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Research paper

Physical characterisation of formulations for the development of two stable freeze-dried proteins during both dried and liquid storage

Stéphanie Passot[a], Fernanda Fonseca[a,*], Muriel Alarcon-Lorca[b], Dominique Rolland[b], Michèle Marin[a]

[a]*UMR de Génie et Microbiologie des Procédés Alimentaires, Institut National de la Recherche Agronomique, Institut National Agronomique Paris-Grignon, Thiverval-Grignon, France*
[b]*Immunoassays and Proteomic R&D, bioMérieux, Chemin de l'Orme, Marcy l'Etoile, France*

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Abstract

The development of stable freeze-dried proteins requires maintaining the physical and biological integrity of the protein as well as increasing the efficiency of the manufacturing process. Our objective was to study the effects of various excipients on both the physical characterisation and the dried and liquid stability of two proteins. Thermo-physical properties of 13 formulations were determined using both differential scanning calorimetry and freeze–drying microscopy. The antigenic activity was evaluated immediately after freeze–drying and after subsequent storage in both dried and liquid state. From the comparison between glass transition (T'_g) and collapse (T_{coll}) temperatures, we concluded that the collapse temperature was a more relevant parameter than T'_g for freeze–drying cycle development and optimisation. One crystalline formulation composed of 4% mannitol and 1% of sucrose protected efficiently both proteins during subsequent storage in dried state (6 months at 25 °C) and in liquid state (3 months at 4 °C after rehydration). However, the freeze–drying behaviour of this crystalline formulation remained difficult to predict and control. On the other hand, two amorphous formulations composed of 4% of maltodextrin and 0.02% of Tween 80, or 5% of BSA preserved antigenic activity during storage in dried state. The glassy character of these formulations as well as their high collapse temperature values (−9 and −12 °C, respectively) should allow simplification and shortening of freeze–drying process.

Keywords: Protein formulation; Storage stability; Crystalline and amorphous bulking agents; Differential scanning calorimetry; Freeze–drying microscopy; Glass transition temperature; Collapse temperature; Freeze–drying

1. Introduction

Freeze–drying is the main process used to produce stable proteins and polypeptides, which are physically and/or chemically unstable in aqueous solution [1]. An appropriate choice of stabilising excipients is needed to protect the protein from denaturation during freezing and dehydration, as well as to provide a glassy matrix required for long-term storage stability in the dried solid [2,3]. Table 1 illustrates the wide number of formulations reported to ensure long-term stabilisation of freeze-dried proteins. Different molecules alone or in combination, at various concentrations, can be used, which makes the formulation step complex and the development of new freeze-dried proteins remains a time-consuming process of trial and error.

To rationalise the choice of excipients, Carpenter et al. [4] have recently proposed a model formulation based on five components: a buffer that does not acidify during freezing (Tris, histidine, citrate), specific pH/ligands that optimise thermodynamic stability of protein, a stabiliser (generally a disaccharide like sucrose or trehalose) to inhibit protein unfolding and provide glassy matrix, a bulking agent (mannitol, glycine, hydroxyethyl starch or bovine serum albumin, BSA) to ensure physical stability and a nonionic surfactant to reduce protein aggregation. The bulking agent not only ensures physical stability of the product but also makes it possible to increase process productivity. Often crystalline bulking agents (like mannitol or glycine) are preferred for lyophilisation because they form stronger

* Corresponding author. Institut National de la Recherche Agronomique, UMR GMPA, 78 850 Thiverval-Grignon, France. Tel.: +33 1 30 81 59 40; fax: +331 30 81 55 97.
E-mail address: fonseca@grignon.inra.fr (F. Fonseca).

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dried cakes, with better dissolution properties than amorphous agents do. Nevertheless, the use of such excipients generally requires an annealing step before sublimation to promote the complete crystallisation of the bulking agent [3]. The amorphous solid systems involving polymers (like dextran, maltodextrin or PVP) exhibit high glass transition temperature values, thus representing an interesting option.

Table 1
Examples of excipients providing an efficient stabilising effect during freeze–drying of protein (activity recovery ≥80% after freeze–drying)

Excipient		Excipient concentration (%)	Proteins	References
Sugars	Glucose	0.1	Catalase	[41]
		0.5	Catalase	[42]
		2	AO	[43]
	Mannose	2	AO	
	Sucrose	0.5	Catalase	[42]
		2	H-ATPase, AO	[43,44]
		3.4	rF XIII, LDH, viral vectors, β-GA	[24,29,45,46]
		5	LDH	[47]
		8	Subtilisin	[25]
		17	LDH, β-GA	[24,29]
	Trehalose	0.5	Catalase	[42]
		2	H-ATPase	[44]
		3.4	rF XIII	[23]
		4	BO	[48]
		8	Subtilisin	[25]
		13.7	PFK	[49]
		17.1	ADH, GDH	[50]
	Maltose	0.1	Catalase	[41]
		2	H-ATPase	[44]
		13.7	PFK	[49]
	Lactose	2	L-asparaginase	[51]
	Maltotriose	0.1	Catalase	[41]
Polyols	Inositol	5.4	β-GA	[52]
		7.2		
		9		
	Mannitol	0.1	Catalase	[41]
		0.9	β-GA	[53]
		1.8	rF XIII	[23]
			L-asparaginase	[54]
			β-GA	[53]
		2.9	β-GA	[54]
		3.6	β-GA	[53]
		7.3	LDH	[54]
		9.1	β-GA	[53]
	Sorbitol	0.1	Catalase	[41]
		0.5	Catalase	[42]
Amino acids	Alanine–glycine	1–5	rhIL-1ra	[55]
Polymers	PEG	1	rF XIII, LDH	[23,29]
		10	LDH	[29]
	Dextran	0.2	Subtilisin	[25]
		0.5	Catalase	[42]
		3.5	rF XIII	[23]
		2, 4 or 8	Aviscumine	[26,56]
		10.0	G6PDH	[57]
	PVP	2	AO	[43]
		10	LDH	[58]
	BSA	1	LDH	
		10		
	Ficoll	1	LDH	[29]
		10		
	PVA	4	β-GA	[59]
	PHEA	4	β-GA, BO	[59]
	HES	8	Aviscumine	[56]
	HP-b-CD	8	Aviscumine	[56]
	Maltodextrin	0.2	Subtilisin	[25]
	Mannosylglycerate	500 mM	ADH, GDH, LDH	[50]

(*continued on next page*)

Table 1 (*continued*)

Excipient		Excipient concentration (%)	Proteins	References
Polymers-augars/polyols	Trehalose-PEG	0.34–1	LDH, PFK	[2]
		0.9, 1.7 or 3.4–1	PFK, LDH	[10]
		0.2 or 0.3–1	LDH	
		3.4–1	rF XIII	[23]
	Sucrose-PEG	3.4–1	rF XIII, β-GA	[23,24]
	Lactose-PEG	0.34–1	LDH, PFK	[2]
		0.9 or 1.7–1	LDH	[10]
	Glucose-PEG	0.5, 0.9 or 1.8–1		
	Sucrose–dextran	10–2	Actin	[60]
		8–0.2	Subtilisin	[25]
	Trehalose–dextran	8–0.2		
		1 or 3–6	Aviscumine	[26]
		1 or 3–5		
	Sucrose–matodextrin	8–0.2	Subtilisin	[25]
	Trehalose–maltodextrin	8–0.2		
	Mannitol–PEG	0.18–1	LDH	[2]
	Mannitol–HES	2–6	Aviscumine	[56]
	Inositol-Dextran	7.2–0.1	β-GA	[52,61]
	Inositol–CMC–Na	7.2–0.1		
Sugars–polyols	Sucrose–mannitol	0.4–0.4	viral vectors	[46]
		1–4	rhIL-1ra	[55]
	Glucose–mannitol	1–2	L-asparaginase	[51]
Sugars–amino acids	Sorbitol–gycine	1–5	rhIL-1ra	[55]
	Trehalose–glycine	1–5		
	Sucrose–glycine	0.5–5		
		1–5		
		1–2		
	Sucrose mannitol glycine	1	rhIL-1ra	[55]
		4		
		2		
Sugars–ions	Trehalose-$ZnSO_4$	1.7–0.016	PFK	[49]
		3.4–0.0145		
	Glucose–$ZnSO_4$	0.9–0.016		
		1.8–0.0145		
	Maltose–$ZnSO_4$	3.4–0.0145		
	Galactose–$ZnSO_4$	1.8–0.0145		
	Glycerol–$ZnSO_4$	0.46–0.016		
	Inositol$ZnSO_4$	0.9–0.016		
Surfactants	Triton X-100	0.02		
	Tween 20	0.002	rF XIII	[23]
	Mannitol-Tween 80	3–0.1	rhIL-1ra	[30]
	Sucrose-Tween 80	3.4–0.1	β-GA	[24]

ADH, alcohol dehydrogenase; AO, ascorbate oxidase; BO, bilirubin oxidase; β-GA, β-galactosidase; GDH, glutamate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; LDH, lactate dehydrogenase; PFK, phosphofructokinase; rF XIII, recombinant Factor XIII; rhIL-1ra, recombinant human Interleukin-1 receptor antagonist; BSA, bovine serum albumin; CMC–Na, Carboxymethyl cellulose sodium; HES, hydroxyethyl starch; HP-b-CD, hydroxyethyl-β-cyclodextrin; PEG, polyethylene glycol; PHEA, α,β-poly(*N*-hydroxyethyl)-L-aspartamide; PVA, polyvinylalcohol; PVP, polyvinylpyrrolidone.

A well-designed freeze–drying cycle not only ensures physical and biological product stability, but also increases the efficiency of the manufacturing process. Therefore, the primary drying as the most time consuming stage of the process, should be carried out at the maximum allowable product temperature (T_{max}), resulting from the operating conditions (shelf temperature and chamber pressure). If the product temperature is higher than T_{max}, the material will undergo viscous flow, resulting in loss of the pore structure obtained by freezing, which is defined as the collapse phenomenon by Pikal and Shah [5]. Collapsed dried products generally have a high residual water content and lengthy reconstitution times and may also present a loss of biological activity. The value of T_{max}, associated to the collapse temperature (T_{coll}), depends on the physical state of the frozen product. For crystalline material, T_{max} corresponds to the eutectic melting temperature of the solute (T_e), whereas for amorphous material, T_{max} is usually associated to the glass transition temperature of the maximally freeze-concentrated phase (T'_g). Differential scanning calorimetry (DSC) and freeze–drying microscopy are the two tools mainly used to characterise the freeze–drying behaviour of pharmaceutical formulation [5–9].

The proteins selected were the toxins A and B of *Clostridium difficile* (a widely nosocomial pathogen, whose toxins cause pseudomembranous colitis), with molecular weight of 308 and 270 kDa, respectively. These two toxins are used as positive control in the diagnostic methods and are general commercialised in liquid or frozen form. This work aimed at developing a freeze-dried protein formulation stable during storage at ambient temperature and also allowing storage at 4 °C in liquid state after rehydration. We have investigated the effect of different excipients on protein stability and on the physical properties determined by DSC and freeze–drying microscopy. Another aim of this study was to identify the most relevant physical properties for freeze–drying cycle development.

2. Materials and methods

2.1. Materials

Sucrose, mannitol, glycine, polyethylene glycol (PEG 4000), Tween 80, were purchased from Prolabo (Paris, France). Maltose, bovine serum albumin (BSA) and a 1 M solution of Tris–HCl were obtained from Sigma (St Louis, MO, USA). Polyvinylpyrrolidone (PVP) (with a reported average molecular weight of 25,000 Da) and maltodextrin (with a dextrose equivalent of 5/8) were obtained from Merck (Darmstadt, Germany) and from Roquette (Lestrem, France), respectively. The concentrated proteins were supplied by bioMérieux (Marcy l'Etoile, France), frozen and stored at −80 °C.

Based on a literature review [4,10–12] (Table 1) we defined 13 formulations by combining four kinds of excipients: 1) a bulking agent either crystalline (glycine (G), mannitol (Mn)) or amorphous (bovine serum albumin BSA (A), PVP (P), maltodextrin (Md); and/or 2) a stabiliser (sucrose (S), maltose (M), maltodextrin; and/or 3) a cryoprotective agent (PEG (E)); and/or 4) a nonionic surfactant (Tween 80 (W)) (Table 2). To obtain reliable crystallisation when using a crystalline bulking agent (mannitol or glycine), their concentration was by at least a factor of three greater than the sum of the concentrations of all other solute components [13]. In the history of protein development, serum albumin has been widely used as an effective cryoprotectant and lyoprotectant [12]. Then, the formulation A involving 5% of BSA was considered as a reference formulation.

The optimum pH for both toxins was previously determined by bioMérieux. This preliminary study also revealed that ligands, which are generally specific to a protein, were not essential for toxins A and B, thus simplifying the formulation.

The concentrated frozen protein solution was thawed in running water at room temperature. Formulations of *Clostridium difficile* toxins were prepared in 10 mM Tris buffer pH 7.8 to a final concentration of 5.14 μg/mL of toxin A and 3.88 μg/mL of toxin B, with various excipients (Table 2).

2.2. Freeze–drying cycle

The formulations were dispensed into 4 mL freeze–drying vials (1 mL final filled volume). The vials were loaded onto a pre-cooled shelf at 4 °C in a SMH 15 freeze drier (Usifroid, Maurepas, France). The following steps were applied: (1) freezing to −60 °C (cooling rate of 1 °C/min) and holding for 2 h; (2) primary drying performed at a shelf temperature (platinum temperature probes immerged in the shelf) of −25 °C for 50 h; (3) secondary drying performed at a shelf temperature of 25 °C for 8 h.

Table 2
Final excipient concentrations of the 13 formulations

	Bulking agent (crystalline)		Bulking agent/stabiliser (amorphous)			Stabiliser (amorphous)		Cryoprotectant	Surfactant
	Glycine G (%)	Mannitol Mn (%)	BSA A (%)	PVP P (%)	Maltodextrin Md (%)	Sucrose S (%)	Maltose M (%)	PEG E (%)	Tween 80 W (%)
A			5						
GS	4					1			
GSE	4					1		1	
GSW	4					1			0.02
GM	4						1		
GMd	4				1				
MnS		4				1			
MdS					4	1			
MdSE					4	1		1	
MdE					4			1	
MdW					4				0.02
PS				4		1			
PM				4			1		

%, w/w for maltodextrin, PVP and BSA; w/v for glycine, mannitol, sucrose, maltose, PEG; v/v for Tween 80. All formulations had a final toxin A and toxin B concentration of 5.14 and 3.88 μg/mL, respectively. The buffer was 10 mM of Tris–HCl (pH 7.8).

The chamber pressure was maintained at 10 Pa throughout the drying process. Thermocouples were placed in the bottom of two different vials to monitor the product temperature. During the sublimation step, the product temperature was maintained at about −34 °C. A water vapor pressure gauge was used to monitor the sublimation step. At the end of the process, the vacuum was broken by injection of dry nitrogen gas, the vials were rapidly stoppered and packed under vacuum in aluminium bags.

2.3. Differential scanning calorimetry

Thermal analysis of solutions and freeze-dried powders was performed using a power compensation differential scanning calorimeter (DSC) (model Pyris 1; Perkin Elmer LLC, Norwallk, CT, USA) equipped with a liquid nitrogen accessory (CryoFill, Perkin Elmer). Temperature calibration was done using cyclohexane (crystal–crystal transition at −87.1 °C), mercury and gallium (melting points at −38.6 and 29.8 °C, respectively) [14]. Approximately 15 μL of solution or 10 mg of dried powder were used. The sample was sealed in an aluminium pan and an empty pan was used as the reference. Cooling and heating rates of 10 °C/min were used. All glass transition temperatures were reported as the midpoint temperature of the heat capacity step associated to the glass transition with respect to the ASTM Standard Method E 1356-91 [15].

Liquid samples were cooled to −120 °C to ensure temperature stability and sample equilibration, and scanned for the first time to 25 °C. This conventional thermal cycle was replicated once to verify the absence of time dependent changes in thermal behaviour. Other thermal cycles were performed to further investigate some liquid samples (described in Section 3).

Dried samples were cooled to −40 °C and then heated to 200 °C. When the glass transition temperature was difficult to identify, other specific thermal treatments were applied: an isothermal annealing or a repetitive sequence of short heat-hold segments (Stepscan DSC software from Perkin Elmer) to eliminate interference from enthalpic relaxation. For the isothermal annealing, the cooling at −40 °C was followed by a first heating up to the annealing temperature (20 °C) and the sample was cooled to −40 °C again. The second heating scan (to 200 °C) was used to determine the characteristic glass transition temperature Tg. When the Stepscan method was applied, the sample was cooled to −40 °C (10 °C/min) and then heated up to 200 °C by steps of 2 °C with a rate of 50 °C/min and isotherms of 0.4 min between steps.

2.4. Freeze–drying microscopy

The collapse temperature (T_{coll}) was measured using a freeze drying cryo-stage (Linkam Scientific Instruments, Surrey, UK) equipped with a liquid nitrogen cooling system and a programmable temperature controller. A 5-μL sample was placed on a 16-mm quartz cover slip and covered with a 13-mm quartz cover slip. A metallic spacer was placed between the cover slips to maintain the sample thickness almost constant between each experiment. Silicone oil was used between the sample and the temperature-controlled plate. Direct observation of the freeze-dried structure was done by using a polarised microscope (Nikon Elipse E600, Nikon, Japan).

The experimental procedure applied was described in a previous publication [16]. The sample was frozen at 10 °C/min to a temperature slightly lower than its T'_g. The system was then placed under vacuum at about 1 Pa and the sample was kept at this temperature for 10–20 min in order to obtain a freeze-dried region large enough to observe the sample structure. The temperature was stepwise increased (generally by 5 °C, or 1 °C when the collapse region approached) until collapse was observed. At each step, the temperature was maintained for about 10 min in order to obtain dried regions of similar width. For the formulation containing a crystalline bulking agent (mannitol or glycine), an annealing step at −20 °C for 20 min was added after the freezing step in order to ensure the bulking agent crystallisation.

The collapse temperature (T_{coll}) so determined corresponds to the lowest temperature of overall loss of the initial frozen structure during freeze–drying [16]. With the defined protocol the precision of the temperature measurements was 1 °C, corresponding to the temperature difference between two steps.

2.5. Water content analysis

In order to check that the water content achieved was sufficiently low to ensure a good physical stability, residual moisture of the freeze-dried products was measured by the Karl Fisher titration method using a Metrohom KF 756 apparatus (Herisau, Switzerland). At least 20 mg of powder were mixed with 2 mL of dry methanol and titrated with Riedel-deHaen reagent (Seelze, Germany) until the end point was reached.

2.6. Enzyme immunoassay

The concentrations of toxins A and B, corresponding to a measurement of the antigenic activity, were assessed by a quantitative enzyme-linked immunoabsorbent assay (ELISA) developed by bioMérieux. The antigenic activity was measured immediately after freeze–drying and after storage of 1, 3 and 6 months at 4 and 25 °C. The antigenic activity recovery was calculated as the percentage of the initial toxin concentration prior to freeze–drying. A stability study in liquid state (after rehydration) was also performed at 4 and 25 °C for 3 months. The coefficient of variation of the titration method was 15% of the measured value.

3. Results

3.1. Thermal analysis of frozen formulations

The thermal properties of the frozen formulations determined by differential scanning calorimetry included glass transition temperature of the maximally freeze-concentrated matrix (T'_g), solute crystallisation temperature (T_{cr}), eutectic melting temperature (T_e) and ice melting temperature (T_{im}).

3.1.1. Crystalline bulking agent

The DSC heating curve of the formulations involving glycine (GS, GSW, GSE, GM and GMd) revealed different thermal events (conventional thermal cycle in Table 3): (i) a glass transition at approximately −70 °C; (ii) several exothermic events in the range of −40 to −20 °C, which were ascribed to the crystallisation of glycine; and (iii) an overlapping eutectic (glycine) and ice melting endotherm. The low T'_g values obtained (between −69 and −72 °C) were close to the T'_g of a quench-frozen solution of glycine reported by Chongprasert et al. [17] (−73 °C/−60 °C). This result clearly suggested that the cooling rate applied (10 °C/min) inhibited totally or partially the crystallisation of glycine, which finally occurred during warming.

A complementary DSC study based on the works of Ma et al. [18] was then performed to promote the maximum crystallisation of glycine. The sample was frozen to −4 °C at 1.5 °C/min, held at −4 °C for 60 min, and then cooled from −4 to −120 °C at 1.5 °C/min. Both the annealing treatment and the slow cooling rate should help glycine to crystallise. The frozen sample was then warmed to 25 °C at a constant rate of 20 °C/min (thermal cycle with annealing in Table 3). In contrast to the data of the conventional thermal cycle, the DSC heating curve did not exhibit any exothermic event suggesting the completion of glycine crystallisation during freezing. The T'_g of the remaining amorphous solution was shifted to a much higher temperature: about −38 °C for the formulations GS, GSE, GSW and GM, and −20 °C for GMd. For all formulations, the ice melting endotherm occurred at approximately −4 °C, the eutectic melting temperature of glycine [7,17]. Besides, the heating scan of GSE exhibited a slight eutectic melting endotherm at −19 °C, probably associated to the melting of PEG crystals (−15 °C, Table 3). It is important to note that the annealing treatment, by promoting the glycine crystallisation, resulted in a decrease of the solute concentration ($\leq$2%) in the amorphous matrix. Therefore, the glass transition signal became weak and could not be readily detectable. Thus, to increase the magnitude of the shift in the baseline, a high heating rate (20 °C/min) was used [8]. The determination of the T'_g was mainly based on the derivative heat flow.

The amorphous fractions of the formulations containing glycine were prepared alone and analysed by DSC. As PEG crystallised during freezing and Tween 80 was introduced at

Table 3
Summary of both the thermal properties of formulations involving glycine or mannitol as crystalline bulking agent (determined during the warming step of different thermal cycles) and the thermal properties of each component of these formulations

	Conventional thermal cycle				Thermal cycle with annealing			
	T'_g (°C)	T_{cr} (°C)	T_e (°C)	T_{im} (°C)	T'_g (°C)	T_{cr} (°C)	T_e (°C)	T_{im} (°C)
GS	−69	−38; −21		−7	−38[a]			−3
GSE	−69	−36; −21		−6	−38[a]		−19	−4
GSW	−69	−57; −36		−4	−39[a]			−4
GM	−69	−37; −20		−7	−37[a]			−4
GMd	−72	−45; −29		−6	−20[a]			−4
MnS	−41; −31	−24(−6.7 J/g)		−1.3	−41; −31[a]	−24 (−2.6 J.g)		−2
					−33; −28[b]	−25 (−3.1 J/g)		−1.9
S	−40 (−41)[p]			−0.4				
M	−38 (−38)[p]			−0.2				
Md	−20 (−23)[p]			−0.07				
Glycine [17]	−73; −60		−4					
Mannitol [21]	−32; −25	−22	−1.5					
Saccharose (5%)	−32							
Maltose (5%)	−30							
Maltodextrin (5%)	−10							
PEG (5%)	−	−51	−15					
Tris–HCl (0.1–1 M)	−60		−14					

T_{cr}, solute crystallisation temperature; T'_g, glass transition temperature of the maximally freeze-concentrated matrix; T_e, eutectic melting temperature; T_{im}, ice melting temperature. The composition of the formulations GS, GSE, GSW, GM, GMd and MnS is given in Table 2. G, glycine; Mn, mannitol; Md, maltodextrin; S, sucrose; M, maltose; E, polyethylene glycol (PEG); W, Tween 80. S, 1% sucrose + 10 mM Tris–HCl; M, 1% maltose + 10 mM Tris–HCl; Md, 1% maltodextrin + 10 mM Tris–HCl. ( )$^p T'_g$ predicted according to a simple linear relationship [19], (J/g), normalised heat of crystallisation.

[a] The sample was frozen to −4 °C (for glycine) or −2 °C (for mannitol) at 1.5 °C/min, held at the subzero temperature for 60 min and then cooled to −120 °C at 1.5 °C/min. The frozen was then warmed at 20 °C/min through 25 °C [18].

[b] The sample was frozen to −120 °C at 10 °C/min, heated to the annealing temperature −30 °C at 10 °C/min and held at −30 °C for 60 min. Then the sample was cooled back to −120 °C at 10 °C/min and reheated to 25 °C at 10 °C/min.

low content (0.02%), we assumed that the thermal properties of the formulations GSE and GSW were similar to the properties of the amorphous fraction of GS. The T'_g values of the solutions S (for GS, GSE, GSW), M (for GM) and Md (for GMd) were close to the T'_g values obtained when an annealing treatment was applied to the glycine formulations (Table 3). Furthermore, the T'_g values of these solutions (S, M, Md) were also estimated using a simple linear relationship [19] based on the T'_g of each binary aqueous solutions (Table 3). The experimental T'_g values were close to the estimated values ($T'_{g\ measured} - T'_{g\ estimated} \leq 3\ °C$). These results further confirmed that the T'_g values of the glycine formulations obtained after annealing treatment, corresponded to the glass transition of the maximally freeze-concentrated matrix after completion of the glycine crystallisation during freezing.

Concerning the formulation MnS involving mannitol as bulking agent (4% mannitol, 1% sucrose and 10 mM Tris–HCl), the DSC heating scans revealed several thermal events. Two weak endothermic events were observed at approximately −41 and −30 °C characterising the glass transition [20,21]. An exotherm event attributable to the crystallisation of mannitol (onset at approximately −24 °C) and an endotherm due to eutectic melting of mannitol and ice (onset at −1.3 °C) were also exhibited. Two thermal treatments were applied to promote mannitol crystallisation (Table 3): (1) freezing with high subzero temperature holding (−2 °C for 60 min); (2) annealing treatment (at −30 °C for 60 min). The thermal treatment at −2 °C did not modify the DSC heating curve. It resulted in a decrease of the normalised heat of crystallisation of mannitol, but did not allow its complete crystallisation. On the other hand, the annealing treatment caused a pronounced change in the DSC heating profile. The first glass transition occurred at a higher temperature (−33 °C instead of −41 °C) and the second glass transition (at −28 °C) appeared more clearly and overlapped with the mannitol crystallisation exotherm. This increase of T'_g values suggested that ice crystallisation occurred during annealing [22]. This complex behaviour of the formulation MnS has been previously described by Cavatur et al. [20] and Pyne et al. [21]. In contrast to the formulations involving glycine, the thermal treatments applied to the formulation MnS did not allow the completion of the mannitol crystallisation and we kept both experimental values of T'_g for the glass transition characterisation.

3.1.2. Amorphous bulking agent

The aqueous formulations involving an amorphous bulking agent (BSA, PVP or maltodextrin) displayed a conventional DSC heating profile revealing a glass transition event and the ice melting endotherm. An eutectic melting endotherm at approximately −20 °C ascribable to the melting of the PEG crystals (E) was observed for the formulations MdE and MdSE (Table 4). The aqueous formulations with PVP gave the lowest glass transition temperature of the of the maximally freeze-concentrated phase, T'_g ($T'_g = -28$ and −24 °C for PS and PM, respectively), whereas the formulations with maltodextrin gave the highest T'_g (−10 and −14 °C for MdE and MdW, respectively). The glass transition temperature T'_g of the formulations was close to the T'_g of the bulking agent used (−15 °C for BSA, −9 °C for Md and −24 °C for PVP), but slightly lower due to the effect of the addition of other solutes like disaccharide and Tris–HCl.

3.2. Freeze–drying microscopy and collapse temperature

The values of collapse temperature T_{coll}, defined as the lowest temperature of overall loss of the initial frozen structure during sublimation, varied between −26 °C for the formulations PS and PM, and −9 °C/−10 °C for MdW

Table 4
Summary of the main thermal properties of the frozen and dried formulations

	Liquid samples				Freeze–dried samples
	T'_g (°C)	T_e (°C)	T_{coll} (°C)	$\Delta T = T_{coll} - T'_g$ (°C)	T_g (°C)
A	−16		−12	4	136±9
MdS	−17		−14	3	86±6
MdSE	−16	−21	−18	−2	123±16
MdE	−10	−18	−15	−5	128±20
MdW	−14		−9	5	144±12
PS	−28		−26	2	89±15
PM	−24		−26	2	98±27
GS	−38		−15	23	75±12
GSE	−38	−19	−18	20	
GSW	−39		−15	24	62±4
GM	−37		−15	22	66±6
GMd	−20		−10	10	153±14
MnS	−41; −31		−20	21; 11	55

The composition of each formulation is given in Table 2. T'_g: glass transition temperature of the maximally freeze-concentrated matrix; T_e: eutectic melting temperature; T_{coll}: collapse temperature; Tg: glass transition temperature of the dried product. Legend: A: bovine serum albumin (BSA); P: polyvinylpyrrolidone (PVP); Md: maltodextrin; S: sucrose; M: maltose; E: polyethylene glycol (PEG); W: Tween 80.

and GMd (Table 4). The addition of PEG (E) resulted in a depression of the collapse temperature (−14 °C for MdS compared to −18 °C for MdSE, −15 °C for GS compared to −18 °C for GSE), which could be ascribed to the melting of the PEG crystals observed at −15 °C (Table 3). The crystalline formulations (involving glycine or mannitol) exhibited collapse temperatures close to the values of the amorphous formulations involving maltodextrin (or BSA).

In Table 4, collapse temperatures (T_{coll}) were compared with glass transition temperatures of the freeze-concentrated phase (T'_g). The amorphous formulations showed collapse temperature belonging to the same temperature range than T'_g (differing by less than 6 °C) [5], whereas the crystalline formulations exhibited a significant higher value of T_{coll} than T'_g, with a difference $T_{coll} - T'_g$ in the range of 10–24 °C.

3.3. Characterisation of the freeze-dried formulations

Visual examination of the 13 freeze-dried formulations did not show any sign of collapse nor shrinkage immediately after the process. The residual water content after freeze–drying was less than 2% for all formulations, which is a standard value for freeze-dried product.

The DSC heating profiles of the dried formulations containing either mannitol or polyethylene glycol (PEG) showed a melting peak at around 150 or 50 °C, respectively (data not shown). These melting peaks confirmed the crystallisation of mannitol and PEG during the lyophilisation cycle. Concerning the formulations containing glycine, no melting peak was observed during the heating scan, which could be explained by the melting of glycine crystals at temperatures higher than the temperature range used. Then, a heating scan was performed at a temperature higher than 200 °C and revealed that glycine began to melt around 230–240 °C. The glass transition temperatures (T_g) of the freeze-dried formulations were reported in Table 4. No T_g was observed for the formulation GSE, certainly because it was overlapped with the melting peak of PEG around 50 °C.

The detection of glass transition events was sometimes delicate due to transitions of very low amplitude for formulations involving low content of amorphous components (crystalline formulations) or spreading out over 10 °C, especially for the formulations involving polymers like maltodextrin or BSA. The software Stepscan DSC was found to be more sensitive to characterise weak glass transition events (Fig. 1). The stepscan signal exhibited a clear glass transition event at about 160 °C, while the conventional DSC signal exhibited no thermal event.

3.4. Antigenic activity recovery during storage in dried state

The antigenic activity recovery of both toxins after freeze–drying and after 3 and 6 months of storage in dried state at 4 and 25 °C were reported in Table 5. Values of activity recovery higher than 100% are not rare in the literature [23–26]. The data were classified according to the results after a 6-month storage at 25 °C in increasing order for toxin A and in decreasing order for toxin B. Therefore, the most efficient formulations for the protection of both toxins appeared in the middle of the table.

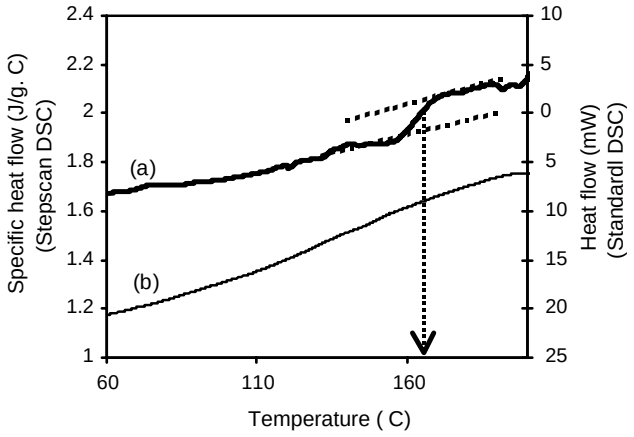

Fig. 1. DSC heating scan of the freeze-dried sample GMd obtained with: (a) Stepscan DSC (heating by steps of 2 °C with a rate of 50 °C/min and isotherms of 0.4 min between steps); (b) standard DSC (heating rate of 10 °C/min).

Considering the coefficient of variation of the ELISA test (15%), the antigenic activity of both toxins was well preserved for all formulations after freeze–drying and a 3-month storage, whatever the storage temperature. Only two formulations MdS and GMd (involving maltodextrin) displayed an activity recovery for the toxin A lower than 70% after 3 months of storage at 25 °C. After 6 months of storage the loss of antigenic activity remained moderate: less then 30 and 40% at 4 and 25 °C, respectively. Nevertheless, the differences between the formulations were more pronounced.

Considering the toxin A, the formulations MdW, A, PS, PM and MdSE ensured the most effective protection after a 6-month storage at 25 °C (with an activity recovery higher than 85%), while the formulations MdS, GMd, GM, GSE and GSW appeared as the least effective (with an activity recovery lower than 75%). When comparing the formulations MdS, MdSE and MdE involving maltodextrin (Md), sucrose (S) and PEG (E), the storage stability increased in the order MdS < MdE ≤ MdSE.

The behaviour of the toxin B during storage appeared to be different than the toxin A one. In fact the antigenic activity of the toxin B significantly decreased between three and 6 months of storage. The formulations GSW, MdW and A gave the highest activity recovery after 6 months at 25 °C (>85%), while the formulations PS, MdSE and PM gave the lowest activity recovery (<75%).

Finally, two amorphous formulations A and MdW involving BSA (A), or maltodextrin (Md) and Tween 80

Table 5
Antigenic activity recovery (%) of both toxins during 6 months of storage in dried state at 4 and 25 °C

		After FD	3 months		6 months	
			4 °C	25 °C	4 °C	25 °C
Toxin A	MdS	105	81	69	90	60
	GMd	83	82	67	86	67
	GM	98	91	78	76	71
	GSE	96	99	80	91	72
	GSW	97	100	89	93	74
	MnS	103	100	90	95	81
	MdE	112	95	84	103	83
	GS	94	93	74	92	84
	MdSE	112	96	90	105	88
	PM	106	89	89	99	90
	PS	110	98	93	108	92
	A	93	102	91	97	93
	MdW	108	91	87	96	95
Toxin B	GSW	111	111	125	76	101
	MdW	117	131	136	95	93
	A	108	108	103	104	89
	GMd	111	105	107	76	83
	MnS	114	85	108	74	81
	GS	111	102	96	84	80
	GSE	107	94	96	72	77
	MdE	114	88	105	69	77
	MdS	128	106	104	95	76
	GM	103	97	91	76	75
	PM	110	91	101	75	68
	MdSE	123	85	102	76	65
	PS	112	89	105	76	62

Values are reported as percentage of initial antigenic titration prior to freeze–drying as assayed by ELISA methods and are the means of two measures. The coefficient of variation of the ELISA test is about 15%. The composition of each formulation is given in Table 2. Legend: FD: freeze–drying; G: glycine; Mn: mannitol; A: bovine serum albumin (BSA); P: polyvinylpyrrolidone (PVP); Md: maltodextrin; S: sucrose; M: maltose; E: polyethylene glycol (PEG); W: Tween 80.

(W) provided an activity recovery higher than 85% for both toxins. Two crystalline formulations GS and MnS involving sucrose (S), glycine (G) and mannitol (Mn) ensured an effective protection of both toxins during storage (with an activity recovery $\geq 80\%$).

3.5. Antigenic activity recovery during storage in liquid state

After rehydration, the antigenic activity was measured during 3 months of storage at 4 and 25 °C (Fig. 2). The data were classified according to the results after 3 months at 4 °C, in decreasing order from left to right of Fig. 2 (respectively, Fig. 2A and B for toxin A and B). When formulations gave close values of activity recovery (in particular for toxin A), the results of the liquid stability at 25 °C were considered. For example, the formulation MdE showing a high activity recovery for the toxin A (96%), was considered inferior to the formulations GSE, GS and GSW, since MdE exhibited a poor stability at 25 °C for this toxin. The most efficient formulations for the protection of both toxins appeared in the left side of the figure.

After 1 month at 4 °C, the antigenic activity was well preserved (data not shown) for all formulations, except for one toxin A sample (A) and one toxin B sample (MdW). After 3 months of storage, nine formulations (PS, GM, PM, MnS, GSW, GS, GSE, MdE and MdSE) for toxin A and six (GSW, PS, MnS, GSE, GM and A) for toxin B showed a recovered antigenic activity higher than 70%. The results at 4 °C revealed a quite high liquid stability of the *Clostridium difficile* toxins, which is in agreement with the works of Freeman and Wilcox [27]. The authors reported that storage at 4 °C has no discernible effect on *C. difficile* cytotoxin (stored in faecal emulsion).

During storage at 25 °C we observed a sharp decrease of the antigenic activity, especially for toxin B. The high temperatures can accelerate both physical (like aggregation) and chemical (like oxidation, deamidation) degradation [28]. Only three formulations GS, GSW and PS ensured an activity recovery of the toxin $B \geq 50\%$ after 1 month at 25 °C, whereas seven formulations (PS, GM, PM, GSW, GS, A and MnS) fully preserved the toxin A. Furthermore, for the worst-case storage condition (3 months at 25 °C) GS presented an activity recovery higher than 30% for both toxins, while PS fully preserved the toxin A.

Finally, if we consider 3 months at 4 °C as a suitable stability criterion, the formulations PS, MnS, GSW, GM and GSE appeared as the most interesting ones.

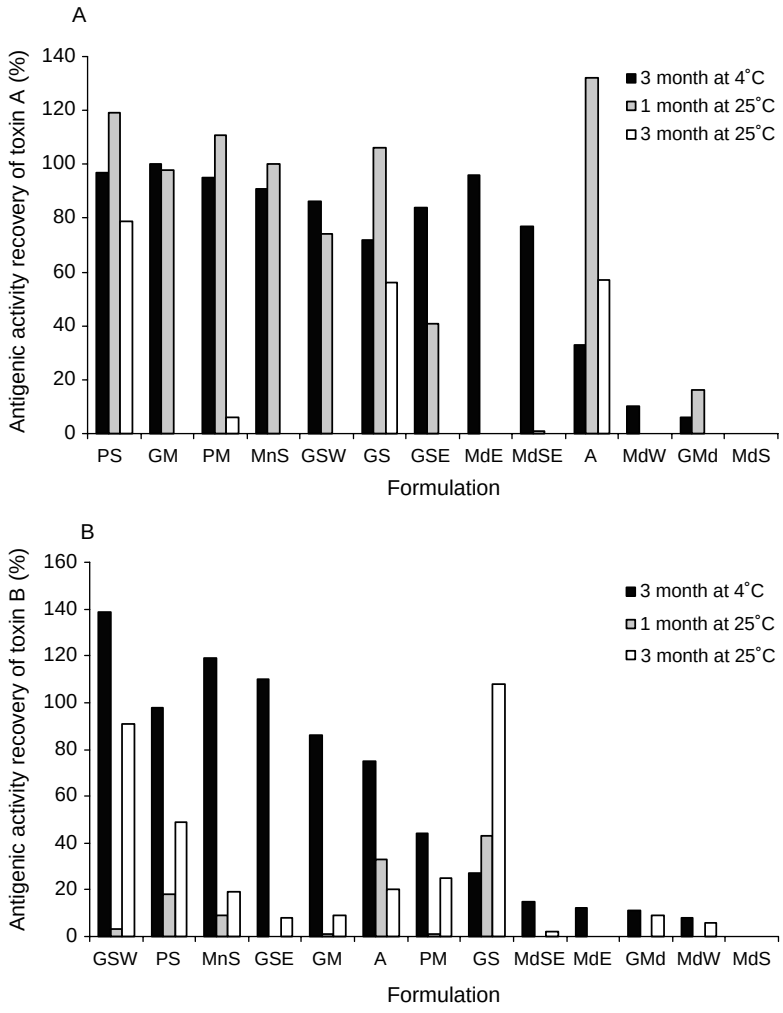

Fig. 2. Antigenic activity recovery (%) of toxin A (A) and of toxin B (B) during 6 months of storage after rehydration at 4 and 25 °C. Values are reported as percentage of initial antigenic titration prior to freeze–drying as assayed by ELISA methods and are the means of two measures. The coefficient of variation of the ELISA test is about 15%. The composition of each formulation is given in Table 2. G, glycine; Mn, mannitol; A, bovine serum albumin (BSA); P, polyvinylpyrrolidone (PVP); Md, maltodextrin; S, sucrose; M, maltose; E, polyethylene glycol (PEG); W, Tween 80.

4. Discussion

The stabilisation of proteins during freeze–drying and storage requires adding specific excipients [4]. Sugars such as disaccharides (sucrose, trehalose…) are widely used to stabilise protein conformation against denaturation due to water removal. Bulking agents such as mannitol, glycine or bovine serum albumin are commonly used to ensure physical stability of the cake. The results of storage stability in dried state suggest that other protective additives than sugars could be necessary. The use of Tween 80 in complement or instead of disaccharide (see Table 5: results of the formulations MdW or GSW compared to MdS or GS) resulted in an equivalent protective ability of the antigenic activity of both toxins during storage at 4 °C and better during storage at 25 °C. In literature [29], the beneficial effects of nonionic surfactants have been explained by two mechanisms. The surfactant may compete with protein for access to air/water, ice/water or vial/water interfaces, where adverse protein-surface interaction may occur, resulting in diminished damage [30]. Additionally, surfactant binding to protein surfaces may sterically block sites for formation of non-native aggregates resulting in higher recovery of native protein [31]. On the other hand, when comparing the ability of the formulations MdS, MdSE and MdE, involving maltodextrin (Md), sucrose (S) and PEG (E), to protect the toxin A, the storage stability increased in the order $MdS < MdE \leq MdSE$. Differential scanning calorimetry indicated that, in the dried samples, PEG was crystalline and the bulking agent (maltodextrin Md or maltodextrin-sucrose mixture MdS) was amorphous. Therefore, since PEG is reported to be a cryoprotective agent [32], it could stabilise the toxin A during the freezing step by the preferential exclusion mechanism [2,10], and maltodextrin and sucrose could protect the toxin during dehydration by hydrogen bonding with the toxin in the place of lost water

[10,33]. The greater stabilising effect of the two formulations MdSE and MdE compared to MdS suggested that the toxin A is sensitive to the freezing stresses.

Optimal storage stability requires that lyophilisation-induced perturbation be minimised and the glass transition temperature Tg of the dried product be maximised. The only way to increase Tg is to introduce polymers (PVP, maltodextrin, dextran…) or proteins (BSA, HSA…) in the formulation. The formulations A (involving BSA) and MdW (involving maltodextrin) exhibited Tg values higher than 100 °C (136 and 144 °C, respectively), while fully preserving the antigenic activity of both toxins during storage at 25 °C. Such results were in agreement with the stabilising effect of dextran and disaccharide mixture observed on Actin by Allison et al. [11]. In this work, the addition of dextran also increased glass transition temperature without affecting the capacity of sugar to inhibit protein unfolding during lyophilisation and resulted in improved storage stability. A lower Tg increase was observed in this work due to a lower ratio sucrose:polymer used (1:1 compared to 1:4 in our work). Nevertheless, even if the 13 formulations displayed glass transition temperatures greatly exceeding the storage temperatures, the lower efficiency (antigenic activity recovery $<75\%$) of some formulations (MdS, GMd for toxin A, and MdSE, PS and PM for toxin B) confirmed the postulate that the glassy state represents a necessary condition but not sufficient for stabilising proteins during freeze–drying and storage [34–36].

When considering lyophilisation of diagnostic or analytical reagents, the long-term liquid stability after rehydration could appear as a complementary and strategic criterion of quality. The majority of the tested formulations ensured an efficient protection of both toxins during storage at 4 °C after rehydration. The most widely accepted mechanism of protein stabilisation in aqueous solution is preferential interaction of proteins [37,38]. In the presence of a stabilising excipient, a protein prefers to interact with water (preferential hydration) and the excipient is preferentially excluded from the protein domain (preferential exclusion). The excipients used in this study are reported to be preferentially excluded from the protein surface [38] and thus making it possible to stabilise the two toxins in solutions at 4 °C. Besides, the results of the liquid stability revealed that maltodextrin used as either bulking agent or stabiliser appeared not to be effective for liquid stability. This polymer stems from the acidic and/or enzymatic hydrolysis of starch and it may be contaminated by trace amounts of enzymes. Hence, its poor effect on liquid stabilisation of toxins A and B could be explained by an instability of the polymer against chemical and/or enzymatic degradation [12,39]. Similar effects of contaminants on polymers stability were observed by Shahrockh at al. [39] on hydroxyethylcellulose (in this case the contamination came from the protein). The range of formulations ensuring protection during both a 6-month storage at 25 °C in dried state and a 3-month storage at 4 °C in liquid state becomes restricted: PS, PM, MnS, GS, MdE, MdSE for toxin A; GSW, MnS and A for toxin B; and consequently MnS for both toxins.

The design and development of an optimised freeze–drying cycle rely on a thorough and relevant physical characterisation of the formulation. During the primary drying (the most time-consuming phase of a lyophilisation cycle), the product temperature should be kept below the maximum allowable product temperature (T_{max}) permitted by the formulation: the eutectic temperature of crystallised solutes or the T'_g for the amorphous matrix. A wrong estimation of this maximum allowable product temperature would result either in damaging of the product or in a too long processing time. The two main ways of increasing T_{max} were applied in this work: using a crystalline bulking agent (mannitol or glycine) with high eutectic temperature or an amorphous bulking agent with high T'_g (or T_{coll}) such as maltodextrin or PVP. Table 3 compared the thermal transition temperatures during warming after different freezing protocols. The results from the DSC work demonstrated the tendency of formulations involving glycine or mannitol to undergo a 'non eutectic' freezing at high cooling rate (10 °C/min). When a high subzero temperature holding (−4 °C for 60 min) or a slow freezing (cooling rate of 1 °C/min) was applied, glycine crystallisation was completed during freezing with no further residual crystallisation during warming and the glass transition temperature of the freeze-concentrated phase T'_g was increased and corresponded essentially to the T'_g of the amorphous components (sucrose, maltose or maltodextrin). On the contrary, the different thermal treatments applied did not permit the completion of mannitol crystallisation. DSC results revealed thus that the behaviour of some formulations (especially those containing crystalline bulking agent) could be difficult to predict and control at full scale. The crystallisation of the crystalline bulking agents must be complete during the freezing step, otherwise their low T'_g will endanger the freeze–drying cycle and the product quality. Therefore, to ensure the total crystallisation of the crystalline bulking agent before sublimation, an adapted annealing step should be added. In this case, formulations including an amorphous bulking agent may give better results. The formulations MdW, A and MdE ensured an efficient protection of both toxins during a 6-month storage at 25 °C and a moderate storage in liquid state (inferior to 1 month).

The comparison between the glass transition temperature T'_g and the collapse temperature T_{coll} (measured by freeze–drying microscopy) pointed out two behaviours depending on the nature of the bulking agent used. The collapse temperature of formulations involving only amorphous components can be reliably predicted by the T'_g, which was not the case for formulations involving any crystallised solute. The crystalline matrix composed of glycine or mannitol exhibited T'_g widely lower than the collapse temperature. Some of formulations (GSW, MnS, GS) were

freeze-dried at a product temperature higher than T'_g but lower than T_{coll} and provided a high storage stability (especially at 4 °C in dried state). Besides, if T_{coll} is used to fix the product temperature during sublimation instead of T'_g, the sublimation time will be significantly reduced. In this sense, Pikal [40] reported that a product temperature increase of 1 °C would result in a 13% reduction of primary drying time. Therefore, the collapse temperature (determined by freeze–drying microscopy) could be defined as a more relevant parameter than T'_g (determined by DSC) for process development and optimisation.

5. Conclusion

This study investigated the thermo-physical properties of 13 combinations of excipients to rationalise the development of two stable freeze-dried proteins during storage at ambient temperature. The interest of adding other specific molecules than sugars was highlighted. For example, a nonionic surfactant (Tween 80 at 0.02%), a cryoprotective agent (PEG at 1%), or a polymer (maltodextrin or PVP at 4%) increasing the glass transition temperature, provided beneficial effect on the storage stability of the dried product. Five formulations (MdW, A, GS, MdE and MnS) ensured high storage stability at ambient temperature for both toxins. The liquid stability after rehydration was also considered as a complementary criterion for the final choice of formulation. And in this case, only one formulation MnS, involving 4% of mannitol and 1% of sucrose made it possible to fully preserve the antigenic activity of both toxins during a 3-month storage at 4 °C in liquid state, as well as during a 6-month storage at 25 °C in dried state.

We have determined the thermo-physical properties, T'_g, T_e, T_{coll} of the formulations in a frozen temperature range for the purpose of optimising freeze–drying process. As a result, the collapse temperature determined by freeze–drying microscopy appeared as a more relevant parameter than T'_g or T_e for the development of the primary drying stage (to fix the maximum allowable product temperature). On the other hand, differential scanning calorimetry provides useful information on the freezing behaviour of multi-components solutions, as the incomplete crystallisation of a bulking agent. The use of a polymer as bulking agent made it possible to reach the same collapse temperature values as when using a crystalline bulking agent. Furthermore, the thermal behaviour of an amorphous formulation is easier to predict during an industrial process than the crystalline formulation ones. If an increase of process productivity is considered as more important than the liquid stability, we will prefer to select the formulations MdW or A (involving maltodextrin or BSA), exhibiting high collapse temperatures.

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