DEVELOPMENT OF NOVEL CONTAINMENT SYSTEMS

FOR FREEZE-DRYING

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ABSTRACT

This thesis investigates the novel use of paper and Tyvek sterilisation pouches as containment systems to perform various applications of freeze-drying. Their effect on the freeze-drying of a pharmaceutical protein, mass and heat transfer, sterile freeze-drying and containment of microorganisms were examined.

Ovine derived Immunoglobulin G (IgG) specific for fluorescein was used as a model biopharmaceutical protein and a range of assays developed to quantify its biological activity, aggregation, turbidity, residual moisture and reconstitution time. IgG was formulated using different carbohydrates and analysed using freeze-drying microscopy to allow precise development of optimum freeze-drying cycles. The IgG was freeze-dried using these cycles and the function and structure of the IgG was shown to be unaffected. Freeze-drying cycles were modified to investigate the effect paper and Tyvek pouches had on the IgG which showed no change in activity or structure. However, pouches decreased sublimation rates and increased process time.

Resistance to water vapour, and the subsequent effect on mass and heat transfer during the freeze-drying process, imposed by paper and Tyvek sterilisation pouches, Gore Lyoguard, product dry layer and freeze-drying stoppers were investigated. Pouches presented greater resistance to water vapour movement than the dry product layer, Lyoguard and stoppers. Increasing resistance decreases mass transfer, increases pressure inside the pouches thereby increasing the heat transfer coefficient.

A process simulation using nutrient rich media was performed where vials were packaged aseptically and transported to a freeze-drier in a normal laboratory environment. The vials showed no growth of contaminating microorganisms after incubation. In addition, pouches containing nutrient rich media were challenged with aerosolised Saccharomyces cerevisiae and also showed no growth of contaminating microorganisms after incubation. Finally, pouches were demonstrated to be able to contain Escherichia coli during freeze-drying thus preventing contamination of the freeze-drier and the environment.
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<tr>
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<tbody>
<tr>
<td>$A_s$</td>
<td>Cross sectional area of vial</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>ePTFE</td>
<td>Expanded polytetrafluoroethylene</td>
</tr>
<tr>
<td>FDM</td>
<td>Freeze-drying microscopy</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein iso-thiocyanate</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>$K_c$</td>
<td>Heat conduction from shelf contact</td>
</tr>
<tr>
<td>KF</td>
<td>Karl Fischer</td>
</tr>
<tr>
<td>$K_g$</td>
<td>Gas conduction of heat form shelf to vial base</td>
</tr>
<tr>
<td>$K_r$</td>
<td>Radiative heat transfer</td>
</tr>
<tr>
<td>$K_v$</td>
<td>Vial heat transfer coefficient</td>
</tr>
<tr>
<td>LAF</td>
<td>Laminar air flow</td>
</tr>
<tr>
<td>LRV</td>
<td>Log reduction value</td>
</tr>
<tr>
<td>MTM</td>
<td>Manometric temperature measurement</td>
</tr>
<tr>
<td>NMWCO</td>
<td>Nominal molecular weight cut-off</td>
</tr>
<tr>
<td>OD$_{280}$</td>
<td>Optical density at 280 nm</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>$P_c$</td>
<td>Chamber pressure</td>
</tr>
<tr>
<td>$P_o$</td>
<td>Vapour pressure of ice</td>
</tr>
<tr>
<td>$P_p$</td>
<td>Pressure inside pouch</td>
</tr>
<tr>
<td>R</td>
<td>Sum of resistances to mass transfer</td>
</tr>
<tr>
<td>$R_b$</td>
<td>Barrier resistance</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative light units</td>
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Rs : Stopper resistance
RTD : Resistance temperature detection
SEC : Size exclusion chromatography
Tc : Collapse temperature
Teu : Eutectic point
Tg : Glass transition temperature (dry state)
Tg' : Glass transition temperature (frozen state)
TN TC : Too numerous to count
Tp : Target product temperature
Ts : Shelf temperature
TSA : Tryptone soya agar
TSB : Tryptone soya broth
Tv : Temperature at base of vial
TVC : Total viable counts
ΔHs : Latent heat of sublimation
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This work is dedicated to my wife Rebecca
CHAPTER 1: INTRODUCTION
1.1 A history of freeze-drying

Similar accounts of the history of freeze-drying are given by Mellor (1978), Franks et al. (2007) and Rey (2010). All authors mention, as one of the earliest forms of the process, the production of a potato flour by the Incas of South America. In the high altitude of the Andes, they naturally froze potato tubers overnight and then allowed them to warm, without exposure to the sun, facilitating removal of ice by sublimation in the rarefied atmosphere.

As discussed in Flosdorf’s (1949) chapter on the early history of freeze-drying, the term sublimation was first used by William Hyde Wollaston in 1813 when, during a lecture to the Royal Society, he showed the relationship between vapour pressure, temperature and the cooling effects of evaporation. During the late 19th and early 20th century freeze-drying was used as a technique for the preparation of tissues for microscopy by Altmann in 1890 and as a preservation technique for tissues intended for transplantation by Carrel in 1912. Rey (2010) cites a paper by Bordas and d’Arsonval in 1906 as the first description of drying a product from the frozen state under moderate vacuum. However, it was not until the advent of World War II, that the greatest advances in freeze-drying for pharmaceutical applications were made. The need for blood plasma and penicillin caused a massive development in techniques and apparatus for freeze-drying and workers such as Greaves in the UK and Flosdorf in the US emerged as pioneers of these processes and equipment. It was during this time that Flosdorf was first credited with the use of the term “lyophile” from the Greek to make “solvent liking”.

The emerging pharmaceutical industry was quick to realise the importance of freeze-drying in the preservation of antibiotics and biological materials and further
developments followed rapidly. Franks et al. (2007) specifically demonstrates this by listing the number of scientific publications in the periods up to and following World War II. Thus, there were only 10 publications pre 1930, rising to 350 over the period 1930-1945 and reaching about 600 in 2000.

From the 1970’s onwards important techniques emerge such as the development of the freeze-drying microscope by Mackenzie and also Rey which enabled the investigation of the thermal properties of formulations and the description of the phenomena of collapse and its significance. This led to further investigations into the physical chemistry of formulations and the prediction of their behaviour during freeze-drying by workers such as Franks (1991). During the 1980’s Pikal emerges as one of the leading researchers in the field, unifying the mass and heat transfer properties of freeze-drying and explaining the subsequent effect this has on the behaviour of materials.

1.2 Applications of freeze-drying

Today freeze-drying is used by many different industries for the preservation of labile, biological materials. However, the manufacturers of foodstuffs and pharmaceuticals are the primary industries that have adopted freeze-drying. Although industrially relevant the preservation of foodstuffs is outside the scope of this thesis and more information about this field can be sought in Mellor (1978). To give an idea of the variety of foodstuffs preserved by freeze-drying Mellor talks about meat, fish, vegetables and fruit, although readers will be more familiar with freeze-dried coffee in their day to day lives.
For the purpose of this study sterile freeze-drying, with particular emphasis on its use within the pharmaceutical industry, will be explored with some additional investigation into the preservation of microorganisms. The pharmaceutical industry has been preserving drugs, vaccines, antibodies and enzymes for systemic therapeutic administration for many years. These products not only need to have their biological activity preserved during freeze-drying they must also be sterile to allow their safe injection into patients. Sterile freeze-drying presents interesting challenges to be overcome and has found convention in compliance with pharmaceutical manufacturing regulations. This convention can be expensive in its application and the early part of this chapter seeks to review current practice, investigate previously attempted alternatives and suggest future more cost effective options.

1.2.1 Applications of non-sterile and sterile freeze-drying

As a generalisation to aid this discussion, products to be freeze-dried can be placed into one of four groups.

i. The first group comprises aqueous solutions of biological products which pose no risk to health and need no protection from potential environmental contamination. Examples include labile proteins, such as monoclonal antibodies for use as diagnostic reagents. This category of products can be preserved by freeze-drying in a non-sterile facility, using non-sterile equipment and would not require special conditions to protect the environment and staff.
ii. Group two consists of non-hazardous products which need to be protected from contaminating microorganisms. A specific example would be an antibody product intended for therapeutic (as opposed to diagnostic) use since it is mandatory that that medicinal products administered systemically are sterile (EMEA, Annex 1, 2008). Other examples are human blood products such as factor VIII and human growth hormone (hGH) to name but a few. During conventional freeze-drying these products must be processed in a sterile environment during filling, transfer to the freeze-drier and freeze-drying until the process is complete and they can be stoppered. This is both technically demanding and expensive in application (Renzi, 2009).

iii. The third group is categorised by products that do not require protection from environmental contaminants but are, themselves, potentially hazardous and cannot, therefore, be allowed to contaminate the environment. Examples include cytotoxic drugs or biological samples which may contain pathogens since it is known that during freeze-drying large numbers of viruses and bacteria can be entrained in the sublimating water vapour (termed ablation) leading to severe contamination of the freeze-drier (Adams, 1991b) and potentially to its operators and the environment. Such products can be filled into vials in a non-sterile but contained area. However, their freeze-drying must take place in a contained facility, such as a negative pressure isolator or room and be followed by rigorous decontamination of the freeze-drier and the facility. This could be a serious problem in a multiproduct facility if freeze-drying a hazardous product of this group is followed by freeze-drying a sterile or
medicinal product. Indeed, the regulatory authorities would consider the risk of cross contamination unacceptable in this situation and insist that separate freeze-driers and facilities be employed with a resultant doubling of costs (Isberg, 2009).

iv. The fourth and final category comprises potentially hazardous products that must also be protected from contamination. Their freeze-drying combines the need for sterility and for contamination protection throughout, to be followed by thorough decontamination. An example is the freeze-drying of Clostridium botulinum toxin.

This study is concerned mainly with sterile pharmaceuticals, specifically proteins that fall into group two of this classification and form the basis of chapters two and three. During the study it also became apparent that it would be possible to investigate applications that fell into group three and these form the basis of chapter four.

1.2.2 Problems with conventional sterile freeze-drying

As described in 1.5.2 a typical pharmaceutical freeze-drier comprises a chamber containing shelves that can be heated or cooled which is connected to a condenser (separated by a valve) with both chamber and condenser connected to a downstream vacuum pump. This is the basic equipment required to perform freeze-drying of products considered in the first group. However, to perform sterile freeze-drying the equipment and facility list increases considerably. As described by Trappler (1995), Snowman (1995) and Akers (2010) in relation to products in the second group, a sterile freeze-drier will normally be housed inside a cleanroom and must be sterilised by some means (usually
clean steam). This sterilisation and cleaning must be qualified from batch to batch as the regulatory authorities require. Therefore, sterile freeze-drying requires stringent facilities for containment, extra utilities to provide steam and pure water for cleaning and thorough validation of these processes. These points will be discussed in further detail in the following section.

1.2.2.1 Operation of sterile filling and freeze-drying

Aside from the high capital cost of setting up a cleanroom manufacturing facility including water for injections plant, clean steam utility and the freeze-drier itself there are certain strict guidelines set by the pharmaceutical regulators that must be observed. These regulators include the European Medicines Agency (EMEA) in Europe and the Food and Drug Administration (FDA) in the United States. The EMEA set out their guidelines in “Annex 1, Manufacture of Sterile Medicinal Products, 2008” and the FDA publish in more depth their “Guide to Inspections of Lyophilisation of Parenterals, 7/93”. Both regulators highlight the same specific areas of processing but the FDA guidelines have been chosen as a basis for this examination because they are more strict.

The extent of a sterile freeze-drying operation goes further than consideration of the drying process itself and commences with the initial sterile filling of the product into vials. Environmental conditions required are discussed further in chapter four but all sterile products are required to be filled in the highest quality EU Grade A or Class 100 conditions achieved by a filtered laminar air flow. Also the FDA specifically states the vials that are filled and semi-stoppered must be maintained within Grade A during transportation from the filling line to the freeze-drier. This reflects concern that during
transportation, as the vials are not sealed, there is potential for contamination of the product. As a result, it is essential during this transportation that the vials are covered by a primary barrier or laminar flow system. Hence, during the design of a filling and freeze-drying unit it is essential to have the freeze-drier adjoining the filling line under laminar flow or a barrier system that can maintain Grade A during transportation.

In their ‘Guide to Inspections of Lyophilisation of Parenterals, 7/93’ the FDA mention sterilisation of the freeze-drier as “one of the more frequently encountered problems noted during inspections”. It is generally accepted that sterilisation by steam under pressure, similar to autoclaving, is the recognised method although ethylene oxide can also be used. Usually temperature probes are placed inside the freeze-drier to ensure the required temperatures are achieved. In addition, there is a requirement that a steam sterilisation process be validated annually by placement of biological indicators or spore strips throughout the freeze-drier. These test if steam penetration has been adequate to kill a set number of microorganisms inside the freeze-drier. Failure of this test has obvious implications but also contributes to down time of the freeze-drier when no manufacturing can occur.

1.2.2.2 Batch to batch contamination

As touched upon in 1.2.1, during freeze-drying it is possible for small amounts of product to be lost from the vials. This is termed ablation and defined by Adams (1991b) as the condition where “Friable product can also be entrapped and lost in the vapour flow evolved from the drying cake and this phenomenon is termed ablation”. Loss of substrate by ablation during freeze-drying was first described by Stein et al. (1950) who detected
contamination of the condenser by pathogenic microorganisms. It was attributed to small amounts of product being carried out in the vapour stream and coming to rest in the condenser. Since then many more studies have been performed and a more in depth examination is given to this process in chapter four. This is obviously a concern to staff working with microorganisms that could result in accidental infection of operators and contamination of the environment. The potential for protein products to escape by ablation during freeze-drying also exists and can lead to possible batch to batch contamination of a pharmaceutical freeze-drier. Thus, it is normal practice for a freeze-drier to be cleaned and sterilised between batches whereby any carryover of ablated product would be eliminated. However, this again needs to be proved to the regulators and cleaning validation studies are performed by swabbing critical hard to clean areas inside the freeze-drier. These swabs are then taken and analysed for any low levels of product adding to the cost, downtime and expense of the process (Korczynski et al., 1995).

1.3 Freeze-drying containment systems

Current research investigating the use of novel containers for freeze-drying is in its infancy (Patel et al., 2011) with the most recent investigation being into the characterization of syringes (Patel et al., 2010). Flexibility and process economics, influencing time to market, are important issues in the current biopharmaceutical market (Novais et al., 2001) and use of disposable manufacturing equipment for upstream processes has been proven to reduce equipment, utility and labour costs. Furthermore, costs for cleaning validation, sterilization and the turnaround of process equipment are
considerably reduced or negligible (Rao et al., 2009) where disposable manufacturing equipment is used. In fact, it is common to see disposable units replacing traditional stainless steel equipment in biopharmaceutical plants (Farid et al., 2007).

This study sets out to investigate the use of disposable containment systems for sterile pharmaceutical freeze-drying and to show that it is possible to perform sterile freeze-drying in an open non-sterile laboratory environment. Using this approach there is no need to house a freeze-drier in a controlled environment, steam sterilize the freeze-drier or perform extensive validation for sterilisation and cleaning.

The concept of sterile freeze-drying in an unclean environment has been investigated using an aluminium box incorporating a system for vial stoppering and air filtration (Taylor et al., 1978). The filter system allowed exchange of water vapour during freeze-drying of serum and was shown to exclude an aerosol of *Serratia marcesens* in challenge testing. More recently W. L. Gore & Associates, Inc. developed the Gore Lyoguard which utilizes an expanded polytetrafluoroethylene (ePTFE) membrane allowing water vapour movement during freeze-drying. In a recent study this membrane was shown to be able to retain 1 µm latex beads and also prevented contamination of nutrient rich media contained within the apparatus when externally challenged with microorganisms (Gassler et al., 2004).

Wikol et al. (2008) recognised that one benefit of containment in freeze-drying was the restriction of product ablation which can cause contamination of the freeze-drier (Barbaree et al., 1982; Adams, 1991b). Therefore, effective use of containment during vial freeze-drying in a multiproduct pharmaceutical facility could prevent batch to batch contamination, expensive cleaning validation operations would not be required, and turnaround times would be greatly reduced between batches.
To develop freeze-drying containment systems a review was made of the currently available devices to identify their design features and requirements. Different designs were conceived to address these and the design candidate best fitting the features and requirements selected for testing and characterisation.

1.3.1 The Porton box

The first description of a freeze-drying containment system in the literature was by Taylor et al. in 1978. His research team was based at the then Microbiological Research Establishment, Porton Down, hence the name ‘Porton box’ used in this study to differentiate between containment systems. Aluminium sheet was fabricated to form a box and welded to form a leak tight unit (Figure 1.1). Rubber seals were used between the lid and the base to ensure a seal and two pleated glass fibre filters were attached to the side of the box. The filters were chosen so that microorganisms could not enter the box but passage of water vapour was allowed during the freeze-drying process. To allow product temperature to be monitored without compromising the aseptic internal environment of the box metal posts were fixed running through the wall and thermocouples soldered to these posts. The lid containing a stoppering mechanism is held in place with four spring loaded clips. The stoppering mechanism consists of a plate fastened to five plungers that are able to be depressed by the normal action of compression of the freeze-drier shelves, thus inserting stoppers into vials (Figure 1.2).
Figure 1.1 The Porton Box with cutaways showing thermocouple posts, stoppering plungers, filters and fasteners (Taylor et al., 1978).
Prior to transfer to a sterile isolator or room the Porton box was first wrapped and autoclaved. The box was then unwrapped and loaded with sterile product filled into vials that were semi-stoppered. Prior to sealing thermocouples are placed in the centre vial to measure product temperature. The box can then be removed from the sterile area and freeze-drying can proceed in a laboratory environment without fear of contaminating microorganisms infecting the sterile product. Following freeze-drying the shelves can be used to stopper vials prior to crimping. The Porton box was used to successfully freeze-dry 1440 vials filled with serum. During this process Taylor and colleagues (1978) reported an increase in product temperature of 4 °C compared to serum when freeze-dried conventionally.

To test the ability of the box to protect a sterile product from contaminating microorganisms the boxes were filled with nutrient rich growth media and placed inside a freeze-drier. The freeze-drier was then exposed to an aerosolised culture of *Serratia marscesens*. Finally, the media inside the box was incubated and no growth of contaminating microorganism was observed.
The Porton box demonstrated that it is possible to perform sterile freeze-drying in a non-sterile environment. However, an increase in product temperature is observed due to resistance to water vapour passage offered by the filters affecting the steady state of mass and heat transfer (discussed in more depth in 1.5.4).

The Porton box was designed to perform contained freeze-drying of vials. This reflects a conventional sterile freeze-drying process where vials are filled, semi-stoppered (to allow passage of water vapour), dried and then stoppered (usually under vacuum) by shelf compression of stoppers into the vials. It would seem feasible that the Porton box could be used for bulk freeze-drying of large volumes of material. However, if a product intended for systemic administration is put in contact with aluminium there is a possibility that small amounts of aluminium could leach into the product contaminating it and leading to potential adverse effects in patients.

1.3.2 The Gore Lyoguard

The Gore Lyoguard is the most recent development in freeze-drying containment and is the only commercially available apparatus for this purpose. It has been designed for bulk freeze-drying of large amounts of material for transport or further processing. Bulk freeze-drying is usually performed in open stainless steel trays where one of the major drawbacks is loss of product by ablation. The Lyoguard has similar dimensions to a standard stainless steel tray of 400 x 270 mm but is made of a lightweight, chemically inert polypropylene frame. Its underside is a thin film with the internal layer coated with polypropylene. The upper side of the Lyoguard is made up of a permeable ePTFE membrane developed specifically for freeze-drying having a high vapour transfer rate. A
port with a screw top on the upper part of the tray allows the Lyoguard to be filled with liquid product (Figure 1.3). The Lyoguard tray has been designed specifically for freeze-drying with the thin film base allowing effective heat transfer and a specially designed membrane providing minimal interference to mass transfer.

According to Gassler et al. (2004) the Lyoguard was found to provide faster and more uniform heat transfer when compared to stainless steel trays. However, some resistance to mass transfer was observed with higher product temperatures recorded and an increase in processing time. However, membrane resistance was found to be less than that of dry product layer using a fill depth greater than 10 mm. Furthermore, the membrane has been shown to be able to retain 1 µm latex beads and, therefore, would contain ablated product.

**Figure 1.3** The Gore Lyoguard. The whole upper surface incorporates an ePTFE membrane for vapour exchange with a screw top port on one corner to allow filling of liquid product.

Microbial challenge testing was also performed by Gassler et al. (2004) whereby the Lyoguard was filled with nutrient rich media and the membrane was challenged with
an aerosol and powder suspension of microorganisms. No growth of contaminating microorganism was observed. Although these tests show that the Lyoguard is able to prevent contamination of a sterile product, Gore do not appear to make any claim that the Lyoguard is able to perform sterile freeze-drying in a non-sterile environment.

Recently, the author was able to use Lyoguard trays to perform sterile bulk freeze-drying of an antibody product and was able to critically appraise its use for sterile processing. In practice the use of the filling cap was found to be difficult and did not lend itself readily to good aseptic technique. The trays were also found to be flimsy and difficult to transport to the freeze-drier as the base and membrane sagged when full of liquid product. To provide support to the Lyoguard frame and to prevent the membrane from sagging and contacting the liquid surface when in the freeze-drier, a modification was required. Plastic strips of the width of the Lyoguard were cut and used as supports to the polypropylene frame (Figure 1.4). When dry it was easy to handle the product in the Lyoguard and following drying the trays were quickly sealed inside bags to prevent moisture re-absorption. However, even with this precaution some anomalies were observed in the residual moisture results and moisture re-absorption of dry products through the ePTFE membrane cannot be ruled out and is, indeed, likely.

Thus it can be seen that the Lyoguard represents further development in the application of contained freeze-drying providing a system that allows effective water vapour exchange and ablation containment using the ePTFE membrane. However, it can only be used for bulk freeze-drying and has been demonstrated to be difficult to aseptically fill and transport. Therefore, it would appear that scope exists to improve the design of freeze-drying containment systems for both bulk and vial freeze-drying processes.
1.4 Design considerations and selection of containment systems for study

Common design features become apparent when considering the Porton Box and the Lyoguard and new freeze-drying containment systems will require these basic design features. Firstly a bacterially retentive medium that allows the free passage of water vapour is essential. The materials of construction require excellent heat transfer properties, be sufficiently inert to allow product contact and most importantly be inexpensive. Finally, any design should accept temperature probes to help monitor and maintain control of the freeze-drying process.

Because of the different requirements for bulk and for vial freeze-drying the first box designs consisted of a universal base with a choice of lids. The lids would incorporate
a stoppering mechanism, similar to the Porton Box, for vial freeze-drying but would be absent for bulk freeze-drying. Six designs were investigated and represented in Figures 1.5 – 1.9.

**Figure 1.5** The ‘Porton Box’ replica consisting of a sheet aluminium box with a hermetically sealing lid, designed to be reusable, with a vial sealing mechanism in the lid. Exchange of water vapour is via gas mask filters in the lid.
**Figure 1.6** The ‘Tupperware Box’ consisting of a polypropylene box with a hermetically sealed lid. A vial sealing mechanism could be incorporated into the lid and water vapour exchange would be via an integral or detachable membrane. This container was designed to be reusable.
Figure 1.7 The ‘Takeaway’ consisting of a thin sheet aluminium foil material similar to take away containers. Vial sealing would be made possible by use of concertina style crushable sides that would compress (shown above). Water vapour exchange would be achieved by an integral or detachable membrane fixed to the lid. Using relative inexpensive materials the system would be disposable.
Figure 1.8 The ‘Shower Cap’ consisting of a sheet aluminium tray to which a flexible film cap is hermetically sealed. Vial closure would be possible through the flexible cap. Water vapour exchange would be through a membrane incorporated into the flexible film cap. This system could be reusable or disposable.
The ‘Envelope’ would be a purely disposable system consisting of a thin porous material that would form part of the membrane for water vapour exchange. Products would be filled into trays or vials and placed inside the envelope and sealed. If required two envelopes could be used to ensure that a secure seal is formed. The flexible nature of the envelope would allow vial closure and also bulk freeze-drying in trays if fabricated at the correct size.
Figures 1.5 to 1.8 were variations around a box enclosure with a base and a lid. The lid could incorporate a vial stoppering system for vial freeze-drying or be used without the stoppering mechanism for bulk freeze-drying. The lids would also have an integral bacterially retentive membrane for water vapour exchange. The major differences between the designs in Figures 1.5 – 1.8 were the materials of construction and their stoppering mechanism.

The ‘Porton box’ replica (Figure 1.5) was easily dismissed from further investigation. Apart from being previously investigated by Taylor et al. (1978) it was bulky and would have been expensive and time consuming to re-engineer. Similarly, the ‘Tupperware Box’ (Figure 1.6) was dismissed because it would have been difficult and expensive to put together. The design also lacked originality and was comparable to the Lyoguard.

Incorporating a base of thin aluminium foil to the ‘Takeaway’ (Figure 1.7) would ensure excellent heat transfer. A lid with an integral membrane could then be fixed to this base. The innovation in this design was that a single container could be used for both bulk and vial freeze-drying. The ‘Takeaway’ was designed to allow the sides of the base to compress, through a concertina design, thereby allowing the shelves of the freeze-drier to stopper vials. Another advantage was that the materials of construction were inexpensive and an economical disposable box could be manufactured. However, it was difficult to visualise a robust method to hermetically seal the lid and base and it was also thought that the aluminium foil might be prone to splitting. It was concluded that this design may be appropriate to the food processing industry but would not be applicable for sterile pharmaceutical processing.

The ‘Shower Cap’ (Figure 1.8) investigated the idea of a flexible lid further and was also versatile enough to allow the freeze-drying of both bulk products and vials. Again
the innovative flexible top allowed the shelves to compress the stoppers into the vials at the end of freeze-drying. It was thought that existing aluminium or stainless steel trays already available to freeze-dry both bulk or vials could be used and a flexible lid stretched over them. This idea was soon rejected when the complexity of creating a hermetic seal between the tray and flexible lid was discussed.

The ‘Envelope’ design (Figure 1.9) was found to be the most versatile, economic and simplest system to satisfy all of the required design features. Envelopes would be easy to manufacture from two layers of thin material of which one would allow free passage of water vapour. Pre-existing trays for vials or bulk product could be sealed inside the envelope and as the envelope is flexible it would allow shelf stoppering of vials. For extra sterility assurance the trays could be double bagged and thermocouples could be inserted through the seal of the envelope. Finally these envelopes could be mass produced cheaply and supplied sterile for a fraction of the cost of any of the other designs considered.

It proved straightforward to find a product that could be adapted easily for freeze-drying containment. Chevron style self sealing sterilisation pouches were already available from most laboratory suppliers in a range of sizes. These pouches are designed for the sterilisation of medical devices or equipment using super heated steam in an autoclave or by ethylene oxide gas. Nolan (2004) provides the fundamental principles behind package type, selection and use in the medical device industry. Their design features two halves bonded together, one half is a clear polymer film and the other a gas permeable layer that is able to exclude microorganisms. Thus, a pre-existing system was found that satisfied all of the requirements for freeze-drying containment. These items were already designed for pharmaceutical use and were manufactured from compliant
materials. The base of the pouches is a thin polymer that would not interfere with heat transfer and thermocouple probes could be sealed easily into the pouch.

1.4.1 Sterilisation pouches

Two types of chevron style self seal sterilisation pouches were obtained, one formed from latex impregnated medical grade paper with a laminated polyester base and the second from Tyvek 1073B with an identical laminated polyester base. The chevron pouch gets its name from the peaked shape end of the package present to indicate and assist the splitting of the two halves to open the package. The two halves are welded together to form a hermetic seal. Packages of this design are low cost, are able to provide a sterile enclosure, have a visible side and are easily opened.

Pouches made from medical grade paper impregnated with latex have been used for many years for this application. Paper is a versatile and cheap material that is readily disposable. However, paper has low tear resistance which can be a particular problem if the package gets wet. Although specifically treated with a latex coat, paper can also still shed particles. Particle shedding needs to be closely monitored during pharmaceutical manufacture as a potential product contaminant.

Recently the use of Tyvek has become more widespread as a material for the porous layer. Tyvek is a spun bonded olefin which forms a fibrous web material made up entirely of very fine strands of high-density polyethylene. This structure allows rapid gas and vapour transmission but excludes microorganisms (5.2 LRV). In addition Tyvek is strong, water resistant and exceptionally tear resistant. Importantly Tyvek is low particle
shedding when compared to medical grade paper (DuPont, 2009). However, Tyvek is slightly more expensive than paper.

1.4.2 Application of sterilisation pouches to sterile freeze-drying

Having carefully considered containment systems for freeze-drying, it was concluded that sterilisation pouches represent the primary candidate for investigation in this study. The final part of this chapter considers briefly some of the fundamental principles of freeze-drying. These principles are referred to throughout this work and an understanding is essential to successfully apply sterilisation pouches to sterile pharmaceutical freeze-drying processes.

1.5 Fundamental principles of freeze-drying

Freeze-drying or lyophilisation is a complex technique involving many different fields including biochemistry, physical chemistry, pharmacokinetics and engineering (Franks et al., 2007). The freeze-drying process is normally split into three distinct parts. First the product is frozen using a specific method dictated by the selected formulation and the product to be dried. Next crystalline water (ice) is removed by direct conversion to water vapour under vacuum (sublimation), and is termed primary drying. Finally any water that is associated by direct interaction with the protein is then removed by heating the product, still under vacuum, by what is termed secondary drying. Each stage has different effects upon the product which can be controlled by careful selection and characterisation of the formulation. As will be discussed, the formulation selection and
characterisation for a product is potentially the most important part of this process and influences both the processing parameters and long term storage stability.

1.5.1 The physical properties of water

To understand the processes of freeze-drying it is essential to understand the physical properties of water and how these are affected by temperature and pressure (Atkins, 1989). These are best described using the phase diagram (Figure 1.10) that shows water can exist as a solid (ice), a liquid or a vapour at differing temperatures and pressures. At atmospheric pressure the equilibrium melting point of water is 0 °C (i.e. the temperature at which water converts from a solid to a liquid) and the equilibrium boiling point (at which it converts from a liquid to a vapour) is 100 °C. These equilibrium points can be shifted by changes in pressure.

Sublimation involves the movement of water directly from the solid (ice) into the vapour phase, critically missing out the liquid phase. Reference to the phase diagram (Figure 1.10) shows that to manifest this jump from solid to vapour the process must occur below the triple point of water that is the temperature and pressure where all three phases (solid, liquid and gas) can exist in thermodynamic equilibrium. The triple point of water exists at 0.01 °C and 0.006 atm and it is only below this temperature and pressure that freeze-drying (or water sublimation) can occur (Atkins, 1989; Greaves, 1954 and Perry, 1985). It is apparent that the physical conditions required to drop below the triple point are extreme and can often be obtained only within a laboratory environment.
Figure 1.10 Phase diagram of water indicating the temperatures and pressures where phase changes occur e.g. the change of phase from liquid to gas at 100 °C and 1 atm. The Triple Point (0.01 °C and 0.006 atm) is shown below which ice changes phase to vapour or gas.

The freeze-drying process works by freezing aqueous samples below 0.01 °C and then applying a vacuum below 0.006 atm. Below these points (triple point) ice will sublimate to water vapour. However, the water vapour still needs to be removed from the freeze-drying chamber otherwise the system will reach equilibrium and sublimation will cease. Such removal is generally achieved by a connecting vacuum chamber kept a few degrees colder than the freeze-drying chamber. If this neighbouring chamber is kept under the same vacuum conditions but at a lower temperature, for example -20°C, the vapour pressure of ice will be $1.05 \times 10^{-4}$. As the water vapour encounters the lower temperature and vapour pressure conditions that exist, it changes phase back into ice that forms on the exposed surface of the condenser. The differences in the vapour pressures between the freeze-drying chamber and the condenser are important and can be considered as the driving force for the freeze-drying process (Pikal, 1994).
1.5.2 Freeze-drying apparatus

A freeze-drying apparatus is able to achieve temperature and pressure below the triple point of water therefore allowing ice to change phase directly to vapour. Older laboratory freeze-driers involve the sample being frozen to a very low temperature in a flask, vial or ampoule using a bath containing a cryogen. During the freezing process the container can be rotated to decrease the freeze-drying time, termed shell freezing by Greaves (1954). The flask containing the frozen material is then connected to a vacuum manifold leading to a condenser and vacuum pump allowing sublimation to proceed. This description is similar to the famous Flosdorf and Mudd ‘Lyophile’ apparatus introduced in 1935. Using this apparatus it is assumed that the latent heat of sublimation or the energy used by the endothermic sublimation process will keep the product cold enough to prevent thawing. Apparatus of this type have been used for many years to dry laboratory scale samples but are crude and lack fine control during the freezing and primary drying stages. Furthermore, it is difficult to perform secondary drying as no adequate system is available to heat the dry product to remove the bound water.

Modern freeze-driers from laboratory to manufacturing scale consist of a freeze-drying chamber housing shelves that can be either heated or cooled. Initially these shelves are used to freeze the product prior to primary drying. They also allow heat to be input overcoming latent energy losses due to sublimation and can be precisely controlled to allow the sublimation process to occur at the highest possible rate without damage to product. Later, these shelves can be heated to higher temperatures allowing secondary drying to proceed until optimum residual moisture levels of the product are achieved. This chamber is connected to a refrigerated condenser that is able to trap evolved water vapour. All chambers are connected to a vacuum pump, sitting at the end of the stream,
to supply the required low pressures (Figure 1.11). Finally a microprocessor is used for system control and data capture.

**Figure 1.11** Schematic of a typical modern shelf freeze-drier showing the interconnection of the chamber and condenser terminating in the vacuum pump to facilitate the removal of water vapour. Shelves can be isolated from the condenser during the freezing stages by the use a connecting valve. The chamber shelves and condenser temperatures are controlled by refrigeration and heating units.

### 1.5.3 Freezing, supercooling and collapse

According to Wang (2000) freeze-drying creates a number of stresses that can denature proteins and therefore affect their biological function. He lists specific freezing stresses as freeze concentration effects; increased ionic strength; and altered pH. These are just some of the stresses associated with the freezing process of freeze-drying. The
additional drying stresses encountered by proteins are considered in later sections of this chapter.

During the freezing stage liquid water is crystallised to form solid ice with the objective of converting this ice to vapour during the sublimation or primary drying phase. It is therefore apparent that the formation of ice will define the microstructure of the frozen solution and subsequently that of the dried product (Hottot et al., 2004). As Pikal (1994) states this is important as it determines the rehydration or reconstitution rate and the freeze-dried cake appearance. However, as the product to be freeze-dried is normally a very pure, heterogeneous system the freezing process is neither simple nor straightforward.

Depending upon the constituents of the freeze-dried product freezing can be considered as either crystalline or amorphous. Before the meaning of these terms can be considered in more depth it is essential to consider what goes on in solution during the freezing process. As discussed by Franks (1992) before ice can form in a chilled solution, sub-microscopic water aggregates must be generated to grow into ice crystals. This complex process is termed ice nucleation and in reality normally occurs around sub-microscopic particulate impurities in solution. However, pharmaceutical solutions are normally exceptionally pure (being filtered to at least 0.2 µm to remove microorganisms) which leads to a paucity of sub-microscopic particles and therefore, points to initiate ice nucleation, which leads to a phenomenon termed supercooling.

Supercooling is defined as the difference between the equilibrium ice nucleation temperature and the actual temperature at which ice crystals nucleate and form (Kasper et al., 2011). Due to supercooling, pharmaceutical solutions can freeze at 10 – 15 °C below the equilibrium freezing point of water. The degree of supercooling also dictates the rate of ice nucleation and therefore determines the number and subsequently the
size of ice crystals formed (Rambhatla et al., 2004). A severe supercooling will produce smaller ice crystals than a mild supercooling (Galan, 2010). Smaller ice crystals will leave smaller channels in the freeze-dried cake and will therefore slow the rate of freeze-drying and also reduce the porosity of the dry cake layer. Hottot et al. (2004) studied ice crystal morphology in detail and concluded that larger ice crystals are desirable as they give the most porous dry structure accompanied by the shortest primary drying times.

Once nucleation has begun ice crystals grow rapidly and water is converted into ice. According to Franks (1992), the system then consists of an ice phase dispersed in a very concentrated aqueous mixture. Here, Franks (1992) is describing the freeze concentration of the water soluble constituents in the material to be freeze-dried which can have serious consequences for a pharmaceutically active protein. These consequences are represented by a model used by Pikal (1990) and Franks (1992; 2007) of 0.9 % saline solution (Figure 1.12). As the ice nucleates in the 0.9 % saline solution at -15 °C it can be seen that the percentage liquid water decreases over time due to ice formation. However, the NaCl present remains in the ever decreasing liquid phase and concentrates as the water is converted to ice. This continues until the temperature becomes low enough for the remaining solution (associated with the solute) to freeze. This is the point when complete freezing or solidification of the system occurs and is termed the eutectic point ($T_{eu}$) for crystalline systems or the glass transition ($T_{g'}$) temperature for amorphous systems. The crystalline sodium chloride of the supercooled eutectic system in the model crystallises at -26 °C when the NaCl has reached a concentration of around 6 molar. Thus, a pharmaceutically active protein formulated in benign isotonic saline, at ambient temperature, and then frozen to -26 °C will encounter potentially damaging salt concentrations during freezing.
Figure 1.12  Freeze concentration effect shown for 0.15 M or 0.9% saline (Pikal, 1990).

An additional effect observed during super-saturation of freeze concentration occurs to buffer salts and can cause drastic and damaging shifts in pH. An example is detailed by Bhatnagar et al. (2007) where in a sodium phosphate buffer system (for example phosphate buffered saline) the dibasic salt is less soluble than the mono-basic salt leading to its precipitation upon freezing. Removal from solution of one component of the buffer reduces buffering capacity and causes a decrease in pH of around three units. Again, this has serious implications for a pharmaceutically active protein when frozen in a phosphate buffer.

When considering the freezing of a product for freeze-drying two important events are taking place during this process. Firstly, the bulk of the water nucleates and forms ice crystals. As the temperature is reduced further the remaining solutes and water freeze or solidify at the eutectic temperature for crystalline materials or the glass transition temperature for amorphous materials. It is rare for a protein system to follow
a purely crystalline behaviour and the majority of pharmaceutical products freeze to form
an amorphous glass. However, the eutectic temperature or the glass transition
temperature is important during the freeze-drying process as it is related to product
collapse.

Adams (1992) discusses the importance of freezing prior to drying by sublimation
and the subsequent effect on product collapse. He states that heterogeneous
pharmaceutical formulations containing proteins, sugars, salts and water will rarely follow
a eutectic freezing pattern. Thus after the water has frozen the remaining components
usually form an amorphous concentrated glass. Below the glass transition temperature
the freeze concentrated components form a glass or highly viscous liquid. During primary
drying when product temperatures can be increased this glass can soften and deform
similar to rubber (Franks, 1989 and 1991). If the temperature increases too far above the
T_g' structure can be lost completely and the drying cake will collapse leaving a residue (not
a cake) inside the vial rendering the product useless. Product collapse temperature (T_c) is
determined using freeze-drying microscopy (FDM) which is discussed in depth in chapter
two (2.1.2.1). Here a microscope is used to view the product collapsing and enable
determination of the temperature at which it occurs. Once the T_c is known the product
temperature can be kept below the T_c during sublimation thus preventing cake collapse
during drying.

During freezing it may also be necessary to perform some thermal treatment or
annealing of components. Clearly, the freezing process is a highly complex phase of the
freeze-drying process involving many complex chemical interactions causing some
crystalline components to behave as amorphous materials. This process has been
discussed by Lu (2004) in relation to a mannitol additive. Here, using a variety of
techniques, it was found that crystalline mannitol when frozen quickly to a low
temperature will form a glassy amorphous structure. This caused a problem when the product temperature was increased during primary drying and the amorphous mannitol crystallised and expanded to such an extent as to cause vial breakage. To prevent this the product is first frozen below the $T_c$ and then temperature is increased to -23 °C, held for a short period of time to allow full crystallisation of the mannitol, and then cooled back to below the $T_c$. Thus annealing or re-crystallisation prevents any further re-crystallisation of mannitol during primary drying and subsequent vial breakage.

1.5.3.1 Shelf freezing

Two problems exist during the shelf freezing of aqueous pharmaceutical products. The first is a freeze concentration effect which has largely been overcome today with our advanced knowledge of the behaviour of excipients during freezing (1.5.6). The second problem is the control of ice nucleation during shelf freezing which is a direct consequence of supercooling due to the purity of pharmaceutical products (1.5.3).

As discussed in 1.5.3 freeze concentration can have drastic effects upon the biological activity of a protein exposing it to high salt concentrations or shifts in pH to mention but a few. Careful selection of buffers and lyoprotectants can overcome this issue and help maintain the protein through this stage of processing. However, it should be noted that during shelf freezing ice has a tendency to begin nucleation at the base of the container or vial, closest to the cold source or shelf. When initiated, ice nucleation begins rapidly and spreads upward moving away from the cold source and causing the product to freeze concentrate with an ice front that moves rapidly upwards towards the top of the container. This can form a plug of increasing product concentration and
density at the surface of the container (Franks, 1992). In extreme conditions or using a badly formulated product this concentrated plug at the top of the container can form a skin or film on the dry product that can impede mass transfer and may also create problems during reconstitution (Rey, 2010).

The second and more current problem faced during the freezing process is that of controlled ice nucleation. A highly pure pharmaceutical solution will tend to supercool during freezing leading to freezing temperatures lower than equilibrium. However, a solution filled into vials and shelf frozen will not have all vials freezing uniformly at exactly the same temperature. This is due to differing numbers of sub-microscopic particles being present in each vial and also to a non-uniform distribution of temperature across vials on a shelf and the random nature of ice nucleation. As the ice nucleation temperature defines the size, distribution and morphology of ice crystals (Kasper et al., 2011) differing nucleation temperatures across the batch will lead to differing ice formation. Thus, with different ice structures, different cake structures will be formed leading to intra-batch variation and potential heterogeneity of product.

To induce ice nucleation and bring about uniform freezing several methods have been employed. Researchers working with Pikal initially came up with an ice fog technique (Rambhatla et al., 2004) where cold nitrogen gas was introduced into the chamber forming an ice fog that penetrates into the vials causing seeding and subsequent freezing. Later this was refined by Patel et al. (2009) by using ice fog with low pressure causing nucleation to occur across a considerably narrower temperature range. More recently a technique was published that pressurises the chamber with an inert gas and subsequent rapid evacuation causes instantaneous ice nucleation (Konstantinidis et al., 2011), however, the mechanism of this process is not fully understood. The most recent study (Geidobler et al., 2012) involves a controlled depressurisation of the freeze-drier
followed by a repressurisation through the condenser. This cycle causes some water vapour to form in the condenser during depressurisation that then travels back to the chamber as ice crystals when repressurised subsequently seeding the vials and inducing immediate ice nucleation.

The control of ice nucleation currently attracts a high level of interest in the field of freeze-drying. The random and non-uniform nature of ice nucleation causes problems in batch to batch consistency and also during scale up. Moreover, control of ice nucleation will ensure uniform ice structures that provide the least resistance to sublimating water vapour, therefore, reducing primary drying times. Although interesting and relevant in terms of the manufacture of consistent pharmaceutical products the controlled nucleation of ice does not fall within the scope of this study. However, due to the prominence of the subject at this time it was thought by the author to be worthy of mention.

1.5.4 Primary drying

After the product has been frozen to below the $T_g'$ of the particular formulation and all components have been fully crystallised by annealing primary drying can commence. This is the removal of the ice crystals (formed during freezing) by sublimation. As discussed in 1.5.1 sublimation occurs below the triple point of water but pharmaceutical products also need to be below the collapse temperature ($T_c$). In reality $T_c$ is normally much lower than the triple point of water and is the governing parameter during primary drying to ensure an elegant cake remains after freeze-drying.
Many researchers have published detailed studies into primary drying with Pikal (1983, 1984, and 1985) emerging as today’s lead researcher in the field with some of the first detailed investigations into the process. In these publications Pikal is the first to accurately describe primary drying as “a problem in coupled mass and heat transfer which can be satisfactorily described using a steady state model where the heat flow is given by the product of the mass flow and the heat of sublimation”. In essence, the sublimation process is an endothermic process that loses energy through latent heat losses when ice changes phase to vapour. Thus, the product cools during primary drying and will continue to cool until finally reaching the temperature of the condenser (and equilibrium) where sublimation will cease (Adams, 1991a). To overcome the latent heat losses heat is put into the system (or product) through the shelves thereby maintaining the steady state and the sublimation process. Hence, there is a coupling of mass transfer or sublimation rate to heat transfer input through the shelves to maintain primary drying.

It is important to regulate the shelf temperature to maximise the primary drying rate and maintain the product below the collapse temperature. The product temperature should be as high as possible without exceeding the target product temperature which should be several degrees below the collapse temperature. Figure 1.13 illustrates the primary drying process as it occurs in the vial and the temperature variations that come about during primary drying. It can be seen that as primary drying proceeds the drying top layer increases whilst the lower frozen layer decreases. Sublimation takes place at the ice front or interface between these two layers and moves from top to bottom. Due to the latent heat losses the sublimation front is the coldest part of the product (shown by the scale on the right hand side). The temperature scale also shows that to achieve the required target product temperature for the highest rate of sublimation a high shelf
temperature may be required and as discussed by Tang et al. (2004) a 5 °C increase in shelf temperature will only bring about a 1 – 2 °C increase in product temperature.

![Diagram of vial temperature distribution during primary drying](image)

**Figure 1.13** Vial temperature distribution during primary drying (The Parenteral Society, Technical monograph No 10, 1998).

Mass and heat transfer are coupled in Equation [1.1] (Patel et al., 2010 and Searles, 2010),

\[
\frac{dQ}{dt} = \Delta H_s \frac{dm}{dt} \tag{1.1}
\]

where the latent heat of sublimation of ice, \(\Delta H_s = 2.97 \text{ kJ/g} \) (Searles, 2010)

The vial mean heat transfer coefficient \(K_v\) (Pikal, 1985 and Hottot et al., 2005) or the flow of heat from shelf to vial is defined by,

\[
\frac{dQ}{dt} = K_v A_s (T_s - T_v) \tag{1.2}
\]

where \(T_s\) is the temperature of shelf coolant, \(T_v\) is the temperature at the base of the vial and \(A_s\) is the cross sectional area of a vial.
From the steady state hypothesis an overall value for \( K_v \) can be calculated based on sublimation rate data (Searles, 2010 and Hottot et al., 2005),

\[
K_v = \frac{\Delta H_s (dm/dt)}{A_s (T_s - T_v)} \tag{1.3}
\]

\( K_v \) is the sum of three contributing factors (Patel et al., 2010) radiative heat transfer, conduction and convection (Figure 1.13). Radiative heat is absorbed by the product from the freeze-drier chamber walls, doors and upper shelf and can be considerable if low product temperatures are required. However, the main contributors to heat transfer are from direct conduction of heat from the shelves to the product and gas convection in air spaces between the vial and shelf. There may be a number of these interfaces that heat will need to overcome to reach the product such as coolant to shelf surface, shelf surface to vial base, vial base to frozen product and frozen product to sublimation front. These are termed resistances to heat transfer and account for the typically large temperature distribution from shelf to sublimation front.

The mass transfer or sublimation rate is influenced by the resistance encountered by water vapour as it is evolved. Figure 1.13 shows that this is mainly resistance encountered from the increasing dry product layer and serves as a reminder as to the importance of the freezing process dictating the cake structure. Resistance is calculated by Equation [1.4] (Patel et al., 2010; Pikal, 1985 and Pikal et al., 1984)

\[
\frac{dm}{dt} = \frac{P_o - P_c}{R} \tag{1.4}
\]

where \( dm/dt \) is the sublimation rate, \( P_o \) the vapour pressure of ice, \( P_c \) the chamber pressure, \( R \) the sum of total resistance. Resistance to mass transfer can have considerable effects on the sublimating product and if high will decrease the latent heat
loss from sublimation. This in turn will cause the product temperature to rise which potentially can cause the product to collapse if the collapse temperature is exceeded.

When the shelf temperature and heat transfer parameters have been optimised sublimation should proceed at the target product temperature without incident. The product should dry at a temperature below the shelf temperature until all of the ice has been removed by sublimation. The completion of sublimation is indicated by a rise in product temperature until it becomes equal to the shelf temperature.

1.5.5 Secondary drying

Following primary drying about 2 – 20 % water still remains in the product. This is the water associated with the product that did not freeze (Tang et al. 2004). Secondary drying aims to remove this residual moisture to a level of around 1 %. During secondary drying the shelf temperature is slowly increased to about 40 °C, still under vacuum, until the remaining water is desorbed. The required level of residual moisture required for optimal stability must be determined experimentally and the secondary drying times then determined and optimised by time course study.

1.5.6 Formulation for freeze-drying

Excipients are normally added to active protein drugs that are to be freeze-dried for a number of reasons that are discussed by Pikal (1990). Mannitol (and sometimes glycine) can often be added as bulking agents that readily crystallise leaving a good support for the protein in the form of an elegant cake. These materials generally have
high eutectic temperatures and freeze-dry quickly at high relative temperatures. Buffers are added to maintain pH and NaCl is often used to ensure isotonicity. The relative benefits of these additives have already been discussed in 1.5.3. Other sugars such as trehalose and sucrose are added as lyoprotectants to enhance the stability of the protein during freeze-drying and long term storage. Both of these sugars are amorphous in nature and will, therefore, exist in the same phase as that of the protein drug. The disadvantage of using these sugars is that they have low collapse temperatures and can increase drying times.

Damage can occur to proteins during freezing, drying and when dried in the solid state. The degradation processes and reactions are considered by Carpenter et al. (1997) as physical degradation such as denaturation and aggregation or precipitation and chemical degradation such as deamidation and oxidation. These can be limited or prevented by the addition of protectants that are thought to function through two mechanisms: The water replacement hypothesis and the glass dynamics or vitrification hypothesis (Chang et al., 2005; 2009). To maintain stability through the retention of native conformation the water associated to the surface of the protein by hydrogen bonding is replaced by the excipient during drying. As a result the native structure of the protein is preserved preventing any un-folding or damage. The glass dynamics hypothesis brings about stability of the protein by immobilisation in a rigid, glass matrix. At low dilutions of protein, molecular mobility is greatly restricted preventing unfolding and molecular collisions that lead to degradation reactions.
1.5.6.1 Characterisation of freeze-drying formulations

Two important techniques exist to help understand and characterise formulations for freeze-drying and help predict their stability in the solid state. In chapter two (2.1.2.1) the use of freeze-drying microscopy to determine the collapse temperature of formulations and thus determine a safe temperature to freeze-dry is discussed in depth. Related to the collapse temperature is the glass transition temperature where materials cease being a hard glass (highly viscous liquid) and make the transition to a rubber like state where it is able to flow, usually within 2-3 °C of the $T_c$. As with any change in state, for example freezing, there are also latent heat events characteristically accompanied by a release or use of energy. This energy release or use can be determined by a technique called differential scanning calorimetry (DSC). Wang (2010) gives an excellent account of how DSC can be used to determine $T_g$ of formulations and the subsequent cycle development.

DSC is also an important tool to determine the glass transition temperature of the dry protein product and can be used to specify the optimal residual moisture required by secondary drying and also the optimum long term storage temperature (Franks et al., 1991). In summary this is related to the vitrification hypothesis and the fact that a protein must be held in a rigid glass structure for optimal stability. Similarly to the frozen product, the dry solid protein will exist as a glass above a certain temperature and therefore, will be most stable above this $T_g$. Below the $T_g$ the product will be rubber and able to flow and the potential for collisions and reactions between molecules will be higher, therefore reducing stability. This is related to the final moisture content of the dry product as more water will enable greater mobility of the molecules leading to increased collisions, increasing the likelihood of degradative reactions and loss of activity.
1.6 Summary

It can be seen that freeze-drying is an industrially accepted technique used to preserve pharmaceutical products that lack long term stability in the liquid state. The processes of freezing, primary drying and secondary drying are complex and technically demanding requiring a high degree of knowledge and experience. Further complexity and expense are added when products for systemic administration require sterile processing. Therefore, an opportunity exists for the development of novel containment solutions that would allow the processing of small batch sizes of sterile products in a non-sterile environment. It is envisaged that these would assist producers of orphan and niche products reducing capital costs and potentially increasing available treatments to patients.

Previously Taylor et al. (1978) developed the Porton box to enable sterile vial freeze-drying in a non-sterile environment. More recently Gassler et al. (2004), working with W. L. Gore and Associates, developed the Lyoguard for contained bulk freeze-drying that would also potentially allow a sterile product to be freeze-dried in a normal laboratory environment. Although able to protect sterile product, both of these apparatus are limited by their specific function to perform either vial or bulk freeze-drying, their lack of user friendliness and cost. Current research indicates that scope exists for the development of containment systems for sterile pharmaceutical products and freeze-drying cultures of microorganisms. Patel and Pikal (2011) highlight the advantages of containment, when appraising the Lyoguard, in that a product can be freeze-dried sterile and that ablation can be contained. In addition, Adams (1994) describes how Taylor’s (1978) box could have been used to contain microorganisms during preservation by freeze-drying.
The development of the Porton box and the Gore Lyoguard demonstrate that it is possible to consistently freeze-dry sterile products in a non-sterile environment. As discussed earlier in this chapter chevron style sterilisation pouches provide economic containment systems that offer an alternative to the Porton box and Lyoguard that are readily available and more user friendly. Pouches offer the major advantage of being able to perform both vial and bulk freeze-drying with little change to an existing process. However, to improve upon the previous studies by Taylor et al. (1978) and Gassler et al. (2004) better characterisation in terms of their effect on mass and heat transfer when processing a pharmaceutical product is required and is a specific criticism of the Lyoguard raised by Patel and Pikal (2011). In addition, more focus is required to evaluate containment systems for sterile pharmaceutical processes that would be acceptable to regulatory authorities.

A model pharmaceutical protein was developed for evaluating pouches in chapter two. An antibody candidate was formulated with different carbohydrates that have been shown to have beneficial properties and interactions with proteins during freeze-drying. These were analysed following accepted methods to determine their performance during freeze-drying and these data used to develop novel freeze-drying protocols. Furthermore, a range of assays and tests were developed to establish any effects the freeze-drying process may have had on the structure and function of the protein.

The performance of the pouches during freeze-drying was then studied in chapter three. Pure water was used to observe how the pouch materials affected mass and heat transfer during sublimation. Using these data the freeze-drying protocols developed in chapter two were modified to allow the model pharmaceutical protein to be freeze-dried in the pouches. The protein was then analysed to determine if freeze-drying in pouches had had any detrimental effect on its structure and function. In addition, the
performance of the pouches was compared to that of the Gore Lyoguard and other factors that are known to affect the efficiency of freeze-drying.

Finally the efficiency of the pouches to prevent contamination of a sterile product and to contain microorganisms during preservation by freeze-drying was investigated in chapter four. Here, pouches were filled with nutrient rich growth media and challenged to determine their ability to prevent entry of contaminating microorganisms. Furthermore, freeze-drying cycles to preserve live microorganisms were developed using methods derived in chapter three. Following freeze-drying the pouches were analysed with a range of microbiological methods and their ability to contain ablated microorganisms and, therefore, prevent contamination of the freeze-drier and environment established.

1.7 Aims and objectives

The aims and objectives of this study are intended to provide a thorough understanding of the application of novel pouch containment systems to freeze-drying and are listed below.

- Aim 1: To verify that it is possible to sterile freeze-dry a model pharmaceutical protein using paper or Tyvek pouches.
  - Objective: Determine any effects pouch freeze-drying may have on structure and function of a pharmaceutical protein model.
  - Objective: Determine the effect pouches may have on mass and heat transfer during freeze-drying.
• Objective: Determine if pouches are able to prevent contamination of a sterile product during a simulated sterile freeze-drying process.

- Aim 2: To verify if paper and Tyvek pouches are able contain microorganisms during preservation by freeze-drying.

  o Objective: Understand if pouches are able to retain ablated microorganism and prevent contamination of the freeze-drier and environment.
CHAPTER 2: FORMULATION, CHARACTERISATION AND DEVELOPMENT OF FREEZE-DRYING CYCLES
2.1 Introduction

Prior to the development and testing of freeze-drying containment systems it was important to have a characterised model pharmaceutical protein to help understand the effects, if any, that containment may have on freeze-drying this type of product. It is already apparent that freeze-drying is a complex and highly technical undertaking and that the behaviour of protein macromolecules can be difficult to predict even before the effects of containment are considered.

Immunotherapeutic products in the form of polyclonal antibodies (supplied by MicroPharm Ltd) were selected as a model pharmaceutical protein for the purpose of this research. Immunotherapy is used to treat envenomation, drug overdose and infection. The antibodies used for this study were produced using the same methods as for therapeutic antibodies but were specific for fluorescein, thereby enabling them to be assayed accurately by measurement of the quenching of the fluorescence of fluorescein (2.1.3.1).

Using historical and published data, novel freeze-drying antibody formulations were developed using common carbohydrates. The collapse temperature ($T_c$) of these novel formulations was determined using freeze-drying microscopy (FDM) and, using $T_c$, optimal freeze-drying cycles were derived. Then freeze-dried antibody formulations were evaluated for any structural and functional changes. Using these data the effects of containment on the freeze-drying cycles and IgG were explored and described in chapter three.
2.1.1 Polyclonal antibodies for immunotherapy

For over one hundred years polyclonal antibodies have been used as immunotherapy to treat infections such as tetanus and diphtheria (Parish, 1965), as well as snake and spider envenomation (Newcombe et al., 2007). In addition, polyclonal antibodies have also been applied to treat drug overdose (Heath, 1995). Polyclonal antibodies for immunotherapy are produced by stimulating the immune system of donor animals, usually sheep or horses. The animals are immunised by repeated injection of non lethal doses of the toxic molecules or antigens causing a humoral immune response that generates a mixed population of antibodies to multiple epitopes on an antigen (Theakston et al., 1995). This mixed population distinguishes polyclonal antibodies from monoclonal antibodies where only one antibody exists for a single epitope on one antigen (Landon et al., 1995). When the antibodies in the blood reach adequate levels, the animal is bled and the serum separated. The majority of antibodies present in the serum will be immunoglobulin G (IgG) that accounts for ~75 % of the serum immunoglobulin (7-19 g/L) (Poulton and Hay, 1995). IgG is a Y shaped molecule (Figure 2.1) consisting of two heavy and two light chains (total MW ~160 kDa) arranged as regions or domains held together by disulphide bridges.
Figure 2.1 Structure of IgG showing the central heavy chains and the outer light chains. Each heavy and light chain is made up of constant regions which are the same for the population of IgG. The N terminus of both light and heavy chains make up the variable regions which are different for each IgG and are specific for epitopes on the antigen and, therefore, are responsible for antigen binding. The C terminus is responsible for cellular interactions during the immune response. In some cases IgG can be cleaved by proteolytic enzymes at the central hinge region. This yields two Fab molecules and one Fc molecule.

Early immunotherapies were administered to patients in the form of serum and caused a majority to develop serum sickness – a reaction to foreign serum proteins (Landon et al., 1995). The incidence of such reactions was reduced by purifying the IgG fraction from serum using ammonium sulphate and sodium sulphate to precipitate IgG or octanoic (caprylic) acid to precipitate albumin (Jones and Landon, 2003; Mpandi et al., 2007).
The ovine antibodies in this study were raised against fluorescein isothiocyanate (FITC) conjugated to keyhole limpet haemocyanin (KLH). Due to the small size of fluorescein (Figure 2.4) it is conjugated to a larger protein (KLH) to increase immunogenicity and to bring about an adequate immune response (Liddell, 2005). The antibodies were purified from serum using caprylic acid precipitation (to 95% purity) and the IgG fraction formulated in 20 mM citrate buffered saline at a concentration of 25 g/L using a scaled down version of MicroPharm’s manufacturing process.

2.1.2 Selection of Freeze-drying Formulations

Immunotherapies produced for Africa by MicroPharm are now presented in a liquid form in 20mM citrate buffered saline (0.9% saline, pH 5.8-6.2). Prior to the development of a stable aqueous formulation these products were routinely freeze-dried (Al-Abdulla et al., 2013). It was found that ovine derived Fab products were successfully freeze-dried with the addition of 5% mannitol. However, this protocol was established many years ago and it is apparent from the recent literature (Wang, 2010) that the field of freeze-drying formulation has developed and many other excipients are being used to protect proteins from the stresses encountered during freeze-drying (1.5.3).

As discussed previously in chapter one (1.5.6) mannitol acts as a crystalline excipient and can be considered as a support or bulking agent for proteins. Recently, Wang (2000) indicated that up to 10% sucrose or trehalose are amongst the most commonly used excipients for therapeutic monoclonal antibody products. These sugars are amorphous in the solid state and are considered to function as protectants for proteins during freeze-drying (Wang et al., 2009; Draber et al., 1995). The properties of
the different excipients are exploited to provide the best long term stability of biologically active products. Therefore, mannitol, sucrose, trehalose and NaCl were selected as excipients for ovine anti FITC in 20 mM citrate buffer and investigated in this study.

Up to 10 % (w/v) (275 mM) sucrose and trehalose have been selected for other products due to their isotonicity at this concentration. In 2001 Cleland et al. showed that lower concentrations of mannitol, sucrose or trehalose of up to 60mM were able to protect a monoclonal antibody for over two years at 40 °C. Using smaller amounts of excipient speeds the freeze-drying process by lowering the dry layer resistance to sublimating water. For this study, concentrations of excipients at 5 % (w/v) were selected which fall midway between isotonic levels and those suggested by Cleland and, therefore, ought not to impede sublimation greatly or be unsafe for human systemic use.

Furthermore, Chang et al. (2005) concluded that an IgG1 monoclonal antibody has 495 water binding sites on its surface and these must be replaced during drying for optimal long term stability. It has been assumed that an ovine IgG molecule will have a similar number of sites and, therefore, to protect the molecule adequately, a molar ratio of 1:500 IgG to protectant is required. This is equivalent to 3 % w/v and if mixed with 2 % mannitol the effectiveness of either trehalose or sucrose formulation as protectant with mannitol as a bulking agent can be evaluated. Details of the selected formulations are presented in 2.2.1.2.

2.1.2.1 Freeze-drying microscopy and determination of collapse temperature

Wang (2010) provides an excellent description of the history and uses of FDM. Essentially it consists of two components, an optical microscope and a freeze-drying
stage. The freeze-drying stage allows thin film samples, suitable for microscopy, to be frozen to a low temperature (~ -40 °C). A vacuum is applied and the temperature of the stage is increased, normally at 1 °C / min. During the temperature increase the behaviour of the sample can be observed in real time allowing determination of events such as collapse. This is discussed further in 2.2.1.3.

As discussed in 1.5.3 and 1.5.6.1, to develop optimal freeze-drying cycles two items of information about the product are critical. These are the glass transition temperature ($T_g'$) and the collapse temperature ($T_c$) (Carpenter et al., 1997). It was not possible to gain access to differential scanning calorimeter (DSC) to determine $T_g'$, however, it was possible to determine the collapse temperature using freeze-drying microscopy (FDM) were it is normally found that the $T_c$ is about 2-5 °C higher than the $T_g'$ (Meister et al., 2009). The $T_c$ is a dynamic measure of the point at which the freeze-dried cake collapses. This occurs when the product loses structure and starts to flow causing the whole cake to collapse in upon itself and is a similar phenomenon to the $T_g'$. Using FDM the $T_c$ can be observed under the microscope during actual freeze-drying. After determination of $T_c$ the target product temperature is calculated and maintained by controlling the shelf temperature of the freeze-drier during primary drying at 2-5 °C below the $T_c$.

2.1.2.2 Theoretical derivation of freeze-drying cycle parameters

As well as the $T_c$, other parameters are required to help develop effective freeze-drying cycles. Tang and Pikal (2004) give guidance on the initial estimates required for
freeze-drying parameters. Application of this guidance in the selection of freeze-drying parameters for the six formulations under study follows.

The protocol for freezing and, therefore, ice formation is important due to its influence upon the final structure and morphology of the freeze-dried cake (Hottot et al., 2004; Kasper et al., 2011). Furthermore, when using crystalline materials such as mannitol it is essential to make sure that it is fully crystallised during the freezing process. Thus, if mannitol is not fully crystallised during freezing as the temperature of the product increases during primary drying mannitol may re-crystallise causing an increase in volume that can lead to vial breakage (1.5.3). To prevent this mannitol is usually annealed during freezing to ensure full crystallisation. Freezing protocols used for each formulation were selected depending upon the crystalline or amorphous characteristic of each formulation. The freezing protocols for amorphous excipients and crystalline excipients were similar except for an annealing step. For the two mixed formulations a freezing protocol for an amorphous excipient was selected as this was the dominant constituent of the system.

Using the collapse temperature for each formulation obtained from FDM the target product temperature for primary drying was derived. This is straightforward and is taken to be optimally 2-3 °C below the \( T_c \). In this study the target product temperature was set at 3 °C below the collapse temperature.

It is possible to calculate a guide value for required shelf temperature to achieve \( T_p \). However, this is complex and requires the input of parameters that can be difficult to derive experimentally unless complex freeze-drying systems are available. Typically, the difference between shelf temperature and target product temperature is 10-14 °C. Using this initial estimation of target product temperature the chamber pressure can be derived in terms of the partial pressure of water at the target product temperature. Ideally the system should run at 10-30 % of the partial pressure of water. These data have been
simplified and expressed graphically by Tang and Pikal (2004) from where the required values were extrapolated for this study.

A chamber pressure well below the vapour pressure of ice at the target product temperature will allow a high sublimation rate. The vapour pressure of ice at the $T_p$ is taken from “Vapour Pressure of Ice” tables (Wagner et al., 1994). The driving force for sublimation is the pressure difference between the vapour pressure of ice of the product at $T_p$ and the chamber pressure. In most practical applications chamber pressure varies between 50 – 200 mTorr and Equation [2.1] can be used to derive an optimal chamber pressure. The Edwards Supermodulyo freeze drier used for this study has only a crude control for adjustment of chamber pressure. This involves a pressure leak system where a small leak is set from the minimum attainable chamber pressure to achieve the required chamber pressure. For the purpose of this study it was decided to run the drier at the minimum attainable chamber pressure for every test. This was done for simplicity and to obtain the highest possible sublimation rates.

$$P_c = 0.29 \times 10^{(0.019 \times T_p)} \quad [2.1]$$

Where $P_c$ = chamber pressure (Torr) and $T_p$ = target product temperature ($^\circ$C)

Primary drying times can also be estimated prior to setting up a freeze-drying run. Again these estimates are based on a number of contributing factors that are unique to the product and formulation under investigation. Factors that have been discussed such as $P_c$ and $T_s$ affect the primary drying time and product dry layer resistance also influences primary drying. Therefore, due to the number of factors governing primary drying time predicted figures have again been extrapolated from another graph in Tang and Pikal (2004).
Secondary drying times are dependent upon the crystalline or amorphous nature of the excipients used. Thus, crystalline excipients ensure that there is less residual moisture after primary drying than with amorphous excipients. In general it is better to secondary dry at higher temperatures for a short time. With less residual moisture, crystalline excipients are able to tolerate a rapid temperature increase without collapse and require less time to dry to less than 1%. Amorphous excipients must be heated up more gently to prevent collapse and held for longer times to reduce residual moisture to required levels (Franks et al., 2007).

2.1.3 Techniques for the analysis of antibodies

The freeze-drying process can be considered a relatively aggressive treatment of naturally derived proteins that have evolved to function in an aqueous physiological environment. By drying to very low levels of residual moisture the protein is placed in an atypical state that can have detrimental effects on structure and function. Excipients are used to preserve the native structure and function of the protein but this retention of form and biological activity must be verified. To determine if the freeze-drying process has had any effect on the protein a series of tests and assays were developed.

As previously discussed antisera containing IgG specific for fluorescein conjugate was selected for purification, formulation and freeze-drying. An assay was developed to measure the ability of the antibody to quench the fluorescence of fluorescein testing the binding of the antibody to the fluorescein thus rendering it unable to fluoresce. Using this method any damage that the freeze-drying process may have caused to the antigen specific binding portion of the IgG could be correlated to a lower binding to fluorescein
thus increasing detectable fluorescence. It is also understood that proteins, when freeze-
dried, can undergo some aggregation in the solid state. Size exclusion chromatography
was used to determine the purity of the IgG and whether any aggregation had occurred
during the drying process.

The long term stability of freeze-dried proteins is related to the extent of drying of
the product and the amount of residual moisture present. It is accepted that a protein
should be dried to between 1 – 5% residual moisture for optimum stability but this must
be considered on a case by case basis. There are several methods available to determine
residual moisture in pharmaceutical freeze-dried products (May et al., 1982) including
gravimetric, thermo-gravimetric, or chemical analysis, such as the Karl Fischer reaction.
Karl Fischer was selected for this investigation due to its sensitivity and ease of use. In
addition the IgG was subjected to several spectrophotometric tests to determine the
protein concentration and turbidity of the samples.

Finally the samples were tested for their time to reconstitute after freeze-drying
(defined as the time it takes to fully re-dissolve the freeze-dried material). If a product
has been freeze-dried to a high standard a highly porous structure or cake should remain
that allows rapid re-dissolution. Time to reconstitute is of importance for emergency
medicines because, in a critical situation, a patient requires rapid administration of the
required treatment.
2.1.3.1 Determination of specific antibody by fluorescence quenching

2.1.3.1.1 Principles of fluorescence

The fundamental principles of fluorescence are presented by Atkins (1989) and involve the sequence of steps shown in Figure 2.2. Initial absorption of a photon of light with the appropriate energy or wavelength excites a molecule to a higher electronic state. The molecule is excited from its ground state \( S_0 \) to higher electronic singlet states \( S_1, S_2 \) where electrons remain paired but an outer one is promoted to a higher energy level. The molecule then rapidly loses excess energy (non-radiatively as heat) as the electron steps down the upper levels and relaxes to the lowest vibrational level of the lowest excited state \( S_1 \). Fluorescence occurs when the molecule returns to its ground state \( S_0 \) and a photon of light is emitted.

The rates of the emission process, the various non-radiative degradation routes and the ability of solvent molecules to accept large amounts of energy to enable a molecule to return to \( S_0 \) determine if a molecule will fluoresce in solution. Thus, when competing deactivation processes are relatively inefficient the molecule will fluoresce. The quantum yield is defined as the ratio of the number of photons emitted to the number of photons absorbed and has a theoretical maximum of one. If the absorption spectrum was monitored it would look like Figure 2.3 with the excitation and emission occurring over a band of wavelengths termed the excitation and emission spectrum. The difference between the maxima of the two spectra is referred to as the “Stokes shift”.

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Figure 2.2 The fundamental principle of fluorescence. After initial absorption and excitation of outer electrons the upper states undergo radiationless decay to $S_1$ followed by a radiative transition (photon emission) to the ground state.

Figure 2.3 Typical absorption and emission spectra of a fluorescent molecule depicting the Stokes shift. For fluorescein the absorption maxima is 490 nm and the emission maxima is 520 nm. The Stokes shift is the difference between the emission and absorption maxima.
In a conventional fluorimeter (Zander, 1968; Sidki, 1984), solution containing a fluorescent molecule is excited with light of an appropriate wavelength. Within a microsecond a steady state is reached where the rate of fluorophore excitation is balanced by the combined rates of radiative and non-radiative degradation to the ground state. Here the total of fluorescence emitted by radiative degradation is proportional to the extinction coefficient of the fluorophore, the intensity of exciting light, the concentration of fluorophore and its fluorescence quantum yield. These become important experimental parameters when fluorescence (or the quenching of fluorescence) is used experimentally.

A typical fluorimeter consists of a light source, a set of optical filters for selection of the correct wavelength of light for excitation and emission, a system for focussing light onto a sample in a compartment, a photomultiplier detector and some sort of amplification and recording system. Modern systems use a PC for control and data acquisition and samples are prepared on 96 well plates for ease of use.

Various fluorescent molecules exist of which one of the least expensive and common is fluorescein. When in its double anionic form, which predominates in alkaline conditions (Figure 2.4), it has a quantum yield close to the maximum theoretical of one and a high peak extinction coefficient for the absorption of photons. Flourescein absorbs blue light, accounting for its orange colour, and emits green fluorescence falling within
the range of detection of laboratory fluorimeters. However, with an absorption maxima of 490 nm and an emission maxima of 520 nm fluorescein has a small Stokes shift (Figure 2.3). Fortunately modern fluorimeters are sufficiently efficient to overcome any light scattering interference from solute molecules and can sensitively detect emission.

![Structure of fluorescein](image)

**Figure 2.4** Structure of fluorescein. Under aqueous alkali conditions the hydroxyl and carboxylic acid groups exchange their protons become negatively charged. This doubly ionised fluorescein is its most fluorescent form.

### 2.1.3.1.4 Fluorescence quenching and antibodies to fluorescein

Changes in the aqueous environment of fluorescein can affect its quantum yield. This is termed fluorescence quenching and can be determined when the fluorescence signal decreases. This can be caused by factors such as pH or temperature and also by interaction of the fluorescein with specific quenching species. This study takes advantage of the quenching effects of specific antibodies binding to fluorescein to develop a simple fluoroimmunoassay for ovine derived polyclonal antibodies.

Antibodies to fluorescein conjugates have been used in studies of antigen-antibody binding since the early sixties. The quenching effect was exploited to develop
fluoroimmunoassays by Nargessi (1979). The extent of fluorescence quenching of fluorescein, expressed as the residual percentage of signal remaining after complete binding by antibody has been reported as between 8 and 14 % (Nargessi and Landon, 1981) for rabbit antisera and less than 3 % was reported by Samuel (1988). From unpublished data, Smith (2000) found ovine antibodies raised against fluorescein could quench fluorescein signal to 13.7 % of free signal and also to have high affinities in the region of $10^{10}$ L/mol. These data suggest that the fluorescence quenching system gives a reliable measure of the amount of specific anti-fluorescein antibody.

2.1.3.2 Karl Fischer Titration

The fundamental principles of Karl Fisher titration have been presented by Scholz (1984). Further to this, May (2010) describes the application of this technique, and others, for the determination of residual moisture in freeze-dried pharmaceutical products.

Karl Fischer titration is a widely accepted analytical technique for quantifying water in many different products. The titration is based on the Bunsen Reaction between iodine and sulphur dioxide in an aqueous medium:

$$2\text{H}_2\text{O} + \text{SO}_2 + \text{I}_2 \rightarrow \text{H}_2\text{SO}_4 + 2\text{HI}$$

[2.2]

In 1935 Karl Fischer realised this reaction could be used for the determination of water in a non-aqueous system in an excess of sulphur dioxide with liberated acid.
neutralised with an organic base. The reagent Karl Fischer developed was composed of sulphur dioxide and iodine in pyridine and methanol. Subsequently it was found the reaction proceeded with alcohol reacting with sulphur dioxide and organic base forming an intermediate alkylsulphite salt which is then oxidised by iodine to an alkylsulphate salt with the oxidation reaction consuming water:

\[
\text{CH}_3\text{OH} + \text{SO}_2 + \text{RN} \rightarrow [\text{RNH}]\text{SO}_3\text{CH}_3 + \text{H}_2\text{O} + \text{I}_2 + 2\text{RN} \rightarrow [\text{RNH}]\text{SO}_4\text{CH}_3 + 2[\text{RNH}]\text{I}
\] [2.3]

Later it was also found that pyridine was unimportant and merely acts as a buffer. Furthermore pyridine is a noxious carcinogen, thus modern reagents are pyridine free and instead contain imidazole or primary amines as an organic base.

Consideration of the Karl Fischer reaction shows that both water and iodine are consumed at a 1:1 ratio. Therefore, when all of the water has been consumed by the reaction an excess of iodine indicates the end point of the titration. The amount of water present in the sample tested can be calculated based on the concentration of iodine or the titre in the KF reagent and the amount of KF reagent used in the titration.

Development of equipment and techniques has resulted in three standard methods for KF titration:

1. Volumetric titration using a one component reagent
2. Volumetric titration using a two component reagent
3. Coulometric determination

Each of these methods has specific advantages depending on their application.
2.1.3.2.1 KF analysis of freeze-dried products

KF moisture determination of freeze-dried products, specifically pharmaceuticals, include the volumetric methods above either using visual or voltametric endpoint detection. These methods usually require around 50 mg of sample, whereas coulometric methods only require about 10-20 mg. The test method is selected on the basis of the amount of sample available, which could be of high value in the case of pharmaceuticals, and also on the amount of water present in the sample. For optimum stability pharmaceutical products are normally freeze-dried to a residual moisture level of between 1 and 5 % (FDA, 1990). These low levels of moisture are close to the lower end of detection for the volumetric methods of KF titration. Coulometric KF, being better at detecting lower levels of moisture is normally selected to determine residual moisture levels of freeze-dried pharmaceuticals.

2.1.3.2.2 Coulometric determination

Coulometric Karl Fischer produces iodine from an iodide containing solution by anionic oxidation:

\[ 2I^- - 2e \rightarrow I_2 \]  \[\text{[2.4]}\]

The reaction cell (Figure 2.5) is made up of a large anode compartment and a small cathode compartment separated by a diaphragm. The anode and cathode compartments
each have a platinum electrode which conducts current through the cell. When current is applied to the stirred anolyte, iodine is produced at the anode which immediately reacts with water present in the compartment. The diaphragm is in place to prevent migration of the iodine generated at the anode to the cathode where it would be converted back to iodide. When all of the water has been consumed and an excess of iodine occurs the current is switched off. The amount of water present is calculated by a microprocessor from the amount of current consumed to produce enough iodine to remove all the water (water and iodine react at a 1:1 ratio during the KF reaction).

**Figure 2.5** Coulometric titration cell consisting of a large anode compartment and a small cathode compartment separated by a diaphragm. Iodine is generated at the anode and reacts with water present in the anolyte. The diaphragm prevents migration of iodine to the cathode where it would be converted back to iodide.
Coulometric KF uses minute amounts of current to generate iodine for the reaction. This makes the technique very sensitive and able to detect micro amounts of water. However, this sensitivity means that atmospheric moisture must be excluded to prevent errors. The anode and cathode chambers are sealed to prevent this and samples are injected using a dry syringe (through a septum) into the anode chamber. In addition, both anolyte and catholyte must be dehydrated by pre-electrolysis prior to use.

2.1.3.3 Size exclusion chromatography

Size exclusion chromatography (SEC) separates molecules on the basis of their size as they pass through a gel medium. This is a heterogeneous system consisting of an aqueous liquid phase contained within the pores of a solid phase (gel medium). The solid phase pores can be controlled to a size selected to be similar to the size of molecules that are to be separated. Small molecules can diffuse into the gel whereas larger molecules are excluded (Striegel et al., 2009).

Gel media are packed into a column to produce a separation bed and the aqueous phase (normally a buffer) is pumped through the column. Samples for separation are added to the top of the column or bed and form a plug as they pass through the column. During its passage small molecules can diffuse from the sample into the media delaying their progress whilst larger molecules are excluded and pass through the bed unimpeded. Thus a separation is obtained with the largest molecules leaving the column first (Titchener-Hooker, 1998). When analysing a protein solution the elution of the different molecules is detected by determining their optical density at 280nm. This is normally
represented by a trace showing distinct peaks where each peak correlates to a separated protein.

2.1.3.4 Spectrophotometric methods

2.1.3.4.1 Optical Density at 280nm

The aromatic side chains of the amino acids phenylalanine, tyrosine and tryptophan of proteins absorb ultraviolet (UV) light strongly in the region of 270-290 nm. Therefore, absorption of UV light at 280 nm is commonly used to measure protein concentration. A spectrophotometer is used with a quartz cuvette of known width or pathlength (L) which is placed in a beam light with radiation intensity (I₀). The emerging intensity of light will be decreased to a value of (I) because of absorption of part of the radiation. The absorbance at wavelength (λ) is defined as:

\[ A_\lambda = \log (I_0/I) \]  \hspace{1cm} [2.5]

This expression is related to (L) and the concentration (c) by the Beer Lambert law:

\[ A_\lambda = \epsilon_\lambda Lc \]  \hspace{1cm} [2.6]
Here ($\varepsilon_\lambda$) is the extinction coefficient at wavelength $\lambda$ (280nm) for the particular protein being studied. The extinction coefficient for a particular protein can be determined by measuring the absorbance of a known concentration of protein and use of the Beer Lambert law (Mathews and van Holde, 1990).

### 2.1.3.4.2 Turbidity Assessment

Turbidity is a measure of the haze or cloudiness of a solution. For protein solutions this is usually an indication of the aggregation of molecules with a loss of conformational integrity and activity (Al-Abdulla et al., 2003). Turbidity was accessed using a spectrophotometer at 600 nm and 350 nm. The degree of clarity of the protein solution is correlated to the light scattering properties of the solution which is dependent upon the amount of insoluble aggregates present (Eckhardt et al., 1994).

### 2.2 Materials and Methods

<table>
<thead>
<tr>
<th>Equipment / material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edwards Supermodulyo pilot scale freeze-drier</td>
<td>Edwards, Crawley, West Sussex, UK</td>
</tr>
<tr>
<td>Lyostat3 freeze-drying microscope</td>
<td>BTL Ltd, Winchester, Hampshire, UK</td>
</tr>
<tr>
<td>BMG Labtech Polarstar fluorimeter</td>
<td>BMG Labtech, Offenburg, Germany</td>
</tr>
<tr>
<td>Aquamax KF coulometric titrator</td>
<td>GR Scientific Ltd, Ampthill, Bedfordshire, UK</td>
</tr>
<tr>
<td>Equipment / material</td>
<td>Supplier</td>
</tr>
<tr>
<td>----------------------------------------------------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>AKTA purifier liquid chromatography system</td>
<td>GE Healthcare Ltd, Hertfordshire, UK</td>
</tr>
<tr>
<td>Superose 12 HR 10/30 size exclusion column</td>
<td></td>
</tr>
<tr>
<td>Beckman Du Series 660 spectrophotometer</td>
<td>Beckman Coulter Ltd, High Wycombe, Buckinghamshire, UK</td>
</tr>
<tr>
<td>Beckman J6B centrifuge</td>
<td></td>
</tr>
<tr>
<td>Sartorius BP100 Analytical Balance</td>
<td>Sartorius-Stedim, Epsom Surrey, UK</td>
</tr>
<tr>
<td>20mm freeze-drying stoppers C1404</td>
<td>Stelmi, Paris, France</td>
</tr>
<tr>
<td>Schott type I borosilicate 10mL tubular glass vials</td>
<td>Adelphi HC, Haywards Heath, West Sussex, UK</td>
</tr>
<tr>
<td>Black 96 well plates</td>
<td>Greiner Bio-One Ltd, Stonehouse, Gloucestershire, UK</td>
</tr>
<tr>
<td>Quartz Cuvettes (1cm pathlength)</td>
<td>VWR Ltd, Lutterworth, Leicestershire, UK</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovine sera specific to fluorescein isothiocyanate</td>
<td>MicroPharm Ltd, Carmarthenshire, UK</td>
</tr>
<tr>
<td>Sterile water for irrigation</td>
<td>Baxter Healthcare, Compton, Berkshire, UK</td>
</tr>
<tr>
<td>Sterile saline for irrigation</td>
<td></td>
</tr>
<tr>
<td>Sodium azide</td>
<td>VWR Ltd, Lutterworth, Leicestershire, UK</td>
</tr>
<tr>
<td>Reagent</td>
<td>Supplier</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Tri-sodium citrate dihydrate USP</td>
<td>Merck Chemicals Ltd, Nottingham, UK</td>
</tr>
<tr>
<td>Citric acid monohydrate USP</td>
<td></td>
</tr>
<tr>
<td>Sodium chloride USP</td>
<td></td>
</tr>
<tr>
<td>Sodium hydroxide USP</td>
<td></td>
</tr>
<tr>
<td>Octanoic acid</td>
<td></td>
</tr>
<tr>
<td>Fluorescein, sodium</td>
<td>Sigma Aldrich Poole, Dorset, UK</td>
</tr>
<tr>
<td>Sodium tetraborate decahydrate</td>
<td></td>
</tr>
<tr>
<td>Bovine γ globulins</td>
<td></td>
</tr>
<tr>
<td>Anolyte: Hydranal Coulomat A</td>
<td></td>
</tr>
<tr>
<td>(Methanol, chloroform, imidazole,</td>
<td></td>
</tr>
<tr>
<td>sulphur dioxide with proprietary iodide)</td>
<td></td>
</tr>
<tr>
<td>Catholyte: Hydranal Coulomat CG</td>
<td></td>
</tr>
<tr>
<td>(Methanol, ethanol, 2, 2’-iminobis-,</td>
<td></td>
</tr>
<tr>
<td>hydroiodide,</td>
<td></td>
</tr>
<tr>
<td>sulphur dioxide)</td>
<td></td>
</tr>
<tr>
<td>Sucrose EP</td>
<td>Fischer Scientific UK Ltd, Loughborough, UK</td>
</tr>
<tr>
<td>Trehalose</td>
<td>Acros, Geel, Belgium</td>
</tr>
</tbody>
</table>
2.2.1 Methods

2.2.1.1 Antibody purification

IgG was purified from ovine antiserum using octanoic acid to precipitate albumin and other serum proteins (Al-Abdulla, un-published). Serum was thawed, allowed to reach ambient laboratory temperature and filtered through a 0.2 µm filter (Sartorius Stedim Ltd, Epsom, UK). The filtered serum was then diluted by 50 % with 0.9 % saline. Octanoic acid (equal to 6 % of the initial volume of serum) was added to the diluted serum and mixed for 30 min. Following this, saline equal to the initial volume of serum was added and the precipitated serum was allowed to stand for 1 h at ambient temperature before being centrifuged for 1 h @ 4000 rpm. The supernatant was decanted and pooled and the pellet discarded. The supernatant was stored at 2 – 8 °C overnight.

Following overnight storage the IgG containing supernatant was 0.2 µm filtered. The filtered supernatant was concentrated by restrictive tangential flow filtration using a 0.1 m² Pellicon mini 30 KDa NMWCO ultrafiltration system (Millipore UK Ltd, Watford, Hertfordshire, UK) and diafiltered against 10 volumes of 20 mM sodium citrate buffer, pH 5.8 – 6.2. The concentration of the diafiltered IgG was determined by OD$_{280}$ and diluted to 25 g/L. The resultant IgG was >95 % pure as confirmed by SEC chromatography. The IgG was stored at 2-8 °C until required. For freeze-drying experiments the IgG was formulated in the required amount if excipient and 0.2 µm filtered prior to use.
2.2.1.2 Antibody Formulations

The bulk formulated IgG in 20 mM citrate buffer produced in 2.2.1.1 was removed from storage at 2 – 8 °C when required. It was then formulated by adding the required excipient from Table 2.1 at the % w/v for study. When fully dissolved the material was filtered through a 0.2 µm filter prior to analysis before freeze-drying experiments.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Protein</th>
<th>Buffer</th>
<th>Excipients</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sheep anti FITC (25g/L)</td>
<td>20mM citrate in 0.9% saline</td>
<td>5% mannitol (crystalline)</td>
</tr>
<tr>
<td>2</td>
<td>Sheep anti FITC (25g/L)</td>
<td>20mM citrate</td>
<td>5% mannitol (crystalline)</td>
</tr>
<tr>
<td>3</td>
<td>Sheep anti FITC (25g/L)</td>
<td>20mM citrate</td>
<td>5% trehalose (amorphous)</td>
</tr>
<tr>
<td>4</td>
<td>Sheep anti FITC (25g/L)</td>
<td>20mM citrate</td>
<td>5% sucrose (amorphous)</td>
</tr>
<tr>
<td>5</td>
<td>Sheep anti FITC (25g/L)</td>
<td>20mM citrate</td>
<td>3% trehalose, 2% mannitol (mixed)</td>
</tr>
<tr>
<td>6</td>
<td>Sheep anti FITC (25g/L)</td>
<td>20mM citrate</td>
<td>3% sucrose, 2% mannitol (mixed)</td>
</tr>
</tbody>
</table>

Table 2.1 A variety of antibody novel formulations testing different physical forms and stability mechanisms (1.5.6) designed to protect proteins (2.1.2) during freeze-drying.
2.2.1.3 Freeze-drying microscopy

The Lyostat 3 consists of a freeze-drying cryostage, a microscope, temperature control system, digital camera, vacuum pump and associated vacuum control equipment such as Pirani gauges. A 2 µL sample was pipetted onto the cryostage, covered with a cover slip and the cryostage lid screwed down to seal the sample and allow a vacuum to be established. When the product was frozen the vacuum pump was switched on to start drying. The sample temperature was slowly increased at 1 °C / min and the sample monitored to observe collapse. When the sample is sealed into the chamber it can be viewed on the computer control screen. Using the PC control software the freezing protocol can be entered and annealing steps can be included if crystalline excipients are used. The onset of collapse is clearly visible on the image and the temperature for this event is recorded.

The $T_c$ was determined for all the formulations listed in Table 2.1 and used for subsequent freeze-drying cycle development. Thus, after determination of $T_c$ using FDM, the target product temperature is calculated and maintained to prevent collapse of the product ensuring that a satisfactory, pharmaceutically elegant cake is formed. The target product temperature was maintained by controlling the shelf temperature of the freeze-drier during primary drying and in this study held 3 °C below the $T_c$. 
2.2.1.4 Freeze-drying cycle parameters

The following freezing protocols were used for amorphous trehalose or sucrose excipients:

1. Load vials onto the shelf and allow to come to 5 °C, hold for 15 – 30 min.
2. Cool to -5 °C without ice formation and hold for 15 to 30 min.
3. Lower shelf temperature to a final temperature of -40 °C at 1 °C / min.
4. Hold for 1 h to 2 h.

This freezing protocol was also used for the two mixed formulations as the amorphous component was the dominant constituent of the system.

For crystalline mannitol the following freezing cycle was used as it incorporated an annealing step and allows full crystallisation:

1. Run steps 1 – 3 above.
2. Increase product temperature to 10 – 20 °C above T_c (but below the eutectic melt temperature) and hold for 2- 3 h.
3. Run steps 3 and 4 above.

After determination of the collapse temperatures (2.3.1) for the formulations it was possible to use these data and the guidance published by Tang and Pikal (2004) to derive theoretical freeze-drying cycle parameters (2.3.2). These data were used to perform trial freeze-drying runs using the Edwards Supermodulyo freeze-drier. In this freeze-drier the vacuum of the shelf chamber and the condenser are pre set to 40 mTorr and 25 mTorr minimum respectively and were not adjusted since the values were well below those calculated as being required to obtain the P_c. Primary drying times were programmed into the Supermodulyo control system that were in excess of the theoretical. This
allowed observation of the actual end of primary drying when $T_p$ was equal to $T_s$. This is also accompanied by a drop in Pirani pressure as the atmosphere changes from predominately water vapour to nitrogen (3.2.1) indicating no further evolution of water vapour and therefore the end of primary drying (Patel and Pikal, 2009). When primary drying was complete the freeze-drier was advanced to secondary drying. All formulations were heated to 40 °C during secondary drying with the crystalline formulations being held for 4 h and the amorphous for 6 h. The ramp rates to achieve this hold were 0.2 °C / min for crystalline formulations and 0.1 °C / min for amorphous formulations.

2.2.1.5 Fluorescence quenching

The recovery of the specific IgG antibody following freeze-drying was assessed by measurement of the quenching of fluorescein fluorescence. Samples were diluted 100 fold in 10 mM sodium borate buffer, pH 9.2, containing 1 g/L bovine gamma globulins and 1 g/L sodium azide. From this stock solution 10 serial doubling dilutions were prepared and 200 µl of each dilution was pipetted onto a black 96 well micro titre plate and 100 µl of 30 nM working fluorescein solution (diluted in sodium borate buffer) added to give a final fluorescein concentration of 10 nM. The plates were incubated in the dark for one hour. Fluorescence intensity was measured using a BMG Labtech Polarstar fluorimeter and plotted against final sample dilution. From this the final dilution (D, Equation [2.7]) that corresponded to 50 % quenching of the fluorescein fluorescence was determined. Figure 2.6 shows a typical antibody dilution curve obtained from the BMG Polarstar. The EC50 value calculated from the curve was used as the 50 % binding dilution.
At 50% dilution, 5 nM fluorescein is bound to the antibody and, assuming that antibody binding sites are essentially saturated with fluorescein, the concentration of bivalent IgG antibody is therefore 2.5 nM. Using a molecular weight of 160 kDa for sheep IgG, the estimated content of specific antibody in the sample was:

\[(2.5 \times 10^{-9}) \times (0.16 \times 10^{6}) \times D \text{ g/L} \]  \hspace{1cm} [2.7]

where D was the dilution factor.

**Figure 2.6** Typical dilution curves obtained using the BMG Labtech Polarstar. Normal sheep serum was run as a positive control. In addition, two standard sheep anti FITC samples were routinely analysed along with duplicate samples.

### 2.2.1.6 Coulometric Karl Fischer

The anode chamber was filled with 100 mL of anolyte solution and the cathode chamber filled with 5 mL of catholyte solution. Electrodes were connected to the anode and cathode chambers and the injection septum and desiccant tube installed to seal the
titrator preventing entry of atmospheric moisture. The reagents were then preconditioned to remove any moisture prior to sample injection.

As large numbers of vials were produced during cycle development individual vials of freeze-dried product could be taken for moisture analysis. For accuracy, the dry reagent could be added directly to material that was in the vial that was sealed under vacuum, thus preventing any exposure to atmospheric water giving errors to the results. The vials and product were weighed, 3 mL of dry solvent was added and after the product had dissolved about 700 µL was added to the titrator and analysed for residual moisture (determined in µg by the titrator). The vials were washed, dried and weighed and the weight of freeze-dried solid calculated. Then by calculating the amount of freeze-dried solid added to the titrator a percentage residual moisture could be determined.

2.2.1.7 Size exclusion chromatography

Purity of IgG was assessed by size exclusion chromatography (SEC) using a GE Superose 12 HR 10/30 column (Rawat et al., 1994) which was equilibrated and eluted with 20 mM citrate buffer, pH 5.8-6.2, in 0.9 % saline at a flow rate of 0.5 mL/min. Samples were diluted to 2.5 g/L in citrate buffer and injected into a 1 mL loop prior to loading. The eluted protein was monitored by absorbance at 280 nm. Figure 2.7 shows a typical SEC chromatogram.
Figure 2.7 A typical Superose 12 HR 10/30 SEC chromatogram the IgG peak is shown at retention volume 9.46 mL and a smaller IgM peak is shown at retention volume 6.38 mL. This chromatogram represents a typical sample of greater than 95 % purity.

2.2.1.8 Optical density at 280nm

Samples were diluted to 0.5 g/L using the required buffer. Total protein concentration was estimated by optical density at 280 nm using an extinction coefficient (1 g/L, 1 cm path length) of 1.5 for ovine IgG (Curd et al., 1971).

2.2.1.9 Turbidity assessment

Antibody turbidity was assessed using the two spectrophotometric methods before freeze-drying and after reconstitution of freeze-dried material in sterile water for irrigation. Apparent optical density was recorded at 600 nm and also apparent optical density between 340 nm and 360 nm was measured and expressed as an average at 350 nm. Both methods used quartz cuvettes with a 1cm path length. The results were then
used to determine the degree of turbidity when compared to the freshly 0.2 µm filtered product prior to freeze-drying.

2.2.1.10 Reconstitution time

Freeze-dried IgG was reconstituted by pipetting 3 mL of sterile water into the vial. The time for total re-dissolution of the cake was recorded.

2.3 Results

The collapse temperatures for the novel formulations studied were determined and used to estimate additional required freeze-drying parameters. These data were then used to develop actual freeze-drying cycles for the formulations from which the freeze-dried antibody products were then analysed using the described methods. This determined if there were any damaging effects on the antibodies and enabled comparison of the formulations ability to protect antibody structure and function during freeze-drying.

2.3.1 Collapse temperatures

The collapse temperature of the six formulations was determined by FDM (Table 2.2). These events were filmed using the Lyostat 3 FDM to enable interpretation of data gathered and accurate determination of $T_c$. Using formulation 3 [ovine IgG, 20 mM
citrate and 5 % (w/v) trehalose] as an example Figure 2.8 shows the frozen product prior to collapse, Figure 2.9 shows the onset of collapse and Figure 2.10 shows complete collapse of the product.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Buffer</th>
<th>Excipients</th>
<th>Collapse Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20mM citrate in 0.9% saline</td>
<td>5% mannitol (crystalline)</td>
<td>-34°C</td>
</tr>
<tr>
<td>2</td>
<td>20mM citrate</td>
<td>5% mannitol (crystalline)</td>
<td>-7°C</td>
</tr>
<tr>
<td>3</td>
<td>20mM citrate</td>
<td>5% trehalose (amorphous)</td>
<td>-20°C</td>
</tr>
<tr>
<td>4</td>
<td>20mM citrate</td>
<td>5% sucrose (amorphous)</td>
<td>-26°C</td>
</tr>
<tr>
<td>5</td>
<td>20mM citrate</td>
<td>3% trehalose, 2% mannitol (mixed)</td>
<td>-29°C</td>
</tr>
<tr>
<td>6</td>
<td>20mM citrate</td>
<td>3% sucrose, 2% mannitol (mixed)</td>
<td>-31°C</td>
</tr>
</tbody>
</table>

**Table 2.2** The effect of antibody / excipient formulation on collapse temperature. Each value for $T_c$ was derived from a single FDM run starting at -40 °C and ramping at 1 °C per minute until collapse was observed (under vacuum). The interpretation of the collapse temperature can be subjective and the results above were confirmed by several experienced operators.
The onset of collapse can be observed as a change in the structure of the dry layer of the product. For each photograph a clearly defined lighter and darker layer can be seen with the lighter layer being the frozen product and the darker the dry product. The interface between the two is the drying front from which water is sublimating. The onset of collapse is the loss of structure first observed in the darker dry layer indicated by the formation of cracks or holes in the dried layer next to the sublimation front (Meister, 2009).

Figure 2.8  Formulation 3 (20mM citrate with 5% trehalose) well below $T_c$ at -29.9 °C drying with no collapse observed (x 10 magnification). At this temperature no fissures or cracks can be observed in the drying layer.
Figure 2.9  Onset of collapse for formulation 3 (20mM citrate with 5% trehalose) at -20 °C (x 10 magnification). At this temperature the onset of collapse can be observed by the appearance of small cracks in the dry layer.

Figure 2.10  Formulation 3 at -18 °C showing collapse along the sublimation front (x 10 magnification). At this temperature large voids are observed in the dry layer indicating complete collapse.
2.3.2 Freeze-drying cycles

The $T_c$ found by FDM was used to derive the $T_p$ for each formulation and, as discussed in 2.1.2.2, $T_p$ was set 3 °C below $T_c$ (Table 2.3). These data enabled all freeze-drying test runs to proceed without product collapse and gave elegant freeze-dried cakes for each polyclonal antibody novel formulation. Comparison of the theoretical data derived for primary drying times (Table 2.3) with the actual experimental primary drying times (Table 2.4) show some variation with a general trend for the actual primary drying times to be slightly longer than the estimated times.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Target Product Temp ($T_p$)</th>
<th>Vapour pressure of ice at $T_p$ (mTorr)</th>
<th>Chamber Pressure ($P_c$) (mTorr)</th>
<th>Required Shelf Temp ($T_s$)</th>
<th>Estimated Primary Drying Time</th>
<th>Secondary Drying Time at 40°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-37°C</td>
<td>134.7</td>
<td>57.46</td>
<td>-31°C</td>
<td>~25hr</td>
<td>4hr</td>
</tr>
<tr>
<td>2</td>
<td>-10°C</td>
<td>1949.3</td>
<td>187.24</td>
<td>0°C</td>
<td>~25hr</td>
<td>4hr</td>
</tr>
<tr>
<td>3</td>
<td>-23°C</td>
<td>578.7</td>
<td>106.02</td>
<td>0°C</td>
<td>~12hr</td>
<td>6hr</td>
</tr>
<tr>
<td>4</td>
<td>-29°C</td>
<td>316.2</td>
<td>81.55</td>
<td>-15°C</td>
<td>~18hr</td>
<td>6hr</td>
</tr>
<tr>
<td>5</td>
<td>-32°C</td>
<td>231.2</td>
<td>71.55</td>
<td>-22°C</td>
<td>~25hr</td>
<td>6hr</td>
</tr>
<tr>
<td>6</td>
<td>-34°C</td>
<td>186.8</td>
<td>65.52</td>
<td>-26°C</td>
<td>~25hr</td>
<td>6hr</td>
</tr>
</tbody>
</table>

Table 2.3 Estimates of freeze-drying cycle parameters. $T_p$ is 3 °C below $T_c$ (Table 2.2).

The vapour pressure of ice at $T_p$ has been taken from vapour pressure of ice tables (Wagner et al., 1994). Chamber pressure has been calculated using Equation 2.1.

Estimated shelf temperatures and estimated drying times have been extrapolated from graphs given by Tang and Pikal (2004). Secondary drying times are based on the crystalline or amorphous nature of the excipients and were also taken from Tang and Pikal (2004).
The secondary drying temperature was fixed at 40 °C for all formulations. Crystalline formulation drying times were kept short at four hours with a 3 h ramp. Times for amorphous or mixed amorphous/crystalline were longer (due to the greater residual moisture) at 6 h with a ramp of up to 7 h. The end point of secondary drying can be found only by determining the final residual moisture of the freeze-dried cake (Table 2.5).

### 2.3.3 Antibody Analysis

The ability of the antibodies to quench the fluorescence of fluorescein was investigated before and after freeze-drying with no observed losses in concentration of antibodies specific to fluorescein (Table 2.5). A two tailed paired t-test was applied to determine that no there was no significant change in activity following freeze-drying and was applied to all the samples tested without consideration of formulation effects. The t-test gave a p value of 0.31 that was greater than 0.05 demonstrating no significant change in antibody activity after freeze-drying. To determine that two samples are significantly different a p value must be below 0.05.
<table>
<thead>
<tr>
<th>Formulation</th>
<th>Specific Antibody Concentration</th>
<th>Specific Antibody Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>pre Freeze-drying</strong></td>
<td><strong>post Freeze-drying</strong></td>
</tr>
<tr>
<td>1</td>
<td>3.77 ± 0.06 g/L</td>
<td>3.76 ± 0.01 g/L</td>
</tr>
<tr>
<td>2</td>
<td>4.52 ± 0.05 g/L</td>
<td>4.10 ± 0.12 g/L</td>
</tr>
<tr>
<td>3</td>
<td>4.65 ± 0.13 g/L</td>
<td>4.34 ± 0.25 g/L</td>
</tr>
<tr>
<td>4</td>
<td>4.70 ± 0.37 g/L</td>
<td>4.10 ± 0.04 g/L</td>
</tr>
<tr>
<td>5</td>
<td>3.40 ± 0.04 g/L</td>
<td>4.01 ± 0.08 g/L</td>
</tr>
<tr>
<td>6</td>
<td>5.16 ± 0.01 g/L</td>
<td>5.02 ± 0.01 g/L</td>
</tr>
</tbody>
</table>

**Table 2.5** Specific antibody activity determined by fluorescence quenching before and after freeze-drying. Results are expressed as means taken from duplicate tests with standard deviation. A two tail, paired t-test was applied to determine if freeze-drying caused any significant change to specific antibody concentration. Using this test a p value of 0.31 was obtained indicating there has been no significant change in specific antibody activity after freeze-drying.

Table 2.6 shows the residual moisture determined by coulometric Karl Fisher analysis where all figures for residual moisture are below 1% except for formulation 6. Residual moisture levels can affect protein activity (2.1.3.2.1) and these results indicate that at residual moisture levels of up to 1.64 % the ability of the specific antibody to quench the fluorescence of fluorescein remains unaffected.
<table>
<thead>
<tr>
<th>Formulation</th>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
<th>Step 4</th>
<th>Step 5</th>
<th>Step 6</th>
<th>Step 7</th>
<th>Step 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cool to 5°C in 6min and hold for 30min.</td>
<td>Cool to -10°C in 18min and hold for 30min.</td>
<td>Cool to -40°C in 30min and hold for 2hr.</td>
<td>Warm to -15°C in 24min and hold for 2hr.</td>
<td>Cool to -31°C in 24min and hold for 2hr.</td>
<td>At -31°C apply vacuum and hold for 36hr.</td>
<td>Warm to 40°C in 3hr and hold for 4hr (under vacuum).</td>
<td>Cool to 18°C in 1hr and stopper under vacuum.</td>
</tr>
<tr>
<td>2</td>
<td>Cool to 5°C in 6min and hold for 30min.</td>
<td>Cool to -10°C in 18min and hold for 30min.</td>
<td>Cool to -40°C in 30min and hold for 2hr.</td>
<td>Warm to -15°C in 24min and hold for 2hr.</td>
<td>Cool to -10°C in 24min and hold for 1hr.</td>
<td>Warm to 0°C in 6min apply vacuum and hold for 15hr.</td>
<td>Warm to 40°C in 3hr and hold for 4hr (under vacuum).</td>
<td>Cool to 18°C in 1hr and stopper under vacuum.</td>
</tr>
<tr>
<td>3</td>
<td>Cool to 5°C in 6min and hold for 30min.</td>
<td>Cool to -10°C in 18min and hold for 30min.</td>
<td>Cool to -40°C in 30min and hold for 2hr.</td>
<td>Not required.</td>
<td>Not required.</td>
<td>Warm to 0°C in 30min apply vacuum and hold for 18hr.</td>
<td>Warm to 40°C in 6.7hr and hold for 6hr (under vacuum).</td>
<td>Cool to 18°C in 1hr and stopper under vacuum.</td>
</tr>
<tr>
<td>4</td>
<td>Cool to 5°C in 6min and hold for 30min.</td>
<td>Cool to -10°C in 18min and hold for 30min.</td>
<td>Cool to -40°C in 30min and hold for 2hr.</td>
<td>Not required.</td>
<td>Not required.</td>
<td>Warm to -15°C in 30min apply vacuum and hold for 23hr.</td>
<td>Warm to 40°C in 6.7hr and hold for 6hr (under vacuum).</td>
<td>Cool to 18°C in 1hr and stopper under vacuum.</td>
</tr>
<tr>
<td>5</td>
<td>Cool to 5°C in 6min and hold for 30min.</td>
<td>Cool to -10°C in 18min and hold for 30min.</td>
<td>Cool to -40°C in 30min and hold for 2hr.</td>
<td>Warm to -15°C in 24min and hold for 2hr.</td>
<td>Not required</td>
<td>Cool to -22°C in 24min apply vacuum and hold for 28hr.</td>
<td>Warm to 40°C in 7hr and hold for 6hr (under vacuum).</td>
<td>Cool to 18°C in 1hr and stopper under vacuum.</td>
</tr>
<tr>
<td>6</td>
<td>Cool to 5°C in 6min and hold for 30min.</td>
<td>Cool to -10°C in 18min and hold for 30min.</td>
<td>Cool to -40°C in 30min and hold for 2hr.</td>
<td>Warm to -15°C in 24min and hold for 2hr.</td>
<td>Not required</td>
<td>Cool to -26°C in 24min apply vacuum and hold for 30hr.</td>
<td>Warm to 40°C in 7hr and hold for 6hr (under vacuum).</td>
<td>Cool to 18°C in 1hr and stopper under vacuum.</td>
</tr>
</tbody>
</table>

Table 2.4 Summary of cycle development result
<table>
<thead>
<tr>
<th>Formulation</th>
<th>Residual Moisture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.44 ± 6 x 10^{-4} %</td>
</tr>
<tr>
<td>2</td>
<td>0.52 ± 2 x 10^{-3} %</td>
</tr>
<tr>
<td>3</td>
<td>0.5 ± 6 x 10^{-4} %</td>
</tr>
<tr>
<td>4</td>
<td>0.57 ± 5 x 10^{-4} %</td>
</tr>
<tr>
<td>5</td>
<td>0.98 ± 3 x 10^{-3} %</td>
</tr>
<tr>
<td>6</td>
<td>1.64 ± 2 x 10^{-3} %</td>
</tr>
</tbody>
</table>

Table 2.6  Residual moisture of formulations after freeze-drying determined by coulometric Karl Fisher titration (samples tested in duplicate). Results are expressed as means of the duplicates with standard deviation.

The potential effects that freeze-drying could have on the purity or aggregation of the antibody was determined using SEC. Again samples were tested before and after freeze-drying and chromatograms were analysed for peaks indicating possible breakdown products of the antibodies. For all formulations run using SEC, both before and after freeze-drying, one major protein peak was observed that consistently represented 95% of the total protein. No peaks were seen indicating breakdown products or aggregation.

The total protein concentration of all samples was investigated by determining the optical density at 280nm both before and after freeze-drying (Table 2.7).
### Table 2.7 Protein concentration (OD$_{280}$) of samples before and after freeze-drying.

Results are expressed as means taken from triplicate tests with standard deviation. To determine that freeze-drying (without consideration of formulation) caused no significant change in protein concentration a two tail, paired t-test was applied. Using this test a p value of 0.44 was obtained indicating that there has been no significant change in protein concentration after freeze-drying.

These results appear to indicate a net loss of total protein which is not reflected in the fluorescence quenching results for specific antibody. It is thought that it might be due to some interference to the optical density measurements by the excipients used as dilution buffers. A paired t-test was used to determine that there has been no significant change in protein concentration following freeze-drying. This returned a p value of 0.44 indicating that freeze-drying has caused no significant change in protein concentration. Again, the paired t-test was applied without consideration to differing formulation.
Possible aggregation was also monitored in terms of turbidity of the samples prior to and after freeze-drying. Two different techniques were employed using different wavelengths of light in the visible range. The samples tested prior to freeze-drying were 0.2 µm filtered and immediately analysed. Samples tested after freeze-drying were analysed immediately after reconstitution (Table 2.8).

When reconstituted using sterile water, all formulations completely re-dissolved in less than 30 s. For all freeze-dried formulations there was an increase in turbidity upon reconstitution which appears to be greater in the purely crystalline formulations.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>600nm Pre FD</th>
<th>600nm Post FD</th>
<th>350nm Pre FD</th>
<th>350nm Post FD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.008</td>
<td>0.045</td>
<td>0.1</td>
<td>0.232</td>
</tr>
<tr>
<td>2</td>
<td>0.01</td>
<td>0.135</td>
<td>0.114</td>
<td>0.391</td>
</tr>
<tr>
<td>3</td>
<td>0.014</td>
<td>0.041</td>
<td>0.125</td>
<td>0.189</td>
</tr>
<tr>
<td>4</td>
<td>0.016</td>
<td>0.051</td>
<td>0.125</td>
<td>0.182</td>
</tr>
<tr>
<td>5</td>
<td>0.026</td>
<td>0.048</td>
<td>0.162</td>
<td>0.222</td>
</tr>
<tr>
<td>6</td>
<td>0.012</td>
<td>0.051</td>
<td>0.123</td>
<td>0.215</td>
</tr>
</tbody>
</table>

Table 2.8 Turbidity of samples before and after freeze-drying. The pre freeze-drying sample was tested immediately after 0.2 µm filtration and the post freeze-drying sample was tested immediately after reconstitution. Only single samples were tested.
2.4 Discussion

The polyclonal antibody novel formulations used in this study were selected to investigate their behaviour following freeze-drying. Currently no data has been reported for the optimum formulation for freeze-drying this type of product despite polyclonal therapies, such as antivenoms, being often destined for tropical regions where cold chain preservation of heat labile proteins is difficult. However, the aim of this study was to develop and characterise a freeze-dried model pharmaceutical product to understand how containment systems might potentially affect the freeze-drying process and product.

For this reason freeze-drying formulations used for therapeutic proteins were selected for testing. These include crystalline formulations (1 and 2) where the excipient acted as a bulking agent to support the amorphous protein and which provides little protection to protein structure but ensuring excellent cake structure. Amorphous formulations (3 and 4) where the excipient acts as a protectant to the protein during freeze-drying are glass formers and may not yield as elegant cake formations as crystalline products. Finally a mixture of both crystalline and amorphous formulations (5 and 6) was tested to protect the protein and provide a good support.

The behaviour of each type of formulation in terms of the major influence of components was partly demonstrated using FDM. However, it is rare for products to exhibit purely crystalline or amorphous behaviour in a mixture of materials (Pikal, 1990), viz, for amorphous protein, crystalline citrate plus excipient. For example, formulation 1 contained mannitol as a support with IgG, citrate buffer and sodium chloride and had the lowest $T_c$ (-34 °C). This was brought about by the presence of sodium chloride, a crystalline product with a very low eutectic melt temperature (Franks, 1992). It was the
influence of sodium chloride that caused the lowering of collapse temperature inferring that any formulation containing NaCl would have a characteristically low $T_p$. Formulation 2 was identical to formulation 1 apart from the absence of NaCl and a considerably higher collapse temperature of -7 °C was seen. Wang (2000) showed that the collapse temperatures of pure amorphous formulations of trehalose and sucrose were different, with sucrose being lower than trehalose. Although direct comparison is difficult with a protein formulation, formulation 3 demonstrated a $T_c$ of -20 °C and formulation 4 a $T_c$ of -26 °C. Interestingly, the combination of trehalose and sucrose with mannitol in formulations 5 and 6 showed the effect of mixing amorphous and crystalline excipients was to lower the $T_c$ to -29 °C and -31 °C respectively.

The selection of excipient had a clear effect on the target product temperature that subsequently impacted upon the primary drying time and rate. Formulation 1 with the lowest $T_c$ has the longest primary time of 36 h whilst formulation 2, with the highest $T_c$, has the shortest primary drying time of 15 h. The estimates in Table 2.3 for primary drying times were lower than the primary drying times determined experimentally (Table 2.4), except for formulation 2 that was difficult to extrapolate due to the high $T_p$. However, the initial estimates were only intended to act as a guide to the end of primary drying where the true indication is taken as a drop in Pirani gauge pressure and $T_p$ becoming equal to $T_s$ (Patel and Pikal, 2009). Both these indications are easily read in real time and the cycles were allowed to continue until these were achieved.

The formulation also has an effect of the secondary drying times since, depending if the product is amorphous or crystalline, differing amounts of moisture will remain after primary drying. Usually a crystalline excipient will complete primary drying with around 5% (w/w) moisture and an amorphous excipient with up to 30% (w/w) moisture (Tang and Pikal, 2004). In general, the drier the product the more stable it is over the long
term. Residual moisture of less than 1% is considered optimal for stability (Greiff, 1971). However, some products can become unstable if allowed to become too dry. Secondary drying temperatures were kept at 40°C for all formulations and were based upon an estimate from the type of formulation used. Crystalline drying times were kept short with a quick ramp to the required temperature. Due to the greater residual moisture, times for amorphous or mixed amorphous/crystalline were longer with a longer ramp. The end point of secondary drying can only be found by determining the final residual moisture of the freeze-dried cake. All figures for residual moisture were in the region of 0.5 % except for formulation 5 and 6 which were 0.98 % and 1.64 % respectively. Therefore, it would appear that the mixture of crystalline and amorphous excipients retain more residual moisture after primary drying than amorphous alone.

The importance of FDM and the derivation of $T_c$ cannot be over-emphasised as a tool for the effective development of freeze-drying cycles. It provides a clear starting point for the accurate estimation of all required parameters for an effective freeze-drying cycle. Its use in this study has enabled freeze-drying cycles for six formulations to be developed quickly and efficiently. Without determining this information prior to cycle development an empirical approach would have been adopted and it would have taken many months longer to achieve the same results.

Interpretation of the fluorescence quenching and SEC results shows that there has been no major reduction in specific antibody activity or purity of antibodies following freeze-drying for all formulations. This is also true for formulations 5 and 6 with slightly higher residual moisture. Therefore, it can be concluded that freeze-drying has had no effect on antibody structure and function, regardless of the formulation tested, over short term storage. In addition the formulations are stable with a residual moisture content of up to 1.78 % (Table 2.6). No loss of total protein is shown by OD$_{280}$, although
the results are quite varied, suggesting that the different carbohydrate formulations could be interfering with optical density measurements. The only notable difference found in the antibody analysis is in the turbidity of the formulations after reconstitution showing an increase in turbidity for formulations 1 and 2. This was also apparent from visual inspection of the reconstituted samples of these formulations which were slightly opaque in comparison to 0.2 µm filtered product. The combined formulations 5 and 6 showed a slight degree of turbidity and the amorphous formulations 3 and 4 showed a minor difference and it is suggested that this phenomena could be studied further using a greater sample size with the application of statistical analysis.

Even though SEC showed no aggregation and the products are all ~95 % pure, it is thought that some minor damage to the protein may have occurred during freeze-drying producing an insoluble aggregation product causing an increase in turbidity. This is so small as not to be detected in the purity or activity tests but can cause an increase of turbidity of the products. The greater increase in turbidity of the crystalline than the amorphous formulations would seem to support the literature that some protein protection was provided by the latter. These results could have implications for the clinical use of these products as it is potentially dangerous for a turbid sample to be used for systemic treatment.

The characterisation of the formulations and development of freeze-drying cycles provide a variety of model pharmaceutical products available to investigate any effects of contained freeze-drying in pouches. This is an advantage over Taylor et al. (1978) and Gassler et al. (2004) as no such model was available or used to test the Porton box or the Lyoguard. By comparing the antibodies freeze-dried using pouches to the antibodies freeze-dried in this chapter any effects on structure or conformation brought about containment can be determined and quantified.
CHAPTER 3: CHARACTERISATION OF STERILISATION POUCHES DURING FREEZE-DRYING
3.1 Introduction

The concept of contained freeze-drying was first introduced by Taylor et al. (1978) when his group successfully freeze-dried serum using the Porton box. It was not until much later that any further work in this field was undertaken when a group working with W. L. Gore & Associates Inc introduced the Gore Lyoguard freeze-drying containment system (Gassler et al., 2004). Both studies showed that a bacterially retentive filter could be devised that allowed the passage of water vapour during the primary and secondary drying stages of freeze-drying but that the filters impede water vapour flow thereby increasing product temperature. However, only Gassler and colleagues (2004) mentioned that primary drying time was increased. No further investigations have been performed to understand the reasons for these observations, although it would appear that the resistance imposed by the filters to water vapour movement is similar to that of the dry product layer (1.5.4) and is affecting the mass and heat transfer.

In chapter one the specialised function of the Porton box and Lyoguard were considered and a design process undertaken to investigate freeze-drying containment. This provided an understanding of the features of both systems that are essential requirements for contained freeze-drying. These ideas were then used to develop designs for novel approaches to allow sterile freeze-drying of vials or bulk products to be performed in a non-sterile environment. The designs drawn up were for a range of apparatus that allowed the passage of water vapour during freeze-drying through a bacterially restrictive medium. Detailed consideration was also given to the component materials of the equipment, whether they were to be re-usable or disposable and their possible influence and effect on mass and heat transfer during freeze-drying. After a
careful review, pouches constructed of medical grade paper and Tyvek were selected as best fitting the design criteria as well as being inexpensive and readily available.

This chapter details the characterisation of these pouches in order to help understand their influence on the freeze-drying process. Specifically, experimental work was undertaken to determine the effect on mass and heat transfer. By modifying techniques used in the literature it was possible to develop a method to determine the resistance of particular barriers to water vapour flow during sublimation and subsequently the role barrier resistance played in mass transfer. Using the same approach methods were also developed to investigate the effect on heat transfer processes during sublimation. As Pikal (1985) states “The theoretical description of primary drying is a problem in coupled mass and heat transfer” and these experiments were designed to investigate the effect barriers had upon coupled mass and heat transfer. The pouches were then compared to the Gore Lyoguard in terms of resistance to water vapour movement and heat transfer. Resistance to water vapour flow was also calculated for other potential barriers including product dry layer and semi-stoppered vials. Finally, the pouches were used to freeze-dry the model pharmaceutical protein developed in chapter two to determine any effects containment had on the structure and function of ovine anti FITC.
### 3.2 Materials and Methods

<table>
<thead>
<tr>
<th>Equipment / material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edwards Supermodulyo pilot scale freeze-drier</td>
<td>Edwards, Crawley, West Sussex, UK</td>
</tr>
<tr>
<td>Paper chevron style self seal sterilisation pouches 305 mm x 381 mm</td>
<td>Marathon Laboratory Supplies, London, UK</td>
</tr>
<tr>
<td>Tyvek 1073B chevron style self seal sterilisation pouches 305 mm x 381 mm</td>
<td>MET Ltd, Ashford, Kent, UK</td>
</tr>
<tr>
<td>152 mm square aluminium trays of 0.7 mm thickness with a capacity for 39 x 10 mL vials</td>
<td>Not known</td>
</tr>
<tr>
<td>Gore Lyoguard R&amp;D style container</td>
<td>W L Gore and associates, Elkton, MD, USA</td>
</tr>
<tr>
<td>Schott type I borosilicate 10mL tubular glass vials</td>
<td>Adelphi HC, Haywards Heath, West Sussex, UK</td>
</tr>
<tr>
<td>20mm freeze-drying stoppers C1404</td>
<td>Stelmi, Paris, France</td>
</tr>
</tbody>
</table>

Reagents used for these experiments have already been listed in chapter two, section 2.2.
3.2.1 Methods

The Edwards Supermodulyo pilot scale freeze-drier (housed in the open laboratory) was used for all tests. It was fitted with calibrated resistance temperature detection (RTD) probes and calibrated Edwards active Pirani gauges. Data were captured using a Eurotherm Chessell (Worthing, East Sussex, UK) Model 346 chart recorder. Using the RTD probes and specially adapted stoppers it was possible to record the product temperature at the base of the vial (to ensure \( T_p \) can be measured at the end of primary drying). Pirani gauges were used to measure chamber pressure and their readings were adjusted to obtain absolute pressure results. Pirani gauges measure the thermal conductivity of the gas and capacitance manometers measure absolute pressure or vacuum independent of gas composition. The thermal conductivity of water vapour is 60\% higher than for a predominantly nitrogen atmosphere therefore, during sublimation, Pirani gauges read 60\% higher than capacitance manometers (Patel et al., 2009) where water vapour predominates. Since capacitance manometers give more accurate vacuum values Pirani gauges were adjusted accordingly. Data were collected after two hours sublimation for every resistance determination and heat transfer experiment to ensure steady state had been reached. Pikal (1985) defines steady state as when the product of the mass flow and the heat of sublimation gives the heat flow and is normally established after half an hour of sublimation. Sublimation was allowed to run to completion to derive total sublimation time (based on when product temperature was equal to shelf temperature and a drop in Pirani pressure was observed, Patel et al., 2009). Sublimation rates were determined gravimetrically by weighing the containers using an external balance prior to sublimation and then every two hours until completion.
Ovine derived polyclonal immunoglobulin G (IgG) specific to fluorescein isothiocyanate (FITC) as described in chapter two was used as a model pharmaceutical product. Ovine anti FITC was formulated in 20 mM sodium citrate buffer (pH 5.8-6.2) at 25 g/L with either 5% w/w mannitol or 5% w/w trehalose (crystalline or amorphous excipients, respectively). Table 2.3 shows that $T_p$ is also different for each of these formulations enabling a range of temperatures to be investigated.

3.2.1.1 Ice Sublimation tests

All sublimation tests were performed with vials containing 3 mL fill volume (representing a 10 mm fill depth) of water. Vials were semi-stoppered and placed onto aluminium trays with each tray tightly holding 39 vials in a close hexagonal pattern. Such semi-stoppered vials were studied either non-contained or double wrapped (one pouch within another) inside paper or Tyvek pouches (Figure 3.1). The Gore Lyoguard was filled with 30 mL of water to obtain a 10 mm fill height (taken as equivalent to 10 vials of a 3 mL fill) and placed directly on the freeze-drier shelf.

Rambhatla (2003) shows there is heterogeneity in heat transfer rates between vials with those at the edge of the tray receiving more heat that the others during sublimation causing an increase in the rate of sublimation. Therefore, vials containing the RTD probes were positioned in the centre vial of each tray to represent the slowest sublimating vial. The RTD probes were also positioned on the base at the centre of each vial using specially adapted stoppers that held the probes in place until the product was frozen. A hole was drilled into the side of the Gore Lyoguard allowing insertion of an RTD probe at the base and centre and then hermetically sealed with silicon mastic (Figure 3.2).
Figure 3.1 Method used to double wrap trays of vials in sterilisation pouch.

The effect on sublimation of the different barriers was studied using a shelf temperature of either 0 °C or -10 °C. The freezing stages for both shelf temperatures were identical and were: ramp to 5 °C in 30 minutes, held for 30 minutes; ramp to -5 °C in 30 minutes, held for 30 minutes and ramp to -40 °C in 1 hour, held for 2 hours. Sublimation was performed at a chamber pressure of 55 mTorr (shelf temperature of 0 °C) and at a chamber pressure of 45 mTorr (shelf temperature of -10 °C) until sublimation was completed. The Gore Lyoguard was tested only at shelf temperature 0 °C using the same freezing stages and chamber pressure 31 mTorr.
Figure 3.2 The Gore Lyoguard Lyo R&D container showing the RTD probe inserted at the base and centre and hermetically sealed with silicon mastic. The Lyo R&D container consists of two parts with the membrane welded onto a screw cap lid that attaches to the base. Note the thin film base to permit effective heat transfer.

3.2.1.2 Heat and mass transfer characterisation

Data derived from the sublimation experiments were used to determine figures for resistance to sublimation for each barrier and also any effects on heat transfer.
Resistance was calculated by Equation [3.1] (Patel et al., 2010; Pikal, 1985 and Pikal et al., 1984)

\[
\frac{dm}{dt} \frac{P_o - P_c}{R_b + R_s} \quad [3.1]
\]

where \(dm/dt\) was the sublimation rate, \(P_o\) the vapour pressure of ice, \(P_c\) the chamber pressure, \(R_b\) the barrier resistance and \(R_s\) the stopper resistance. \(P_o\) was calculated from the temperature recorded for the product (Pikal, 1985) and \(P_c\) was read from the chart recorder trace. \(R_b\) was calculated by subtracting \(R_s\) from the total resistance determined experimentally for barrier systems. \(R_s\) was determined experimentally testing semi-stoppered, non-contained vials.

The coupling of heat and mass transfer (Pikal et al., 2010 and Searles, 2010) is shown by Equation [1.1] in 1.5.4. The vial mean heat transfer coefficient \(K_v\) or the flow of heat from shelf to vial (Pikal, 1985 and Hottot et al., 2005) is defined by Equation [1.2] in 1.5.4.

From the steady state hypothesis an overall value for \(K_v\) can be calculated based on sublimation rate data (Searles, 2010 and Hottot et al., 2005)

\[
K_v = \frac{\Delta H_s (dm/dt)}{A_v(T_s - T_v)} \quad [3.2]
\]

where \(T_s\) was the temperature of shelf coolant, \(T_v\) was the temperature recorded at the base of the vial and \(A_v\) was the cross sectional area of the vial (4.6 cm\(^2\)).

Therefore using the data generated from the sublimation experiments it was possible to derive figures for barrier resistance from Equation [3.1] and then the vial mean heat transfer coefficient \(K_v\) from Equation [3.2].
3.2.1.3 Contained freeze-drying of a model protein

Collapse temperatures for the IgG formulations were determined using a Lyostat3 freeze-drying microscope (2.3.1) and used to develop the freeze-drying cycles in Table 2.4. Formulation 2 cycle was used for the mannitol formulated IgG non-contained, paper and Tyvek contained vials. The cycle parameters detailed in Table 2.4 were kept exactly the same except primary drying was monitored and extended for vials contained in paper or Tyvek pouches as required. Formulation 3 cycle was used for the trehalose formulated IgG non-contained and paper contained vials. The cycle parameters detailed in Table 2.4 were kept exactly the same except primary drying was monitored and extended for vials contained in paper pouches as required. Formulation 3 cycle was also used for the trehalose formulated IgG Tyvek contained vials except a Ts of -10 °C was used for primary drying. Again the primary drying times were monitored and extended for vials contained in Tyvek pouches until completion.

Both contained (paper and Tyvek) and non-contained vials were dried together. Primary drying times were extended until the temperature of the contained vial RTD was equal to the shelf temperature (accompanied by Pirani pressure drop) before secondary drying was commenced. Secondary drying times were specific for each formulation and kept constant for both contained and non-contained vials. At the end of each drying cycle vials were stoppered by shelf compression under vacuum. Dry layer resistance of the protein was calculated at two hours using the same method for contained, water filled vials.
3.2.1.4 Assessment of freeze-dried model protein

The antibodies were accessed following freeze-drying using the techniques detailed in chapter two. The recovery of the specific IgG antibody was assessed by measurement of the quenching of fluorescein fluorescence. Total protein concentration was estimated by absorbance at 280 nm using an extinction coefficient (1 g/L, 1 cm path length) of 1.5 for ovine IgG. Purity of IgG was assessed by size exclusion chromatography (SEC). Antibody turbidity was assessed using two spectrophotometric methods before freeze-drying and again after reconstitution of freeze-dried material using sterile water. Apparent optical density was recorded at 600 nm and measurement of apparent optical density between 340 nm and 360 nm (both 1 cm path length), expressed as an average at 350 nm was recorded. Residual moisture content of the freeze-dried material was determined using a coulometric Karl Fischer titrator.

3.3 Results

3.3.1 Non-contained, paper and Tyvek barrier ice sublimation tests

3.3.1.1 Sublimation time

Containment in either paper or Tyvek pouches caused an increase in the sublimation time compared with non-contained vials. Vials contained in Tyvek pouches took longer to complete sublimation than vials contained in paper pouches at shelf temperature ($T_s$) of 0 °C (Figure 3.3) or at both 0 °C and -10 °C (Table 3.1). Thus, the
Sublimation times were 12.5% longer in paper and 25% longer in Tyvek when compared to non-contained vials at T_s 0 °C. At T_s -10 °C sublimation times were 27% longer in paper and 43% longer in Tyvek when compared to non-contained vials.

**Figure 3.3** Effect of barriers on sublimation time of pure water at T_s = 0 °C. Sublimation is still able to proceed when vials are contained in paper or Tyvek pouches at a slower rate thus increasing sublimation time. Individual trays were weighed every two hours until sublimation was complete.
Table 3.1 Effect of barriers on sublimation time, sublimation rate and product temperature. All readings were taken in triplicate except for non-contained vials at $T_s = 0$ °C where $n = 4$. The results are expressed as means with standard deviation.

3.3.1.2 Sublimation rate

Gravimetric sublimation rates determined at two hours were normalised as a sublimation rate per vial. Values for non-contained vials were higher compared to vials contained in paper which in turn were higher than in Tyvek pouches (Table 3.1) at shelf temperature.
temperatures \((T_s)\) of 0 °C and -10 °C. As expected, sublimation rates decreased with decreasing shelf temperature.

3.3.1.3 Product temperature

Product temperature \((T_p)\) determined at 2 h sublimation was lowest for non-contained vials, with vials contained in Tyvek pouches having the highest temperature (Table 3.1). This was consistent for \(T_s = 0 \text{ °C}\) and -10 °C with product temperature marginally decreasing with shelf temperature.

3.3.1.4 Resistance to sublimation

The resistance to water vapour evolved during sublimation for different barriers, defined by Pikal (1985) as the ratio of pressure difference to mass flow, was calculated using Equation [3.1]. Barrier resistance \((R_b)\) was determined at 2 h sublimation where non-contained vials showed the least resistance (Figure 3.4) and Tyvek pouches the highest resistance to sublimation. Resistances for paper and Tyvek are shown with stopper resistance \((R_s)\) subtracted. The resistance values appear to be independent of shelf temperature, even given some variability of the results. It must be emphasised that the resistance shown is for a double layer of water vapour permeable material.
Figure 3.4 Effect of barriers on resistance to sublimation showing resistance increasing with different barriers. All readings were taken in triplicate except for non-contained vials at $T_s = 0^\circ C$ where $n = 4$. The results are expressed as means with error bars indicating standard deviation.

3.3.1.5 Heat transfer ($K_v$)

$K_v$ is a measure of the efficiency with which the vial transfers heat from source to sink and is defined by Pikal (1985) as the ratio of the heat flux to temperature difference. Normally heat has to overcome three barriers namely the interface between the shelf surface and the bottom of the aluminium tray; the interface between the tray and the vial bottom; and the ice between the bottom of the vial and the sublimation front. Furthermore, $K_v$ is the sum of three contributions namely $K_c$ from direct conduction from shelf to vial; $K_r$ from radiative heat transfer; and $K_g$ from gas conduction between the shelf and vial (Patel et al., 2010 and Pikal et al., 1984). For different containment systems
$K_v$ was determined using Equation [3.2] and compared to non-contained vials (Figure 3.5). Non-contained vials had the lowest $K_v$ and Tyvek had the largest observed increase in $K_v$. The same increase and order was observed at $T_s = 0 \degree C$ and -10 \degree C. As would be expected, for non-contained vials, $K_v$ is independent of shelf temperature. The effect of a change in shelf temperature is not so clear for the contained systems due to the greater variability in the results.

![Figure 3.5](image-url)  

**Figure 3.5** Effect of barriers on vial mean heat transfer coefficient ($K_v$) showing $K_v$ increasing with different barriers. All readings were taken in triplicate except for non-contained vials at $T_s = 0 \degree C$ where $n = 4$. The results are expressed as means with error bars indicating standard deviation.

### 3.3.2 Sublimation tests using the Gore Lyoguard

The Gore Lyoguard (Lyo R&D container) was tested in direct contact with the freeze-drier shelf without the use of an aluminium tray. Sublimation rate, product temperature, resistance and heat transfer was determined at $T_s = 0 \degree C$ only (Table 3.2).
Table 3.2 Characteristics of the Gore Lyoguard resembling the results obtained for non-contained vials with product temperature 1 °C higher (Table 3.1), low sublimation rates, double the resistance compared to non-contained vials (Figure 3.4) and lower $K_v$ (Figure 3.5). However, $K_v$ cannot be compared to non-contained vials as the Lyoguard was in direct contact with the shelf. The results are expressed as means with standard deviation where n = 3.

3.3.3 Effect of containment on IgG formulations

3.3.3.1 Freeze-drying cycles

As discussed in chapter two, freeze-drying microscopy was used to determine the collapse temperatures ($T_c$) of amorphous (trehalose) and crystalline (mannitol) formulations of IgG. $T_c$ was -20 °C for the former and -7 °C for the latter. The sublimation time for IgG was monitored and extended until completion. $T_c$ and, therefore, $T_p$ was monitored carefully to ensure $T_p$ stayed several degrees below $T_c$.

The effect of containment systems on the freeze-drying behaviour of the antibody sample tested reflected that observed for pure water (Table 3.3). Primary drying time and product temperature increased with increased resistance to water vapour passage of
each barrier used. The same order of barrier resistance was found as for ice sublimation tests. It is important to note that when using the Tyvek pouches with the trehalose formulation the shelf temperature was decreased to -10 °C to avoid primary drying above the collapse temperature. However, reduction of $T_s$ to -10 °C gave a $T_p$ dangerously close to $T_c$ although the drying proceeded without collapse.

<table>
<thead>
<tr>
<th>Containment</th>
<th>Mannitol</th>
<th>Trehalose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-contained</td>
<td>Paper</td>
</tr>
<tr>
<td>Primary Drying Time (h)</td>
<td>18.5</td>
<td>20</td>
</tr>
<tr>
<td>Secondary Drying Time (h)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Shelf Temp (°C)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Product Temp (°C)</td>
<td>-32</td>
<td>-23</td>
</tr>
</tbody>
</table>

Table 3.3 Freeze-drying cycle parameters for amorphous and crystalline IgG formulations showing the effects of containment in paper and Tyvek pouches compared to non-contained vials.

### 3.3.3.2 Dry layer sublimation rates and resistances

To establish an overall comparison of resistance to water vapour during sublimation the dry layer resistance of the amorphous and crystalline formulations were
determined using the described methods (Table 3.4). Figure 3.6 shows that dry layer resistance is important and is greater than the resistance offered by a stopper and interestingly also the Lyoguard ePTFE membrane. However, dry layer resistance is considerably less than that of the paper or Tyvek barriers even when considered as an average for the higher resistance amorphous formulation shown in Figure 3.6.

<table>
<thead>
<tr>
<th></th>
<th>Mannitol</th>
<th>Trehalose</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sublimation Rate (g/h/vial)</strong></td>
<td>0.238 ± 0.01</td>
<td>0.131 ± 0.003</td>
</tr>
<tr>
<td><strong>Resistance (Torr h g⁻¹ vial⁻¹)</strong></td>
<td>0.579 ± 0.09</td>
<td>0.988 ± 0.16</td>
</tr>
</tbody>
</table>

**Table 3.4** Comparison of sublimation rate and dry layer resistance (during primary drying) of crystalline and amorphous formulations of IgG. The results are expressed as means with standard deviation where n = 3.

**Figure 3.6** Comparison of resistance to sublimation presented by the different barriers to water vapour flow determined during this study. The results are expressed as means with error bars indicating standard deviation.
3.3.3.3 Effects of contained freeze-drying on a model protein (ovine IgG)

Fluorescence quenching showed that the freeze-dried antibody samples in non-contained freeze-dried vials (Table 3.5), vials double wrapped in paper pouches (Table 3.6) and vials double wrapped in Tyvek pouches (Table 3.7) exhibited no notable loss of biological activity and remained >95 % pure with no major loss of total protein following freeze-drying. Residual moisture for trehalose and mannitol formulations was ≤ 1.1 %. Some increase in turbidity was noted upon reconstitution. However, this was only relevant for the mannitol formulation and only a minor increase in trehalose turbidity was found compared to pre drying. The degree of turbidity increase was comparable for all samples, contained or non-contained, and was an effect of the freeze-drying process, not of containment, as demonstrated in chapter two.
<table>
<thead>
<tr>
<th></th>
<th>Trehalose</th>
<th>Mannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre Freeze-</td>
<td>Post Freeze-</td>
</tr>
<tr>
<td></td>
<td>Drying</td>
<td>Drying</td>
</tr>
<tr>
<td>Reconstitution</td>
<td>N/A</td>
<td>&lt;30 s</td>
</tr>
<tr>
<td>Time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Turbidity (600nm)</td>
<td>0.022</td>
<td>0.058</td>
</tr>
<tr>
<td>Turbidity (350nm)</td>
<td>0.139</td>
<td>0.213</td>
</tr>
<tr>
<td>OD$_{280}$</td>
<td>26.8 g/L</td>
<td>26.1 g/L</td>
</tr>
<tr>
<td>Size Exclusion</td>
<td>&gt;95 % pure</td>
<td>&gt;95 % pure</td>
</tr>
<tr>
<td>Chromatography</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sp AB (Fluorescence</td>
<td>4.5 g/L</td>
<td>4.5 g/L</td>
</tr>
<tr>
<td>Quenching)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual Moisture (KF)</td>
<td>N/A</td>
<td>1.11 %</td>
</tr>
</tbody>
</table>

**Table 3.5** Effects of freeze-drying on IgG (non-contained vials). Turbidity results show an increase brought about by freeze-drying that appears to be greater for the mannitol formulation compared to trehalose. Total protein concentration and specific antibody concentration appear similar both pre and post freeze-drying and SEC indicates no change in purity brought about by freeze-drying. N/A = not applicable.
<table>
<thead>
<tr>
<th></th>
<th>Trehalose</th>
<th>Mannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre Freeze-Drying</td>
<td>Post Freeze-Drying</td>
</tr>
<tr>
<td>Reconstitution Time</td>
<td>N/A</td>
<td>&lt;30 s</td>
</tr>
<tr>
<td>Turbidity (600nm)</td>
<td>0.022</td>
<td>0.039</td>
</tr>
<tr>
<td>Turbidity (350nm)</td>
<td>0.139</td>
<td>0.182</td>
</tr>
<tr>
<td>OD&lt;sub&gt;280&lt;/sub&gt;</td>
<td>26.8 g/L</td>
<td>26.0 g/L</td>
</tr>
<tr>
<td>Size Exclusion</td>
<td>&gt;95 % pure</td>
<td>&gt;95 % pure</td>
</tr>
<tr>
<td>Chromatography</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sp AB (Fluorescence Quenching)</td>
<td>4.5 g/L</td>
<td>4.3 g/L</td>
</tr>
<tr>
<td>Residual Moisture (KF)</td>
<td>N/A</td>
<td>0.81 %</td>
</tr>
</tbody>
</table>

Table 3.6 Effects of freeze-drying on IgG in vials that were semi-stoppered and double wrapped in paper pouches. Turbidity results show an increase brought about by freeze-drying that appears to be greater for the mannitol formulation compared to trehalose.

Total protein concentration and specific antibody concentration appear similar both pre and post freeze-drying and SEC indicates no change in purity brought about by freeze-drying. N/A = not applicable.
<table>
<thead>
<tr>
<th></th>
<th>Trehalose</th>
<th>Mannitol</th>
<th>Trehalose</th>
<th>Mannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre Freeze-Drying</td>
<td>Post Freeze-Drying</td>
<td>Pre Freeze-Drying</td>
<td>Post Freeze-Drying</td>
</tr>
<tr>
<td>Reconstitution Time</td>
<td>N/A</td>
<td>&lt;30 s</td>
<td>N/A</td>
<td>&lt;30 s</td>
</tr>
<tr>
<td>Turbidity (600nm)</td>
<td>0.0298</td>
<td>0.0407</td>
<td>0.022</td>
<td>0.1013</td>
</tr>
<tr>
<td>Turbidity (350nm)</td>
<td>0.1595</td>
<td>0.194</td>
<td>0.1433</td>
<td>0.3309</td>
</tr>
<tr>
<td>OD&lt;sub&gt;280&lt;/sub&gt;</td>
<td>19.84 g/L</td>
<td>26.99 g/L</td>
<td>27.38 g/L</td>
<td>26.59 g/L</td>
</tr>
<tr>
<td>Size Exclusion</td>
<td>&gt;95 % pure</td>
<td>&gt;95 % pure</td>
<td>&gt;95 % pure</td>
<td>&gt;95 % pure</td>
</tr>
<tr>
<td>Chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sp AB (Fluorescence Quenching)</td>
<td>4.01 g/L</td>
<td>4.1 g/L</td>
<td>3.84 g/L</td>
<td>3.81 g/L</td>
</tr>
<tr>
<td>Residual Moisture (KF)</td>
<td>N/A</td>
<td>0.7 %</td>
<td>N/A</td>
<td>0.68 %</td>
</tr>
</tbody>
</table>

Table 3.7 Effects of freeze-drying on IgG in vials that were semi-stoppered and double wrapped in Tyvek pouches. Turbidity results show an increase brought about by freeze-drying that appears to be greater for the mannitol formulation compared to trehalose. Total protein concentration and specific antibody concentration appear similar both pre and post freeze-drying and SEC indicates no change in purity brought about by freeze-drying. N/A = not applicable.
3.4 Discussion

As shown by Taylor and his colleagues (1978) and by Gassler et al. (2004) containment increases both primary drying times and product temperature. This is caused by barriers affecting the mass transfer and heat transfer characteristics during sublimation. Containing semi-stoppered vials decreases the rate of mass transfer between the aqueous product and the chamber / condenser which slows the overall sublimation time thereby impacting on the product temperature and associated vapour pressure of ice within the containers (Tang et al., 2004).

The sublimation process uses energy through the latent heat of sublimation. To balance this energy demand, heat energy is put into the system via shelves which stop the product cooling and reaching equilibrium with the condenser where sublimation would cease (Adams, 1991a). The reduced sublimation rates induced by the barriers reduced the heat loss through sublimation but the heat input remains the same through constant $T_s$. This resulted in a substantial increase in product temperature for the contained vials.

The degree of barrier resistance for the contained systems reflects the increase in product temperature. Resistances appear to be independent of shelf temperature and broadly constant. Stoppers posed the least resistance and had the shortest sublimation time and highest rate. Tyvek has the longest sublimation time and most reduced sublimation rate with paper between these two.

The use of the paper / Tyvek barriers would clearly have an impact on the heat transfer characteristics of the contained vials and an interesting effect on $K_v$ was observed. The mean heat transfer coefficients for the barrier systems increased relative to the non contained vials. Using the same trays and vials it would be expected that $K_v$
would remain the same with constant $T_s$. As Pikal et al., (1984) demonstrated vial heat transfer coefficients increase with increasing container / chamber pressure. It appears that increased resistance caused by the double layer of barrier results in an increase in pressure inside the pouch causing $K_v$ to increase. This is demonstrated in Figure 4.7 and from Equation [3.1] where the decrease in sublimation rate is proportional to an increase in resistance to water vapour flow causing an increase in the pressure inside the containment system. Further evidence that resistance and, therefore, the pressure increase is responsible for $K_v$ increase is that these data are independent of surface area of the barriers. The pouches have the greatest surface area of any of the barriers used at $870 \text{ cm}^2$ which should provide the fastest rate of exchange of water vapour. Moreover, two differing materials (paper and Tyvek) with the same surface area have quite different sublimation rates. Thus, it is suggested that Equation [3.2] to calculate $K_v$ could be re-written to consider pressure and resistance when barriers to sublimation are used. This is considered in more depth in chapter five.

This pressure dependent increase in $K_v$ in the pouch may suggest that the dominant contribution to $K_v$ would be from $K_g$ as $K_c$ and $K_r$ are both independent of pressure (Patel et al., 2010 and Pikal et al., 1984). However, minor changes in geometry and set up can impact $K_v$. For example, no attempt was made in this study to insulate the front of the drier with aluminium foil to limit $K_r$ which in turn would affect $K_c$ and $K_g$ (Rambthatla et al, 2003). Furthermore, an extra double layer, attributable to the polymer film of the pouch, was added between the shelf and the base of the aluminium tray creating yet another heat transfer interface along with the double layer of water vapour permeable barrier. Further work would be required to determine exactly the heat transfer mechanisms at work with the barriers studied. Manometric temperature measurement (MTM) is considered to be a more advanced technique to calculate these
data (Tang et al., 2006 and Tang et al., 2005) but unfortunately this technology was not available for this study. MTM involves isolating the freeze-drying chamber from the condenser by means of a connecting valve available on most freeze-driers. The rise in pressure, attributable to the generation of water vapour through sublimation is measured and, through a series of computer algorithms, is correlated to the vapour pressure at the sublimation interface. From this the computer software is able to calculate figures for resistance using methods similar to Equation [3.1]. It has also been shown that \( K_v \) can be investigated with MTM and the different contributions to \( K_v \) from \( K_{pg} \), \( K_c \) and \( K_r \) can be determined from pressure rise data. Future investigation might involve MTM along with a more detailed analysis to determine the contributing factors of heat transfer.

The ePTFE membrane of the Gore Lyoguard functioned effectively as a water vapour permeable barrier during sublimation and the sublimation rates were comparable to non-contained vials whose resistance has been taken as negligible in previous studies (Tang et al., 2006). The high sublimation rate was also accompanied by a low product temperature indicating a low resistance to water vapour flow. This is reflected by studies undertaken by Gassler et al. (2004) where the product temperature during sublimation of ice increased only by 3-4 °C with the use of the ePTFE membrane. Although the figures measured for heat transfer cannot be compared directly to non-contained vials, paper or Tyvek pouches (due to direct shelf contact), the data for sublimation rate, \( T_p \), resistance and Gassler’s (2004) data indicate that a comparable \( K_v \) would be low.

Other researchers have reported that dry layer resistance is greater than the resistance offered by a stopper (Kuu et al., 2006 and Johnson et al., 2010) and to date, dry layer resistance has been the highest resistance determined during sublimation. Data from this study show that dry layer resistance is, indeed, important and is greater than the Lyoguard resistance. This was also demonstrated by Gassler et al. (2004) where dry
product layer resistance was greater than the ePTFE membrane when freeze-drying 5% mannitol with a 10mm fill height. However, dry layer resistance is substantially less than that of the paper or Tyvek barriers where it must be remembered that a double layer has been used throughout. Hence, it is important to highlight that freeze-drying is possible using barriers that pose a greater resistance to water vapour flow than product dry layer.

When comparing the Gore Lyoguard to medical grade paper and Tyvek pouches the Lyoguard exhibited minimal resistance to water vapour. This could be assumed to be the case for Taylor’s (1978) Porton Box where a 4 °C increase in product temperature was observed when freeze-drying serum. This is comparable to Gassler’s (2004) temperature rise data, for the Lyoguard, which had minimal resistance to water vapour flow when compared to paper or Tyvek pouches. However, the minimal resistance to the passage of water vapour was due to the use of a bulky gas mask filter or a relatively complex and expensive membrane. When selecting disposable equipment, minimum expenditure is desired. The Lyoguard is several orders of magnitude more expensive than pouches and, therefore, pouches would appear a more appropriate choice for disposable packaging for sterile freeze-drying of niche pharmaceutical products.

The effect of containment systems on the freeze-drying behaviour of the antibody reflected that observed with pure water. Primary drying time and product temperature increased with increased resistance to water vapour passage of each barrier used and the same ranking of resistance is found as for ice sublimation tests. Using amorphous and crystalline formulations of the model pharmaceutical protein, primary drying times were increased and shelf temperature was adjusted to prevent collapse. The resulting products, when compared with non-contained freeze-dried IgG, showed no effect on antibody activity or structure. Residual moisture levels were also comparable indicating that containment did not prevent bound water removal during secondary drying.
Furthermore, it could also be inferred that no structural changes occurred to the freeze-dried cake as reconstitution times are the same. However, scanning electron microscopy of the cakes is required to prove this deduction.

It can be seen that it is possible to contain the freeze-drying process in a number of different equipment designs. These can range from a bulky aluminium box to a disposable pouch. However, whatever the type of container used the selection of the water permeable membrane is crucial to performance during freeze-drying because of its effects on mass and heat transfer. More resistant materials such as Tyvek increase the process time and product temperature the most whereas the less resistant materials such as the ePTFE in the Gore Lyoguard increase these parameters the least. As demonstrated in this work, even if the increase in $T_p$ and process time have no effect on the structure and function of ovine IgG specific for fluorescein (demonstrated by retention of the antibodies ability to bind fluorescein and quench fluorescence) it would still be desirable to complete the process in the least time possible. Clearly future research leading to a combination of a flexible package system with a very low resistance water permeable media would provide a versatile freeze-drying containment system with the least impact on processing time and temperature.
CHAPTER 4: MICROBIOLOGICAL EVALUATION OF FREEZE-DRYING CONTAINMENT SYSTEMS
4.1 Introduction

Prevention of contamination of a sterile product contained inside the Porton box was investigated by Taylor et al. (1978) using an aerosol challenge test. Similarly, aerosol challenge but also talc challenge tests were performed by Gassler et al. (2004) on the Lyoguard. Both researchers aseptically filled their containers with sterile nutrient rich growth medium and then exposed the containers to high concentrations of live microorganisms. After exposure, the containers were surface decontaminated, incubated and inspected for growth of specific contaminating microorganism. Both groups showed that the Porton box and the Lyoguard were able to prevent contamination of sterile media by preventing the passage of microorganisms.

As discussed in chapter one, loss of substrate by ablation can occur when preserving microorganisms by freeze-drying and there exists a paucity of studies detailing methods for containment of microorganisms during freeze-drying. A preliminary study indicated that ablation of microorganisms during freeze-drying could be contained within a glass chamber (Adams, 1991b). Another study using the Porton box, demonstrated its ability to substantially reduce loss of microorganisms, however, some contamination was detected around the seals of the box after freeze-drying (Adams, 1994). Thus, it is clear that considerable scope exists for the evaluation of pouches to fulfil this containment function.

This chapter investigates the effectiveness of pouches to prevent contamination of a sterile product using comparable aerosol challenge and also a freeze-drying process simulation. Tests were performed under dynamic conditions that were as close as possible to those experienced during sterile filling and freeze-drying processes. The
ability of the pouches to contain ablation of live microorganisms was also investigated in the latter part of this chapter. Two different types of microorganisms were freeze-dried and their containment assessed using a range of microbiological methods. Specially developed freeze-drying protocols were also derived to overcome the effects of barrier resistance on mass and heat transfer imposed by pouches. These were developed from data and expertise obtained in chapter three.

4.1.1 Aerosol challenge testing of the Porton box and Gore Lyoguard

Taylor et al. (1978) introduced an aerosol of Serratia marscesens at a concentration of $10^{10}$ cfu / mL through continual loss of vacuum into a freeze-drier containing a Porton box filled with sterile nutrient rich media. After the introduction of aerosolised microorganism the chamber was held at a low vacuum for 48 h. No growth of Serratia marscesens was observed on the media contained inside the Porton box while controls situated outside the Porton box indicated growth of contaminating microorganism.

Gassler et al. (2004) performed an aerosol challenge test and a talc challenge test on the Lyoguard. Lyoguard trays were filled with sterile soybean casein digest broth and were then exposed to an aerosol of Bacillus subtilis var. niger, de-contaminated and incubated at 37 °C for 7 days. The microorganism challenge level was given as 2500 – 23,800 cfu / cm², assumed to be a challenge of cfu / cm² of Lyoguard membrane. No growth of contaminating microorganism was observed while controls exposed within the aerosol chamber indicated growth. The talc challenge test was performed by filling the Lyoguard with molten, sterile trypticase soy agar and allowing it to solidify. The trays
where then placed in a dust chamber and exposed to a high concentration of \( B. \) \textit{licheniformis} spores (Gassler et al., 2004). The trays were decontaminated and incubated at 35 °C for 7 days. No growth was observed within the trays while positive controls indicated growth of contaminating microorganism.

The different units used to express the initial challenge of microorganisms make it difficult to compare the results obtained in these two studies. The Porton box received a large inoculum of microorganisms for challenge testing at \( 10^{10} \text{ cfu / mL} \) but the Lyoguard initial challenge was given as \( \text{cfu / unit surface area of the Lyoguard membrane} \) not the total number of organisms used. If the surface area of the Lyoguard membrane is taken as \( 1014 \text{ cm}^2 \) this would mean that the membrane would have been challenged with \( 2.5 \times 10^6 – 2.4 \times 10^7 \text{ cfu / tray} \) which is several orders of magnitude lower than one millilitre of the Porton box initial challenge. It can be seen the Lyoguard may not have undergone the highest possible challenge when compared directly to Porton box. However, both were able to prevent contamination of sterile media when challenged with aerosolised microorganisms. To demonstrate equivalence to these devices by prevention of contamination of sterile media, pouches were also subjected to a similar aerosol challenge.

4.1.2 Aerosol challenge testing

As discussed by Nolan (2004) guidance standards for aerosol challenge are well established and published in many forms. PDA Technical Report No. 27, Pharmaceutical Package Integrity (1998) gives useful methodology and was used as the fundamental basis on which to develop test protocols during this study. The aerosolised microbial challenge
test involves placing packages containing media into an aerosolisation chamber and charging the chamber with aerosolised microorganisms. Following challenge the packages are removed, decontaminated, incubated and then inspected for growth of contaminating microorganism.

The PDA Technical Report (1994) states that Bacillus subtilis spores are typically used for aerosol challenge testing and this was the organism selected for the aerosol challenge testing of the Lyoguard (Gassler et al., 2004). The reasons for selection of Bacillus subtilis for this type of study are that the spores are non-pathogenic, exist as discrete cells, produce readily measurable colonies and are robust (Tallentire and Sinclair, 1996). However, due to their robust nature they are exceptionally resistant to inactivation and their clean up can be difficult (Sagripanti et al., 2007). Therefore, due to concerns of long term laboratory contamination with Bacillus spores and to minimise potential health risks to personnel a method based on Dunkelberg’s (2006, 2009) work using Saccharomyces cerevisiae was considered a safer option for this study.

Dunkelberg (2006) developed a method to determine the sterile integrity of a wrapped medical product at a probability of re-contamination of 1:10^6. Working to prevent nosocomial infections, 90 mm Sabouraud agar plates were packaged in chevron style pouches (similar to those used in this research and used to contain sterile medical instruments). These were then subjected to an aerosol of Saccharomyces cerevisiae under vacuum cycles and incubated to recover any viable yeast cells that had breached the package. A concentration of 10^7 – 10^8 organisms / mL was nebulised using a Pari Juniorboy nebuliser.

For the purpose of this study an apparatus was constructed (Figure 4.1) that would be able to mimic the low vacuum conditions encountered during freeze-drying. In the freeze-drier it was observed that the pouches inflated under vacuum and deflated
when the vacuum was released due to the resistance of the pouch material as described in chapter three. It was decided that a sterile product contained within pouches would be at most risk of contamination from ingress of airborne contaminating microorganisms during this inflation / deflation cycle. Therefore, the media containing pouches were placed within a vacuum chamber and subjected to a vacuum release cycle with live microorganisms being introduced through a nebuliser during the vacuum release in a similar manner to Dunkelberg’s (2006, 2009) methodology.

![Figure 4.1](image)

**Figure 4.1** Aerosol challenge test apparatus showing component parts.

### 4.1.3 Process simulation using sterile media

Sterile pharmaceutical processes are subject to strict regulation to ensure that products intended for systemic use are safe and efficacious. To help ensure compliance pharmaceutical regulators publish guidance for manufacturers performing sterile processes. The FDA state in their ‘Guide to inspections of lyophilisation of parenterals (7/93)’ that “*Validation of filling operations should include media fills and the sampling of*
critical surfaces and air during active filling (dynamic conditions)”. The EMA publish more
detail in ‘Annex 1, Manufacture of Sterile Medicinal Products, 2008’ specifying “Validation
of aseptic processing should include a process simulation test using a nutrient rich medium
(media fill). The process simulation test should imitate as closely as possible the routine
aseptic manufacturing process. The target should be zero growth in all containers”. Thus,
a pharmaceutical manufacturer would ensure regulatory compliance by mimicking filling
and freeze-drying processes using sterile nutrient rich media.

By their nature media simulations mimic specific sterile process and for this
reason were not used to test the Porton box or Lyoguard. Furthermore, it is usual for
suppliers of equipment designed for sterile processing to limit their testing purely to
functional tests, such as the aerosol and talc challenge tests, leaving process specific
testing to sterile product manufacturers. Therefore, a media process simulation was
thought to be the most appropriate challenge to validate a pharmaceutical sterile process
using pouches. A small scale filling and freeze-drying process was designed and mimicked
using sterile nutrient rich media in exactly the same manner a pharmaceutical
manufacturer ensures regulatory compliance using the cited guidance.

Careful consideration as to what stages are simulated is required as the freeze-
drying process can potentially affect the viability of sterile media and therefore its ability
to support growth of microorganisms and the freezing process is capable of reducing
microbial contamination. Thus, it can be assumed that the same would be true of a
sterile pharmaceutical product where the main risk of contamination would be during the
filling and transportation steps when the product is liquid and at ambient temperature. It
is for these reasons that the FDA specifies in their ‘Guide to inspections of lyophilisation
of parenterals (7/93)’ that “media fills should primarily validate the filling, transportation
and loading aseptic operations”. Following this guidance the pouches were used to
simulate the filling, transportation and loading processes. One reason that pouches are being investigated as containment systems for sterile freeze-drying processes is to fulfil a transport role. Therefore, it was essential that these steps were challenged during this media simulation.

4.1.4 Containment of ablation

Loss of microorganisms during freeze-drying was first reported by Stein and Rogers (1950) when *Bacillus anthracis* was recovered upon analysis of the water retained in their freeze-drier condenser. Their work involved the use of pathogenic organisms and highlighted the need to protect workers from accidental infection. Following this Reitmen *et al.* (1954) and Busby (1959) showed that the presence of inline cotton filters between the drying microorganism and the condenser prevented carryover and recovery of microorganism in the condenser. Thus, the inclusion of a crude filter went some way to prevent accidental infection of personnel and provides an early justification for the adoption of containment for this type of work. During a more recent study Barbaree and Sanchez (1982) freeze-dried two different bacterial cultures of *Pseudomonas aeruginosa* and *Proteus mirabilis* together in the same freeze-drier. When the dried cultures were reconstituted and cultured, growth of the *Pseudomonas* was detected contaminating vials that originally contained only *Proteus* and *vice versa*. They concluded that during the drying process organism had ablated, cross contaminating vials in the freeze-drier chamber. *Escherichia coli* was also used as a trace organism to investigate ablation during preservation by freeze-drying (Adams, 1991b). In that study *E. coli* was cultivated, harvested and re-suspended at high cell densities before being freeze-dried in a vial inside
what was termed a ‘mini-chamber’. After freeze-drying the vials were sealed and the mini chamber rinsed and then cultured indicating that considerable numbers of microorganisms had ablated from the dried product.

As Adams (1996) suggests, protective containment devices could prevent ablation of attenuated vaccines during freeze-drying and it was postulated that the pouches could contain microorganisms during the freeze-drying process. This was initially challenged, unsuccessfully, using cultures of *Saccharomyces cerevisiae*. Latterly these experiments were repeated with *Escherichia coli* JM 109 using a similar method to that developed by Adams (1991).

**4.1.4.1 Containment of microorganism ablation during freeze-drying of *Saccharomyces cerevisiae***

An extensive literature search revealed that the containment of ablation during the freeze-drying of *Saccharomyces cerevisiae* had not been tested previously, so suitable methods for this study had to be developed. Specifically these methods had to incorporate medical grade paper and Tyvek sterilisation pouches.

As discussed by Morgan *et al.* (2006), high initial cell concentrations of greater than $10^8$ cfu / mL are nearly always reported when freeze-drying microorganisms for preservation. High initial population sizes are essential to ensure survival of sufficient numbers of microbial cells for detection as the bulk of cells die during the freezing, drying and storage processes. A method for production of large cell concentrations ($1.0 \times 10^9 – 5.0 \times 10^9$ cfu / mL) of *Candida sake* yeast strain that were preserved by freeze-drying was found that was suitable for this study (Abadias *et al*., 2001).
Selection of the correct suspending medium (freeze-drying formulation) to ensure viability of microorganism was also found to be as important. Working with *S. cerevisiae*, Berney *et al.* (1991) tested 93 separate suspending medium for their ability to preserve viability during freezing and freeze-drying and found that using 10% skimmed milk with 10% trehalose 96% of the inoculum was recovered following freeze-drying. Furthermore, Abadias *et al.* (2001) and Potomska *et al.* (2012) had similar recoveries with other strains of yeast using skimmed milk powder and trehalose. Therefore, 10% skimmed milk and 10% trehalose was selected as a preservation medium.

The freezing process (described in 1.5.3) has been demonstrated to cause damage to protein products by freeze-concentration and pH changes. This is also the case for microorganisms but in addition the formation of ice crystals is also known to damage cells (McLellan and Day, 1995). Therefore, any freezing regime used for freeze-drying requires careful selection to maximise survival of microorganism. Berney *et al.* (1991) found that 3 °C / min was the most effective cooling rate to preserve viability of *S. cerevisiae* freeze-dried at -38 °C. Abadias *et al.* (2001) also found that gentle freezing to -20 °C preserved viability better than aggressive freezing using liquid nitrogen. Kolkowski and Smith (1995) used a slightly different method with their shelf freeze-drier and pre-cooled the shelf to -35 °C prior to placing the product on the shelf. Only one study cited (Berney *et al.*, 1991) utilised *S. cerevisiae* and as this was the organism under consideration for this study this freezing method was considered the most appropriate for use.

Few methods exist in the literature for the preservation of yeasts and fungi by freeze-drying. Again the freeze-drying process drastically affects their survival and careful consideration was required to derive the best protocol. Kolkowski and Smith (1995) state that $T_s$ must be kept low at -35 °C to ensure that drying occurs below the melting point of the sample. The cells must also be kept below the freezing point of the fungal cytoplasm.
that is between -15 and -20 °C until unbound water is removed during sublimation. It seemed wise to dry below the temperatures suggested as Kolkowski and Smith (1995) appear to be describing a similar phenomenon to collapse temperature observed when freeze-drying protein samples.

Freeze-drying cycle development was further complicated by the additional effects of the pouches on the process. When freeze-drying ovine IgG in chapter three it was found that the resistance offered by the pouches changes the mass and heat transfer causing sublimation rates to decrease and product temperature to rise. Therefore these published methods had to be adapted to compensate for the effects of the pouches.

Uncertainties also arose around the selection of recovery methods to enumerate ablated microorganism in this study. Adams (1991b) rinsed the inside of his containment system with re-suspending fluid to recover ablated microorganism and then plated this out to enumerate the number of microorganisms present. This method could not be used during this study due to the nature of the pouch material. When recovery solution was applied to the surface of paper pouches it was absorbed and was impossible to collect, serial dilute and plate out for enumeration. Therefore, it was decided to use contact plates applied directly to the surface of pouches and swabbing to recover any viable cells. Additionally, the 3M Clean-Trace surface ATP analyser swabs were used to detect contamination in terms of relative light units (RLU). Contact plates were intended to cover a specific internal area of the pouch and were enumerated as cfu / 24 cm² (area of the contact plate). The surface area of the contact plate is less than the total pouch surface area exposed to microorganisms, therefore, when no microorganism was detected by a contact plate results were given as <1 cfu / 24 cm² to reflect the potential for non-detected microorganisms. Swabbing with sterile recovery solution was intended to qualitatively detect microorganisms. Swabs analysed with the 3M Clean-Trace surface
ATP analyser give results in RLU which is proportional to the amount of ATP present and therefore gives a measure of cleanliness of a surface (Corbitt, 2000), the higher the RLU the more contaminated a surface is. According to the manufacturer’s instructions (3M, 2013) to determine surface contamination using the 3M Clean-Trace surface ATP analyser a surface can be considered contaminated if a reading of >250 RLU is obtained.

4.1.4.2 Containment of microorganism ablation during freeze-drying of *Escherichia coli*

Adams (1991b) freeze-dried 1 mL of *E. coli* as a trace organism in vials contained within in a mini chamber. The mini chamber was a larger vial cut into two that could be sealed to contain a smaller vial containing suspensions of *E. coli*. A known amount of organism in differing suspending mediums was added to the smaller vial and freeze-dried inside the mini chamber. To determine the extent of ablation, debris was recovered and enumerated to determine the amounts of microorganism present. It was found that varying amounts of organism could be recovered depending upon the formulation used and also that semi-stoppering reduced the losses of bacteria during drying. This approach seemed suitable for this study and instead of mini chambers, vials containing *E. coli* JM109 were double wrapped in either paper or Tyvek pouches.

Importantly the *E. coli* was deliberately re-suspended using 0.9 % sodium chloride that performs badly during freeze-drying due to its low $T_{eu}$. Adams (1991b) intentionally designed a freeze-drying process that ran above the eutectic temperature of the sodium chloride and caused blow out from the vial neck. This blow out causes debris to be ejected from the neck of the vial contaminating the local area. Furthermore, if the filled
vials were not semi-stoppered maximum ejection of ablated microorganism could be achieved.

Before sealing the smaller vials inside the mini-chamber Adams (1991b) froze samples to -40 °C for 4 h. The mini-chambers were then freeze-dried for 16 h with a shelf temperature 4 °C and a vacuum of 13.3 – 8.0 Pa (60 – 100 mTorr). This cycle was adapted for the Edwards freeze-drier and for pouches where the T_s had to be customised to compensate for the effects on mass and heat transfer as characterised in chapter three.

4.2 Materials and Methods

<table>
<thead>
<tr>
<th>Equipment / material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>20mm freeze-drying stoppers C1404</td>
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</tr>
<tr>
<td>Schott type I borosilicate 10mL tubular glass vials</td>
<td>Adelphi Healthcare Packaging,</td>
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<tr>
<td>Schott type I borosilicate 2mL tubular glass vials</td>
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<tr>
<td>Astell AMA240 Autoclave</td>
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</tr>
<tr>
<td>152 mm square aluminium trays of 0.7 mm thickness with a</td>
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<tr>
<td>capacity for 39 x 10 mL vials</td>
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<tr>
<td>Paper chevron style self seal sterilisation pouches</td>
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<td>305 mm x 381 mm</td>
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<td>Equipment / material</td>
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<tr>
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<tr>
<td>Tyvek 1073B chevron style self seal sterilisation pouches 305 mm x 381 mm</td>
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<tr>
<td>Baxa repeater pump</td>
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<td>Sterile Baxa fluid transfer tube set</td>
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<td>HERA Safe KS12 Class II safety cabinet</td>
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<td>3M Clean-Trace surface ATP analyser</td>
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<td>3M Cleantrace swabs</td>
<td></td>
</tr>
<tr>
<td><strong>Equipment / material</strong></td>
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<tr>
<td>--------------------------</td>
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<tr>
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<td>90mm sterile Petri dishes</td>
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<td>Edwards 5 vacuum pump</td>
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<table>
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<tr>
<th><strong>Reagent</strong></th>
<th><strong>Supplier</strong></th>
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<tr>
<td><em>Saccharomyces cerevisiae NCPF 3178</em></td>
<td>HPA Culture Collections, Porton Down, Salisbury, UK</td>
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<td><em>Escherichia coli JM109</em></td>
<td>Promega UK, Southampton, UK</td>
</tr>
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<td>Skim milk powder</td>
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</tr>
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<td>Reagent</td>
<td>Supplier</td>
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<td>----------------------------------------------</td>
<td>---------------------------------------------------------</td>
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<tr>
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</tr>
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<td>Sterile 55mm TSA plates (supplied sterile, pre-poured)</td>
<td></td>
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<td>Sterile 15mL TSA slopes (supplied sterile, pre-poured)</td>
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<td>Sabouraud agar</td>
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<td>Tryptone soya agar</td>
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<td>Maximum recovery diluents (MRD)</td>
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<td>Sodium chloride USP</td>
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</tbody>
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### 4.2.1 Aerosol challenge testing

Sets of 15 Sabouraud agar plates were double wrapped in paper and Tyvek pouches. The vacuum apparatus was set up inside a class II safety cabinet as shown in
Figure 4.2. An overnight culture of *Saccharomyces cerevisiae* was grown in 100 mL of sterile liquid Sabouraud media by inoculating with one colony taken from a stock plate and incubating at 30 °C without agitation. Following incubation the overnight culture was gently mixed to obtain homogeneity and approximately 6 mL of the live culture was added to the Pari LC Sprint Junior nebuliser. Pouches were placed inside the vacuum chamber along with an open Sabouraud agar plate to act as a positive control. The vacuum pump was switched on and a vacuum of around 70 mTorr was applied to the chamber (roughly equivalent to the vapour pressure of ice at -2 °C) and the chamber isolated from the vacuum pump. The nebuliser was then pressurised with compressed nitrogen to 1.5-2.0 bar giving a flow rate of 6 L / min that, according to the manufacturer, would give an aerosol output of 0.23 mL / min with a median diameter of particles of 3.9 µm. The vacuum was released into the chamber through the nebuliser in 3-5 min until atmospheric pressure was re-established. The plates were left inside the chamber for 5 min before surface decontamination and incubation for 48 h at 30 °C. This cycle was repeated until 15 paper and 15 Tyvek pouches had been similarly processed. Positive controls were placed in the chamber with pouches 1, 4, 7, 10, 13, and 15 (for both paper and Tyvek). In addition, at the same points, the packages were swabbed with the 3M Clean-Trace surface ATP analyser according to the manufacturer’s instructions to determine surface contamination (4.1.4.1). The total viable count of the inoculum was determined by serial decimal dilution in Maximum Recovery Diluent (MRD), plating onto Sabouraud agar and incubating at 30 °C for up to 48 hours.
4.2.2 Media process simulation

A small scale freeze-drying run of 312 vials was simulated using nutrient rich media according to FDA and EU guidelines. A total of eight trays of 39 vials were autoclaved at 121 °C for 30 min. Freeze-drying stoppers, control vials and paper and Tyvek pouches were autoclaved at 121°C for 15 min. Vials were then aseptically filled in a horizontal laminar flow cabinet in a clean room, semi-stoppered by hand using pre-sterilised forceps, double wrapped and sealed in pouches. Four trays were sealed in paper and four in Tyvek pouches. In addition, seven positive control vials were filled and semi-stoppered. Five positive control vials were transferred to the Edwards Supermodulyo Freeze-drier housed in the laboratory and the remaining two positive control vials were deliberately contaminated by skin contact. Two of the paper and two of the Tyvek packages were transferred to a Virtis freeze-drier housed in the cleanroom.
The remaining two paper and two Tyvek packaged vials were transferred to the Edwards Supermodulyo Freeze-drier housed in the open laboratory. The packaged vials and positive control vials were left un-stoppered for four hours in the respective driers prior to being stoppered by shelf compression and incubated for two weeks at 35 °C. During the filling and transfer the cleanroom environment was sampled for viable particles using an SAS active air sampler and for non-viable particles using a Climet CI500 laser particle counter. In addition, the surfaces of the laminar flow and the clean room and laboratory freeze-driers were swabbed and analysed using a 3M Clean-Trace surface ATP analyser.

### 4.2.3 Development of freeze-drying cycles to determine ablation during freeze-drying of *Saccharomyces cerevisiae*

To determine the effects of barriers on the freeze-drying process non-sterile trays of vials were prepared containing 39 vials with a 1 mL fill of 10 % w/v skimmed milk / trehalose and double wrapped. Into the two centre vials RTD probes were placed to monitor the $T_p$ during drying. The RTD probes monitored the changes in $T_p$ that the barriers induce during sublimation. As discussed in chapter three barriers decrease sublimation rate and increase $T_p$. The $T_s$ for the freeze-drying method was then adjusted to ensure that the correct $T_p$ was obtained during drying. It was found that barriers interfered so greatly with the mass transfer using the Kolkowski and Smith (1995) method that it was abandoned. Instead the Potomska *et al.* (2012) method III was taken and adapted were the product was frozen to -35 °C over two hours, a vacuum was applied and $T_s$ held at -35 °C for 1 h; the shelf was then heated to -20 °C and held for 15 h, finally the shelf was heated to -10 °C and held for 4.5 h.
4.2.4 Containment of microorganism ablation during freeze-drying of *Saccharomyces cerevisiae*

A starter yeast culture was grown up by inoculating two 100mL lots of sterile Sabouraud broth, each with one colony from a culture plate using an inoculation loop. These were then incubated at 30 °C overnight. The following day the two 100 mL starter cultures were used to inoculate two 500 mL volumes of Sabouraud broth (made up in 5 L sterile conical flasks). A sterile flea was added to each culture, placed on a magnetic stirrer, stirred gently and incubated overnight at 30 °C. The following day the cultures were decanted aseptically into two 1000 mL autoclaved centrifuge pots and spun at 4200 rpm for 35 min at 10 °C. The supernatant was poured off and 10 mL of 0.2 µm filtered phosphate buffered saline (PBS) was added to each pot. The cells were re-suspended, combined into one pot and then spun at 4200 rpm for 35 min at 10 °C. The resultant supernatant was discarded and the cells re-suspended in 10 % of their initial volume using suspending medium. A TVC was performed on the re-suspended cells to determine the number of cells recovered prior to freeze-drying.

Solutions of 10 % skim milk and trehalose were made up in Baxter sterile water for irrigation. The skimmed milk powder was autoclaved at 121 °C for 15 min for the first set of experiments and then Tyndallised for the second set of experiments. Trehalose 10 % solution was 0.2 µm filtered into a sterile container prior to use.

Following the final centrifugation and cell re-suspension in 10 % w/v skimmed milk and trehalose the cells were aseptically filled into vials. Two trays of 39 vials were prepared by autoclaving at 121 °C for 30 min and 1 mL of cell suspension was pipetted
into each vial. The trays were then double wrapped in sterile pouches and transferred to the freeze-drier for processing.

Vials were placed onto the Edwards freeze-drier shelf at ambient temperature and the product was frozen to -35 °C over two hours, vacuum was applied and T₁ held at -35 °C for 1 h; the shelf was then heated to -20 °C and held for 15 h, finally the shelf was heated to -10 °C and held for 4.5 h.

To monitor the sterile freeze-drying of yeast an extra non-sterile tray of vials was prepared containing 39 vials with a 1 mL fill of 10 % w/v skimmed milk / trehalose only and double wrapped. Into the two centre vials of this tray RTD probes were placed to monitor the Tₚ. The non-sterile RTD probes would contaminate the sterile yeast cultures and were therefore placed inside these non-sterile pouches.

When freeze-drying was complete the trays of vials were removed from the freeze-drier. The outer layer of the outer package was sampled with Sabouraud agar contact plate. The outer pouch was then opened and the inner surface sampled with a Sabouraud agar contact plate. The same procedure was also performed on the inner package but the inside of the inner pouch was sampled with three Sabouraud agar contact plates. Also the outer and inner layers of the internal pouch were sampled with the 3M Clean-Trace surface ATP analyser. A sterile swab wetted with sterile PBS was used to sample the whole surface of the inner pouch (870 cm²) and was plated out on a Sabouraud agar plate.

Finally two freeze-dried vials of yeast were reconstituted with 1 mL of sterile water, combined and TVC performed to determine the total number of viable organisms after freeze-drying.
4.2.5 Development of freeze-drying cycles to determine ablation during freeze-drying of *Escherichia coli*

The freeze-drying method originally developed by Adams (1991b) was repeated using vials containing a 1 mL fill of 0.9 % saline. The shelves of the Supermodulyo were pre-cooled to -40 °C and vials (un-wrapped) placed on the shelves and held at -40 °C for 4 h. The shelf temp was ramped to 4 °C in 1 h and then held for 16 h with a vacuum of 7 Pa (52.5 mTorr) being used. The experiment was then repeated firstly double wrapping the trays in paper followed by Tyvek pouches. To compensate for the higher T_p that barrier resistance causes, the shelf temperature was reduced during drying with a T_s of 0 °C used for paper pouches and a T_s of -5 °C used for Tyvek pouches.

4.2.6 Containment of microorganism ablation during freeze-drying of *Escherichia coli*

The *E. coli* JM 109 was grown up on tryptone soya agar (TSA) slopes by inoculating with one colony from a stock culture plate and incubating at 37 °C for 16 hours. The bacterial lawn was re-suspended in 10 mL of sterile 0.9 % saline (prepared by 0.2 µm filtration) and the cell density was determined by TVC. Aliquots of 1 mL of bacterial suspension were then aseptically pipetted into seven autoclaved 3 mL vials. The vials were held in close hexagonal packing in the centre of on autoclaved aluminium tray using aluminium strips. Two duplicate trays were prepared and double wrapped using paper pouches for experiment one and Tyvek pouches for experiment two. The trays were freeze-dried using specially developed methods to allow for the changes brought about in
product temperature and process time by the pouches using shelf temperatures of $T_s = 0$ °C for paper and $T_s = -5$ °C for Tyvek.

When freeze-drying was complete the trays of vials were removed from the freeze-drier. The outer layer of the outer package was sampled with a 3M Clean-Trace surface ATP analyser swab and a TSA contact plate. The outer pouch was then opened and the inner surface sampled with a 3M Clean-Trace surface ATP analyser swab and a TSA contact plate. The same procedure was also performed on the inner package. In addition, the inside of the inner pouch was swabbed with a sterile swab (wetted with sterile PBS) and plated out on a TSA agar plate. To recover debris that had blown out from the vials the trays were rinsed with 1 mL of sterile water and TVC performed to determine the number of organisms present.

4.3 Results

4.3.1 Aerosol challenge tests

An aerosol of *Saccharomyces cerevisiae* at an initial challenge concentration of $8.3 \times 10^6$ cfu/mL was introduced into the vacuum chamber containing pouches and controls. This high concentration of microorganism when tested with the 3M Clean-Trace surface ATP analyser gave a positive control result of 33,524 RLU demonstrating results several orders of magnitude greater than the contaminated surface indication of >250 RLU recommended by 3M (4.1.4.1). The results for all positive controls and contaminated surfaces tested during this study (using the 3M Clean-Trace surface ATP analyser) reflected this large range of detection.
Positive control 90 mm plates placed within the vacuum chamber and deliberately exposed to the aerosol all showed growth with varying numbers of cfu detected on each plate (Figure 4.3). The colonies on each plate were counted and then expressed as an average challenge of cfu per 90mm plate (Table 4.1). At the same positions as the positive control plates the 3M Clean-Trace surface ATP analyser swabs of the outer exposed surface of each pouch demonstrated microbial contamination. Data was expressed as an average challenge per pouch (Table 4.1). Of the fifteen paper pouches exposed, no growth of contaminating *Saccharomyces cerevisiae* was observed on the contained Sabouraud agar plates (Figure 4.4). This was also true for the fifteen Tyvek pouches exposed (Figure 4.5).

<table>
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<tr>
<th>Pouch material</th>
<th>cfu / 64 cm² mean</th>
<th>RLU / swab mean</th>
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<tr>
<td><strong>Paper</strong></td>
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<td>5388</td>
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<tr>
<td><strong>Tyvek</strong></td>
<td>43</td>
<td>2462</td>
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**Table 4.1**  Mean cell recovery of positive controls and mean RLU values (n = 6) from pouch exterior following aerosol challenge testing
Figure 4.3  *S. cerevisiae* (positive control for aerosol challenge test) cultivated on a 90 mm Sabouraud agar plate at 30 °C for 48h.

Figure 4.4  An example of a 90 mm Sabouraud agar plate double wrapped in paper pouches after exposure to an aerosol of *Saccharomyces cerevisiae* and incubation at 30 °C for 48 h showing no contaminating microorganism. Note that the plates were packaged so that the exposed agar faced towards the porous paper side of the pouch.
Figure 4.5 An example of a 90 mm Sabouraud agar plate double wrapped in Tyvek pouches after exposure to an aerosol of *Saccharomyces cerevisiae* and incubation at 30 °C for 48 h showing no contaminating microorganism. Note that the plates were packaged so that the exposed agar faced towards the porous paper side of the pouch.

### 4.3.2 Media Simulation

The two vials filled with TSB media and deliberately contaminated as positive controls became turbid after 24 h incubation at 35 °C (Figure 4.6) indicating microbial growth, as expected. From the 5 positive control vials transported to the laboratory freeze-drier one vial showed growth after 48 h incubation (Figure 4.7). After two weeks incubation the eight packages of vials were opened and each individual vial compared to a positive control. All of the 312 vials in the packages remained clear and were negative for microbial contamination (Figure 4.8). The remainder of the bulk sterile media used to fill the vials was incubated and this also showed no evidence of growth.
Environmental monitoring data showed that the laminar flow maintained an EU grade A environment (EMD Annex 1, 2008). This is <3520 particles at 0.5 µm and <20 particles at 5.0 µm per cubic metre of non-viable air sample and <1 cfu / m³ of viable air sample and <1 cfu / 4 hours on a 90mm settle plate. The clean room also maintained an EU grade A environment conforming to the previous specifications. No colonies were recovered from all settle and viable air sample plates during operation. In addition, 0 particles were detected in the LAF at 0.5 and 5 µm with 1 particle detected at both 5 and 0.5 µm as a background in the room.

3M Cleantrace surface ATP analyser swabs of surfaces within the laminar flow and the Virtis freeze-drier (housed in the cleanroom) gave luminescence readings between 10 - 20 RLU well below the pass value of 250 RLU given by the manufacturer (4.1.4.1). However, the Edwards freeze-drier (housed in the general laboratory) gave counts of 101 and 320 RLU. Compared to a heavily contaminated swab, such as that for a live microbial culture (which would be in excess of 30,000 RLU), these counts are relatively low. However, there is an indication that the laboratory drier carries marginally more contamination than the cleanroom drier having a reading classed as a failure by being > 250 RLU. Therefore, slightly greater potential for contamination of a sterile product exists in the laboratory freeze-drier.
Figure 4.6 The two positive control vials deliberately contaminated by skin contact during the media simulation. Both vials are turbid indicating microbial growth.

Figure 4.7 Five positive control vials exposed to the environment during transportation. Vials one to four remained clear indicating no microbial growth. Vial five is turbid indicating microbial growth.
Figure 4.8 A tray of vials from the media simulation experiment (un-wrapped after incubation) indicating no microbial growth and demonstrating that double wrapping can prevent contamination of a sterile product when mimicking a small scale freeze-drying process.

4.3.3 Freeze-drying cycles for containment of *Saccharomyces cerevisiae*

As discussed in 4.2.3 the Kolkowski and Smith (1995) protocol was first selected to freeze-dry the *S. cerevisiae*. The method was initially tested without microorganisms using vials filled with the 10 % w/v skimmed milk / trehalose suspension medium and double wrapped in paper. The product was frozen to -35 °C in two hours, vacuum was applied when $T_p = T_s$ and held at - 35 °C for 8 hours to allow for the slower sublimation caused by the pouches (this was considered the primary drying stage of the process). Following this the $T_s$ was increased to 10 °C over 9 hours and held for 12 hours to complete secondary drying. During primary drying it was observed that the $T_p$ remained at around -40 °C whilst $T_s = -35^\circ$C. $T_p$ then rose with $T_s$ during the ramp to 10 °C
completing sublimation when $T_s = 0 ^\circ C$. The process was repeated with Tyvek pouches and the same result was observed. This would indicate that the resistance provided by the pouches was limiting sublimation to such an extent that primary drying was unable to reach completion after 8 h. The completion of primary drying (indicated by a drop in Pirani pressure and $T_p = T_s$, 3.2.1) was observed at 0 °C during the ramp for secondary drying. This was too large an excursion from the conditions set by Kolkowski and Smith (1995) that viability of the $S. cerevisiae$ could not be guaranteed, therefore, the Potomska (2012) method was tested as an alternative.

The Potomska (2012) method was performed first using vials filled with the 10 % w/v skimmed milk / trehalose suspension medium without containment. The run was successful and primary drying completion (3.2.1) was observed after 13.5 hours of the 15 hour hold at $T_s = -20 ^\circ C$. Vials double wrapped in paper were then dried using the same method. The primary drying was monitored and extended to 18 hours to allow for the slower sublimation caused by the pouches. The shelf temperature was kept constant at -20 °C where an increase in $T_p$ to -28 °C was observed (open vials dried at $T_p = -32 ^\circ C$). This increase in $T_p$ was considered acceptable as there was no product collapse. Being below the melting point of fungal cytoplasm of between -15 and -20 °C (4.1.4.1) it was assumed not to be a threat to the viability of the $S. cerevisiae$. The same method was repeated using vials double wrapped in Tyvek. In this case, primary drying at $T_s = -20 ^\circ C$ had to be extended to 19 hours until completion. The observed product temperature was similar to that for paper at -28 °C. No collapse was observed and the product had stayed below recommended limits again indicating there would be no threat to the viability of the $S. cerevisiae$. All of the subsequent freeze-drying runs using live $S. cerevisiae$ were performed using these protocols.
4.3.4 Containment of microorganism ablation during freeze-drying of *Saccharomyces cerevisiae*

Two freeze-drying runs were performed using the *S. cerevisiae* each with two duplicate trays of 39 vials re-suspended in 10 % w/v skimmed milk / trehalose. Analysis of the trays following both freeze-drying runs failed to detect *S. cerevisiae* ablation using contact plates (Table 4.2) the 3M Clean-Trace surface ATP analyser swabs (Table 4.3), and sterile swabs (Table 4.4). Both experiments yielded a lower than expected concentration of microorganism following culture and re-suspension prior to freeze-drying. Furthermore, loss of *S. cerevisiae* viability was also seen following freeze-drying (Table 4.5). During the first freeze-drying run only 0.075 % of cells were recovered and this increased to 0.57 % for the second attempt. Elegant cakes were formed in the vials and no debris attributed to ablation was detected visually. These experiments were only performed using paper pouches as no ablation was detected during the first experiment a repeat was performed to verify this result.
### Table 4.2

Enumeration of contamination of paper pouch surfaces following freeze-drying of *S. cerevisiae* determined using Sabouraud agar contact plates incubated for 5 days at 30 °C. The results indicate no detection of microorganism per unit surface area of the contact plate and are therefore expressed as <1 cfu / 24 cm².

<table>
<thead>
<tr>
<th></th>
<th>Run 1</th>
<th>Run 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exterior (outer pouch)</td>
<td>Exterior (outer pouch)</td>
</tr>
<tr>
<td>Tray 1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Tray 2</td>
<td>1</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>Interior (outer pouch)</td>
<td>Interior (inner pouch)</td>
</tr>
<tr>
<td>Tray 1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Tray 2</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>Exterior (inner pouch)</td>
<td></td>
</tr>
<tr>
<td>Tray 1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Tray 2</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>Interior (inner pouch)</td>
<td></td>
</tr>
<tr>
<td>Tray 1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Tray 2</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Run 1</td>
<td>RLU on the exterior surface of the inner pouch</td>
<td>RLU on the interior surface of the inner pouch</td>
</tr>
<tr>
<td>-------</td>
<td>-----------------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Tray 1</td>
<td>26</td>
<td>12</td>
</tr>
<tr>
<td>Tray 2</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td>Shelf</td>
<td>Upper Shelf</td>
<td>Control (Hand)</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>20</td>
</tr>
<tr>
<td>Run 2</td>
<td>RLU on the exterior surface of the inner pouch</td>
<td>RLU on the interior surface of the inner pouch</td>
</tr>
<tr>
<td>Tray 1</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td>Tray 2</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>Shelf</td>
<td>Upper Shelf</td>
<td>Control (Hand)</td>
</tr>
<tr>
<td></td>
<td>74</td>
<td>12</td>
</tr>
</tbody>
</table>

**Table 4.3** Level of contamination of paper pouch surfaces following freeze-drying of *S. cerevisiae*. Data given as RLU using 3M Cleantrace swabs. The manufactures recommend that a figure of >250 RLU indicates a contaminated surface. The results above indicate no detection of contamination.
Run 1 | Interior (inner pouch)  
---|---  
Tray 1 | <1  
Tray 2 | <1  
Run 2 | Interior (inner pouch)  
Tray 1 | <1  
Tray 2 | <1  

Table 4.4 Analysis of swabs of the interior of the inner pouch following freeze-drying *S. cerevisiae* in paper pouches (data given in cfu / 24 cm²) indicating no contamination.

| Run 1 | cfu / mL  
---|---  
Pre freeze-drying | 2.01 x 10⁷  
Post freeze-drying | 1.5 x 10⁴  
Run 2 | cfu / mL  
Pre freeze-drying | 7.5 x 10⁵  
Post freeze-drying | 4.25 x 10⁴  

Table 4.5 Cell densities of *S. cerevisiae* before and after freeze-drying. Run 1 pre freeze-drying and Run 2 post freeze-drying are expressed as means where n=2.

4.3.5 Freeze-drying cycles for containment of *Escherichia coli*

Repeating the freeze-drying method originally developed by Adams (1991b), without containment, caused blowout from the vials coating the shelf above with debris
During this experiment a $T_s$ of 4 °C was used yielding an initial $T_p$ of -28 °C that slowly increased until the completion of drying (taken as $T_p = T_s$) with a total drying time of 9 h. The experiment was then repeated double wrapping the trays in paper and using the lower $T_s$ of 0 °C. Similar results were observed with debris covering the inner layer of the inner pouch. Importantly $T_p$ was around -28 °C and total drying time was 9h indicating the vials had experienced the same conditions as during the previous experiment. Repeating the experiment with Tyvek pouches, $T_s$ was dropped to -5 °C, and again blowout was observed, $T_p$ was around -26 °C and drying time was around 9 h. Thus, a consistent freeze-drying process yielding observable blowout with paper ($T_s$ of 0 °C) and Tyvek ($T_s$ of -5 °C) pouches was obtained. This was then used for all subsequent experiments using live microorganism.

![Figure 4.9](image)

**Figure 4.9** Blowout coating the upper shelf observed when freeze-drying 0.9 % saline.

### 4.3.6 Containment of microorganism ablation during freeze-drying of *Escherichia coli*

Two freeze-drying runs were performed using paper and Tyvek pouches, each being performed using duplicate sets of vials. Visual inspection of the trays following the
first freeze-drying run using paper pouches detected no debris in tray 1 and considerable debris deposits in tray 2. Additionally, the interior surface of the inner pouch of tray 2 showed some absorption of deposited salt crystals. Further inspection of the exterior surface of this inner paper pouch showed some of the crystalline debris had penetrated through the paper layer. During run 2 both Tyvek pouch duplicates yielded debris deposits contained within the pouch and trays. Again, the interior surface of the inner Tyvek pouches showed some absorption of deposited salt crystals. However, this had not penetrated through the Tyvek material as observed with the paper tray 2. Where ablation was observed, paper tray 2 had the largest amount of debris deposited.

Results obtained using the 3M Clean-Trace surface ATP analyser swabs (Table 4.6) confirmed the debris distribution observations. Where large amounts of debris were observed on the inner layer of the interior packages (paper tray 2, and Tyvek trays 1 and 2) high RLU readings (indicative of a high level of contaminants) were also noted (Table 4.6). The paper tray 1, where no ablation was observed, showed only background readings. Testing the exterior of the inner pouches showed background readings except for the paper tray 2 where a high background reading was seen. This correlated with the visible breakthrough of crystalline material and could be attributable to small amounts of contamination. For paper and Tyvek trays the interior of the outer pouches produced low background readings. The exterior of the outer pouches showed only low background counts except tray 2 of the Tyvek pouches which had a slightly higher background count. These layers had been exposed to the environment during transport to and from the freeze-drier and it would, therefore, be usual to detect background levels of contamination. Analysis of the freeze-drier shelves showed similar low background counts usually observed in general laboratory areas. Positive controls were taken as a swab of the palm of the hand where the RLU figures indicated a contaminated surface.
The swabs collected from the interior of the inner pouch with sterile PBS all showed substantial levels of *E. coli* for paper tray 2 and Tyvek trays 1 and 2 only tray 1 paper showed no growth of contaminating microorganism (Table 4.7). The detection of microorganisms using the swabs corresponds to the observation of debris observed and the RLU figures obtained.

<table>
<thead>
<tr>
<th>Paper</th>
<th>RLU of exterior surface of the outer pouch</th>
<th>RLU of interior surface of the outer pouch</th>
<th>RLU of exterior surface of the inner pouch</th>
<th>RLU of interior surface of the inner pouch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tray 1</td>
<td>94</td>
<td>72</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>Tray 2</td>
<td>17</td>
<td>25</td>
<td>891</td>
<td>31,231</td>
</tr>
<tr>
<td>Shelf</td>
<td></td>
<td>Upper Shelf</td>
<td>Control (Hand)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>61</td>
<td>80</td>
<td>15,098</td>
<td></td>
</tr>
<tr>
<td>Tyvek</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tray 1</td>
<td>91</td>
<td>15</td>
<td>18</td>
<td>24,275</td>
</tr>
<tr>
<td>Tray 2</td>
<td>295</td>
<td>56</td>
<td>51</td>
<td>31,189</td>
</tr>
<tr>
<td>Shelf</td>
<td></td>
<td>Upper Shelf</td>
<td>Control (Hand)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>57</td>
<td>4,467</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.6 Ablated *E. coli* following freeze-drying in paper and Tyvek pouches determined using Cleantrace swabs. The exterior of the Tyvek tray 2 can be considered as a contaminated surface with an RLU reading of >250. In addition, the exterior of the inner pouch of paper tray 2 can also be considered a contaminated surface.
<table>
<thead>
<tr>
<th>Paper</th>
<th>Interior (inner pouch)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tray 1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Tray 2</td>
<td>140</td>
</tr>
<tr>
<td>Tyvek</td>
<td>Interior (inner pouch)</td>
</tr>
<tr>
<td>Tray 1</td>
<td>TNTC*</td>
</tr>
<tr>
<td>Tray 2</td>
<td>TNTC*</td>
</tr>
</tbody>
</table>

Table 4.7  Ablated *E. coli* following freeze-drying in paper and Tyvek pouches determined using sterile swabs of the interior of the inner pouch after plating out and 48 hours incubation at 37 °C (data given in cfu / 64 cm²).

*TNTC = too numerous to count

Contact plates applied to all layers of the pouches (paper and Tyvek) directly above where the vials were situated in the trays indicated low levels of contamination for the outer layers that had been exposed to the environment (Table 4.8). The inner layers of the exterior pouches (paper and Tyvek) showed no growth of contaminating microorganism. The external layer of the inner pouch of paper tray 1 and Tyvek tray 2 also indicated no growth. However, 1 cfu / 24 cm² was detected on paper tray 2 and Tyvek tray 1. The interior surface of all the inner pouches (exposed directly to any debris) showed large numbers of contaminating microorganism and the relative positions of the close hexagonal packing of the seven vials was clearly visible (Figure 4.10). Tray 1 paper pouch was the exception. Here, 5 cfu / 24 cm² was detected indicating that only minor ablation had occurred. The reason for this was unknown.
<table>
<thead>
<tr>
<th></th>
<th>cfu Exterior (outer pouch)</th>
<th>cfu Interior (outer pouch)</th>
<th>cfu Exterior (inner pouch)</th>
<th>cfu Interior (inner pouch)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tray 1</td>
<td>4</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>5</td>
</tr>
<tr>
<td>Tray 2</td>
<td>3</td>
<td>&lt;1</td>
<td>1</td>
<td>TNTC*</td>
</tr>
<tr>
<td>Tyvek</td>
<td>cfu Exterior (outer pouch)</td>
<td>cfu Interior (outer pouch)</td>
<td>cfu Exterior (inner pouch)</td>
<td>cfu Interior (inner pouch)</td>
</tr>
<tr>
<td>Tray 1</td>
<td>2</td>
<td>&lt;1</td>
<td>1</td>
<td>TNTC*</td>
</tr>
<tr>
<td>Tray 2</td>
<td>1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>TNTC*</td>
</tr>
</tbody>
</table>

*Table 4.8* Ablated *E. coli* following freeze-drying in paper and Tyvek pouches determined using contact plates after 48 hours incubation at 37 °C (data given in cfu / 24 cm²).

*TNTC = too numerous to count

![Figure 4.10](image)

*Figure 4.10* Contact plate taken from the inner surface of the inner package of paper tray 2 illustrating recovery of multiple colonies of *E. coli*. The pattern of the colonies recovered on the plate reflects the close hexagonal packing of the vials.
The initial cell densities of the inocula for the ablation experiments were equivalent to those obtained by Adams (1991b) of $2.25 \times 10^9 - 1.35 \times 10^9$ cfu / mL. However, the number of cells recovered in the debris (Table 4.9) could not be compared to Adams (1991b) as a larger number of vials were freeze-dried.

<table>
<thead>
<tr>
<th></th>
<th>Tray 1 (cfu / mL)</th>
<th>Tray 2 (cfu / mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre freeze-drying</strong></td>
<td>$1.67 \times 10^9$</td>
<td>$1.17 \times 10^9$</td>
</tr>
<tr>
<td><strong>Post freeze-drying</strong></td>
<td>Not detected</td>
<td>$1.0 \times 10^6$</td>
</tr>
<tr>
<td><strong>Tyvek</strong></td>
<td>Tray 1 (cfu / mL)</td>
<td>Tray 2 (cfu / mL)</td>
</tr>
<tr>
<td><strong>Pre freeze-drying</strong></td>
<td>$1.5 \times 10^9$</td>
<td>$1.5 \times 10^9$</td>
</tr>
<tr>
<td><strong>Post freeze-drying</strong></td>
<td>$4.17 \times 10^3$</td>
<td>$3.33 \times 10^3$</td>
</tr>
</tbody>
</table>

**Table 4.9** Cell densities of *E. coli* before and after freeze-drying. A single TVC was performed per sample.

### 4.4 Discussion

The Paper and Tyvek layers used in the construction of the pouches were selected on the basis of their ability to prevent the passage of microorganisms during sterilisation processes (DuPont, 2009). This was also proven for aerosol challenge tests, process simulation of a small batch size vial freeze-drying process and ablation experiments. The aerosol challenge test showed the ability of the selected pouches to prevent entry of contaminating microorganism. When pouches were subjected to a high concentration of aerosolised microorganisms they were able to prevent the contamination of nutrient rich
media held inside the pouches to a level of 100 %, although a smaller sample size was used when compared to Dunkelberg (2006). The process simulation qualified the ability of the pouches to prevent contamination of nutrient rich media and was performed following guidelines set down by both European and US regulators for the validation of sterile pharmaceutical freeze-drying processes. Thus, it can be claimed that pouches prevent the contamination of sterile products by microorganisms under high challenge conditions and when simulating a sterile filling, transport and loading process. Therefore, it is possible to freeze-dry sterile products in a non-sterile laboratory environment by double wrapping in either Tyvek or paper pouches (where double wrapping is used to provide extra security to the sterile product). The performance of the pouches in comparable aerosol challenge tests performed using the Porton box and the Gore Lyoguard show equivalent results in their ability for excluding microorganisms. However, the innovative application of the pouches to perform both vial and bulk freeze-drying processes distinguishes them from the other two containment systems.

This research highlights a new method for the preservation of microorganisms by freeze-drying. As discussed, the freeze-drying of microorganisms can lead to contamination of the freeze-drier. This can be exacerbated if collapse is observed, leading to the formation of aerosols distributing microorganisms around the freeze-drier (Morgan et al., 2006). Using lower initial cell concentrations is a strategy to limit the amount of contaminating microorganism released during ablation. However, this is likely to affect the long term viability of preserved cultures by a reduction in cell survival. This study indicates that it is possible to contain high initial concentrations (10^9 cfu / mL) of *Escherichia coli* when formulated in a medium that collapses by using a double layer of pouch containment.
Due to the experienced gained handling *S. cerevisiae* during the aerosol challenge testing it was decided to also use it for ablation experiments. However, with no other published studies detailing the ablation of *S. cerevisiae* during freeze-drying it proved complex to develop all the different processes required and after carrying out two experiments using the *S. cerevisiae* no ablated microorganism could be detected. Hence it was decided to follow the Adams (1991b) method (using *E. coli*) where successful detection of ablated microorganism had been reported.

Many factors may have contributed to the unsuccessful detection of ablated *S. cerevisiae* during the execution of these experiments. However, it is thought that the loss of cell viability following freeze-drying, where recoveries of less than 1% were obtained, was the major cause. With the loss of 99% of viability there would be few remaining cells available to contaminate by ablation. Compounding this, the low initial concentration of cells obtained following culture (2 x 10⁷ for experiment 1 and 7.5 x 10⁵ for experiment 2) reduced the chances of recovering viable cells following freeze-drying. When these initial cell concentrations are compared to the recommendations made by Morgan *et al.* (2006) of at least 10⁸ cfu / mL it appears that inadequate numbers of organism were present prior to freeze-drying to ensure survival.

The freeze-drying cycles developed from the literature may have also played a role in the loss of viability of the *S. cerevisiae*. As Berney *et al.* (1991) demonstrated freezing rates prior to freeze-drying were critical, although slower rates were better than faster rates. The freezing rates used in this study could have had potentially damaging effects on the microorganism with the most probable explanation being formation of intracellular ice crystals. Moreover, the Tₚ used for freeze-drying was based upon a figure supplied by Kolkowski and Smith (1995) for the freezing temperature of fungal cytoplasm.
This is, however, not optimised for *S. cerevisiae* and represents another area where potential damage to the cells could have occurred.

The preparation of sterile skimmed milk for re-suspension and protection of cells during freeze-drying also proved difficult for both experiments. The skimmed milk used in experiment 1 was autoclaved at 121 °C for 5 min were it caramelised turning a pale pink colour. This decolourisation was initially thought to be usual. However, it was subsequently discovered that it was attributable to denaturation of the skimmed milk. To avoid this, prior to experiment 2, the skimmed milk was Tyndallised by exposure to three 30 min heating cycles to 100 °C over three days. However, during the third 100 °C cycle decolourisation of the skimmed milk was again observed. This decolourised skimmed milk was again used and low viabilities of cells were recovered. It is thought that decolourisation of the milk damaged its ability to protect cells during freeze-drying and could also be partly responsible for the loss of viability of the cells.

Another reason that ablation of *S. cerevisiae* was not detected was thought to be due to the excellent quality of the cakes produced after freeze-drying. There was no evidence of cake collapse where the skimmed milk / trehalose combination produced elegant and pharmaceutically acceptable cakes. During the *E. coli* experiment the cakes were designed to collapse and produce debris that was ejected outside the vial whereas the skimmed milk / trehalose formulation was selected for its ability to preserve viability of freeze-dried yeasts. It is postulated that this selection did not induce ablation and is a further reason why *S. cerevisiae* was not detected. Repeating this exercise using 0.9 % saline as a suspending medium for *S. cerevisiae* might prove more successful for detection of ablated microorganism, so long as viability following freeze-drying was not diminished in this suspending medium. Further effort to optimise experimental conditions may have been rewarded.
Finally, the relatively large size and behaviour of the *S. cerevisiae* may have played a role in the prevention of ablation during freeze-drying. Cells of *S. cerevisiae* are known to be in the range of 5 – 10 µm in diameter and readily group together to form clusters. It is possible that these clusters were too big to be carried in the sublimating water vapour.

When freeze-drying *E. coli* in 0.9 % saline collapse was observed and large amounts of debris were deposited inside the inner pouch of double wrapped trays. Using high initial cell concentrations of $1.17 \times 10^9$ - $1.67 \times 10^9$ recoveries of $3.33 \times 10^3$ - $1.0 \times 10^6$ cfu / mL were obtained from the debris when using both paper and Tyvek containment pouches. During these experiments a crystalline deposit was also observed on the internal layer of the inner pouches. When this deposit was analysed with contact plates and sterile swabs it was also found to contain viable *E. coli*. Also the 3M Clean-Trace surface ATP analyser swabs detected surface contamination of between 24,273 – 31,231 RLU, similar to the levels of 33,524 RLU found when analysing a culture of *S. cerevisiae* (cell concentration $8.3 \times 10^6$). Testing the external layer of the internal pouch (paper tray 1 and Tyvek trays 1 and 2) using contact plates showed a considerable reduction in the number of organisms detected with no more than 1 cfu / 24 cm$^2$ found and a reduction in 3M Clean-Trace surface ATP analyser readings. However, this was not true for tray 2 paper where a Clean-Trace surface ATP analyser reading of 891 RLU was detected (considered a contaminated surface at >250 RLU). This was the package were the largest collapse was observed causing penetration or breakthrough of crystalline material. It is thought that, due to the semi-absorbent nature of the paper material, some of the aerosolised collapsed material had potentially soaked through the paper. This was not observed using the Tyvek packages as there hydrophobicity would have prevented this.

It can be seen that the inner pouches containing the trays of collapsed *E. coli* are able to contain the ablated microorganism. However, this cannot be considered absolute
retention of *E. coli* as 1 cfu / 24 cm$^2$ was detected for both paper and Tyvek pouches on the exterior surface of the inner pouch (although this could have been chance contamination as colonies were not formally identified). Sampling of the inner layer of the outer pouches (paper and Tyvek) with contact plates and background readings using 3M Clean-Trace surface ATP analyser swabs failed to demonstrate bacterial contamination. Importantly, this result indicates that the small amount of contamination detected on the exterior layer of the inner pouch (paper tray 2) has been contained by the exterior pouch and prevented from reaching the environment. The exterior surface of the outer pouches indicated minor levels of contamination as detected by contact plates and 3M Clean-Trace surface ATP analyser swabs attributable to exposure to the environment during transportation from the aseptic area to the freeze-drier and back again.

Thus it can be seen that both paper and Tyvek pouches are able to contain ablation of *E. coli* during freeze-drying, therefore, preventing potential contamination of the freeze-drier and environment. However, it is essential that double layers are used as a single layer does not provide enough security for absolute containment of *E. coli*.

It is not known why tray 1 paper had minor ablation and no debris. The data suggest that the vials did not undergo collapse to the same extent as tray 2. Prevention of collapse could have come about if the product was able to sublimate at a much lower $T_p$ than that derived during the cycle development tests. It is not known how this could have been possible as both trays were treated as duplicates and freeze-dried upon the same shelf. However, it is postulated that if the tray did not have proper contact with the shelf during freeze-drying heat transfer would be reduced and, due to the latent heat of sublimation, the $T_p$ would be lower. When freeze-drying vials in pouches it has been the norm to place the clear polymer layer at the base to be in contact with the shelf.
Therefore, it is not possible to confirm visually the position of the vials or trays in relation to the shelf during loading. Thus, it is possible that this tray may not have had proper contact with the freeze-drier shelf effecting the $T_p$ and subsequent ablation.

Unfortunately, it was not possible to repeat this test and the single data point obtained using paper pouches will have to be relied upon.

During the aerosol challenge testing it could be argued that the selection of a relatively large $S.\ cerevisiae$ may not represent the fairest challenge to the ability of the pouches to prevent contamination of sterile media. A smaller microorganism presenting a greater challenge would have been a better selection, although the selection of $S.\ cerevisiae$ was based on laboratory safety and available equipment and not the size of the microorganism. Given the development of the experimental techniques during this research a repeat aerosol challenge using $E.\ coli$ JM109 would seem logical. However, after the use of $E.\ coli$ JM109 for the successful containment of ablation and prevention of contamination of the freeze-drier it would seem plausible to suggest this retest to be unnecessary. During this study it was demonstrated that both paper and Tyvek pouches were able to retain $E.\ coli$ during freeze-drying at high levels of organism challenge. Therefore, if the pouches are able to retain high levels of microorganisms it would seem logical that the pouches would also be able to prevent their entry.

At the start of this research the testing of the hypothesis of performing sterile freeze-drying (vials and bulk products) in a non-sterile environment using paper or Tyvek sterilisation pouches was the primary aim. Furthermore, the development of this novel process for the manufacture of sterile pharmaceutical products was considered the main objective. However, as this research has developed over time, it has become apparent that this technology also has important implications in bio-safety, particularly the containment of microorganisms during freeze-drying and the prevention of
contamination of the apparatus and environment. For example, Cammack et al. (1985) described the loss of virus titre by ablation when freeze-drying vaccines and Adams (1994) also discusses the use of containment systems to prevent the contamination of a freeze-drier by ablation when processing bio-hazardous products. Therefore, it would seem that there is potential scope for the use of pouches to contain microorganisms when preparing vaccines, stock cultures of microorganisms or potentially pathogenic microorganisms.
CHAPTER 5: FINAL DISCUSSION, CONCLUSIONS AND FUTURE WORK
5.1 Final discussion, conclusions and future work

The concept of using containment to perform sterile freeze-drying in an un-clean environment was first investigated by Taylor et al. (1978) using the Porton box. More recently Gassler et al. (2004) reported on the performance of the Gore Lyoguard, developed for bulk freeze-drying by W. L. Gore and Associates Incorporated. Critical evaluation of these studies indicated scope existed for a reappraisal of freeze-drying containment systems. Specifically, improvements to the design of containment systems, characterisation of their effects on pharmaceutically active proteins and on mass and heat transfer and validation of their ability to protect a sterile product from contamination would contribute considerably. The objective of this research was to develop and characterise novel containment systems suitable for pharmaceutical manufacturers of neglected and orphan drugs to freeze-dry products without having to invest in expensive sterile processing equipment and facilities. It is likely the considerable expense of sterile processing currently prevents the manufacture of some neglected drugs such as antivenoms. The resultant cost savings that containment systems offer might enable these low volume drugs to be produced to the benefit of patients suffering from niche conditions.

The designs of the Porton box and Gore Lyoguard were studied and the specific features required for contained, sterile freeze-drying were identified. Common to both systems is a bacterially retentive membrane that allows the passage of water vapour during the freeze-drying process. Additionally, the materials used for construction must have excellent heat transfer properties. This seems logical as freeze-drying can be considered basically as a problem in coupled mass and heat transfer (Pikal, 1985). A range of different designs were conceived that satisfied these criteria but were also
versatile enough to perform both vial and bulk freeze-drying. The simplest and least expensive of these was an envelope design that allowed trays of vials or bulk product to be sealed inside and then freeze-dried (chapter one). The flexible envelope allowed the shelves of the freeze-drier to be used to compress stoppers into the necks of vials.

Readily available chevron style sterilisation pouches fitted the envelope design exactly and incorporated a bacterially restrictive layer of porous medical grade paper and Tyvek with a thin film polymer layer that would not interfere with heat transfer.

To determine if containment systems caused damage to the conformation or function of proteins during freeze-drying a model pharmaceutical protein was developed. Ovine IgG specific for fluorescein was formulated using excipients selected for their ability to protect or support a protein during drying. A range of sensitive methods were used to assess any loss of biological specificity and to detect any changes to the conformation of the IgG. The IgG formulations were also studied using freeze-drying microscopy that enabled efficient and accurate design of freeze-drying cycles. When the antibodies were freeze-dried and tested no loss of biological activity or structure was found. Previous testing of the Porton box and the Lyoguard (Mayeresse, 2009) determined the physical effects that a protein would be subjected to but not how a protein would behave. The original IgG formulations and freeze-drying cycles developed were then used to investigate the effects that pouches may have on the protein during contained freeze-drying.

Prior to proving performance of the pouches using the model protein, the effects on mass and heat transfer were investigated. No such characterisation was performed on the Porton box. However, both Taylor et al. (1978) and Gassler et al. (2004) reported that containment caused an increase in product temperature during freeze-drying suggesting that resistance to mass transfer is affecting the steady state. Resistance was partially
investigated by Gassler et al. (2004) who demonstrated that the Lyoguard PTFE membrane provided 10-15% more resistance to mass transfer during sublimation. More recently, Patel and Pikal (2011) discuss the relative benefits of the use of the Gore Lyoguard and highlight the importance of the “quantitative characterisation” of mass and heat transfer using the Lyoguard trays. With consideration to these aspects, the novel concept of freeze-drying containment using disposable sterilisation pouches was investigated. Using available equipment, novel methods were developed to investigate the effects that these barriers had on mass and heat transfer during freeze-drying. These were then compared to stopper resistance, product dry-layer resistance and the Gore Lyoguard membrane resistance to derive an overall analysis.

It was found that the resistance of the barrier to sublimating water vapour was the most important factor affecting the mass transfer (chapter three). A ranking was defined whereby increasing resistance to water vapour movement during freeze-drying, imposed by a barrier, was found to decrease the sublimation rate and subsequently increase product temperature and process time. The resistance imposed by the Lyoguard membrane was low and comparable to stopper resistance as hypothesised by Patel and Pikal (2011). It was also less than the product dry layer resistance as Gassler et al. (2004) confirmed. Resistances determined for stoppers compare favourably to those obtained by Pikal et al. (1984 and 1985) and are represented in Figure 5.1. It is often difficult to compare these data with that of other researchers as different stopper designs have differing geometries and, therefore, varied resistance to water vapour flow. However, when stoppers used for this study are compared to Pikal’s (1985) 13mm stoppers (with similar geometries) similar values for resistance are observed confirming the validity of the experimental approach to determine data of the correct order of magnitude.
Figure 5.1 Reciprocal of stopper resistance against pressure determined at $T_s = 0$ (circle) and -10 °C (square). These data are plotted next to Pikal’s (1985) published linear function (line) determined by regression analysis. Given the variation in stopper geometries data from this study shows agreement with Pikal (1985).

Pouches made of paper and Tyvek imposed a greater resistance than that of the dry product layer but still allowed sublimation to proceed, albeit at a slightly slower rate. Previously the dry product layer had been the highest reported resistance encountered by water vapour during freeze-drying. The greatest resistance determined in this study was imposed by the Tyvek pouches and was four times the dry product layer resistance. When tested, this reduced mass transfer rate had no effect on the activity or structure of a model pharmaceutical product. Thus, it is possible to impose a far greater resistance to mass transfer than dry product layer and still freeze-dry a model protein with no deleterious effects.

The resistance of the barrier also increased the heat transfer coefficient of the system with higher resistance increasing the heat transfer coefficient by increasing the pressure around the system. The higher the pressure imposed the larger the increase in heat transfer coefficient suggesting that gas conduction ($K_g$) is the heat transfer factor
being affected since it is the only factor that is pressure dependent. However, this pressure effect was not proven experimentally and will be discussed in more detail later in this section.

An important feature in the selection of paper and Tyvek pouches for this study was their ability to prevent the passage of microorganisms. Hence their current uses to package medical devices, instruments and other items that require sterilisation by steam or gas. However, their ability to perform this function during a simulated freeze-drying process was unknown. It was decided to prove this in a manner that would be most applicable to pharmaceutical manufacturers and, therefore, a process simulation of a small scale filling and freeze-drying process was undertaken. These tests are routinely performed by pharmaceutical manufacturers to validate in house sterile processes, and testing of this nature was not performed on the Porton box or Lyoguard. The paper and Tyvek pouches were found to prevent contamination of sterile media and would therefore prevent ingress of microbial cells and subsequent contamination of a sterile product. Yet the probability that a product might be contaminated during a manual filling process prior to freeze-drying remains. It is due to this that producers of large volume (and value) sterile products introduce robotic systems for these processes thereby removing the risk of accidental contamination by operators which is obviously beyond the means of a small producer. If a small manufacturer were to introduce a contained freeze-drying process careful consideration into the balance of reducing manufacturing costs and regulatory compliance would be required. To this end it would be strongly recommended that further media simulations be performed allowing capture of more data points to determine if significant risk to a sterile product does exist. It is generally accepted by manufacturers and regulators alike that three successful media simulation runs are the minimum to prove the asepsis of a process.
The aerosol challenge tests carried out also show that pouches are able to prevent a high challenge of aerosolised microorganisms from contaminating a contained sterile product. This approach was also used by Taylor et al. (1978) and Gassler et al. (2004) using similar tests. However, the differing test methods and sample size used make direct comparison of results obtained for each containment system difficult (4.1.1). Moreover, using relatively large *S. cerevisiae* may not have represented as rigorous a challenge as a smaller microorganism such as *B. subtilis*.

Many studies (dating back to Stein et al., 1950) relate to contamination problems encountered whilst freeze-drying microorganisms. Early research in this field highlighted the benefits of crude filters to prevent contamination of the freeze-drier itself and of the environment (Reitman et al., 1954; Busby, 1959). Furthermore, it has been shown that cross contamination can occur within the freeze-drying chamber during a drying process (Barbaree et al., 1982). This contamination of the freeze-drier and environment, attributable to ablation, is well documented (chapter four) and shown to be prevented by the Gore Lyoguard membrane (Gassler et al., 2004). During this study it became apparent that pouches performed well as containment systems and were able to protect a sterile product from contamination. It was then postulated that pouches would also be able to contain microorganisms and thus prevent contamination of the freeze-drier and environment by ablation. This was successfully demonstrated when freeze-drying high initial concentrations of *E. coli* JM 109 deliberately formulated to induce collapse (representing the worse case for microorganism ablation). To achieve this, results indicated that a double layer of pouches are required for absolute containment. The research carried out into the containment of microorganisms during freeze-drying using pouches cannot be considered trivial. Many citations used throughout this study have emphasised that microorganisms released by ablation during freeze-drying cause
contamination but no method exists for containment and prevention of contamination. The need for such a system is suggested by Adams (1994) when describing a hypothetical processing chain for a bio-hazardous product. Thus the product could be filled into vials and could “be contained within filter boxes designed to reduce ablation and spillage” before being loaded into the freeze-drier. Therefore, it would seem appropriate that this study represents the first, novel solution to the problem of contamination of the freeze-drier by ablation of microorganisms.

For reasons previously discussed, double wrapping trays can only be considered applicable to the processing of small scale batches of products, such as orphan and niche pharmaceutical products. The most obvious solution for a manufacturer of these types of products is to avoid expensive and complex freeze-drying completely and develop a liquid form of the final product (Al-Abdulla et al., 2013). However, this may not always be possible for products that are inherently unstable in the liquid phase. In such a situation the use of sterilisation pouches to freeze-dry would seem appropriate. Moreover, the pharmaceutical industry tends to be risk averse and the adoption of new technology can be slow, unless the financial rewards are great, due to the extra regulatory scrutiny and justification the introduction of a new process demands. Furthermore, the importance of freeze-drying containment to industry must be considered objectively. It has been thirty five years since the concept of sterile freeze-drying in a non-sterile environment was first tested and since then only the Gore Lyoguard has been developed. If such an apparatus was of major importance to the pharmaceutical industry then it is reasonable to suppose that further research would have been undertaken. Thus the main interest in the use of sterilisation pouches for contained freeze-drying would initially seem limited to small volume, unstable orphan or niche products or to academia. However, potential bio-
safety applications offer exciting alternative applications that could be more relevant industrially.

The development of original polyclonal antibody formulations (chapter two) formed the foundation work for this study and they were used to investigate any potentially detrimental effects contained freeze-drying may have had on proteins. However, no data has been reported for the optimisation of formulations and freeze-drying cycles for polyclonal antibody products. This is surprising as polyclonal antibody immunotherapies are used to treat envenomation that is endemic in underdeveloped, tropical regions where cold chain preservation can be difficult. The use of freeze-dried antivenom would not require a cold chain and, therefore, presents a viable alternative. However, it has been demonstrated that polyclonal antibodies and their fragments are stable in liquid phase at higher temperatures (Al-Abdulla et al., 2003; 2013) and freeze-drying may add to the final costs of antivenoms that are destined for some of the world’s poorest countries. It is intended that the formulations, freeze-drying cycles and details of long term stability be reported. Samples that were freeze-dried using containment systems several years ago remain and it is likely they are still intact. If shown to be intact these data can then be used to report on the technique of contained freeze-drying and its applicability to antivenom manufacture in terms of reduction of sterile processing costs. With some further development, containment offers the opportunity to reduce the cost of freeze-drying apparatus considerably. Thus antivenom producers in developing countries would be able to separate the freeze-drying process by freezing pouches of sterile product outside of a freeze-drier. Frozen pouches could then be placed into a vacuum chamber (connected to a refrigerated condenser) where the maintenance of product temperature be achieved through latent heat losses during sublimation, similar to older freeze-drying equipment (Flosdorf, 1949). Without temperature controlled
shelves the hardware costs of freeze-drying are reduced providing economically accessible freeze-drying for pharmaceutical manufacturers in the developing world.

The use of pouches to contain the ablation of microorganisms during preservation by freeze-drying is another area that represents new opportunities. A similar pouch freeze-drying process was attempted unsuccessfully by researchers based at the National Institute for Biological Standards and Controls (NIBSC) (private communication) with the objective of developing contained freeze-drying processes for the preservation of microbial standards. It is proposed to write up this work and report the findings obtained when freeze-drying *E. coli* JM 109. It is hoped that this will stimulate other laboratories to investigate the use of pouches to freeze-dry a range of microorganisms varying in size and pathogenicity. For example, DNA plasmid could be formulated and freeze-dried and ablation assayed for using PCR. In terms of relative sizes, this could be considered a virus model for a freeze-drying process and would address Adams (1996) requirement for a containment device to prevent ablation of attenuated vaccine.

It has been demonstrated that the factor governing mass and heat transfer during freeze-drying when using barriers to water vapour is the resistance of that barrier to water vapour movement. It has been shown that there are materials available, for example the ePTFE of the Gore Lyoguard, which provides minimal resistance to water vapour movement and therefore minimal effects on mass and heat transfer. However, the ePTFE is expensive, delicate and liable to rupture when compared to robust paper or Tyvek. The ideal next generation freeze-drying pouch would be made of a material that has low resistance to water vapour movement and, therefore, less impact on the freeze-drying process but also has the strength and tear resistance of paper or Tyvek. Materials to be used for the porous layers of freeze-drying pouches can be considered simply as filtration media and many types of filtration media are available from suppliers. To
investigate the variety of filter materials available it is proposed to develop an apparatus similar to a vial that would be able to accept a thermocouple or RTD probe and be hermetically sealed. To the neck of this device a screw type o-ring seal designed to accept standard discs of filter media will be fitted. When filled with water, similar sublimation experiments to those detailed in chapter three could be performed that would determine the resistance to water vapour flow during sublimation and enable a thorough survey of the behaviour of filter materials. These data can then be used to select the best type of filter media for specialist freeze-drying containment pouches or envelopes.

It is also recommended that further investigation be undertaken using MTM and computer modelling simulations to better understand and characterise the effects of barrier resistance on the freeze-drying process. Interestingly, Patel and Pikal (2011) also recommend computer simulation be carried out on conventional freeze-drying processes. Specifically, computer simulation correlated to experimental observation would provide information as to surface area requirements in relation to throughputs of the barrier layer and could be used to predict effective surface area requirements. Therefore, if a membrane material existed that provided little or no resistance to water vapour but was relatively expensive it could be sized correctly. This would mean that pouches could be designed with small windows containing the exact surface area of required material keeping costs to a minimum. Furthermore, if more fragile membranes were used small windows could be incorporated into a flexible pouch and protected by some sort of protective cage system preventing accidental rupture.

To investigate the effects that pouches have on the heat transfer coefficient, in particular the pressure increases that their resistance causes, further data analysis and experiments can be performed. The application of Equation [5.1] taken from Pikal (1985)
has enabled the determination of the pressure inside the pouches using experimental data obtained in chapter three (Table 5.1).

\[
\frac{R_b}{R_s + R_b} = \frac{(P_p - P_c)}{(P_o - P_c)}
\]  

Equation [5.1]

Where \( R_b \) is barrier resistance, \( R_s \) is stopper resistance, \( P_p \) is the pressure inside the pouch, \( P_c \) is the chamber pressure and \( P_o \) is the vapour pressure of ice.

<table>
<thead>
<tr>
<th>Barrier material</th>
<th>( P_p ) (pressure inside pouch) Torr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyvek</td>
<td>1.047</td>
</tr>
<tr>
<td>Paper</td>
<td>0.578</td>
</tr>
<tr>
<td>Lyoguard</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Table 5.1 Values determined from experimental data (chapter three) for actual pressures inside the pouches or containment system, increasing resistance increases internal pressure.

Table 5.1 shows that it is possible to determine the pressure inside the pouch during freeze-drying and confirms the experimental observation where pouches expanded due to the resistance to water vapour passage. This caused an increase in pressure inside the pouch causing inflation. These pressures are greater than the chamber pressures for each experiment but less than the vapour pressure of sublimating ice. With some further work to determine the separation distance between the vial base and the surface upon it rests these pressures can now be used to determine \( K_e \) using equations detailed by Pikal (1985). Furthermore, if the emissivity of the pouches could be determined radiation heat transfer contributions could also be calculated and combined.
with contact terms to provide a complete view of heat transfer effects. These expressions could then be combined, as \( K_v = K_s + K_r + K_c \) (3.3.1.5), and compared to values calculated for \( K_v \) in chapter three.

Finally, the use of pouches for contained freeze-drying to enable sterile freeze-drying in a non-sterile environment is thought to be appropriate for the preservation of tissue samples for transplant. Amniotic membrane has been shown to be effective in the treatment of pterygium by Nakamura et al. (2006) and for the re-construction of the ocular surface by Libera et al. (2008). Both of these researchers used amniotic membrane preserved by freeze-drying. Human amniotic membrane was obtained from donors undergoing caesarean section, washed with buffer and antibiotics, freeze-dried and then gamma irradiated. According to Nakamura et al. (2006) the material preserves well and readily rehydrates. It is postulated that these samples could be collected and washed in the same way but packaged under the sterile conditions of the operating theatre into freeze-drying containment pouches. These samples could then be freeze-dried in the laboratory and then stored within the same packages until required. Bone allografts are another tissue used for transplants that are preserved by freeze-drying. Jackson et al. (1988) details methods for this procedure but again it would seem plausible that samples could be packaged in freeze-drying containment pouches under sterile conditions, freeze-dried in the laboratory and stored until required.

In conclusion, comparison of data derived using pouches during this study to the Porton box and Lyoguard indicates a contribution to the characterisation of apparatus for contained freeze-drying. The most innovative aspect has been the development of a simple, inexpensive freeze-drying containment system that allows both sterile bulk and vial freeze-drying to be performed using a flexible barrier while allowing shelf stoppering, if required.
The pouches provide resistance to water vapour movement causing a lowering of sublimation rate (mass transfer), increased process time, an increase in product temperature and an increase in heat transfer coefficient. However, these changes are shown to have no effect on a model pharmaceutical product. It is also evident from the microbiology work carried out in chapter four that pouches prevent contamination of a sterile product allowing sterile freeze-drying to be performed in a normal laboratory environment. These findings satisfy the objectives set in section 1.7 and achieve this study’s primary aim to demonstrate that it is possible to perform sterile freeze-drying of pharmaceutical products using pouches.

In addition, another novel application for these pouches has been described where it has been demonstrated that pouches are able to contain ablation of microorganisms during a freeze-drying process. Previous studies have found potentially dangerous contamination of the freeze-drier and condenser when preserving microbes by freeze-drying caused by particles ablating from vials. The use of pouches as barriers contains the ablated microorganisms, therefore, preventing contamination, and achieving the secondary aim of this study.


U.S. FDA. Lyophilisation of parenterals (7/93), guide to inspections of lyophilisation of parenterals.


