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APPLICATIONS OF DSC IN THE DEVELOPMENT OF IMPROVED FREEZE-DRYING PROCESSES FOR LABILE BIOLOGICALS

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The stabilities of protein based products in the freeze-dried state depend critically on the formulation and process variables temperature, pressure, time and moisture content. The principal determinants of biological stability are the glass/rubber transitions of the freeze concentrate and of the dried product and its residual moisture content. This paper describes the role of DSC in the investigation of the component processes which together form the basis of effective freeze-drying: a maximisation of yield, an optimum shelf life and a cost-effective process.

Introduction

In recent years considerable progress has been made in the isolation and purification of materials of biological origin. However, the post-separation stability of many of these materials in aqueous solution in such that, to enable their potential to be fully exploited, they need further processing to give them an acceptable shelf-life. There are many techniques that can be employed but probably the most common is freeze-drying (lyophilisation).

Freeze-dried biologically active labile materials, e.g. enzymes should have a number of advantages over materials stabilized by other techniques. A good product should have a small volume, a high degree of stability and be easily and rapidly reactivated by rehydration. Unfortunately, total or partial inactivation during processing is not uncommon. Indeed, with the high purity products that can now be produced by improved separation procedures it is becoming a regular occurrence.

The traditional approach to solving these stability problems is to add stabilizers. This is usually performed in a rather haphazard manner. Add a stabilizer, see what happens; if no improvement, add another and so it

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continues, possibly over many years. This type of approach is expensive, both in time and resources, but is not surprising when the concensus of opinion from experts in the field can be express as 'Freezing and drying data can only be determined empirically by the trial and error method i.e. by way of testing the finished product' [1].

The quality of a product

The quality of a freeze-dried product (Fig. 1A) is assessed by its shelf-life, rehydratability and activity although secondary features such as appearance cannot be overlooked. These quality features are related back to the moisture content of the product throughout processing. Moisture content is in turn dependent on the settings of the process parameters that can be controlled in the freeze-dryer; temperature, pressure and time. We have considered the biophysical processes that underline freeze-drying (Fig. 1B). Although all the factors need to be taken into account when determining an optimum freeze-drying cycle, probably the most important is vitrification.

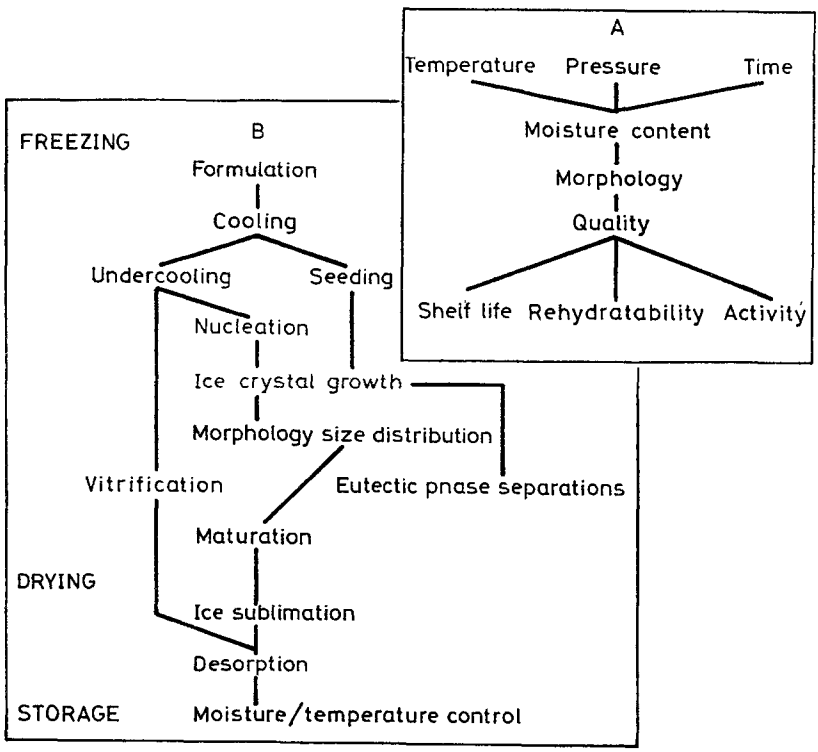


Fig. 1 (A) Experimental variables which determine the quality of the product. (B) Physical parameters that influence freezing and drying behaviour (Taken from reference 2)

Vitrification and freeze-drying

Figure 2 shows a plot of temperature against mol fraction of solids, in this case sucrose. Depending on starting concentration, two possible situations can arise on cooling. In dilute solutions (e.g. 40% by weight sucrose) ice will form as the temperature is reduced to below the liquidus curve. This will result in a two phase system consisting of pure ice and a freeze concentrate (solid and unfrozen water). As the temperature is further reduced more ice will crystallize and the freeze concentrate will be further concentrated. Eventually the freeze concentrate will become so concentrated (for sucrose circa 80 weight percent) that it will undergo a sharp increase in viscosity and form a glass – that is, it will vitrify. The temperature at which this glass transition takes place is termed T_g'. In solutions that are initially highly concentrated (e.g. sucrose >80% by weight) freezing will not occur and on cooling, the preparation will form first a rubber and then a glass.

Freeze drying starts with a dilute solution. As described for dilute solutions, on cooling ice crystallizes out, resulting in a concentration of the remaining material. With further reductions in temperatures more ice crystallizes and the material becomes more and more concentrated until it forms a glass at T_g', the temperature of maximum freeze concentration where the viscosity is such that no more ice can form. Under such conditions any labile

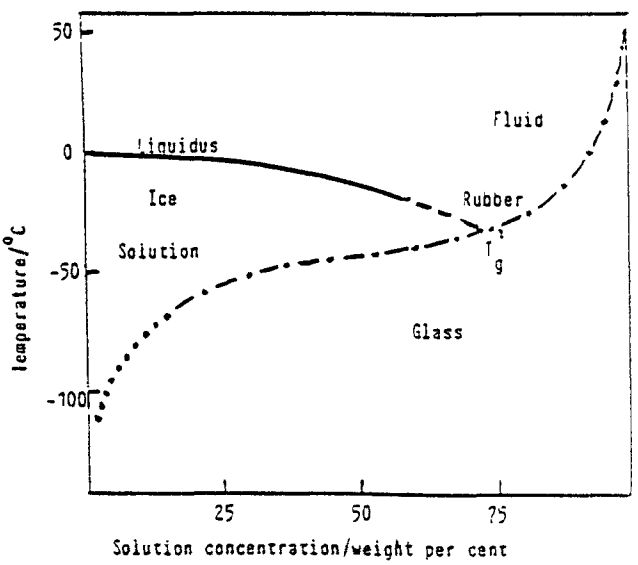


Fig. 2 State diagram for the binary system sucrose+water, showing the equilibrium liquidus and the non-equilibrium glass transition curve: all points on this curve have the same viscosity

material that was in the preparation (e.g. enzyme) will be trapped in the glass and effectively stabilized by immobilization.

In freeze-drying the ice phase is next removed by sublimation (primary drying). In order to protect the protein as described above this must be performed at a temperature $<T_g'$. Knowing the rate of ice sublimation at T_g' the duration of this stage of processing can be calculated. At the end of primary drying the remaining material is an amorphous glass containing typically 50 weight percent water. This is then heated and further water removed (secondary drying). For this stage, the rate of temperature increase and duration of processing can be calculated from the rate of diffusion of the residual moisture from the partially dried matrix. At the end of second-ary drying the product has a low moisture content and an enhanced T_g. Provided the material is then stored below T_g in a glassy state, its stability should be maintained.

A predictive approach to freeze drying

There are three measurements that provide sufficient information that a predictive model of an optimum freeze-drying cycle can be produced. They are, the temperature of maximal freeze concentration, (T_g'), the amount of unfrozen water at this temperature (W_g') and T_g of the dried product, the latter providing critical information on the quality and storage stability of Differential scanning calorimetry (DSC).

If a typical solution, as used in freeze-drying, is cooled to some subzero temperature, a DSC trace as shown in Fig. 3 is obtained upon reheating. The area under the endotherm corresponds to the melting of ice. From the area the amount of frozen water in the sample can be calculated. Since, the initial amount of water and solid in the sample is known, W_g' (the amount of unfrozen water at T_g') can be derived. This is the mass of water that has to be removed during secondary drying. It is also possible to determine T_g' by measuring the peak areas and melting temperatures for a range of different solid water ratios and extrapolating back to zero frozen water, i.e. T_g'. This is time consuming and prone to errors in area measurements due to difficulty in accurately determining the baseline for an area measurement, especially at low water concentration. A better method is to determine T_g' directly.

Computers provide an ideal tool to do this, enabling among a number of things the capability to zoom in on sections of the curve and analyse them in detail. If we do this here we find the second order glass/rubber transition associated with T_g' (Fig. 3).

The software system we used was specially written. Although there are a number of commercially available systems few are able to effectively perform the full range of analyses required. For example, many have a data 'correction' facility which is supposed to remove noise from incoming data.

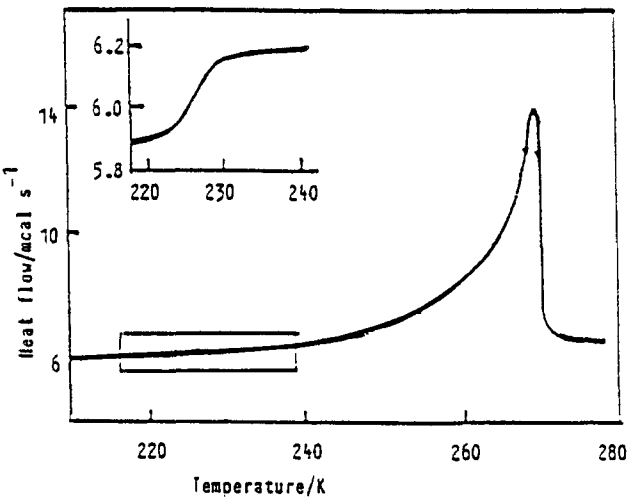


Fig. 3 The complete melting trace of a frozen sucrose solution subject to a typical freeze-thaw process. Inset is an expansion of the area in the rectangle. The glass transition at 227 K can be clearly seen

However, the relative magnitudes of the small glass transition to the large melting peak often results in the obliteration of the lower transition by the 'correction' software. Our system (DARES-DSC) has been reported in detail elsewhere [3], its main features are listed in Table 1.

The third variable that can be measured in the glass transition temperature of the dried product. On first heating a characteristic glass transition temperature with some relaxation is observed. On the second run the relaxation is removed. Sometimes, as is the case in Fig. 4, the relaxation can be deceptive so care needs to be taken and at least two runs made. Figure 4, shows a plot of heat flow *vs.* temperature for freeze-dried product. The first scan, if seen in isolation, would appear to be the first order endothermic transition. In this case it could not be, as the material was a pure sucrose glass. If the sucrose had crystallized, a melt would be expected above 420 K. The second run confirmed this, showing T_g at the same temperature as the thermal event obtained on the first run. The first run is therefore a glass temperature with an extremely high degree of relaxation.

Table 1 Major features available within the DARES-DSC program [3]: selection is from menus with the aid of a mouse

Visual aids	Calculations	Output facilities	Input/edit facilities
Extrapolate line	Area calculation	Print graph as displayed	Take data from calorimeter
Scale display	Transition temperature	Print-out of data	Input stored file from disk
Mark transition temperature	Slope gradient	Output to disk as ASCII file	Edit user variable fields
Overlay traces	Differntiate (any order)	Print index of disk contents	
Level trace	Subtract traces	Backup disk	
	Average traces	Delete files	
	Divide traces	Write calorimeter data to disk	
		Write edited data to disk	

The importance of the measured variables in freeze-drying

During primary drying the sample must be kept below T_g' so that the enzyme is stabilized in an amorphous glass and protected from damage. If the product temperature is allowed to rise above T_g' then some of the ice will melt back into the non-ice phase forming a highly concentrated solution.

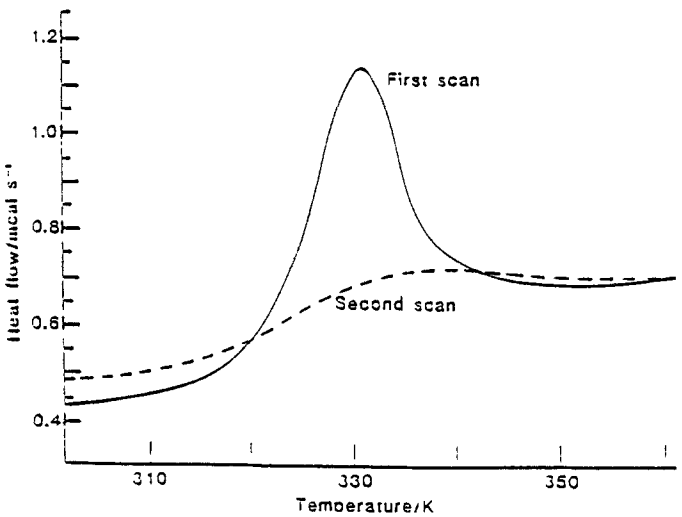


Fig. 4 The overlayed traces obtained on the first and second heating scans of a freeze-dried product that is expected to show a second order (glass) transition. The implications are discussed in the text

Under these conditions the enzyme will be prone to inactivation and the product matrix in danger of collapse due to a weakening of its structure. Since ice sublimation rates are much more rapid at higher temperatures, it is desirable that the products to be freeze-dried have a high T_g'. The addition of stabilizers that themselves have high T_g's is preferred since these will raise the T_g' of the preparation as a whole. A list of possible stabilizers is shown in Table 2. It can be seen that materials such as maltotriose (T_g' = 249.5 K) should be used. Glycerol is unfavourable because it has a low T_g'. In general it can be considered that low molecular weight species (e. g. salts) will suppress T_g' while large molecules, e. g. carbohydrates and, very often, the active material itself, will raise T_g'. The overall T_g' of a preparation will be related to the mass ratio of the solid components.

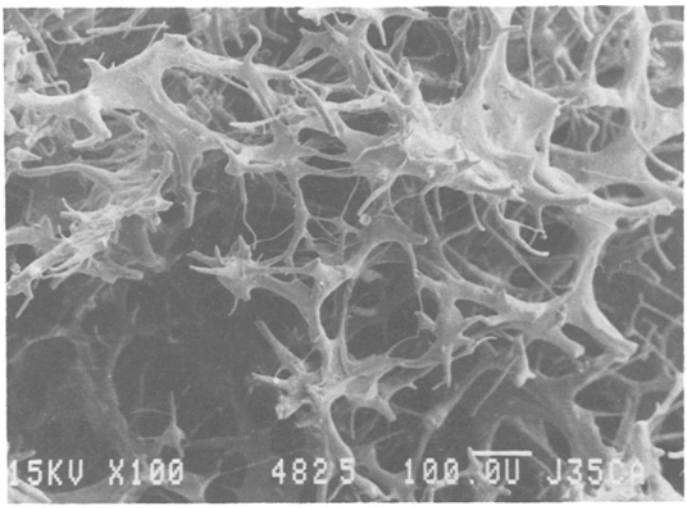
Table 2 Glass/rubber transitions of anhydrous (T_g) and freeze-concentrated (T_g') carbohydrates and water contents of the freeze-concentrates. Data from Ref. [4, 5]

	T_g, K	T_g', K	wt% water
glycerol	180	208	46
xylitol	234	226.5	42.9
ribose	263	226	33
xylose	282	225	31
glucitol (sorbitol	270	229.5	18.7
glucose	312	230	29.1
mannose	303	232	25.9
galactose		232.5	45
fructose	286	231	49
maltose	316	243.5	20
cellobiose	350		
trehalose	350	243.5	16.7
sucrose	330	241	35.9
glucose/fructose (equimolar)	293	230.5	48
maltotriose	349	249.5	31

W_g' is the amount of water that needs to be removed during secondary drying. This should be as low as possible because it then enables a more rapid secondary drying. A low W_g' also reduces the probability of product collapse.

Combining T_g' and W_g' requirement, the best types of stabilizers are those such as maltose and sucrose. Sucrose has a further advantage in that it is inexpensive.

a)



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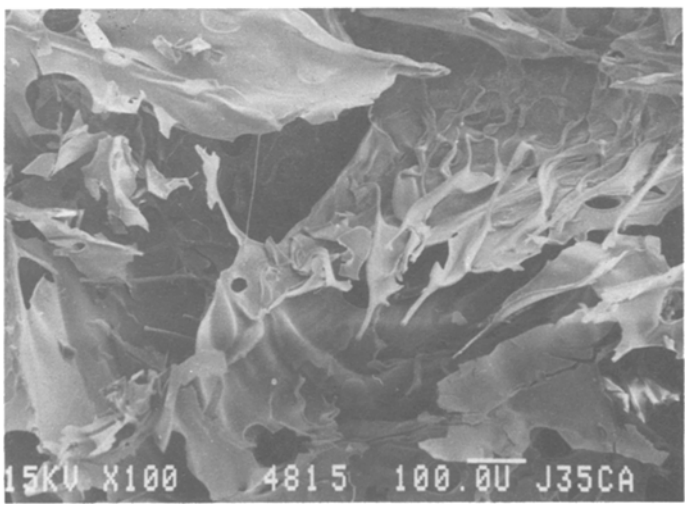

Fig. 5 Scanning electron micrographs of freeze-dried therapeutic products which had been frozen to –50°C. Sample (a) had a $T_g' > -50°C$ and (b) a $T_g' < -50°C$. The structure of the freeze-dried (b) is poor due to incomplete freeze concentration during primary drying

The T_g value of the dried product gives the maximal safe storage temperature. If kept below T_g, the enzyme will be in an amorphous glass, physically protected from decay. If stored for extended periods above this temperature, it is prone to activity loss.

An example of the importance of T_g' in a real situation

The product was a mixture of protein, sucrose and salt. It was routinely frozen to -50° then freeze-dried. However, there was a marked variance in batch quality. Figure 5a shows an electron micrograph of a good product, the open structure indicates good drying. Following indifferent drying, the open structure is lost (Fig. 5b). This product is difficult to rehydrate and has poor stability. The only difference in the formulation and processing specifications between the batches was the protein content. We examined both a high and low protein concentration preparation. The former has a T_g' of 226 K (Fig. 6a), that is, above the freezing temperature of -50°. The product should be stable during primary drying since the active protein will be 'trapped' in a glass. The low protein preparation had T_g' below the freezing temperature (Fig. 6) and therefore was still in a rubbery state during primary drying. This resulted in partial collapse during processing, giving a poor product.

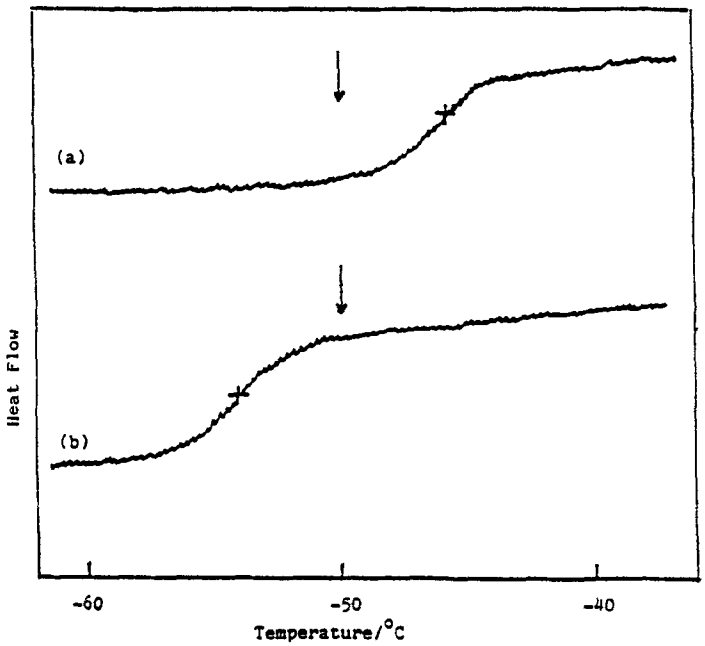

Fig. 6 DSC heating scans of the preparations shown in Fig. 5. The routine freezing temperature is indicated by the arrows and T_g' by +. The two preparations differ only in the protein concentration of the solutions. The sample with the lowest protein concentration has the lowest T_g'

In order to ensure that the product is of a constant high quality, regardless of protein concentration, it must be ensured that the product is always in the

form of a glass during primary drying; it must always be frozen to below T_g'. There are a number of ways of doing this but probably the most effective is to compensate for the drop in T_g' caused by the reduction in protein concentration by adding extra sucrose.

Conclusion

DSC can be used to measure the physical constants that are important for freeze-drying. These measurements can then be used to define the optimum processing parameters – time, temperature and pressure. This approach is more efficient in terms of time and cost than deriving the process parameters by trial and error. Additionally, the measurement of T_g of the dried product gives an immediate indication, directly after the completion of processing, as to its long-term stability. A more detailed discussion of how the concepts reported here can be used to improve freeze-drying is given in references 2 and 6.

References

1 H. Willemer, Joint Meeting IIR Commission C1 and C2 Karlsruhe (1977) 1.
2 F. Franks, Cryo-Letters, 11 (1990) 93.
3 R. H. M. Hatley, F. Franks and M. Green, Thermochim. Acta, 156 (1989) 247.
4 H. Levine and L. Slade, Pure and Appl. Chem., 60 (1988) 1841.
H. Levine and L. Slade, Cryo-Letters, 9 (1988) 21.
5 L. X. Finegold, F. Franks and R. H. M. Hatley, J. Chem. Soc. Faraday Trans., I 20 (1989) 2945.
6 F. Franks, Process Biochem., Feb. 1989 Supplement.

Zusammenfassung – Die Eigenschaften und die Stabilität proteinhaltiger Produkte im gefriergetrockneten Zustand sind durch die gewählten Prozessbedingungen und durch die physikalischen Eigenschaften der Endprodukte vorgegeben. Insbesondere sind die Glasumwandlungstemperaturen des Gefrierkonzentrates und des getrockneten Produktes als auch der endgültige Wassergehalt von Bedeutung. In der vorliegenden Arbeit werden DSC-Untersuchungen zur Optimierung des Gefriertrocknungsprozesses als auch Studien zur Stabilität der Produkte dargestellt.