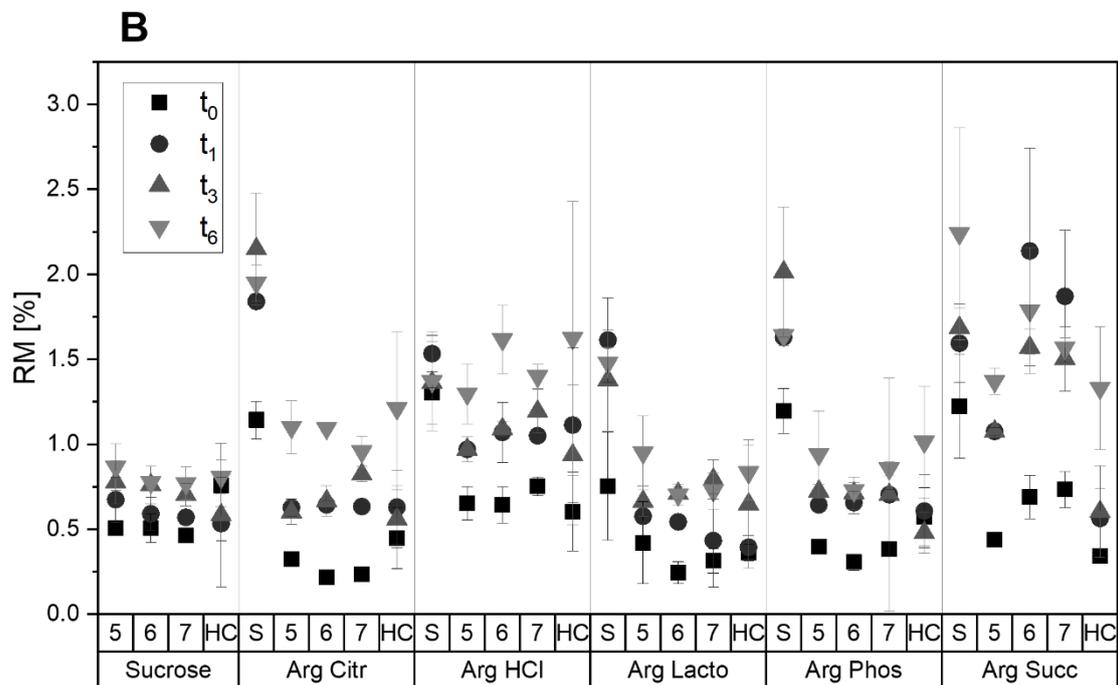
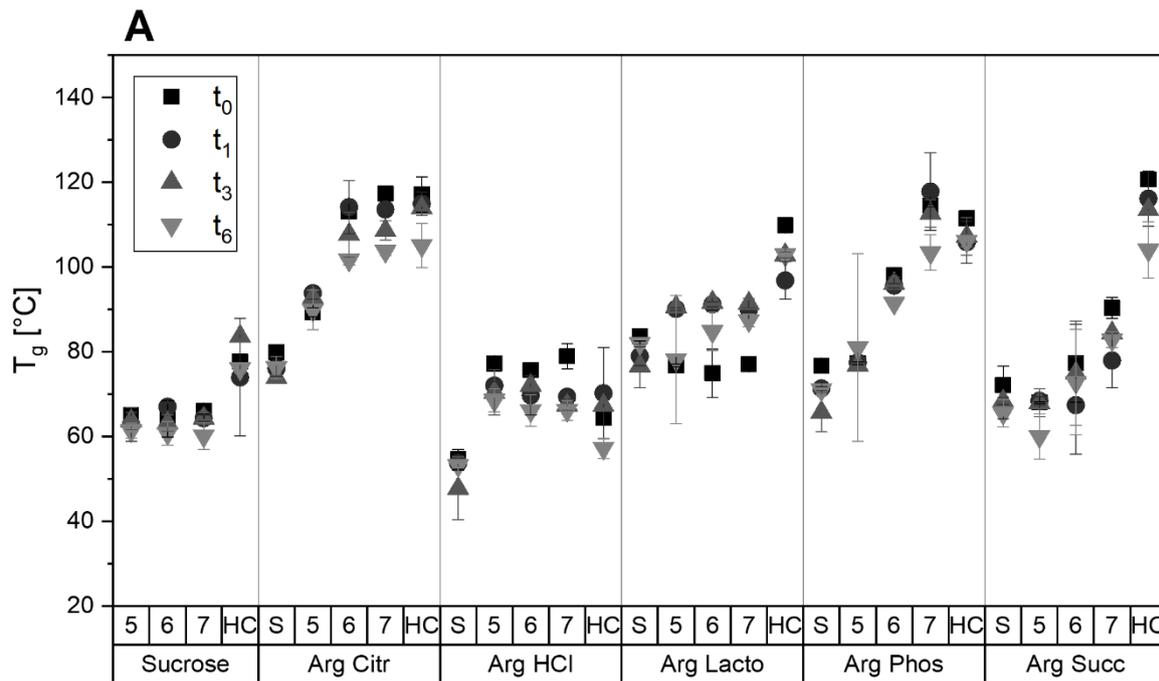


Fig. 4. T_g (a) and RM (b) of sucrose and arginine based 2 mg/mL mAb lyophilisates at pH 5, 6 and 7 (5, 6, 7) stored at 50 °C, as a mix with sucrose at pH 6 (S) and at pH 6 with 50 mg/ mL mAb (HC) after lyophilisation (t_0) and upon storage at 40 °C for 1 (t_1), 3 (t_3) and 6 months (t_6).

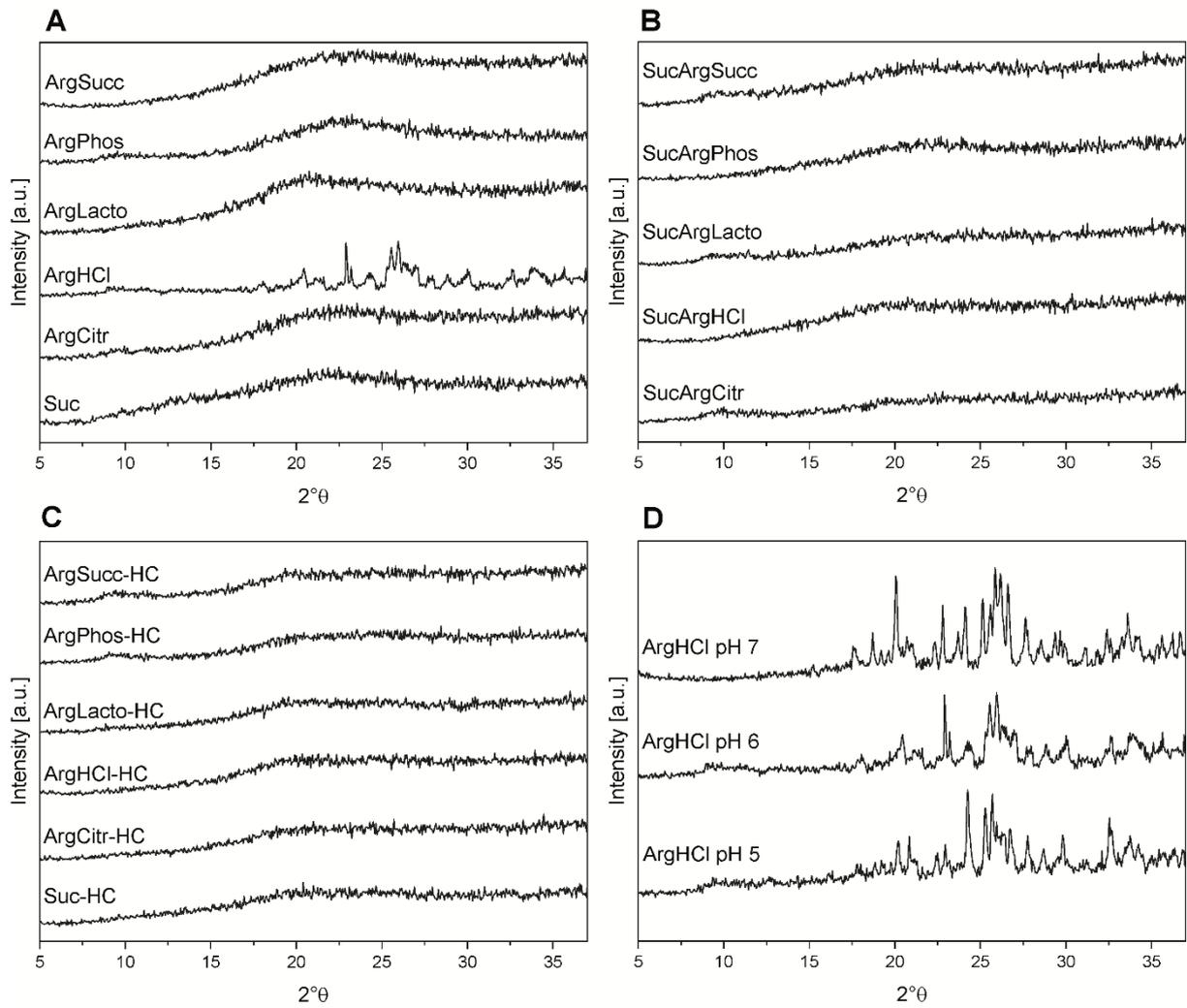


Fig. 5. XRPD of (a) 2 mg/mL mAb pH 6 samples after 6 months storage at 50 °C, (b) mixtures of arginine and sucrose pH 6 samples after 6 months storage at 40 °C, (c) 50 mg/mL mAb pH 6 samples after 6 months storage at 40 °C, and (d) ArgHCl pH 5, 6, and 7 after 3 months storage at 50 °C.

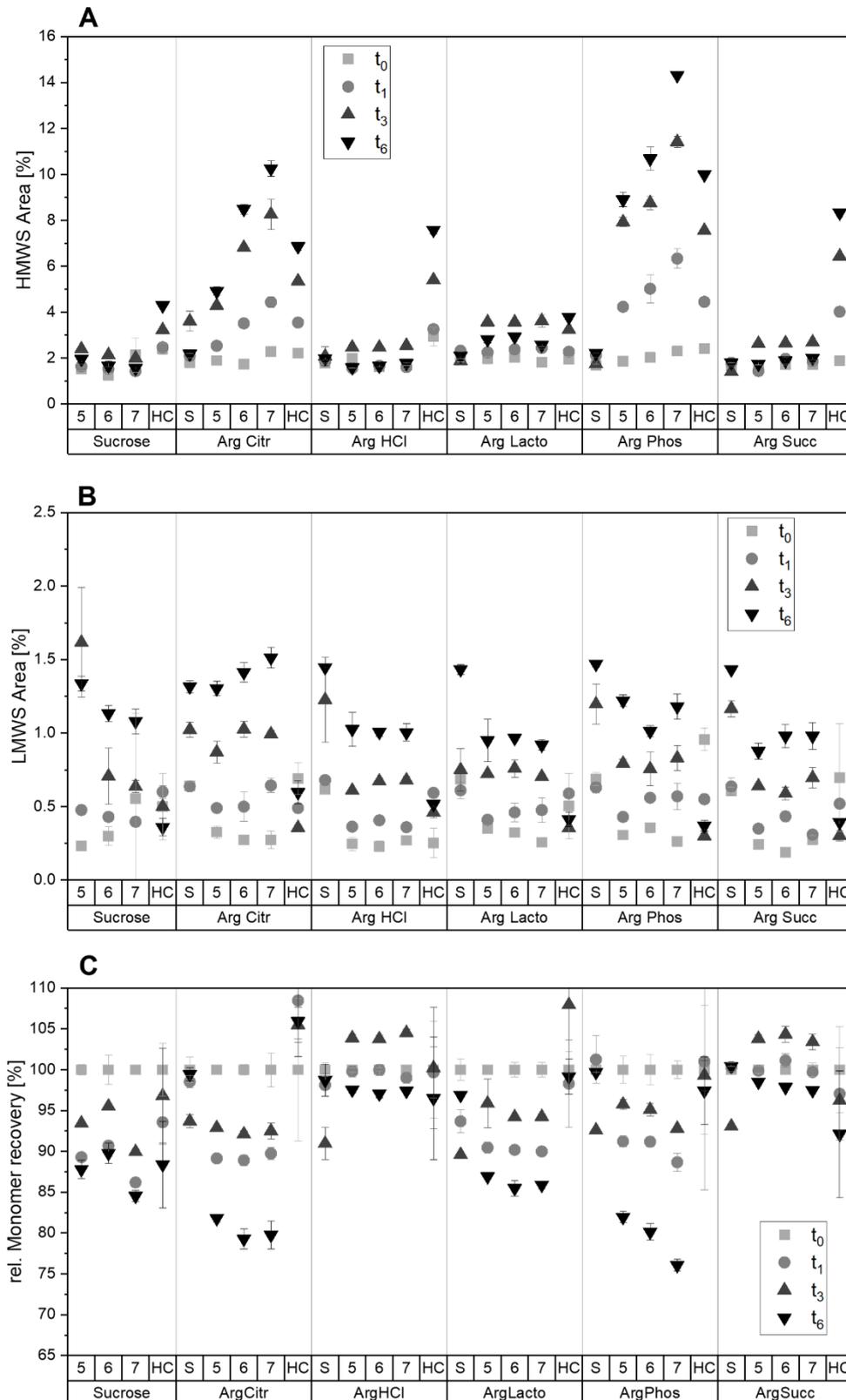


Fig. 6. (a) Higher molecular weight species (HMWS), (b) lower molecular weight species (LMWS), and (c) relative monomer recovery of sucrose and arginine based 2 mg/mL mAb lyophilisates at pH 5, 6 and 7 (5, 6, 7), as a mix with sucrose at pH 6 (S) and at pH 6 with 50 mg/mL mAb (HC) after lyophilisation (t₀) and upon storage for 1 (t₁), 3 (t₃) and 6 months (t₆).

Table 1: Freeze-drying cycles used.

Step	Ramp [°C/min]	Shelf temperature [°C]	Pressure [μbar]	Hold time [h]
<i>Cycle 1: Sucrose, ArgCitr, ArgLacto, ArgPhos (pH 5 – 7)</i>				
<i>ArgCitr-HC, ArgHCl-HC, ArgLacto-HC, ArgPhos-HC, ArgSucc-HC, Suc-HC (pH 6)</i>				
Freezing	1.0	-50	-	1.5
Primary Drying	0.5	-20	60	54.5 / 64.7 (HC)
Secondary Drying	0.4	+40	60	8.3
<i>Cycle 2: ArgHCl, ArgSucc (pH 5 – 7)</i>				
Freezing	1.0	-50	-	1.5
Primary Drying	0.5	-20	40	49.5
Secondary Drying	0.4	+50	40	5
<i>Cycle 3: SucArgCitr, SucArgHCl, SucArgLacto, SucArgPhos, SucArgSucc (pH 6)</i>				
Freezing	1.0	-50	-	1.5
Primary Drying	0.5	-20	60	46.5
Secondary Drying	0.4	+30	60	8.3

Table 2: T_g' values of sucrose and arginine formulations with mAb.

Formulation	T_g' [°C]				
	pH 5	pH 6	pH 7	with sucrose (pH 6)	high conc (pH 6)
Suc	-32.5 ± 0.3	-33.5 ± 0.3	-31.3 ± 0.6	--	-31.6 ± 0.4
ArgCitr	-26.4 ± 0.1	-27.1 ± 0.1	-27.2 ± 0.03	-30.4 ± 0.03	-23.1 ± 0.1
ArgHCl	-46.4 ± 0.1	-46.3 ± 0.2	-46.4 ± 0.1	-38.2 ± 0.1	-32.5 ± 0.3
ArgLacto	-26.2 ± 0.3	-25.6 ± 0.05	-25.2 ± 0.4	-28.8 ± 0.2	-21.4 ± 0.1
ArgPhos	-30.6 ± 0.01	-28.7 ± 0.3	-27.3 ± 0.1	-32.1 ± 0.2	-23.8 ± 0.03
ArgSucc	-37.7 ± 0.1	-38.0 ± 0.1	-37.9 ± 0.1	-33.8 ± 0.3	-23.6 ± 0.02

Table 3: Subvisible particle count and turbidity data of sucrose and arginine based 2 mg/ml mAb lyophilisates at pH 5, 6, and 7 (5, 6, 7) stored at 50°C, as a mix of sucrose at pH 6 (S) stored at 40°C, and at pH 6 with 50 mg/ml mAb (HC) after lyophilisation (t₀) and after 6 months storage (t₆) at 40°C. Turbidity data marked with * were analysed after 3 months storage.

		Subvisible particle count $\geq 1 \mu\text{m/ml}$		Turbidity [FNU]	
		t ₀	t ₆	t ₀	t ₆
Sucrose	5	23,425 \pm 947	8,003 \pm 1,257	3.3 \pm 0.4	3.1 \pm 0.1
	6	24,623 \pm 2,334	53,550 \pm 7,280	3.3 \pm 0.8	7.7 \pm 0.3
	7	28,520 \pm 2,926	90,296 \pm 17,487	4.6 \pm 1.5	11.7 \pm 1.0
	HC	15,516 \pm 2,372	62,851 \pm 16,681	13.3 \pm 1.1	14.8 \pm 0.4*
ArgCitr	S	31,045 \pm 9,252	42,826 \pm 9,904	2.7 \pm 0.5	6.4 \pm 0.8
	5	60,562 \pm 18,570	118,557 \pm 22,679	4.9 \pm 3.6	12.9 \pm 0.7
	6	15,705 \pm 3,704	144,738 \pm 25,431	2.8 \pm 0.7	11.5 \pm 1.7
	7	14,450 \pm 1,439	144,651 \pm 24,362	4.6 \pm 2.2	11.9 \pm 0.4
	HC	15,951 \pm 5,481	71,050 \pm 21,407	11.3 \pm 0.4	13.2 \pm 0.9*
ArgHCI	S	10,559 \pm 1,281	12,726 \pm 3,208	2.6 \pm 0.4	4.8 \pm 1.0
	5	4,638 \pm 716	23,947 \pm 7,182	3.4 \pm 1.1	4.2 \pm 0.5
	6	1,311 \pm 475	41,502 \pm 4,358	2.4 \pm 0.1	5.6 \pm 0.3
	7	1,685 \pm 229	74,419 \pm 10,053	3.4 \pm 1.1	7.1 \pm 0.02
	HC	7,590 \pm 4,864	15,447 \pm 5,027	4.2 \pm 0.2	5.2 \pm 0.3*
ArgLacto	S	23,017 \pm 5,325	54,532 \pm 10,957	3.1 \pm 0.3	7.0 \pm 0.2
	5	58,353 \pm 26,010	101,698 \pm 32,743	5.7 \pm 1.1	14.5 \pm 1.2
	6	56,680 \pm 13,404	113,148 \pm 36,471	6.7 \pm 1.3	16.9 \pm 0.7
	7	42,961 \pm 12,292	122,398 \pm 40,258	3.9 \pm 0.7	18.3 \pm 0.7
	HC	12,870 \pm 5,141	42,001 \pm 14,172	8.8 \pm 0.5	9.3 \pm 0.1*
ArgPhos	S	13,315 \pm 3,783	29,408 \pm 4,107	2.6 \pm 0.4	5.9 \pm 0.1
	5	53,938 \pm 12,009	144,320 \pm 16,079	6.0 \pm 1.2	17.0 \pm 1.1
	6	30,348 \pm 5,273	135,814 \pm 20,690	3.9 \pm 0.5	13.1 \pm 1.1
	7	46,976 \pm 5,529	116,887 \pm 17,356	5.6 \pm 0.7	11.7 \pm 0.2
	HC	10,911 \pm 5,049	76,135 \pm 22,258	10.2 \pm 0.2	12.4 \pm 0.7*
ArgSucc	S	14,294 \pm 2,929	33,445 \pm 4,962	2.5 \pm 0.3	7.9 \pm 1.1
	5	16,852 \pm 3,368	48,648 \pm 9,856	4.2 \pm 0.2	5.8 \pm 0.4
	6	9,692 \pm 1,903	112,868 \pm 18,386	2.8 \pm 0.5	8.1 \pm 3.6
	7	9,238 \pm 2,327	116,937 \pm 18,206	2.8 \pm 0.6	16.3 \pm 1.0
	HC	15,966 \pm 2,923	73,258 \pm 23,688	12.2 \pm 0.3	13.9 \pm 0.9*