TECHNOLOGICAL ENHANCEMENTS IN FREEZE DRYING

ANDREW JAMES INGHAM
Doctor of Philosophy

UNIVERSITY OF LONDON SCHOOL OF PHARMACY
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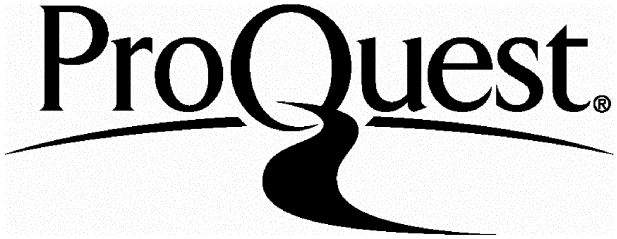

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Abstract

The aims of this thesis are to tackle some of the newer areas of lyophilisation protection including enhancing technology to better determine the primary drying length of a freeze drying cycle. An attempt has been made to focus research from the perspective of a research freeze-dryer when used for scale up studies. It is hoped that the data gathered due to this approach can be applied across a broad range of formulations.

Control of the freeze drying cycle is examined, relating a formulation's physical change induced during a freeze-drying cycle to both the freezing and drying stages. Changes occuring to the size of a liposome population with narrow polydispersity using laser light scattering are used to examine the effects of freezing rate, low temperature processing and rehydration; aggregation during both conventional and non-conventional freeze-drying cycles is also investigated.

The relationship between protein protection and sucrose recrystalisation was determined with a combination of modelling and sequence analysis. Sucrose recrystalisation in the dry state was examined with a variety of enzymes, unifying existing research by allowing the prediction of losses in enzyme activity by examination of the amino acid sequence. Enzymes studied included: catalase, lactate dehydrogenase, lysozyme, asparaginase, adenosine deaminase, β-galactosidase, fructose-6-phosphate kinase (phosphofructokinase), β-amylase, glucose oxidase, ascorbate oxidase and ribonuclease.

Keywords: Primary drying, liposomes, lyophilisation, protein, aggregation, freeze drying, sucrose, mannitol.

Thanks Dad, Mum and my brother.

Technological Enhancements in Freeze Drying.

1.1 List of freeze drying cycles

1.2 List of figures

1.3 List of tables

2 Introduction

2.1 History of freeze-drying

Bordas and d'Arsonval in a paper for the Academie des Sciences (1906) described the desiccation of a product from a frozen state under vacuum. This process was later termed "freeze-drying" and has been heavily used for storage of foods and pharmaceuticals. The term 'lyophilisation' originated from the ability of a product that had been freeze-dried to "love solvent", to "love rehydration". Although not an exact translation of what happens during the process of freeze-drying, European use has lead to the definition of lyophilisation as the complete process of freeze-drying. Britain's contribution to the current status of freeze-drying was fuelled by the need for blood plasma during the Second World War. As a result Britain produced much of the scale up equipment currently in use worldwide. Europe's funding of process research and resultant publications means that today the most commonly used term is that of 'lyophilisation'. Engineers and processing personnel prefer to use the term freeze-drying, while microbiologists may call the process 'cryodesiccation'.

2.2 The main components of the freeze-drying process

Any freeze dryer provides conditions to match those of Figure 2-1. Combining this figure with any pharmaceutical freeze dryer allows three distinct components to be defined that are essential for the processing conditions of freeze drying to be produced. These are the:

i. Shelf (to lower the temperature)
ii. Vacuum (to lower the pressure) and
iii. Condenser (to trap water vapour).

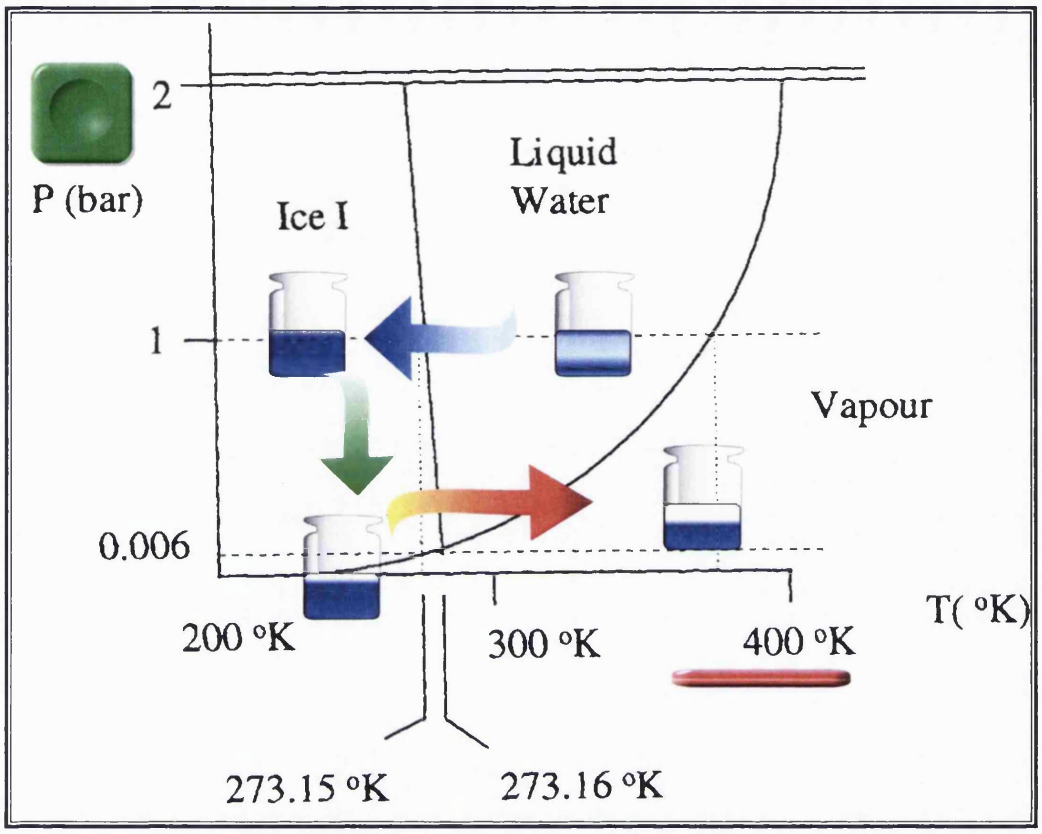


Figure 2-1 : Phase diagram for the freeze drying process. Stages of drying process are shown from the liquid state of a formulation. Adapted from Ward, (1997).

2.2.1 Shelf

The shelf can either provide energy or remove it from the product. The normal functioning range of a shelf is between –60 °C and +80 °C. Certain freeze-dryers appear to have no shelf as the room's own temperature takes the place of the 'shelf' supplying the energy for sublimation. To control the shelf temeprature silicon oil or a gas based refrigerant provides precise alterations in shelf temperature across the whole surface of the shelf. Despite precise control, major changes in freeze-dried product can result from shelf to shelf variation in the case of a multi-shelf freeze dryer. Product variation has also been shown to occur with the location of product on the shelf. Studies involving shelf mapping at least predict damage to product in specific shelf locations. Bindschaedler (1999) showed using micrograms of active protein, large variation in product activity according to the time individual vials were loaded onto the freeze-dryer shelves. This was done to demonstrate the problems associated with large multi-shelve freeze-dryers where

the first tray loaded could be cooled for periods approximately 20 minutes longer than the last tray.

Temperature plays an important role in freeze-drying and is measured for many different reasons in several areas of the apparatus. Commonly, measurements would be taken of the coolant 'fluid in' and 'fluid out' for each individual shelf with specific limits defining the shelf's temperature. Product temperatures are measured often to monitor the process of a cycle and are sometimes involved in meeting GMP requirements. Table 2-1 below shows the most common methods for measurement of temperature within a freeze drier.

Table 2-1 : Commonly used equipment for temperature determination during freeze-drying

Thermocouple	-196 °C to +170 °C multiple meters required to measure this non-linear range.
Resistance thermometer	Linear over wide temperature range. Very accurate but bulky.

2.2.2 Vacuum

The second essential component of any freeze drier is the vacuum. Vacuums can be produced by many different types of pump but pressures must be maintained low enough to produce sublimation of water. Industrial freeze dryers often use very powerful pumps, forcing air bleeds into the chamber otherwise sublimation would actually slow down. The reasoning for a specific type of vacuum pump for a freeze-dryer is then normally based on resistance to solvent or other processing requirement such as sterilization. Figure 2-2 shows the possible pressures that can be produced using some common pumps. Note the vacuum required (given range) for freeze-drying.

Vacuum (bar)	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	1	10	100	1000
Water ring pump														
Steam injector														
Mechanical rotary														
Roots booster														
Molecular drag (air)														
Molecular drag (water)														
Turbo-molecular														
Diffusion														
Cryopump														

Figure 2-2 : Pumps with the potential to provide pressures required for freeze drying. The vacuum (bar) required to achieve the triple point for ice sublimation is shown as vertical green area. From Wood (2000) with adaptations.

Pressure is simply the measurement of the vacuum within the product chamber. Freeze-dryers use the SI unit 'mbar' or 'mTorr, which covers the pressure range of interest.
The most commonly used gauges found in freeze-dryers are shown in Table 2-2 below.

Table 2-2 : Pressure monitoring methods found within freeze-dryers.

Type	Pressure range
Pirani	+/- 6 % between 10^{-2} and 10 mbar
Capacitance manometer	+/- 1 % (or better) between 10^{-4} and 1000 mbar

2.2.3 Condenser

The condenser is the third essencial component to create the conditions for freeze drying, in most cases the condenser provides the rate-limiting step with regard to the maximum speed with which water can be driven from a formulation. Ultimately the collection or condensation of ice onto a condensers surface controls the maximum processing speed for a particular formulation. The lower the condenser temperature the greater the difference allowed in temperature between the shelf and condenser, thus allowing higher

rates of water sublimation and collection. Low temperature condensers also protect the vacuum pump from organic solvents or water vapour damage. Liquid nitrogen having only been proposed for use within a condenser several years ago is installed in many prominent industrial plants to allow the lowest possible condenser temperatures (≅-190 °C).

2.3 The pharmaceutical application of freeze-drying

For a formulation to be freeze dried it must proceed through several stages. The first stage must be the formation of solid water which is performed with a freezing stage. A formulation is then dried using sublimation before often been heated above ambient temperatures to further lower moisture content. The final (secondary drying) stage can be likened to vacuum oven drying. Before a formulation is freeze-dried it would be hoped that several aspects of the formulation have been tailored to suit the transition first to the frozen state and then to the dried state. If rehydration of the dry powder is required for administration then this too must be considered before processing. The sections that follow draw together specific aspects of a formulation that it may be ponient to have considered before development of a freeze drying cycle.

2.4 The typical freeze-dried formulation

In many instances freeze-drying is not a leading consideration in a formulation scientist's mind. Many freeze-drying cycles have been produced using a "trial-and-error" approach, some very successfully but most with only basically defined process parameters. The most common current practice if a problem occurs is to increase the concentration of a disaccharide sugar until 'protection' is achieved. A formulation scientist should actually consider many aspects before approaching the freeze- dryer. For example physical examination of the product can provide information on high temperature exposure or passage through critical processing temperatures. But before these temperatures are discussed it is important to describe some of the possible ways that formulations can be damaged so a critical temperature can be applied correctly. It is not a correct assumption that all damage is caused by one factor or indeed by all the factors. Individual formulations must be examined in isolation as even slight changes in composition and

occasionally purity are known to cause large changes in many areas of control for freeze drying.

2.5 In what way can a formulation be damaged during freeze drying?

2.5.1 Considerations for the liquid state of a formulation

2.5.1.1 Concentration of active component

In some cases protein components have been found to exert self-protection (Ward, 1997); for example L-asparaginase at 3, 5, 12, 24 mg/ml concentrations showed 15, 42, 70, 78 % retained activity respectively. Self-protection in this case was defined as the ability of a component when increased in concentration to inhibit its own damage. Asparaginase was suggested to protect itself with the formation of a dimer or tetramer.

The case of a protein formulation having the ability to self-protect is unusual; for protein and other formulations there are several ways the same problem regarding a loss of activity can be approached. These methods can all be broadly split into two groups. It is apparent a combination of the methods in many cases is the most appropriate to provide the best levels of protection:

- Those requiring changes in freeze drier processing parameters
- Those requiring changes to the initial product formulation

2.5.1.2 The solvent extracted from a formulation

The most commonly sublimed solvent is water. But recently there have been increasing attempts to extract organic solvents from the frozen state too (Ni *et al.* 2001). Solubilization in high or low solubility solvents affects how a product progresses to the frozen state. This is also a major consideration for freeze dryer specification and control since the vapour point and condensation temperatures of most organics are greatly different to those for water. In cases the calculated condenser temperatures for an organic solvent are almost unachievable and the required surface areas for the condensers to trap the solvent in a small amount of time are unusually large.

According to theories for protein protection (Wang, 1999) a residual water or solvent level must be present for proteins to maintain a hydration shell, without which they are suggested to be unable to maintain their tertiary structure. In the case of an organic solvent providing this hydration shell, GMP standards work against the maximum theoretical activity requiring the lowest levels of solvent possible if the product is intended for patient administration. Unfortunately in this case the aim to reduce an organic solvent may be in direct opposition to protein stability. It is accepted that levels of 2 %w/v moisture in the powdered cake provide stability to the majority of proteins (Wang, 1999) reductions below 2 %w/v moisture reduce activity by the removal of moisure from protein hydration shells (Wang, 1999). If an organic solvent was to be completely removed it would often require extended drying producing levels lower than 2 %w/v of moisture.

2.5.1.3 Additional components within a formulation

Any solutions containing a mixture of solutes (including substrates for enzymes) can be scrutinized to see if a component is required within a solution or if it could be added at the reconstitution stage. Considerations such as moving components from the freeze dried formulation to the diluent should be given serious consideration, since the benefit to the required processing conditions in temperature and the resultant length can be of great benefit both to the patient in terms of purity and activity of the drug but also to the cost of processing. The example of an enzyme substrate solution is used to illustrate the appropriate scrutinization of a mannitol 2-dehydrogenase solution below.

Mannitol 2-dehydrogenase (Klimacek *etal*, 2003) interconverts mannitol and fructose. If mannitol 2-dehydrogenase was to be stored using the freeze drying process, it should be possible to examine the components within the formulation to better design it for processing. A typical solution may be considered to have equal levels of both mannitol and fructose (1 %w/v : 1 %w/v) with a low concentration of enzyme (100 ug/ml).

Examining this solution, mannitol is a known crystallising sugar and upon crystalisation has been shown to cause damage to enzyme activity (Carpenter *etal*, 1993). Removal of the substrate in the mannitol 2-dehydrogenase formulation may improve the system for freeze-drying; by reducing the mannitol levels the levels of crystallization during freezing would be reduced. The maintance of an enzymes activity results in an economic benefit but may also enhance dosage control for the final active pharmaceutical ingredient. Complete removal of all solutes leaving the enzyme on its own without bulking agent would be unlikely to provide any protection for the enzyme, very dilute concentrations of enzyme do not provide structure to a freeze dried cake and collapse occurs often resulting in large activity losses. If fructose was to be chosen for protecting the enzyme it does not tend towards crystallization preferring to be in the amorphous state when frozen (Suzuki and Komatsu, 1996). Fructose is a wise choice as it is already present in the mannitol 2-dehydrogenase formulation; it is one of the enzyme's products and because of this would be unlikely not to appear to some degree in the final solution. From the clinical perspective its increase in concentration is unlikely to be unacceptable from an overall formulation view point since in may already be present in a lower level. In this case the most sensible method to freeze dry mannitol 2-dehydrogenase would be to let the enzyme exhaust the mannitol substrate and then freeze.

The low levels of mannitol would mean the solution would be unlikely to crystallize which in this case is shown to be an advantage for mannitol 2-dehydrogenase. However an advantage of solution crystallization should be considered since crystallization is not always a negative factor when freezing a material. Crystallised solutions generally benefit from increased drying rates due to stronger structure allowing higher temperatures during primary drying and having an obvious cost benefit due to a reduction in drying time. In the case of the mannitol 2-dehydrogensase solution the gain in dehydrogenase enzyme recovery by using a non-crystallising solution should more than compensate for the extension in drying time.

2.5.1.4 The addition of protective agents

In sections 2.4 and 2.5.1.3 the use of different lyoprotectants had been suggested to protect product activity or appearance during freeze drying. Lyoprotectants are added to protect against a broader range of characteristics of an unsuccessful freeze-drying cycle. A lyoprotectant may protect:

i. The physical appearance of a formulation
ii. The activity of a component
iii. Against freezing stresses
    a. the lyoprotectant is acting either solely as a cryoprotectant or may act as a combination
iv. Against the cycle conditions
    a. In cases mannitol is used in a formulation not because of its protection or appearance benefiting enhancements but to increase the rate of drying and therefore protect against shortened drying cycles.
v. By means of its presence or concentration (either mass or molar) in driving the formulation to a specific solid state (see section 2.5.2.2).

It can be seen that lyoprotection is a very broad term and in many cases encompasses the larger group of compounds called cryoprotectants as freezing is also an essential stage in a freeze drying process.

A full list of lyoprotectants used during freeze-drying will not be included here. But a detailed lyoprotectant review including some of the common properties of interest can be found in the literature (Wang, 1999).

2.5.1.5 The formulations viscosity

Viscosity could be discussed in terms of the summation of the individual constituents, but is very important in several theories for protection during freeze-drying so it is viewed here seperately. According to the particle isolation hypothesis (Allison, Molina et al., 2000; Anchordoquy, Alison et al., 2001) maintaining a dispersion of particles within a solution could be the key to protection and for this reason changes in viscosity could have

varying effects on the stability during freeze-drying. High viscosity solutions are hypothesised to slow particle movement during freezing allowing the glass state to form without forcing of particles together where they must aggregate.

2.5.2 Freezing

There is no direct correlation between the structure of a frozen product and its current temperature. Most, if not all, characteristics of a frozen mass are determined by its thermal history. Knowledge of the current temperature of a frozen solution is therefore not enough. The example below shows clearly the influence of variations in thermal history on a simple NaCl system.

NaCl at -25 °C has two possible states depending on its thermal history:

i. A sponge ice network – when slow cooled to -25 °C
ii. Crystallization as a eutectic – when fast cooled to -40 °C then warmed to -25 °C.

Freeze-drying protocols always intend to freeze a solution but the temperatures involved are not always sufficiently low and are not always held for a sufficient period to convert all fluid to a solid phase. In the example above for sodium chloride cooling to -20 °C may produce a solid structure, since ice could make up upto 99 %w/v of the solution. A frozen proportion of this solution would appear completely solid without proper investigation. The small (1 %w/v) sodium chloride phase would exist within the solids structure as a liquid.

Once ice is formed, a degree of super-cooling must occur to freeze hypertonic concentrated fluids which in most cases will contain the active substance. This point of complete freezing has been referred to as T_{cs} (maximum temperature of complete solidification).

It is obvious from these statements that the rate and length of freezing are vital to know the product's 'thermal history'. Products that have undergone annealing are more complex; they have used the thermal history of the frozen material to impart specific characteristics on the frozen material. Annealing varies the temperature during a freezing

procedure and in cases it may produce a skin on the surface of the product. A skin can be formed when water is allowed under an increased temperature to re-crystallize. This involves small ice particles becoming larger, made possible by an increase in the available energy. Any other (still solid) phase is pushed to the surface by this action as the largest ice crystals form at the base of a vial. On complete freezing two often-distinct divisions in the appearance of the frozen material are visible and this is termed the skin. Long-term storage of samples in the frozen state produces the same effect; even at –70 °C some energy is available for movement of the smaller ice crystals and skin formation is likely.

2.5.2.1 Freezing rate effects on a formulation

The thermal history of a product has been shown to be generated by alterations in a formulations temperature which must be conducted over a specific period of time. The envolvement of the rate of change can provide alterations in properties with influence during freeze drying.

The most commonly considered rate of change is that during the freezing of a formulation. To freeze a formulation 'fast' on the laboratory scale either submersion in liquid nitrogen or a dry ice- acetone bath are both considered appropriate. In contrast, industrial freeze-drying varies greatly not in what techniques are available but in which techniques are economical. Dry ice and liquid nitrogen are seen as highly expensive during industrial production, especially when industrial freeze-dryers are seen to be capable of shelf temperatures as low as -90 °C and lower. The important difference to be noted is that industrial freeze-dryers reach low temperatures at a much slower rate than the laboratory equivalent. Freezing on a shelf is slower than liquid nitrogen or dry ice and acetone. This 'small' change in protocol will have effects on thermal history (see NaCl example, section 2.5.2).

The thermal history records for a formulation are vitally important. Reproducing the thermal history accurately will give products entering the freeze-dryer the same solid-

state properties which are required to prevent batch to batch variability within a formulation.

2.5.2.2 Solid-state properties

A frozen drug solution gains some advantages as well as some disadvantages from its new solid state. These properties, termed the solid state properties (Table 2-3), clearly have great influence on the next stage of processing which is sublimation of the ice (or freeze-drying).

Solution crystallization during freezing is widely studied and many systems have been characterized, the most detailed been those for ice formed within ice-cream (Faydi, Andrieu et al., 2001). Each system characterised still remains formulation specific and so characterisation on this level is not justified for large scale screening of potential lyophilisation formulations. The addition of even the smallest component will affect solid-state properties. Polyethylene oxide polymer complexes loaded with drug exhibit major changes in their lamellar and crystal structure. The quantity of a drug encapsulated within the polymer complex varies the proportion of amorphous to crystalline content (Shekunov and Taylor, 1999).

Additives commonly used to protect active components also have their properties altered. Slow cooling of the disaccharide additive lactose results in production of single crystals of α-lactose monohydrate. Rapid cooling produces microcrystal aggregates of anhydrous α- and β- lactose. The rapid cooled microcrystal example of a solid-state property is used to increase the crushing strength of tablets (Shekunov and York, 2000).

Table 2-3 : Solid state properties for frozen drug solutions (Shekunov and York 2000)

Solid-state properties	Effect on drug substances and/or drug product
Structural [According to Shekunov and York (2000) & Suryanarayanan and Mitchell (1984) the one state model defines no sharp distinction between crystallinity and the totally amorphous state.]	
Crystallinity Amorphous	Physical and chemical stability
Polymorphs	% Relative humidity profile is altered (hygroscopicity)
Solvates (hydrates)	Solubility profile and dissolution rate
Salts	Aspects of processing
Crystal defects	Cracking as water escapes the cake structure
Dimensional	
Particle size distribution	Process behaviour: bulk density, agglomeration, flow/rheology, compaction
Particle morphology	Particle permeability (e.g. potentially how adsorption to particle is affected)
Particle surface structure	Bio-availability (drug absorption), consistency and uniformity of the dosage form
Chemical	
Organic and inorganic impurities, residual solvent and decomposition products	Toxicity
Chiral forms and chiral separation sterility (microbial limits)	Chemical, physical and enantiomeric stability
Mechanical	
Brittle/ductile transitions, fracture stress, indentation hardness, stress/strain relaxation, yield pressure.	Milling and tableting behaviour
Electrical	
Electrostatic charge distribution	Agglomeration and flow properties

2.5.2.3 Water crystallization

Having seen the effect on drugs entering a frozen solid state there is always at least one other phase formed during freezing (either a second amorphous phase or a crystalline one). The second phase is most commonly 'bulk' water (although it could be an alternative solvent, for example t-butanol). Bulk water is not combined in any way with the drug's phase, but is instead free. The percentage of bulk water is seen as increasingly important as new high activity drug compounds are handled in larger and larger amounts of bulk water. The final density of any remaining product after freeze drying is controlled

by the size of any secondary phase and the relationships to surface area can be hypothesised to be further linked to loss of activity and solubility of any dry powder.

Allison, Molina *et al.*, (2000) proposed the 'particle isolation hypothesis' that damage to macrostructures (including DNA and protein) within solution can be prevented by stopping self-interaction. With the use of liposomes they showed vitrification (Liapis and Pikal, 1996) during freezing of a solution to be unimportant and that spatial separation of particles within unfrozen structures prevents aggregation. Assuming this to be correct leads to the belief that sufficient dilution of any active component would result in its protection but this is not always the case (see section 8.2.3.1).

Results from a recent study on direct observation of ice crystal size (Faydi, Andrieu et al., 2001) suggests a possible alternative: dilution in this case was found to reduce ice crystal size. The control of ice crystal size is well known as a freeze-dryer variable. Should it have been proposed that a reduction in the size of ice crystals would reduce interaction? It could be that the use of common sugars is only required to nucleate ice. Anchordoquy's previous findings (Anchordoquy and Carpenter, 1996; Anchordoquy, Carpenter et al., 1997; Allison, Molina et al., 2000) had shown that virtually any excipient is capable of offering similar protection during freezing.

Extensive work has been conducted on water crystallization (Vippagunta and Britain, 2001) and its affects on freeze-drying. The relationship between cooling rate and diameter of ice crystals is well characterised. Generally, vials cooled at a rate of 0.5 to 5.0 °C/min result in single spherical ice crystals. The higher the cooling rate, the smaller the ice crystal.

The freeze drying industry has targeted increased rates of sublimation by controlling the formation of ice which allows the industry to provide significant cost reduction due to cycle length reduction. Has it overlooked the protection aspects provided by suitable ice crystal structure? Has the industrial need for slow freezing in concentrated solutions led to a clouded view around aspects of activity and protection?

The answer is that the idea of initiating and controlling nucleation of ice is not new, but we know much about reproducing certain frozen states and not always why they are successful. Growth of ice and other eutectic components has been used to increase drying as well as restructure formulations prior to freeze drying. Glass beads have been added during freezing to produce instantaneous nucleation, thus standardizing the time of freezing across ranges of solutions (Franks, 2001). Pharmaceutical formulations, especially aseptic formulations, are problematic to freeze because nucleation generally occurs round microscopic impurities or aggregates and aseptic solutions contain few of these. If preparation aseptically reduces solution impurities to such a level that the largest impurity is that of the active protein, nucleation is unlikely to maintain any tertiary structure of the protein. In these solutions it is particularly difficult to achieve high levels of nucleation to allow small ice crystal formation so it may be that the choice of a particular ice crystal state is not always possible.

2.5.2.4 Water nucleation versus crystal growth

Detailed studies of solution behaviour prior to nucleation have shown (Hobbs, 1974) nucleation is caused by reducing the temperature of small particles (impurities) that become super cooled before the main mass and act as the centre for an ice crystal formation. Eventually after many nucleations, a point is reached when the viscosity of the mass is so high nucleation is inhibited.

Solutions in a freeze-drying cycle undergo nucleation during freezing in what appears to be a random fashion. Nucleation is however, not random just very difficult to predict (Faydi, Andrieu et al., 2001).

In contrast to nucleation, crystal growth is encouraged by increasing temperature and would continue to reduce viscosity of the mass with maintained energy until the melting point of the frozen mass is reached and it melts. This method is used successfully in annealing.

It is possible for water to undergo homogenous nucleation (spontaneously nucleate). In practice this does not happen and heterogeneous nucleation always predominates due to impurities in the solution.

2.5.2.5 The primary drying processing collapse temperature

The collapse temperature (Tc) is a processing parameter specific to an individual formulation. Products exceeding their formulations Tc undergo a structural softening within the frozen solid (see Figure 5-3 page 38). As the sublimation front passes through solid material below its Tc the structure of the solid dry cake that is regularly left behind is disrupted and is said to have 'collapsed' (Figure 7-31 page 108). Tc is a subjectively determined end point to the loss of structure within a freeze-drying formulations structure and is currently best visualised with the use of a freeze drying microscope. The use of such a technique is disadvantageous because operator influence and experience is required to characterise formulations quickly and in an appropriate and reproducible manner. However, since collapse is a structural phenomenon it does not have only a single influencing factor. To use an analogy, during the construction of a building, the cementing of bricks in the correct manner is not the only factor which controls the success of its structure. The quality of materials and bricks in combination with the implementation of a design or plan and the surrounding conditions all combine to structural success. In the same way the subjective nature of microscope analysis when performed with experience allows combinations of all factors (section 2.5) to be examined and a determination of a specific formulations controlling collapse factor can be made.

Knowledge of transitions such as: glass transitions in the solid state (Tg), glass transitions in the frozen state (Tg'), eutectic melts (T_{eu}) and temperatures of collapse (T_c) both within the frozen solid state and dried solid state should not be ignored. However, it is often the case that exposure above one of these distinct temperatures does not cause structural collapse leading to a collapsed freeze-dried cake. In an individual freeze-dried formulation the success of drying does not always have the same controlling factor which will determine its measured success after freeze drying.

The same philosophy of not trusting a single predictive value can and does apply to the reliance on a single value of Tc. A clear morphology of structure is formed for formulations freeze-dried with product temperatures below their individual Tc temperature. This however, does not transform into protection of an individual component (section 3.1.1.2) or phase; micro-collapse was characterised in formulations with product temperatures below Tc. It is simple to suggest that structural changes or softening of components within a phase of a frozen solid could lead to areas which undergo collapse while other areas provide strong supporting structure to give the overall formulation the appearance of no collapse. Indeed, this is the case with the proposed mechanism of action for mannitol and one of the first solids in which micro-collapse was reported (Johnson, Kirchhoff et al., 2002).

2.5.3 Cycle damage

2.5.3.1 Transition between different drying cycle stages

Bulk water is removed during primary drying using sublimation. The rate of sublimation and therefore the period that a product undergoes primary drying is dependent on several factors (section 6.7 page 68). The point at which primary drying is completed is significant since it allows the use of higher shelf temperatures during the period named 'secondary drying' thus allowing significant cost reductions. These reductions due to the energy saved because of the difference in shelf temperature and time are estimated to be up to $100/ °C (Ward, 2004).

Melt back is the process of switching between these two phases too early and causing the solid solution to melt, generally melt back results in the rehydration of any dry material and damage to at least the formulation's appearance.

With a carefully controlled switch, secondary drying can begin without melt back as the whole formulation is a dry powder. Using best design methods this transition to secondary drying is completed by ramping the shelf temperature. Ramping is the process of arriving at a new shelf temperature in a controlled manner which is normally by a very

slow temperature increase over time (e.g. 0.5 °/min). The exact defining line for the beginning of secondary drying and the end of primary drying can be somewhere on the shelf's ramp depending on how the cycle was designed. Literature defines secondary drying in unspoken terms as the 'time when the button is pressed'. Although better definitions are attempted (Bindschaedler, 1999) authors still displays hesitation as to any defined beginning or end of a stage.

2.5.3.2 Stoppering of a freeze-dried product for removal

The final stage of a freeze drying cycle is often the sealing of product vials to prevent contamination or degradation during further handling.

A discussion of all aspects of stoppering is not appropriate here and extensive reviews are available in literature (Hora and Wolfe, 1999). The stoppers allow not only water to escape but also the complete sealing of the vial; stoppers therefore have two distinct states to allow appropriate positioning (Adams, 1991). Sealing within the chamber of a freeze-dryer allows storage not only under vacuum but also under inert gas and in a sterile environment if required.

Opening of a freeze-dryer chamber with products in an un-sealed state will allow the highly hygroscopic material to absorb back around 1 % moisture (Willemer, 1999). Previous discussions (section 2.5.1.2 page 18) have pointed to 2 % w/w moisture as suitable for storage of lyophilised products. Back filling with inert gas or vacuum sealing is therefore essential for prolonged storage experiments where a hydroscopic powder is to be produced.

Differing stopper design can affect final product temperature by up to 3 °C through a reduction in vapour flow from the vial (Willemer, 1999). This however is insignificant in actual processing of a formulation as product temperatures varies to a greatly extent due to shelf temperature and the temperature map of the shelf. It may prove to be significant if vials are to be sealed with a thin film or other stoppers used in research, or and when novel devices such as syringes are expected to be processed through a freeze dryer.

3 Freeze drying formulation models

3.1 Introduction

The broad range of freeze dried materials which are studied today (Wang, 1999) is more diverse than ever. However the items being processed have been reduced to a smaller marked segment as the cost of alternate drying methods such as spray drying and fluidised-bed drying become more appropriate for the drying of food stuffs and low value items. Early work within the freeze drying field concentrated on the speed of drying and was aimed mainly at food processing (Mellor, 1978). In contrast, the work being conducted in the field today concentrates on changes of a structural nature often within the conformation of proteins, or morphology changes of small carriers and the effect this has on delivery vehicles.

3.1.1 Drying particle delivery systems

Particulate carrier systems include a diverse range of constructs such as microspheres, nanoparticles, and self-assembly systems (e.g. micelles and liposomes). Each of these systems is designed to modulate the pharmacokinetic and/ or the tissue/ cell distribution in a beneficial way. Morphological as well as dimensional changes affect distribution heavily. When liposome size was found to increase post freezing drying it was seen as a positive effect by some (Antimisiaris, Jayasekera et al., 1993) but a negative by others (Marion, Allison et al., 2001) due to the effect it had on localisation within the targeted body tissue. Liposomes greater than 0.4 μm in size were seen to remain at the injection site while those smaller moved into the systemic circulation (see section 3.1.1.3). The investigations conducted within this thesis concentrate on the size maintenance of a given structure; this is the situation most lyophilisation scientists will encounter when a formulation scientist approaches them to store a liquid formulation.

3.1.1.1 Liposomes

Liposomes are sealed bilayer constructs composed of amphipathic phospholipid molecules. These amphipathic phospholipids, a good example of which is

phosphatidylcholine (PC) (lecithin), contain a hydrophilic polar head-group and a hydrophobic tail (such as a diglyceride containing fatty acid with 14- 24 carbon atoms). Hydrophobic 'tail groups' face towards each other, excluding water, and are held together by Van der Waals forces. The polar hydrophillic 'head groups' are aligned together and face out towards the water. The net result is a closed lipid bilayer capable of entrapping aqueous material at its core.

Over the last 30 years liposomes have evolved as carriers (Strom and Crommelin, 1998) for improved delivery of: chemotherapeutic agents (e.g. Caelyx^{TM} - Doxorubicin), imaging agents (e.g. radio labelled amino acids (Stefanidakis and Koivunen, 2004)), antigens (e.g. alum), immunomodulators, chelating compounds, heamoglobulin and co-factors and more recently genetic material (e.g. Plasmids (Yong, Debs et al., 1995)) . The main advantage of liposomes is that they are simple structures and form a stable base for many additional modifications (e.g. polymer coats to target cancer).

Liposome subclasses exist with more specific uses and characteristics; these are continuously expanded as new uses are found for the liposome.

Table 3-4: Liposome subclasses (adapted from (Strom and Crommelin, 1998))

Liposome type	Major application
Conventional liposomes	Macrophage targeting Local depot Vaccination
Long-circulating liposomes	Selective targeting to pathological areas Circulating micro reservoir
Immunoliposomes	Specific targeting
Cationic liposomes	Gene delivery

3.1.1.2 Liposome use in gene and vaccine delivery

The use of liposomes to induce an immune response is of particular interest for vaccination strategies. Their ability to carry antigen for prolonged periods is

advantageous in providing long-term presentation thus facilitating an immune response. Liposome adjuvanticity applies to a large variety of bacterial, viral, protozoan, tumoural and other antigen models (Gregoriadis and McCormack, 1994). Unfortunately many researchers when employing freeze-drying to obtain a solid product do not pay much attention to the detail and overlook freeze-drying simply defining the complete process as:

"Freeze-dried overnight under vacuum (<0.1 Torr) in a hetosicc freeze-dryer" (Gregoriadis and McCormack, 1994)

Recent studies such as Taschner, Muller et al. (2001) are finding that dramatic effects on activity can be the result of small changes caused during lyophilisation. Taschner et al, (2001) describe the case of anti-idiotypic antibody (MMA 383), which substantially lost its immunogenicity despite undergoing no apparent degradation during drying. The group found increased flexibility between two of the molecule's Fab fragments as a variable and hypothesised its link to a loss in activity drawing the following conclusion:

"The subtle but reproducible structural changes induced by lyophilisation may be related to the loss of *in vivo* immunogenic properties"

The exact nature of the strucutural change was not determined.

So if freeze drying can result in damage why do it? The answer is that the result of long-term storage in the liquid state, ready for administration is far worse, or that storage in the liquid state is simply uneconomical. This happens in the case of liposome- DNA complexes where formulations have such a short half-life that they must be prepared at the bedside. The equivalent freeze-dried product would be shelf stable at room temperature. Patented technology is now allowing freeze-dried formulations to be administered without reconstitution and by unskilled staff (Roser, Garcia de Castro et al., 1999; Roser, 2001). This is possible through the suspension of a freeze dried material in a

non-aqueous solvent with inherent bacterial action and a high degree of biocompatibility (perfluorocarbon – PFC).

3.1.1.3 The importance of liposome size

Liposome size is the most important factor that determines the extent of lymphatic liposome absorption from a potential injection site (Allen and Hansen, 1993, Patel, 1998 , Tumer and Kirby, 1983). For small (< 0.1 μm), neutral liposomes, lymphatic absorption can reach 70 % of the injected dose whereas; larger sizes remain at the injection site, for example 80 % of 0.4 μm vesicles (Oussoren and Strom, 2001).

It is an obvious concern knowing that freeze-drying can result in a ten fold increase in liposome size and broaden any particle size distribution (Harrigan and Madden, 1994). When fusion of vesicles occurs it is not only the targeting of liposomes that is affected but also the entrapped drug. Winden and Crommelin, (1999) showed that at temperatures below T_g, 50 % of aqueous entrapped drug can be lost along with an increase in vesicle size.

4 Materials

4.1 Chemicals

A list of chemical used within the thesis is provided below. Water was distilled and filtered (0.2 μm) or of the AnalaR (BDH) type. Details of the enzymes used in chapter 7 are detailed within this chapter (Table 7-13 page 90).

Chemicals	Notes	Supplier	Country
Alginic Acid High viscosity	Sodium salt [A7128] From Macrocystis pyrifera (Kelp) Viscosity @ 2 % 25 °C 14,000 cps	Sigma	UK
Alginic Acid medium viscosity	Sodium salt [A2033] From Macrocystis pyrifera (Kelp) Viscosity @ 2 % 25 °C 3,500 cps	Sigma	UK
Alginic Acid High viscosity	Sodium salt [A2158] From Macrocystis pyrifera (Kelp) Viscosity @ 2% 25 °C 250 cps	Sigma	UK
Calcium chloride dehydrate	99.0 % [C3381]	Sigma	UK
Chloroform	99.8 % [C312]	Sigma	UK
Cholesterol	99 % [C8667]	Sigma	UK
Egg phosphatidylcholine	Kind gift. Based on the dry weight it was certified as 96.2 % pure.	Lipoid	GERMANY
Ethylenediaminetetraacetic acid (EDTA)	99.4- 100.6 % Free acid [E9884]	Sigma	UK
Mannitol (D)	SigmaUltra, [M9546]	Sigma	UK
Melezitose (D)+ Hdrate	99 % [M5375]	Sigma	UK
Raffinalose Pentahydrate	SigmaUltra, [R0514]	Sigma	UK
Sodium Citrate	99.0 % [S4641]	Sigma	UK
Stearylamine	98 % [S9273]	Sigma	UK
Sucrose	SigmaUltra, [S7903]	Sigma	UK
Trehalose (D)+	SigmaUltra, [T9531] reduced metal ion content	Sigma	UK
Water	Analar	BDH	UK

Electronic components used in design (chapter 6) were purchased from individual manufactures as detailed with basic components supplied by Maplin UK, Farrnel electronics UK.

Glass manipulation was done by Pullers LTD of London; small pieces of steel wire were used to manipulate glass componets as well as connect differing glass types together.

5 Methods

5.1 Particle size analysis

The two methods commonly employed to analyse particle size and distribution are **L**ow **A**ngle **L**aser **L**ight **S**cattering (LALLS) and **P**hoton **C**orrelation **S**pectroscopy (PCS).

Both machines are manufactured by Malvern Instruments, UK but measure particle size using different principles. The MasterSizer uses LALLS, where small particles (0.5 – 4000 μm) scatter the sizer's laser to a lesser extent than larger particles. The sizer displays values equivalent to the volume of a sphere that equates to the same size as the particle. The PCS measures Brownian motion, which is based on the theory that large particles undergo slower Brownian motion than smaller particles. PCS returns values of intensity which are converted to volume.

In the studies presented in this thesis LALLS was used throughout, due to the large changes in particle size following freeze-drying. Measurement of particles with the PCS system is not appropriate, since particle sizes >1 μm cannot be measured with a high degree of accuracy.

5.1.1 Low Angle Laser Light Scattering

Light to scatter is supplied by a low power Helium-Neon laser; the beam is typically 18mm in diameter. The power of the laser is unimportant, however the higher the wavelength of the laser the smaller the particle size that can be measured and the greater

the resolution of an individual population. In this case a Helium-Neon laser was satisfactory for study use. The light beam, also known as the analyser beam, is passed through a sample holder or presentation device and any particles present will scatter light. The Fourier transformation property applies to the spread of particles in this narrow plane of particles.

Scattered light is detected by diodes at defined controlled angles (generally 90 ° but the full range [0-360 °] is possible over a very short period (10 μs). This provides a "snapshot" of particles; often as many as 2000-5000 are required for representative sampling.

Mie theory is then used to calculate scattering intensities from particle size distributions. The MasterSizer software produces many scattering intensity graphs from particle size distributions (almost randomly) until it finds a match, and then refines its match until it achieves a perfect fit. The software then displays the population distribution which would produce the scattering pattern detected (Rawie A., 2002). It uses the residual value to confirm how well the software's particle distribution graph matches the actual one recorded for the sample. Residuals for a match can be accepted as a true representation of the population if less than 1 %. Figure 9-49 (page 175) shows a sample Malvern MasterSizer data report for a large population (modal size 178.68 μm) characteristic of a 1μm liposome population which has been freeze-dried.

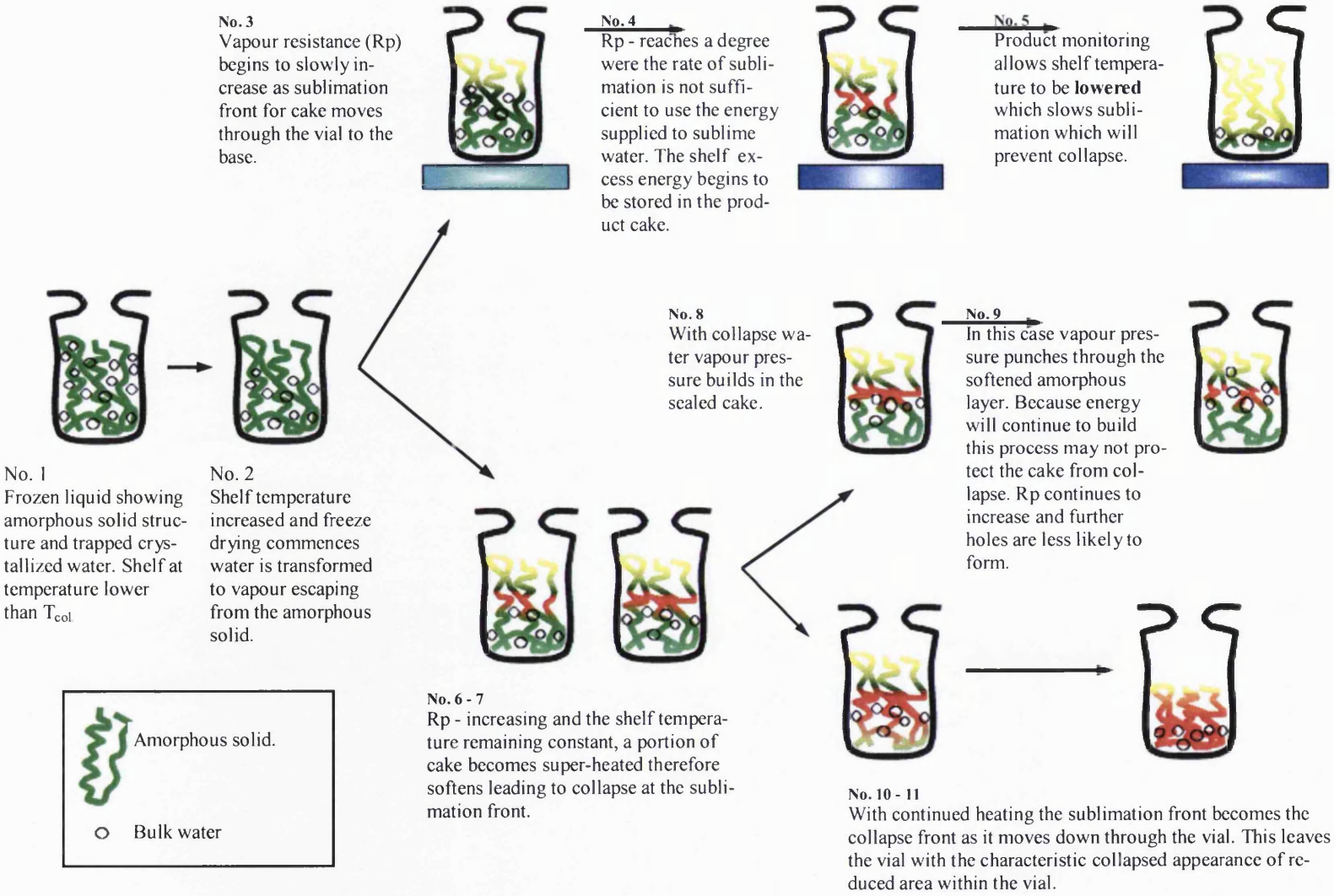


Figure 5-3 : Possible routes leading to collapse in a freeze dry cake.

5.1.2 Practical use of Low Angle Laser Light Scattering

All particles (e.g. liposomes) are dispersed in 0.2 μm filtered water unless otherwise stated and measured at an obscuration value always above 6.5 % but never greater than 50 %. Obscurations greater than 50 % increase the possibility of multiple scattering as laser light refracts more than once from differing particles. The presentation factor and algorithm are set as standard WET-3OHD [Particle RI = 1.53 Particle Absorption = 0.1 Dispersant RI = 1.33] and the Fraunhofer presentation respectively. A background check of obscuration divisible by 200 must be passed before measurement is allowed. All readings use the small sample dispersion unit with the range lens (300 mm). Each sample was allowed to disperse evenly (this may take time and monitoring is provided by the inspect graph) and was subsequently measured three times sequentially.

All readings are checked for residuals below 1 % to confirm correlation with the laser light scattered model and either the mean of the volume distribution or modal size cited.

5.1.2.1 Annotation of laser light scattering results

The use of volume of distribution is limited in this report. Modal size is used since the mean volume of distribution is thought to be a misleading interpretation of the population's most common particle sizes. In the case of freeze-dried particle sizes measured with LALLS particles have been seen to change after freeze-drying in an 'all or nothing' manner. Particles (1 μm) either retain there original size increasing only slightly (1.3 μm) or become relatively large (>10 μm). This could be linked to the proposed methods of size increase where aggregation is known to occur. If the volume of distribution was to be used it is possible to have values suggesting that the population is perhaps 7μm when in fact there are no particles of this size, since the mean of volume is indeed 7μm but the number of particles at this size is different. Simply a few very small particles became very large and by doing so pulled the mean volume to the centre of the distribution when in fact most particles by number were still of the original size.

It is the protection of the original population which is of most interest in freezing drying. Whether the formulation is microparticles or a protein formulation it is the retention of the original size or structure which is often most requested and the reason this report uses modal sizes throughout.

The modal size is simply the most common volume diameter within a population distribution. Its sensitivity to change is limited to following the main components within the solution which is ideal for the freeze drying systems that were seen in these studies.

5.2 Liposome preparation

Individual freeze-drying studies depending on the localization target (see section 3.1.1.3) have required liposomes with specific characteristics. This has included narrow population distributions (small polydispersity) and specific species of liposomes including single unilamellar vesicles (SUV) and multilamellar vesicles (MLV). Narrow distributions (small polydispersity) are generally preferred for the more specific targeting of a location based on size distribution. Both MLV and SUV can have several uses but generally SUV have been found to have reduced entrapment and quicker release characteristics. Liposomes for studies in this thesis were of the neutral type detailed below. Neutral liposomes have the largest size changes upon freeze-drying and were therefore chosen for study due to the large effect this would have on the potential distribution if they were to be clinically administered. The large size changes on freeze drying were hypothesised to be a result of the loss in benefit gained from the repulsive charge interactions when cationic or anionic liposomes are dried under the same conditions.

5.2.1 Thin film hydration method for production of a liposome suspension

The thin film hydration method was initially used to prepare liposomes. To form a thin film that upon hydration will form liposomes, lipid (phosphatidylcholine) was first dissolved in chloroform; depending on the formulation of the liposomes being prepared

additional components such as cholesterol (a packing molecule used too increase the rigidity of bilayers) and stearylamine (used to impart a positive charge on the lipoosomes) were also dissolved in the solvent. A typical molar ratio for positively charged liposomes would be 16:8:4 (Molar ratio) phosphatidylcholine, cholesterol, stearylamine respectively. Neutral liposomes were produced without the addition of stearylamine.

The dissolved lipid components were transferred to a 150 ml round bottom flask, and the chloroform evaporated in a rotary evaporator, under vacuum on its minimum rotation setting in a 30 °C water bath for one hour. The remaining chloroform was removed by purging under a stream of nitrogen gas for 5 minutes.

De-ionised water was added to produce a final lipid concentration of 7.8 mg/ml. Initially addition was dropwise while vortexing, with the remainder added before rotary shaking the flask (37 °C 60 R/min) for 1 hour.

5.2.2 Extrusion of liposomes for obtaining- multilamellar vesicles or single unilamellar vesicles

The extrusion technique can be used to produce multi-lamellar vesicles (MLV) or single unilamellar vesicles (SUV) as well as to narrow the size distribution and give a smaller polydispersity index (Allen and Hansen, 1993). Extrusion within an extruder (Northern lipids inc LIPEX) involves forcing the liposomes through an extrusion filter made of polycarbonate (WHATMAN, UK) with holes of diameter 100 μm- 0.1 μm depending on the final liposome size required; this could be further repeated until the required polydispersity index (LALLS or PCS determined) was obtained.

The in-house extruder is capable of extruding volumes of 50 ml in one cycle. Each liposome suspension (DRV or thin film produced) was forced through a polycarbonate extrusion membrane (Fischer Scientific UK). To produce the liposomes used in chapter 8 a 1 μm polycarbonate membrane was required. The smaller the pore size of the membrane the more difficult extrusion becomes. For membranes with pore <0.6 μm mild

heating of the liposome suspension in a 30 °C water bath (to exceed Tg) was required before extrusion to prevent blockage of the filter. Nitrogen pressure suppied to the extruder was supplied at a constant 4 bar. Each 50 ml sample volume was passed a minimum of ten times through an individual membrane to achieve a suitable size. .

5.3 Differential scanning calorimetery

Differential scanning calorimetery (DSC) uses controlled heating of a reference and sample to measure endothermic and exothermic transitions as a function of temperature. When a formulation passes through a physical or chemical change a corresponding alteration in enthalpy can be observed. The two main types of DSC are the heat flux DSC and power compensation DSC. Both measure the same heat stimulated changes but differ in the method for application of the stimulus. Power compensated DSC uses two individual heating sources to maintain identical sample and reference temperatures and calculates the energy required to maintain these matching temperatures. The DSC used for studies is detailed in section 7.3.1.3 page 91.

5.3.1 Heat flux DSC

Heat flux DSC uses the equation for power consumption (P) to calculate the energy supplied in order to maintain reference and sample pans at a constant temperature, and is shown in the following equation.

$$P = dQ/dt = Cp.dT/dt + f(t,T)$$

P – power supplied to maintain pan temperatures. (J)
Cp – heat capacity (J/°C)
dT/dt – applied heating rate (°C/sec)
$f(t, T)$ – is the heat flow from kinetic processes (°C/sec)

Heat flow (dQ/dt) can be plotted against a reference temperature for thermal events to be visualised (e.g. Figure 7-34 & Figure 7-35).

5.3.2 DSC Experimental preparation for analysis

Sample preparation for DSC analysis was conducted at 0-1 % relative humidity by breaking the vial seal and transferring small quantities of sample (≅3 mg) to hermetically sealed DSC pans (Hill and Craig, 1999) within a chamber. Sample consistency was produced by first breaking the freeze dried vial seal then grinding the material into a fine free flowing homogenous powder.

Using a modified version of the model 8230 Rigaku Themro Plus (Rigaku, Japan) with external cooling and increased sensitivity samples were analyzed according to the method of Imamura et al., (1998). The specific DSC in use had been altered to allow multiple copper shields to be placed around the samples providing resistance against event modest temperature changes. This provided the equipment with a very stable and sensitive baseline. In order to maintain sensitivity and provide resolution of any events high ramp rates greater than those in use (6 °C/min) were not recommended due to the relatively slow changes in temperature found within the layered copper housing. Provision for temperatures below ambient (Japan 22 °C) were made possible with the use of a controlled flow liquid nitrogen system

Baseline correction was performed from empty hermetically sealed pans. Temperature calibrations using the calibrants *n*-octadecane (Tm=28.24 °C) and indium (Tm=156.60 °C) were performed every two days during analysis.

Interpretation of the DSC data was performed by extrapolation from the midpoint of any transition using pre and post transition baselines (Figure 7-34 page 112).

5.4 Freeze-dryer operation

The freeze-drier used throughout was either the Virtis Advantage EL with the Mentor control system or the Virtis Advantage with Wizard control system (VIRTIS inc, USA). The vacuum system for both machines was of the BOC Edwards (EDWARDS, UK) type. Data logging was performed for each freeze-drying cycle using an external computer

interface. Acceptance of a successful cycle was allowed if within ± 1 °C of the inputted parameter.

Monitoring of process parameters within the machine was conducted with thermocouples of the 'exposed junction coated T type'. Unless other wise stated freeze drying vials were a tubular glass type with 1 port butly stoppers and placed in direct contact with the shelf. Cooling from ambient temperatures and all thermal treatments always took place on the drier's shelf and was recorded as part of the complete freeze drying cycle.

5.5 Primary dry switch operation V3

The Priamry drying switch was developed as part of a study to monitor the length of primary drying. Since the trigger mechanism of the switch required differing methods for setup at different phases of development it is the method for the final and 'production' design which is presented here.

The principles behind the operation of a primary dry switch can be found in chapter 6, the final model (section 6.6 page 68) is based on a commercial switch from Assemtech (RI-46A) with alterations by a small scale glass supplier (PULLERS,UK). The final design can be seen in Figure 6-7 and was connected to a suitable timing circuit (Figure 6-10(b)) with defence standard 61-12 Part 29/1 wiring. The switches use to measure the end point of drying for high concentrations of solute in a solution was demonstrated in section 6.7 (page 68) and it was used throughout several protein studies including those in section 7.3.1.2 (page 90) to prevent extending the primary drying cycles un-necessarily.

Each switch requires an individual test to confirm correct operation before it can be used in the freeze-drying process. The reasoning behind this test is demonstrated in section 6.6, page 68. Using the timing circuit or resistance meter activation of the switch with a magnet would confirm correct manufacture.

Positioning of the switch takes place within either a modified vial or manual fixing of its position. In the case of a switch been used to measure the conclusion of primary drying it would be positioned at the base of the vial to detect the absence of the last of bulk water.

Using a small volume pipette, the inner portion of the switch is washed with a small volume of distilled water. In cases where a switch has become contaminated, a suitable agent should be used to clean the switch. Use of strong cleaning agents is possible; dilute acids and mild surfactants are all appropriate. The switch requires retesting before each use.

Drying of the switch is required in a moderate environment (+30 °C). On completion of drying the switch is loaded with 3 µl of deionised and filtered water. Use of t-butanol is not recommended due to its expansion on freezing. Removal of 2 µl of water leaves a small volume (approximately 1 µl) of water separating individual switch pins, this is shown under the light microscope in Figure 6-8 on page 63.

Loading of the experimental formulation takes place to the container of the switch before the usual freezing procedure. Only at this point would both the primary circuit and magnet be connected to the switch. Detection of incorrectly prepared switches can be made at this point since premature activation would cause the circuit to already be complete. Initial time data would then be recorded and the experiment allowed to continue.

5.6 Freeze-drying microscope analysis for collapse point determination

Analysis of freeze drying under the microscope was performed using the Lyostat1 (Biopharma Technology Limited, UK) microscope system. Small volumes of solution (6 µl) were pipetted onto the quartz covers positioned on the copper stage. Using video capture footage of the sample freeze-drying was recorded at different temperatures while a high vacuum (>200 mTorr) was maintained in the chamber.

The freeze drying microscope was used to determine the characteristic collapse temperatures (Tc) of a drying solution, based on the disruption of the freeze-dried cake's structure at differing temperatures (-120 to +60 °C).

5.7 Karl Fischer moisture level determination

Moisture content was determined on conclusion of freeze-drying using a volumetric Karl Fisher titrator (Mettler Toledo, model CL31). Karl Fischer titrations are performed by a reaction of free iodine with water.

$$I_2 + H_20 + SO_2 \longrightarrow HI + SO_3$$

Detection of free iodine in the system indicates the end point of the titration and the quantity of water neutralized with Karl Fischer reagent can be expressed as % of dry mass.

Before analysis calibration of Karl fisher volumetric reagent was performed against known volumes (0.5 µl) of AnalaR grade water. The determined calibration factor was then used for all subsequent analyses. Samples were injected through the stopper with 3 ml of the titration solvent extracted from the titrator vessel. After brief vortexing sample volumes of 1 ml were extracted from the sample vial and injected into the titrator vessel and titration began. The colourless hydrogen iodide is titrated with Hydranal composite solution until the presence of iodine is detected.

5.8 Dialysis of protein solutions

The dialysis of individual enzymes required in chapter 7 to allow the separation of stock enzymes from any salts or buffers (page 89) used 3500 molecular weight cut-off dialysis tubing from Visking. Each 8cm section of tubing was first heated with 2 %w/v sodium bicarbonate and 1mM EDTA at 80 °C for 30 minutes then left to magnetically stir in filtered and distilled water. If stored the membrane was transferred to a solution of 0.1 %w/v sodium azide and refrigerated.

Actual dialysis was always performed against filtered and distilled water over a period not exceeding 12 hours in an agitated volume not less than 500 ml.

5.9 Protein quantification

Protein quantification was performed using the micro-bicinchoninc acid (BCA) assay (Smith, rohn et al., 1985) (PIERCE Biotechnology, UK). The assay provides quantification based on the macromolecular structure, the number of peptide bonds, and the cysteine, tryptophan and tyrosine residues within an individual protein (Wiechelman, Braun et al., 1998). Using the micro-plate technique samples were analyzed against the original stock solution of the protein using the standard kit protocol.

Standard curves were produced with known amounts of protein using serial dilution from 40 μg /ml down to 2 μg /ml. 150 μl of each unknown sample was then serially diluted in a similar manner before incubation at 37 °C for 2 hours or until the plate colour (green changing to purple) was suitable for reading. The microplate was returned to room temperature and measurements taken with a spectrophotometer at 540 nm. A best fit curve was used to quantify the unknown solutions against the standards for individual proteins and they were also compared to a known standard curve for BSA. Individual protein standard curves did show differences to BSA curves specifically at higher concentrations but this difference was never greater than a standard deviation of ±0.31 μg and was seen as acceptable for the experimental concentrations in use.

6 Endpoint determination for the primary drying period of freeze drying

6.1 Background

Freeze drying of any pharmaceutical is an expensive process which is 4-8 times higher costs than air-drying (Ratti, 2001). During the freeze drying process products must be maintained at a pressure of at least 0.006 bar and below 273.15 °K. In most cases this temperature must be exceeded due to physical damage that could be caused to formulations dried at higher product temperatures. Low temperatures of 253 °K are

common place and fluctuations in pressure require vacuums to have a set point less than that of the minimum (0.006 bar). Otherwise the chance of accidental change to the liquid phase is just too great.

The cost of using the freeze drying process is often justified by the benefit of a new solid state imparted by freeze drying. Obviously tailoring a freeze-dried pharmaceutical can be of benefit to either the product or the 'pocket', unfortunately it is rarely both. Either processing can be done very cheaply with comparative poor product quality (e.g. strawberry food products) or using the more expensive processing parameters (low temperature shelves/ condenser) and gain products with a higher degree of product regularity and reproducibility. Pharmaceutical products often require lower temperatures to increase stability and product quality, working against the economic savings which require increases in temperature for cost reduction.

The following chapter describes a method to determine the primary drying end point, by allowing this determination to be conducted with greater precision it is hoped further cost savings can be sort through a reduction into total cycle length. Before discussing this, it is important to have an understanding of the currently available methods used for end point determination and the reasoning for careful determination of this point.

6.2 Primary drying

It was concluded by Ratti (2001) that improvements to classical vacuum freeze drying should address the following key areas following his assaertion that the 'energy cost' for freezing, vacuum, sublimation, condensation were 4, 26, 45 and 25 % respectively:

i. Improve heat transfer to help sublimation.
ii. Reduce drying times (reduce vacuum costs).
iii. Avoid condensation costs.

The energy cost was that associated with running the individual components of the freeze drier for that found in a typical freeze drier. Using the length and temperatures of a typical freeze drying cycle (-20 °C 56 h +20 °C 12 h, 6 μbar, -80 °C condenser) it has been calculated that the cost of freezing is relatively insignificant when contrasted to the

costs of sublimation (the energy inputted to cool the freeze driers shelves) (Figure 6-4). Its apparent then that in line with Ratti's studies that the reduction in a freeze-drying cycle length would result in a cost reduction for all the categories shown (excluding those of freezing).

A typical freeze-drying cycle when subdivided between the freezing, primary drying and secondary drying divisions highlights the relative volume of energy involved in performing the different stages of the cycle. It is clear then that reductions in the length of primary drying would have the most significant affect at reducing the total energy of a freeze-drying cycle.

Speaking form an economic point of view, it can be seen that most money is spent within the sublimation section (Figure 6-4) and that the largest portion of this sublimation money is spent within the primary drying phase of a cycle. It should therefore economically be the aim of a freeze drying scientist to improve classical freeze drying by reducing the length of primary drying in preference to other stages of the cycle. This has been a key target for industrial manufactures; however it is important to keep in mind that a reduction in cycle time may not always have benifical affects on the quality of a formulation (section 2.5.2.3 page 25).

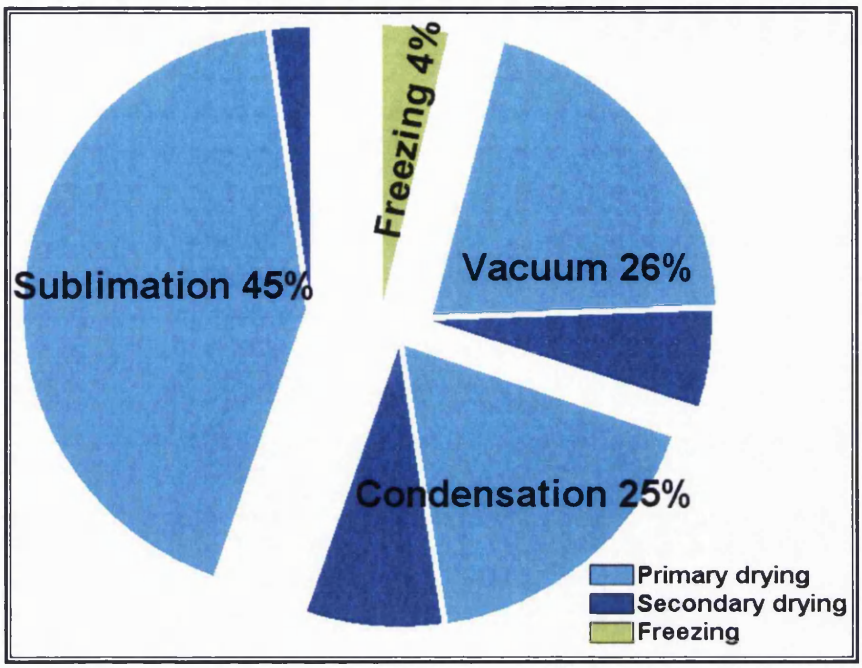


Figure 6-4 : An energy breakdown for the freeze drying process. Categories are subdivided with percentage energy used by the stages of a typical freeze drying cycle (-20°C 56 h +20°C 12 h, 6μbar, -80°C condenser).

6.2.1 Measuring primary drying

Currently there is little consensus as to the end point of primary drying since it is so interwoven with that of the secondary drying stage (i.e the passage from lower temperature for sublimation to high temperature for desorbtion during drying). A number of authors frequently comment that "Despite the practical importance of determining the point where primary drying terminates, there is no easy or universally recognized method" (Rey and May, 1999). It is expected that if primary drying time is shortened the time needed for secondary drying must be increased (assuming an unaltered cycle length).

Currently primary drying is best determined when the freeze dryer design allows pressure rise tests (PRT) to be preformed. This test is performed with a simple isolation of the condenser from the chamber which allows chamber pressure fluctuation to be monitored. If water is leaving the product the pressure in the chamber will rise; if it is not leaving as is the case at the end of primary drying no pressure increase will be seen.

Freeze dryers must have isolation valves between the chamber and condenser to allow the pressure rise test to be preformed. In fact this is the case for most industrial freeze dryers as condenser and chamber units are completely separated occasionally in different buildings. There is no such isolation available on benchtop research or small scale freeze dryers (<£20,000). When using the pressure rise test it is suggested that damage due to collapse can occur with condenser isolation >10 seconds. This is due to an increase in product temperature as a result of slowing the sublimation rate. Obviously a single thirty second test is not a concern but multiple end point determinations in a product cycle may be more likely to result in product collapse if the test is negative. This has a higher probability than might be expected since pressure rise tests all take place within a very short amount of time surrounding what is estimated to be the end point of primary drying.

When the pressure rise test is not available the most common option is estimation from freeze-dryer cycle records like the one shown in Figure 6-12 (page 72). It is easy to see that determination of even the simplest formulations primary end point determination is difficult. A typical approach would be to observe a change in:

i. Product temperature,
ii. Change in slope of chamber pressure and
iii. Change in slope of the condenser temperature trace.

The three end points (i, ii, iii) generally show a degree of agreement. In cases when the end points do correlate, it is known that these predictions lead to somewhat shorter estimates than the real duration of primary drying (Rey and May, 1999).

In the case of many freeze-dryers used in the laboratory environment prediction can be even more restricted. In these cases chamber pressure cannot be monitored due to either uncontrolled vacuum leaks or the continuous running of a vacuum pump. The use of externally routed product probes also causes difficulty, as insertion into the chamber often resulting in large vacuum leaks.

6.2.2 Defining primary drying

If the reader requires a basic understanding of all divisions pertaining to the process of lyophilization they are directed to Mellor (1978).

Defining primary drying is a difficult task. Theoretically if primary drying is the point at which there is no unbound water, it can be said that the top layer has finished primary drying once the sublimation front has passed in which case it begins secondary drying. A question that should be asked is has the complete product finished drying? No of course not. This is because the lower layers of frozen material are still loosing water and all progressively entering secondary drying. A definition of primary drying must incorporate an actual end point rather than allowing confusion to be generated from this gradual entry to secondary drying. It is however universally accepted that the endpoint of primary drying should be that at which 'bulk' or unbound water has been removed completely from the vial. This definition is universal because unbound water has serious effects on product quality (section 2.5.3) if the product is allowed to melt back.

6.3 The design of the Primary Dry Switch (PDS)

The primary dry switch has been developed to overcome the problems related to work with a small scale or benchtop research freeze dryer or mixed load large scale drying. It has been designed to assist in the measuring of primary drying end points specifically for high solute liquids although has been found to have the same function as existing methods with the more typical dilute solution.

6.3.1 Requirements from a suitable measuring device for primary drying end point determination

Table 6-5 summarises different methods of measuring the end point of primary drying and illustrates specific limitations of individual methods for each method of measurement.

The requirements set for development of the primary drying switch were developed around the foundation needs both within the industry for optimization (section 6.2 page

48) and the realistic scale equipment that is available to such an industry to conduct such research (section 6.2.1 page 50).

Specifications for development of the primary drying switch:

i. Determination of the primary drying end point
ii. Measurement of multiple vials
iii. Cost effective
    a. Re-usable
    b. Disposable
iv. Use in all freeze dryers from research to commercial dryers
v. Minimum interference to a formulation's freezing and drying process

6.3.2 Identification of a detection technique to be used in the design of the primary drying switch

The majority of freeze dried pharmaceutical products are designed for filling into vials but large amounts of freeze drying in other industries including the food and the reclamation industries takes place in trays or on continuous flow beds. Within the last 5 years there has also been a drive for more novel devices to be freeze dried. These have including the prefilled syringe (VETTER) where both dry powder and diluent are stored together and the Zydis^{TM} tablet tray systems (CARDINAL HEALTH) which involves components to allow quick dispersion of dried matrix. The plastic or glass housings associated with these uncommonly shaped devices are becoming more frequent in freeze drying as marketing of a new product requires more than the functionality of the typical ampule vial. Pressure to provide ease of reconstitution and control contamination on manipulation of a product has lead to the need for improved packing design.

However, most of the existing probes to monitor drying are used in parenteral vial situations. Monitoring takes place in the centre of the vial base as this has been shown to be the last position to undergo sublimation of unbound water. The device developed during this project is similar and will concentrate on measurement of unbound water in the same location.

Many devices involve interpretation of a graphical plot to give the end point of a cycle. In the case of thermocouple data traces, these are very subjective and operator bias is a major disadvantage. Authors have defined alternative methods for prediction from the same graphical data, with final drying times differing by ± 4 hour. An example of such a situation can be seen in Figure 6-12 (page 72). Use of the average product temperature recorded with thermocouples provides very differing results to those that would be predicted by using the first responding temperature probe. It's also clear that the rise in product temperature at the end of primary drying (labelled C) is dependent on the level of inflection which is arbitrarily set at 5 % in this case. Setting this level of fluctuation in product temperature to for example 10 % would cause a lengthing in the predicted time for the primary drying end point.

To prevent such variations, a project defining the end point of primary drying with an "all or none" nature was needed. A mechanical signalling system was chosen as it provided this on or off state, at all points the mechanism was either not activated or activated. This approach removed the interpretive nature completely from the end point detection.

Table 6-5 : Details of different methods used for primary drying end point detection.

Method	Description	Advantages	Disadvantages	Acceptance	References
Pressure rise test (PRT)	• Isolation of the condenser from the main chamber causes an increase in pressure when drying is incomplete	• Assessment as a whole of primary drying stage • Repeat determinations can take place. Allowing monitoring throughout cycle • Determination has high accuracy • Non invasive, quick and very easy to carry out	• Vial numbers > 1 cannot be monitored individually • Repeat determinations are required to determine endpoint which could result in product variations • Leak monitoring (vacuum calibration) is an expensive prerequisite. • Requires condenser to be isolated from the drying chamber. Expensive • Optimization runs are required to determine a suitable 'pressure rise'. This is often determined using Karl Fischer	• Industrially accepted as method of choice • Smaller scale GMP dryers may use alternate methods as described in the product licence	(Mellor J.D., 1978; Rey and May, 1999; Oetjen, 1999; Andrieu, 2004)
Thermocouple determination	• A thermocouple probe is placed within the vial (at the base) and temperature increases above 5 % of the stable baseline temperature are indicative of the end point.	• Relatively cheap. • Easily installed within a vial. • Ability to measure multiple vials (6+ is common) any number is possible.	• Accuracy is not high and has high subjectivity of the operator • Precise positioning of probe is very difficult. • Thermo probe location in vial causes tunnelling of water in the individual formulation. • Products monitored must generally be disposed of. • Over estimation is common, although some allow for this in a calculation.	• Highly used as a research tool. • Often the second line choice for industrial measurement or confirming technique. • Most industrial cycles if possible use thermocouples although due to placement and sterility this is soften not possible.	(Mellor J.D., 1978; Rey and May, 1999; Oetjen, 1999)

Method	Description	Advantages	Disadvantages	Acceptance	References
Near Infra red. (NIR)	• NIR probe end is positioned parallel with vial to produce a continuous scan of the vial base.	• Whole product can be visualized and monitored throughout process. • Formulation changes maybe visualized. • Non-invasive to the vials formulation. • Possible low water content determination during secondary drying.	• Exact vial base monitoring does not take place. Product treated as whole. • Calibration is difficult • Interpretation of water influence is debatable. • Expensive. • Placement interferes with vial and shelf thermal conditions.	• The NIR technique is well respected within pharmaceutics but its place in freeze drying is not too clear.	(Bechtold-Peters, Bassarab et al., 3 A.D.; Corver, 2004)
Electronic balance	• A high accuracy balance is placed on the shelf. Monitoring of vial until its weight is constant indicates primary drying.	• Non invasive to the vials formulation.	• Over estimation is apparent. • Measurement of drying below -40°C is not possible. • Placement interferes with vial and shelf temperature	• Has been available to researchers for a number of years but has had a limited reception. • No known use commercially.	(Bechtold-Peters, Bassarab et al., 3 A.D.; Pikal, Shah et al., 1983; Xiang, Heye et al., 2004)
X-ray	• Expose of the vial to X-rays causes a photographic film to show contrast in the areas where water is localized.	• Non invasive to the vials formulation. • High accuracy.	• Expensive • Placement interferes greatly, in cases vials are removed though an airlock system	• Very limited use.	(schelenz, Engel et al., 1995)
Karl fisher	• Repeat sampling and moisture determination using the Karl Fischer test on a batch of samples can be used to approximate a primary drying end point.	• Can be linked with sampling for secondary drying. • High water content accuracy.	• Sampling of vials • Alteration of sample before analysis. May melt back. • Test is not reusable, as well as requiring high numbers of disposable samples.	• Very low use for determination of primary drying.	(Rey and May, 1999)

Method	Description	Advantages	Disadvantages	Acceptance	References
Impedance analysis	• Involves monitoring the difference in conductivity during drying.	• Measurement during drying • Cost effective	• Still experimental • Sensitivity not known	• Very limited use • Published information suggests impedance is of greater importance than conductivity.	(Rey and May, 1999) (Ward, 2004)
The design of the Primary Dry Switch detailed in section 6.3					
Primary drying switch Prototype V 1 (PDSAIv1)	• Electrically open switch which on removal of ice crystals is allowed to close.	• Proves concept of mechanical closure switch • Non subjective	• Large in size • Unreliable • Difficult to operate with a large amount error caused by the technique. • Only practical use with water or other sublimed solvent		
Primary dying switch Prototype V 2 (PDSAIv2)	• Electrically open switch which on removal of ice crystals closes. Magnetic assistance allows less pure samples to be used. Use at base of vial determines end point.	• Reduced size • Improved reliability • Comparable with interference caused by temperature probe	• Battery failure common • Generation of false positives • Short product life		
Primary drying switch Prototype V 3 (PDSAIv3)	• Electrically open or closed switch with small volumes of water, magnetic closure provides endpoint determination.	• Standardized • Side magnetic triggering reduces interference to product caused by measurement • Possibility to measure large numbers • Good agreement with experimental findings	• Short product life • Generation of false positives		

6.4 Primary Dry Switch (type: AIv1)

6.4.1 Design

The mechanical signalling system chosen for detecting the endpoint of primary drying was a two position switch. Prototype 1, shown in Figure 6-5, displays an initial working design for the switch. In this switch two contacts were separated by a small amount of ice (approximately 10 mg), when the ice sublimed a contact was made completing a circuit. By positioning this system at the centre and base of a vial we could monitor the point at which the last amount of ice would be sublimed.

In this initial very early design, a 9 v open circuit was created with a light emitting diode (LED) in series. A contact within the switch caused completion of the circuit and the LED would illuminate this simple design is shown in Figure 6-6 (page 62).

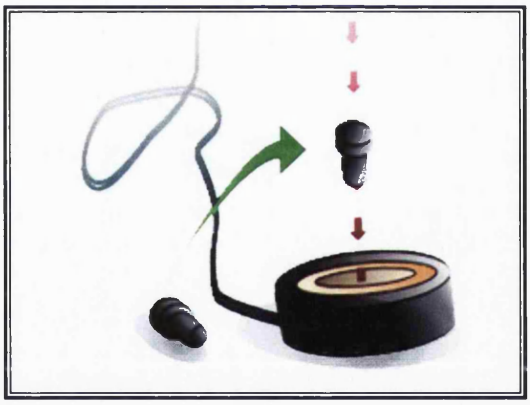


Figure 6-5 : PDSAIv1 – Initial prototype switch showing small T type plunger on the left and switch encased in plastic disc to protect contacts (orange). Red arrows show direction of movement caused by magnetic field.

6.4.2 Method and discussion

Five millilitres of 0.2 μm filtered water was placed in individual vials and the switch positioned at the base of each vial. Freezing to -45 °C was conducted on the freeze dryer shelf and drying initiated at a shelf temperature of -10 °C. Table 6-6 shows the number of successful switch activations for two types of plunger.

Activation of the primary drying switch within the freeze dryer was very low (5 %). A slow increase in contact force between the two circuit portions caused by the slow sublimation of ice, produced contacts that improperly connected. This high resistance junction in the closed circuit > 900 Ω was insufficient to carry current to activate the circuit. The standard method employed in the design of a convention double pole switch to overcome this difficulty is to use a spring loaded system. Pressure greater than a specific force causes the triggering of a far greater force in the switch mechanism. This extra force is normally applied to from above the source of the switches contacts so would be called 'pushing the switch closed' can be imagined as a spring that constantly forces the switch closed.

It was thought that use of a similar design within PDSAIv1 would be inappropriate; the large increase in size caused by adding such a spring loaded system would make the already large switch even larger and impractical for use in a small freeze drying vial. Further investigation of switches revealed commercial micro-switches were not suited; micro-switches of a similar single or double pole design are generally built for high pressure activation (equivalent to the pressure applied by the human finger). Instead PDSAIv1.2.2 used the simple basic design of version 1 with the type 2 steal pin plunger and a ferrite magnet (BHmax. 8 MGOe) positioned at the base of the vial. It then employed a magnet to pull close the switch rather than 'pushing the switch closed' as in a conventional switch.The use of a magnet was thought appropriate because:

- It provided increased force between pole contacts overcoming the switch activation problem.
- It did not act at low field strength (Ken-ichi, Inaba et al., 2002; Shotaro, Ken-ichi et al., 2003) on properties of the sample (in cases where iron content was minimal) preventing changes in the frozen structure of the formulation
- It provided good practical positioning of the switch at the base of the vial during both setup and freezing when ice formation was seen to lift the switch from the base of the vial.

PDSAIv1.2.1, v1.2.3, v1.2.4, v1.2.5 used magnets of ceramic (5 MGOe), AlNiCo (24 MGOe), AlNiCo (27 MGOe), NdFeB (35 MGOe) respectively. Each magnet provided a different degree of activation. NdFeB provided an increase in activation of 46 % compared with the Ferrite versions ($P< 0.05$) although due to its high flux density it was not used further.

6.4.3 Results

The results for successful switch activation are shown in Table 6-6.

Table 6-6 : PDSAIv1 successful activations with differing switch arrangements.

Prototype	Plunger type	Successful Activations (%) (n=13)	Magnet
PDSAIv1.1		15	NA
PDSAIv1.1.1		15	Ferrite
PDSAIv1.2		15	NA
PDSAIv1.2.1		23	Ferrite
PDSAIv1.2.2		23	Ferrite
PDSAIv1.2.3		31	Aluminium, Nickel Cobalt
PDSAIv1.2.4		54*	Aluminium, Nickel Cobalt
PDSAIv1.2.5		69*	Neodymium Iron Boron
* - P<0.05 when compared with <PDSAIv1.*.3			

6.4.4 Conclusions

The use of a mechanical switch within the freeze dryer has been demonstrated. The limited success with a basic switch system where contacts between switch poles was producted by the loss of water on sublimation was overcome with use of a magnetic field. It has been demonstrated that the use of a magnetic field can enhance the contact force for activation of a switch under the conditions required for sublimation.

Practically PDSAIv1 is limited to timing pure water and organic solutions. Although a magnetic flux density could be created at the height of the plug to crush any dried solute

in-between the plug and the switches bottom connector, it would be impractical and potentially a safety hazard. This would also contradict one of the main aims of this study namely to avoid interference with the formulation during drying as any formulation in-between the plug and bottom connector would be significantly altered.

6.5 Primary Dry Switch (type: AIv2)

6.5.1 Design

The second prototype shown in Figure 6-7 (page 63) and Figure 6-9 was based on a miniaturized version of PDSAIv1.2.2. In this version it was possible to rely on the strong flux density of a magnet within the lower portion of a vial to close the switch, use of a heavy plunger was not necessary. Contacts could be held a fixed distance apart by fixing them in glass allowing further miniaturization. On application of a suitable flux density the Fe-contacts would bend and thus touch completing the circuit. This technique was developed in early neural networks where one magnetic field would increase in size triggering a varying number of channels (Murphy and Sayegh, 1995). For the purposes of this study the operating conditions and scale would be significantly different to those employed in neural networks.

An RF Relay (G5Y Omron) uses a magnetic switching system to perform 1 activation every 10 ms and is only 30 mm in length. To be able to perform reliably it is sealed under vacuum with the magnets that cause its activation based on the flux density of an individual batch of magnets and sealed within the same housing. Production to this type of specification can only be achieved for the electronics industry. The tolerances involved in even a high precision freeze-drying vial would mean separation of the field source and the switch contacts, this would be too greter variation to successfully operate this type of switch, even if it could be operated under 300 mTorr vacuum found within most of these switches.

PDSv2 is constructed from a soft glass with two iron containing wires positioned < 1 mm apart (Figure 6-8). Two manufactures (Assemtech and Crydom) were identified producing fixed position wire contacts. Magnets with a Maximum Energy Product

(BHmax) of 10-20 MGOe are all suitable for PDS activation. By positioning the magnet towards the end of one contact the flux was found to act along its length providing sufficient force to close the switch.

To measure the length of primary drying a timing circuit was triggered by relay in a secondary circuit (Figure 6-10).

During initial testing (data not shown) high current had been hypothesised to have a positive effect within the primary (switch) circuit allowing greater chances of successful switch activation. This high current (50 mA) had however been the result of several false positives and a reduction was made with the addition of a 14 Ω resistor which provided a more acceptable compromise.

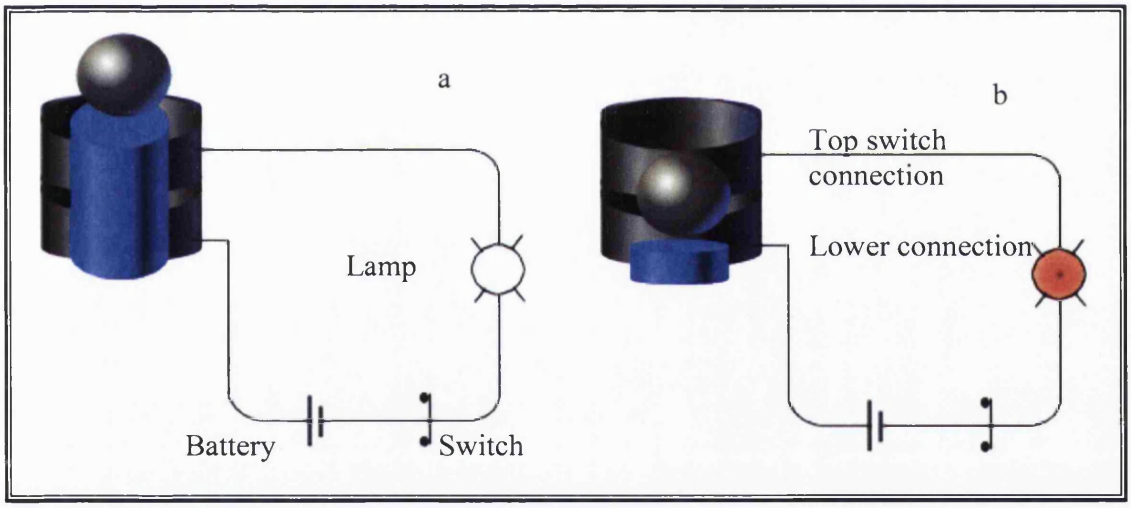


Figure 6-6 : Diagrammatic demonstration of PDSAIv1 mode of action and its associated circuitry. The main components (from left to right are) the Primary drying switch, battery, on/off switch, and lamp. (a) Shows the switch in the loaded state. (b) Shows the switch activated during freeze drying as both connections are joined by the metal sphere.

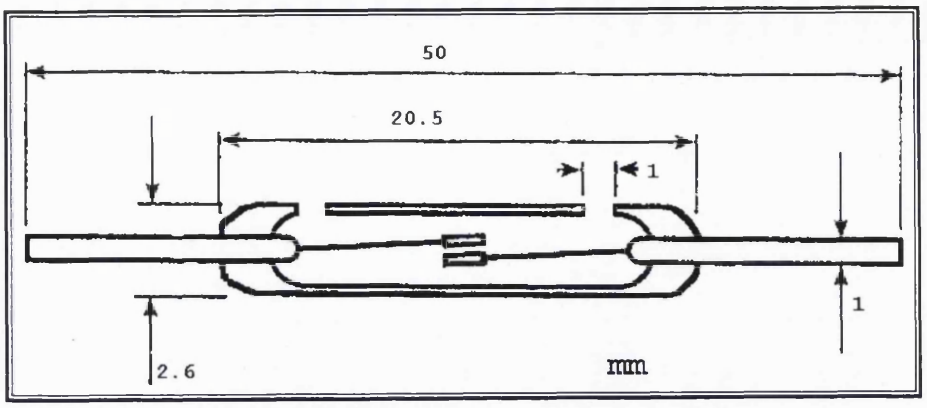


Figure 6-7 : Diagram showing the details for PDSAIv2.2.1 – (dimensions in mm)

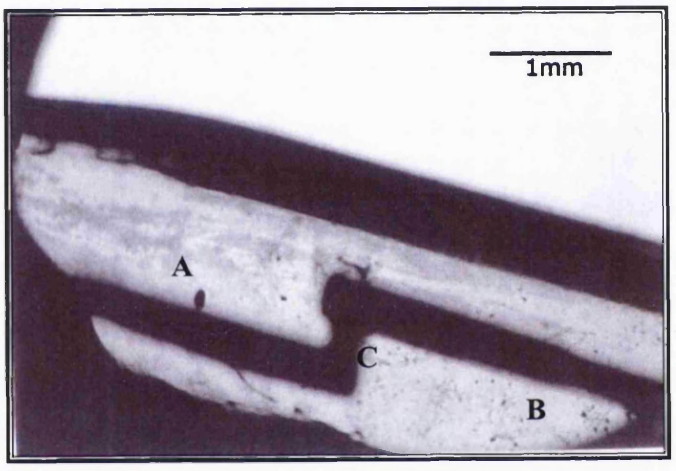


Figure 6-8 : Light microscopy image of PDSAIv2 – shown with side casing removed. Note in this case liquid joins both Fe- Contacts. (A&B) Fe contacts (C) liquid joins contacts.

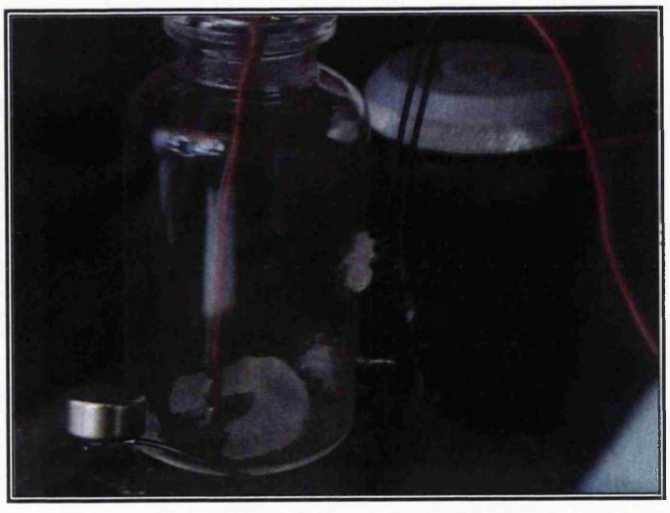


Figure 6-9 : Freeze-drying vial with PDSAIv3.1.1 (Ferrite magnet) and thermocouple inserted. Towards the back of the shelf is a magnetic trigger base (Neodymium Iron Boron).

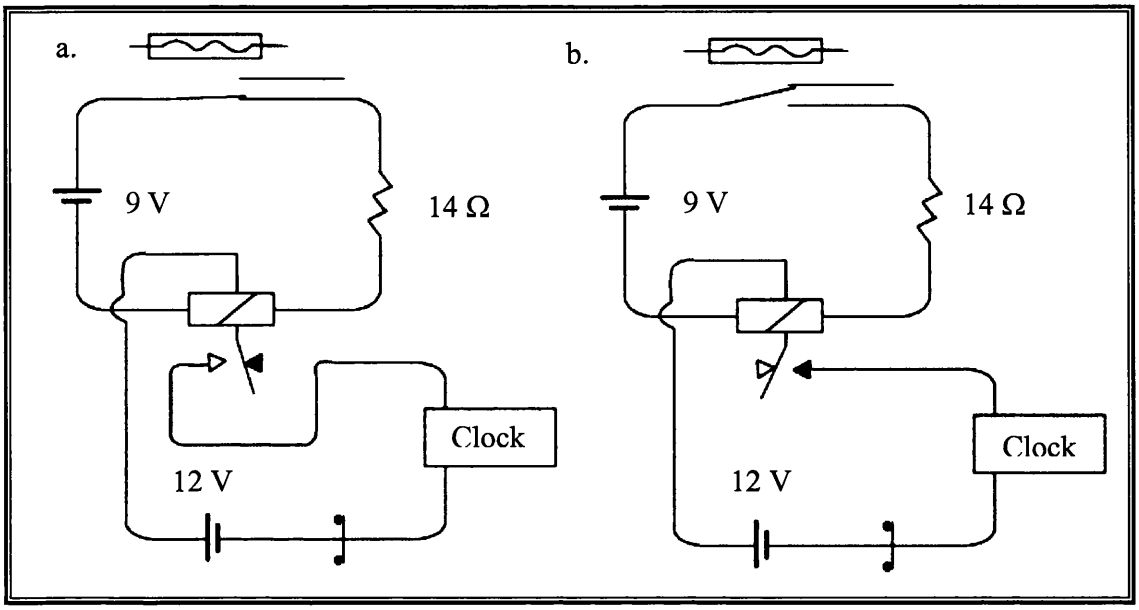


Figure 6-10 : Primary Drying Switch type: AIv2 (a & b) & AIv3 (b) circuit design. Lower circuit is timing circuit triggered by a relay (secondary circuit). The switch circuit (primary circuit) has one of two states (a) "Always on" switch setup. (b) "Switch off clock off" setup.

6.5.2 Method and discussion

The standard general protocol for setup of the PDSAIv2 was followed in each case (Section 5.5). Modifications are as dictated by an individual switch type and the ciruitary in use (Figure 6-10). Initially reliability was the main driver and optimization for the ideal conditions for the switch took place. Initial data (not shown) provided several modifications to the protocol such as suitable aqueous volumes for loading to each switch, cleaning and loading hole diameter (shown maximum recommended in Figure 6-7). Table 6-7 shows data for the number of successful activations based on the sublimation of 2 ml of 0.2 μm filter water from a 3ml vial. Positioning of the magnet flux provided by a ferrite magnet gave a method of increasing correct activation of the switches.

Table 6-7 : Primary Drying Switch V2 lifespan and chance of correct activation.

Supplier part reference	Magnet position	Number of : positives	false positives	fails	(n=)	Repeat activations
Crydom						
CRC200H		4	2	3	10	>15
		2	2	6	10	
DRA283		0	1	2	3	4
		0	0	4	4	
MRA200		5	0	2	8	7
		4	2	3	9	
Assemtech						
RI-46A		7	0	3	10	>40
		8	1	1	10	
		2	2	6	10	
RI-07AA		6	1	3	10	>15
		7	0	3	10	
		0	1	6	7	
0551 form C		0	0	9	9	>15
		2	3	4	9	
		1	2	2	5	

Magnet position key:

	Magnet base was used to trigger the switch with magnet flux directly underneath the vial (Figure 6-9)
	Magnetic flux was provide to the switch poles down the length of the switch (Figure 6-9)
	To provide suitable magnetic flux to cause switch activation a ring magnet surrounding the connecting wire was moved down the wire into an activating position once the formulation had frozen significantly (not shown)

Definitions:

Positives	Those switches illuminating an LED in a simple loop circuit.
False positives	Those switches activating <2 minutes into primary drying.
Fails	Switches not activating or those > 3 standard deviations from the mean.

Repeat Activations	The average life of a switch before it was unable to undergo the testing phase within the setup protocol.

Table 6-8 : Effect of a magnetic base on product temperature

2 %w/v NaCl Formulation. Surface location of vial	Switch within vial	Programmed shelf temperature (°C)	Surface shelf temperature (°C)	Surface temperature of magnetic trigger base (Neodymium Iron Boron) (°C)	Product temperature (°C)	Lag time to reach –30°C (min)
Shelf	No	-40	-40.1 (± 0.4)	-40.3 (± 0.4)	-41.2 (± 0.6)	3
Shelf	Yes	-40	-40.2 (± 0.5)	-40.3 (± 0.8)	-39.9 (± 0.3)	3
Magnetic base.	Yes	-40	-40.3 (± 0.5)	-38 .2 (± 0.6)*	-38.4 (± 0.2)*	6 (± 2)*

* Probability < 0.05 comparable with those of shelf based.

6.5.3 Results

Type AIv2 primary drying switches reduced several distinct disadvantages that were apparent with previous designs. The main advantage was the reduction in physical size of the switch which greatly improved practical placement within a conventional vial.

By precisely loading a formulation to an individual vial (with a switch) it was possible to prevent formulation entry to the switch workings because of the reduced size of the switch workings. This allowed the switch to work without contacts becoming separated by freeze dried solute. Although magnets with large flux densities were investigated to close switches which had been contaminated by dried solute. It was found contamination at the levels required to prevent activation was rare. In the case of switches loaded with solute containing liquid, physical damage to the switch (including permanent magnetization) resulted before magnetic flux strength was great enough to crush the powdered solute after freeze drying. By providing a very small entry to the switch (<1 mm) it was believed possible that the surface viscosity prevented contamination of the switch workings.

Removal of the magnetized base Figure 6-9 (page 63), which consisted of a machined 50 mm diameter aluminium block of 10 mm in height, has prevented the statistically significant differences occurring in product temperature. Additionally, temperature lags caused in the vials of the previous switches (Table 6-8) were also eliminated.

Using the open circuit design (Figure 6-10 (b)) and providing power with a battery, produced false positives and failed to activate the circuit. Battery failure was common, with the secondary circuit never being activated or the circuit being reset before completion of the experiment. The solution was the removal of the battery and replacement with a mains powered transformer.

Using the form C type of switch it was possible to connect a third circuit to produce a signal confirming the proper operation of the unit during all stages of preparation and throughout drying. A form C switch provides connections both in the open and closed position of the switch allowing its current state to be monitored at all times. However because of the poor reliability of this type of switch (Table 6-7 - 0551) and the size increase it was not appropriate for further development of the Primary Drying Switches of v3. It was also noted when form C switches (only one shown Table 6-7) were used in the circuit design A (Figure 6-10), either because of the poor reliability of switches or the ability of ice formation to push contacts apart, secondary circuit activation was exceptionally low. Form C switches due to the use of an closed circuit at all times could; during preparation be seen 'welding' themselves closed as the aqueous surroundings acted as very high resistance. This process appeared to be similar to the reaction at anode or cathode during electrophoresis, with excessive bubbling within the switch.

6.5.4 Conclusions

The use of a small (20mm) semi-sealed mechanical switch can be used repeatedly to detect the loss of water by sublimation from within the unit. Use of a switch in the open position prevented damage to the switch in a liquid environment and prevented switch failures caused during ice formation within the switch. Magnetic flux applied after freezing was able to act along the length of the switch this allowed the the switch to be

positioning directly on the freeze drier shelf without need for a base or any type of support.

6.6 Primary Dry Switch (type: AIv3)

6.6.1 Final Design – Taken forward for experimental measurement

Based on the part RI-46A (Assemtech) with alterations by a specialist small scale glass supplier (PULLERS, UK) a batch of 15 of the V3.1.1 switches were manufactured within the tolerances given in Figure 6-7. It is worthy of note that the lengths of wire used to connect the primary circuit to the secondary circuit are defence standard 61-12 Part 29/1 and as such are capable of operation to −75 °C and in excess of +60 °C (covering the range for most freeze dryers). The primary and secondary circuits in all cases are the type shown in Figure 6-10(b) and timing was provided by a timing mechanism accurate to a single minute. This was the production unit used throughout other studies.

6.7 Predicting the length of primary drying with PDSAIv3

6.7.1 Introduction

Primary drying switches activate when ice separating contacts are removed. As such they can be positioned anywhere within the freeze-drier vial and are not restricted to measuring just the end of primary drying. A switch could for example be positioned at the top of a vial to measure the onset of the drying cycle. In the main part of these studies they are used at the base of the vial to give a reproducible end point without the need to interpretate a graphical plot or other data that could be interpretated subjectively.

Predicting the end point of primary drying using thermocouple data recorded at the base of a freeze dry vial is by far the most common method of predicting the end point of primary drying. Several attempts at reducing the amount of subjective influence that can be applied to this endpoint data have been made. Many of these take the form of mathematical representations of a group of formulations or models for only very low fill depth formulations. A well used model is that of Steinbach (6.7.1.1) which, although

suggesting it predicts primary drying time, is most useful for adjustment of times predicted with thermocouple data.

The reason these models are required is that although the temperature shift of thermocouples should always represent the end point of primary drying, the influence of dried product means they never do. The hypothesis behind the use of thermocouple temperature measurement is that once all water has sublimed, energy from the freeze dryer shelf preferentially heats the thermocouple causing a sudden change in the product temperature. It has been shown that this is not always the case and that energy is often absorbed by the probe tip throughout the final stages of primary drying. The thermocouple rises in temperature in some cases well before the end of drying. Positioning of a thermocouple tip at the centre and base of a freeze dry vial is not simple - during ice formation even the most carefully placed tip can be moved. During experimentation with magnets it was observed tips can be positioned highly accurately, using a suitable magnetic field.

6.7.1.1 Steinbach mathematical model (Osai)

An interpretation of the Steinbach model in (Oetjen, 1999) was used with modifications due to some translation errors and the addition of further work by Oetjen (Oetjen, 1999) to provide mathematical predictions of the end point of primary drying. This has made it possible without using the pressure rise test to calculate all required values from a standard laboratory research scale freeze drier.

Important simplifications to note involved in both the Steinbach and Oetjen models are:

- The solid has no sides and energy can only be lost from its uppermost surface
- Sublimed water must travel through the dried cake
- Frozen water is not porous
- There is no heat conducted to a dried layer

A calculation preformed with the modified Osai model can be seen in Table 6-10 (page 82). The Steinbach model functions by allowing the calculation of heat energy reaching

the sublimation front Ktot (Figure 11 below). Should the energy needed for sublimation be generated within the model, ice is transformed into a gas and the resistance generated by existing dry material is calculated (TermD). The rate of sublimation and any delay required for its vapour to leave the drying substance allows the T_{md} (end point of primary drying) to be manipulated based on properties of the formulation including: the porosity of dried material (TermD), the surface area available for sublimation (TermB), the thermal conduction to the sublimation front (TermC) and the level of solute (TermA).

Figure 11: The Osai equations used in the prediction of primary drying time.

$$K_{tot} = \frac{(m_{ice} LS)}{(T_{md} F)\dfrac{1}{(\mathrm{T_{Sh}} - \mathrm{T}_{ice})}}$$

$$TermA = \frac{Pg(\xi \mathrm{w}\ \Delta \mathrm{m}\ \mathrm{LS}\ \mathrm{d})}{(\mathrm{T_{Sh}} - \mathrm{T}_{ice})}$$

$$TermB = \frac{1}{K_{tot}}$$

$$TermC = \frac{d}{2\lambda g}$$

$$TermD = \frac{d}{(2LS \quad b/\mu)}$$

$$\text{Predicted Tmd} = TermA(TermB \quad TermC \quad TermD)$$

Key:

m_{ice}	Mass of ice
F	Used shelf area
d	Thickness of product area
T_{sh}	Temperature of shelf
T_{ice}	Temperature of ice at sublimation front
$P_{h20,ch}$	Partial vapour pressure in the chamber during primary drying
Ps	Equilibrium vapour prerssure at T_{ice}
T_{md}	Time of main drying
LS	Sublimaion energy of ice
ξw	Part of water in inital product
Pg	Density of frozen product
Δm	Part of freezable water
λg	Heat conductivity in the frozen product
b/μ	Mass transport coefficient
Ktot	Heat transfer cooefficient by conduction and by convection from the shelf to the sublimation front
T_{md}	Length of primary drying

Section 9.1.1 (Appendix, Page 144) presents a computer Perl script to conduct calculations based on a modified approach calculating equilibrium vapour pressures from the temperature of the frozen solid during main drying (T_{ice}) (Oetjen, 1999). The routine is capable of storing data so that individual modifications to parameters can be examined. For example a change in surface area or of product density can be examined by repeating calculations and varying only the individual investigated property. Warnings within the program related to incorrect data entry may help an inexperienced user prevent errors involved with some of the lesser used units in this calculation.

6.7.1.2 Using thermocouple data

Specific knowledge of a formulation's thermocouple data is commonly used in process validation (Bindschaedler, 1999) where the practicalities of a freeze-drier may mean that, even a time minutes (or hours) from the true primary drying end point will have no influency on the cycle development. Movement into the secondary phase of drying must be slow and gradual, any powder with a high degree of moisture content exposed to a high temperature quickly may allow the glass transition (Tg) temperature to be exceeded and as a result a portion of the product may collapse. Transfers to the secondary drying stage correctly will allow products with primary drying still incomplete to finish during the slow temperature rise to secondary drying temperatures.

The graph in Figure 6-12 shows a typical thermocouple spread across a product undergoing lyophilisation (original source data from Bindschaedler (1999)). It should be noticed the standard deviation involved in making a prediction for the end of primary drying (average 13.25 hours) is ±1.1 hours.

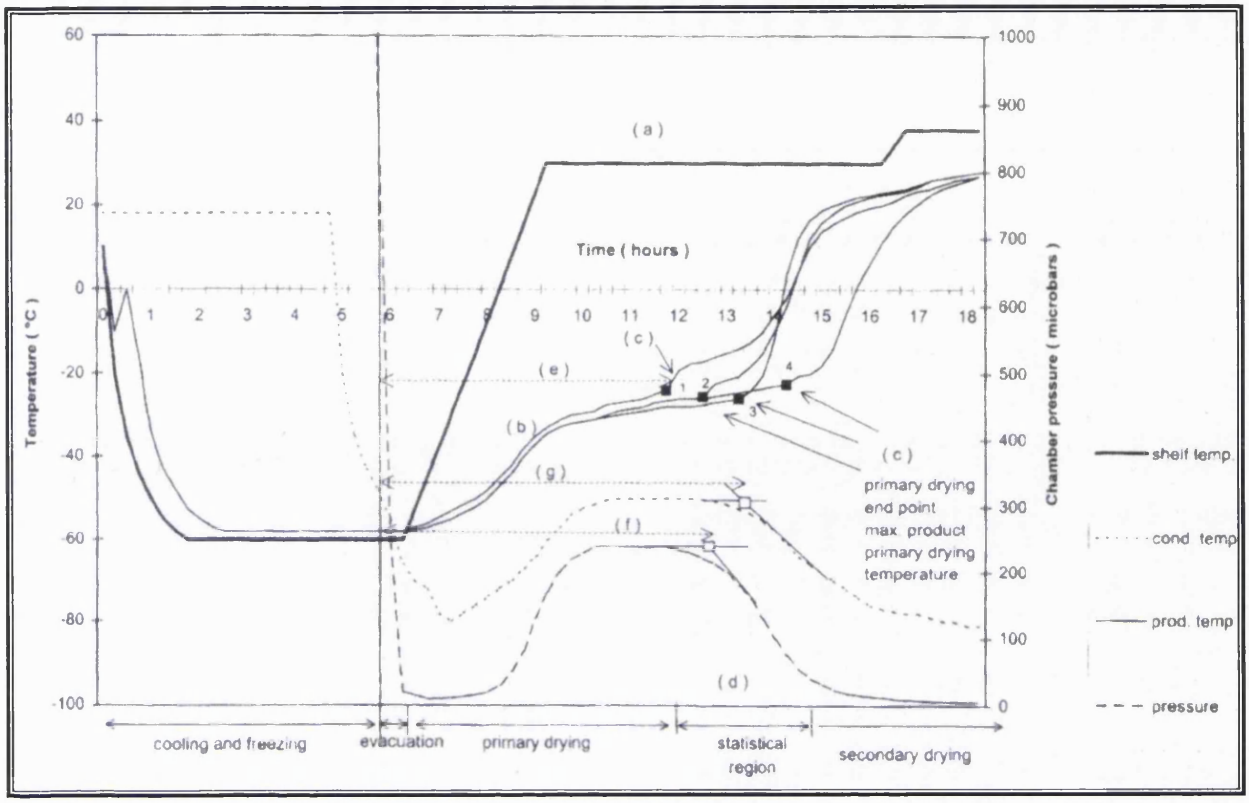


Figure 6-12 : Graph depicting the key end points for primary drying from recorded thermocouple data. The statistical region (end of E to point C) shows the spread of product end points marked 1-4. Image reproduced from Rey and May (1999).

6.7.2 Study aims

The purpose of this part of the study was to demonstrate the ability of V3 PDS to detect end points for primary drying in line with those of existing predictive and direct measurement methods. In doing so it was also hoped we could highlight some of the limitations which exist with current methods relying on product temperature measurements with the thermocouple.

We chose to investigate sucrose due to its wide use as a lyoprotective agent over broad concentration ranges. The levels of sucrose required for protection of varing active components often means by mass it is has the highest concentration within a formulation (Wang, 1999). Knowledge of this rate controlling components affect on primary drying time is of particular interest for future lyoprotection formulation development. The use of

isotonic levels of sucrose (727 mM) provides an indication of cycle lengths for cells and those formulations indicated for IV injection using sucrose as a sole agent to balance isotonicity.

6.7.3 Materials and methods

PDS V3 were used in all cases with a solid ferrite magnet mounted to the side of the switch Figure 6-9. The standard protocol was followed for preparing the switch and loading. Sucrose solutions were prepared to 50, 100, 200, 400 and 727 mM.

6.7.3.1 Freeze drying

Aliquots of 10 ml of either sucrose solution or water were loaded into one of two switch vials. Thermocouples were frozen into the centre of the vial or fixed with thermal putty to the drier shelf near the vial. The drier was programmed with Cycle 6-1 . Before initiation of primary drying magnets were put in place and the primary and secondary circuits for timing connected.

Cycle 6-1: -10 °C constant shelf temperature primary drying. Vials removed under atmospheric conditions at a time point determined by the experiment.

Shelf temperature (°C)	Time (min)	Control condition
≅15	0	Hold
Formulations loaded		
-50	150	Ramp
-50	60	Hold
Primary drying		
-10	2000	Hold

The vacuum is maintained at a constant 80 (± 10) μbar during both primary and secondary drying.

6.7.4 Results and Discussion

Results for switch activation time are presented in Table 6-9 and shown graphically in Figure 6-18 (page 83). Logged freeze dry cycle data for a representative individual cycles are shown for water only (Figure 6-13 page 77) and 50 mM (Figure 6-14 page 77), 200 mM (Figure 6-15 page 78), 400 mM (Figure 6-16 page 78), 727 mM (Figure 6-17 page 79) sucrose solutions.

6.7.4.1 Thermocouple endpoint

Both PDS and thermocouple data show a degree of agreement for distilled water. As the solution has its solute concentration increased, we find the degree of disagreement between the two techniques grows (Table 6-9).

The 200 mM sucrose solution has a primay drying end point (t_{MD}) of 782 minutes (Table 6-9 page 82) the level of disagreement between the thermocouple and PDS becomes greater as the concentration of sucrose in solution is increased. At a sucrose concentration of 727 mM the disagreement between the two techniques is greater than 3 hours. If a prediction of the primary drying end point from thermocouple data for a 727 mM sucrose solution was made from the data presented in figure Figure 6-17 (page 79) it would be incorrect since it would predict a quicker drying time than a solution that was less solute dense (400 mM Sucrose - Figure 6-16). The case of the high sucrose solution drying first would not make logical sense. Indeed the result of samples removed at the predicted end point times (Table 6-9) show that qualitatively samples of 727 mM sucrose are wetter than those at the predicted endpoint for 400 mM sucrose (Table 6-12 page 84). This would indicate primary drying was not complete at these end points, the level of water still present indicates that a significant underestimation of the length of primary drying had been made.

To explain this apparent error involved in the use of thermocouples at high sucrose concentrations it has been hypothesised that the dry sucrose mass influences the subliming ice temperature.

It was also hypothesised that such a result of high sublimation temperature could be caused by the formation of a surface skin in high concentrations of sucrose. However, this is unlikely, as the hypothesis further developed it was realized it would be most likely to cause the opposite of the presented observations i.e. a skin would have the affect of extending the predicted drying time and not reducing it. Although the surface of the 727 mM sucrose formulation appears to form a skin during freezing (Table 6-11 : Appearance of Cycle 6-1 dried cakes) it does not act to increase the rate of drying. Skins

are formed when solute during freezing is concentrated at the surface of a freezing solid. During drying the increased localization of solute at the top of the drying cake means the resistance to vapour flow provided by the top of the dried cake is much greater than the rest of the formulation, hence is termed a surface skin. A surface skin formed in such a way would extend primary drying times to varing degrees but would not have the effect of reduction in drying time certainly to the extent that had been seen for sucrose.

It is hypothesised that the lowering in basline value during primary drying for the 727 mM sucrose concentration is a result of a skin formation and that an alternate event must be responsible for the early onset of temperature rise within the formulation. The thermocouple traces for 727 mM of sucrose are representative of such a skin formation. Initially high product temperatures are seen during primary drying. Energy is building in the cake and not being used to sublime water. Eventually such a case would cause collapse of the cake, but in this case the skin must be broken (Figure 5-3 vial image No9. Page 38). Product temperatures after the breaking of a skin return to a lower value suggesting sublimation is continuing and that it requires less units of energy to convert the same amount of water (assuming the rate limiting stage is always the sublimation rate of the ice).

Having described a possible explanation for the initial product temperature changes in Figure 6-17 for 727 mM sucrose the explanation for the early onset of the primary drying end point can be related to the increase in solute concentration. One of the important assumptions of the Osai equation is that there is no heat conduction to the dry layer. This is also applicable to the interpretation of all thermocouple data. It is hypothesized that in the case of sucrose as the concentration is increased the conduction of energy to the dried layer is increased too; this is due to the increase in heat capacity of the dried cake (when compared with lower concentration dry cakes). The temperature of the combined dried cake and frozen product increases, signifing the end of primary drying artificially early. Once the thermal energy stored in a dried cake reaches its maximum which would occur almost instantaneously as it is passed by the sublimation front, only the rate of sublimation could remove this energy from the system. Since sublimation is at its

maximum at all points this thermal energy is constantly held in the dried cake. At the point when the ice block has less thermal store than the dried cake, energy is transferred from the cake to the ice. This gives the ice two sources of heat energy; the shelf, and the dried product, the thermal energy stored within its solid dry structure reaches a maximum and begins to feed back to the ice block translating the product temperature suddenly upwards and causing the characteristic end of primary drying sudden temperature increase. The bulk water is still present but now has an increased temperature. Should this temperature approach collapse, the products final physical appearance would be altered.

In a less concentrated solution the point when the dried cake reaches its maximum store of thermal energy is still the same but its effect to a sudden change in temperature is far less, since it will be able to have less impact on the product temperature. This resistance of a solute to release energy would be magnified in high fill depth vials with high solute density like those of the sucrose used in this study.

Examining Figure 6-13, Figure 6-15 and Figure 6-16 (pages 77- 78) for changes in thermocouple endpoint may suggest this effect in even the lowest concentration solutions. In several cases two portions to a thermocouples data trace rise can be made out. If we take the 200 mM sucrose trace (Figure 6-15) as an example an initial small rise (550 min) leading to a sharper rise (620 min) followed by a secondary much broader rise (620 min to 1000 min) can be seen. It is this first portion (small rise) where end points are predicted by both the primary dry switch and the Osai equation for low concentration solutions while at higher concentrations only the thermocouple data suggests this is the primary drying end point. This would suggest the cakes must be affected by the heat effects of the dry layer. It is clear that cakes removed at the point of a very sharp temperature rise will often melt back demonstrating that a high degree of bulk water must still be present (Table 6-12 page 84). The true end point of primary drying must be completed at some point after this sharp rise.

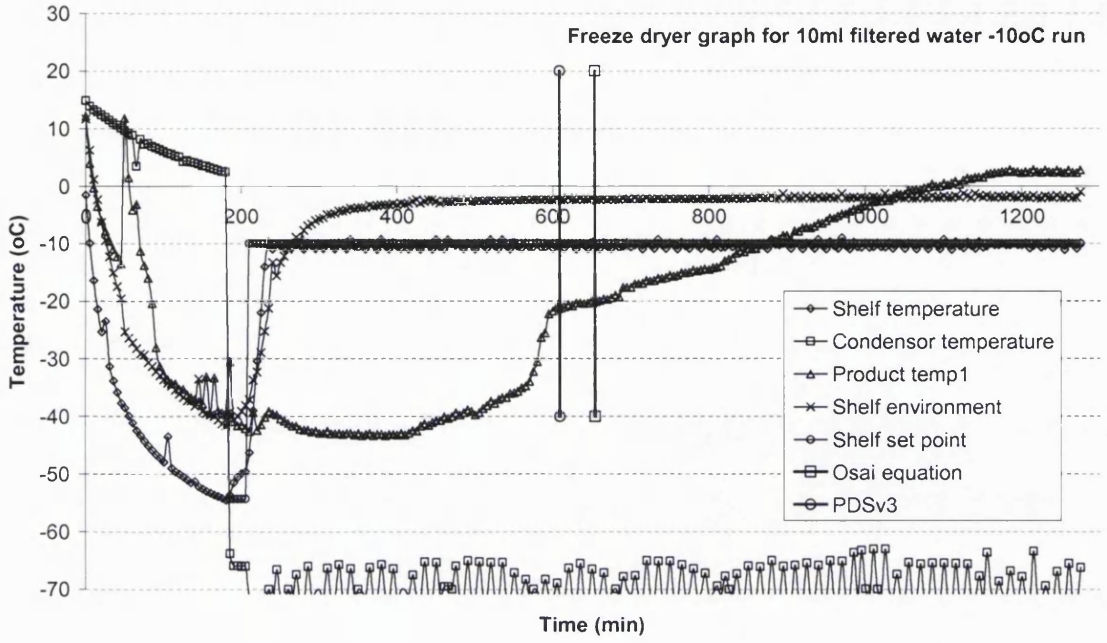


Figure 6-13 : Graphical display of logged data for 10 ml of 0.2 μm filtered water freeze-dried using Cycle 6-1: -10 °C constant shelf temperature primary drying. Vials removed under atmospheric conditions at a time point determined by the experiment. Temperature probes monitor both product temperature and the surface conditions of the shelf. The result of PDSv3 triggering is shown for the probed vial as is the predicted value for primary drying using the Osai equation based on the average of 4 vials.

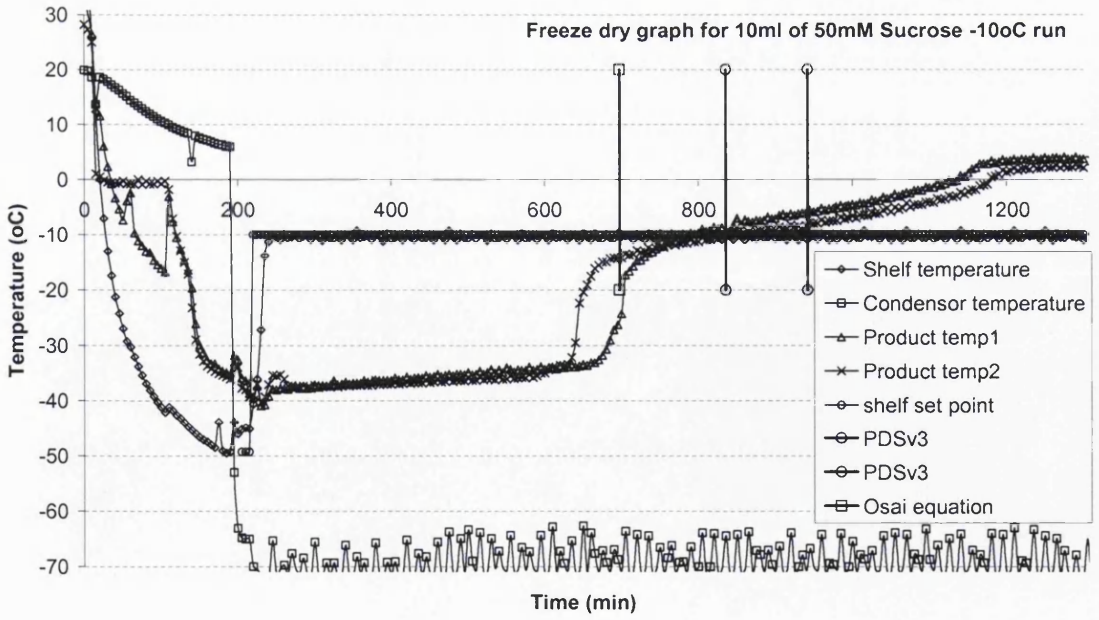


Figure 6-14 : Graphical display of logged data for 10 ml of 50 mM sucrose freeze-dried using Cycle 6-1: -10 °C constant shelf temperature primary drying. Vials removed under atmospheric conditions at a time point determined by the experiment. Temperature probes are monitoring product temperature only and are positioned in separate identical vials. The result of PDSv3 triggering is shown for the probed vials as is the predicted values for primary drying using the Osai equation based on the average of 4 vials.

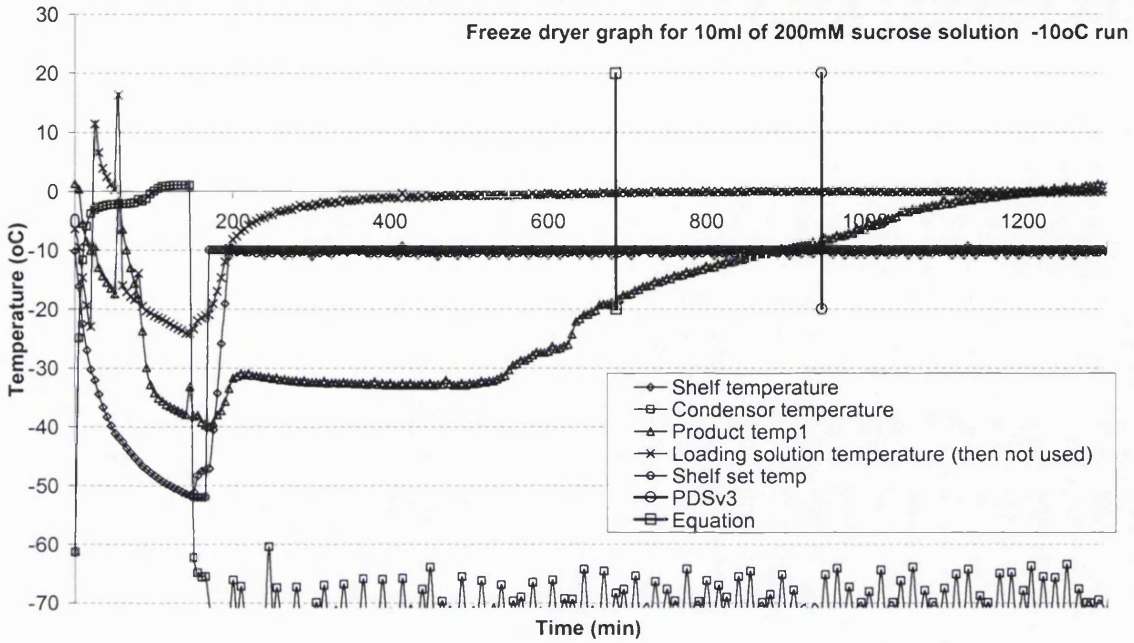


Figure 6-15 : Graphical display of logged data for 10 ml of 200 mM sucrose solution freeze-dried using Cycle 6-1: -10 °C constant shelf temperature primary drying. Vials removed under atmospheric conditions at a time point determined by the experiment. Temperature probes are monitoring both product temperature and the surface conditions of the shelf. The result of PDSv3 triggering is shown for the probed vial as is the predicted value for primary drying using the Osai equation based on the average of 4 vials.

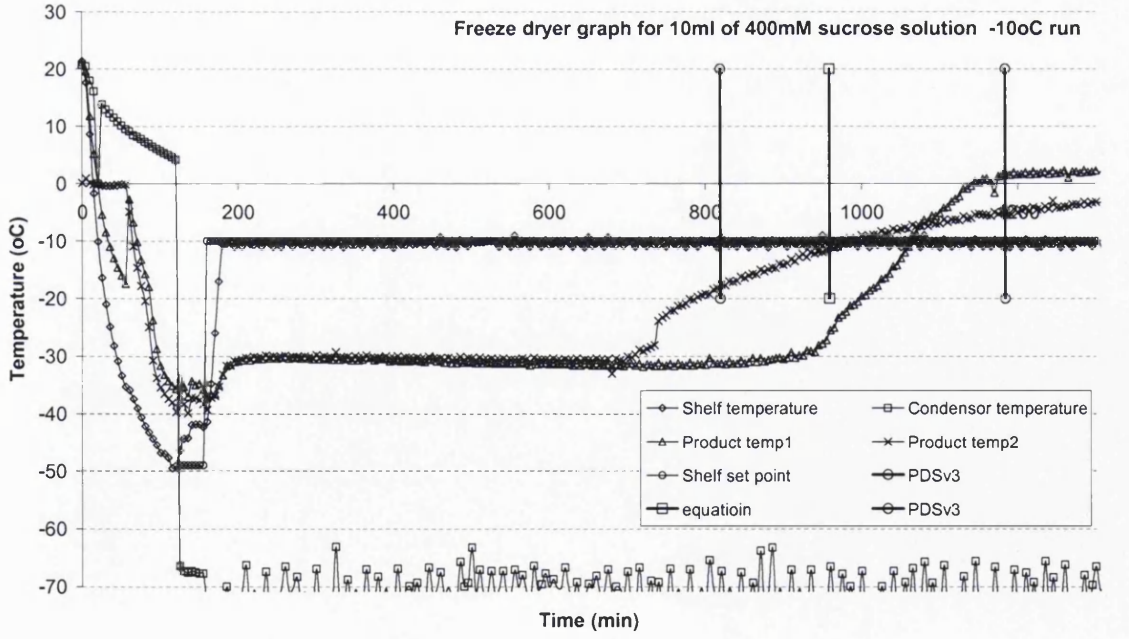


Figure 6-16 : Graphical display of logged data for 10 ml of 400 mM sucrose solution freeze-dried using Cycle 6-1: -10 °C constant shelf temperature primary drying. Vials removed under atmospheric conditions at a time point determined by the experiment. Temperature probes are monitoring product temperature only and are positioned in separate identical vials. The result of PDSv3 triggering is shown for the probed vials as is the predicted values for primary drying using the Osai equation based on the average of 4 vials.

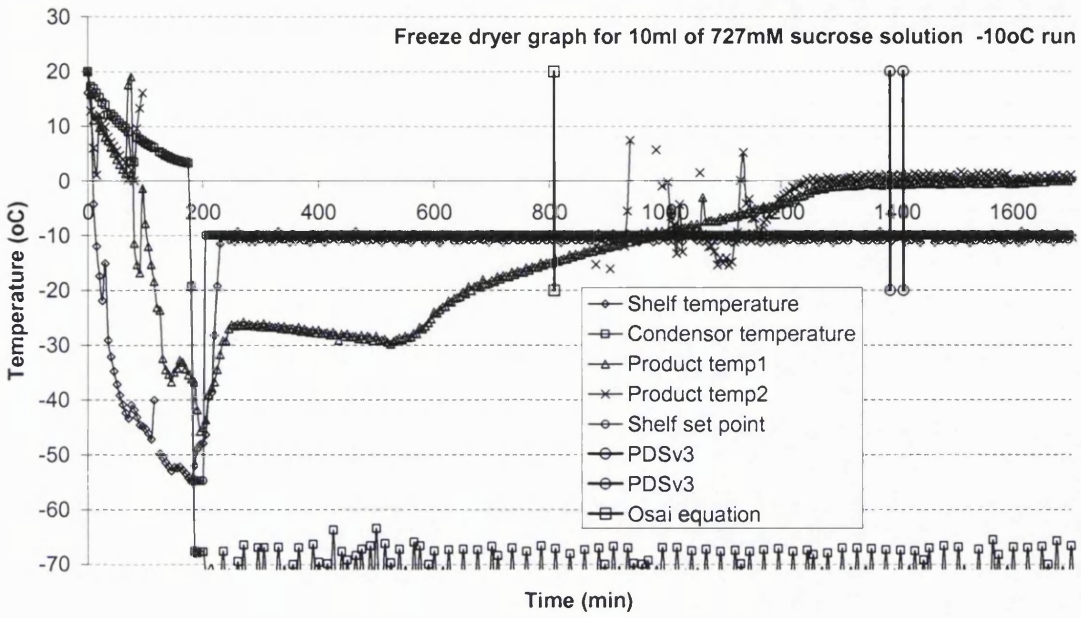


Figure 6-17 : Graphical display of logged data for 10 ml of 727 mM sucrose solution freeze-dried using Cycle 6-1: -10 °C constant shelf temperature primary drying. Vials removed under atmospheric conditions at a time point determined by the experiment. Temperature probes are monitoring product temperature only and are positioned in separate identical vials. Product temp2 in this case is only partially recorded. The result of PDSv3 triggering is shown for the probed vials as is the predicted values for primary drying using the Osai equation based on the average of 4 vials.

6.7.4.2 Osai equation

The modified version of the Steinbach equation developed by Oetjen uses thermocouple data strongly to predict its primary drying endpoint. The measurement of the Osai T_{md} value is based on the 5 % fluctuation from baseline of product temperatures. T_{md} points generated in this way are more reproducible but, as discussed, not always strongly related to the true primary drying end point. The strong reliance of the equation on T_{md} values predicted from temperature probes means errors produced here can be transferred to the predicted value very easily. Figure 6-18 demonstrates this strong reliance clearly. At low sucrose concentrations the Osai equation predicts times less than that predicted by the conventional thermocouple data (Table 6-9 : Predicted drying times for sucrose solutions using PDS, thermocouples & the Osai equation). As sucrose concentration is increased this difference becomes less until in the final sucrose concentration the Osai equation predicts times longer than the thermocouple data. This would follow the theory of high

solute concentration causing thermocouple bias described (section 6.7.4.1 page 74) and would demonstrate the use of the Osai model to correct errors generated in this manor.

6.7.4.3 Primary Dry Switch Endpoints

A primary drying switch has distinct advantages over thermocouple data and therefore the Osai equation, a primary drying switch is not affected by solute concentration in any way. From a practical point of view high solute concentration actually assists in loading product because solutions with higher viscosity are generally less likely to penetrate the switch workings and cause switch failure.

It can be seen that for sucrose the near linear relationship created by use of a primary dry switch is more significant than that produced with information gained from the thermocouples (Figure 6-18). We have shown at high sugar concentrations (400 mM – 727 mM) thermocouple data becomes unreliable as there is a greater degree of subjective interpretation. The use of a calculation to correct such data is possible but even with well characterized formulations it will only result in minor alterations.

Use of predictive equations is limited in many cases by the assumptions involved (6.7.1.1) which is linked largely to the number of uncontrollable factors involved in loading a freeze-dryer's shelf. Published predictive methods appear successful only on the most well characterized formulations and with very low fill depths (1-5 mm) (Schoen, Braxton et al., 1995; Oetjen, 1999; Zhai, Taylor et al., 2003). Even when a calculation method has proved successful in many cases, formulations presented for freeze-drying are not well characterized and a lyophilisation scientist would still find theselves reliant on feed back data gained from optimization cycles.

Physical examination of vials removed at the predicted primary drying time end points (Table 6-12) demonstrate that at points when the switch predicts primary drying to be over no bulk water is visible. It is possible that this is an over estimate of the primary drying times, specifically for low concentrations of sucrose. This is demonstrated when

vials without any bulk water (without melt back) can be detected at earlier time points (PDSv3, 657 min 885 min Table 6-12).

Thermocouple predictions (Table 6-12) perform significantly worse with melt back being common at most of the predicted primary drying time points. This indicates that primary drying is not complete when predicted with this method.

6.7.5 Conclusions

Chapter 6 has described the development of an alternative method for the detection of primary drying end points. Primary drying times of solutions with high fill depth and high product density can be measured with greater accuracy than those of the conventional thermocouple method.

Some of the limitations of the modified Steinbach Oetjen equation have been outlined but it is also clear that use of such an equation to modulate thermocouple data gives more accurate time predictions than thermocouple alone.

The use of a primary dying switch within a sucrose solution produces a linear relationship between concentration increase and primary drying time over a wide concentration range 0 – 727 mM.

6.7.6 Additional work

Further chapters in this thesis discuss the practical uses of the PDS and show some of its potential applications. In many cases it is synergistic to use both the switch and thermocouple data together.

The use of a porous plastic seal to the switch is a target for future investigation. Although the current switch is disposable reuse is prefereable because of its cost. Changes to the location of electronic connections could reduce this cost further. Although the case of the switch has minimal effects on freezing (Table 6-8) and drying of a formulation it is envisaged that its effects could be minimized further by reduction in its overall size to minimize its nucleation inducing effects.

Table 6-9 : Predicted drying times for sucrose solutions using PDS, thermocouples & the Osai equation

Formulation	Primary drying time			
	PDSv3 (min) (n= 4)	Thermocouple (min) (n= 4)	Steinbach and Oetjen equation (Osai)(min)	Difference (min) (Osai - Thermocouple)
Distilled water	467 (± 61)	369 (± 18)	434.04	65.4
Sucrose 50 mM (1.7 %w/v)	690 (± 55)	480 (± 28)	502.44	22.4
Sucrose 200 mM (6.9 %w/v)	782 (± 56)	367 (± 40)	515.82	148.8
Sucrose 400 mM (13.7 %w/v)	949 (± 219)	645 (± 97)	799.20	154.2
Sucrose 727 mM (24.9 %w/v)	1207 (± 16)	409 (± 39)	603.30	194.3

Table 6-10 : Values used in the prediction of drying time using the Osai equation (Table 6-9 : Predicted drying times for sucrose solutions using PDS, thermocouples & the Osai equation)

Term		Formulation				
Value	Units	Distilled water	Sucrose 50 mM	Sucrose 200 mM	Sucrose 400 mM	Sucrose 727 mM
m_{ice}	kg	0.01005	0.01005	0.01005	0.01005	0.01005
F	$X10^{-4}$ m^2	5.02725	5.02725	5.02725	5.02725	5.02725
d	m	0.02	0.02	0.02	0.02	0.02
T_{sh}	°C	-10	-10	-10	-10	-10
T_{ice}	°C	-43.2	-36.3	-32.9	-30.6	-27.2
$P_{h20,ch}$	mbar	0.118813	0.118813	0.118813	0.118813	0.118813
P_s	mbar	0.0898	0.1937	0.2764	0.3515	0.5017
t_{MD}	h	5.816667	6.583333	6.45	11.5	6.65
LS	kj/kg	2805	2805	2805	2805	2805
ξ_w	kg/kg	1	0.982885	0.93154	0.86308	0.751148
ρ_g *	kg/m^3	975.35	982.896	1006.599	1039.857	1089.087
Δm		0.95	0.95	0.95	0.95	0.95
λ_g **	kj/m h °C	8.481	8.4672191	7.768516	6.836913	5.313741
b/μ	kg/m h mbar	-1.185	0.406	0.197	0.074	0.079
K_{tot}	$KJ/m^2h°C$	290.37	323.87	379.64	236.70	490.25
term A		1565.70	1957.68	2182.27	2321.90	2534.81
term B		0.0034	0.0031	0.0026	0.0042	0.0020
term C		0.0011	0.0012	0.0013	0.0015	0.0018
term D		-3.0075	8.79x10-6	1.81x10-5	4.77x10-5	4.54x10-5
t_{MD}	h	7.234	8.374	8.597	13.32	10.055

* (@-35°C) extrapolation based on (Bubnik, Kadlek et al., 1995)

** extrapolated based on (Saad and Scott, 1996)

Continued over

Key:

m_{ice}	Mass of ice
F	Used shelf area
d	Thickness of product area
T_{sh}	Temperature of shelf
T_{ice}	Temperature of ice at sublimation front
$P_{h20,ch}$	Partial vapour pressure in the chamber during primary drying
Ps	Equilibrium vapour prerssure at T_{ice}
T_{md}	Time of main drying
LS	Sublimaion energy of ice
ξw	Part of water in inital product
Pg	Density of frozen product
Δm	Part of freezable water
λg	Heat conductivity in the frozen product
b/μ	Mass transport coefficient
Ktot	Heat transfer cooefficient by conduction and by convection from the shelf to the sublimation front
T_{md}	Length of primary drying

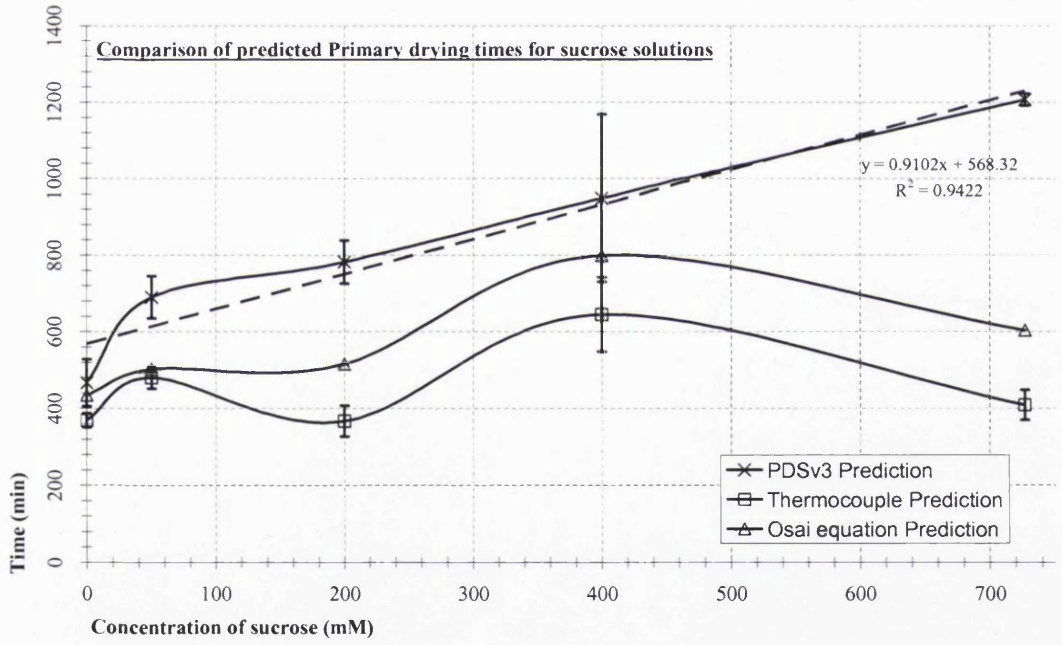


Figure 6-18 : Comparison of the predicted drying times using PDSv3, thermocouple data and the Osai model for a range of sucrose concentrations.

Table 6-11 : Appearance of Cycle 6-1 dried cakes with a total drying time of 1397 minutes.

Formulation	Visual cake appearance
Distilled (0.2 μm filtered) water	No cake
50 mM sucrose	Good cake
100 mM sucrose	Good cake
200 mM sucrose	Slight splitting, no shrinking
400 mM sucrose	Good cake
727 mM sucrose	Surface skin, slight splitting, no shrinking

Table 6-12 : The degree of melt back for a vial (2.5Ø) of different concentrations of sucrose solutions with 10ml fill depth, removed from the drying Cycle 6-1.

Time (min) until removal from cycle	Degree of melt back apparent in the vial after 10 minutes at room temperature.				
	Distilled water	Sucrose 50 mM	Sucrose 200 mM	Sucrose 400 mM	Sucrose 727 mM
PDSv3					
657	*	*	++	+++	+++
885	+	*	*	+	+++
952	*	*	*	++	+++
1139	*	*	*	*	+++
1397	*	*	*	*	*
Thermocouple					
559	*	*	+++	+++	+++
650	*	*	+++	+++	+++
557	*	*	+++	+++	+++
835	*	*	*	++	+++
599	*	*	+++	+++	+++
Osai					
434	*	+	+++	-	++++
502	*	*	+++	-	+++
515	*	*	++	+++	+++
799	*	*	*	++	+++
603	*	*	++	+++	+++

Key:
* = no significant melt back
+ = cake pinch from the wall of the vial
++ = Pinch of the cake wall at base on vial (also characteristics of +)
+++ = Water apparent in vial dissolving partially or completely freeze dried cake (also characteristics of ++)
++++ = approximately 50% or greater of water remaining (also characteristics of +++)

7 Predicting protein freeze-drying properties

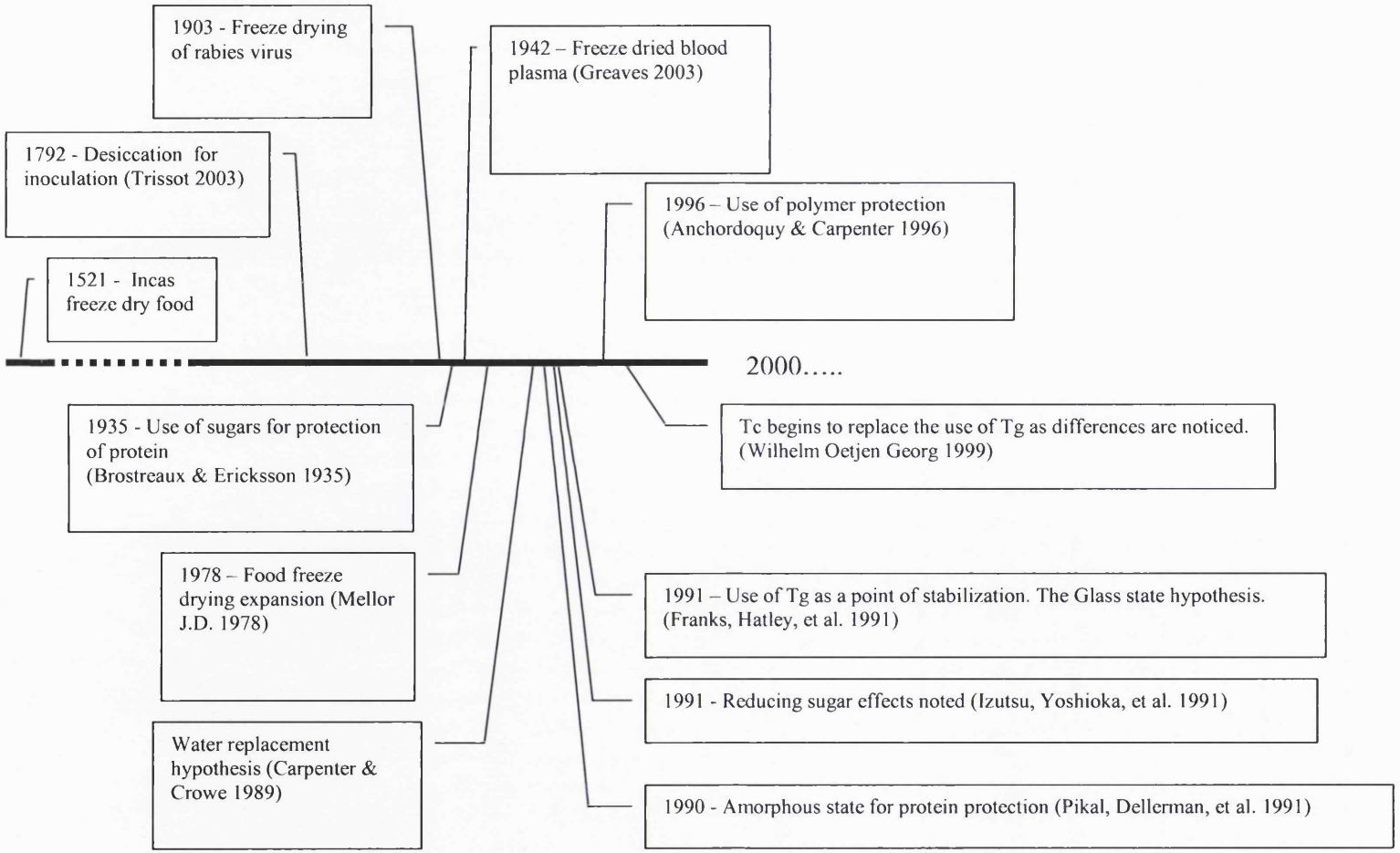
7.1 Background

Freeze-drying is slowly becoming the long term storage technique for a new market of biopharmaceutical drugs, these drugs are therapeutic proteins with precisely controlled purity and activity. The previous main drive for freeze drying had been the small molecules and organic drug compounds but these are less efficiently stored as lyophilized powders. Techniques such as spray drying are becoming increasingly more popular, allowing product production with greater cost effectiveness and freedom from being tied to a batch production process. Spray drying ends with essentially the same product as freeze drying which in most cases is an amorphous powder; it is seen as a major competitor to freeze drying and is definitely reducing the market share of products that would have been freeze dried in the past. Such products have included milk and high value fruit flavouring slurries from strawberries and blackberries.

Freeze-drying proteins is not novel in anyway; the first use of freeze drying recorded was by the Incas in the High Andes who dried fish and other foods. But the level of attention to the recovery of specific proteins has become heightened in the past 20 years. Knowledge of pharmaceutical protein therapeutics requires specific international units (IU) of activity after a cycle and in almost all cases prolonging of any storage shelf life.

The development of protein lyophilisation has taken the path illustrated in Figure 7-19. Knowledge related to proteins is gained on a case by case basis and often comparison of the individual protein experiments is complicated; involving large numbers of variables. An investigator is left to question a specific cycle condition or particular thermal conductivity parameter such as glassware or sample volume. This has not stopped lyophilisation science for individual enzymes evolving and models such as lysozyme and catalase are now well characterized (Figure 7-33, page 110) due largely to this approach.

Figure 7-19: The evolution of protein freeze drying.



In this chapter the investigations are centred on freeze drying from the point of view of the proteins and not the freeze drying cycle. Differences in protective agents are continuously investigated (Wang, 1999) but one question remains: how much information can be gained from the structure of a protein itself? Clear structural differences (Figure 7-33, Figure 7-23) are apparent, but how can we apply these to lyophilization protection of proteins? It would be hoped that information generated in this manner could then allow the design of freeze drying cycles where parameters influence the formulation in a known manner without the need to speculate as to the freeze drying cycle design or indeed alter the formulation to fit a specified cycle.

7.2 Protein temperatures of denaturation (T_d)

The T_d is the point at which half the protein molecules in a solution are unfolded and half are still in their native folded state (Kang, Jiang et al., 2002). When a sugar is added to a protein the temperature of denaturation is seen to rise, this has been related to maintenance in the activity of lysozyme (McGarvey, 2003). It is generally accepted that an increase in the conformational strength of a protein's tertiary structure will increase thermal stability (Hageman, 1992; Jiang, Woo et al., 2002). Sucrose, trehalose and mannitol can all be seen to have a stabilizing effect in this manner (McGarvey, 2003).

Detection of T_d by DSC is only possible for enzymes with low sugar content (<100mg/ml): larger sugar contents mask the T_d with the exothermic recrystallisation of the sugar (McGarvey, 2003). It is these larger concentrations that are however so often of practical use in protein protection during lyophilisation (McGarvey, 2003; Ibanoglu, 2005).

7.2.1 Protein protection during dehydration

It is accepted that an amorphous excipient is benifical in protecting proteins during dehydration (Topp, 2004; Chang, 2004). The exact method of protection is uncertain. It is important to realize protection during a complete freeze drying cycle includes freezing and lyophilisation stresses. In many cases the formulation is then rehydrated which induces its own range of often forgotten stresses.

7.2.1.1 Amorphous glass formation

Mechanical immobilization in an amorphous glass solid state during freezing is thought to prevent protein unfolding and spatial separation of protein components (Franks, Hatley et al., 1991). Although protein alone would form such a glass state on its own it is suggested that the protein must be locked in this glass state in a disperse nature. Protein bulking agents such as bovine serum albumin (BSA) have demonstrated this property (Anchordoquy and Carpenter, 1996). It is the use of a protective agent such as sucrose which in most cases is suggested to provide this dilution of the protein while still maintaining the glass state.

7.2.1.2 The water replacement hypothesis

Although it is suggested to be a separate mechanism, similarities and overlapping between the glass state theory and water replacement hypothesis are clear. The water replacement hypothesis suggests protein - water hydrogen bonds are replaced within the glass state by those that can be formed with a lyoprotective agent.

In lysozyme, carboxylate hydrogen bonds were seen to reduce after freeze drying when not in the presence of an agent to replace them (Carpenter and Crowe, 1989).The use of sucrose to replace such hydrogen bonds was later demonstrated in α- lactalbumin (Prestrelski, Arakawa et al., 1993).

7.3 Amino acid frequency study

In 1999 Carpenter and his colleagues Rondolph and Izutsu commented that "it's not clear as to why the efficacy of general protein stabilizers often varies depending on the protein being studied". In this study we hoped to discover if amino acid content or sequence would affect the hypothesised stability inferred from the temperature of recrystallisation, and therefore infer something about how the protein effects its own protection with the sugar sucrose.

7.3.1 Method introduction

The T_d of a protein has been linked with structural strength and activity after freeze drying (section 7.2); alterations of T_d by lyoprotective agents can be hypothesized to be linked to the proteins likely activity after a *suitable* freeze-drying cycle (7.3.1.2).

A 500mM concentration of sucrose is required in many cases for successful protection of a protein formulation (Kerwin, 2004). The recrystallisation of sucrose obscures direct measurement of T_d; however the effect on the end point of recrystallisation can be monitored and related to T_d (McGarvey, 2003).

Changes in the end point of recrystallisation were measured for 8 different enzymes (detailed in Table 7-13) at variable concentrations (7.3.1.1). Since protein concentration was determined by mass before freeze-drying it was possible to relate the total sequence content with the effect on recrystallisation of a known amount of sucrose.

Once differences in a protein's sequence were tabulated they could be transformed and contrasted with recrystallisation data. The large amount of data generated at this point meant this must be done in an automated manner because a single protein could produce more than 100,000 points for data contrast.

Because it is unlikely any relationship is related to the frequency of a single amino acid, both amino acid type and sequence groups are covered by the software. Secondary and tertiary structure are outside the bounds of this software; although they are important considerations for protein science in general, in-depth computations of these characteristics will not be considered.

7.3.1.1 Protein preparation

Proteins were selected on the basis of existing literature with respect to freeze-drying. Initial comparisons were made based on size and enzyme similarity to exclude enzymes with a very similar structure or sequence dependence. A summary of structural similarity is given in Figure 7-33 (page 110) and details of supplier in Table 7-13.

Dialysis of all enzymes was performed against water with a 3500 molecular weight cut-off membrane (Visking, UK) to remove excess salts, then protein quantity confirmed using bicinchoninic acid (BCA) determination (5.8 & 5.9 page 47). Loss during this process could be as high as 65 % by activity. BCA of the dialysis membrane showed levels equivalent to 60 % of these proteins being lost to the membrane or container, thus indicating a large amount of absorption possibly due to the pH -7 conditions during this dialysis.

Table 7-13 : Supply details for enzymes

Enzyme name	Details of origin.	Supplier	EC (number if available).	Lot:
Catalase	From human erythrocytes	SIGMA UK.	1.11.1.6	60628-
Lactate dehydrogenase	From *Bacillus strearothermo*	SIGMA UK.	1.1.1.27	59023-
Lysozyme	From chicken egg white	SIGMA UK.	3.2.1.17	L4631-084k6111
Asparaginase		SIGMA UK.	3.5.1.1	A4887-113k0948
Adenosine deaminase	From bovine spleen	SIGMA UK.	3.5.4.4	A5043-015k7018
β- Galactosidase	From *Escherichia coli*	SIGMA UK.	3.2.1.23	G6017-113k6049
Fructose -6- phosphate kinase(Phosphofructokinase)	From *bacillus searothermophilus*	SIGMA UK.	2.7.1.11	F0137-054k1477
β- Amylase	From Barley	SIGMA UK.	3.2.1.2	A2771-034k0645
Glucose oxidase	From *Asperigillus niger*	SIGMA UK.	1.1.3.4	G7146-074k6036
Ascorbate oxidase	From *cucurbita sp*	SIGMA UK	1.10.3.3	I1136-044k4789
Ribonuclease	From *aspergillus clavatus*	SIGMA UK	3.1.27.5	R6398-113k1345

7.3.1.2 Freeze drying cycle design

Each of the 4 differing enzyme concentrations (78, 156, 312 and 625 μg/ml) were freeze dried with 500mM Sucrose in the manner detailed in Cycle 7-2 and Cycle 7-3 (page 113)

as appropriate. To provide the best case freeze drying cycle, high concentration enzyme solutions were measured for their Tc using the Freeze drying microscope (Lyostat 1). The effect of enzyme concentration on the collapse point of sucrose was determined as -32.0 °C and was insignificant in respect to shelf conditions at concentrations <2500 μg/ml (results not shown). Primary drying was conducted to allow product temperatures below the lowest measured Tc, *i.e.* amylase Tc = -38 °C. PDSv3 (section 6.6) were also used in 5 cases to correct the primary drying length which as this cycle was not needed for efficiency was given 10 % additional time to make sure of complete primary drying in all cases. Cycle alterations were conducted for secondary drying until moisture levels (determined with Karl Fischer) of 1.8 % (±0.5) mean average (by mass of dry powder) were produced for all samples.

7.3.1.3 Differential Scanning Calorimetery

Measurement of recrystallisation exotherms was conducted in the Faculty of education laboratories of Chiba University Japan, under the supervision of Professor Inaba. On an experimental DSC model 8230 Rigaku Themro Plus. The method used was that of (Imamura, Suruki et al., 1998). Sample preparation is that detailed in 5.3.2 DSC Experimental preparation for analysis (page 43).

7.3.1.4 Software design

The software element of this study consisted of three distinct programs developed with the following aims:

- Produce all possible protein sequences to be checked. (EAASC - 7.3.1.4.1).
- Using a sequence, check individual proteins for the frequency with which the sequence appeared (ESFC - 7.3.1.4.2).
- Correlate the frequency of a sequence in several different proteins with its effect on the end point of re-crystallization (ESA - 7.3.1.4.3).

All the programs were developed for the Perl (v5.0) interpreter as this programming language was developed with string handling at its forefront and it allows for a reduced coding time and a reduced number of routines.

7.3.1.4.1 EAASC – e. Amino Acid Sequence Creator

The 'extra'amino acid sequence creator (EAASC) uses an attic counting algorithm for the appropriate number of amino acids (9 amino acids would be counted in the 9^{th} attic number set). All possible combinations of the IUB/ IUPAC code can be produced as well as several other wild card letters or group specific letters (covered at the interpretation phase 7.3.1.4.2). Importantly it is case sensitive. The full code is given in the appendix 9.2.1 page 152.

7.3.1.4.2 ESFC – e. Sequence frequency counter

The 'extra'sequence frequency counter (ESFC) is capable of stripping raw FASTA formated protein sequence files extracting only the sequence (shown for Ribonuclease in Figure 7-28) and then proceeding to count sequences retrieved from an input file (generally these are from EAASC but it is not limited to these).

In the current version of ESFC (V2) the definitions found in Table 7-14 are used to specify a position in a sequence that should be treated as any amino acid or one of a specific group. For example the sequence AahAG is interpreted as the amino acid residues [alanine] [any amino acid] [any hydrogen bonding amino acid] [alanine] [glycine]. This allows combinations of amino acids to be constructed not just with specific residues but based on a type or functional combination. Table 7-14 shows the complete definitions for these search strategies.

Each protein needs to be processed separately and an output file listing the sequence and frequency of that sequence is obtained. The full code for ESFC is given in the appendix 9.2.2 page 156.

7.3.1.4.3 ESA – e. Statistical correlator

The 'extra' statistical correlator (ESA) uses multiple ESFC files to correlate a set of matching data supplied by the user. Using up to three data transformations, data is contrasted against the frequencies extracted from each of the protein ESFC files. The following statistical data is calculated for each set of frequency data:

- mean
- sumsquares
- sum(X*Y)

and derived from this the:

- slope of a line
- intercept
- deviance
- X variance
- Y variance
- covariance
- r
- r^2
- critical value

Output is in the comma separated value (CSV) format (using space as the delimiter) and allows easy interpretation by many spreadsheet packages. The full code is given in the appendix 9.2.3 page 159. An example of output is shown in Figure 7-20 although it would be usual to view this output in a spreadsheet package such as Excel where presentation is much clearer.

Figure 7-20 : Example of ESA comma separated output file – first line shows the start date of the calculation followed by the protein files in use, the figures for correlation are then displayed for the individual proteins.

```
# start time 18:12 19/7/2004.
# protein-sequence-frequency files in use : -------> 5112003sucrose.txt
51120031NNS_fastaAsp.txt 51120031dp0_fastaGal.txt 51120031ByB_fastaAmy.txt
51120031A5P_fastarib.txt 51120031LDb_fastaLac.txt
# checking data against values : -------> 100.8 110.2 112.97 104.2 108.1 103.25
#
# The next line is a definitions line- space separated import
# into any standard spreadsheet will produce labelled columns of data.
#YonXorXonY(regression_line) sequence n meanx meany sumsquaresX sumsquaresY sum(X*Y)
sumSxy sumSxx sumSyy slopeofline intercept deviance Xvariance Yvariance COvariance r
r2 Criticalvalue PercentagePoints75 90 95 97.5 99 99.5 99.75 99.9 99.95 Significance
(1=significant) rest_are_0
>SKIP G  frequencies all equal 0 skipped measurement
XY C  6.0000 13.6667 106.5867 82.0000 639.5200 9151.1800 411.0733 3067.3333 106.4083
0.1340 104.7551 51.3177 511.2222 17.7347 68.5122 0.7195 0.5177 2.0722 0.7180 1.4400
1.9430 2.4470 3.1430 3.7070 4.3170 5.2080 5.9590 1.0000 1.0000 1.0000 0.0000 0.0000
0.0000 0.0000 0.0000 0.0000 X  0  4  64  6  6  2 Y 100.8 110.2 112.97 104.2 108.1
103.25
YX C  6.0000 106.5867 13.6667 639.5200 82.0000 9151.1800 411.0733 106.4083 3067.3333
3.8632 -398.0956 1479.2877 17.7347 511.2222 68.5122 0.7195 0.5177 2.0722 0.7180 1.4400
1.9430 2.4470 3.1430 3.7070 4.3170 5.2080 5.9590 1.0000 1.0000 1.0000 0.0000 0.0000
0.0000 0.0000 0.0000 0.0000 Y  0  4  64  6  6  2 X 100.8 110.2 112.97 104.2 108.1
```

Table 7-14 : ESFC interpretation of sequences input'ed to the program for counting. Allows selection of a category or group of amino acids that are to be counted as a continous set.

**** DEFINITIONS **** These are the current definitions in v2 of sequence counter : CASE SENSITIVE	
a	Any amino acid
r	Aromatic amino acid (FWYH)
h	Hydrogen bonding (CWNQSTYKRHDE)
c	Hydrophilic (NQSTKRHDECY)
n	Charged at neutral pH negative /acidic (DEC)
e	Essential amino acids - (ABDCEQGPSY)
l	Aliphatic alkane side chains(GAVLIP)
i	Ionisable amino acid (DEHCYKR)
b	Hydrophobic (GAVLIPYFWMC)
s	Sulphur containing (CM)
p	Charged at positive pH positive /basic (KRH)
q	Non -essential amino acids - (RHILMFTWV)

7.3.1.5 Analysis of protein sequence frequency data

The recrystallisation endpoint values of proteins were correlated since the T_d of a protein would change this point most significantly for sucrose (McGarvey, 2003)(section 7.3.1). The effect of lysozyme concentration in this study on sucrose recrystallisation can be seen in Figure 7-35.

Using an appropriate ESA output file (Figure 7-20) the end point of the recrystallisation exotherms at 312 µg/ml (Table 7-17) were correlated for the 5 proteins:

i. L-asparaginase II
ii. Lactate dehydrogenase
iii. Beta-galactosidase
iv. Beta-amylase
v. Ribonuclease a.

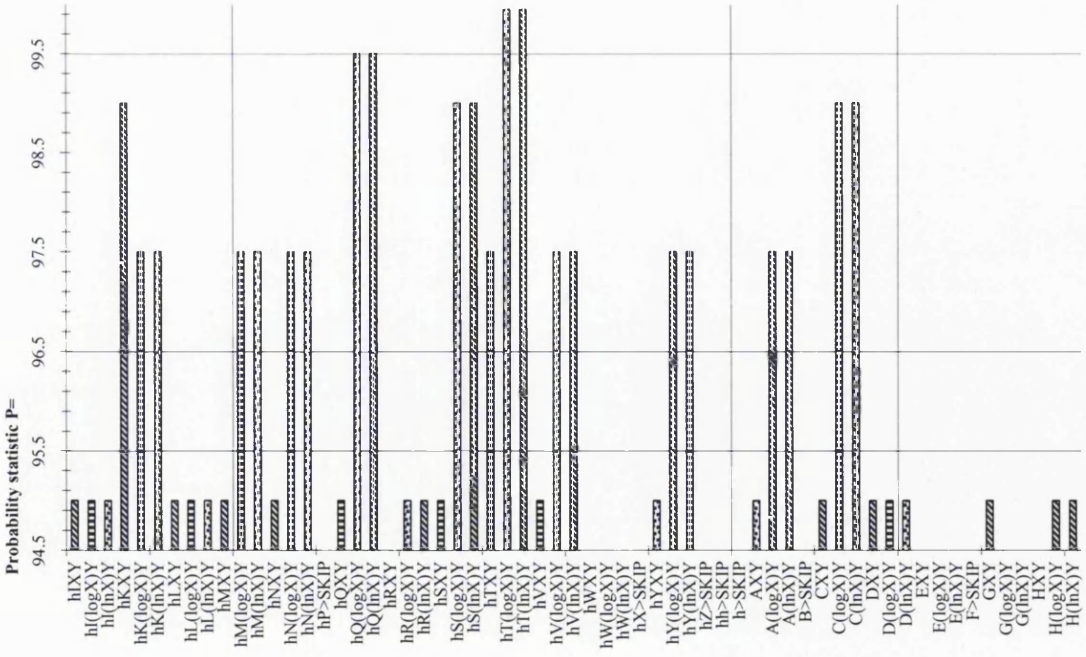


Figure 7-21 : Graphical display of a small region (61 of >7000) ESA correlation data sets. The good correlation of DSC recrystallisation end point and specific frequencies of amino acids is show by high *p* values. *p* values indicate that H= 0 can be disregarded with in some cases > 99 % certainty (linear relationships are therefore significant). Sequence definitions are given in Table 7-14

EAASC was used to create all single and pair amino acid combinations of both individual amino acids and all combinations of groups (Table 7-14). Over 7000 pairs were created and correlated.

The graphical plot of an individual sequence's statistical significance is shown Figure 7-21 above. It shows a higher degree of correlation for sequences containing hydrogen bonding amino acids. The accepted hydrogen bonding amino acids are CWNQSTYKRHDE (Table 7-14) which can be seen structurally in Figure 7-23 (page 98).

The sequence showing the highest correlation is that of hT ([hydrogen bonding amino acid] + [threonine]) p = 99.9. The hT individual data set is shown in Figure 7-22 and alternate sequences with high correlation are shown in Table 7-15.

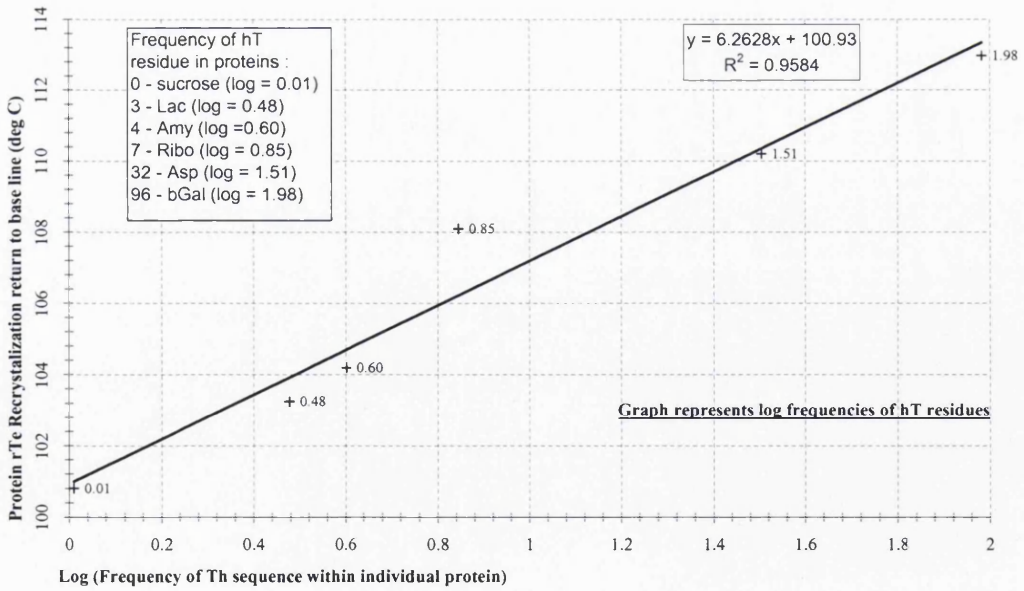


Figure 7-22 : Correlation of hT sequence (hydrogen bonding amino acid and threonine amino acid) with rTe (End point of recrystallisation exotherm). using data for the enzymes asp; l-asparaginase II, lac; lactate dehydrogenase, bgal; beta-galactosidase, amy; beta-amylase, ribo; ribonuclease A.

Table 7-15 : Sequences found to have high correlation of frequency with the recorded data for rTe (recrystallisation end point). Sequence defined according to Table 7-14 and standard IUB code.

Amino acid sequence	Level of significance (P value)
hT	99.9
TM	99.75
TF	99.75
Ch	99.75

7.3.2 Discussion

The identification of a sequence related to T_d could allow us to make predictions of enzyme stability in a sucrose solution prior to freeze drying; this prediction has been made for the enzymes considered in this chapter in Table 7-16 (page 103). ESFC is capable of calculating for individual proteins the total content of the hT ([hydrogen bonding amino acid] + [threonine]) sequence in minutes allowing it to be inputted into the relationship generated by ESA. The relationship between Td and hT frequency was found to be:

$$y = 6.2628 (\log \{sequence\ frequency\}) + 100.93$$

(Figure 7-22, above)

With a combination of T_d knowledge (Franks, Hatley et al., 1991) (Hageman, 1992; Imamura, Suruki et al., 1998; Kang, Jiang et al., 2002; McGarvey, 2003) and the frequency data from ESFC prediction, the drying success of an unknown protein in a sucrose solution could be attempted. At present this should be limited to hT ([hydrogen bonding amino acid] + [threonine]) frequencies within the range of 0-96.

Since preliminary studies with the Rasmol protein molecular modeller software revealed that threonine residues were generally confined to the surface of the tertiary structure of a range of proteins (Figure 7-23 below). It was encouraging to learn that sucrose as a ligand could have access to most of these potencial hydrogen bonding sites; it could therefore use the proposed hydrogen bonding sites as suggested by the current literature as mechanisms for sugar protection during freeze drying (covered in section 7.2.1).

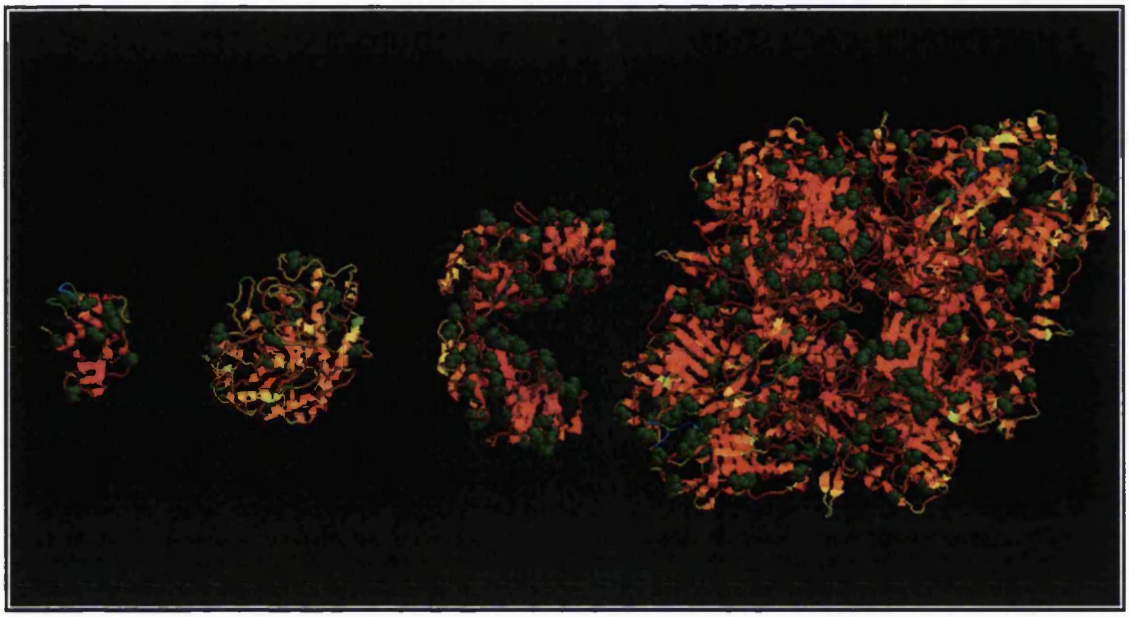


Figure 7-23 : Molecular models for (left to right) Ribonulease A, Beta - Amylase (e.c.3.2.1.2), L-Asparaginase II, Beta – Galactosidase. Imaged in Rasmol 2.7.2 (Sayle and Milner-White, 1995) Structure shown as ribbon, with colouring demonstrating charge (red indicates negative charge green is more neutral and blue is positive). Threonine (Thr) residues are shown in green as molecular orbitals.

7.3.2.1 Further work

Further work would involve the development of a routine based on the work of (Lee.B and Richards, 1971) to predict the exposed residues of a protein's sequence (Figure 7-28). This could be added to ESFC to allow pre-filtering of FASTA data to allow only those residues present in an exposed state to be considered available for interaction with an external solute (v3).

Since threonine is itself a hydrogen bonding amino acid it is hypothesized that sucrose requires two very closely positioned hydrogen bonds as a 'strong holding site' at which it can fix the tertiary structure of the protein. This should not lead to the belief that sucrose is the only molecule capable of bonding to these or any other site on a proteins surface; just that sucrose by chance has acquired the ability to bind important changing areas in the proteins structure, which would otherwise irreversibly change on freeze drying. Threonine is often involved in *O*-glycosylation of a protein's non-fixed conformational

portion; 65-85 % of a proteins weight can become carbohydrate in this manor (Abeijon and Hirschberg, 1987; Jentoft, 1990). It is suggested that the localization of sucrose to these non-fixed threonine rich areas may be responsible for imparting tertiary structural protection against lyophilisation stresses.

7.4 Docking studies

To further investigate the likelihood of sucrose localizing to these 'threonine high hydrogen bonding areas' docking studies were undertaken using sucrose as the ligand. Threonine had been shown to be prevalent on the surface of a range of the enzymes understudy. It was hypothesised that sucrose would be found to preferencially dock at sites containing threonine and hydrogen bonding amino acids.

Docking calculations were carried out using the automated docking program, AutoDock3 (Morris, Goodsll et al., 2004), on a personal computer (Pentium II 550 MHz) running Red Hat 9 Linux (patched) and using the ADT (AutoDock Tools) GUI (graphical user interface) controls for visualization. The program was used with the Genetic algorithm (GALS) (Morris, Goodsell et al., 1998) in combination with a grid based energy evaluation method where a grid map of 102 points square with 0.375 A spacing was used.

Cluster analysis was used to categorize all 1000 docked configurations into groups. The configurations which had root-mean-squared deviation (rmsd) values less than 1A were grouped together. In each group, the lowest energy configuration was selected as representative of that group. Our attention focused on the group with the highest percentage occurrence or ``the most occurring configuration". This configuration represented the highest probability of occurring in the real system.

7.4.1 Molecule preparation

7.4.1.1 The Macromolecule

The macromolecule ribonuclease A was prepared from the standard PDB file available from www.rcsb.org/pdb (Berman, Westbrook et al., 2000) PDB ID : 1A5P (Pearson, Karplus et al., 1998)(Figure 7-33 page 110). All bound water was removed and the hidden hydrogen's displayed. It was chosen because of its low molecular weight since this would reduce overall processing time for processor intensive calculations.

7.4.1.2 Ligand

The sucrose ligand was first modelled in ChemDraw Ultra V8.0 and optimized to minimize its energy (RMS minimum gradient 0.1) in Chem3D Pro before export in the PDB file format. Using the ADT GUI for Autodock3 all hydrogen atoms were added and using the standard default, Gasteiger charges were assigned to the ligand.

7.4.1.3 Results

Four areas of high docking were found on the surface of Ribonuclease (Figure 7-26). The highest occurrence was that to a portion with the sequence [TGS] (Figure 7-24- Figure 7-28). This area also had the lowest docking and binding energy (Figure 7-25).

Although the Auto docking calculations did not show binding to the sequence hT as had been hypothesized, they did suggest binding to the region [TGS]. Binding did take place at a threonine residue; we further found that the main bonding points took place between threonine and serine residues, but not to the glycine residue. The glycine residue at this point was seen to bend the protein (Figure 7-24) it was also likely that it was buried from the surface (Figure 7-25). In this case the sequence 'actually seen' by the ligand is that of [TS] (Figure 7-28) which would fit the hypothesis of [hT] if viewed from the correct direction.

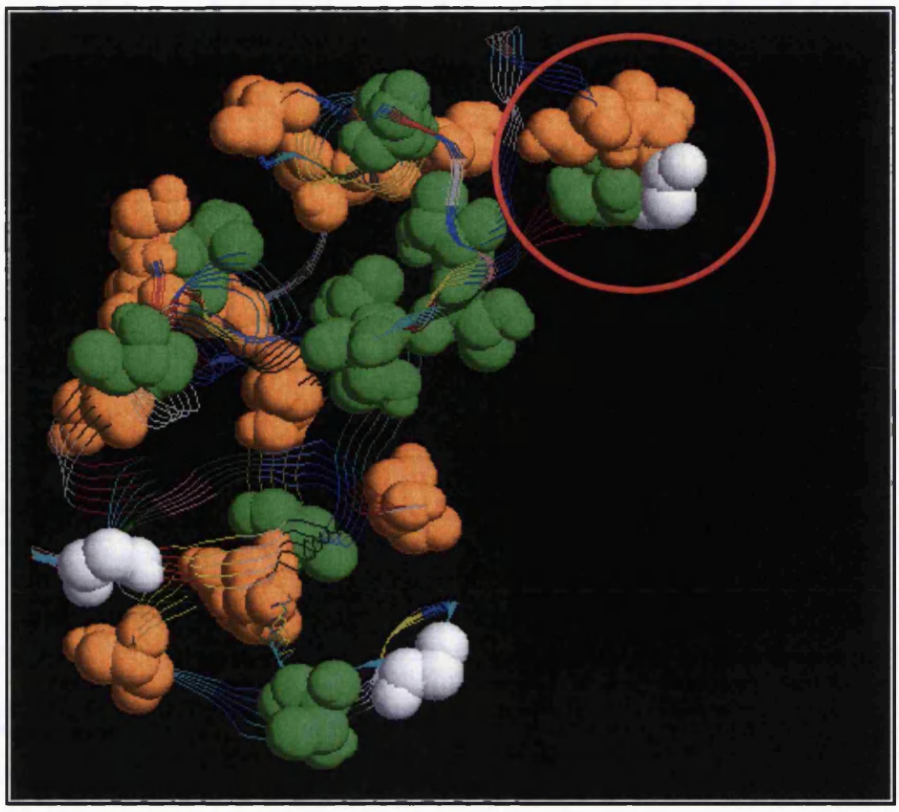


Figure 7-24 : Ribonuclease A, modelled in strand display (Rasmol). Serine, threonine, glycine residues shown coloured as orange, green and white respectively (non-standard colouring). Circled area-shows the sequence [TGSS]

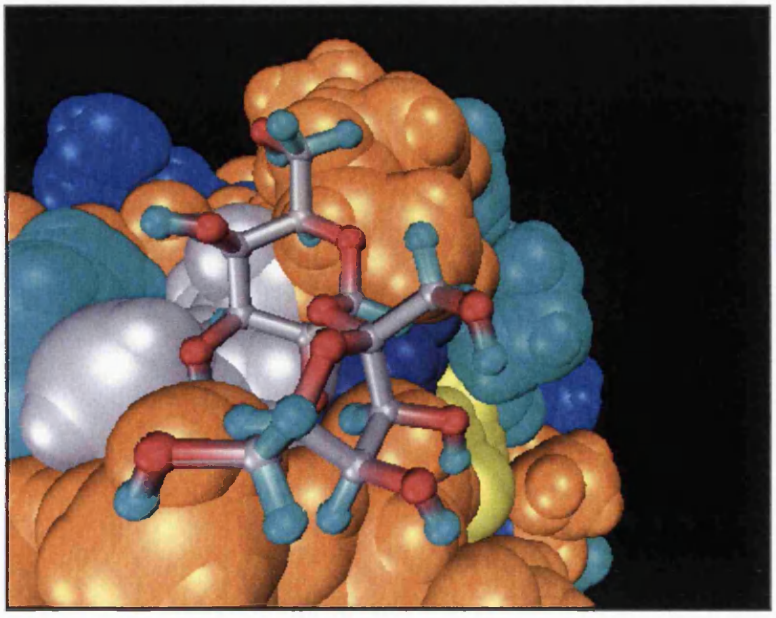


Figure 7-25 : The sucrose ligand docking to ribonuclease. Model is rendered in autodock3 using the Rasmol standard colour scheme (Figure 7-27) for amino acid residues in the space filled model type. Sucrose is modelled as the stick filled type with full hydrogens pictured. The Energy of binding and the docking value are at the lowest calculated in this position -1.49 & -2.93 respectively (calculated n=1000). It can be seen sucrose is docking between threonine and serine residues of the same chain (Figure 7-27).

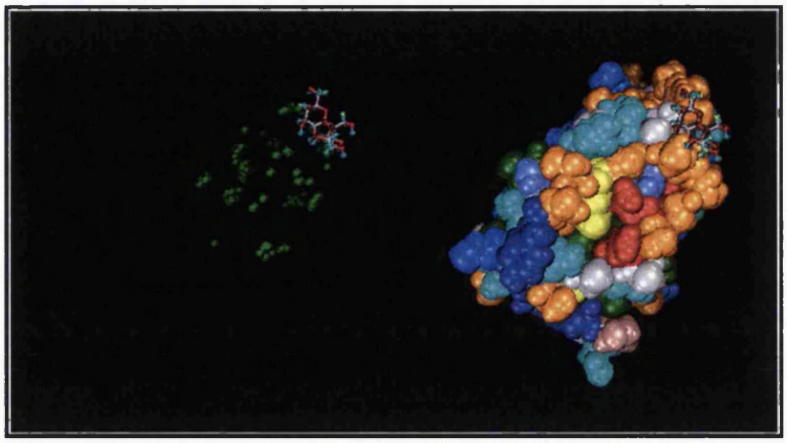


Figure 7-26: Full view of ribonuclease molecule (right) with sucrose ligand as Figure 7-25. On the left are cluster groupings of the ligand docking (with the Ribonuclease molecule removed for clarity). Those areas of high SER and THR residues have lower docking energies with more frequent docking.

ASP, GLU		GLY	
LYS, ARG		LEU, VAL, ILE	
CYS, MET		ALA	
SER, THR		TRP	
PHE, TYR		HIS	
ASN, GLN		PRO	
others/ unknown			

Figure 7-27 : Standard Rasmol colour coding for amino acid residues.

KETAAAKFERQHMDSSTSAASSSNYCNQMMKSRNLTKDRAKPVNTFVHESLADVQA
VCSQKNVACKNGQTNCYQSYSTMSITDCRETGSSKYPNAAYKTTQANKHIIVACEG
NPYVPVHFDASV

Figure 7-28 : IUB sequence of ribonuclease A, as seen by ESFC. Highlighted areas are those containing [hT] residues as defined by Table 7-14 (yellow). TGSS sequence shown partly in red. Green area and all other underlined areas is an implementation of an exposure routine – v3 pre-beta.

7.4.2 Discussion

Both docking and sequence correlation suggest the involvement of hydrogen bonding within a freeze-dried cake.

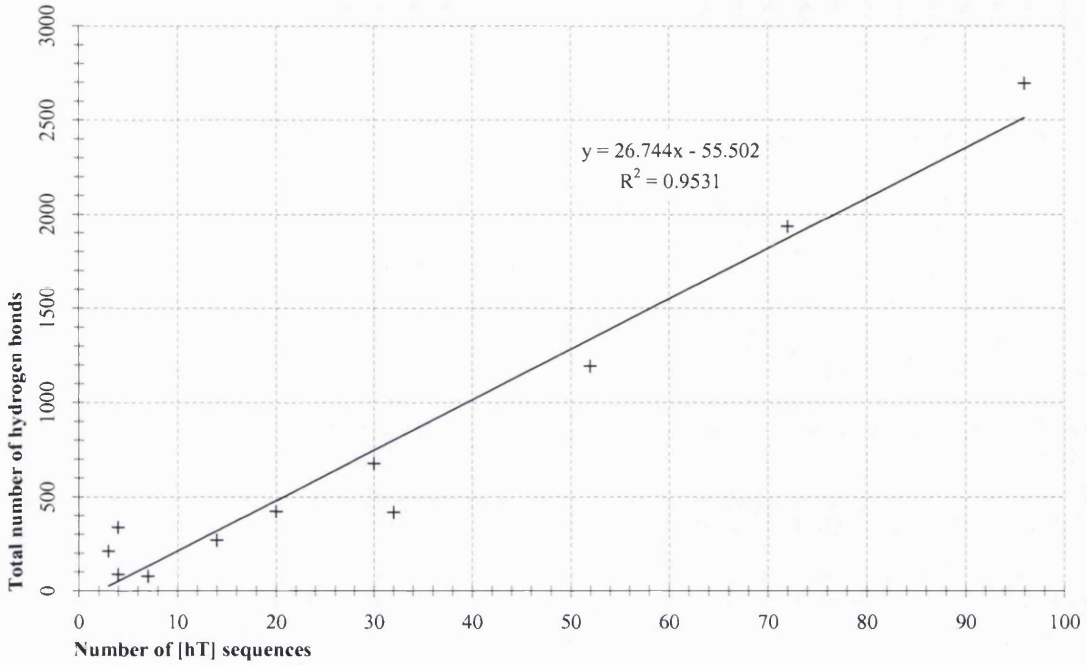


Figure 7-29 : Relationship between total number of hydrogen ions and total number of [hT] sequence, for the enzymes contained within Figure 7-33.

It has been suggested that increased T_d (McGarvey, 2003) and the subsequent increase in apparent sucrose recrystallisation temperature provides an increase in stability. Since we have related the T_d to an increase in hydrogen bonding (Figure 7-22 page 96, Figure 7-29 above) through the end point of re-crystallization of sucrose, we can create a table of predicted stability (Table 7-16 below).

Table 7-16 : Hypothesized stability for a group of enzymes commonly used in freeze drying.

HYPOTHESIZED STABILITY.	ENZYME
MOST STABLE	BETA-GALACTOSIDASE
↓	PHOSPHO- FRUCTOKINASE
↓	A CATALASE
↓	ASPARAGINASE
↓	ASCORBATE OXIDASE
↓	GLUCOSE OXIDASE
↓	ADENOSINE DEAMINASE
↓	RIBONUCLEASE A
↓	BETA-AMYLASE*
	LYSOZYME *
LEAST STABLE	LACTATE DEHYDROGENASE

* SIMILAR STABILITY.

Careful selection of enzymes allowed for comparison with a large source of well described protein literature. Allowing the predictions made in Table 7-16 to be tested to a small degree. Jiang and Nail, (1998) shows for a 10 μg/ml concentration of β-Galactosidase and lactate dehydrogenase (LDH) a relative activity recovery of 45 % and 25 % respectively. Results for catalase suggested very little loss of activity after freeze drying with all concentrations showing activities >85 % on recovery although it is note worthy that the enzyme concentration is one thousand times greater than either β-Galactosidase or LDH. Comparison of the freeze-drying cycles used in these experiments requires careful examination as the relationship would be predicted to be dependent on a suitable freeze drying cycle.

Failure to account for gross controlling factors such as exposure to collapse exposes the protein to a completely different set of destructive properties. Anchordoquy and Carpenter (1996) and Anchordoquy, Izutsu *et al.* (2001) have shown LDH recovery to be as low as 15 % but when the solution was bulked with bovine serum albumin (BSA) recovery could be increased to >85 %. This is also true for amylase which when bulked with human serum albumin (HSB) responds to a freeze drying cycle in a similar manor (Gemma Gubern., Francesca Canalias. et al., 1996).

Using low sugar concentrations of 0.025 M (Anchordoquy and Carpenter, 1996) reported recoveries of LDH of 60.4 ±7.5 % while Xiang, Heye et al. (2004) using almost the same concentration (0.03 M) but focusing on cycle optimization reported recoveries of intact enzyme from 94.7 to 107.3 %. It is apparent from literature that preparation of an enzyme specifically with relation to concentration can be vital. For example, although Xiang investigated freeze drying cycles with annealing, differing freezing rates and rates of drying, his lowest recoveries (94.7 %) were still far greater than those reported by Anchordoquys *et al* freeze drying cycle. It is probable that this was due to the 'self protection effect' as described for asparaginase (Ward, Adams et al., 1999).

We suggest that hydrogen bond cross linking between individual proteins is responsible for self protection and that the addition of sugar allows further links to be produced

enhancing the protective effects (Figure 7-30 page 106). Where long chains of hydrogen bonding sugar molecules are generated, enhancement of protection is much reduced as spatial separation on the scale of a proteins size interaction has a much higher probability with sugar chains flexing to a far greater extent. This is suggested to be responsible for the enhanced effects of polymers in combination with protein and sugars. Polymers allow far less movement of the macromolecular proteins while acting as tether points for shorter sucrose chains. This would place a whole glass state both during its formation and on formation in a state of far greater rigidity.

By having an increase in hydrogen bonding positions certain proteins are able to produce more sites for hydrogen bonding to sucrose. Better spatial separation combined with the ability of sucrose to replace the proteins hydration shell provides a state less prone to movement as any glassy state forms. This is in line with the hypothesized stability of proteins in Table 7-16 (page 103).

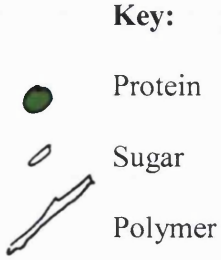
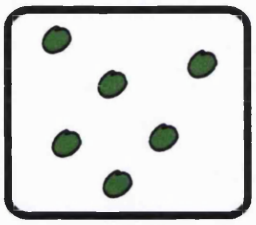
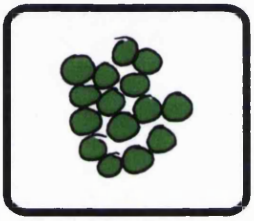


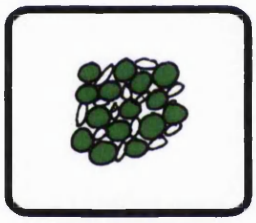
Figure 7-30 : Hypothesis for protein protection based on a hydrogen bonding relationship.



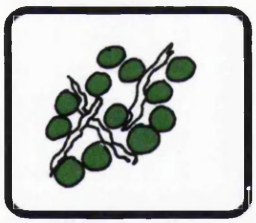
Protein at a dilute concentration. Hydrogen bonds formed between molecules other than water are hard to form, and so are easily damaged during both freezing and drying.



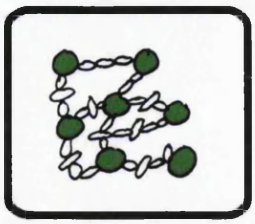
Concentrated protein. Hydrogen bonding is possible with neighbouring protein molecules and is competitive with that of any surrounding water.



Concentrated protein with sugar. Hydrogen bonds with other protein molecules and water are further competed for by sugar molecules. Small spaces within he structure can be filled with sugars which are capable of increasing hydrogen bonding further than in a concentrated solution alone.



Dilute protein with polymer. Hydrogen bonding of the protein is possible with the polymer as well as itself. The polymer provides spatial separation and strength to the freeze dried cake.



Dilute solution with sugar. Although sugar hydrogen bonds to the surface of the molecules because of the distances involved it is not able to successfully separate the molecules and does not spatially separate the protein. The constant breaking and reforming of hydrogen bonds keeps the solution fluid.

Dilute solution of protein, sugar and polymer. The polymer provides structure to the solution by spatially separating the molecules and allowing sucrose to form in small chains and in the pockets, thus preventing damage from stresses on eventual drying.

7.4.3 Conclusion

From the experimental data produced in this study, it may be concluded that enzymes affect the T_d and position of recrystallisation of sucrose. For the enzymes L-asparaginase II, lactate dehydrogenase, β-galactosidase, β-amylase and ribonuclease A a statistically significant relationship between the frequencies of the amino acid sequence [hT] was established at a concentration of 312 μg/ml (p= 99.9 %).

Docking studies showed four main clusters of binding with one cluster more probable than the others. The most probable binding site found during the simulations was to the sequence [TGS] where the energy of binding and the docking value was -1.49 and -2.93 respectively.

7.4.4 Further work

Expansion of the protein set would allow the correlation to be further investigated, while studies with other sugars may show differences in alteration of the T_d value and consequently different protective activities. We have already discussed the importance of having detailed information about individual proteins (7.1 page 85) but characterization of secondary even tertiary structure may allow development of processing parameters for freezing, drying and reconstitution.

Due to experimental limitations enzyme activity could not be measured in all cases. Study of the recovered activity is certainly a key factor and further development would concentrate in this area.

Although the docking examples shown were performed on the smallest proteins available larger proteins are more difficult to study. Study of β-galactosidase as a model protein did not show similarities with those results gained for ribonuclease; large clusters of binding sites appeared inter domain within its structure and without any apparent sequence or spatial relationship. It has also been hypothesized (7.4.2, Figure 7-30) that combinations of multiple sucrose ligands may provide the most efficient method of binding and

therefore protection, but current methods to study these possibilities are unclear. Modelling of multiple ligand situations increases modelling time exponentially and incurs a high level of uncertainty throughout.

An extension of the ESFC software is possible in the light of the new results. Counts that take place in both directions along the chain allow inclusion of otherwise overlooked inverted sequences which could be included. The addition of an exposure routine has already been approached (Figure 7-28) based on previous work in this area (7.3.2.1). Both these extensions could provide further feature enhancements to the software.

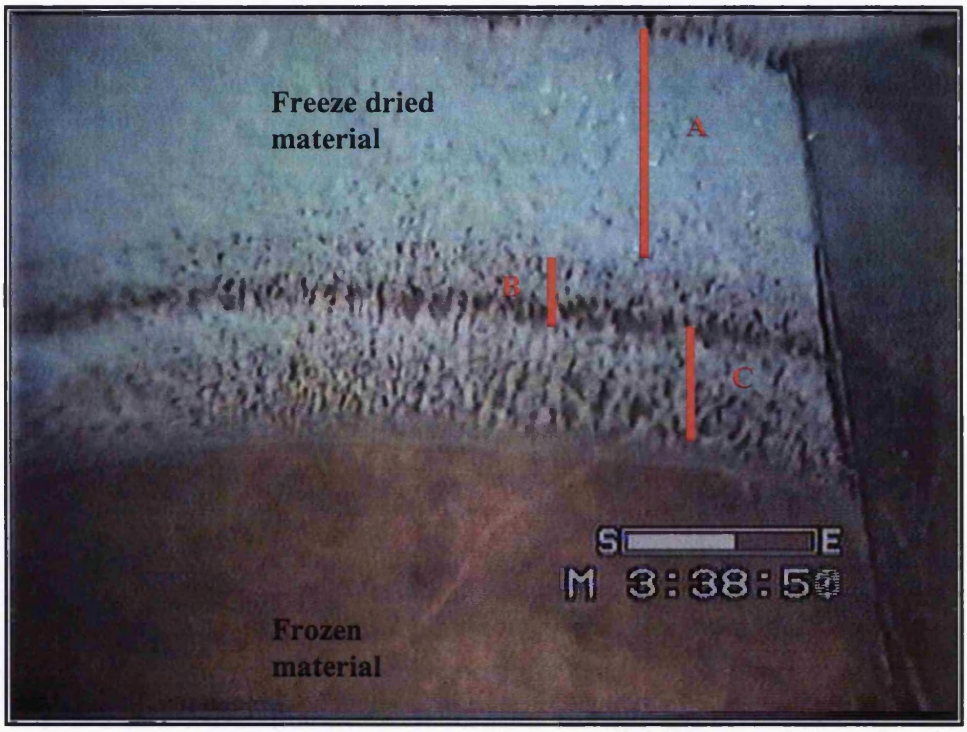


Figure 7-31 : Freeze-drying microscope (Lyostat1) image of Trehalose 50 mM solution undergoing freeze drying labelled regions A (-35 °C), B (-20.5 °C), C (-27.0 °C) show good cake structure, collapse, and weak structure respectively.

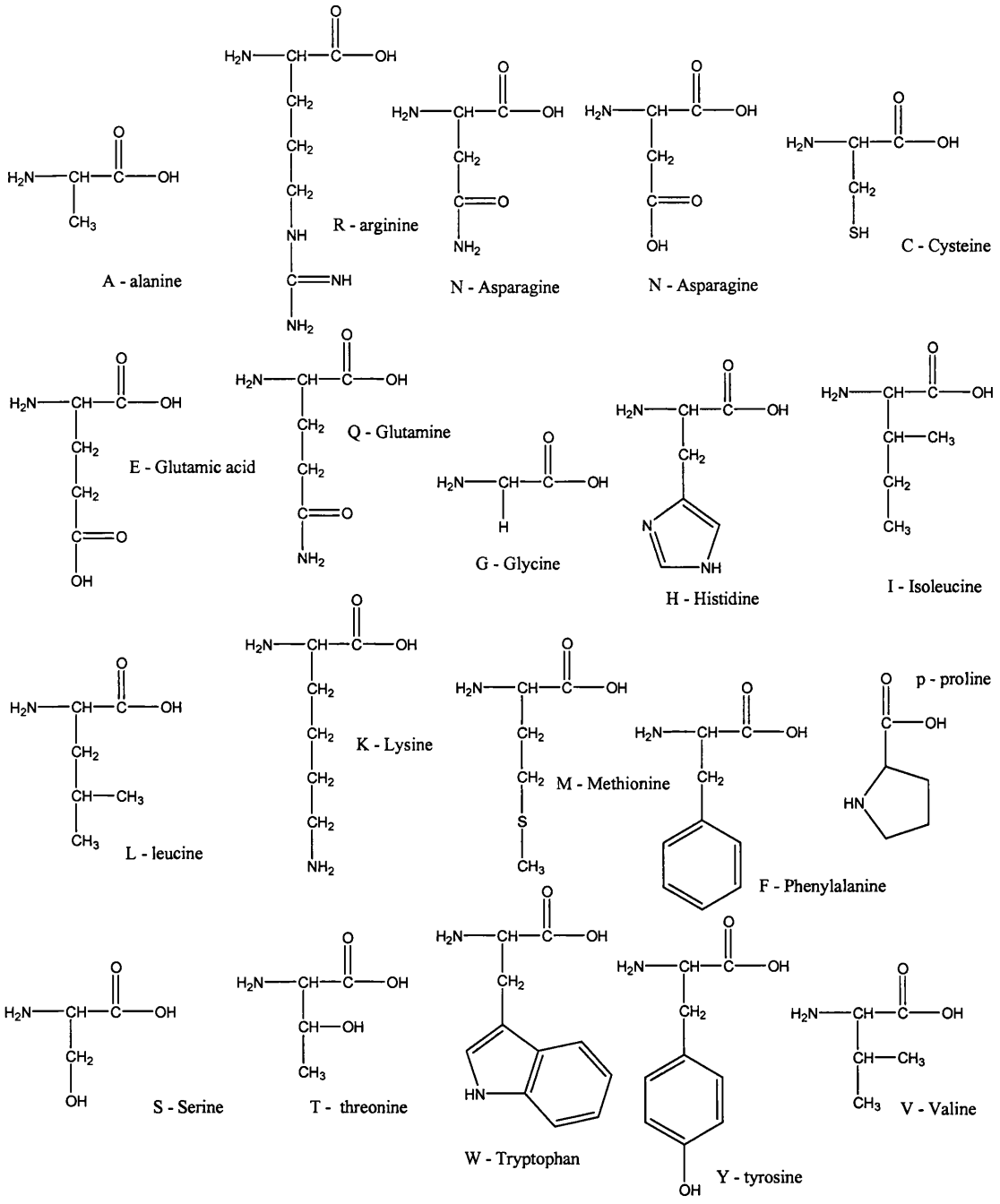


Figure 7-32 : Chemical structures of the 20 amino acid side chains labelled with IUB/ IUPAC code. The remaining IUB/IUPAC code includes X – unknown, Z – glutamic acid or glutamine, B – aspartic acid or asparagine.

Figure 7-33: Tabulated characteristics of enzymes previously investigated for their state during or after freeze drying.

Enzyme	PDB CODE	CLASSIFICATION	The number of								
			CHAINS	GROUPS	ATOMS	BONDS	BRIDGES *	H-BONDS *	HELICES	STRANDS	TURNS
ASPARAGINASE †	1NNS	Hydrolase	3	652	4862	4952	2	417	30	28	0
A CATALASE ††	1DGB	Oxidoreductase	11	1991	16066	16849	0	1193	81	54	0
LYSOZYME #	132L	Hydrolase	2	129	1001	1033	4	87	4	3	15
LACTATE DEHYDROGENASE ##	1LDB	Oxidoreductase	2	294	2277	2327	0	211	7	12	0
ADENOSINE DEAMINASE ‡	1KRM	Hydrolase	3	349	2788	2874	0	269	19	7	0
BETA-GALACTOSIDASE ‡‡	1DP0	Hydrolase	16	4044	32500	33783	0	2695	92	256	0
GLUCOSE OXIDASE §	1GAL	Oxidoreductase (Flavoprotein)	2	581	4452	4745	1	423	13	17	0
BETA-AMYLASE §§	1BYB	Hydrolase (O-Glycosyl)	2	491	3925	4085	0	339	12	8	0
PHOSPHO-FRUCTOKINASE ¤	1MTO	Transferase	9	2552	19200	19616	0	1933	105	95	0
ASCORBATE OXIDASE ¤¤	1AOZ	Oxidoreductase (oxygen acceptor)	5	1104	8732	9046	6	677	-	-	-
RIBONUCLEASE A |	1A5P	Hydrolase	2	124	949	969	3	78	3	7	0

* calculated with (Sayle and Milner-White, 1995)(Sayle and Milner-White, 1995), † (Hellman, Miller et al., 1983; Ward, Adams et al., 1999), †† (Zamocky and Koller, 1999), # (Perez, De Jesus et al., 2002; Elkordy, Forbes et al., 2002), ## (Izutsu, Yoshioka et al., 1995; Anchordoquy and Carpenter, 1996; Mazzobre, Pilar Buera et al., 1997; Anchordoquy, Izutsu et al., 2001; Hillgren and Alden, 2002), ‡ (Wang, 1999; Bota, Gella et al., 2001), ‡‡ (Burin, Jouppila et al., ; Ohtakara and Mitsutomi, 1987; Ken-ichi, Sumie et al., 1991; Ken-ichi, Sumie et al., 1993; Mazzobre and Pilar Buera, 1999; Perez, De Jesus et al., 2002; Elkordy, Forbes et al., 2002; Burin and Pilar Buera, 2002) § (Berman, Westbrook et al., 2000) §§ (Ongen, Yilmaz et al., 2002) ¤ (Carpenter, Crowe et al., 1987) ¤¤ (D'Andrea, Salucci et al., 1996)

Table 7-17 : DSC data obtained for enzyme and sucrose powders – 31 to 190 °C scan at 5°C/min, crimp closed pans – all samples lyophilized achieving 1.9 % w/w moisture by dry mass (± 0.5 %) (Cycle 7-2 & Cycle 7-3).

Enzyme Conc (µg/ml). in 500 mM Sucrose soln	Recrystallisation Onset (rTo) °C	Recrystallisation maximum (rTp) °C	Recrystallisation ΔH (rΔH) (j/g)	Recrystallisation return to base line (rTe) °C	Melt onset (mTo) °C	Melt maximum (mTm) °C	Melt ΔH (mΔH) (j/g)	Melt return to baseline (mTe) °C
ASP								
312 (n=3)	105.0 (±13.4)	108.0 (±11)	0.168 (±0.4)	110.2 (±10)	177.8 (±0.2)	181.0 (±0.1)	-24.6 (±3.8)	181.3 (±0.0)
625 (n=3)	Not detected	Not detected	Not detected	Not detected	176.5 (±0.9)	181.0 (±0.0)	-53.0 (±9.4)	181.3 (±0.1)
CAT								
78 (n=3)	71.2 (±0.9)	78.6 (±0.5)	27.0 (±5.8)	85.1 (±0.7)	135.4 (±9.9)	153.1 (±13.7)	-27.5 (±9.0)	170.2 (±0.9)
156 (n=3)	69.2 (±0.9)	78.7 (±0.5)	21.5 (±10.0)	86.3 (±0.3)	135.4 (±1.6)	159.3 (±2.1)	-16.2 (±7.0)	168.7 (±0.7)
LYOS								
625 (n=3)	83.1 (±2.4)	89.2 (±2.2)	67.2 (±7.0)	93.8 (±1.2)	147.6 (±0.9)	163.1 (±0.4)	-57.8 (±8.6)	171.6 (±0.3)
LAC								
78 (n=3)	80.7 (±0.1)	87.4 (±0.6)	64.5 (±1.3)	92.7 (±1.1)	151 (±1.9)	164.2 (±1.2)	-48.6 (±3.2)	172.6 (±1.1)
156 (n=3)	83.1 (±0.4)	89.2 (±0.7)	67.2 (±4.5)	93.8 (±0.9)	147.6 (±0.6)	163.1 (±0.2)	-57.8 (±1.8)	171.6 (±0.4)
312 (n=2)	75.6 (±0.4)	80.5 (±0.2)	55.6 (±2.4)	103.3 (±8.6)	144.1 (±0.4)	157.9 (±0.1)	-46.2 (±7.5)	167 (±1.4)
ADEN								
GAL								
312 (n=3)	81.3 (±1.8)	95.0 (±1.1)	16.3 (±1.6)	113.0 (±4.0)	136.4 (±2.3)	148.6 (±28.0)	-15.1 (±1.1)	175.2 (±0.3)
625 (n=1)	74.2	84.2	16.2	92.5	141.0	164.1	-12.7	172.2
OXI								
AMY								
312 (n=3)	86.9 (±5.5)	96.0 (±7.3)	38.2 (±6.7)	104.2 (±7.2)	160.8 (±8.6)	169.1 (±4.2)	-24.6 (±10.8)	174.1 (±2.7)
625 (n=2)	92.5 (±4.2)	106.2 (±6.8)	61.6 (±5.4)	112.6 (±4.3)	170 (±6.7)	171.7 (±3.8)	-47.2 (±11.3)	176.7 (±3.0)
1250 (n=1)	90.6	105.3	35.5	119.4	141.9	162.3	-25.9	171.3
PHOSF								
78 (n=2)	68.4 (±1.0)	77.8 (±0.9)	31.9 (±3.6)	88.9 (±2.3)	135.0 (±1.2)	158.6 (±1.8)	-22.9 (±7.6)	167.4 (±1.7)
ASCOR								
RIBO								
312 (n=3)	84.0 (±1.9)	97.4 (±1.5)	60.3 (±1.7)	108.1 (±2.3)	149.4 (±14.4)	160.9 (±5.5)	-51.2 (±20.0)	170.5 (±1.2)
625 (n=3)	84.7 (±2.0)	99.9 (±2.5)	56.9 (±5.7)	111.4 (±2.3)	143.6(±0.6)	157.5 (±0.2)	-43.9 (±10.9)	169.1 (±0.2)
No enzyme								
0 (n=3)	84.6 (±1.2)	95.3 (±0.7)	67.1 (±4.2)	100.8 (±0.36)	159.4 (±0.53)	163.8 (±2.4)	-60.7 (±1.2)	175.1 (±1.8)

Abv – ASP; L-ASPARAGINASE II, CAT; A CATALASE, LYOS; LYSOZYME, LAC; LACTATE DEHYDROGENASE, ADEN; ADENOSINE DEAMINASE, GAL; BETA-GALACTOSIDASE, OXI; GLUCOSE OXIDASE, AMY; BETA-AMYLASE, PHOSF; PHOSPHOFRUCTOKINASE, ASCOR; ASCORBATE OXIDASE, RIBO; RIBONUCLEASE A.

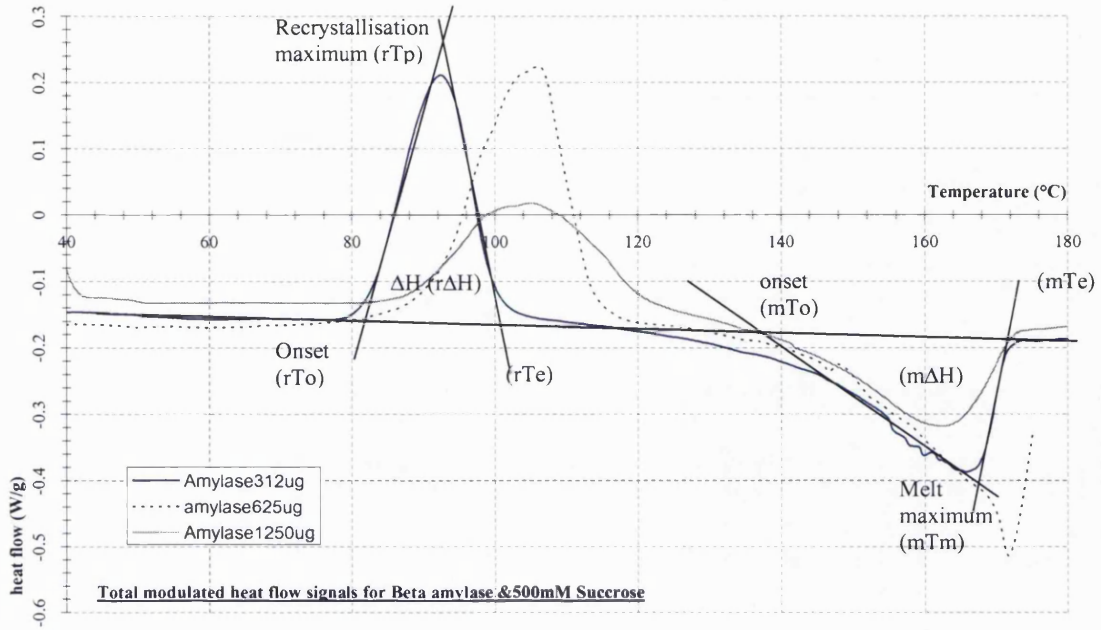


Figure 7-34 : The total modulated heat flow signals for varying concentrations of β - Amylase enzyme with 500 mM sucrose post freeze-drying. Construction lines shown with abbreviations from Table 7-17

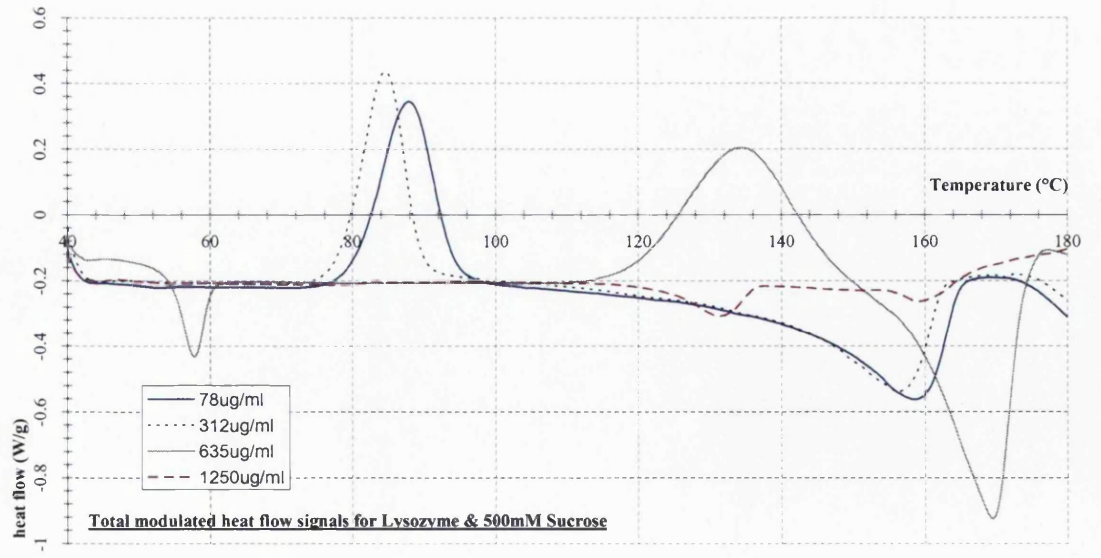


Figure 7-35 : The total modulated heat flow signals for varying concentrations of lysozyme enzyme with 500 mM sucrose post freeze-drying.

Cycle 7-2 : Freeze-drying cycle for sucrose and enzymes l-asparaginase, A catalase, lysozyme, lactate dehydrogenase, adenosine deaminase, beta-galactosidase, phospho-fructokinase, ribonuclease A <626 µg/ml.

Shelf temperature (°C)	Time (min)	Control condition
≅15	0	Hold
Formulations loaded direct onto shelf		
-50	150	Ramp
-50	60	Hold
Primary drying		
-10	1300	Hold
Secondary drying		
+20	150	Ramp
+20	230	Hold
** The vacuum is maintained at a constant 80 (± 10) µbar during both primary and secondary drying Vials are 23mm∅ 1 port stopper 8ml max fill with a sample volume of 1ml. Stoppered under 80 µbar vacuum and crimp sealed.		
Optimized 3 times, with an aim of a final moisture content of 2.5 % by mass.		

Residual moisture by Karl Fischer % by dry mass. Final batch for	
l-asparaginase	2.1 (± 1.3)
A catalase	2.0 (± 1.9)
lysozyme	1.9 (± 1.2)
lactate dehydrogenase	2.6 (± 0.8)
adenosine deaminase	1.8 (± 0.6)
beta-galactosidase	1.7 (± 1.1)
phospho-fructokinase	1.4 (± 0.7)
ribonuclease A	2.3 (± 1.1)
Karl Fischer is mean of 3 samples one from each concentration.	

Cake appearance notes	Generally: White, no shrinkage, occasional cracked powder cake. Phosphofructokinase has slight yellow coloration after drying.

Cycle 7-3 : Freeze-drying cycle for the enzymes l-asparaginase, A catalase, lysozyme, lactate dehydrogenase, adenosine deaminase, beta-galactosidase, phospho-fructokinase, ribonucleaseA >626 µg/ml. glucose oxidase, beta-amylase♦, ascorbate oxidase > 0µg/ml.

Shelf temperature (°C)	Time (min)	Control condition
≅15	0	Hold
Formulations loaded direct onto shelf		
-50	150	Ramp
-50	60	Hold
Primary drying		
-10	1300	Hold
Secondary drying		
+20	150	Ramp
+20	345	Hold
See also Cycle 7-2 **		
Optimized 4 times, with an aim of a final moisture content of 2.5 % by mass. ♦ required total of 630 minutes secondary drying		

Residual moisture by Karl Fischer % by dry mass. Final batch for	
l-asparaginase	1.4 (± 0.9)
A catalase	1.0 (± 1.2)
lysozyme	1.3 (± 1.5)
lactate dehydrogenase	1.8 (± 1.3)
adenosine deaminase	2.2 (± 0.9)
beta-galactosidase	2.5 (± 1.0)
phospho-fructokinase	0.9 (± 0.5)
ribonuclease a	2.3 (± 1.1)
glucose oxidase	2.5 (± 1.2)
beta-amylase	2.4 (± 1.3)
ascorbate oxidase (asc)	2.6 (± 1.2)
Karl Fischer is mean of 3 or 5 samples one from each concentration within cycle.	

Cake appearance notes	No shrinkage and good cakes. Asc is a brown colour before and after drying.

8 Freeze-drying cycle optimization

8.1 Introduction

It is known that the freeze-drying process causes damage to many formulations including separation of emulsions, denaturation of proteins, morphological damage of microparticles and many other structural alterations which in some cases can be reversed (Elkordy, Forbes et al., 2002) and in others cannot (Harrigan and Madden, 1994).

In the following studies the effects of a range of common bulking agents are studied when processing under a range of different freeze drying conditions. The parameters adjusted within the different freeze-drying cycles were:

- Freezing rate
- Product temperature during freeze-drying
- Temperature of secondary drying
- Rehydration on completion of freeze-drying

Using a liposome model examination of the effects of cycle conditions on morphology and size was undertaken.

Further to the the study of typical bulking agents and common lyoprotection agents, two hypotheses were developed around key aspects of cycle damage were investigated. The concept that freeze drying damage could be divided into that which was a result of cake collapse and that which was a result of exceeding a critical non-collapse related temperature was emphasised with the development of a formulation unable to collapse at even very high processing temperatures (Section 8.4 page 134). It is suggested that collapse is a gross change of structure which because of its large over riding effect on cake structure is responsible for an equivalently large proportion of damage within the formulation. In the case of a formulation which does not collapse a second mechanism becomes the cause of damage of any damage. This second point of damage which may be of an equivalent level in destructive nature to any activity is proposed to be caused by an

alternate method representative of the formulations lower level functions and not the gross ability to hold physical structure as is the proposed case for collapse.

The second hypothesis for activity loss was suggested to be that formulations when rehydrated were exposed to forces such as hyper concentration, which at the very instant of water addition produced a tendency to aggregate. The very first drops of water to rehydrate a hygroscopic formulation were therefore responsible for inducing degrees of aggregation within the powdered formulation as it attempted to solubalise it. To prevent this in the studies presented here water was sublimed back into the cake structure using a reverse freeze drying technique (Section 8.5 - page 139) from which samples could be rehydrated with less aggregation tendency.

8.1.1 Drying stage challenges

8.1.1.1 Freezing

Freezing of liposomes has been well documented with freeze-thaw being used in several protocols for vesicle preparation or alterations in size (Castile and Taylor, 1999) as well as drug loading of liposomes. Although generally not applied to freeze-drying, the act of freezing is well characterized with knowledge of ice crystal formation being well characterized for known freezing cycles (Faydi, Andrieu et al., 2001).

Since freezing is known to increase the size of a population of liposomes, freeze-drying must protect the formulation during both freezing and drying, hence the use of cryo and lyoprotectants respectively. In many cases lyoprotectants also provide cryoprotection allowing the addition of only one additive. In the case of lyoprotectant use it is believed that glass state formation is the protective mechanism, and that individual lyoprotectants can be used to protect differing formulations with the same level of action. It is this pattern that has lead to the acceptance of the disaccharide group of sugars as the first line choice in lyoprotectant.

8.1.1.2 Product temperature

Product temperature during primary drying is receiving a large amount of interest from both product aesthetics (the appearance of a dry powder) and the ability for the correct drying temperature to prevent activity loss. But undoubtedly the main interest, as previously discussed, must be the economics of drying (section 6.1 – page 47). Defining key temperatures of a formulation is important for storage of a frozen formulation as well as the processing during freeze-drying. The freeze-drying process itself and the continued processing or storage of any freeze-dried powder has a variety of critical temperatures associated at differing stages, from the liquid formulation where the the primary glass transiton (Tg`) and temperature of collapse (Tc) are important, to the solid powdered form where the glass transition (Tg) and moisture levels (% H_20) control activity. The glass transition (Tg) temperature has long been the critical temperature most commonly discussed but more recently the temperature of collapse as well as information of crystalline and amorphous content on larger scale volumes has become more relavant. Techniques such as resistance analysis are used to detect the onset of events to provide early warning of recrystallization events which may or may not be the preferred state in the frozen solid. Although analysis of the dry powder after freeze-drying has in the past played a key role in prediction of stability, new techniques concentrating on the liquid formulation are rightfully providing insights to the most relevant critical temperatures.

The Tc has now replaced the Tg` as the critical temperature of choice for primary drying product temperatures. In the case of liposomes, the initial concentration of the macro-structures effects Tc. Thus even structural changes by way of concentration of solute in a formulation will affect the structure on drying.

8.1.1.3 Secondary drying

Secondary drying has a key role in final product moisture content; the method of achieving this can be made up of a variety of conditions including varying the length of drying, the temperature and the pressures used. Rarely is it suitable to seal a formulation at the temperature of primary drying and so even the most conservative freeze drying cycles will have had some form of secondary drying.

8.1.1.4 Rehydration of a formulation prior to measurement of a retained property

Occasionally freeze dried formulations are never rehydrated. They are used in the final powdered state; this would be the case with a powdered inhalation device. For most formulations however the freeze dried powder is rehydrated meaning at some point the powdered formulation is expected to interact with a solvent. Rehydration of formulations other than those conducted with standard salt solutions (producing known humidity) is not a widely studied area. We choose to investigate the process using sublimation of gas to ice within a powdered solid. Rather than produce absortion of the water to a structure the described alternate technique would presented ice to powdered solid at very close proximity using sublimation. It was hypothesised fom this concept that it may be possible to produce an alternate environment for rehydration. This environment would preferencially rehydrate formulations (when thawed) which would reduce aggregation forces between particulate matter in a solution. Particulates in this context would include nano and micro structures including protein and liposomes.

8.2 Cycle controls effect on liposome size

This study examined the effect of freeze drying processing parameters on liposome size. To tackle the size altering effects of freeze drying liposomes several approaches have been developed including methods which do not involve drying at all (Barry, 2004). The most common approach however is the addition of a lyoprotectant to stabilize the structure and activity of the liposomes. We looked at the effect of freezing rate, drying temperature and the addition of an annealing stage on a group of common lyoprotectants: sucrose, trehalose, mannitol and two less common potential protectants: raffinalose, melezitose (both tri-saccaride sugars).

8.2.1 Method

Egg phosphatidylcholine (EPC) was rehydrated (7.8 mg/ml) from a thin film containing cholesterol (EPC 16: 8 cholesterol) to produce multilamellar vesicles. This colloid was

then extruded (Section 5.2.2 page 41) to form large unilamellar vesicles of 1μm, before the addition of varying concentrations of sugars. Liposomes were then incubated at room temperature for 30 minutes and sized using laser light scattering. Freeze drying was conducted in the different cycle condictions as detailed in Cycle 8-5 through Cycle 8-9.

On completion of freeze drying samples were reconstituted to their original weight and samples extracted for laser light scattering which were then available for comparison to the initial size.

8.2.2 Results

The size of liposomes on reconstitution after a freeze drying cycle are displayed in Figure 8-36 through Figure 8-40. In the case of a formulation having two distributions of liposomes the largest (by volume) was chosen to represent the change in the solution. Since size change was deamed to be of an 'all or nothing' nature. Initial liposome population was either changed or remained largely unchanged; allowing categorisation using this method to be deemed appropriate for interpretation of these results. Initial liposome size had a modal size distribution of 1μm in all cases.

8.2.3 Discussion

8.2.3.1 Defining the experimental Limit Tc value

Initial investigation of the collapse temperatures of liposomes revealed a relationship with concentration. Concentration has previously been assumed to be independent of the collapse temperature since the Tc is dependent on the final frozen state and thermal history of a formulation and not the concentration of the eventual phase. To have structure, a formulation must have mass and density to create a volume of freeze dried cake, the density of a formulation which has undergone sublimation will then determine the level of cake structure. The cake must be significant enough to be visually classified as a suitable freeze dried cake; the exceeding of this point can be triggered from two perspectives which can be classified as follows:

i. Passing of the conventional Tc where the structure of the formulation exceeds a specific temperature and its structure is weakened and destroyed. This leaves an apparent empty volume on the freeze dry microscope analysis.
ii. Dilution to the limit that the density of structural product will not allow a volume of dried cake to be formed which can be classified as structural (limit Tc). In this case of dilution no structure is seen on cake formation.

It was this effect of limit Tc which was seen within liposome formulations. A population of liposomes of concentration 7.8 mg/ml was seen to collapse at -38 °C, 3.9 mg/ml and at -42 °C, 1.95 mg/ml. At 0.975 mg/ml the formulation had no discernable structural to its freeze-dried cake even at temperatures < -60 °C. The effect of approaching limit Tc can be seen firstly as an apparent reduction in Tc, which is likely to be of a subjective nature were the microscope operator perceives a difference in structure which may be viewed differently by different operators. Small distinctions of structure are exaggerated as the effective dried density appears reduced and small changes previously ignored are exaggerated. It can be seen that limit Tc should be used with a unit of concentration and potentially a temperature at which no structure could be detected. In the case of the suspension of 1μm liposomes the limit Tc was 0.0975 %w/v (@-60 °C). It should therefore be clear that freeze drying a formulation below its Tc will not always result in good freeze dried cake structure. The volume of cake produced exceeding the limit Tc temperature is insufficient to be described as structural and Tc becomes less valid. It is suggested Tg may be a better determinant of the most appropriate state at these low concentrations.

8.2.3.2 The different types of cycles and control

Since liposomes were used at a concentration of 7.8 mg/ml and the solution bulked with sugars limit Tc did not apply to any of the formulations under discussion in Cycle 8-5-Cycle 8-11 since the concentrations of sugar alone were significant enough to provide structure to the formulation.

Cycle 8-5 demonstrates a freeze drying cycle with high primary drying shelf temperatures. This type of cycle may be used for fast product production, or high fill depth products where production time is deemed to be too slow to dry at a more ambient temperature.

During Cycle 8-5 product temperatures were seen to exceed Tc for the sugar Melezitose (Tc= -22.2 °C), 90 % of all the liposome formulations had the physical appearances of collapse. The level of protection for the different sugars was then quite surprising since protection was expected to be minimal. Figure 8-36 (page 124) shows the level of protection imparted by differing levels of sugars to a 1μm liposome suspension. It can be seen that at levels greater than 75 mM of sugar, protection of the size does not appear to be further enhanced. Both sucrose and trehalose protect the modal size to a similar extent and out perform the two trisaccharide sugars, raffinalose and meleziose during this cycle.

Cycle 8-6 (page 125) demonstrates a freeze drying cycle with shelf temperatures equivalent to those which might be seen with a laboratory freeze drier with uncontrolled shelf temperature. It should be noted however that since both the vacuum level and shelf temperature for this cycle are controlled it should not be taken as a model for the less controllable freeze driers. The level of protection imparted by the sugars is significant at a much lower concentration (Figure 8-37 page125) than previously seen for Cycle 8-5 (Figure 8-36). With the exception of sucrose, modal sizes have a 25 % improvement over Cycle 8-5 with the addition of only 50 mM of sugar and the appropriate cycle modifications.

Collapse temperatures for specific formulations were exceeded in several cases throughout primary drying but did not result in product malformation. Surface abnormalities were apparent and may have been the result of localized collapsed areas within the bulk of the main cake which were not visable externally.

Cycle 8-7 (page126) combines both a low shelf temperature for primary drying with an annealing stage which thermally alters the frozen state of the formulation before freeze

drying. Since the annealing stage shown in Cycle 8-7 provides energy for crystal growth in the frozen state it was expected that a comparative decrease in drying time would occur (when compared with the equivalent -10 °C non annealed cycle). This was hypothesized to result from changes in the resistance to vapour flow b/μ (Table 6-10). This did not prove true and was thought to be a result of surface skin formed during annealing. This crust was likely to have very large resistance to vapour flow responding with a similar effect to the sequence shown in Figure 5-3 (page 38 - vial number 8) although no collapse occurred during Cycle 8-7.

Protection of the liposome population was poor when compared with other low temperature primary drying cycles (Cycle 8-8 & Cycle 8-9). Even those with product temperatures in excess of Tc (Cycle 8-6) still provided significantly better levels of protection. It is suggested that liposomes behave in a similar manor to proteins which have been shown to collect at phase changes because of their amphiphilic nature and are thus damaged as a result (Wang, 1999). Liposomes may become concentrated at the phase changes of the ice with further growth of the ice (induced by annealing) forcing them together.

Annealed formulations of liposomes are likely to be damaged before freeze drying, it appears sugars are unable to protect against this relocation of liposomes within the frozen structure which has important implications for liposome storage in the frozen state prior to freeze drying. Storage at temperatures of -20 °C can be assumed to allow the same annealing effects at a slower rate and as a result could have a heightened level of damage on the conclusion of freeze drying.

Cycle 8-8 (page 127) combines a low primary drying temperature with a relatively slow cooling rate for the formulations. Slow cooling is known to produce larger ice crystals and has therefore been hypothesised to produce formulations similar in structure to those of Cycle 8-7. The dense 'crust' like layer seen in the annealed formulations of Cycle 8-7 was not present for Cycle 8-8. This suggested the level of ice crystal growth was not as great as seen with an annealing stage.

The level of protection for liposome size was seen to be no greater than those provided by Cycle 8-6. The same level of protection was however achieved with much lower concentrations of sugar (75 mM).

Cycle 8-9 (page 128) provides for formulations loaded into vials ready cooled with liquid nitrogen. These vials were in contact with liquid nitrogen which will give them a temperature significantly below -125 °C and provides for extremely fast freezing of the formulation. This was therefore the inverse of Cycle 8-8 where large crystals were expected to form. The suggested production of small ice crystals formed during snap freezing should spatially separate individual liposomes in the frozen state.

Figure 8-40 shows a significant level of protection for liposomes with no lyoprotectant freeze dried under Cycle 8-9 conditions. It further shows the highest levels of protection seen in the freeze drying cycles described here. Sucrose at 125 mM allows a modal size change of only 2.4 μm. It is thought this is a combination of both optimal sugar concentrations and freeze drying parameters working together. Each component of the process (e.g. shelf temperature) or the formulation (e.g. sugar concentration) is able to protect differently orientated or spatially positioned liposomes that would previously have been damaged. This could be those liposomes of the population surrounding the surfaces of the freeze-dried material or those which spend more time conducting thermal energy at the base of a freeze drying vial.

8.3 Conclusion

8.3.1 Cross cycle comparison

It is of note that although disaccharides achieve the best levels of modal size protection for a liposome population, the trisaccharides were more effective in cases where the concentration of sugar was reduced. This can be seen in Figure 8-36, Figure 8-37 and Figure 8-39 where levels of protection are often significantly greater for raffinalose and meletzitose at concentrations of 50 and 75 mM but as the concentration is increased they

are quickly caught and overtaken by the levels of protection provided by disaccharide sugars at their higher concentrations.

It is suggested that trisaccharides may function in a similar manner to the hypothesis suggested for proteins, polymers and sugars (see Figure 7-30, page 106), with the trisaccharide sugar replacing the polymer in this example. Initially trisaccharides would be able to connect liposomes with a lower number of intermolecular connections than a disaccharide sugar which would require additional molecules to bridge the same size gap inbetween individual liposomes. However as the sugar concentration is increased more connections are less likely as steric hindrance prevents further approach in the trisaccarhide system. In contrast the disaccharides are hypothesised to initially be unable to connect liposomes together and keep them spatially separated; but as the concentration of sugar is increased, holding the liposomes apart becomes less important and the pure number of intermolecular bonds prevents quick movement of a liposome in both the frozen and solution state. This hypothesis would allow for the higher degree of protection demonstrated in disaccharide solutions.

Cycle 8-4 : Collapse point temperatures for some selected formulations

Cycle 8-5 : High primary shelf temperature freeze drying cycle.

Shelf temperature (°C)	Time (min)	Vac (µbar)	Control condition
≅ 15	0	-	Hold
Formulations loaded direct onto shelf			
-50	150	-	Ramp
-50	60	-	Hold
Primary drying			
+30	1200	10	Hold
Secondary drying			
+40	30	400	Ramp
+40	90	400	Hold
Vials are 23 mm∅ tubular 1 port stopper 8 ml max fill with a sample volume of 3 ml. Stoppered under 80 µbar vacuum and crimp sealed.			

Collapse temperatures (Tc) Lyostat1 freeze dry microscope	
Sucrose (50 mM)	-30
Trehalose (50 mM) (Figure 7-31 page108)	-26.5
Raffinalose (50mM)	-24
Melezitose (50 mM)	-23.5
Melezitose (50 mM) 15 min anneal -10 °C	-22.2

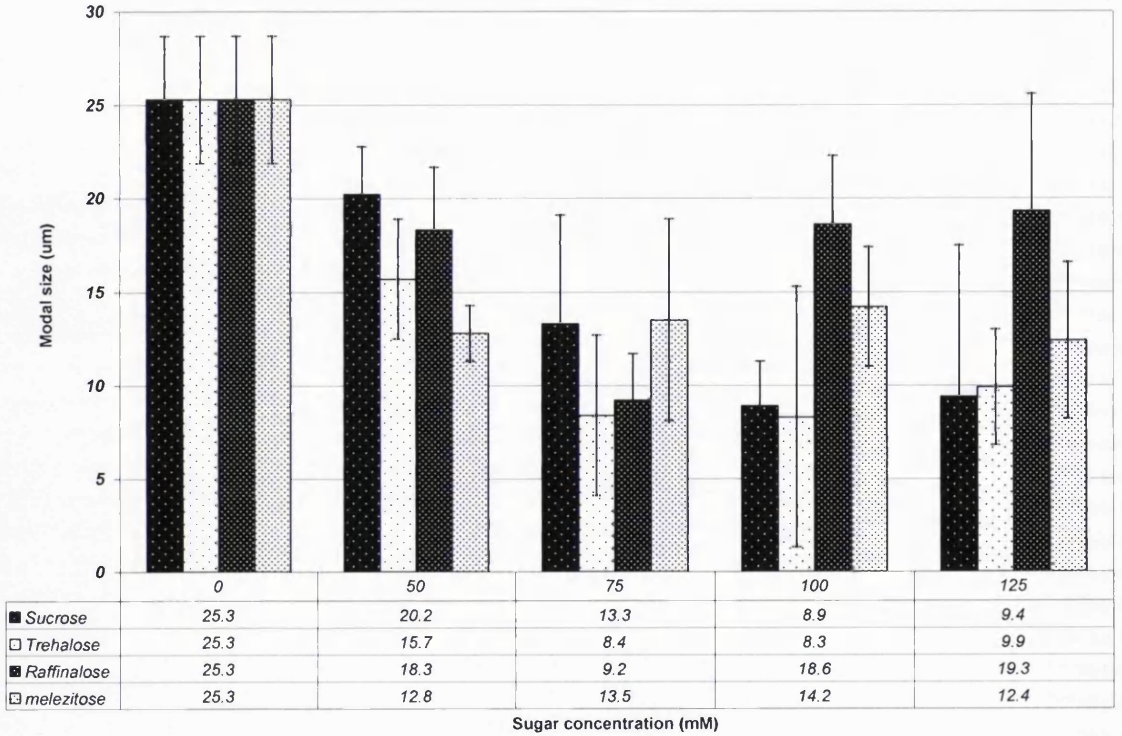
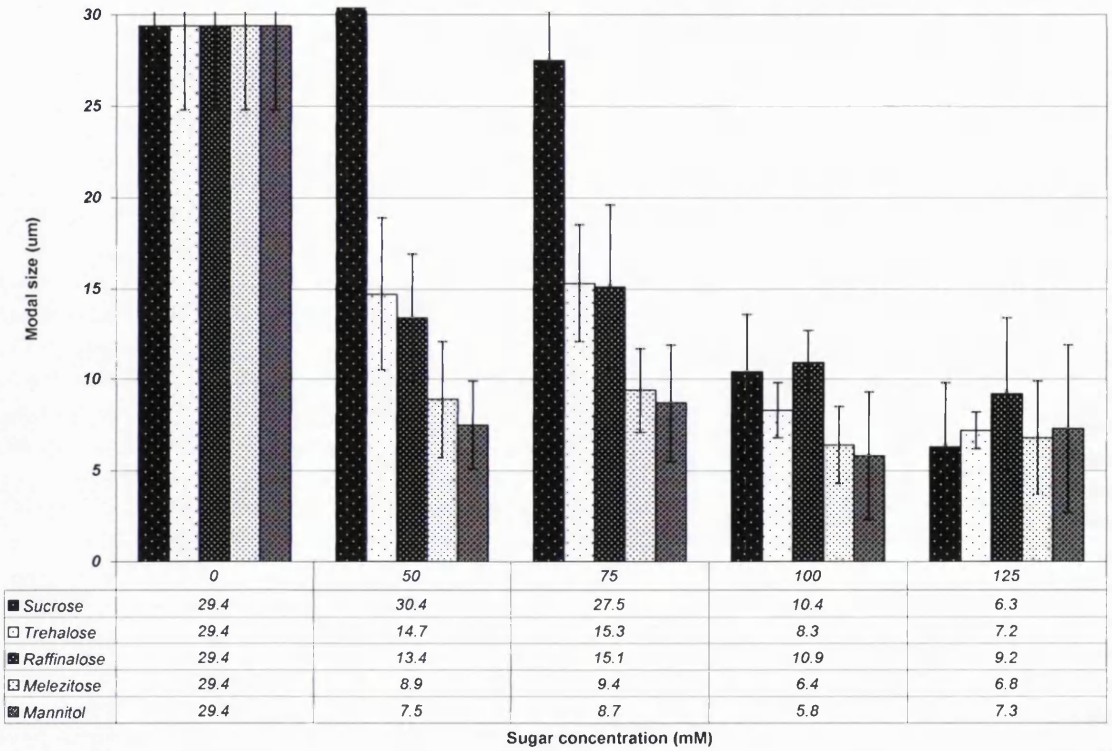


Figure 8-36 : Change in size of a liposome population (1.27 ± 0.3 µm) following freeze-drying with and without combined cryo- and lyo- protectants under the conditions of Cycle 8-5 were n=3.

Cycle 8-6 : Ambient shelf temperature freeze drying cycle.

Shelf temperature (°C)	Time (min)	Vac (µbar)	Control condition
≅ 15	0	-	Hold
Formulations loaded direct onto shelf			
-50	150	-	Ramp
-50	60	-	Hold
Primary drying			
+15	2460	100	Hold
Secondary drying			
+20	30	200	Ramp
+20	90	200	Hold
Vials are 23 mm∅ tubular 1 port stopper 8 ml max fill with a sample volume of 3 ml. Stoppered under 80 µbar vacuum and crimp sealed.			



	0	50	75	100	125
Sucrose	29.4	30.4	27.5	10.4	6.3
Trehalose	29.4	14.7	15.3	8.3	7.2
Raffinalose	29.4	13.4	15.1	10.9	9.2
Melezitose	29.4	8.9	9.4	6.4	6.8
Mannitol	29.4	7.5	8.7	5.8	7.3

Figure 8-37 : Change in size of a liposome population (1.43 ± 0.2 µm modal original size) after freeze drying with and without combined cryo- and lyoprotectants under the conditions of Cycle 8-6.

Cycle 8-7 : Annealed low shelf temperature freeze-drying cycle.

Shelf temperature (°C)	Time (min)	Vac (µbar)	Control condition
≅ 15	0	-	Hold
Formulations loaded direct onto shelf			
-50	150	-	Ramp
-50	60	-	Hold
-10	60	-	Ramp
-10	90	-	Hold
-50	60	-	Ramp
-50	5	-	Hold
Primary drying			
-10	2460	100	Hold
Secondary drying			
+20	30	200	Ramp
+20	90	200	Hold
Vials are 23 mm∅ tubular 1 port stopper 8 ml max fill with a sample volume of 3 ml. Stoppered under 80 µbar vacuum and crimp sealed.			

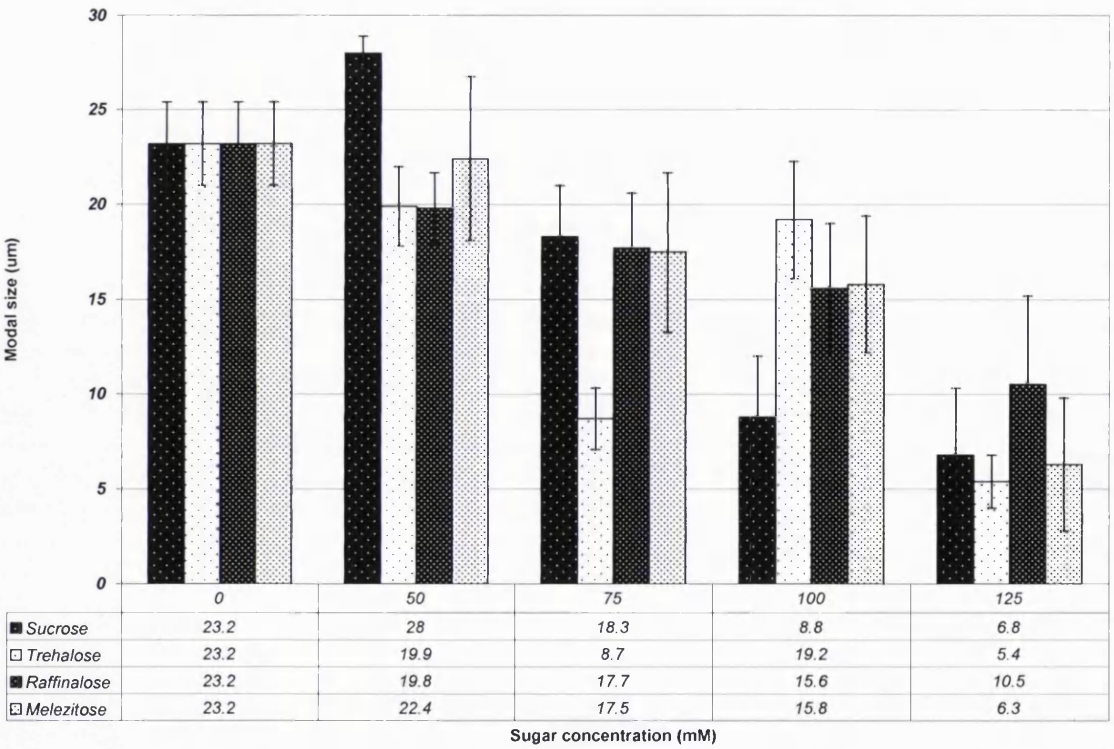
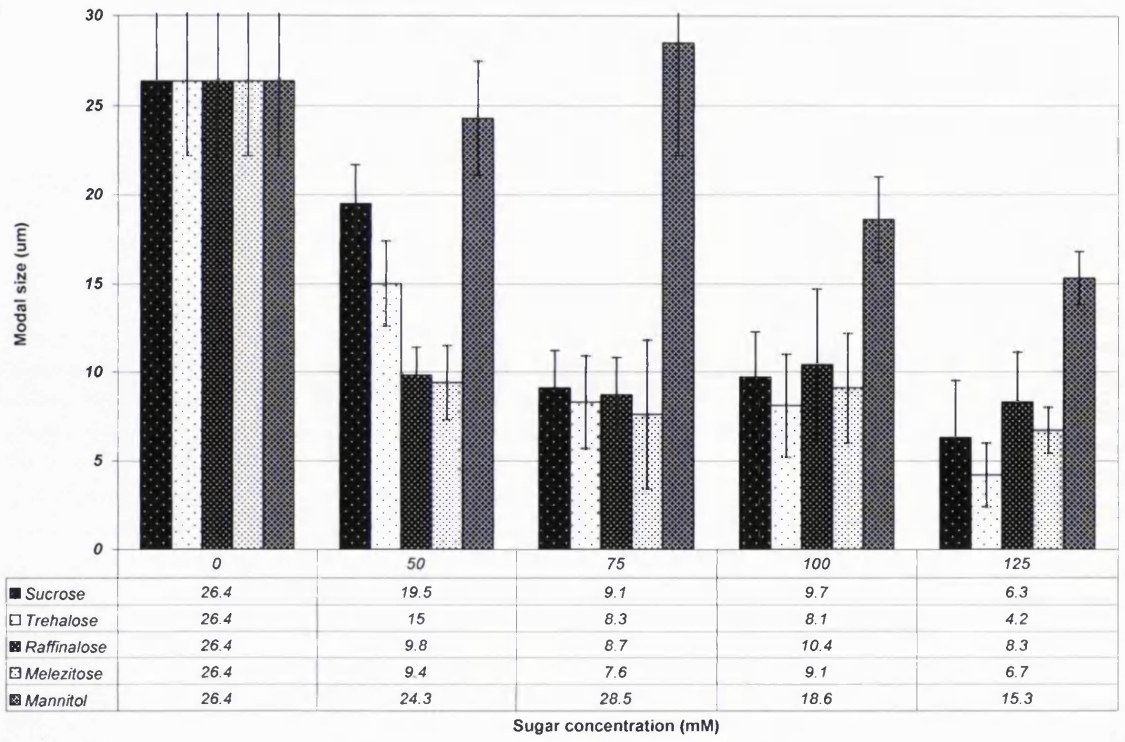


Figure 8-38 : Change in size of a liposome population (1.1 ± 0.1 µm modal original size) after freeze drying with and without combined cryo- and lyoprotectants under the conditions of Cycle 8-7 .

Cycle 8-8 : Low shelf temperature freeze-drying cycle.

Shelf temperature (°C)	Time (min)	Vac (µbar)	Control condition
≅ 15	0	-	Hold
Formulations loaded direct onto shelf			
-60	200	-	Ramp
-60	60	-	Hold
Primary drying			
-30	2460	100	Hold
Secondary drying			
+20	30	200	Ramp
+20	90	200	Hold
Vials are 23 mm∅ tubular 1 port stopper 8 ml max fill with a sample volume of 3 ml. Stoppered under 80 µbar vacuum and crimp sealed.			

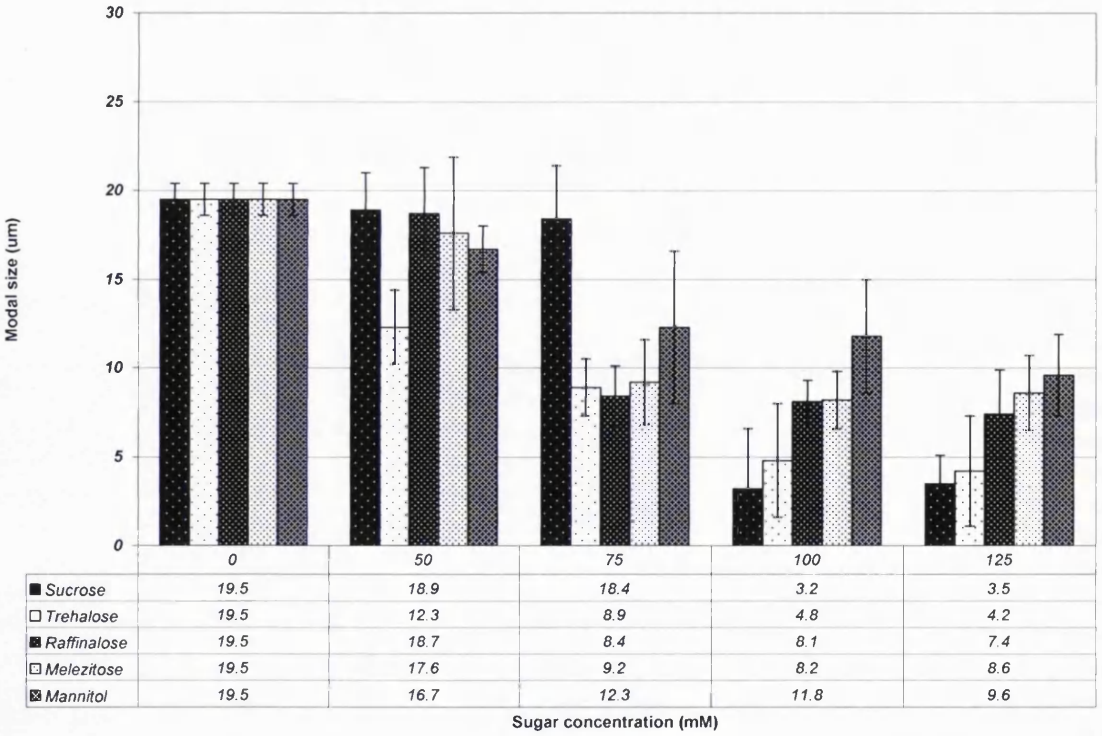


	0	50	75	100	125
Sucrose	26.4	19.5	9.1	9.7	6.3
Trehalose	26.4	15	8.3	8.1	4.2
Raffinalose	26.4	9.8	8.7	10.4	8.3
Melezitose	26.4	9.4	7.6	9.1	6.7
Mannitol	26.4	24.3	28.5	18.6	15.3

Figure 8-39 : Change in size of a liposome population (1.16 ± 0.3 µm modal original size) after freeze -drying with and without combined cryo- and lyoprotectants under the conditions of Cycle 8-8.

Cycle 8-9 : Fast freeze low shelf temperature freeze drying cycle.

Shelf temperature (°C)	Time (min)	Vac (µbar)	Control condition
≅ 15	0	-	Hold
Formulations loaded direct onto shelf♦			
-50	5	-	Ramp
-50	60	-	Hold♦
Primary drying			
-30	2460	100	Hold
Secondary drying			
+20	30	200	Ramp
+20	90	200	Hold
Vials are 23 mm∅ tubular 1 port stopper 8 ml max fill with a sample volume of 3 ml. Stoppered under 80 µbar vacuum and crimp sealed. ♦ Formulations loaded to liquid nitrogen pre-cooled vials <-130 °C. Note: high vial failure rate noted. Formulations pre-chilled to 3-4 °C before addition to vial.			



	0	50	75	100	125
Sucrose	19.5	18.9	18.4	3.2	3.5
Trehalose	19.5	12.3	8.9	4.8	4.2
Raffinalose	19.5	18.7	8.4	8.1	7.4
Melezitose	19.5	17.6	9.2	8.2	8.6
Mannitol	19.5	16.7	12.3	11.8	9.6

Figure 8-40 : Change in size of a liposome population (1.1 ± 0.2 µm modal original size) after freeze drying with and without combined cryo- and lyoprotectants under the conditions of Cycle 8-9.

Cycle 8-10 : Pulse type sublimation rehydration - 8.5 page 139 and Figure 8-44a

Shelf temperature (°C)	Time (min)	Vac (µbar)	Control condition
Initial freeze drying conducted as for Cycle 8-7♦			
-50	360	100	Hold
Formulations direct on shelf			
Condenser cycled on and off. -50°C on, >- 65 °C off.			
Vials stoppered 100 µbar.			
+20	10	-	Ramp
Melt			
+20	30	-	Hold
♦Primary drying was reduced in length to 1000 minutes. Vials are 23 mm∅ tubular 1 port stopper 8 ml max fill with a sample volume of 3 ml.			

Cycle 8-11 : Vial as condenser type sublimation rehydration - 8.5 page 139 and Figure 8-44b

Shelf temperature (°C)	Time (min)	Vac (µbar)	Control condition
Initial freeze drying conducted as for Cycle 8-10			
Formulations mounted inverted onto condenser			
-50	30	-	Ramp
-50	120	-	Hold
Pre-frozen -20°C 300-400ml block of ice loaded to shelf.			
-50	120	-	Hold
-30	120	100	Ramp
-30	240	100	Hold
Vials are 23 mm∅ tubular 1 port stopper 8 ml max fill with a sample volume of 3 ml.			

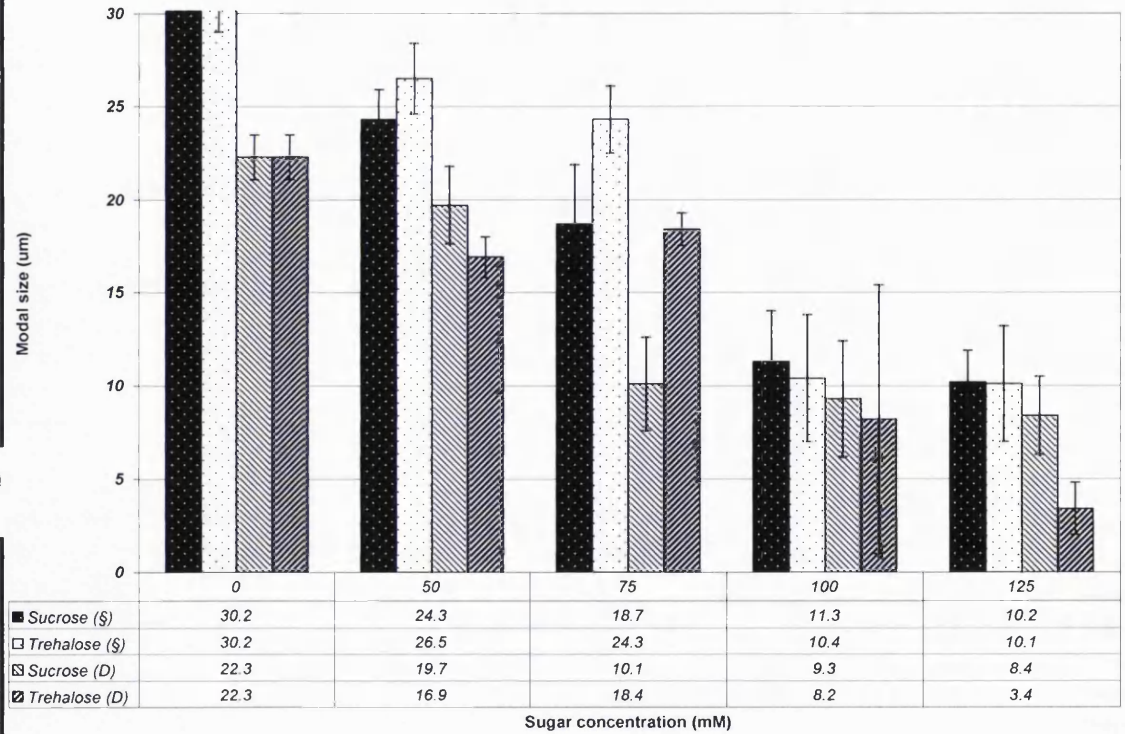


Figure 8-41 : Comparisons of two reverse freeze -drying cycles (sublimation rehydration) effect on liposome size. Initial liposome population with a modal size of 1.0 (± 0.3) µm. Cycle 8-10 (§) condenser pulsing. Cycle 8-11 (D) Vial as condenser. Figure 8-44 shows orientation of reverse freeze drying chamber for § & D.

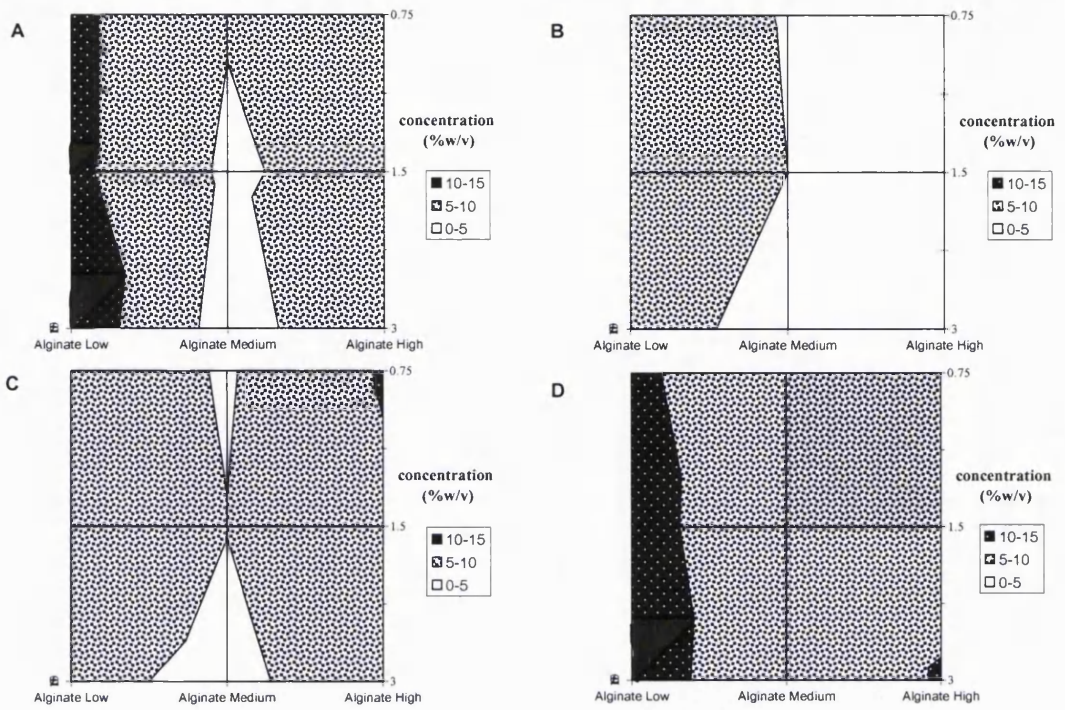


Figure 8-42 : Liposome population modal size after freeze drying through a) Cycle 8-5, b) Cycle 8-6, c) Cycle 8-7, d) Cycle 8-8 with varying grades (low, medium, high viscosity) and concentrations (0.75 – 3 %w/v) of sodium-alginate. Initial population with a modal size of 1.3 (± 0.1) μm. liposome modal size is shown grouped into bands. Those with large modal size 10-15μm are shown as black areas, 5-10 μm are partially shaded, and 0-5 μm are clear areas.

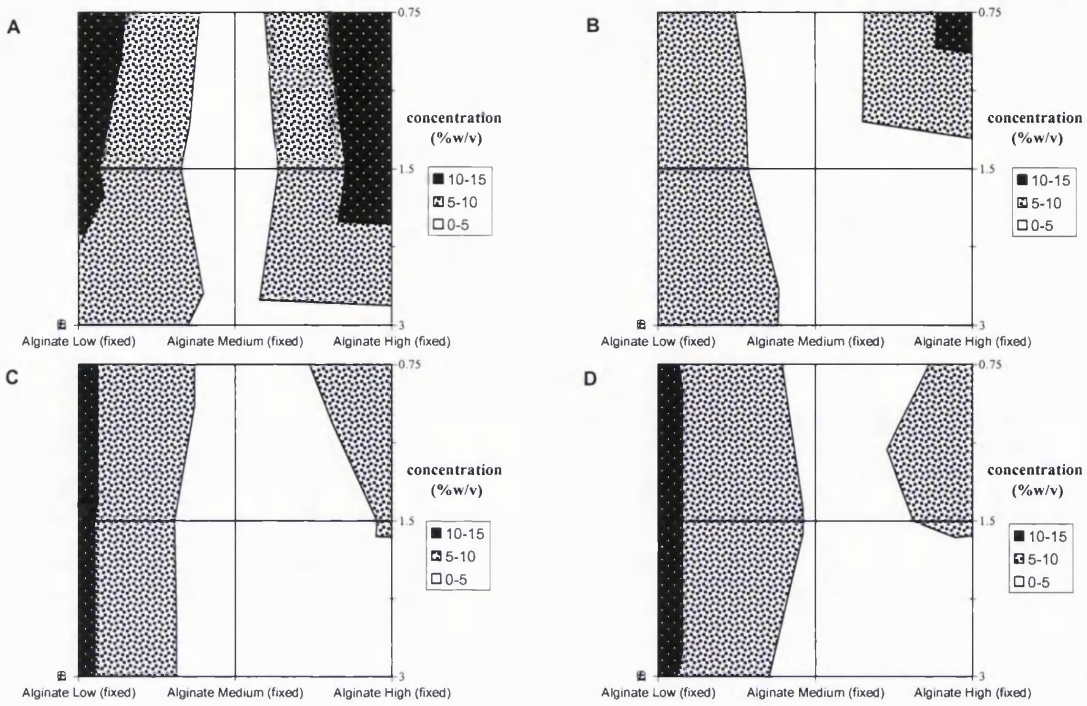
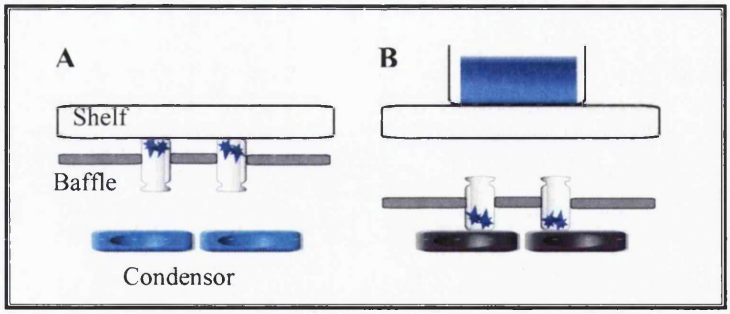


Figure 8-43 : Liposome population modal size after freeze drying through a) Cycle 8-5, b) Cycle 8-6, c) Cycle 8-7, d) Cycle 8-8 with alginate fixed with $CaCl_2$ prior to freeze drying and dissolved with sodium-citrate on completion. Initial population with a modal size of 1.3 (± 0.1) µm. Those with large modal size 10-15 µm are shown as black areas, 5-10 µm are partially shaded, and 0-5 µm are clear areas.

Figure 8-44 : Reverse freeze drying, dryer configuration. A) shows inverted vials with pulsing condenser setup. Water for rehydration comes from the condenser. B) Vial as condenser setup. Water for rehydration comes from a block of ice on the shelf surface.



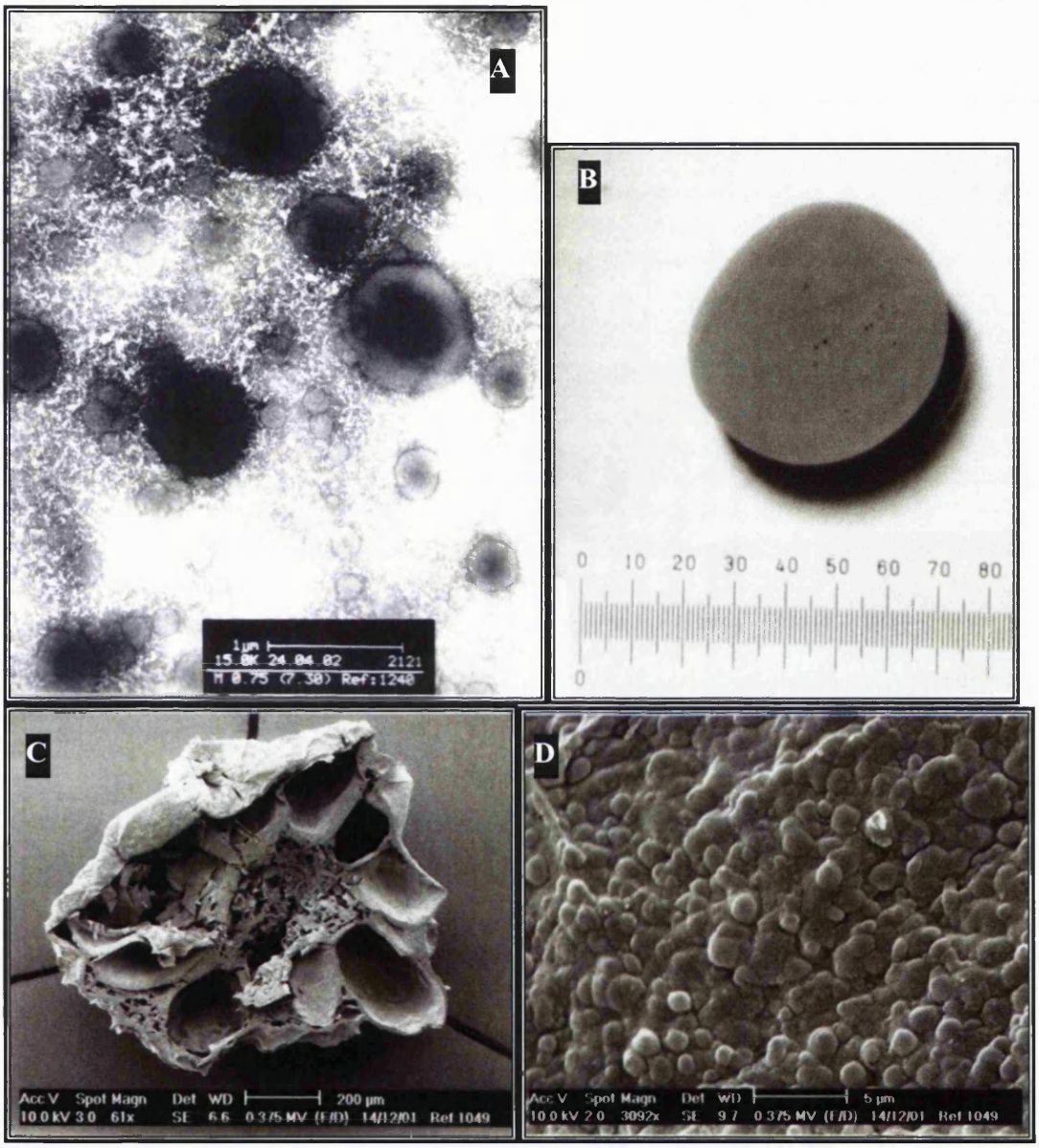


Figure 8-45 : A) TEM negatively stained 1 µm modal volume diameter liposome population in medium viscosity alginate. B) Light microscopy of formed alginate bead containing liposomes prior to freeze-drying (measuring graticule = 0.8 mm). C) Electron microscope image of freeze-dried Na-alginate bead cut to expose core. Contains liposomes fixed throughout its surfaces. D) Magnification of freeze-dried bead surface shows high concentrations of surface abnormalities measuring around 1 µm. phospholipid concentration for all images is 7.8 mg/ml.

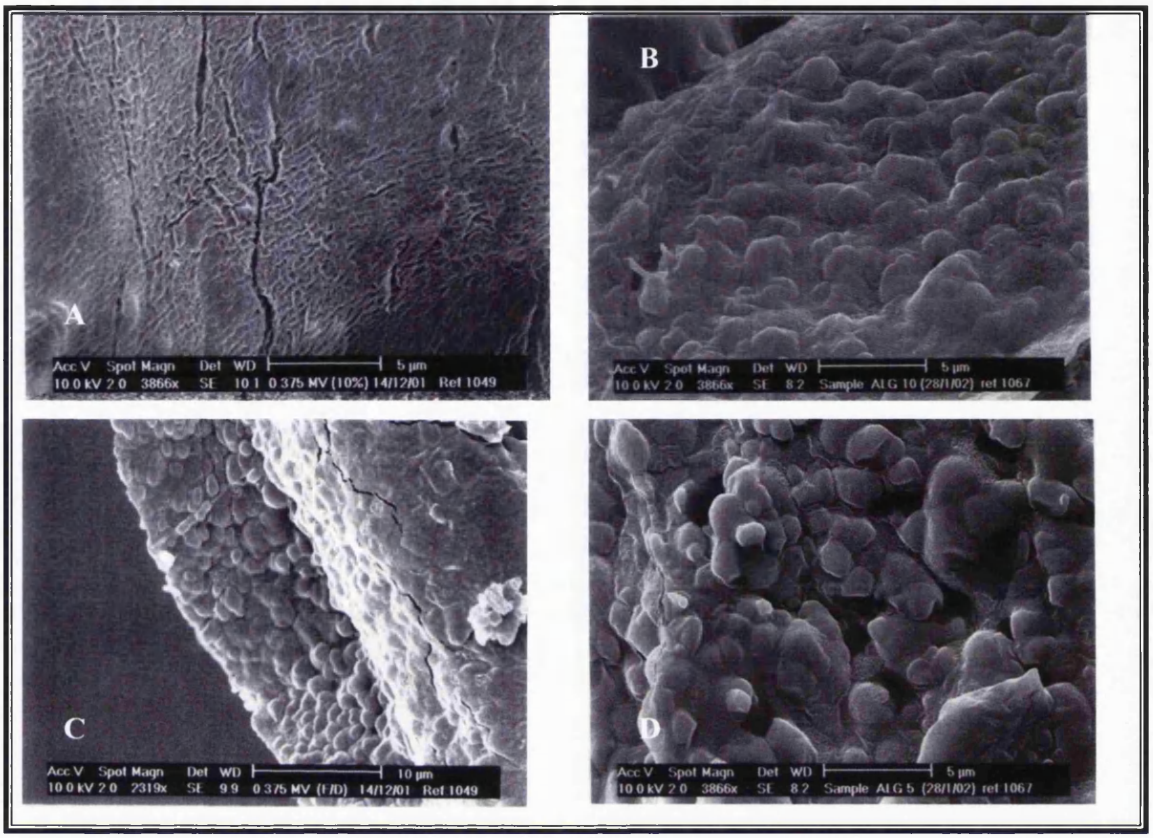


Figure 8-46 : Images represent increasing concentration of liposomes 1 μm (as lipid concentration) onto a fixed concentration of alginic acid. All alginic acid beads are 0.375 %w/v $CaCl_2$ fixed A) no liposomes B) 5.2 mg/ml lipid C) 7.8 mg/ml D) 10.4mg/ml. Size bars are 5 μm, 5 μm, 10 μm, 5 μm respectively.

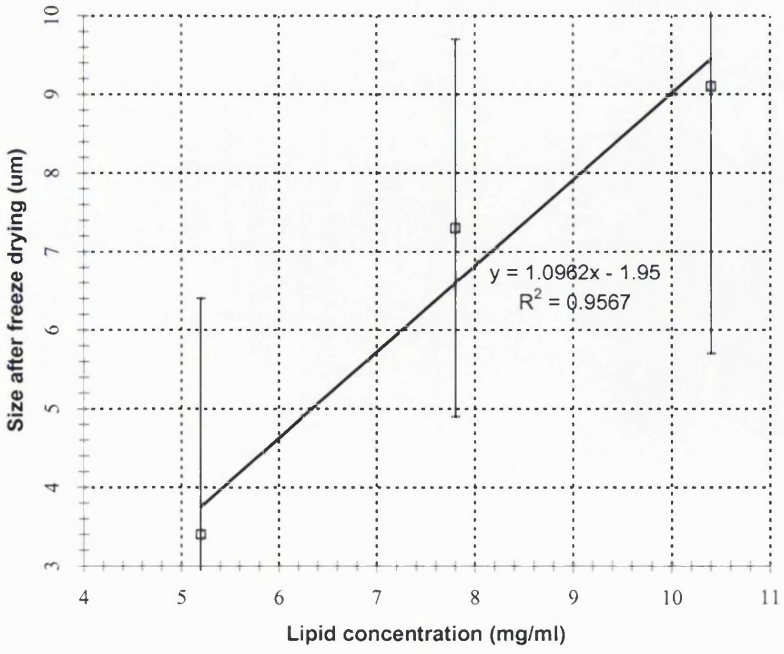


Figure 8-47 : Graph demonstrating effect on 1.27 μm (± 0.9) liposome population of increased lipid loading to medium viscosity alginic acid fixed beads of 0.375 %w/v. After Freeze drying (Cycle 8-7).

8.4 Physical isolation matrix

It was hypothesized that a matrix formed prior to freeze drying may hold individual or small groups of components in a fixed position allowing freezing and subsequent freeze drying to progress without the damage that would be apparent should the system be freeze-dried without this added support. An analogy to illustrate the hypothesis would be the erection of scaffolding to support walls of a building which when complete would have the scaffolding removed before becoming functional.

A liposome model was chosen to test the hypothesis since freeze drying damage to its size is clearly apparent from literature and previous study results (section 8.2). Differing methods to produce a scaffold matrix were experimented with which included the described chitosan crosslinking (Alexakis, Boadi et al., 1995) and starch formation (Artursson, Edman et al., 1984; Laakso and Sjoholm, 1987). The well characterized alginate scaffolds were used for the main studies. Scaffolds with pore sizes suitable to trap a liposome population of 1 μm can be produced in an automated manor with entrapment possible down to a liposome size of 50 nm (Klein, Stock et al., 1983).

8.4.1 Alginate scaffold matrix

Alginic acid is extracted from giant brown seaweed and is a generic name given to many derivatives. A unit of alginate acid is composed of a polysaccharide made up of L-guluronic acid and D- mannuronic acid (King, 1983). We used the L-guluronic acid (G-block) regions of the structure to form solid gel structure with the use of calcium ions. The strongest solid structures could be made with calcium ions, forming structures like that shown in Figure 8-45 (page 132 B).

Of course entrapment of an active component inside a structure such as an alginic acid matrix is no good if the active cannot be recovered after freeze drying. Formation of such a structure is therefore only half the challenge since the requirement to remove the matrix after freeze drying was required. This can be achieved in the case of a fixed alginic acid with the use of a sequestration such as sodium hexametaphosphate,

ethylenediaminetetraacetic acid (EDTA), disodium hydrogen orthophosphate or sodium citrate. Each sequestrate is capable of competitively removing calcium from the G-block binding positions within the gel structure and returning alginic acid back to its solution state.

To use the previous analogy of a building construction, a building would not be much use if the scaffolding used to erect it was actually damaging to the structure. The same can be said for a liposome suspension which is damaged by the erection of an isolating alginic acid matrix. Certain interactions within this system are known and discussed below.

8.4.2 Interaction of alginic acid and liposomes

Use of calcium salts in formation and sodium citrate (in removing the matrix) at the experimental concentrations found in these studies was not seen to effect modal size as determined by laser light scattering within the time period before fixing the alginate beads solid. Calcium has over time been shown to aggregate phosphatidylcholine (PC) liposomes at relatively low concentrations (1.8 mM) (Mosharraf and Taylor, 1995) but since fixing of the alginic acid is almost instantaneous this type of aggregation over a 10 hour period is unlikely to have an effect although the osmotic effect of unbalanced solutions should not be discounted.

Alginate has a characterized interaction with liposomes with a suggested insertion into the membrane resulting in a reduction in size (Cohen, Bano et al., 1991; Bosschetti, Gregooriadis et al., 1995). Comparisons were made of such insertions to the pins inserted in a buildings wall to support tall structures. Once formed by the mixing of a solution of alginate (1.5 %w/v) the liposomes reduce in size by a small degree (~0.2 μm) at their new smaller size they are quite stable (>6 hours) (Figure 8-45 A page 132). If a free flowing solution is required care must be taken to prevent contamination with any form of zwitterions likely to cause gelation, although use of a low level of sequestrate to scavenge contaminates is effective at preventing gellation prior to the gel fixing process.

The reduction in enthalpy of transition (ΔHg) for the glass transition of DPPC liposomes when alginic acid is added can be interpreted as allowing increased fluidity of the liposome but not disrupting its integrity (Bosschetti, Gregooriadis et al., 1995). This may also apply to the EPC system (Masayuki and Jun, 2001). The classification developed by Papahajopoulos (Papahadjopoulos, Moscarello et al., 1975) allows the liposome alginate mixture to be classified as follows:

- Surface binding followed by the partial penetration into the bilayer with enhanced permeability, expansion of the bilayer, and a decrease in both Tg and ΔHg.

8.4.3 Method

To provide a comparison of the effect of alginic acid in its solid state a liposome population was prepared both within an alginic acid matrix and with just alginic acid (without fixing with CaCl).

Egg phosphatidylcholine (EPC) was rehydrated (7.8 mg/ml) from a thin film containing cholesterol (EPC 16: 8 Cholesterol) to produce multilamellar vesicles. This colloid was then extruded to form large unilamellar vesicles of 1 μm, before the addition of varying concentrations of alginic acid at different viscosity grades. Liposomes were incubated at room temperature for 30 minutes and then sized using laser light scattering. Freeze drying was conducted as detailed in Cycle 8-5 through Cycle 8-8 in both the fixed state and unfixed state. The fixed state consisted of bead formation into 1 % calcium chloride solution through a pressurized needle producing beads of varied consistency and size. Once formed beads were washed with distilled water and based on 80 % recovery of liposomes were reconstituted to their original concentration before pipetting as 0.5 ml volumes on to the base of the appropriate freeze dry vial. Non fixed solutions were pipetted as individual volumes of 0.5 ml onto the base of the appropriate vial.

On completion of freeze drying (Figure 8-45 C & D – page 132) samples were stored at room temperature before a mass equivalent to 0.5 %w/v was resuspended in sodium

citrate 0.2 M and incubated. This was followed by repeat analysis of the size of the liposome population with the laser light scattering technique.

8.4.4 Results and discussion

Results are presented for fixed formulations and non fixed formulations in Figure 8-42 (page 130) and Figure 8-43 (page 131) respectively.

Fixed beads are seen to protect the size of the liposome population in almost all cases. Results of electron microscopy for the surface are similar to those previously seen for nanoparticles (Bodmeir, Chen et al., 1989) where the presence of particles is visible as adhered spheres. The level of liposome entrapment at high concentrations does reduce size protection (Figure 8-47) possibly through spatially interfering with crosslinking (increased surface stacking can be seen in Figure 8-46) as was seen on the effect of disintegration by Bodmeir and Chen *et al* (1989).

Use of the medium viscosity fixed gel gives the most significant increases in maintenance of the liposome size. Modal size is almost unchanged when medium viscosity alginate (1.5 %w/v) is used during Cycle 8-5, Cycle 8-6 &Cycle 8-7. Sizes before drying were 1.35, 1.27, 1.2 μm respectively, with sizes after freeze drying and scaffold removal of 1.7, 1.8, 1.2 μm respectively.

The use of an annealing cycle prior to freeze drying (Cycle 8-7 : Annealed low shelf temperature freeze-drying cycle.) provides damaging conditions to a liposome size. This is thought to be due to ice crystal growth forcing liposomes into closer contact where on rehydration they may fuse together. This effect has also been seen in the studies of liposomes and sugar, were the crystallising sugar mannitol with slow cooling performs particularly badly under-performing (at all concentrations) compared to the other sugars (Figure 8-39 – page 127). This hypothesis would also support the large improvement in size maintenance between unfixed and fixed gels within the freeze drying Cycle 8-8. Unfixed beads in this case have a size 5-15 μm for the majority of the grid (Figure 8-42 D) while the fixed solution shows a much larger potential area for the 0-5 μm particle

range (Figure 8-43 D). This is likely as fixed beads can be assumed to slowly recrystallise as they prevent diffusion of ice crystals, while the spherical nature may also provide a comparatively reduced product temperature because of reduced shelf contact. Both conditions would prevent the mannitol sugar crystallization speed.

It was thought the use of high viscosity alginate did not show significant levels of protection for fixed beads in part because of the difficultly in removing Ca^{2+} from the G-block binding sites and therefore the production of small contaminating particulate gel pieces. This would support the observation that at high primary and secondary drying temperatures beads were able to 'over-dry', strengthening the beads and causing a slowing of the dissolution on sequestrate incubation after drying. Both Cycle 8-5 and Cycle 8-6 with relatively high drying conditions showed a worsening of the ability of high viscosity gels to protect a liposome population.

8.4.5 Conclusions

Although the addition of alginate to a liposome formulation may have disadvantages including lowering of entrapment (results not shown) or aggregation due to zwitterion contamination, it can be employed successfully to maintain size distribution during freeze drying. Much of untargeted liposome technology is based on localization controlled by size (Anchordoquy, Carpenter et al., 1997; Castile and Taylor, 1999; Molina, Allison et al., 2002). Since size is such an important controlling factor it is vital this property is protected above others and this makes alginate bead protection a potential method.

It is of significant note that for a medium viscosity alginate bead, protection is higher than 100 mM of the sugars in all the considered freeze drying cycles Cycle 8-5 - Cycle 8-9. In the case of a poorly optimised freeze-drying cycle alginate fixing would be the preferred method.

In the case of oral delivery of liposomes, or delivery to body cavities unfixing of the alginate beads may be un-necessary since alginic acid has proven biocompatibility (Ulubayram, Nur Cakar et al., 2001; Speijers and Van Apeldoorn, 2002) and on

breakdown may release liposomes in an appropriate manner and suitably sized for possible transfection or depot effect (Gombotz and Wee, 1998).

We have developed a system of fixing the position of a small molecule before freeze drying. The scaffold system shows prevention of macromolecular alterations to a population of liposomes fixed within its structure and has greater protective properties than conventional sugars.

8.5 Reverse freeze drying

By modifying the typical orientation of a freeze-dryer to alter the product to become the condenser we were successfully able to sublime water from a frozen state and trap it within the structure of a freeze dried cake. Allowing the controlled melting of this solid provided a novel method of rehydration for a powder.

Freeze drying in its typical mode of operation functions by condensing water onto the surface of the condenser the continued process of water condensation results in the eventual removal of all water which leaves the sample dehydrated. If this process is reversed it can be seen that it is possible to condense water back inside a cooled powder cake. Water in this case is not absorbed as is the case when humidity is used to rehydrate a sample. Instead the powdered sample is actually just in very close contact with solid water, it could be said that the powder still has a low percentage moisture content which would be correct.

It was hypothesised that this type of rehydration may provide conditions that prevented particular types of damage caused or made possible by the process of freeze drying. It was hypothesized during the annealing stage of cycle 7 that liposomes were moved into closer spatial orientation Figure 8-48 (page 140) demonstrates this hypothesis. Step A shows two vials identically frozen, in step B the top vial is simply held at a low temperature while the lower vial is annealed. The annealing process grows the ice crystals size and must move the location of the liposomes. At step C the different vials are freeze dried under identical conditions the top vial has its liposomes distributed

evenly within the powder while the lower vial has the liposomes consentrated in a smaller area and has already caused the aggregation of 2 liposomes. The samples represented in D and G are identical but are to be rehydrated using different methods to demonstrate the hypothesised changes due to rehydration.

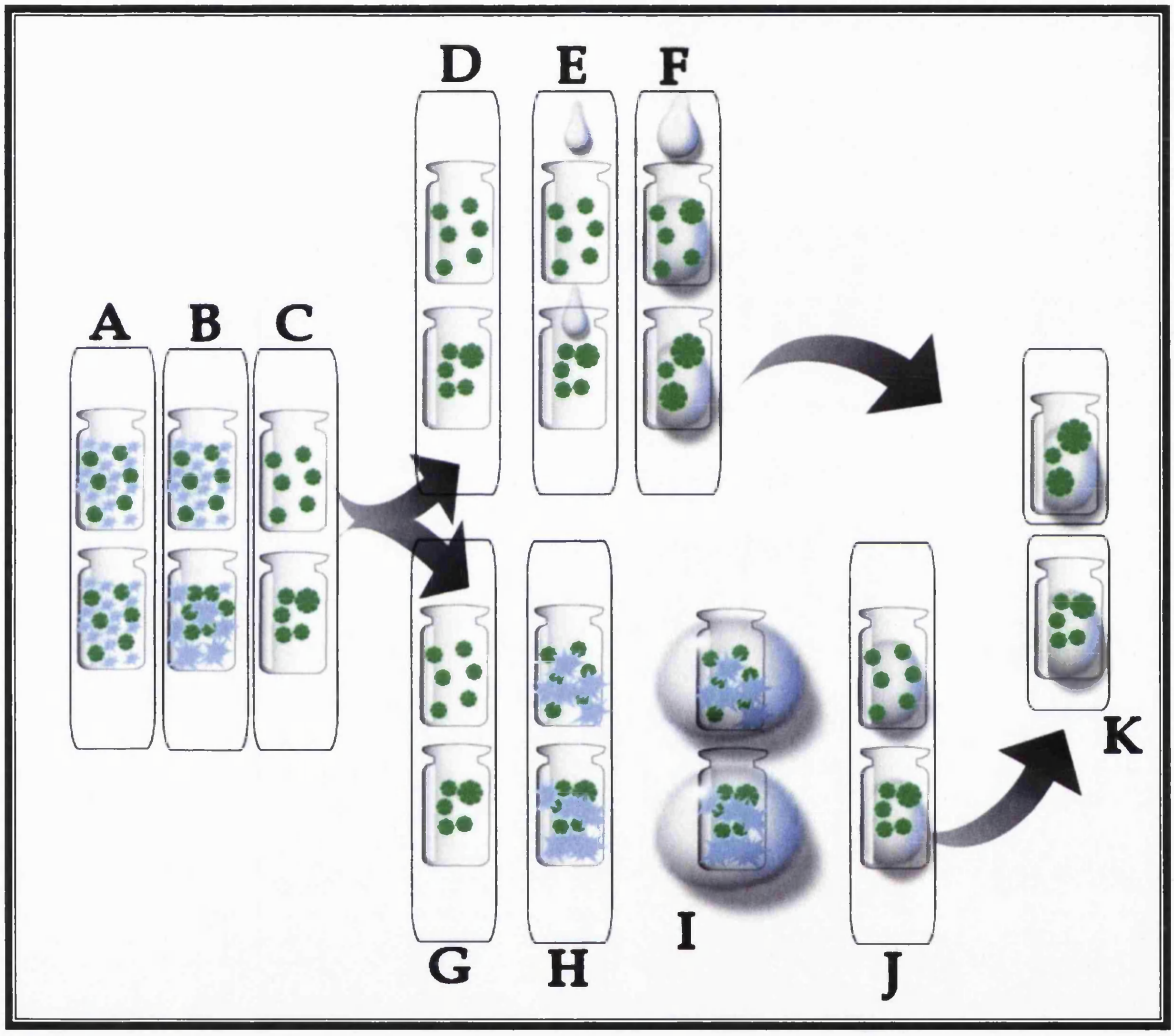


Figure 8-48 : Diagrammatic interpretation of how reverse freeze drying may help an annealed formulation of liposomes avoid excessive aggregation. (A) Vials are frozen in an identical manor – fast freezing produces small ice crystals (blue) and leaves liposomes (green) well dispersed. B – the bottom vial undergoes an annealing step like that of Cycle 8-7, ice crystals are allowed to grow producing larger ice crystals and moving liposomes closer together to do this. (C) After freeze drying liposomes are still held (sugar not shown) in the same position. (D) In this case liposomes are rehydrated with addition of a volume of liquid (12 °C). Those liposomes which were in very close contact (E- bottom) are aggregated (F- bottom) while those with closer proximity were not aggregated (F- top). (G) –(H) liposomes have water sublimed back into the cooled cake (-70 °C) they are then melted using a hot water bath or further addition of hot water (I). Both vials melted using this method have little difference (J) but as can be see in K the greatest difference in protection is seen when annealed vials are compared.

Step D vials are rehydrated conventionally with the drop wise (E) addition of water. The upper vial with distributed liposomes rehydrates with a small degree of aggregation of the liposomes (F). The lower vial because of the close proximity of the liposomes allows liposomes to be drawn together in the initial low levels of water resulting in aggregation.

Step G vials are rehydrated using the reverse freeze drying technique. Solid water is positioned within the powdered sample, the ice does not interact with the powder. By condensing on the surface of the cooled powder it forms within the same channels it was previously sublimed from and since the powder is colder at the base of vial it begins to form here first. Step H shows this addition of ice. To complete the rehydration of the sample the ice formed in step H is melted using a warm water bath (I). Step J shows the different rehydration of the two vials. The upper vial suffers minimal aggregation since at the moment of rehydration each liposome has a large amount of water to suspend in. The lower vial has similar conditions and the only aggregation seen is that which occurred earlier in the process (B). The main point of interest is when the lower (annealed) vials from both step F and step J are compared (this comparison is step K). These vials differ only in the method of rehydration and yet it can be seen that the vial rehydrated with drop wise addition of water has a larger degree of aggregation.

Figure 8-48 serves to demonstrate that the alternate method of rehydration described here is not predicted to cause benefit to all processed formulations rather it may only protect those that have been altered during storage or processed in a particular manner. We have used the process of annealing to demonstrate the system here but it may be just as relavent to formulations with skin forming properties.

To provide conditions suited to reverse freeze drying the powdered sample had to become the lowest temperature in the freeze drying system. By doing this it would become the condenser and collect ice. To provide these conditions samples were positioned to provide for these conditions in two differing arrangements. Using the condenser to cool the product cake, trapping the water from the shelf (Figure 8-44 -B) and using the shelf to

condense water back into the product by defrosting the condenser progressively while samples remained on the shelf (Figure 8-44 –A).

8.5.1 Method and results

Egg phosphatidylcholine (EPC) was rehydrated (7.8mg/ml) from a thin film containing cholesterol (EPC 16: 8 Cholesterol) to produce multilamellar vesicles. This colloid was then extruded to large unilamellar vesicles of 1μm. Both trehalose and sucrose were added at varying concentrations (Figure 8-41) then freeze dried with the recipe for Cycle 8-7 and rehydrated with either Cycle 8-10 or Cycle 8-11. Particle size was determined both before and after freeze drying with laser light scattering. Results are presented graphically in Figure 8-41 (page 129).

8.5.2 Conclusions

When the pulse type rehydration (Cycle 8-10) where the condenser is turned on and off in order to allow water to sublime into the chamber is used, liposome size protection is reduced below the levels seen when liposomes are protected by sugar alone and rehydrated in the conventional fashion. Figure 8-38 (page 126) shows sugar protection alone while Figure 8-41 (page 129) shows the protection to size offered by the two types of reverse freeze drying studied. Considering the experimental limitations of turning the condenser on and off repeatedly the poor performance is likely caused by the lack or control over condenser temperature specifically the temperature of the water at the surface of the condenser may suffer from large temperature swings this may have resulted in large amounts of water vapour in the chamber at specific opints. Melt back caused by the formation of liquid water is difficult to avoid in these conditions and the product appearance was characteristic of such an effect.

When using the vial as the condenser (Figure 8-44 b) and the freeze drier shelf to control rehydration (Cycle 8-11), there is an improvement over the pulse type rehydration method but no significant benefits over those of the sugar formulations with conventional rehydration.

To conclude, a freeze dryer configuration has been developed which is suited to sublimation of ice back into a freeze dried product cake. Although no significant improvement was gained for the protection of liposome size it is suggested the system maybe of use in partial hydration of samples and it is still to be proven that this system would not have a positive effect on activity of enzymes or other system.

9 Appendix

9.1 Endpoint determination for primary drying period

9.1.1 Perl script code for automating the correction of thermocouple data

```
#!/usr/local/bin/perl
# Code is available from www.eatcha.com in a windows and UNIX launch able format. You
should acknowledge this publication in any use of this software.

$Tice = 1;

$micead = "(m>ice) (Kg)";
print ("mass of frozen water (m>ice) (Kg):");
$mice = <STDIN>;
chop ($mice);

$Fad = "(F) (m2)";
print ("used surface area of the shelves (F) (m2):");
$F = <STDIN>;
chop ($F);

$dad = "(d) (m)";
print ("thickness of the product layer (d) (m):");
$d = <STDIN>;
chop ($d);

$Tshad = "(Tsh) (oC)";
print ("temperature of the shelf during MD maximal (Tsh) (oC):");
$Tsh = <STDIN>;
chop ($Tsh);

$Ticead ="(Tice) (oC)";
print ("temperature of the ice at the sublimation front (Tice) (oC):");
$Tice = <STDIN>;
chop ($Tice);

$Ph20chad ="(Ph20,ch) (mbar)";
print ("partial vapour pressure in the chamber during MD (Ph20,ch) (mbar):");
$Ph20ch = <STDIN>;
chop ($Ph20ch);

$Psad ="(Ps) (mbar)";
print ("equilibrium vapour pressure at Tice (Ps) (mbar) (will be calculated- press
enter):");
$Ps = <STDIN>;
chop ($Ps);

$tmdad ="(tmd) (h)";
print ("time of MD (tmd) (h):");
$tmd = <STDIN>;
chop ($tmd);

$LSad = "(LS) (Kj/Kg)";
print ("sublimtion energy of ice (LS) (Kj/Kg):");
$LS = <STDIN>;
chop ($LS);
```

```
$Ewad ="(Ew) (Kg/Kg)";
print ("part of water in the initial product (Ew) (Kg/Kg):");
$Ew = <STDIN>;
chop ($Ew);

$Pgad ="(Pg) (Kg/m3)";
print ("density of the frozen product (Pg) (Kg/m3):");
$Pg = <STDIN>;
chop ($Pg);

$Amad ="(Am) (Kg/Kg)";
print ("part of freezeable water, assumed as (Am) (Kg/Kg):");
$Am = <STDIN>;
chop ($Am);

$Agad="(Ag) (KJ/m h oC)";
print ("heat conductivity in the frozen product as $Agad :");
$Ag = <STDIN>;
chop ($Ag);


# verification of values to be put in equation.

print ("\n\nFigures to be calculated :\n");
if ( $mice <= 0) {
       print ("$micead has been set to 1.243\n");
       print ("please note : this is not advisable -  you should set the weight of
product\n");
       $mice = 1.243;
}else{
       print ("$micead $mice\n");
}

if ($F <= 0) {
       print ("$Fad has been set to 0.2193\n");
       print ("please note : this is not advisable - you should set surface area of
shelf\n");
       $F = 0.2193;
}else{
       print("$Fad $F\n");
}

if ($d <=0){
       print("$dad has been set to 7*10-3\n");
       print("please note : this is not advisable - you should set product height\n");
       $d = 0.007;
}else{
       print("$dad $d\n");
}

if ($Tsh == 0){
       print("$Tshad has been set to +10\n");
       print("please note : this is not advisable - you should set the shelf
temperature\n");
       $Tsh = 10;
}else{
       print("$Tshad $Tsh\n");
}

# care this checks Tice if it is equal to 0 ie no input it asks for non BTM data then
moves on. if it still equals 0 it sets it to -22

if ($Tice == 0){
       print("$Ticead Please set the product temperature at which freeze drying was
initiated this sets Tice in the case that BTM data is unavaliable :\n");
       $TiceNbtm = <STDIN>;
       $Tice = $TiceNbtm;
       if ($Tice <=0){
              print("This is not advised but $Ticead set to -22oC\n");
              $Tice = -22;
              }
```

```
}else{
        print("$Ticead $Tice\n");
}

if ($Ph20ch <=0){
        print("$Ph20chad has been set to 0.245mbar\n");
        $Ph20ch = 0.245;
}else{
        print("$Ph20chad $Ph20ch\n");
}

# Ps     To calculate this value you need reference 1.108 the equation for the graph.

if ($Tice >=-20){
                $eq="Y=6.2661e0.0892x";
                $m=6.266;
                $c=0.0892;
                $R2=0.9996;
        }elsif ($Tice >=-40){
                        $eq="Y=8.6315e0.1046x";
                        $m=8.6315;
                        $c=0.1046;
                        $R2=0.9995;
                }elsif ($Tice >=-60){
                                $eq="Y=19.211e0.1242x";
                                $m=19.211;
                                $c=0.1242;
                                $R2=0.9995;
                        }elsif ($Tice >=-80){
                                        $eq="Y=92.701e0.1499x";
                                        $m=92.701;
                                        $c=0.1499;
                                        $R2=0.9987;
                                }elsif ($Tice >=-100) {
                                        $eq="Y=1449.2e(0.1839x)";
                                        $m=1449.2;
                                        $c=0.1839;
                                        $R2=0.9992;
                        }
#calculation from the equation to go in here to give Ps

$power = $c*$Tice;

$Ps=$m * exp($power);

print("Ps has been set to $Ps based on a Tice of $Tice using equation $eq with an R2 value
of $R2\n");


if ($tmd <= 0){
        print("$tmdad has been set to 2.5h\n");
        print("please note: this is not advisable\n");
        $tmd = 2.5;
}else{
        print("$tmdad $tmd\n");
}


if ($LS == 2805){
        print("$LSad $LS\n");
                }elsif ($LS >=1) {
                        print("$LSad equals $LS    \n");
                        print("please note: you have specified a value other than 2805
kJ/kg\n");
                        }else{
                                print("please note: no value was entered set to 2805
kJ/kg\n");
                                $LS = 2805;
                                }

if ($Ew <=0){
```

```
        print ("$Ewad $Ew\n");
        print ("assumed $Ewad set to 0.931\n");
        $Ew = 0.931;
}else{
        print ("$Ewad $Ew\n");
}

if ($Pg <=0){
        print("$Pgad is set to 900\n");
        $Pg = 900;
}else{
        print("$Pgad $Pg\n");
}

if (Am <= 0){
        print("$Amad is set to 0.9\n");
        $Am = 0.9;
}else{
        print("$Amad $Am\n");
}

if (Ag <= 0){
        print("$Agad is set to 6.28\n");
        $Ag = 6.28;
}else{
        print("$Agad $Ag\n");
}

# values of equation have been listed and some calculated (as required)
# values logged as follows
#
# mice
# F
# d
# Tsh
# Tice
# Ttot  (need)
# Ph2och
# Ps
# Ap (need)
# tmd
# LS
# Ew
# Pg
# Am
# Ag
# we still need to calculate the above (need)

# calculation of Ktot
print("\n");
$Ttot=$Tsh-$Tice;

# fixin g  the calculation   - introduce the fixings bellow to correct the equation to
othat within page 65 text.
#$Ttot=43.88;
# fixing over

$Ap= $Ps-$Ph20ch;

#fixing
#$Ap=0.605;
#over fix

print("Ktot \n");
$Ktot=($mice*$LS)/($tmd*$F)*1/($Ttot);
print("(Mice * LS) / (Tmd*F) 1/ (Ttot) = Ktot\n");
print("($mice * $LS)/($tmd*$F)*1/($Ttot)=$Ktot\n");

# calculation of b/u
print ("\nb/u \n");
$bu=$mice/($tmd*$F) *($d/2/$Ap);
```

```
print("(mice/(tmd*F)(d/2/Ap)= b/u\n");
print("($mice/($tmd*$F)($d/2/$Ap)= $bu\n");

#calculation of term A,B,C,D
#A
#Define main equation AD definitions
$termAad="Pg(Ew*Am*LS*d)/Ttot";
$termBad="(1/Ktot)";
$termCad="(d/2 Ag)";
$termDad="(d/2 LS b/u)";
print ("\nmain equation used:\n $termAad { $termBad +$termCad +$termDad } \n\n");
print ("termA= $termAad\n");
print ("termB= $termBad\n");
print ("termC= $termCad\n");
print ("termD= $termDad\n\n");

$termA= $Pg* ($Ew*$Am*$LS*$d)/ $Ttot;
print("$termAad\n");
print("$Pg($Ew*$Am*$LS*$d)/$Ttot = $termA\n");
print("termA= $termA\n");

#termB
$termB= 1/$Ktot;
print ("\n$termBad\n1/$Ktot\n");
print ("termB= $termB\n");

#termC
$termC= $d/(2*$Ag);
print ("\n$termCad\n$d/(2*$Ag)\n");
print ("termC= $termC\n");

#termD
$termD= $d/(2*$LS*$bu);
print ("\n$termDad\n$d/(2*$LS*$bu)\n");
print ("termD= $termD\n");

#Tmd main drying calculated

$Tmd= $termA*($termB+$termC+$termD);

print ("\nmain equation used:\n $termAad { $termBad +$termCad +$termDad } \n\n");
print ("$termA*($termB+$termC+$termD)=$Tmd");

print ("\n \n \n Primary drying = $Tmd h");

#--------------REPEATER !---------------

do {

        print ("\n\nPress 1 to exit - Otherwise a repeat using the current data will be
performed\n\n>");
        $again = <STDIN>;
        chomp ($again);

        if ($again == 1) {exit};


        print ("mass of frozen water (m>ice) (Kg):");
        $mice2 = <STDIN>;
        chop ($mice2);

        if ($mice2 <= 0){
                print("left as $mice\n");
        }else{
                $mice = $mice2;
                print ("changed to $mice\n");
        }

        print ("used surface area of the shelves (F) (m2):");
        $F2 = <STDIN>;
        chop ($F2);
```

```
if ($F2 <= 0){
                print("left as $F\n");
        }else{
                $F = $F2;
                print ("changed to $F\n");
        }

        print ("thickness of the product layer (d) (m):");
        $d2 = <STDIN>;
        chop ($d2);

if ($d2 <= 0){
                print("left as $d\n");
        }else{
                $d = $d2;
                print ("changed to $d\n");
        }


        print ("temperature of the shelf during MD maximal (Tsh) (oC):");
        $Tsh2 = <STDIN>;
        chop ($Tsh2);

if ($Tsh2 == 0){
                print("left as $Tsh\n");
        }else{
                $Tsh = $Tsh2;
                print ("changed to $Tsh\n");
        }


        print ("temperature of the ice at the sublimation front (Tice) (oC):");
        $Tice2 = <STDIN>;
        chop ($Tice2);

if ($Tice2 == 0){
                print("left as $Tice\n");
        }else{
                $Tice = $Tice2;
                print ("changed to $Tice\n");
        }


        print ("partial vapour pressure in the chamber during MD (Ph20,ch) (mbar):");
        $Ph20ch2 = <STDIN>;
        chop ($Ph20ch2);

if ($Ph20ch2 <= 0){
                print("left as $Ph20ch\n");
        }else{
                $Ph20ch = $Ph20ch2;
                print ("changed to $Ph20ch\n");
        }


        print ("equilibrium vapour pressure at Tice (Ps) (mbar):");
        $Ps2 = <STDIN>;
        chop ($Ps2);

# Ps     To calculate this value you need reference 1.108 the equation for the graph.

if ($Tice >=-20){
                $eq="Y=6.2661e0.0892x";
                $m=6.266;
                $c=0.0892;
                $R2=0.9996;
        }elsif ($Tice >=-40){
                        $eq="Y=8.6315e0.1046x";
                        $m=8.6315;
                        $c=0.1046;
```

```
                $R2=0.9995;
        }elsif ($Tice >=-60){
                $eq="Y=19.211e0.1242x";
                $m=19.211;
                $c=0.1242;
                $R2=0.9995;
        }elsif ($Tice >=-80){
                $eq="Y=92.701e0.1499x";
                $m=92.701;
                $c=0.1499;
                $R2=0.9987;
        }elsif ($Tice >=-100) {
                $eq="Y=1449.2e(0.1839x)";
                $m=1449.2;
                $c=0.1839;
                $R2=0.9992;
        }
#calculation from the equation to go in here to give Ps

$power = $c*$Tice;

$Ps=$m * exp($power);

print("Ps has been set to $Ps based on a Tice of $Tice using equation $eq with an R2 value
of $R2\n");

print ("time of MD (tmd) (h):");
$tmd2 = <STDIN>;
chop ($tmd2);


if ($tmd2 <= 0){
        print("left as $tmd\n");
}else{
        $tmd = $tmd2;
        print ("changed to $tmd\n");
}


print ("sublimtion energy of ice (LS) (Kj/Kg):");
$LS2 = <STDIN>;
chop ($LS2);

if ($LS2 <= 0){
        print("left as $LS\n");
}else{
        $LS = $LS2;
        print ("changed to $LS\n");
}


print ("part of water in the initial product (Ew) (Kg/Kg):");
$Ew2 = <STDIN>;
chop ($Ew2);

if ($Ew2 <= 0){
        print("left as $Ew\n");
}else{
        $Ew = $Ew2;
        print ("changed to $Ew\n");
}



print ("density of the frozen product (Pg) (Kg/m3):");
$Pg2 = <STDIN>;
chop ($Pg2);

if ($Pg2 <= 0){
        print("left as $Pg\n");
}else{
```

```
                $Pg = $Pg2;
                print ("changed to $Pg\n");
        }


        print ("part of freezeable water, assumed as (Am) (Kg/Kg):");
        $Am2 = <STDIN>;
        chop ($Am2);

if ($Am2 <= 0){
                print("left as $Am\n");
        }else{
                $Am = $Am2;
                print ("changed to $Am\n");
        }


        print ("heat conductivity in the frozen product as $Agad :");
        $Ag2 = <STDIN>;
        chop ($Ag2);

if ($Ag2 <= 0){
                print("left as $Ag\n");
        }else{
                $Ag = $Ag2;
                print ("changed to $Ag\n");
        }

#   now just a repeat from the main intro portion

# calculation of Ktot
print("\n");
$Ttot=$Tsh-$Tice;

# fixin g  the calculation   - introduce the fixings bellow to correct the equation to
othat within page 65 text.
#$Ttot=43.88;
# fixing over

$Ap= $Ps-$Ph20ch;

#fixing
#$Ap=0.605;
#over fix

print("Ktot \n");
$Ktot=($mice*$LS)/($tmd*$F)*1/($Ttot);
print("(Mice * LS) / (Tmd*F) 1/ (Ttot) = Ktot\n");
print("($mice * $LS)/($tmd*$F)*1/($Ttot)=$Ktot\n");

# calculation of b/u
print ("\nb/u \n");
$bu=$mice/($tmd*$F) *($d/2/$Ap);
print("(mice/(tmd*F)(d/2/Ap)= b/u\n");
print("($mice/($tmd*$F)($d/2/$Ap)= $bu\n");

#calculation of term A,B,C,D
#A
#Define main equation AD definitions
$termAad="Pg(Ew*Am*LS*d)/Ttot";
$termBad="(1/Ktot)";
$termCad="(d/2 Ag)";
$termDad="(d/2 LS b/u)";
print ("\nmain equation used:\n $termAad { $termBad +$termCad +$termDad } \n\n");
print ("termA= $termAad\n");
print ("termB= $termBad\n");
print ("termC= $termCad\n");
print ("termD= $termDad\n\n");

$termA= $Pg* ($Ew*$Am*$LS*$d)/ $Ttot;
print("$termAad\n");
```

```
print("$Pg($Ew*$Am*$LS*$d)/$Ttot = $termA\n");
print("termA= $termA\n");

#termB
$termB= 1/$Ktot;
print ("\n$termBad\n1/$Ktot\n");
print ("termB= $termB\n");

#termC
$termC= $d/(2*$Ag);
print ("\n$termCad\n$d/(2*$Ag)\n");
print ("termC= $termC\n");

#termD
$termD= $d/(2*$LS*$bu);
print ("\n$termDad\n$d/(2*$LS*$bu)\n");
print ("termD= $termD\n");

#Tmd main drying calculated

$Tmd= $termA*($termB+$termC+$termD);

print ("\nmain equation used:\n $termAad { $termBad +$termCad +$termDad } \n\n");
print ("$termA*($termB+$termC+$termD)=$Tmd");

print ("\n \n \n Primary drying = $Tmd h");

} while ( $again == n);

# ------------------- end of code -----------
```

9.2 Software for predicting protein lyophilisation properties

9.2.1 EAASC – e. Amino Acid Sequence Creator

```
#!/usr/local/bin/perl
# Code is available from www.eatcha.com in a windows and UNIX launch able format. You
should acknowledge this publication in any use of this software.
#!/usr/local/bin/perl
print "*****************************\n*  Amino Acid combination   *\n*         Andrew
Ingham     *\n*        www.eatcha.com     *\n*****************************\n\n\n";
# 8703v4.pl
# v5 - case sensitivity added.
# v6 - added new digit.
#
# This program was created by Andrew Ingham,
# All rights are reserved. except those specifically held by other copyright holders.
# you cannot copy this code.
#
#                                          Amino Acid combination algoritham,
#
#

# This program is capable of combining Aa into a specific sequence lenght to cover all
combinations
# At present it can combine upto 30 Aa and has a maximum sequence lenght of 6.
# Easy modifications make it possible to add both Aa and sequence lenght.

# UPdated definitions file!!
```

```
$currentdefinitions = "These are the current definitions in v2 of sequence counter : CASE
SENSITIVE
a  Any amino acid                    l  Aliphatic alkane side chains(GAVLIP)
r  Aromatic amino acid (FWYH)        i  Ionizable amino acid (DEHCYKR)
h  Hydrogen bodning (CWNQSTYKRHDE)   b  Hydrophobic (GAVLIPYFWMC)
c  Hydrophilic (NQSTKRHDECY)        s  Sulphur containing (CM)
n  Charged at neutral pH negative /acidic (DEC)
p  Charged at positive pH positive /basic (KRH)
e  Essential amino acids - (ABDCEQGPSY)
q  Non -essential amino acids - (RHILMFTWV)";


#
# To add Aa. add the line at ********** element conversion point ***** simply increase
both numbers in the list
# remember always have the high numbers at the top, other wise conversion takes place
multipuli and messes up.
#
#
# To add sequence lenght capabilities make sure digits are long enough ie. 100, 1000,
10000 so on
# above this maker repeat the if loop block changing figures with respect to pattern.
# *************digits for sequence length counting **********************
# you must also change this block
# ************ digit zero ******************
# you must have one more digit zero than you have if loops for! remember this or you will
get sudden stops.
#

print "Please enter - individual Amino acids (as one line CASE SENSITIVE) or enter \n 1
for IUB standard \n 2 for IUB advanced\n 3 for individual Amino acids with definitions \n
: ";
$Aa=<stdin>;chop $Aa;

if ($Aa == 1)
       { print "  (using IUB/IUPAC Aa code)
 A - Alanine
 B - Aspartic acid or Asparagine (not used)
 C - Cysteine
 D - Aspartic Acid
 E - Glutamic Acid
 F - Phenylalanine
 G - Glycine
 H - Histidine
 I - Isoleucine
 K - Lysine
 L - Leucine
 M - Methionine
 N - Asparagine
 P - Proline
 Q - Glutamine
 R - Arginine
 S - Serine
 T - Threonine
 V - Valine
 W - Tryptophan
 X - Unknown
 Y - Tyrosine
 Z - Glutamic acid or Glutamine\n\n";

@Aa                                                                                =
('A','B','C','D','E','F','G','H','I','K','L','M','N','P','Q','R','S','T','V','W','X','Y','
Z');
}elsif ( $Aa == 2) {

print "\n **** ADVANCED **** \n Set to use IUB/IUPAC Aa code : \n ABCDEFGHIKLMNPQRSTVWXYZ
\n $currentdefinitions \n\nEnter any additions : ";

$additionals = <stdin>;
$A = "ABCDEFGHIKLMNPQRSTVWXYZ";
$Aa = $A.$additionals;
```

```
@Aa = split ( '', $Aa);

}elsif ($Aa == 3) {

print "**** DEFINITIONS ****\n ";
print $currentdefinitions;
print "\nEnter Amino acid sequence : ";
$Aa=<stdin>;
chomp $Aa;
@Aa = split ( '', $Aa);


}else
       { print "**specified Amino Acids in use**\n\n";
@Aa = split ( '', $Aa);

};


print "@Aa \n";
print "you have specified ";print scalar @Aa; print " amino acids\n\n";

# slimit is vital for the attic count. When it is reached the next digit is counted.
$limit = scalar @Aa;

#
# this program will count in several different attic counting systems eg. 4444
#
# The value $limit is how many amino acids you have.
# The sequence is controlled by the digits. The user specifiys which digit (sequence
length will be the top digit) #
# with Save function - to file.



#specificy sequence length.
print "please enter the length of the sequence : ";
$topdigit = <stdin>; chomp $topdigit;
print "**sequence length set**\n";

# this sets all the variables to 0 for the start. always use one greater than maximum used
(memory problem)

# ************ digit zero ******************

$firstdig=0;
$seconddig=0;
$thirddig=0;
$fourthdig=0;
$fivedig=0;
$sixdig=0;
$sevendig=0;
$eightdig=0;

# ************ digit zero ******************



# request file name for storage
print "please enter file name for output (.txt) : ";
$outputfile = <stdin>; chomp $outputfile;

#Readies an outputfile. remember >> means append to this file (add on) not reset.

unless ( open(FILE,  ">>$outputfile")){
print "cannot open the file ";
exit;
}


#Main loop.
```

```
do {

$firstdig++;


if ($firstdig ==$limit) {
if ($topdigit ==1) {$end=1}
        $firstdig=0;
        $seconddig++;
}
if ($seconddig ==$limit) {
if ($topdigit ==2) {$end=1}
        $seconddig=0;
        $thirddig++;
}
if ($thirddig ==$limit) {
if ($topdigit ==3) {$end=1}
        $thirddig=0;
        $fourthdig++;
}
if ($fourthdig ==$limit) {
if ($topdigit ==4) {$end=1}
        $fourthdig=0;
        $fivedig++;}


if ($fivedig ==$limit) {
if ($topdigit ==5) {$end=1}
        $fivedig=0;
        $sixdig++;}

if ($sixdig ==$limit) {
if ($topdigit ==6) {$end=1}
        $sixdig=0;
        $sevendig++;};

if ($sevendig ==$limit) {
if ($topdigit ==7) {$end=1}
        $sevendig=0;
        $eightdig++;};

# *************digits for sequence length counting *********************

# this portion allows the numbers (of which ever attic to be converted) otherwise whould
have problems with eg 13 rather than 9

@array = ($eightdig,$sevendig,$sixdig,$fivedig,$fourthdig,$thirddig,$seconddig,$firstdig);

#useful  bug checking print statement
#print @array, "\n";

#   ***************** element conversion point for extra Aa's ************
foreach $element (@array) {
$element =~ s/30/$Aa[30]/g;
$element =~ s/29/$Aa[29]/g;
$element =~ s/28/$Aa[28]/g;
$element =~ s/27/$Aa[27]/g;
$element =~ s/26/$Aa[26]/g;
$element =~ s/25/$Aa[25]/g;
$element =~ s/24/$Aa[24]/g;
$element =~ s/23/$Aa[23]/g;
$element =~ s/22/$Aa[22]/g;
$element =~ s/21/$Aa[21]/g;
$element =~ s/20/$Aa[20]/g;
$element =~ s/19/$Aa[19]/g;
$element =~ s/18/$Aa[18]/g;
$element =~ s/17/$Aa[17]/g;
$element =~ s/16/$Aa[16]/g;
$element =~ s/15/$Aa[15]/g;
$element =~ s/14/$Aa[14]/g;
```

```
$element =~ s/13/$Aa[13]/g;
$element =~ s/12/$Aa[12]/g;
$element =~ s/11/$Aa[11]/g;
$element =~ s/10/$Aa[10]/g;
$element =~ s/9/$Aa[9]/g;
$element =~ s/8/$Aa[8]/g;
$element =~ s/7/$Aa[7]/g;
$element =~ s/6/$Aa[6]/g;
$element =~ s/5/$Aa[5]/g;
$element =~ s/4/$Aa[4]/g;
$element =~ s/3/$Aa[3]/g;
$element =~ s/2/$Aa[2]/g;
$element =~ s/1/$Aa[1]/g;
$element =~ s/0/$Aa[0]/g;
}

# puts the array into a single long string
$prin = join ('', @array);


@prin = split('', $prin);

#This section chops the waste of the front of the number. (i.e 6 digits to 3 etc.)

until ( $topdigit == scalar @prin) {
shift @prin;
}

#prints the final sequences

print @prin, "\n";



# Puts the information into the open output file. note difference to print above removes
spaces from inbetween letters

print FILE @prin;
print FILE "\n";


# this big until loop makes sure you only count attic to the relavent number, then stops
it here.
# use @prin to to see if should finish if all is equal to last letter of @Aa then
finished!.
$fin = 200;
$fin = join ('',@prin);
$endletter = @Aa[scalar @Aa];
$fin  =~ s/$endletter/0/g;



} until ($end == 1);

#put in sequences
#closes the text file when everything is finished.

close (file);
# ------------------- end of code -----------
```

9.2.2 ESFC – e. Sequence frequency counter

```
#!/usr/local/bin/perl
# Code is available from www.eatcha.com in a windows and UNIX launch able format. You
should acknowledge this publication in any use of this software.
#!/usr/local/bin/perl
print "*****************************\n*       Sequence counter    *\n*         Andrew
Ingham     *\n*        www.eatcha.com     *\n*****************************\n\n\n";
```

```
#
#Protein count sequences
#
# V2 31-7-2003, added all sequence modifys for protein possibles.




print "Enter the filename of the [FASTA] protein sequence (.txt) : \n";
$proteinfilename = <stdin>;

chomp $proteinfilename;

unless (open(fasta, $proteinfilename)) {
       print "cannot open file \"$proteinfilename\"\n\n";
       exit;
}


print "\nFileopen\n";

@protein = <fasta>;

print "File contents are :\n";
print @protein, "\n\n";

# this portion gets rid of all the useless lines and creates a single $string with
sequence data.

foreach $element (@protein) {
       if ($element =~ /^\s*$/) {next;
}elsif($element =~ /^>/) { next;
}elsif($element =~ /^\s*#/) { next;
}else{$string .=$element;
}
}
$string =~s/\s//g;

print "File sequence is : \n";
print $string, "\n\n";

# now to check for the data.

# First get data

print "Enter the filename of the sequence store you wish to search with (.txt) : \n";
$sequencefilename = <stdin>;

chomp $sequencefilename;

unless (open(sequen, $sequencefilename)) {
       print "cannot open file \"$sequencefilename\"\n\n";
       exit;
}

#file open
print "opened sequence data \n";

#would you like more than one protein processed?
#if yes
#ask for names by pushing onto @moreproteins
# once complete string is empty.
# print @proteins
# check we can open all files, give names based on the element ie $element
# once all are open



# this next bit is the file to save to.
```

```
#
$savefilename =$sequencefilename.$proteinfilename;
print "\n\n\nThe combined search data will be called : ", $savefilename, "\n";
$outputfile = $savefilename;
#Readies an outputfile. remember >> means append to this file (add on) not reset.

unless ( open(FILE,  ">>$outputfile")){
print "cannot open the file ";
exit;
}


#reading one line at a time check for sequence
print "\n\n\n\nNow ready to count from sequence file ", $sequencefilename, " in protein ",
$proteinfilename, "\n\n";
#print " If you would like to check for more than one protein enter them  now or enter to
begin ";
$go = <stdin>;



$count = 0;

#foreach $protein (@proteins)

do{
$seq= <sequen>;
chomp $seq;
# now protect the string - from the mod changes
$seqprotect = $seq;



########################################
# v2 - this mod allows you to search for anything at a specific position or
# a type of base - eg a hydrophobic - hydrophilic - ones begin with A lol!!
# each has its own - note - case sensitivity is important.

# this allows the matching of any particular base. it is 'a'
# putting in a chain of input amino acids like this AbCaaa - allows three unknowns in each
sequence.
$seq =~ s/a/[A-Za-z0-9]/g;            #       'a'
# this allows matching of Aliphatic (alkane) side chains: 'l'
$seq =~ s/l/[AGILPV]/g;               #       'i'
# this allows matching of Aromatic :          'r'
$seq =~ s/r/[FWYH]/g;
# this is Ionizable -                 'i'
$seq =~ s/i/[DEHCYKR]/g;
# h-bonding groups -                  'h'
$seq =~ s/h/[CWNQSTYKRHDE]/g;
# the hydrophobic -                   'b'
$seq =~ s/h/[GAVLIPYFWMC]/g;
# the hydrophilic -                           'c'
$seq =~ s/c/[NQSTKRHDECY]/g;
# sulphur containing -                        's'
$seq =~ s/s/[CM]/g;
# charged at neutral pH negative /acidic -    'n'
$seq =~ s/n/[DEC]/g;
# charged at neutral pH positive /basic -     'p'
$seq =~ s/p/[KRH]/g;
# essential amino acids -                     'e'
$seq =~ s/e/[ABDCEQGPSY]/g;
# non - essencial amino acids - (including FMR)      'q'
$seq =~  s/q/[RHILMFTWV]/g;
#### please update this definitions file and move into to the sequence production.pl ####
$currentdefinitions = "These are the current definitions in v2 of sequence counter : CASE
SENSITIVE
a  Any amino acid                    l  Aliphatic alkane side chains(GAVLIP)
r  Aromatic amino acid (FWYH)        i  Ionizable amino acid (DEHCYKR)
h  Hydrogen bodning (CWNQSTYKRHDE)   b  Hydrophobic (GAVLIPYFWMC)
```

```
c  Hydrophilic (NQSTKRHDECY)          s  Sulphur containing (CM)
n  Charged at neutral pH negative /acidic (DEC)
p  Charged at positive pH positive /basic (KRH)
e  Essential amino acids - (ABDCEQGPSY)
q  Non -essential amino acids - (RHILMFTWV)";


# counting
while ($string =~ /$seq/gi ) { $count++;}

print $seqprotect, " ", +$count, "\n";

# add the counted sequence to new file.

print FILE $seqprotect, " ", +$count, "\n";

#reset the count
$count =0;
#until $seq is empty ie end of file.
}until ($seq =~ /^\s*$/ );

close $sequencefilename;
close $FILE;
close $proteinfilename;
# ------------------- end of code -----------
```

9.2.3 ESA – e. Statistical correlator

```
#!/usr/local/bin/perl
# Code is available from www.eatcha.com in a windows and UNIX launchable format. You
should acknowledge this publication in any use of this software.
#!/usr/local/bin/perl

#@jim = ('30','22','1','0');
#@jime = ln_sub (\@jim);
#print "@jim", " ";
#print "\n";
#print "@jime", " ";

print "*****************************\n* Correlation confirmation  *\n*         Andrew
Ingham     *\n*        www.eatcha.com     *\n*****************************\n\n\n";
# 8703v4.pl
# This program was created by Andrew Ingham,
# All rights are reserved. except those specifically held by other copyright holders.

#v7 IMPORTANT UPDATE TO MATHS 21-7-2003.
#v8 IMPORTANT loop update to fix - output errors 22-7-2003
#v9 modified the error loops so they are more careful.
#checks for arrays of 0 entering calculation routines.
# also checks sequences if they are not the same error fails (maybe it should skip and
continue?)
#v10 small mods to output file,
#v11IMPORTANT protection against zeros into Log routines added.
#v12 cosm changes, but also ln transformation added.
#v12b Ln and Log sub routine errors causing 0 to show corrected. Now logs correct numbers
instead of reset to 1 been caused.

# multiply file opening and slurp reading of input files,
# reording into appropriate arrays.
#


# Each sequence data file for protein is opened
# first line is readed.
# this is then split to contain the two values sequence, and a number.

# these numbers then become X values,
# while user input provides Y values,
```

```
# statistics provides correlation data,
# and output of hitme_sub
# this then loops for second line of each file.


#########################################################
#
#              program begins
#
#########################################################

print "Input operator initals : "; $initals = <stdin>; chomp $initals;

# open many files and load the information into filehandles
print "\n\nNow enter protein-sequence-frequency files to be used (enter to end) : \n";
@filename = multifileopen_sub(\$_);
$filetotal = scalar @filename;
print "The program has ", scalar @filename, " protein-sequence-frequency files\n" ;
# on less than 2 files there is no value in doing any of this
if ( $filetotal < 3) {
        print "\n\n *** too few protein files ***\n\n";
        exit;
        }


# please enter the values which will be compared with these files, in the order displayed
above.
print "\n\n";
@freqy = getexptdata_sub (\@filename);

# this extracts 1 line of data from each filehandle and puts it one array. it needs to
know how many files to do this from. In this case this is done by counting the file names
(first line)
# rem- file total already exists above.
@filedata = newdataline_sub (\$filetotal);

# screads the data into pieces separating the two portions, it retains one copy of the
sequence in [0].
@freqx = strip_sub (\@filedata);

# now remove the sequence from front of X data. PROTEIN GOES IN AS X freq.
$sequence = shift (@freqx);

# Now request the percentage point data that maybe appropriate for small - n
distributions.
@p = (75,90,95,97.5,99,99.5,99.75,99.9,99.95);
@infinatepercentagepoints = (0.674,1.282,1.645,1.960,2.326,2.576,2.807,3.090,3.291);
print "\n\nNow enter the percentage points for the t-distribution. \nIf T has a
distribution v (Tv) The v value for your files is = ", scalar @freqy,"\n";
print"Or press enter to use 'infinate' values (below) : \n\nP(%) 75    90    95    97.5 99
99.5  99.75 99.9 99.95\n";
print " --> ", "@infinatepercentagepoints", " <--\n";
foreach $element (@p) {
        print " value for $element : ";
        $numb = <stdin>;
                if (not $numb =~ /^\s*$/) {
                        chomp $numb;
                        push (@percentagepoints, $numb);
                }else{
                         @percentagepoints = @infinatepercentagepoints;
                        last;
                }
}

# you have the option of looking for linear correlation with original data and log, ln
(natural loged) transformed values
print "\n\nYou have the option of looking for linear correlation \nwith the original data,
log10, and ln (natural log) transformed data,\nthe default (enter) is all : \n";
print "Please note 0 is treated as 1 for all log calculations.\n";
print "1 (on) 0 (off)\n";
```

```
# lets do it the tidy way and put everything in an array in case we decided to add more!
@onoroff = ('original data','log10','ln (natural log)');
foreach $element (@onoroff) {
        print " turn $element : ";
        $numb = <stdin>;
                if (not $numb =~ /^\s*$/) {
                        chomp $numb;
                        push (@regressionrequired, $numb);
                }else{
                         @regressionrequired = (1,1,1);
                        last;
                }
}

# tell the user all the data for the first sequence,
print "\n\nThese are the frequences of seq $sequence in the protein-sequence-frequency
files (freqX values) -->\n","@freqx","\n";
print "This is the contrasting experimental data --> \n", "@freqy", "\n\n\n";


# Do they agree the program is ready to go.
print "I am ready to begin processing the remaining data if you agree with the sample
above PRESS ENTER, otherwise enter N to exit\n";
$go =<stdin>;
chomp $go;
if (not $go =~ /^\s*$/ ) { print "\n\n *** stopped *** \n\n"; exit;};

##################################################
#
#               input from user is over.
#
##################################################



# now do stats on first line and output to newly opened file. and organised file.
#opening the save file:
# use date for filename.

($sec, $min, $hour, $mday, $mon, $year, $wday, $yday, $isdst) = gmtime;
$year += 1900 ;
$mon ++;

# this was standard portion to set up the clock. This next bit opens an output file
# you should notice that the file name is based on the date - and initals
# by using this method if the user creates this file then breaks and redoes the program
again
# within a minute the program assumes their wish to replace the file (this is probable as
a run must have been started by mistake!)

$outputfile = "$mday$mon$year-$min-$initals.txt";
unless ( open( savefile, ">$outputfile")) {
        print " Could not open a save file \n\n\n please check access rights\n ";
        exit;
        }

# setup inital file contents
# file output is specially formated for delimited (space) import & perl re-use to
spreadsheet program using alt 255!

print savefile "# Andrew Ingham, All rights Reserved.\n#
Copyright 2003 by Andrew Ingham.\n#\n# visit www.eatcha.com for information\n#
start time $hour:$min $mday/$mon/$year.\n";
print savefile "# protein-sequence-frequency files in use : -------> ", "@filename", "\n";
print savefile "# checking data against values : -------> ", "@freqy", "\n";
print savefile "#\n";
print savefile "# The next line is a definitions line-
 space separated import \n# into any standard spreadsheet will produce labelled columns of
 data. \n";
print savefile "#YonXorXonY(regression_line) sequence n meanx meany sumsquaresX
sumsquaresY sum(X*Y) sumSxy sumSxx sumSyy slopeofline intercept deviance Xvariance
```

```
Yvariance COvariance r r2 Criticalvalue PercentagePoints75 90 95 97.5 99 99.5 99.75 99.9
99.95 Significance (1=significant) rest_are_0\n";
# logs starttime
($sec, $min, $hour, $mday, $mon, $year, $wday, $yday, $isdst) = gmtime; $year += 1900 ;
$mon ++;
print "\n\n **** Started **** \n run start : $hour:$min $mday/$mon/$year ";


#####################################################################
#       Primary data string.
#               For notes on this section see main loop.
#####################################################################
#now print in the first lot of data important this happens before we begin loop.

$isitzero = sum_sub (\@freqx);
if ($isitzero == 0) {
print savefile ">SKIP $sequence frequencies all equal 0 skiped measurement\n";
       #do nothing to data
       # main loop will deal with second line.
} else {
       # do the calculation on first set of data.



               if ( @regressionrequired [0] == 1) {

                       @firstcalcxy = hitme_sub (\@freqx, \@freqy, \@percentagepoints);
                       print savefile "XY $sequence ","@firstcalcxy"," X ","@freqx"," Y
","@freqy","\n";
                       # this may look strange - but if you put the values in the other
way you can find the second line. In this case when you should use the
                       # output with what ever is fixed first - ie Y is always variable,
or X - is variable.
                       # in our case for proteins if we asumme the sequence is fixed we
look at Y on fixed X (so this second regression line).
                       @firstcalcyx = hitme_sub (\@freqy, \@freqx, \@percentagepoints);
                       print savefile "YX $sequence ","@firstcalcyx", " Y ","@freqx"," X
","@freqy","\n";
                       }
               if ( @regressionrequired [1] ==1) {

                       #LOG HAPPENS HERE
                       # calculation with loged values, - for notes see MAIN LOOP.
                       @freqxlog = @freqx;
                       @freqxlog = log_sub(\@freqxlog);

                       # this is required for a zero check again ! why? because logs of 1
will produce zero - therefore enter 0 into calculation again!
                               $isitzero = sum_sub (\@freqxlog);
                               if ($isitzero == 0) {
                                       print savefile ">SKIP $sequence
frequencies for the Logs equal 0 skiped measurement\n";
# this may be thought to need next in next loop ! it doesnt if you want it to check for
other transformations (I know ln 1 still equals 0) but if you add others it could take
ages to track this little f down.
                                       #do nothing to data
                                       # main loop will deal with second line.
                               } else {
                                       # do the calculation on first set of data. If not
all zero.

                               # this is basically the log routine - rest is just
protection.

                               # values are logged see if they are linear.
                               @logcalclxy = hitme_sub (\@freqxlog,  \@freqy,
\@percentagepoints);
                               print savefile "(logX)Y $sequence ","@logcalclxy"," X
","@freqxlog"," Y ","@freqy","\n";
       print "I'm here 2\n";
```

```
                                # now YonX
                                @logcalcylx = hitme_sub (\@freqy, \@freqxlog,
\@percentagepoints);
                                print savefile "(Y(logX) $sequence  ","@logcalcylx"," Y
","@freqxlog"," X ","@freqy","\n";
        print "just before Y logs\n";
                                # Y loged x-not logged.
                                # note how all arrays are preserved- xly, ylx, lxy, lyx
                                @freqylog = @freqy;
                                @freqylog = log_sub(\@freqylog);
        print "I dont think Ill fuk up\n";
                                # values are logged to see if they are linear
                                @logcalcxly = hitme_sub (\@freqx, \@freqylog,
\@percentagepoints);
                                print savefile "X(logY) $sequence ","@logcalcxly"," X
","@freqx"," Y ","@freqylog","\n";
                                # now logY on X
                                @logcalclyx = hitme_sub (\@freqylog, \@freqx,
\@percentagepoints);
                                print savefile "(logY)X $sequence ","@logcalclyx"," Y
","@freqx"," X ","@freqylog","\n";
                                }
                        ##########################################################
                        # this is the natural ln
                        ##########################################################

        if ( @regressionrequired [2] ==1) {
                @freqxln = @freqx;
                @freqxln = ln_sub(\@freqxln);

# this is required for a zero check again ! why? because logs of 1 will produce zero -
therefore enter 0 into calculation again!
                                $isitzero = sum_sub (\@freqxln);
                                if ($isitzero == 0) {
                                        print savefile ">SKIP $sequence
frequencies for the ln equal 0 skiped measurement\n";
# this may be thought to need next ! it doesnt if you want it to check for other
transformations (I know ln 1 still equals 0) but if you add others it could take ages to
track this little f down.
                                        #do nothing to data

                                } else {



                        # values are ln see if they are linear.
                        @logcalclxy = hitme_sub (\@freqxln,  \@freqy, \@percentagepoints);
                        print savefile "(lnX)Y $sequence ","@logcalclxy"," X ","@freqxln","
Y ","@freqy","\n";
                        # now YonX
                        @logcalcylx = hitme_sub (\@freqy, \@freqxln, \@percentagepoints);
                        print savefile "(Y(lnX) $sequence  ","@logcalcylx"," Y
","@freqxln"," X ","@freqy","\n";

                        # Y ln x-not lned.
                        # note how all arrays are preserved- xly, ylx, lxy, lyx
                        @freqyln = @freqy;
                        @freqyln = ln_sub(\@freqyln);

                        # values are ln to see if they are linear
                        @logcalcxly = hitme_sub (\@freqx, \@freqyln, \@percentagepoints);
                        print savefile "X(lnY) $sequence ","@logcalcxly"," X ","@freqx"," Y
","@freqyln","\n";
                        # now lnY on X
                        @logcalclyx = hitme_sub (\@freqyln, \@freqx, \@percentagepoints);
                        print savefile "(lnY)X $sequence ","@logcalclyx"," Y ","@freqx"," X
","@freqyln","\n"
                        }
                }

        }
```

```
}

#################################################################
#
#              main loop.
#
#################################################################



# now enter loop to complete files;
while ( $sequence == 0){

############################Most code just below here if all freqx are 0 skip to next

        # newdata line
        @filedata = newdataline_sub (\$filetotal);
        # strip
        @freqx = strip_sub (\@filedata);
        # shift the freqx - take sequence of front of array.
        $sequence = shift (@freqx);



                #end loop - when sequence is zero. all 0 that is or blank - must exit early
because otherwise will find a divid by zero in one of the statistic routines.
                # finish off and close file
                # check for end first (before check for all zeros).
                if ($sequence =~ /^\s*$/) {
                        ($sec, $min, $hour, $mday, $mon, $year, $wday, $yday, $isdst) =
gmtime;
                        $year += 1900 ;
                        $mon ++;
                        print "\n\n\n **** Finished **** \n run end : $hour:$min
$mday/$mon/$year ";
                        print savefile "\n\n\n **** Finished **** \n\n run end :
$hour:$min $mday/$mon/$year";
                        close (savefile);
                        exit;}

                # this if statment checks to see if the freqx is all zeros (would fail
stats) - if it is it doesnt bother with the statistics and just sends you back to top of
the mainloop - updating the savefile accordingly.
                # bad code I know -  just keeps it happy.
                $isitzero = sum_sub (\@freqx);
                $zero = 0;

                if ($isitzero =~ $zero) {

                        # get next information - tell savefile what is going on. skiping
stats for this.
                        print savefile ">SKIP $sequence
frequencies all equal 0 skiped measurement\n";

                        next;   #return to top of while loop, ie. ignore this set of
figures! or zeros!

                }


        # statistics.


                ################################################################
                #               calculation with Input values.
                ################################################################
        # notes:
        #               freqx data is PROTEIN DATA, (this would be fixed - assumed)
        #               freqy data is INPUT DATA, (this is measured and variable)
        #       X-has a  non-random value set
        #       Y-has a  random value set.          page 540.
```

```
	#

	#
	#	Special comment: have printed the raw data on end- is represented from the
view of the stats!- X is non variable Y- variable.
	#	when  they are re-arranged only the catogories are i.e method of input to
hit_me.
	#	Y,X are from view of stats, if you plot them in excel, you should get
straight line of that in data line.
	#
	if (@regressionrequired [0] == 1) {
		@firstcalc = hitme_sub (\@freqx, \@freqy, \@percentagepoints);
		# output to file.
		print savefile "XY $sequence ","@firstcalc"," X ","@freqx"," Y
","@freqy","\n";
		#second regression line, as described previous.
		@firstcalcyx = hitme_sub (\@freqy, \@freqx, \@percentagepoints);
		print savefile "YX $sequence ","@firstcalcyx"," Y ","@freqx"," X
","@freqy","\n";
		}


		#################################################################
		#		calculation with Loged values.
		#################################################################

	# to do, ask user, if they want these additions doing.- even if they wish to use -
linear regression,
	# so linear yes, ln yes, Log yes, square (dont know about this one should be ok).
	# note there is no point logging both sets of values fool!! (B.A. the A-team).
	# logged X - although we normally think of fixed (there still fixed just a
transformation).

	#
	#	Special comment: have printed the raw data on end- is represented from the
view of the stats!- X is non variable Y- variable.
	#	when  they are re-arranged only the catogories are i.e method of input to
hit_me.
	#	Y,X are from view of stats, if you plot them in excel, you should get
straight line of that in data line.
	#
	if ( @regressionrequired [1] ==1) {
		@freqxlog = @freqx;
		@freqxlog = log_sub(\@freqxlog);

# this is required for a zero check again ! why? because logs of 1 will produce zero -
therefore enter 0 into calculation again!
			$isitzero = sum_sub (\@freqxlog);
			$zero = 0;
			if ($isitzero == 0) {
				print savefile ">SKIP $sequence
frequencies for the Logs equal 0 skiped measurement\n";
# this may be thought to need next ! it doesnt if you want it to check for other
transformations (I know ln 1 still equals 0) but if you add others it could take ages to
track this little f down.
				#do nothing to data

			} else {


		# values are logged see if they are linear.
		@logcalclxy = hitme_sub (\@freqxlog,  \@freqy, \@percentagepoints);
		print savefile "(logX)Y $sequence ","@logcalclxy"," X
","@freqxlog"," Y ","@freqy","\n";
		# now YonX
		@logcalcylx = hitme_sub (\@freqy, \@freqxlog, \@percentagepoints);
		print savefile "(Y(logX) $sequence  ","@logcalcylx"," Y
","@freqxlog"," X ","@freqy","\n";

		# Y loged x-not logged.
```

```
                        # note how all arrays are preserved- xly, ylx, lxy, lyx
                        @freqylog = @freqy;
                        @freqylog = log_sub(\@freqylog);

                        # values are logged to see if they are linear
                        @logcalcxly = hitme_sub (\@freqx, \@freqylog, \@percentagepoints);
                        print savefile "X(logY) $sequence ","@logcalcxly"," X ","@freqx","
Y ","@freqylog","\n";
                        # now logY on X
                        @logcalclyx = hitme_sub (\@freqylog, \@freqx, \@percentagepoints);
                        print savefile "(logY)X $sequence ","@logcalclyx"," Y ","@freqx","
X ","@freqylog","\n";

                        }

                }

                ###########################################################
                # this is the natural ln
                ###########################################################

if ( @regressionrequired [2] ==1) {
                @freqxln = @freqx;
                @freqxln = ln_sub(\@freqxln);

# this is required for a zero check again ! why? because logs of 1 will produce zero -
therefore enter 0 into calculation again!
                                $isitzero = sum_sub (\@freqxln);
                                if ($isitzero == 0) {
                                        print savefile ">SKIP $sequence
frequencies for the ln equal 0 skiped measurement\n";
# this may be thought to need next ! it doesnt if you want it to check for other
transformations (I know ln 1 still equals 0) but if you add others it could take ages to
track this little f down.
                                        #do nothing to data

                                } else {



                        # values are ln see if they are linear.
                        @logcalclxy = hitme_sub (\@freqxln,  \@freqy, \@percentagepoints);
                        print savefile "(lnX)Y $sequence ","@logcalclxy"," X ","@freqxln","
Y ","@freqy","\n";
                        # now YonX
                        @logcalcylx = hitme_sub (\@freqy, \@freqxln, \@percentagepoints);
                        print savefile "(Y(lnX) $sequence  ","@logcalcylx"," Y
","@freqxln"," X ","@freqy","\n";

                        # Y ln x-not lned.
                        # note how all arrays are preserved- xly, ylx, lxy, lyx
                        @freqyln = @freqy;
                        @freqyln = ln_sub(\@freqyln);

                        # values are ln to see if they are linear
                        @logcalcxly = hitme_sub (\@freqx, \@freqyln, \@percentagepoints);
                        print savefile "X(lnY) $sequence ","@logcalcxly"," X ","@freqx"," Y
","@freqyln","\n";
                        # now lnY on X
                        @logcalclyx = hitme_sub (\@freqyln, \@freqx, \@percentagepoints);
                        print savefile "(lnY)X $sequence ","@logcalclyx"," Y ","@freqx"," X
","@freqyln","\n";

                        }

                }


###################################################################
#                       end of main loop.
###################################################################
```

```
}

# exit for good measure - never required.
exit;

#################################################################
#
#               Program subroutines.
#
#################################################################


########################################
# ln_sub
# this is the natural log of the number put back into the array.
# it has protection against zeros entering it (conversion to 1) - it will not matter if 1
is entered and is allowed to pass the resulting 0 to another routine!

sub ln_sub{
        my ($array) = @_;
        my @logged;
        my $zero = 0;
        foreach $element (@$array){
                if ( $element == 0) { $element = 1;}
                        my $number = log ($element);
                        push (@logged, $number);
                }
        return @logged;
        }


########################################
# log_sub
# This is log to the base 10. element Log'er - this is not with statistics because its not
a stat!
# take array and logs each element.
# this routine needs protection against 0 been inputed!
# coverts 0 to one- but doesn't warn user. (it could be the user should know!)- maybe I
should warn in text.

sub log_sub{
        my ($array) = @_;
        my @logged;
        my $zero = 0;
        foreach $element (@$array){
                if ( $element == 0) { $element = 1;}
                        my $number = log ($element)/log (10);
                        push (@logged, $number);
                }
        return @logged;
        }

########################################
# strip the file data - so it can be read.
# NOTE - this is not designed to work with negative numbers . OR decimal places.

sub strip_sub {
        my ($filedata)= @_;
        my @freqx;
        my @seqcheck;
        foreach $element (@$filedata){
                $element =~ /.*\s/;
                push (@seqcheck, $&);          # builds up array of all the sequence
portions at the start.
                $element =~  /\s\d*/;
                push (@freqx, $&);                    # builds up array full of numbers
from the file. these will be x
                }
        my $check = sequencecheck_sub (\@seqcheck);  # sends to sequence check to see if
sequence is same.
```

```
        if (my $check == 1) {print "\n\n Checking FAIL - sequence is not same\n\n ";
exit;}; # fails if the sequences are not same.

                        #note here one sequence of @seq data ie. AAA is removed and put on
the start of freqx data.
        $onesequence = @seqcheck [0];
        unshift (@freqx, $onesequence);

        return @freqx;
        }

##########################################
# sequencecheck_sub
# checks to see if all items in array are same, by seeing it the first item is repeated
throughout.
# 1 is given if the check fails

sub sequencecheck_sub {
        my ($array) = @_;
        my $check;
        $previous = $$array [0] ;
                foreach $element (@$array) {
                        if ( $element =~ $previous ) { $check= 3;
                        }else { $check = 1; print "\n\n**** The sequences been processed
did not match ****\n**** this was the sequence $previous (assumes first file is
correct)****\n\n"; exit;}

                }
        return $check;
        }


##########################################
# takes data out of files labeled with multiopen.
#
#

sub newdataline_sub {
        my ($filetotal) = @_;
        my $filenumber = 0;
        my @seqfreq;
                do {
                        my $filecontent = <$filenumber>;
                        chomp $filecontent;
                        push (@seqfreq, $filecontent);              #put whole line of
file in array , one from each file will be put in this array.
                        $filenumber++;
                }until ( $filenumber == $$filetotal );
        return @seqfreq;
        }

##########################################
#
# To be honest this is not the best opener - but it opens multi files until no input from
user is entered.
# It checks to see if the file has actually opened.when the user enters a blank space it
moves on.
# don't ask about the if statement - its messy but required, otherwise the numbering gets
messed up or a fail from the open comman.
# now deals with non - numbered file names.
# REM this if files are renamed in a windows session while open (ie on screen) windows
keeps a copy of the old file, which messes up an code references to files, this doesnt
happen in linux :(
#

sub multifileopen_sub {
        my $n =0;
        my $file = -1;
        my @filenames;
                do {
                        $file = <stdin>;
```

```
                if ($file =~ /^\s*$/ ) { $total = $n; $n++; } else {        # extreamely
useful pattern match here !!! simply means nothing entered by user.
                        unless (open ($n, $file)) { print " \n\n cannot open : $file \n\n
this computer may have fish inside it (Japandy)."; exit; };
                        $n ++;                  # remember the first handle is 0, this means
this number to be read correctly must be 1 digit greater, ie. 2 file handles is three
files open (to a person)
                        chomp $file;
                        push (@filenames, $file);   # collects a new array with all
filenames,
                        }
                } until ($file =~ /^\s*$/);

return @filenames;
}

##########################################
#
# getexptdata_sub
# This uses the number of files opened in multifileopen_sub to collect a second array of
data 'experimental data' that will be used to contrast against the frequencies in freqX,

sub getexptdata_sub {
        my ($filenames) =@_;
        my @exptdata;
        print "Please enter the experimental data for each file now (corelation data) :
\n";
                foreach $element (@$filenames) {
                        print $element, " : ";
                        my $exptdata = <stdin>;
                        chomp $exptdata;
                        push (@exptdata, $exptdata);
                        }
        return @exptdata;
        }



###############
# Subroutines #
###############


#############################################################################
# sum_sub
# this is SUM . It adds all parts of an array together.

sub sum_sub {
        my($x) = @_;
        my $sum;
        my $number;
                foreach $number (@$x) {
                        $sum += $number;
                        }
        return $sum;
        }


#############################################################################
# sum_sub
# this is SUM OF SQUARES. It takes ONE array, squares each number then adds them all up.

sub sumsq_sub {
        my($x) = @_;
        my $sum;
        my $total;
        my $number;
                foreach $number (@$x) {
                        $sum = $number * $number;
                        $total += $sum;
```

```
                        }
        return $total;
        }


#############################################################################
# sumxy_sub
# this is SUM X*Y. It takes 2 arrays and multiplys the 1st number in each array together.
It then adds all these together.
# requires TWO input arrays x and y both must be same lenght!!

sub sumxy_sub {
        my ($x, $y) = @_;
        my $xysum;      # this line protects $xysum in subroutine cant protect it in
foreach loop otherwise the subroutine would not have access to it.
                if (scalar @$x == scalar @$y) {
                        my $positionofvalue = scalar @$x;                    # would love to
to use this line but can't get scalar component to work in an equation like $here = $tree/
scalar $@array.
                        my @y = @$y;
                                foreach my $numberx (@$x){
                                        my $numberinx = scalar @$x;
                                        my $position = $numberinx - $positionofvalue ;
                                        my $sum = $numberx * $y[$position];
                                        $xysum += $sum;
                                        $positionofvalue --;                 # this is my
way of keeping the numbers paired up, (links with $position line), as we go through the
foreach loop the position is counted down, allowing use $y[$position] on a pair of
results. Line must be at end, remeber first run of loop you want result to = 1 not 0!
                                        }
                }else{ print "\n sumxy_sub reports : Arrays are incorrect length \n";}
return $xysum;}


#############################################################################
# mean_sub
# this is MEAN. It takes ONE array adds all digits together then divides by the total in
the array.
# it uses       SUM. sum_sub

sub mean_sub {
        my ($array) = @_;
        my $total = sum_sub(\@$array);
        my $numberinarray = scalar @$array;
        my $mean = $total/$numberinarray;
        return $mean;}


#############################################################################
# sxy_sub
# this is Sxy. It takes 2 arrays and pushes them through to calculate the quantity Sxy.
Understanding statistics P524 - ISBN 0-19-914391-9 - Oxford University Press, Graham
Upton, Ian Cook. 1996.
# it uses       sum_sub
#               sumxy_sub

sub sxy_sub {
        my ($x,$y) = @_;
        my $sumxy = sumxy_sub(\@$x, \@$y);
        my $sumx = sum_sub(\@$x);
        my $sumy = sum_sub(\@$y);
        my $numberinarrayx = scalar @$x;
        my $numberinarrayy = scalar @$y;
        my $xy;                                                    #remember be careful
if you move this the subroutine needs access!! important
                if ($numberinarrayx == $numberinarrayy){                     # quick check
to see if arrays will even work. This is future proofing as you will recieve the same
error from sumxy_sub. As it has just checked earlier for same thing.
                        $xy = $sumxy - (($sumx*$sumy)/$numberinarrayy);
                }else{
                        print "\n Sxy_sub reports : Arrays are incorrect length \n";
```

```
                        }
        return $xy;}


#############################################################################
# sxx_sub
# this is Sxx OR Syy they are the same with respect to calculation. Understanding
statistics P524 - ISBN 0-19-914391-9 - Oxford University Press, Graham Upton, Ian Cook.
1996.
# Just feed it ONE array of x or y data.

sub sxx_sub {
        my ($x)  =  @_;
        my $sumsquares = sumsq_sub(\@$x);
        my $sum_x_or_y = sum_sub(\@$x);
        my $numberinarray = scalar @$x;
        my $sxx = $sumsquares - (($sum_x_or_y*$sum_x_or_y)/$numberinarray);
        return $sxx;
        }


#############################################################################
# lineb_sub
# this is the  slope of the line. Y=bx +a
# It requires sxy_sub and sxx_sub (and all there dependents).

sub lineb_sub {
        my ($x, $y) = @_;
        my $sxy = sxy_sub(\@$x, \@$y);
        my $sxx = sxx_sub(\@$x);
        my $b = $sxy/$sxx;
        return $b;
        }



#############################################################################
# intercepty_sub
# this it the intercept of a regression line on the y axis.
# It requires  meany_sub
#              lineb_sub
#              intercepty_sub
# Requirements are extensive for this subroutine and others it is suggested you do not
attempt to remove further routines from this libary.
# TWO arrays

sub intercepty_sub {
        my ($x, $y) = @_;
        my $meany = mean_sub(\@$y);
        my $meanx = mean_sub(\@$x);
        my $b = lineb_sub(\@$x, \@$y);
        my $intercepty = ($meany - ($b*$meanx));
        return $intercepty;
        }


#############################################################################
# deviance_sub
# this calculates the deviance of the line.
# TWO arrays

sub deviance_sub {
        my ($x, $y) = @_;
        my $syy = sxx_sub(\@$y);
        my $sxy = sxy_sub(\@$x, \@$y);
        my $sxx = sxx_sub(\@$x);
        my $deviance = ($syy-(($sxy*$sxy)/$sxx));
        return $deviance;
        }
```

```
###########################################################################
# variance_sub
# will work on x or y as the routines called all work on either of these too, i.e Sxx, Syy
both same.
# ONE array

sub variance_sub {
        my ($x) = @_;
        my $sxx_syy = sxx_sub(\@$x);
        my $variance = $sxx_syy/ scalar @$x;
        return $variance;
        }


###########################################################################
# covariance_sub
# This will calculate the covarience from 2 arrays.
# For simplicity I have not done a check for array size here, if they are wrong previous
routines will display error.
# TWO arrays.

sub covariance_sub {
        my ($x, $y) = @_;
        my $sxy = sxy_sub (\@$x, \@$y);
        my $covariance = $sxy/ scalar @$x;
        return $covariance;
        }



###########################################################################
# rvalue_sub
# This is pearsons correllation (r)
# It is not r squared, it can exist as a positive or negative number ie. -1 to +1 with
either of these been a perfect correlation. see Understanding statistics P547 - ISBN 0-19-
914391-9 - Oxford University Press, Graham Upton, Ian Cook. 1996.
# TWO arrays

sub rvalue_sub {
        my ($x, $y) = @_;
        my $covariation = covariance_sub (\@$x, \@$y);
        my $variancex = variance_sub (\@$x);
        my $variancey = variance_sub (\@$y);
        my $correlation = ($covariation/ (sqrt($variancex*$variancey)));
        return $correlation;
        }


###########################################################################
# rsquared_sub
#
# TWO arrays

sub rsquared_sub {
        my ($x, $y) = @_;
        my $sxy = sxy_sub(\@$x, \@$y);
        my $sxx = sxx_sub(\@$x);
        my $syy = sxx_sub(\@$y);
        my $r2 = (($sxy*$sxy)/($sxx*$syy));
        return $r2;
        }


###########################################################################
# h0orh1_sub
# This tests gives the result to see if the hypothesis Ho is true - no correlation. If its
not true H1 is excepted and there is a correlation
# If the value predicted for the distribution is less than that for the observed there is
no correlation.
# see Understanding statistics P553 - ISBN 0-19-914391-9 - Oxford University Press, Graham
Upton, Ian Cook. 1996.
```

```
# It does not tell you if it correlates, you must check this with tables (statistic
tables).
# TWO arrays

sub h0orh1_sub {
        my ($x, $y) = @_;
        my $r = rvalue_sub (\@$x, \@$y);
        my $r2 = rsquared_sub (\@$x, \@$y);
        my $predicted = ($r*(sqrt((scalar @$x - 2)/(1-($r*$r)))));
        return $predicted;
        }


##########################################################################
# significance_sub
#  is the result significant- returns an array that paired with @percentagep - shows the
level of significance.
# THREE input arrays.
# ONE OUTPUT ARRAY.

sub significance_sub {
        my ($x, $y, $percentagep) = @_;
        my $result = h0orh1_sub (\@$x, \@$y);
        my $location;
        my @numbers;
                foreach my $element (@$percentagep){
                        if (my $position = 0) { $position = scalar @$percentagep};   #1
                        $location = scalar @$percentagep - $position;               #1
                        if ($result <= 0) { $result = $result*-1}   # this line always
makes sure you use a positive number, besacue the stats tables are always for the
positive!
                                if ($result >= $element) {
                                        push (@numbers, 1) }else{
                                                push (@numbers, 0)}
                        $position--;                                    #1 lines marked this
work to count one array in against the other.
                        }
        return @numbers;
}


##########################################################################
# hitme_sub
# One routine that does it all into one array. Including checking on @percentage_points to
see if there is significance. this variable must be set for other distributions.
# includes rounder to make sure to only 4 digits.
# in this order in array,
# With the sequenced shifted on the front it is perfect to be outputed to a file. values
will be space separated.
#
#               distribution tn ie. n
#               mean x
#               mean y
#               sum squares x
#               sum squares y
#               sum (x*y)
#               sum Sxy
#               sum Sxx
#               sum Syy
#               slope of line
#               intercept on y
#               deviance
#               x variance
#               y variance
#               covariance
#               r
#               r2
#               Critical value --- calculated significance value
#               @percentage_points value 75,     # note that after we find if this value
was significant, percentage point value the number 1 designates significance and 0 no
significance.
```

```
#               90
#               95
#               97.5
#               99
#               99.5
#               99.75
#               99.9
#               99.95
#               1
#               1
#               1
#               1
#               1
#               1
#               1
#               0 # this example would show significance to the 99.75% point.
#               0
#
#       Figures are given to only 4 decimal places at this point, this may mean they are
given decimal places if they havent got them. They are calculated to the maximam until
this rounding stage. These values are now only for display or output.
#
#



sub hitme_sub {
        my ($x, $y, $percentagepoint) = @_;
        my $n = scalar @$x;
        my $meanx = mean_sub(\@$x);
        my $meany = mean_sub(\@$y);
        my $sumx = sum_sub(\@$x);
        my $sumy = sum_sub(\@$y);
        my $sumxy = sumxy_sub(\@$x,\@$y);
        my $sxy = sxy_sub(\@$x,\@$y);
        my $sxx = sxx_sub(\@$x);
        my $syy = sxx_sub(\@$y);
        my $b= lineb_sub(\@$x,\@$y);
        my $intercept = intercepty_sub(\@$x,\@$y);
        my $deviance = deviance_sub(\@$x,\@$y);
        my $variancex = variance_sub(\@$x);
        my $variancey = variance_sub(\@$y);
        my $covariance = covariance_sub(\@$x,\@$y);
        my $r = rvalue_sub(\@$x,\@$y);
        my $r2 = rsquared_sub(\@$x,\@$y);
        my $criticalvalue = h0orh1_sub(\@$x,\@$y);
# percentage point array to insert.
        my @grid = significance_sub (\@$x,\@$y, \@$percentagepoint);

        my @resultarray
=($n,$meanx,$meany,$sumx,$sumy,$sumxy,$sxy,$sxx,$syy,$b,$intercept,$deviance,$variancex,$v
ariancey,$covariance,$r,$r2,$criticalvalue);

        push (@resultarray, @$percentagepoint);
        push (@resultarray, @grid);

        my @result;
                foreach my $element (@resultarray) {
                        $number = sprintf ("%.4f", $element); # nice line - $number =
sprintf ("%.4f", $number); converts to only 4 decimals rounding as it goes.
                        push (@result, $number);
                        }

        return @result;
        }

# ------------------- end of code -----------
```

9.3 Method report

Figure 9-49 : Reproduction of a Malvern mastersizer data report. Displays the large population (modal size 178.68µm) characteristic of a 1µm liposome population which has been freeze-dried.

Sample Details

Sample ID: lipobea
Sample File: 210901AI
Sample Path: C:\SIZERS\ANDREW\
Sample Notes:

Run Number: 1
Record Number: 5

Measured: 21 Sep 2001 16:38
Analysed: 21 Sep 2001 16:38
Result Source: Analysed

System Details

Range Lens: 300 mm
Presentation: 3OHD
Analysis Model: Polydisperse
Modifications: None

Beam Length: 14.30 mm
[Particle R.I. = (1.5295, 0.1000); Dispersant R.I. = 1.3300]

Sampler: MS7

Obscuration: 21.3 %
Residual: 0.417 %

Result Statistics

Distribution Type: Volume

Concentration = 0.0017 %Vol
Span = 1.766E+00

Density = 1.500 g / cub. cm
Uniformity = 2.130E+00

Specific S.A. = 1.1854 sq. m / g

A.S.T.M Derived Diameters (um)

N	3	2	1	0
D[4, N]	10.96	6.08	4.47	3.59
D[3, N]		3.37	2.85	2.47
D[2, N]			2.41	2.12
D[1, N]				1.86

Distribution Moments

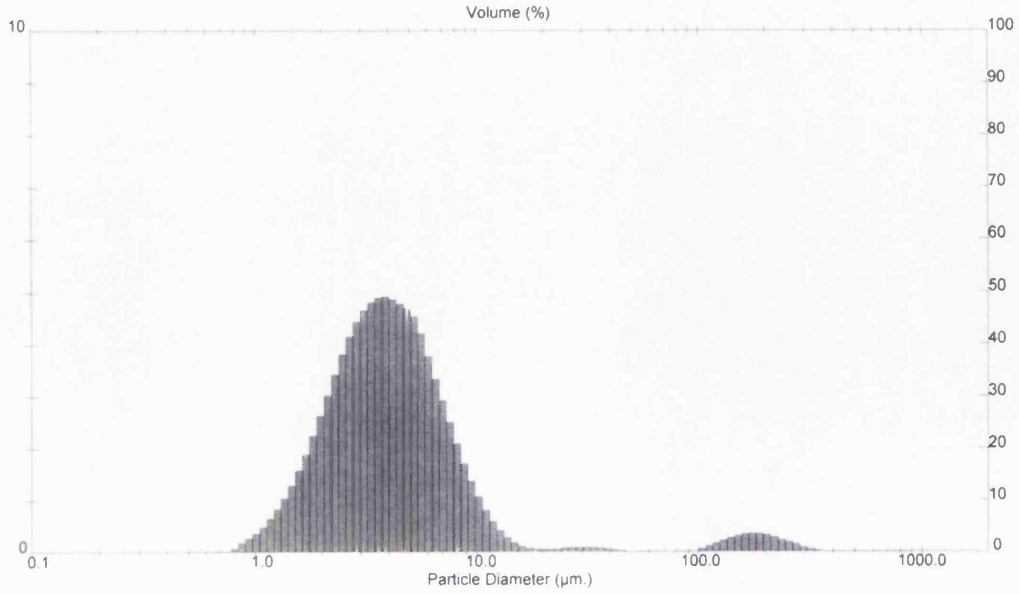
	Mean	Stan. Dev.	Skewness	Kurtosis
Volume	10.96	35.164	5.6634	32.9146
Surface	3.37	5.059	33.0567	1390.6196
Length	2.41	1.523	16.5269	1923.6366
Number	1.86	1.014	2.8450	101.4003

Distribution Percentiles (um) -- Volume

Percentile	Size	Percentile	Size
10.0 %	1.85	80.0 %	6.52
20.0 %	2.39	90.0 %	8.78
50.0 %	3.92	95.0 %	12.99

Distribution Modal Sizes (um)

Mode	Size	Mode	Size
1	3.80	2	178.68



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RI. CM (CR)
SM. JS. KH.
DL. JI. VJ.
RS. RB. VB.
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