Impact of lyophilisation conditions on bio conjugate product quality attributes

A thesis submitted to Dublin City University for the degree of Master of Science

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January 2018

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Acknowledgments

Thank you to my mother Mary and my sister Kim and my late father David for supporting me throughout the years. It's because of all of you that I got this far.

Thank you to my university supervisor, Dr Ciarán Fagan for your patience and guidance throughout these last few years. It's been a pleasure collaborating and learning from you.

Thank you to my work supervisor Dr Kevin Doyle for providing the supportive environment which allowed me to conduct this research. Your technical guidance and your insight into the conjugation process have been invaluable to me. Thanks also to Ms. Eimear Walsh for reviewing the final document and providing many useful suggestions.

Thank you to Dr Neysi Ibarra and Dr Enda Moran for supporting my further education studies.

Thank you to Dr Serguei Tchessalov, whom I have learned most of what I know about freeze drying. I will be always grateful to you for being so generous with your time and for your willingness to share your knowledge.

I would like to thank Dr Brian Moran and Dr Laura Truman for the invaluable support you gave me throughout different aspects of this project. You know what you are guys.

Finally I would like to acknowledge the role of colleagues from whom I have learned a great deal over the years- Mr. Michael Darwent, Dr. Bakul Bhatnagar, Mr. Daniel Dixon, Dr. James Pierce, Ms. Helen McElligott and Ms. Mairead Looby.

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Abbreviations

ANOVA	Analysis of variance
Capacitance manometer	CM
Collapse temperature	Tc
Critcal quality attributes	CQA
Molecular size distribution	Kd
Design of experiments	DOE
Drug Product	DP
Final Container	FC
Freeze drying Microscopy	FDM
Glass transition of a frozen solution	T_g'
Glass transition of a lyophilised powder	T_g
Grams of active ingredient	GAI
Heat transfer coefficient of a vial	Kv
Heat transfer fluid	HTF
High-performance liquid chromatography	HPLC
Karl Fischer	KF
Unit of measure	UOM
Kilo Dalton	kDa
Litre	L
Manufacturing Control System	MCS
Microgram	µg
Milligrams of active ingredient	mGAI
Millilitre	mL
Modulated Differential Scanning Calorimetry	mDSC
Molecular Weight Cut Off	MWCO
Nano mole	nmol
Nanometer	nm
Not Tested	NT
Percentage Relative Standard Deviation	%RSD
Pirani vacuum gauge	PVG
Refractive index	RI
Relative Humidity	RH
Residual moisture	RM
Reverse Osmosis	RO
Saccharide to Protein ratio	SPR
Thermocouple	TC
Ultraviolet	UV

Glossary

$(\frac{\partial m}{\partial t})$ - sublimation rate at given cycle time, g/s S_{heat} - surface of heat conduction, cm^2

K_v - vial heat transfer coefficient, cal/(s*cm^2*K)

T_{shelf} - shelf temperature (typically inlet temperature of heat transfer liquid), K

$T_{product}$ - product temperature (typically measured just above the vial bottom), K

$T_{sublsurf}$ -temperature of sublimation surface, K

ΔH_S =676 cal/g- specific heat of sublimation

S_{out}-external surface of vial, cm^2

S_{in}- internal surface of vial, cm^2

d_{out}- external vial diameter, cm

d_{in}-internal vial diameter, cm

$P_{sublsurf}$ - pressure of water vapour over sublimation surface, Torr

$P_{chamber}$- chamber pressure, Torr

$R(h)_i$ - dry cake resistance at dry layer height, Torr*hr* cm^2/g

Abstract

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Title : Impact of lyophilisation conditions on bio conjugate product quality attributes.

Freeze drying is a unit operation used to remove water to ensure the stability of a pharmaceutical product over its shelf life. It is usually the final processing step in the manufacture of a drug product for intravenous use. In this study, lyophilisation was employed not as a final processing step, but as an intermediate step in the manufacture of a multivalent bio conjugate vaccine. Two chemically activated virulent bacterial capsular polysaccharides, serotypes F and K, were lyophilised above and below their collapse temperatures. The lyophilisation process was subsequently characterized and it was determined that shell freezing conditions, stopper placement and product temperatures during primary drying influence cake appearance, moisture content and glass transition temperature.

The serotype F and K test articles lyophilised above and below collapse were then chemically conjugated to a carrier protein, protein X. Conjugation efficiency was assessed using a number of physiochemical assays. Results for both serotypes lyophilised above and below collapse were within specification for all product quality attributes. For serotype K, freeze dried above collapse, protein unfolding and higher levels of unconjugated saccharide were observed which indicate the lyophilisation above collapse did impact conjugation efficiency.

To assess the impact of lyophilisation conditions on biological function of the antigens, a drug product formulation model was developed. The formulation comprised serotype F and K, along with a number of other conjugated, capsular polysaccharides serotypes. Each conjugated test article lyophilised above and below collapse was formulated into the multivalent formulation. It was determined that the total biological function of the polysaccharide and total adjuvant biological function of serotype F and K; lyophilised at different conditions, met specification and were comparable to each other. It can be concluded that lyophilisation above the collapse temperature had no practical influence on the biological function of the antigen.

1.0 INTRODUCTION

1.1 Lyophilisation of Biologics

Biological products, according to the US Federal Drug Administration (FDA), include a wide range of products such as vaccines, blood and blood components, allergenics, somatic cells, gene therapy, tissues, and recombinant therapeutic proteins. They can be composed of sugars, proteins, nucleic acids or they may be living things such as cells and tissues (Chinna, 2013). According to the latest EvaluatePharma® report, by 2022, 52% of the value of the top 100 pharmaceutical products will come from biologics (EvaluatePharma, 2017). The global lyophilization market for pharmaceutical and biotechnology products was valued at $1.97 billion in 2014 and is expected to reach $2.66 billion by 2019 (Markets and Markets, 2014). Currently, lyophilised products account for about 25% of newly approved parenteral drugs (Gardner, 2015). The current contribution to biologics sales growth is primarily from monoclonal antibodies (mAbs), recombinant proteins, and vaccines, and is expected to venture further into antibody- drug conjugates (ADC), fusion proteins and immune-conjugates (Bhatnagar et al., 2013).

1.2 Lyophilisation overview

Lyophilisation or freeze drying is used within the pharmaceutical industry to stabilize biological products in order to achieve a longer shelf life (Depaz et al., 2016). Most biologics are delivered parenterally: intravenously (injection into a vein), subcutaneously (injection under the skin) or intramuscularly (injection into a muscle). The products are usually formulated as a liquid and because of this they have limited stability. Therefore, lyophilisation is used to stabilize the biologic by converting solutions of unstable materials into solid form by removing water, thus improving long-term storage stability (Patel and Pikal, 2011). The dried product is then reconstituted using water or a saline solution before parenteral administration. Products for lyophilisation are usually filled in to vials, ranging in fill volumes from 2-100 mL. Vials for lyophilisation purposes are manufactured using Type 1 borosilicate glass to ISO 9001 and ISO 15378 and comply with European Pharmacopoeia, US Pharmacopoeia and Japanese Pharmacopoeia international standards (O'Fagain and Colliton, 2017). However, a small percentage of drug products, biological standards, and process intermediates are freeze-dried in non-vial containers such as, bottles, ampoules or trays (Patel and Pikal, 2011).

Freeze drying comprises three main steps (Wang, 2000):

i. Freezing: conversion of water to ice
ii. Primary drying /sublimation: removal of ice by applying heat and low vacuum
iii. Secondary drying/desorption: removal of unfrozen water adsorbed on to the surface of the amorphous solid.

A typical freeze dryer comprises a product chamber, shelves, a condenser, a vacuum pump and a refrigeration system. The chamber is constructed of 316 L stainless steel and houses temperature controlled shelves, on to which partially stoppered vials filled with product are frozen. The shelves are hollow, containing heat transfer fluid (HTF) and are generally capable of heating from -70 °C to 65 °C. The shelves supply the energy via the HTF which allows water to freeze and provides heat energy for drying. The function of the condenser is to collect the water vapour evolved during primary and secondary drying, i.e. it acts as a "cold trap". The condenser is maintained at a temperature below -40 ºC to ensure that the vapour pressure of the solvent collected on the condenser is lower than the vapour pressure of the solvent in the chamber. Once at the condenser, the water vapour is converted to ice. The vapour pressure is the pressure exerted by a gas in equilibrium with the same substance in liquid or solid form (Gooch, 2011). The vacuum pump removes atmospheric gases from the chamber, reducing the pressure in the chamber. To facilitate sublimation during primary drying, the pressure is kept below the vapour pressure of ice. The refrigeration system provides cooling to the shelves through heat exchangers within the shelf HTF system. There are several different types of refrigeration systems available, with the type of configuration depending on a number of factors such as the load capacity, type of refrigerant and size of the freeze dryer (Trappler, 2004a). A schematic of a freeze dryer is presented in Figure 1-1, page 4. At the end of the lyophilisation cycle, nitrogen gas can be introduced into the chamber to provide an inert blanket over the product. The partially stoppered vials are then sealed inside the chamber by compressing the shelves together. The resultant force provided by the stoppering ram presses the stopper into the vial and provides an effective hermetic closure. Failure to fully stopper vials can allow ingress of air in to the product and this may impact the efficacy and long term stability (Curry et al., 2010). At the end of the lyophilisation process, the lyophilised cake should have almost the same size and shape as the liquid that was initially filled into the vial and the cake should have a uniform colour and texture (Patel et al., 2017).

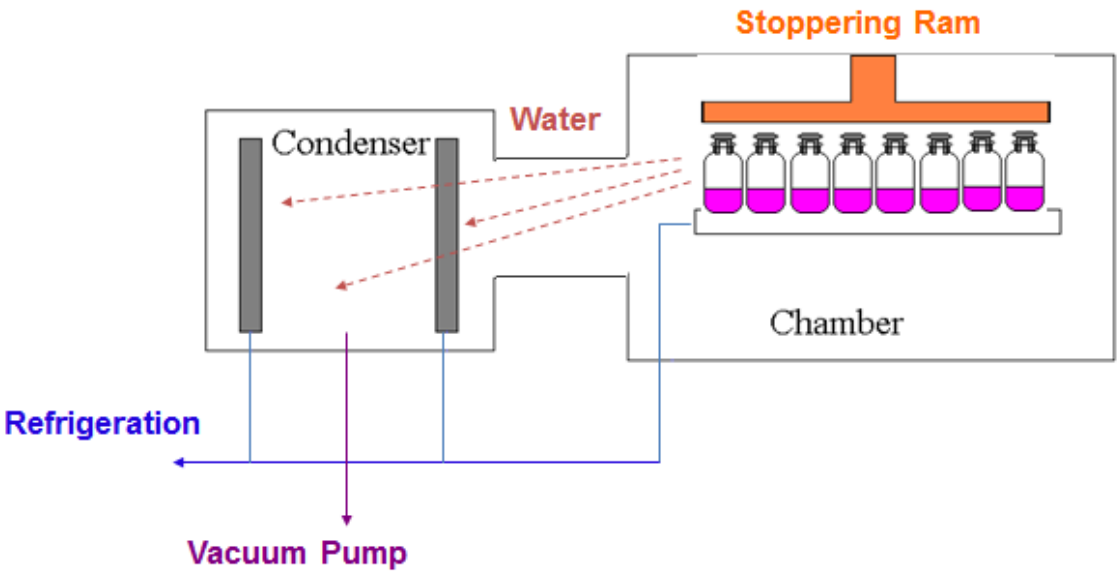

Figure 1-1: Schematic of a freeze dryer. Partially stoppered vials sit on a shelf in the chamber. Sublimed water collects on the condenser as ice. Image courtesy of Daniel Dixon, The company.

1.3 Formulation

The goal of formulation is to create a multicomponent system, which can be freeze-dried in a suitable time frame, whilst at the same time ensuring stability during the product's life cycle (Bhatnagar et al., 2013). When a formulation is cooled during the initial freeze segment of a lyophilisation cycle, the structure that is formed may exhibit crystalline or amorphous properties and in some cases a mixture of both. Excipients may be classed as, but are not limited to, bulking agents, tonicity modifiers, buffers and cryoprotectants/lyoprotectants (Wang, 2000). Each excipient added to a formulation should have a specific function; e.g. amorphous sucrose or trehalose can be used as stabilizers and / or cryoprotectants (Chang et al., 2005) whereas crystalline excipients such as mannitol and glycine are used as bulking agents to improve the elegance of the freeze dried cake (Lu and Pikal, 2004). There are two major hypotheses to describe the stabilisation of proteins by sugars. The first one is the ‘‘glassy dynamics’’ or "vitrification" hypothesis. The vitrification theory describes stabilisation from a kinetic perspective (Mensink et al., 2017a). This theory proposes that the sugar provides a rigid glassy matrix in which the protein is dispersed, and the limited mobility in the glassy matrix reduces molecular motion thereby preventing inactivation reactions (Franks, 1994).The second mechanism is the ‘‘water replacement’’ or "water substitute" hypothesis. This theory describes stabilisation from a thermodynamic perspective (Chang and Pikal, 2009). This theory proposes that the hydroxyl groups of the sugar will form hydrogen bonds with polar sites on the protein's surface; replacing hydrogen bonds between water and the protein thus stabilizing the native structure of the protein during lyophilisation (Carpenter and Crowe, 1989, Allison et al., 1999). Grasmeijer and co-workers showed that as long as there is sufficient vitrification, i.e. a Tg of at least 10–20 ºC above the storage temperature, water replacement is the predominant mechanism of stabilization. However, when the storage temperature is near or above the Tg, vitrification becomes the limiting factor for stability(Grasmeijer et al., 2013). It is generally accepted that while both theories have their merits, they do not fully explain protein stabilization on their own (Mensink et al., 2017a). The vitrification theory has been expanded to discuss the impact that global mobility (α transitions) and local mobility (β transitions) has on protein stability (Yoshioka et al., 2007, Cicerone and Soles, 2004). The water replacement hypothesis has been developed further to show that smaller and more flexible oligosaccharides are better able to stabilize a number of model proteins during storage after freeze drying (Tonnis et al., 2015,

Mensink et al., 2015). Lyophilised formulations typically contain excipients in both amorphous and crystalline forms. Formulations consisting solely of crystalline excipients by themselves are not commonly used for lyophilisation; as the potential for crystallization during storage can remove the stabilizing effects of those excipients (Izutsu et al., 1994, Arakawa et al., 2001). Therefore, the physical state of the freeze-dried cake is usually partially crystalline (amorphous protein and crystalline excipients) or amorphous (amorphous protein and amorphous components)(Bhatnagar et al., 2013). The portion of the excipient matrix added to stabilize the protein should be in the same amorphous phase as the protein for effective stabilisation (Bhatnagar et al., 2013). A comprehensive review of formulation for lyophilisation can be found in the literature (Carpenter et al., 1997, Tang and Pikal, 2004).

1.4 Freezing

The objective of freezing is to create an optimum ice crystal structure to enable the removal of water vapour from the cake. The freezing step influences the performance of the subsequent drying steps and the quality attributes of the final drug product depends on the way in which the liquid was frozen (Jameel and Searles, 2010). The first step of the process involves loading vials containing the product, on to the shelf of the freeze dryer and normally freezing below -40 ºC with the aim of converting water (solvent) to a crystalline ice phase. This ice phase is removed later during the primary drying stage of the cycle. The remaining product and excipients (solutes) remain mainly in an amorphous phase (Patel and Pikal, 2011).The ice nucleation temperature, which is stochastic in nature, is the temperature at which ice crystals first appear. The degree of super cooling is the temperature difference between the thermodynamic ice formation temperature and the actual temperature at which ice begins to form, usually around 10 to 25 °C lower (Tang and Pikal, 2004). It governs the rate of nucleation and determines the number of ice crystals formed; this in turn affects the porosity of the freeze dried cake (Rambhatla et al., 2004). The pores in the cake, remaining after the sublimation of ice are a direct reflection of the size and geometry of the ice crystals formed during freezing (Rambhatla et al., 2004). As described by Patel and co-workers, a higher degree of super-cooling leads to smaller ice crystals being formed, resulting in a smaller pore size. This in turn leads to greater resistance to vapour flow, and longer primary drying times (Patel et al., 2009). In a manufacturing environment there are less nucleation sites available for freezing, due to the low particulate environment; therefore, the degree of super cooling is higher. It is for this reason that manufacturing lyophilisation cycles are longer than those in the laboratory. Proteins are sensitive to extremes in temperature and are stable only in a defined temperature range (Bhatnagar et al., 2013). It is well established in the literature that the freezing step can have a direct influence on the tendency of a protein to denature (Heller et al., 1996, Chang et al., 1996). Similar to denaturation caused by heating, a decrease in temperature can also result in protein unfolding; by a process called cold denaturation (Franks, 1993, Sabelko et al., 1998). Another potential stress a protein can encounter is the freeze concentration of solutes. During freezing, water is converted to crystalline ice and this results in the solutes in the amorphous region become more concentrated (Heller et al., 1999). Buffer salts may concentrate, causing undesirable effects on protein structure (Randolph, 1997). The freezing process can also influence the primary drying rate due to difference in ice nucleation temperature (Searles et al., 2001b). In the case of

protein– saccharide systems, phase separation into protein-rich and saccharide-rich phases can result in protein instability and can also facilitate the crystallization of other components (Bhatnagar et al., 2011). In addition the freezing step has also been shown to influence cake appearance (Esfandiary et al., 2016), and residual moisture content (Searles et al., 2001a). A detailed discussion of the stresses involved during the freezing of proteins can be found in a review (Bhatnagar et al., 2007).

1.4.1 Freezing and lyophilisation in bottles

As mentioned previously, an alternative container for bulk freeze drying is that of bottles. Bottle freeze drying was used during World War II to freeze dry blood plasma. The method described by Greaves consisted of spinning the bottles at a high speed on their vertical axes. This caused a hollow cone to be formed down the centre of each bottle, distributing the plasma evenly round the inside periphery increasing the surface area for sublimation (Greaves, 1941). This method was refined over the years by various researchers and in 1969 Dike and co-workers described a "shell freezing" method to freeze biological material in bottles immersed in a bath filled with industrial alcohol at -40 ºC(Dike et al., 1969). Today, shell freezers commercially available are capable of freezing bottles from 40 mL to 2 litres. For effective shell freezing, the bottle is held on its side (at an angle) and rotated by motor driven rollers in a refrigerated bath resulting in a thin coating (or shell) around the inside walls of the container. The objective of shell freezing today is the same as that described by Greaves in 1941; that is to decrease the thickness of frozen material for a given total mass, thereby increasing the surface area for sublimation. Once shell freezing is completed, the frozen containers can then be transferred to precooled shelves of a lyophiliser for drying.

1.5 Primary drying

After the product has been frozen, the pressure in the freeze dryer is reduced to levels <1 Torr, and the temperature of the shelves raised to facilitate sublimation of ice (Kramer et al., 2009). Sublimation is the conversion of a solid to a vapour without passing through the liquid phase. Primary drying is governed by heat and mass transfer processes. The main heat transfer mechanism is the temperature gradient between the product and the shelf (Tang et al., 2006), whilst the dominant mass transfer mechanism is the pressure gradient between the vapour pressure of ice arising from the product and the chamber pressure itself (Chang and Patro, 2004).

1.5.1 Heat transfer

The temperature difference between the shelf and product, together with the vial heat transfer co-efficient (Kv) ,determines the ice sublimation rate during primary drying (Tang et al., 2006). As heat transfer increases, the sublimation rate increases, resulting in shorter lyophilisation cycle times. During primary drying, the heat flow from the shelf to the vials is equal to the heat consumed by ice sublimation (Tang et al., 2006) and can be described by Equation 1-1, page 9, (Pikal et al., 1984).

$$\frac{dQ}{dt}(in) = K_v \cdot A_v \cdot (T_s - T_b)$$

Equation 1-1 :Heat transfer equation, where dQ/dt is the heat transfer rate (cal hr^{-1} $vial^{-1}$); A_v is vial cross-sectional area (cm^2); T_s is the shelf temperature (°C); T_b is the temperature of the vial bottom (°C), and K_v is the vial heat transfer coefficient

Knowledge of the vial Kv will simplify scale up from laboratory to commercial scale and help to reduce cost. Kv measurements, along with primary drying modelling, allows for the prediction of the drying behaviour of formulations in freeze dryers at different temperatures and pressures (Tchessalov and Warne, 2007). The heat transfer coefficient is defined as the ratio of the area-normalized heat flow to the temperature difference between heat source and heat sink (Pikal et al., 1984). Product temperature during primary drying is not controlled directly by the freeze dryer but is a function of three programmed parameters, shelf temperature, chamber pressure and time

(Pikal et al., 1984). Product temperature during freeze drying can be measured in several ways, with thermocouples being placed in to vials being the most common method. In a seminal paper by Pikal, it was discussed that heat transfer to the vial comes from three mechanisms; conduction, convection and radiation (Pikal, 1985):

i. Conduction: Heat transfer due to direct contact between shelf and vials (Kc).
ii. Convection: Heat transfer due to motion of gas molecules (Kg).
iii. Radiation: Heat transfer from the walls and chamber door of the freeze dryer (Kr).

The Kv quantifies the heat transferred from each of the mechanisms into the frozen liquid in the vials. By combining all three mechanisms, the heat transfer coefficient can be expressed as per Equation 1-2, page 10.

$$K_v = K_c + K_r + K_g$$

Equation 1-2: The vial Heat transfer coefficient, Kv is the sum of three contributions, conduction (Kc), convection (Kg) and Radiation (Kr)

1.5.2 Mass transfer

Mass transfer is discussed in terms of resistance, which is defined as resistance of dry product layer to the mass flow of water vapour formed during sublimation (Pikal, 1985). It has been suggested that there are there are three main barriers to mass transfer (Pikal et al., 1984, Pikal, 1985).

i. The dried product layer
ii. The stopper inserted partially into the vial
iii. The pressure drop from the chamber to the condenser.

The dried product layer is the most significant barrier to mass flow. The dried product layer, is in essence, the dried powder or cake composed of the active ingredient and excipients (Overcashier et al., 1999). It has been demonstrated that as primary drying proceeds, the resistance increases, as a result of the increase in the thickness of the dried layer (Pikal et al., 1983, Pikal, 1985). In other words, it is more difficult to remove water as drying proceeds. The water vapour is removed through the dry layer as the sublimation front moves from the top to the bottom of the vial (Bhatnagar et al., 2013). Pikal also reported that the product resistance decreases sharply as the

product temperature increases toward the collapse or eutectic temperature (Pikal, 1985). Independently, Milton and Overcashier found that the increase in resistance to mass flow correlates with the increase in thickness of the dried layer. Resistance to mass transfer can be described by Equation 1-3, page 11, (Tang et al., 2006).

$$\frac{dm}{dt} = A_p \cdot \frac{P_{ice} - P_c}{\hat{R}_p + \hat{R}_s}$$

Equation 1-3: Mass transfer equation, where dm/dt is ice sublimation rate (g/hour/vial); A_p is internal cross section area of vials (cm^2); P_{ice} is the vapour pressure of ice at the temperature of sublimation surface P_c is the pressure in the freeze drying chamber; and $\hat{R}_p + R_s$ is the sum of the total resistance of the product and the stopper (cm^2 Torr hour/g).

Note that as a material is dried close to its collapse temperature, the resistance remains constant with the increase in the dried-layer thickness (Milton et al., 1997, Overcashier et al., 1999).

The collapse temperature is defined as as the maximum product temperature that allows drying to occur without the loss of porous "cake-like" structure with the dimensions equivalent to those of the frozen solid (Pikal and Shah, 1990).This phenomena will be discussed further in Section 1.5.4, page 13.. The rate of water vapour removal depends on the product resistance, which is a function of how the initial solution was frozen (Rambhatla et al., 2004). Resistance, on the other hand, also depends on the formulation, nature of the excipients and their physical state (amorphous vs crystalline) (Overcashier et al., 1999, Bhatnagar et al., 2013).

1.5.3 Primary drying modelling

Mathematical modelling can be employed for optimization of primary drying and can provide a better understanding of the impact of process and formulation variations on cycle time (Pikal et al., 2005). Over the past 30 years, numerous papers have been published using mathematical models to describe the lyophilisation process. In one paper, a simple steady state model was described which can be utilized to design an appropriate primary drying step (Tchessalov&Warne, 2008). Heat and mass balance at the sublimation surface for each primary drying segment is described by Equation 1-4, page 12. The model is based on the assumption that all transferred heat is used for the sublimation. Microsoft Excel Solver can be used to calculate product temperature throughout the lyophilisation cycle. The product cake resistance as function of cake height, R(h) can also be estimated for the product using the measured product temperature profiles obtained from the lyophilisation cycle. A simultaneous solution of the heat transfer (from the surroundings to the product) and mass transfer (from sublimation surface to the condenser surface) enables estimation of the product temperature and drying time as a function of chamber pressure and shelf temperature (Bhatnagar et al., 2013). The model can be verified by comparing the calculated product temperature to the measured temperature obtained during the lyophilisation cycle (Tchessalov et al., 2017).

$$\left(\frac{\partial m}{\partial t}\right)_i = \frac{S_{in} * \left(P_{Subl} - P_{Chamber}\right)_i}{R(h)_i} = \frac{S_{out} * K_V(P) * \left(T_{Shelf} - T_{product}\right)_i}{\Delta H_S}$$

Mass transfer = Heat Transfer

Equation 1-4: Heat and Mass balance equation during primary drying. Where $\left(\frac{\partial m}{\partial t}\right)_i$ is the sublimation rate at time ,i, S_{in} and S_{out} are inner and outer surface areas of the vials; ΔH_S is a latent heat of sublimation of water; P_{Subl} and $P_{Chamber}$ are pressure over the sublimation front and chamber pressure respectively; T_{Shelf} is the shelf temperature; $R(h)_i$ is the cake resistance at dry layer height h_i., $T_{product}$ is the product temperature at the bottom of the vial (presumably the highest temperature in the frozen layer).

1.5.4 Critical temperatures

Determination of the critical temperature of a product prior to lyophilisation is vital in order to design an efficient and safe cycle for that product. To achieve this, it is essential that the formulation be characterized in order to determine the temperature above which desirable properties of a freeze dried product are lost (Her and Nail, 1994). Modulated differential scanning calorimetry (mDSC) can be used to determine the glass transition temperature, and other transitions such as crystallization,and melting (Wang, 2000). It measures the difference in heat flow to a sample compared to a reference and detects both(i) first-order irreversible/kinetic thermal events such as crystallization and eutectic melt (exotherms or endotherms) and (ii)second-order reversible events such as glass transitions (Jameel and Searles, 2010). For amorphous formulations,the critical temperatures are:(i) the glass transition temperature (Tg' and Tg)[1] and (ii) the collapse temperature (Tc). For crystalline formulations the critical temperature

[1] **T_g'** (pronounced as Tg prime) is used within the pharmaceutical industry to denote the glass transition of a frozen solution whereas **T_g** denotes the glass transition of the final lyophilized product

is the eutectic melt temperature (Teut). A eutectic mixture is a mixture of two or more crystalline materials that are in such close contact that they melt like a pure substance. Eutectic melting of crystalline excipients can lead to a defect called meltback. During the freezing stage,water is converted to a crystalline ice phase. As the temperature drops further,the excipients and protein molecules lose translational mobility and do not have enough energy to form a crystal lattice. Instead, they arrange themselves between the ice crystals in a disorderly amorphous configuration;and are described as glasses (Kalogeras, 2012). Therefore,it is necessary during freezing that the formulation is frozen at a temperature low enough to ensure that all of the formulation components are immobile i.e. the formulation must be frozen below its glass transition temperature, Tg'. The glass transition temperature can be defined as the temperature at which the dynamics of an amorphous system changes from a more mobile phase "rubbery" state to a less mobile "solid-like" glassy state (Shamblin, 2004, Hancock and Zografi, 1997). The temperature at which maximum freeze-concentration occurs is defined as Tg'. Below Tg',a rigid glass with high viscosity and low mobility is formed (Pikal, 1994). It is well established in the lyophilisation literature that the collapse temperature, Tc, is generally a few degrees higher than the glass transition temperature. The collapse temperature is measured using freeze drying microscopy (FDM). This instrument comprises a light microscope with a camera on top and attached is a vaccum pump,a Pirani gauge and a Dewer flask containing liquid nitrogen fixed to the microscope stage. It is essentially a mini freeze dryer, and it allows the user to programme a recipe and then visually identfiy a temperture at which various thermal events such as collapse,onset of crystallization and melting occur. Tc is usually within 1 ºC to 2 ºC of Tg' and both are often used interchangeably (Pikal and Shah, 1990). However,for higher concentration protein formulations,the difference between Tg' and Tc may be larger as protein concentration increases (Colandene et al., 2007). The collapse temperature was defined by Mackenzie as the disappearance or annihilation of the freezing pattern with the passage of the sublimating interface (MacKenzie, 1976). Whereas Levi and Karel defined collapse as loss of structure,a reduction in pore size and volumetric shrinkage of dried materials (Levi and Karel, 1995). During primary drying,when the product temperature exceeds the Tg' of the maximally freeze-concentrated solution,it can result in a loss of the microstructure established by the freezing process (Pikal and Shah, 1990). When a product collapses,it can result in the clogging of the pores formed as a result of sublimation of ice, and this can significantly reduce the rate of

sublimation (Chang and Patro, 2004). It has been reported in numerous papers that freeze drying above collapse should be avoided as it can lead to product with loss of pharmaceutical elegance and higher moisture content (Levine and Slade, 1988, Adams and Ramsay, 1996). Collapse is often refereed to as "total" or "gross collapse" and almost certainly product with a collapsed appearance would be rejected from a commercial process. It has also been demonstrated that proteins lyophilised above the collapse temperature have exhibited aggregation and loss of activity on stability (Izutsu et al., 1994, Passot et al., 2007). Images of acceptable and collapsed cakes are presented in Figure 1-1, page 16.

1.5.5 Microcollapse

There is another type of collapse phenomenon to consider: microcollapse. It has been demonstrated that when amorphous formulations are lyophilised above the collapse temperature, complete collapse does not occur but rather small holes can appear in the cake and these additonal pathways for vapour flow result in a signifcant decrease in cake resistance (Milton et al., 1997, Overcashier et al., 1999). Lyophilisation in this manner has been found to increase the size of pores in the dry layer and reduce their tortuisty,resulting in low resistance to the flow of water vapour which does not increase with dry layer thickness (Johnson et al., 2010). An advantage of drying near the microscopic collapse temperature is that the reduced resistance can result in shorter primary drying times. However concerns have been raised that exposing proteins to the micocollapsed state could affect long term stability (Meister and Gieseler, 2009).

1.5.6 Primary drying above the collapse temperature

Despite the adverse effects of lyophilisation above the collapse temperature and/or the glass transition temperature, recent studies have shown that lyophilisation above the critical temperature of a formulation is possible without compromising product quality or stability. Lyophilisation above the collapse temperature is attractive because for every 1°C increase in product temperature during primary drying, the overall primary drying time can be reduced by 13% (Pikal, 1994). This is a very attractive scenario for drug manufacturers as the cost of production can be reduced. It has been demonstrated that even with total or gross collapse, protein stability either improves or is not statistically different compared with products lyophilised below the critical temperature. In a paper by Schersch, the effect of lyophilisation above and below the collapse temperature on the stability of protein lyophilies containing IgG1

antibodies was studied. The protein was formulated in two amorphous formulations with sucrose and trehalose as well as partially crystalline mannitol–sucrose-based formulations. After lyophilisation, above and below collapse, the formulations were placed on stability at temperatures as high as 50 °C for 15 weeks. It was found that appearance was not altered during storage at 50 °C and stability of the protein, using a range of biochemical assays showed no difference between collapsed and non-collapsed cakes (Schersch et al., 2012). Tchessalov and coworkers lyophilised a capsular polysaccharide serotype in an amorphous formulation above and below the collapse temperature. The product quality of the lyophilised polysaccharide was assessed by the ability of the polysaccharide to conjugate to a carrier protein. It was found in this study that polysaccharides lyophilised above the collapse temperature had comparable product quality when compared to polysaccharide lyophilised below the collapse temperature and cycle time reduced from 35 hours to 8 hours. However, the cake appearance of the lyophile freeze dried above the collapse temperatures was generally poorer in comparison to the lyophile freeze dried below the collapse temperature (Tchessalov et al., 2010).

Figure 1-1: Comparison of an acceptable cake (left) and a collapsed cake (right) for an amorphous formulation. In the lower part of the collapsed cake a "ring" is observed indicating that this part of cake exceeded the collapse temperature and the amorphous material started to flow during primary drying

Images courtesy of Daniel Dixon and Serguei Tchessalov

1.6 Secondary drying

The last stage of a lyophilisation cycle is called secondary drying. The purpose of secondary drying is to reduce the level of moisture to assure maximum stability during storage (Tang and Pikal, 2004). After primary drying, approximately 5-20% of the remaining water resides in the amorphous phase and this water must be removed (Chouvenc et al., 2005). The unfrozen water may be adsorbed on the surface of the crystalline product or is in the solute phase, a crystalline hydrate or dissolved in an amorphous solid to form a solid solution (Pikal et al., 1990).Once ice has been removed during primary drying, the shelf temperature is raised to remove this unfrozen water (Arakawa et al., 2001). The optimal level of moisture in a product is highly formulation and product specific (Bhatnagar et al., 2013) ,but is usually less than 1% w/w for proteins and varies between 2% and 3% for vaccines (Jameel and Searles, 2010). When the optimum moisture level required is unknown ,which is common during cycle development, a slow heating rate(0.1 to 0.2ºC/min) from the end of primary drying to the secondary drying temperature should be used to minimise risk of collapse and shrinkage (Tang and Pikal, 2004, Jameel and Searles, 2010). A secondary drying shelf temperature of 25 ºC to 30 ºC for 3 to 4 hours is a good starting point (Jameel and Searles, 2010). As mentioned above, 5%–20% of residual water will remain at the end of primary drying and the Tg/collapse temperature of the product will be quite low; thus, fast ramping to the elevated temperature of secondary drying carries the risk of product damage in terms of both quality and stability (Jameel and Searles, 2010). In the past, the pressure during secondary drying was, as Pikal said “carried out as low as possible but this practice is without foundation”. Pikal and coworkers demonstrated with both amorphous and crystalline formulations that the rate of secondary drying does not depend on the chamber pressure in the range of pressures typically encountered in the freeze drying of pharmaceuticals, 0 to 200mT (Pikal et al., 1990). In the same paper, they recommended a combination of a higher drying temperature and a shorter drying time as opposed to a low drying temperature for a longer time.

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1.7 Stability

In order to assure maximum stability of a lyophilised product, it is critical that the storage temperature does not exceed the T_g (Hancock and Zografi, 1994). The stability of amorphous materials (both physical and chemical) is related to molecular mobility which in turn is strongly influenced by temperature and moisture content in the lyophilised cake (Shamblin, 2004). Residual or absorbed water can promote physical changes in an amorphous material through its role as a plasticizer. As a plasticizer, water acts as a physical diluent that increases free volume and molecular mobility (Shamblin, 2004). The higher the levels of water present in a lyophilised product, the lower the glass transition temperature. Since molecular mobility decreases with a decrease in the temperature below the T_g, it is considered good practice to store a lyophilised material 50 °C below its glass transition temperature (Hancock and Zografi, 1994). A comprehensive review of correlations between molecular mobility and chemical stability during storage can be found in the literature(Yoshioka and Aso, 2007).

1.8 Bio-conjugation of polysaccharides

1.8.1 Introduction

In 1917 Dochez and Avery showed that virulent pneumococcal bacteria released a "soluble specific substance," that fell out of solution when incubated with type-specific antisera (Dochez and Avery, 1917). They showed that this substance was able to withstand heat, an unusual characteristic for a protein, but they determined that it contained nitrogen, a component of all proteins. Later in two seminal papers, Heidelberger and Avery were able to purify the substance, removing all nitrogen and to determine that the substance was in fact a carbohydrate and that other pneumococcal serotypes of pneumococcus each had a bacterial capsule with a distinct carbohydrate signature (Avery and Heidelberger, 1923, Heidelberger and Avery, 1924). In 1924, Ramon and co-workers showed that horses injected with diphtheria toxoid along with unrelated substances produced higher specific antibody titers (Ramon, 1924). These substances are called adjuvants, from the Latin word adjuvare meaning to aid or help. In that same paper Ramon described adjuvants as "substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone" In 1929 Avery and Goebel demonstrated that the immunogenicity of polysaccharides was enhanced when chemically coupled to a protein. A year later, Francis and Tillet showed that isolated capsular polysaccharides of pneumococci were immunogenic in humans (Francis and Tillett, 1930).

1.8.2 Vaccines

Fast forward to 1950, Heidelberger and coworkers determined that capsular pneumococcal polysaccharides could be used as vaccines providing long lasting immunity against infection (Heidelberger et al., 1950). A vaccine, as defined by the WHO, is made from a weakened or killed form of a microbe which can provide immunity to a particular disease. It stimulates the body's immune system to kill the foreign invader and it produces antibodies that it can call upon at a later time if the body encounters that same pathogen again (WHO, 2017). Despite their potential as effective treatments, the development of chemotherapeutics and antibiotics, i.e. small molecules, led to a loss of interest in polysaccharides as therapies (Vliegenthart, 2006). Renewed interest in vaccines was only brought about by the steady decrease in the effectiveness of antibiotics. In 1983, a bio-conjugate vaccine called Pneumovax was introduced. This was a capsular polysaccharide vaccine derived from 23 pneumonia serotypes. Bio-conjugation is the attachment of one molecule to another, usually through a covalent bond, and in most cases at least one of the molecules is of biological origin or is a fragment or derivative of a biomolecule (Hermanson, 2013). A number of bio-conjugate vaccines against *Haemophilus influenzae type Streptococcus pneumoniae, Neisseria meningitidis*, and *Salmonella enterica serovar Typhi*, have been licensed in the United States since 2000 (Pan et al., 2016). Purified capsular polysaccharides are now one of the major virulence factors of encapsulated bacterial pathogens but are poorly immunogenic due to their inability to engage T cells (Tontini et al., 2013). Polysaccharide vaccines only elicit a B-cell response, so although antibodies are produced, there is no long-term memory(Broker et al., 2017) . To enhance their immunogenicity, they are often chemically conjugated by covalent linkage to a carrier protein, such as diphtheria or tetanus toxoid (Chang et al., 2012). The role of a carrier protein in immunology is to provide T cell help for B cell antibody production to a poor/non-immunogenic antigen (McCool et al., 1999). A high level scheme of the manufacturing process of conjugate vaccines is presented in Figure 1-3, page 20.

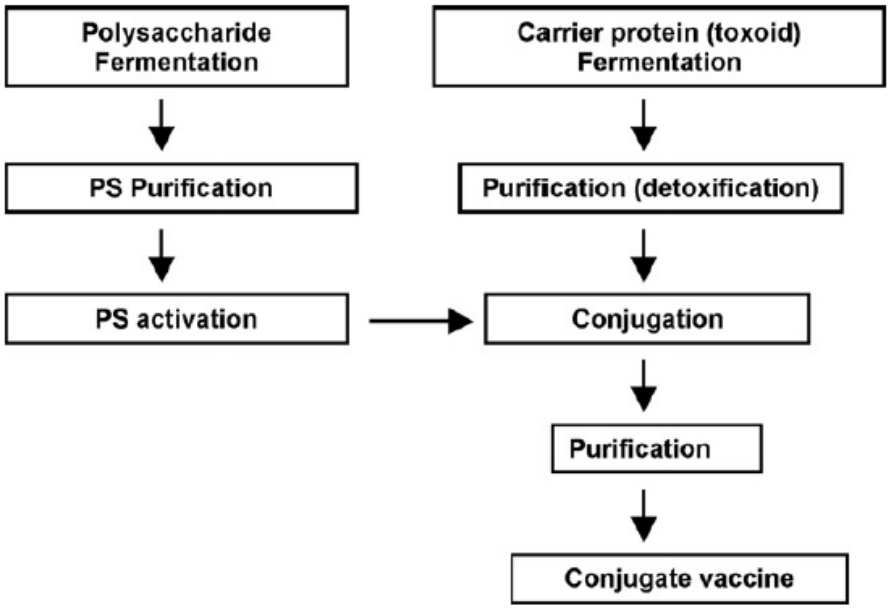


Figure 1-3: A general process for manufacture of a polysaccharide–protein conjugate vaccine. Reproduced from (Frasch, 2009)

1.8.3 Chemistry of polysaccharides

For a polysaccharide to be chemically linked to a protein, it must first be chemically activated. One method involves the use of the oxidizing agent sodium periodate(Gumpert and Sharon, 1978) Sodium periodate oxidizes vicinal diols (two adjacent carbons with hydroxyl groups) into aldehydes (H-C=O) and in the process breaks C–C bonds. The reactive aldehyde groups then condense with free amino groups on the protein in the presence of sodium cyanoborohydride to form a stable secondary amine. Condensation of the aldehyde groups with the epsilon amino groups on the protein lysine residue is a slow process and can run for days. The remaining aldehyde groups are then quenched using sodium borohydride converting them back to hydroxyls(Frasch, 2009). The second activation method is by cyanylation using 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) (Lees et al., 1996). Activation of polysaccharides with CDAP is based on the formation of cyanate esters on polysaccharides hydroxyls. As Frasch explains, CDAP reacts with the polysaccharide, exchanging a cyano group for a hydroxyl hydrogen, creating a highly reactive cyanoester. The cyanoesters react with the epsilon amines of lysine on the carrier protein to form a stable O-alkyl- linkage. Unlike periodate

oxidation, CDAP activation takes only a few minutes and the conjugation is complete in a few hours. The reaction is then quenched using an amino-containing reagent such as glycine (Frasch, 2009).

1.8.4 Filtration of polysaccharides

Filtration is a physical, pressure driven process used to separate components of a process stream using a membrane/filter to accomplish the separation based on differences in size and/or charge of the components within the stream. The two main types of filtration used within the biopharmaceutical industry to purify polysaccharide conjugates are normal flow filtration (NFF) and tangential flow filtration (TFF). Tangential flow filtration also called crossflow filtration or Ultrafiltration/diafiltration (UF/DF), is used for a range of applications as a key unit operation within the biopharmaceutical, food, beverage and chemical process industries (van Reis, 1997 #19). TFF is a widely used technique for the clarification, concentration and purification of biopharmaceutical intermediates and products and is used for concentration (ultrafiltration) and buffer exchange (diafiltration) of biomolecules(Shire, 2009). In TFF, the feed-stream containing the biomolecule of interest is recirculated under pressure and is pumped tangentially (or parallel) to the membrane surface with pressure applied in the direction of the membrane in order to force a partial volume of the process stream onto the membrane surface. The tangential flow to the membranes 'sweeps' a portion of the accumulated components from the surface of the membrane (polarisation layer) and back into the bulk solution (Rathore et al., 2004). The pore size of the membrane is selected based on the size of the target biomolecule e.g. if the membrane has a MWCO of 50 kD, particles larger than 50 kD will be retained (known as the retentate),whereas non target molecules, such as water, unreacted free protein, residual solvent pass through the membrane to waste (permeate or filtrate). Buffer exchange is performed in diafiltration mode where the desired buffer is added to the retentate vessel at the same rate as the permeate/filtrate flow which results in a constant retentate volume. In the NFF, the feed stream moves perpendicular to the membrane and purified liquid passes through the membrane. The biomolecule is filtered through through a 5 µm pre filter in preparation for TFF. The process stream flow and pressure is directed at the membrane surface which results in the larger components of the process stream being retained by the membrane. In NFF mode, the filter can be fouled easily, as the components that are larger than the membrane pore size accumulate at

the surface of the membrane. A schematic of NFF and TFF purification is presented in Figure 1-2, page 22.

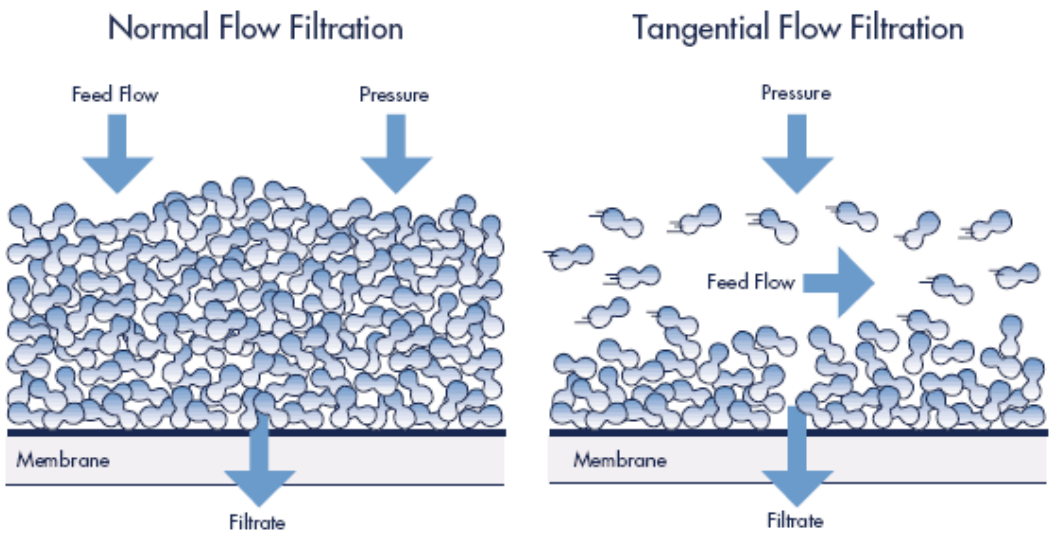


Figure 1-2: Comparison of TFF and NFF(Millipore, 2003)

2.0 PROJECT AIM

Freeze drying is a unit operation used to remove water so as to ensure the stability of a pharmaceutical product over its shelf life. It is usually the final processing step in the manufacture of a drug product for intravenous use. At the end of the lyophilisation process, the product is presented as a dry powder in a vial, which can be easily reconstituted for parenteral administration to a patient. In recent years, a number of researchers have explored freeze drying products above their collapse temperature and have reported that there were no adverse effects on product quality attributes (Passot et al., 2007, Schersch et al., 2010, Tchessalov et al., 2010). Freeze drying above the collapse temperature has traditionally been avoided, as it can lead to product with poor cake appearance, high moisture and can result in failure to meet product quality attributes. On the other hand, freeze drying above the temperature collapse has a major advantage that is attractive to drug manufacturers: shorter lyophilisation cycle times which can reduce the cost of manufacturing.

In this study, lyophilisation was employed not as a final processing step, but as an intermediate step in the manufacture of a multivalent bio conjugate vaccine. Two chemically activated virulent bacterial capsular polysaccharides, serotypes F and K, were lyophilised above and below their collapse temperatures. They were then chemically conjugated to a carrier protein, designated as protein X. Product quality was assessed using a range of physiochemical methods. After conjugation was complete, both serotype F and K conjugates were processed further into a multivalent adjuvant containing formulation. The total biological function and adjuvant biological function of the conjugates were then determined. The primary project aim was to determine if lyophilisation above the collapse temperature of serotype F and K, had any impact on conjugation product quality attributes and on the biological function of the conjugates.

The project was approached in three stages to gain an understanding of:

(i) Factors influencing lyophilisation product quality

(ii) Impact of lyophilisation conditions on conjugation product quality attributes

(iii) Impact of lyophilisation conditions on biological function of serotype F and serotype K conjugates

2.1 Factors influencing lyophilisation product quality

(i) Impact of shell freezing angle, bottle fill weight and ethanol fill depth on residual moisture content and cake appearance.

Chemically activated serotype F was formulated as liquid with an amorphous cryo-protectant. After formulation, the serotype was filled into a 1 L bottle and lyophilised in a large scale freeze dryer. A DoE was designed to understand the impact of varying bottle angle during shell freezing; varying bottle fill weight and varying the ethanol bath fill depth in order to determine their influence on cake appearance and moisture content.

(ii) Develop a cake morphology ranking system to assess degrees of collapse in a lyophilised serotype

A morphology ranking system was developed in order to rank and catalogue the appearance of bottles after lyophilisation, with the aim of correlating cake appearance to varying degrees of collapse/ micro collapse.

(iii) The impact of product temperature during lyophilisation on moisture content and cake appearance

In a large scale freeze dryer, product temperature of serotype F during primary drying was measured and its impact on cake appearance and moisture content was assessed.

(iv) The impact of stopper configuration on product temperature, appearance moisture content.

In a large scale freeze dryer, serotype F was freeze dried. The size of the bottle vapour port for sublimation was modified using specially designed caps. The aperture port of five bottles was controlled using lids with different sized holes to restrict the flow of water vapour: 1.30 cm^2, 0.79 cm^2, 0.50 cm^2, 0.28 cm^2, 0.13 cm^2. The impact of aperture restriction on product temperature, appearance and moisture content of serotype F was assessed.

(v) The influence of aperture restriction during lyophilisation of serotypes F and K in 1L bottles.

In a laboratory scale freeze dryer, two bottles each of serotype F and K were lyophilised in separate lyophilisation cycles. In each cycle, one of the bottles was lyophilised with a stopper placed in the standard configuration: this was the control bottle. The other bottle was freeze dried

with a restricted aperture cap of size 0.28 cm^2: this was the aperture bottle. The impact of aperture restriction on product temperature, appearance moisture content and glass transition temperature was assessed.

(vi) Lyophilisation of serotypes F and K in 50 mL Schott vials above and below the collapse temperature

Serotype F and K were lyophilised above and below their respective collapse temperatures in separate lyophilisation cycles using 50 mL Schott vials. The primary drying step for each cycle was designed using a primary drying modelling tool (Tchessalov and Warne, 2007). Once the lyophilisation cycles were executed, the model was validated by comparing the calculated product temperature and the actual product temperature. After lyophilisation, cake appearance was assessed, and moisture content and glass transition temperature were determined.

2.2 Impact of lyophilisation conditions on conjugation product quality attributes

2.2.1 Conjugation of lyophilised serotypes F and K above and below their collapse temperature

On three independent occasions, serotype F and serotype K were each conjugated to protein X. For each serotype, on each conjugation occasion, three test articles were conjugated: (i) a control bottle, (ii) an aperture bottle (both lyophilised at various degrees above the collapse temperature) and (iii) vials lyophilised below collapse temperature. Using a number of physiochemical assays, the impact of lyophilisation conditions on conjugation product quality attributes was assessed.

2.3 Impact of lyophilisation conditions on biological function of serotype F and serotype K conjugates

2.3.1 Drug product model development

To assess the impact of lyophilisation conditions on total and adjuvant biological function, a drug product model was developed. A number of 120 g drug product multivalent formulations were prepared and these formulations consisted of the serotype F and K, along with a number of other conjugated, capsular polysaccharides serotypes. The model was validated if the total and adjuvant biological function of all serotypes were within specification and within the %RSD of both biological function assays.

2.3.2 Drug product formulation of test articles serotype F and serotype K lyophilised above and below the collapse temperature

Each serotype F and K test article (control, aperture and below collapse) from each conjugation occasion was further processed into individual multivalent 120 g drug product formulations. The total biological function and adjuvant biological function of each test article was assessed in order to determine if lyophilisation conditions had any impact on these quality attributes.

3.0 MATERIALS, EQUIPMENT AND METHODS

3.1 Materials

Prior to commencing this project, serotypes F and K were chemically activated using company proprietary methods. The carrier protein, protein X, was supplied in two forms, a liquid preparation and a lyophilised preparation.

Activated serotype F was formulated with an amorphous cryo-protectant and lyophilised. It was then conjugated to protein X that was previously lyophilised. The dominant excipient in the protein X formulation was the same amorphous cryo-protectant used for the serotype F formulation.

Activated serotype K was formulated with liquid Protein X, and lyophilised. It was then conjugated to protein X.

The quantity of each serotype, protein X and amorphous excipient in each formulation cannot be disclosed for proprietary reasons. However, it can be stated that the quantity of amorphous excipient used in both serotype formulations was well in excess of each serotype added. Materials used in this work are presented in Table 3-1, page 29.

Table 3-1: Materials used for conjugation studies

Materials/Equipment	Manufacturer
Activated polysaccharide serotypes F and K	The company
Lyophilised Protein X	
Liquid Protein X	
Adjuvant	
Amorphous Cryo protectant	
Buffer 1 (Initial TFF buffer)	
Buffer 2 (Final TFF buffer)	
Buffer 3 (Drug product formulation buffer)	

3.2 Lyophilisation equipment

Equipment used for lyophilisation are presented in Table 3-2, page 30.

Table 3-2: Equipment for lyophilisation studies

Equipment	Supplier/Manufacturer
Lyomax 1 freeze dryer	IMA Edwards, NY,USA
Benchmark 500 laboratory freeze dryer	SP Scientific ,NY USA
MKS Capacitance manometer	MKS instruments, MA USA
APG100-XLC Pirani gauge	Edwards Vacuum, West Sussex UK
1 L Kimble bottle	Gerresheimer, Belgium
38 mm EZ Pull stopper	West Pharmaceuticals, Dublin Ireland
FMEL 2 Freezemobile Shell freezer	SP Scientific ,NY USA
Pro 360 Digital protractor	Caulfield Industrial, Galway Ireland
Type T thermocouples	Withnell Sensors, Withnell UK
50 mL Schott vials	Adelphi Healthcare Packaging, West Sussex UK
20 mm Lyotech Stoppers	West Pharmaceuticals, Dublin Ireland
Kaye validator 2000	Kaye, Pforzheim Germany
Karl Fischer 756 coulometer	Metrohm
Differential Scanning calorimeter Q1000	TA Instruments

3.2.1 Freeze dryers

A Lyomax 1 freeze dryer was used for all large scale experiments and a Benchmark 500 was used for all laboratory scale lyophilisation experiments. Lyomax 1 units have two product shelves (useable shelf area of 1.34 m^2, 45 bottles per shelf), see Figure 3-1, page 31. The Benchmark 500 has three product shelves (useable shelf area of 0.426 m^2) capable of holding sixty 50 mL Schott vials on each shelf. The three shelves can be configured to provide one shelf with a nominal shelf clearance during operation of 307 mm. This is to accommodate lyophilisation of 1 L bottles, see Figure 3-2, page 32. Both units have an advanced Allen Bradley PLC (programmable logic controller) system as standard which allows for automatic control of vacuum pressure, shelf temperature and condenser temperature. The software also allows the end user to program a recipe, which is a series of sequential steps that will be followed during the lyophilisation process. The end user inputs the freezing rate, temperature and duration, as well as shelf temperature ramp rates, holding step temperatures, durations and pressures for primary and secondary drying steps.

Figure 3-1: Lyomax 1, two shelves, 45 bottles per shelf.

Figure 3-2: Benchmark 500, three shelves, sixty 50 mL Schott vials and one shelf six 1L bottles.

3.2.2 Shell Freezers

A dual bath FMEL 2 Freezemobile shell freezer was used to freeze 450-550 g of activated polysaccharide filled into 1L bottles; see Figure 3-3, page 33. The shell freezer was filled with 96% ethanol and ramped down to ≤ -70 ± 10 ºC. Once the temperature set-point was reached, the 1 L lyo bottles were loaded manually into each bath. The base of the bottle rested on mechanically driven rollers and the neck of the bottle was suspended by a chain looped around a Teflon roller aid cap placed on top of the bottle. As the bottles rotated, activated polysaccharide froze and coated the inside of the bottle, reducing the thickness of ice thereby increasing the surface area available for sublimation. Once the contents of the bottle were solidified, the bottles

were removed from the bath, placed into aluminium tins and stored in a -75 ºC freezer prior to lyophilisation.

Figure 3-3: FMEL 2 Freezemobile Shell freezer.

3.3 Karl Fischer

In this method, 100 to 300 mg of lyophilised powder was transferred to a 6 mL KF vial (Metrohm, part number 6.2419.007) and dissolved in 2 mL of 2-methoxyethanol. Each vial was then vortexed and transferred to a shaker for 1 hour. Once shaking was complete, the vial was vortexed again and 1 mL of liquid was injected into the Metrohm 756 coulometer. The residual moisture content was reported as a percentage (%)

3.4 Differential Scanning Calorimetry

Modulated DSC (mDSC) measurements were performed using a modulated DSC equipped with a refrigerated cooling system (Model Q1000,TA Instruments). The functionality of the instrument was verified by running an indium metal standard (Tm: 156.60±0.1 °C) and n-decane (Tm: -30 °C) in hermetically sealed crimped aluminium pans (TA Instruments 900793.901) with lids (TA Instruments 900794.901) . Lyophilised samples were prepared in a glove box purged with nitrogen; the relative humidity (RH) in the glove box was measured at 2% RH. Between 3 and 6 mg were delivered to the aluminium sample pans and crimped. Samples were equilibrated at -20 °C and warmed at 2 °C/min to 220 °C and modulated at 0.5 °C/min every 100 seconds. Thermographs were then analysed using Universal Analysis software. All lyophilised samples were analysed in triplicate.

3.5 Conjugation equipment

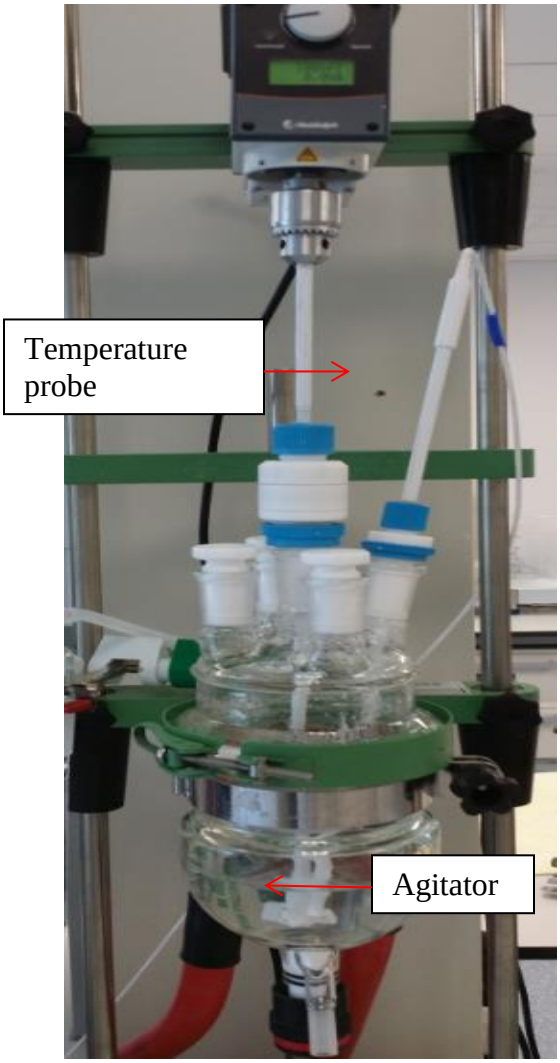
Equipment used for the conjugation process is presented in Table 3-3, page 34.

Table 3-3: Equipment used for conjugation process

Materials/Equipment	Supplier/Manufacturer
ChemRxnHub reaction vessel	GPE, Bedfordshire UK
V max 1L NFF system	Sartorious Stedim, Goettingen Germany
Cogent µScale TFF system	Merck-Millipore France
P1000 Pipette	Gilson
P200 Pipette	

3.5.1 ChemRxnHub reactor

The conjugation reaction was performed in a 1 litre ChemRxnHub reaction vessel. The system was equipped with a heat transfer fluid draining system, a Huber temperature control module (temperature range 120 ºC to 250 ºC) and a Heidolph precision motor for agitation; see Figure 3-4, page 35 and Figure 3-5, page 35.



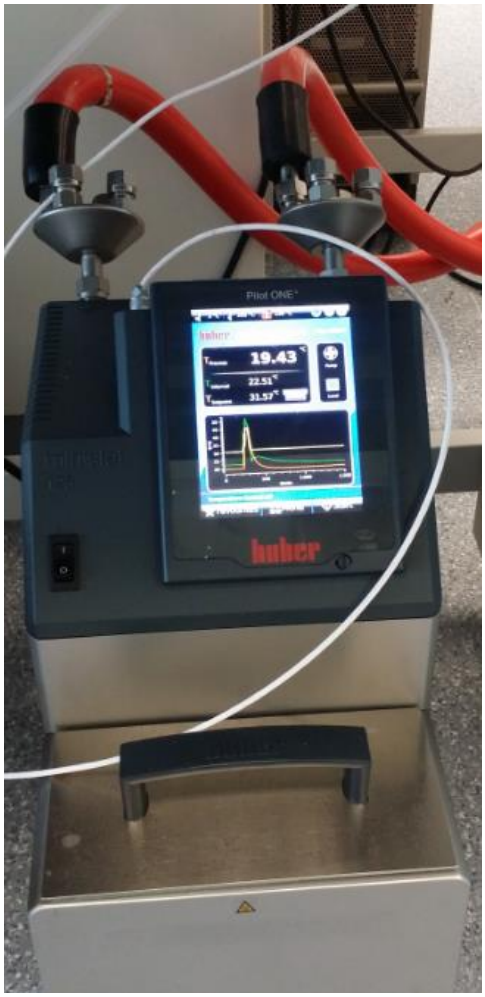


Figure 3-4: Chemglass reactor system

Figure 3-5: Huber temperature control module

3.5.2 Normal Flow filtration (NFF) using a 5 µm filter

The diluted Polysaccharide-Protein X conjugates were poured into a 1 L pressure vessel connected to a head plate, equipped with a process air supply, vent and a pressure gauge. A filter housing was then connected to the outlet valve of the pressure vessel and a 5 µm filter was inserted; see Figure 3-6, page 36. A 0.5 bar pressure was then applied. To wet and bleed the filter, the valve upstream of the filter holder was opened to allow the product to enter the filter holder. In order to collect the filtrate, a second 1 L vessel was placed on a balance directly under

the filter and tared. The valve downstream of the filter holder was then opened and simultaneously Mettler Toledo BalanceLink software was activated. The cumulative volume/weight of the filtrate collected at 3 second intervals was recorded as it passed through the 5 µm filter. Any observed filter blockages were recorded.

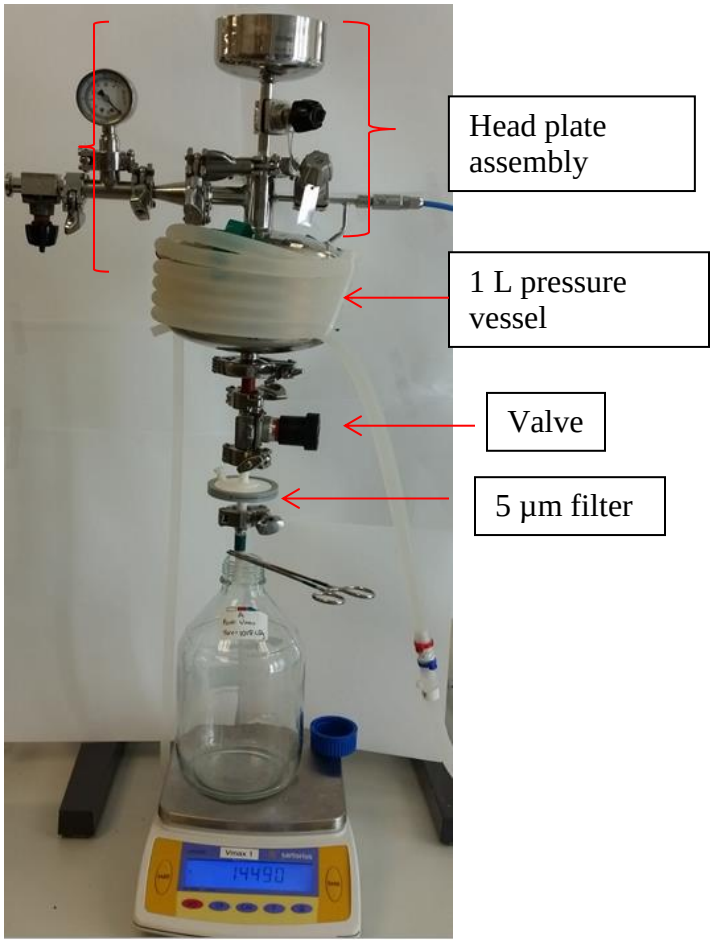


Figure 3-6: NFF system

3.5.3 Tangential Flow Filtration (TFF)

TFF was executed using a Cogent® µScale TFF System, see Figure 3-7, page 38. The Cogent® system consists of a polypropylene retentate reservoir, a drawing syringe, a peristaltic feed pump, manual retentate valve, feed and retentate line pressure transmitters, a balance to measure the permeate volume and an internal data acquisition system. The retentate liquid level was

controlled manually during the concentration step and was maintained constant during diafiltration using an airtight retentate vessel targeting a specific pressure. The crude conjugate was concentrated to a target concentration followed by an initial diafiltration buffer exchange with Buffer 1. The buffer was added to the retentate vessel at the same rate as the permeate flow was removed resulting in a constant retentate volume. After initial diafiltration, a final diafiltration was performed with buffer 2, using the same pressure as the initial diafiltration step. Once final diafiltration was complete, the permeate line was closed, the retentate valve fully opened and the retentate vessel was removed from the system. A second retentate vessel was then connected up to the system and a rinse volume of buffer 2 was added and recirculated for 10 minutes to recover as much product as possible from the membrane. The rinse volume was then added to the original retentate vessel and filtered through a 0.22 µm bio-burden reduction filter into a receiving vessel.

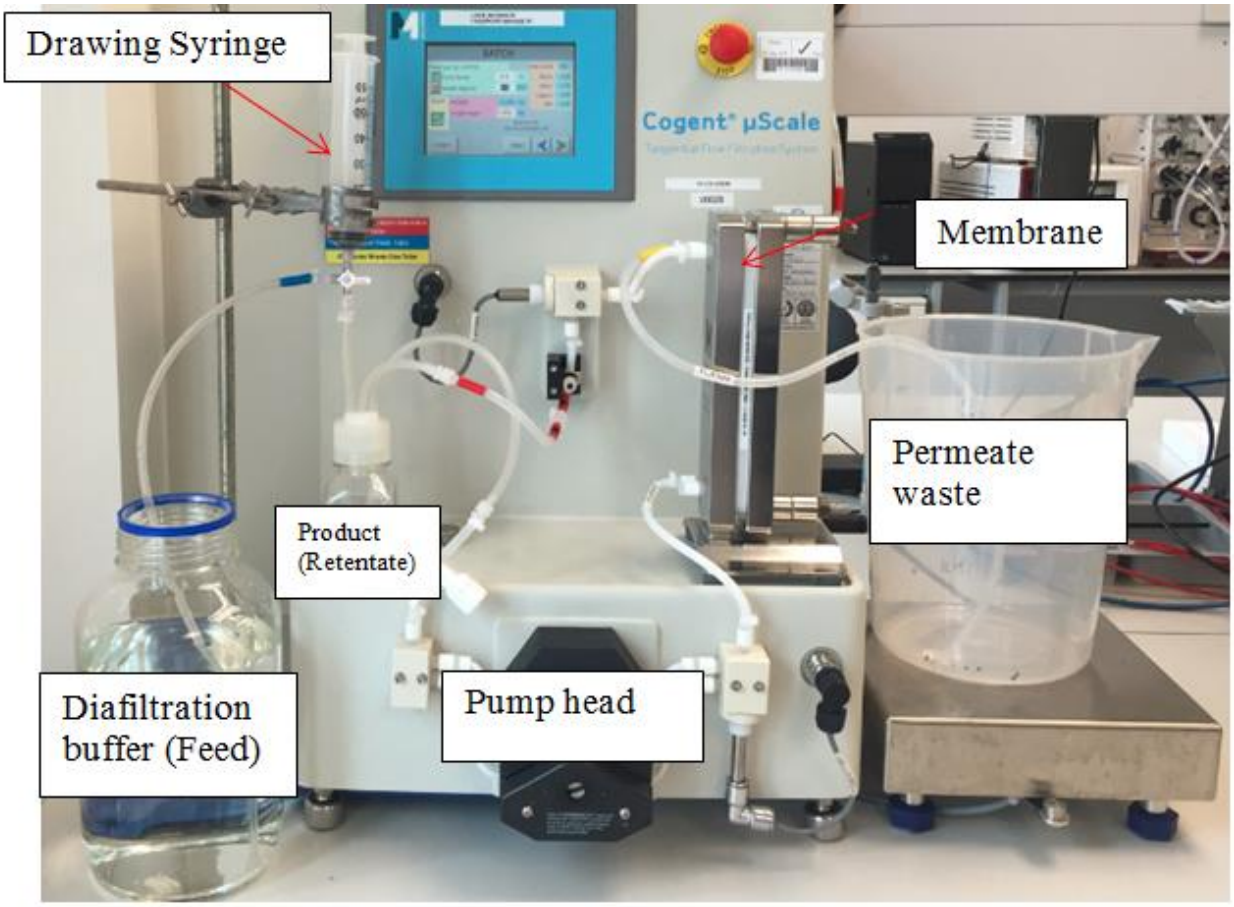


Figure 3-7: Cogent® µScale TFF System

3.6 Analytical methods used to assess product quality of Polysaccharide-Protein X conjugates

A number of different orthogonal analytical techniques were used in this research. Orthogonal methods are defined as the use of a combination or a variety of different analytical methods, with each having its own characteristic measuring principle, for example; size, quantification or structure (Mahler et al., 2009). Immunochemical and physical properties of the conjugate vaccine were determined throughout the conjugation manufacturing process using a range of assays compliant with WHO and European Pharmacopeia guidelines (WHO, 2009, EDQM, 2008). To ensure confidentiality, all analytical assays used to assess product quality attributes cannot be fully disclosed, and all results contained within this document have been normalised. The normalised data are represented as the actual value divided by the specification, multiplied by the numerical value of 10; see Equation 3-2, page 39. All analytical testing was carried out by the company's analytical sciences group using validated analytical methods. All lyophilisation cycle activities, moisture testing, conjugation chemistry, purification steps (TFF, and NFF) and preparation of all DP formulations were performed solely by the Candidate. The output of all analytical methods and the unit of measure is presented in Table 3-4, page 40.

$$\text{Displayed value} = \frac{\text{Actual value}}{\text{Specification}} \times 10$$

Equation 3-2: Formula used for normalisation of data[1]

[1] Analytical method 4, (molecular weight determination) data was normalized relative to the average of a very limited manufacturing data set. This is a characterisation test only and no specifications have been assigned.

Table 3-4: Analytical methods used to assess product quality

Analytical method	Measurement	UOM
1	Determination of protein concentration	mg/mL
2	Determination of polysaccharide concentration	mg/mL
3	Measure of the efficiency of the conjugation reaction between the polysaccharide serotype and Protein X	No units
4	Determination of Molecular weight	kDa
5	Determination of the amount of unconjugated saccharide	%
6	Determination of molecular size distribution	%
7	Determination of: (i) Total Biological activity (ii) Biological activity with adjuvant	(i) µg/mL (ii) %
8	Determination of residual moisture content	%

3.7 Statistical methodology

Where appropriate a design of experiments approach was used for modeling or analysing a problem which may be dependent on one or more factors. The DoE was designed and analysed using Design Expert statistical software version 8.0.6 (Stat-Ease, Inc. Minneapolis, MN 55413, USA). Studies were designed to evaluate the effect of (i) bottle angle during shell freezing (ii) bottle fill weight and (iii) ethanol bath fill depth on residual moisture content and cake appearance.

Other statistical assessments of data were performed using Minitab 17 (Minitab Inc, Pennsylvania 16801-3210). A one way analysis of variance (ANOVA) was used to determine if there was a statistical difference between the means obtained from a given conjugation quality attribute as a function of lyophilisation conditions.

3.7.1 Statistical terminology

In ANOVA testing, before the data are analysed, a null hypothesis is stated. The null hypothesis is a statement that is assumed to be true until evidence to the contrary is presented. In this study, the null hypothesis stated was "freeze drying above the collapse temperature has no practical impact on conjugation product quality attributes". The null hypothesis is a premise which is either rejected or accepted based on the experimental data. One of the outputs of the ANOVA test is the probability value or p value. To determine if there any statistically significant differences between the test article means, the p value is compared to a specified significance level α, usually,0.05(5%). If the p value is lower than this limit, the null hypothesis is rejected and the alternative hypothesis (that conjugation product quality attributes are impacted by lyophilisation conditions) is accepted. The p-value gives the probability of obtaining the present test result—or an even more extreme one—if the null hypothesis is correct (du Prel et al., 2009) A p value below 0.05 means that there is only a 5% risk of concluding that a difference exists when there is no actual difference (Minitab, 2016). If the p value of a factor is <0.05, that factor is deemed statistically significant. Conversely if the p value is >0.05 that factor is deemed not statistically significant. It must be stated at this point that while the use of statistical models is useful in understanding what factors may influence a response, statistical significance is not equivalent to scientific or clinical importance and it should not be used as the sole basis for scientific reasoning (Bangdiwala, 2016). P values are not a replacement for empirical knowledge

and process understanding but are a tool to measure the statistical plausibility of a result (du Prel et al., 2009).

4.0 EXPERIMENTAL METHODS

The purpose of the experiments discussed in section 4.1 and 4.2 was to understand the factors influencing lyophilisation product quality at both large scale and laboratory scale.

4.1 Large scale lyophilisation experiments

A number of lyophilisation cycles were executed in the Lyomax 1 and Lyomax 3 large scale freeze dryers. The experiments carried out and the expected outputs are presented in Table 4-1, page 44.

Table 4-1: Experiments in commercial freeze dryer

Experiment	Purpose	Serotype examined
Large scale experiment 1	Understand the impact on residual moisture content and cake appearance of varying bottle angle during shell freezing, bottle fill weight and ethanol bath fill depth.	Serotype F
Large scale experiment 2	Develop a system to classify cake appearance and develop a morphology ranking system to assess degrees of collapse in lyophilised serotypes.	
Large scale experiment 3	Measure product temperature during lyophilisation. Understand the impact of product temperature during primary drying on cake morphology.	
Large scale experiment 4	Determine the influence of aperture restriction during lyophilisation on product temperature and its effects on cake morphology and moisture content.	

4.1.1 Impact of shell freezing angle, bottle fill weight and ethanol fill depth on residual moisture content and cake appearance on Serotype F.

Studies were designed using Design Expert 8 statistical software to evaluate the effect of (i) bottle angle during shell freezing (8 º, 13 º, and 23 º), (ii) bottle fill weight; (450-550 g), (iii) ethanol bath fill depth (54-76 mm) on residual moisture content and cake appearance. The duration of shell freezing for bottles filled between 450 g to 550 g ranged from 40 minutes to 1 hr 41 minutes. The higher the fill weight, the longer the duration of freezing required. In total eight bottles of serotype F were shell frozen at different conditions; see Table 4-2 , page 45.

Table 4-2: Experimental design for shell freezing study

Bottle #	Fill weight (g)	Ethanol Level (mm)	Bottle Angle (º)
13	450.25	Max: 76	8.8
14	547.1	Min: 54	7.5
15	450.35	Max: 76	19.3
16	551.7	Max: 76	17.4
17	550.4	Max: 76	8.4
18	552.35	Min: 54	17.2
19	449.5	Min: 54	7.3
20	350.80[1]	Min: 54	23.0

As per the DOE, the shell freezers were filled to different levels to understand the impact ethanol fill volume on moisture content and appearance. A stainless steel ruler was used to measure the appropriate ethanol level. After shell freezing, the bottles were transferred to a -75 ºC freezer and lyophilised in a Lyomax 1. After six hours, the bottles were loaded into the lyophiliser with shelf temperature at -50 ºC. Primary drying was performed at a specific target shelf temperature and pressure for a number of hours. Secondary drying (desorption) was then performed at a higher shelf temperature for a number of hours. The purpose of shell freezing was to reduce the

[1] Bottle broke when thawed, resulting in a loss of material

maximum thickness of the material and to increase the surface area available for sublimation. The shell freezing process uses ethanol as heat transfer fluid. The shell freezing procedure used was as follows:

1. Each shell freezer was filled with ethanol. The ethanol was cooled down to a temperature set point of ~ -70 ºC.
2. A plastic roller aid cap was placed on top of the bottle.
3. The bottle was loaded into the shell freezer and secured using a bridge/chain jig apparatus; see Figure 4-1, page 47.
4. The angle of the bottle in the bath was adjusted as per the experimental design presented in Table 4-2, page 45.
5. The angle was measured using a Mititutyo digital protractor; see Figure 4-2, page 47.
6. The bottle sat on stainless steel rollers and was mechanically rotated. The liquid in the bottle was distributed on the inner surface of the bottle creating a "shell" of frozen material.
7. The bottles remained in the shell freezer for a minimum of 1 hour to allow the liquid to solidify.
8. In order to obtain product temperature data during lyophilisation, Type T thermocouples (TC) were placed in some bottles prior to shell freezing. A bottle cap with a pre-drilled hole was placed on the bottle. The TC was threaded through the hole and placed as low as possible into the bottle, ensuring that the TC did not touch the bottom of the bottle. The TC was wrapped around the neck of bottle and secured with tape; see Figure 4-3, page 47.
9. After shell freezing, bottles were placed in aluminum sleeves, see Figure 4-4, page 47 and placed in a freezer at-75 ± 10 °C.

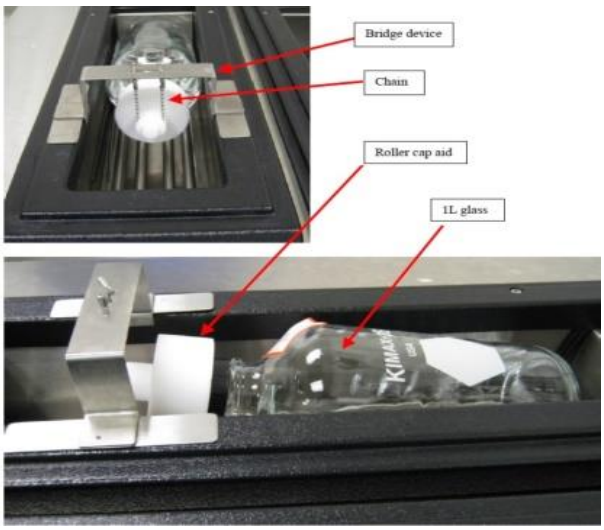


Figure 4-1: Setup for shell freezing

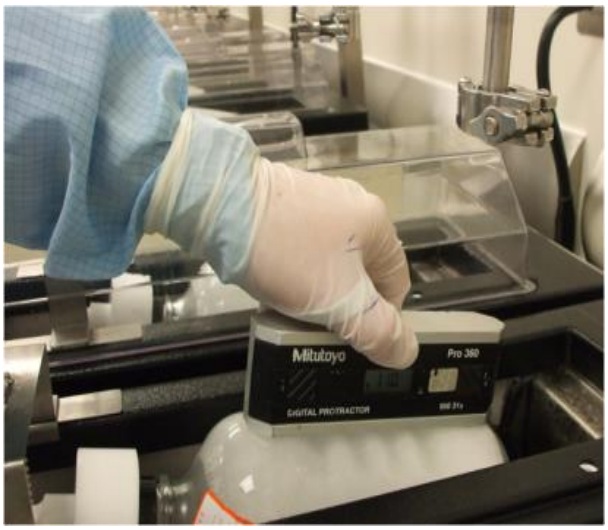


Figure 4-2: Digital protractor measuring bottle angle during shell freezing

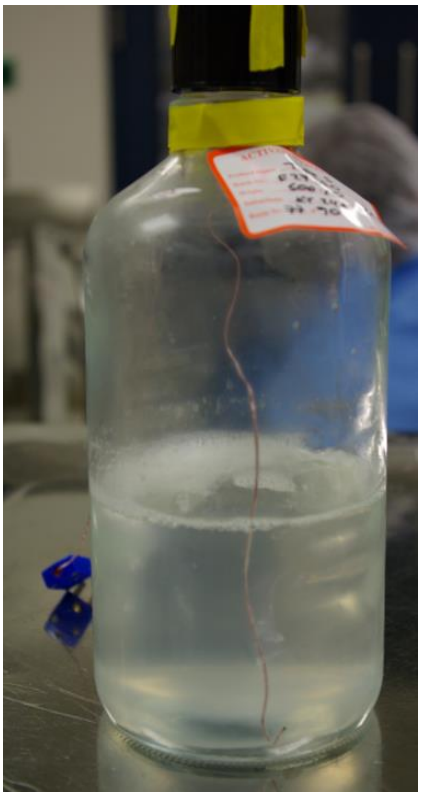


Figure 4-3: Thermocouple placed in 1L bottle.

Figure 4-4: Rolleraid cap, 1L bottle placed in aluminum sleeve after shell freezing

4.1.2 Develop a cake morphology ranking system to assess degrees of collapse in a lyophilised serotype

The measure of cake appearance is a subjective criterion. Sometimes, it is difficult to differentiate between different morphologies. A system was developed in order to rank the appearance of bottles after lyophilisation so as to correlate cake appearance and varying degrees of collapse. A sample of 234 bottles of lyophilised serotype F was visually examined; each bottle was ranked from 1 to 4 in decreasing quality of appearance.

4.1.3 The impact of product temperature during lyophilisation on moisture content and cake appearance

A total of 18 bottles of serotype F out of 58 were shell frozen with a thermocouple in place. After storage at -75 °C for a predefined time, bottles were loaded into to the Lyomax 1 lyophiliser; see Figure 4-5, page 49. The bottles (in aluminum sleeves) were placed into custom made racks which secured the bottles on the shelf of the freeze dryer. During loading, the position of each bottle on the shelf was recorded. When placed on the shelf, the thermocouples were connected into a Kaye Validator via a feed-through gland which was attached on to the port of the lyophiliser; Figure 4-6, page 49. This allowed product temperature data to be recorded during the lyophilisation cycle. These thermal data would reveal if cake appearance was influenced by product temperature. After the cycle was completed and once the chamber reached atmospheric pressure, the door was opened, and the bottles were unloaded and inspected.

Figure 4-5: Bottles loaded on to shelf of freeze dryer. The technician can be seen placing a stopper into a bottle.

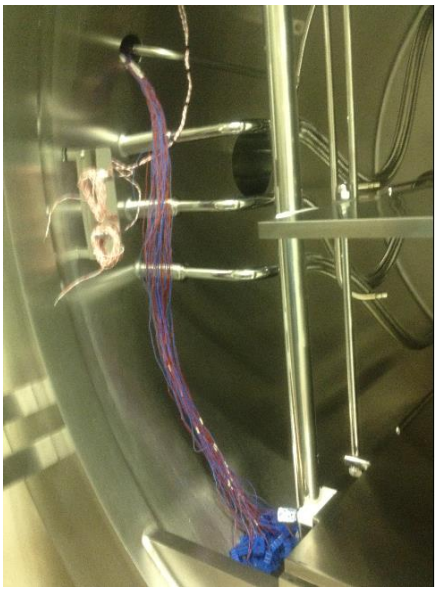


Figure 4-6: Feed-through is attached to blank port of dryer and TC fed into chamber.

4.1.4 The impact of stopper configuration on product temperature, appearance moisture content

The influence of stopper configuration during lyophilisation on appearance and residual moisture was assessed in a study conducted at manufacturing scale; see Table 4-3, page 50. Forty eight bottles of serotype F and 42 bottles of a cryo-protectant excipient were shell frozen, stored at -75 ºC and lyophilised using the standard cycle. The aperture port of five bottles was controlled using lids with different sized holes to restrict the flow of water vapour: 1.30 cm^2, 0.79 cm^2, 0.50 cm^2, 0.28 cm^2, 0.13 cm^2; see Figure 4-7, page 50. Discussion with operators from the manufacturing area revealed that across a number of different serotypes, occasionally (and randomly) during loading, the stopper could "slip" down into the bottle near to the upper grip of the stopper, reducing the aperture size for vapour evolution. To avert this happening, the forks of the stopper on a number of bottles were manually stretched and this appeared to provide more stability to the stopper. The correct stopper placement configuration is shown in Figure 4-8, page 50.

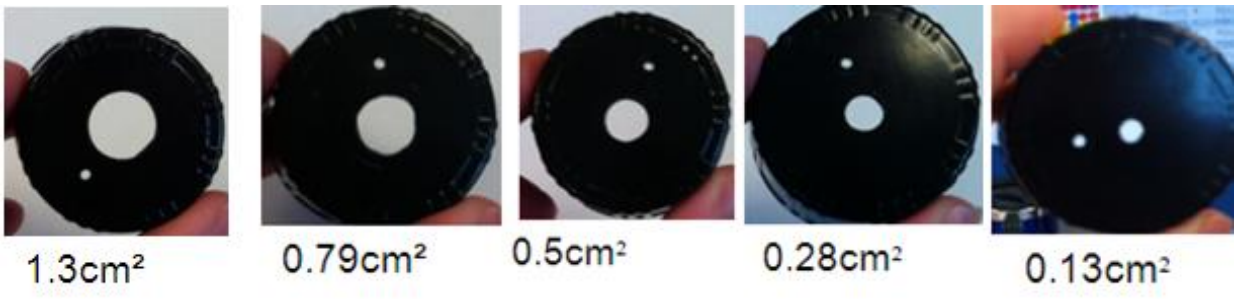


Figure 4-7: Apertures sizes used in Large scale Experiment 3

Figure 4-8: Stopper placement configuration. The stopper is seated at the lower grip in order to ensure the correct aperture port is maintained throughout the lyophilisation cycle.

Table 4-3: Stopper configuration for Large scale experiment 3

Stopper configuration	Number of bottles loaded in to freeze dryer
No stoppers (open bottle aperture of 4.20 cm^2),	16
A restriction effected by sealing with a lid with a defined aperture size (1.30 cm^2, 0.79 cm^2, 0.50 cm^2, 0.28 cm^2, 0.13 cm^2)	5
Standard stopper placement as described in Figure 4-8	17
Stoppers where the forks of the stopper had been 'splayed' or stretched.	10

4.2 Laboratory scale lyophilisation experiments

Experiments at laboratory scale in Benchmark 500 Freeze Dryer freeze dryer were also executed; see Table 4-4, page 51.

Table 4-4: Laboratory scale lyophilisation experiments 1 and 2

Experiment	Purpose	Serotype examined
Laboratory scale experiment 1	Lyophilisation in 1L bottles. To ascertain the influence of aperture restriction during lyophilisation on product temperature and its effects on cake morphology and moisture content.	Serotype K
Laboratory scale experiment 2	Lyophilisation in 50 mL Schott vials above and below the collapse temperature. Primary drying setpoints will be determined using Excel based primary drying modelling tool	Serotype F

4.2.1 Influence of aperture restriction during lyophilisation of serotypes F and K in 1L bottles

Two bottles of serotype F and K were lyophilised in separate lyophilisation cycles in the Benchmark 500 Freeze Dryer. In each cycle, one of the bottles was lyophilised with a stopper placed in the standard configuration; as per Figure 4-8, page 50: this was the control bottle. The other bottle was freeze dried with a restricted aperture cap of size 0.28 cm^2: this was the aperture bottle; see Figure 4-7, page 50 and Table 4-5, page 52. Prior to shell freezing, a type T thermocouple was placed into the control and aperture bottle. After storage at -75 °C, the bottles were then loaded into the freeze dryer with the shelf temperature at -50 ºC. Primary drying was performed at a specific shelf temperature and pressure for a number of hours. Secondary drying (desorption) was then performed at a higher shelf temperature for a number of hours. The impact of aperture restriction on product temperature, appearance moisture content and glass transition temperature was assessed.

Table 4-5: Laboratory scale experiment 1 in 1L bottles

Serotype	Number of bottles lyophilised	Stopper configuration
K	2	Control
		Aperture (0.28 cm^2)
F	2	Control
		Aperture (0.28 cm^2)

4.2.2 Lyophilisation of serotypes F and K in 50 mL Schott vials above and below the collapse temperature

Serotype F and K were lyophilised above and below their respective collapse temperatures in separate lyophilisation cycles using 50 mL Schott vials. The primary drying step for each cycle was designed using the equations described in section 1.5.3. The model is based on the assumption that (i) all transferred heat is used for the sublimation of ice and (ii) the pressure and shelf temperature are controlled precisely. The model calculates a product temperature profile as a function of shelf temperature and chamber pressure and also resistance to vapour flow as a function of dried layer thickness. Once the lyophilisation cycles were executed, the model was validated by comparing the calculated product temperature and the actual product temperature. In each cycle, sixty five x 50 mL Schott vials containing 10 g of each serotype were filled gravimetrically. Filling was performed in a Class 1 Biological Safety Cabinet (BSC) in order to reduce the potential for contamination. Once filled, thermocouples were placed into three vials of each serotype with the thermocouple touching the base of the vial; see Figure 4-9, page 54. The vials were placed in a basket and immersed in the -70 °C ethanol bath for ~ 20 minutes; see Figure 4-10, page 54. Once frozen, the vials were transferred to a -75 °C freezer for a minimum of 6 hours prior to lyophilisation. The vials of each serotype were then placed on a tray and loaded into the lyophiliser at -50 ºC. Vials with thermocouples were placed in the front, and centre rows; see Figure 4-11, page 54. The lyo cycles executed below and above collapse temperature are presented in Table 4-6, page 53 and Table 4-7, page 53 respectively. Primary drying conditions were derived using the model. The same secondary drying temperature and

duration for both the above and below collapse cycles was used. After lyophilisation, cake appearance was assessed, and moisture content and glass transition temperature were determined.

Table 4-6: Lyophilisation serotype F and K below collapse temperature

Cycle Step	Cycle phase	Shelf Temperature	Pressure
Loading	Freezing	-X ºC	Atmospheric
Hold	Freezing	-X ºC for Y hours	Atmospheric
Ramp	Primary drying	-X ºC for Y hours	Z mT
Hold	Primary drying	-X ºC for Y hours	Z mT
Ramp	Secondary drying	-X ºC for Y hours	Z mT
Hold	Secondary drying	-X ºC for Y hours	Z mT
Stoppering			
Aeration			

Table 4-7: Lyophilisation of serotype F and K above collapse temperature

Cycle Step	Cycle phase	Shelf Temperature	Pressure
Loading	Freezing	-50 ºC	Atmospheric
Hold	Freezing	-X ºC for Y hours	Atmospheric
Ramp	Primary drying	-X ºC for Y hours	Z mT
Hold	Primary drying	-X ºC for Y hours	Z mT
Ramp	Secondary drying	-X ºC for Y hours	Z mT
Hold	Secondary drying	-X ºC for Y hours	Z mT
Stoppering			
Aeration			

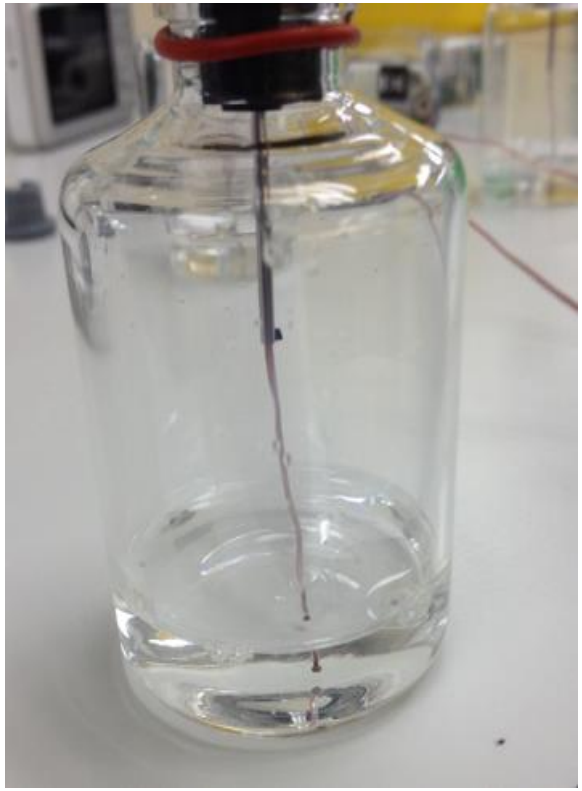


Figure 4-9: TC in placed in vial

Figure 4-10: Vials placed in a basket and immersed in ethanol bath

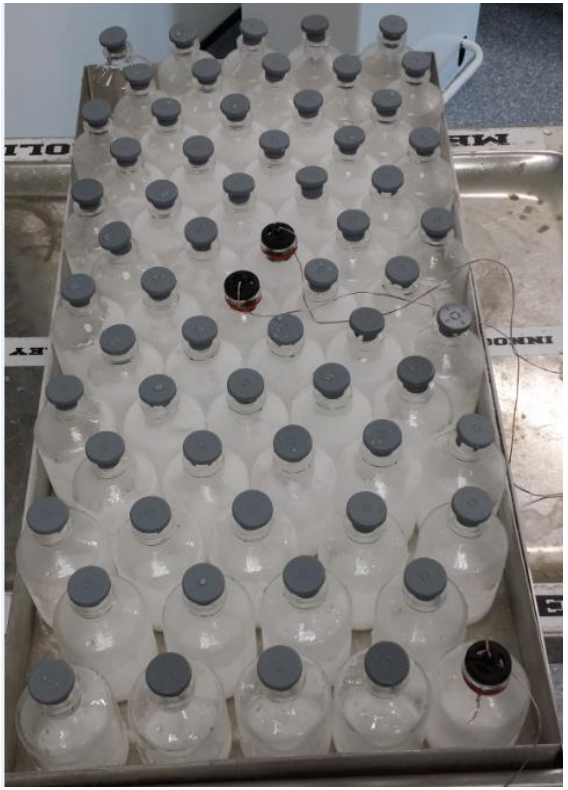


Figure 4-11: Vial configuration on the tray. TC were placed in the centre and front row of the tray

4.3 Laboratory scale conjugation of serotypes F and K

4.3.1 Experiment 1 and 2: Conjugation of serotypes F and K lyophilised above and below their collapse temperature

On three independent occasions using company proprietary methods, serotype F and serotype K were each conjugated to protein X. For each serotype, on each conjugation occasion, three test articles were conjugated: (i) a control bottle, (ii) an aperture bottle (both lyophilised at various degrees above the collapse temperature as described in 4.2.1, page 51) and (iii) vials lyophilised below collapse (as described in section 4.2.2, page 52). Using a number of physiochemical assays, the impact of lyophilisation conditions on conjugation product quality attributes was assessed. An overview of the conjugation process is presented in Figure 4-12, page 57. Across the conjugation process samples were taken for analysis; see Table 4-8, page 58.

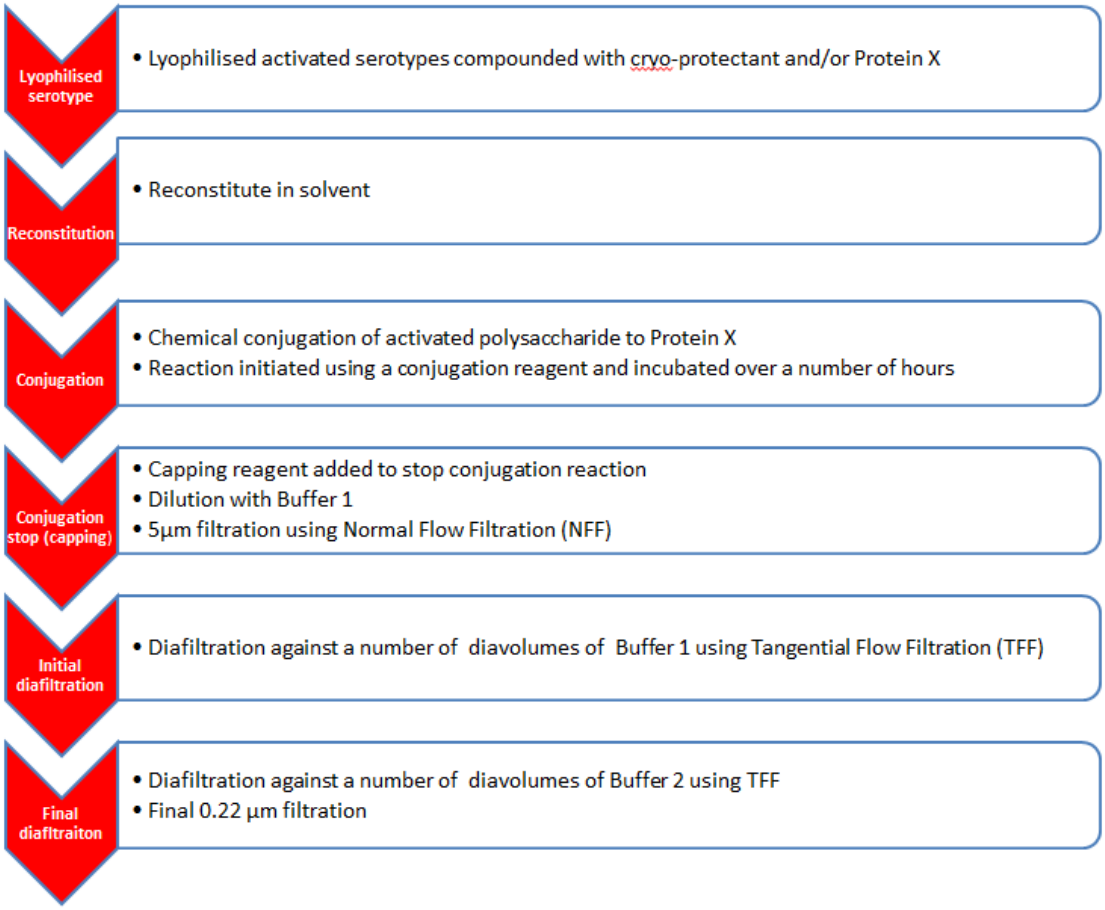


Figure 4-12: Conjugation process flow

Table 4-8: Testing points and conjugation product quality attributes assessed as a function of lyophilisation conditions

Sample point	Analytical methods					
	Analytical method 1 Determination of Protein content	Analytical method 2 Determination of Polysaccharide content	Analytical method 3 Determination of conjugation efficiency	Analytical method 4 Determination of Molecular weight	Analytical method 5 Determination of amount of unconjugated saccharide	Analytical method 6 Determination of molecular size distribution
Post capping reaction (Dialysis)	X	X	X	NT	X	NT
Pre 5 μm filtration	NT	NT	NT	X	NT	NT
Final diafiltration	X	X	X	X	X	X

The conjugation process (using Company proprietary methods) employed was as follows:

1. A quantity of lyophilised powder was removed from each test article in order to provide 200-208 mGAI of polysaccharide for the conjugation reaction.
2. The lyophilised powder was transferred to a 150 mL Duran vessel and reconstituted in a solvent for a predefined number of hours, targeting a specific concentration (X mg/mL); see Figure 4-13, page 60.
3. Once the reconstitution step was complete, the contents of each test article were transferred to a 100 mL ChemRxnHub reaction vessel (this will be referred to as a Chem glass reactor in the remainder of the report) and allowed to mix at a specific agitation speed for a predefined time; see Figure 4-14, page 60.

Note: As Serotype F was lyophilised without Protein X, lyophilised Protein X was reconstituted separately and following reconstitution, both serotype F and Protein X were pooled together in the Chem glass reactor.

4. The conjugation reaction was initiated by the addition of a predefined molar equivalent amount of a "conjugation reagent". The reaction was incubated at a specific temperature for a predefined number of hours.
5. The conjugation reaction was stopped by the addition of a predefined molar equivalent amount of a "capping reagent". The capping reaction was also incubated at a specific temperature for a predefined number of hours.
6. Once the capping reaction was complete, each test article (now a crude conjugate) was removed from the Chem glass reactor and dispensed into a clean 150 mL Duran vessel. From each test article, 5 mL of crude conjugate was removed and transferred to a 100K tube for dialysis purification; see Section 4.3.2, page 62.
7. The remaining crude conjugate was diluted by pouring into a 1L Duran bottle containing Buffer 1 and mixing for a predefined time; see Figure 4-15, page 60. After mixing was complete, 5 mL was sampled from each test article for molecular weight determination.
8. Each test article was then poured in to a Vmax system for 5 µm filtration; see Figure 4-16, page 60.
9. After 5 µm filtration, each test article was purified using a Cogent TFF system; see Figure 4-17, page 61. The filtered conjugate was initially purified against a number of diavolumes of buffer 1.
10. The final diafiltration was performed against a number diavolumes of buffer 2 and product quality attributes such as (i) saccharide to protein ratio, (ii) the amount of unconjugated saccharide, (iii) molecular weight determination and (iv) molecular size distribution were determined.

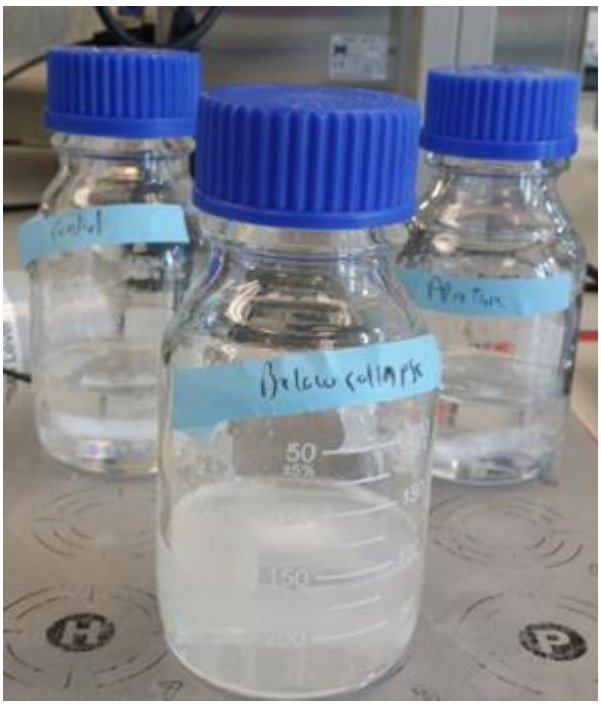


Figure 4-13: A 150 mL glass Duran vessel used for reconstitution. Each test article is reconstituted in solvent for a predefined time

Figure 4-14: Transfer of reconstituted test article to a Chem glass reactor system for conjugation

Figure 4-15: After conjugation was complete, the crude conjugate was poured in to container with Buffer 1 and diluted.

Figure 4-16: Test article poured into NFF system for 5µm filtration and collected in 1 L Duran bottle

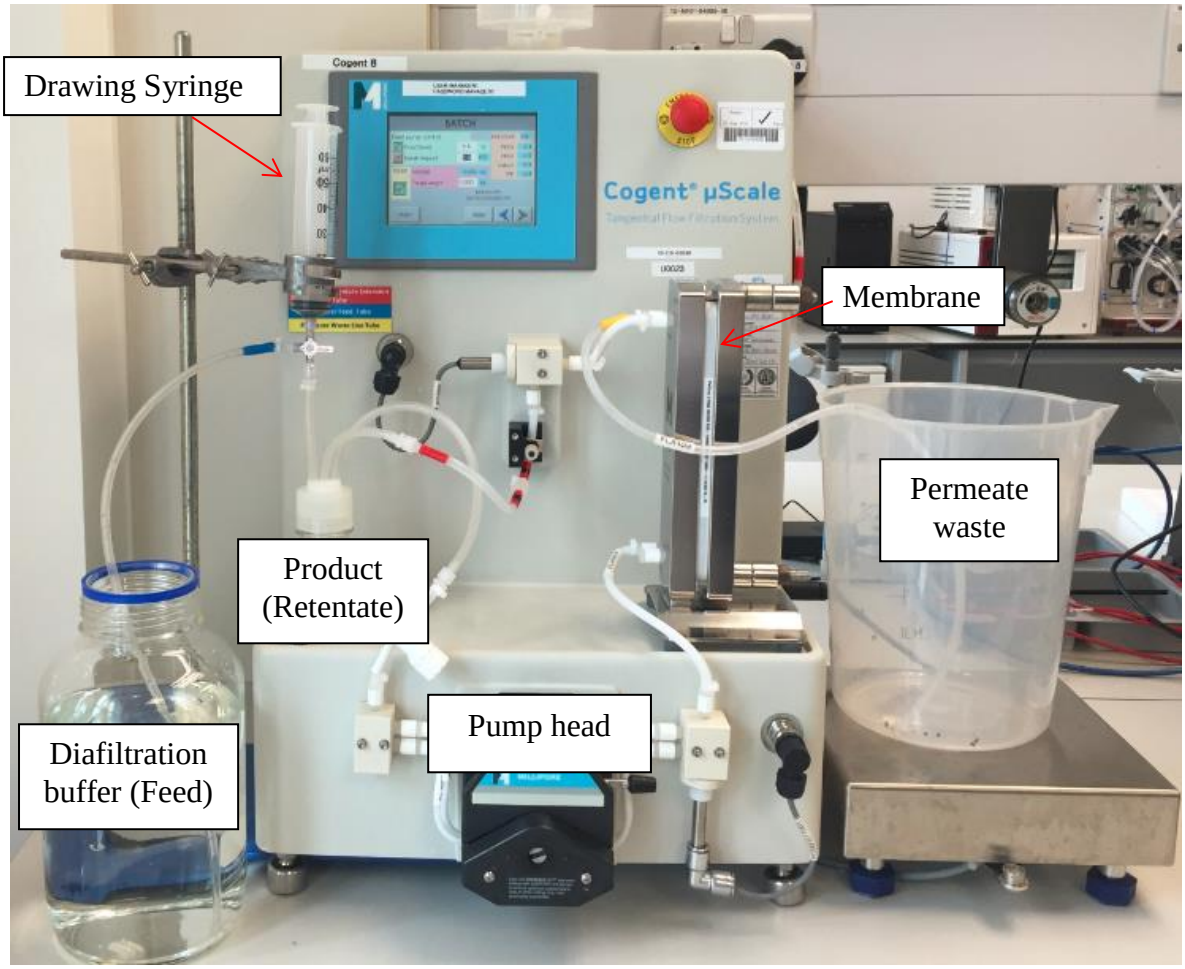


Figure 4-17: Setup for Tangential Flow Filtration

4.3.2 Dialysis purification

Once the capping reaction was complete, 5 mL was sampled from each test article and transferred to a dialysis tube with a specific MWCO. The purpose of this purification step was to assess efficiency of the conjugation reaction and quantify the amount of unconjugated saccharide present, independent of NFF and TFF steps. In dialysis, only molecules that are small enough to fit through the membrane pores are able move through the membrane whilst the sample of interest is retained.

1. The dialysis tubes were placed into a 5 L container filled with buffer 1 and transferred to a stir plate in a fridge at 2-8 °C for agitation at 100 rpm.
2. The buffer was changed a minimum of 8 times, allowing at least 4 hours between buffer changes. For the final three buffer exchanges, buffer 2 was used[4].
3. The product recovered at the end of the dialysis purification was analysed and the SPR was determined along with the amount of unconjugated saccharide.

[4] For serotype F, Buffer 1 only was used for the dialysis step.

4.4 Impact of lyophilisation conditions on biological function of serotype F and serotype K conjugates

The serotype F and K test articles were processed further into a multivalent formulation and the biological function was assessed; see Table 4-9, page 63. The impact of lyophilisation conditions on biological function of the antigen were assessed

Table 4-9: Laboratory scale lyophilisation experiments 1 and 2

Experiment	Purpose	Serotype examined
Biological function experiment 1	To develop a small scale 120g drug product model to determine the impact of lyophilisation conditions on total biological function and adjuvant biological function	Serotype K Serotype F
Biological function experiment 2	Each serotype F and K test article (control, aperture and below collapse) from each conjugation occasion were further processed into individual multivalent 120 g drug product formulations. The total biological function and adjuvant biological function of each test article was assessed in order to determine if lyophilisation conditions had any impact on these quality attributes.	

4.4.1 Drug product model development

A 120 g drug product multivalent formulation was prepared. This multivalent formulation comprised the serotype's F and K, along with a number of other conjugated, capsular polysaccharides serotypes. These polysaccharides were also chemically activated and individually conjugated to Protein X using company proprietary methods. The formulation excipients used were Buffer 3, a quantity of surfactant and a quantity of adjuvant. The target polysaccharide content in the multivalent formulation was 14 µg/mL for all serotypes, except serotype F, which was targeted at 28 µg/mL. Over four discrete occasions, a number of 120 g formulations were prepared. On formulation occasions 1, 2 and 3, three 120 g formulations were prepared. On formulation occasion 4, seven formulations were prepared, giving a total of sixteen formulations. The total biological

function and adjuvant biological function was then measured using an appropriate nephelometry assay.

The acceptance criteria for model qualification were as follows:

- Total biological function and adjuvant biological function results should be within specification.
- The %RSD of each 120 g formulation should be within the %RSD of total biological function and adjuvant biological function assays, ≤ 20 %.

The formulation procedure was as follows:

1. A 250 mL Nalgene container with a stir bar was tared on an analytical balance. This was the drug product formulation container (FC).
2. The required masses of polysaccharide, adjuvant and surfactant were calculated.
3. Using a 0.22 µm filter, Buffer 3 and the surfactant were filtered and added gravimetrically to the FC.
4. An additional 4 g of filtered Buffer 3 was added.
5. Using a P1000 and P100 Gilson pipette, the first conjugate was added to the FC, serotype A.
6. After the serotype was added, the pipette tip was rinsed with between 0.8- 1.00 mL of RO water. This flush volume was added to ensure that as much serotype material as possible has been removed from the pipette tip.
7. The remaining conjugates were then added to the FC.
8. Once all the serotypes were added, the FC was made up to 72 g with Buffer 3.
9. The pooled conjugates in the FC were mixed for a predefined time.
10. In parallel, the adjuvant suspension was mixed for ≥ 30 min prior to addition to the FC.
11. The mass of Buffer 3 required in order to reach a final target product mass of 120 g was calculated and then added to the FC.
12. The mass of adjuvant required to achieve the correct concentration was calculated and then added to the FC.
13. To ensure adequate conjugate-adjuvant binding, the FC was then mixed for a predefined time.
14. The final drug product container is presented in Figure 4-18, page 66.
15. A 15 mL aliquot was removed from each formulation and biological function of the polysaccharide was assessed

4.4.2 Drug product formulation of test articles Serotype F and K lyophilised above and below the collapse temperature

Using the formulation procedure described in Section 4.4.1, page 63, each serotype F and K test article (control, aperture and below collapse) from each conjugation was further processed into individual multivalent 120 g drug product formulations; see Table 4-10, page 65. Once formulated, a 15 mL aliquot was removed from each formulation and biological function of the polysaccharide was assessed.

Table 4-10: Serotype F and K test articles prepared in multivalent 120 g formulation

Serotype	Conjugation occasion	Test article	Number of 120g formulations
F	001	Control	9
	002	Aperture	
	003	Below collapse	
K	001	Control	9
	002	Aperture	
	003	Below collapse	

Figure 4-18: 120 g drug product. The formulation contained polysaccharide/Protein X conjugates, of serotype F and K along with other serotypes. Serotype F and K test articles lyophilised above and below collapse were individually formulated and the impact of polysaccharide biological function was assessed

5.0 RESULTS AND DISCUSSION

5.1 Large scale lyophilisation experiments

5.1.1 Impact of shell freezing angle, bottle fill weight and ethanol fill depth on residual moisture content and cake appearance.

Studies were designed using Design Expert 8 statistical software to evaluate the effect of (i) bottle angle during shell freezing (8 º, 13 º, and 23 º), (ii) bottle fill weight; (450-550 g), (iii) ethanol bath fill depth (54-76 mm) on residual moisture content and cake appearance. The combination of low fill weight and low angle, producing the smallest shell thickness, resulted in lowest moisture (3.28%). In contrast, high fill weight/high angle resulted in higher moisture due to larger thickness of the ice shell. This demonstrates that increased ice thickness is a significant factor influencing residual moisture. However, bottle 19 had high moisture (9.54%) even with low fill weight and low angle. Variation in residual moisture may also be influenced by low ethanol fill level (54 mm). This is due to the limited contact area of the ethanol with the bottle (and the liquid inside the bottle), thus restricting the contact surface for heat removal during shell freezing. Results are presented in Table 5-1, page 68. Another observation is that a combination of high bottle angle (17.2° – 23.0°) and a low ethanol level (54 mm) resulted in bottles that did not pass visual inspection after lyophilisation (Bottles 18 and 20). The serotype F bottles generated in this study using variable shell freezing conditions are shown in Figure 5-1, page 69.

Table 5-1: Results of shell freezing at by varying fill volume, bottle angle and ethanol bath depth

Bottle #	Fill weight(g)	Ethanol Level(mm)	Bottle Angle (º)	Visual Inspection	Residual Moisture (%)
13	**450.25**	**Max: 76**	**8.8**	**Pass**	**3.28±0.02**
14	547.10	Min: 54	7.5	Pass	7.70±0.21
15	**450.35**	**Max: 76**	**19.3**	**Pass**	**2.14±0.40**
16	**551.70**	**Max: 76**	**17.4**	**Pass**	**10.50±0.45**
17	**550.40**	**Max: 76**	**8.4**	**Pass**	**6.08±0.09**
18	552.35	Min: 54	17.2	Fail	8.00±0.17
19	449.50	Min: 54	7.3	Pass	9.54±0.41
20	350.8[5]	Min: 54	23.0	Fail	6.44±0.01

[5] Bottle broke when thawed resulting in a loss of material.

Figure 5-1: Serotype F bottle appearance post lyophilisation. Bottles were frozen with a range of fill weights, ethanol levels and angles. For bottles 13, 15, 16, and 17,acceptable cakes were produced with high ethanol bath depth(76 mm) and high angle(above 17º),. The adverse influence of low ethanol bath depth and high bottle angle are evident in the appearance for bottle 18 and bottle 20. It was observed that cake appearance significantly worsened when a lower ethanol fill depth (54 mm) was used.

The results of low ethanol bath depth (54 mm) were removed from the DOE study due to the low fill weight of one of the design points (bottle 20) and because of the poor appearance of bottle 18. The models were interpreted for serotype F with only bottle angle and fill weight at the fixed 76mm bath depth (bottles 13, 15, 16 and 17, highlighted in bold in Table 5-1). The data show that a reduction in bottle angle and bottle fill weight results in lower reported moisture results; see Figure 5-2, page 70. The output of the DOE showed that bottle angle and bottle fill weight were significant factors, as their p values were below 0.05 (0.03 and 0.01 respectively) ;see Table 5-2, page 70. The adjusted R^2 value of 0.9987 indicates that the data accounts for 99% of the variation in the model.

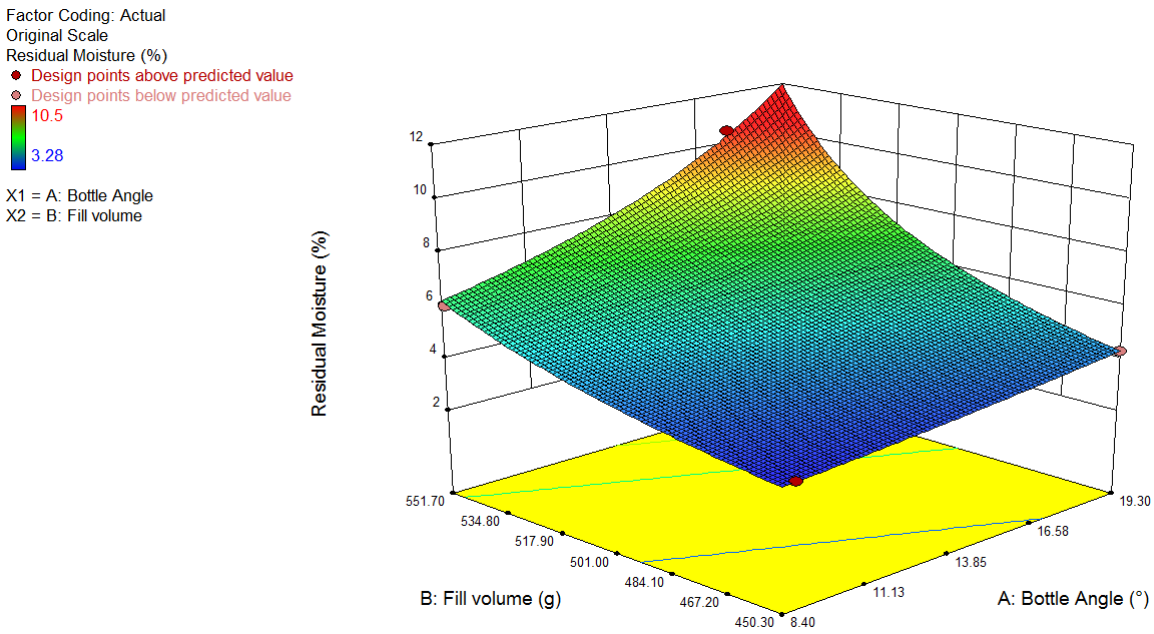


Figure 5-2: Response surface for the interaction between two factors, bottle fill weight (volume in bottle determined gravimetrically) and bottle angle. These factors influence the moisture content (response). As bottle fill weight and angle increases, moisture content increases

Table 5-2: ANOVA results for impact of shell freezing angle, bottle fill weight

Parameter	Range tested	p value	Adjusted R squared
Fill weight	450.30 to 551.70 g	0.03	0.99
Angle	8.80° to 19.30°	0.01	

As the fill weight and the angle are increased, the level of residual moisture increases. The levels of residual moisture can be controlled by reducing these two parameters at an ethanol bath depth of 76 mm. An assessment of the morphology of the bottles also shows that bottle 16 which had a high fill weight (551.7 g) and a steep angle 17.4° has a bottle morphology that is indicative of a greater level of collapse. It was observed that bottle 15, which had a lower fill weight of 450.35 g and which was shell frozen at a steep angle of 19.3 ºC, exhibited better cake morphology. The impact of this difference in morphology can be seen further by comparing the residual moisture results of bottle 15 and 16, 4.28%

and 10.50% respectively. Both bottles were shell frozen under similar conditions (High Angle, and Ethanol level of 76 mm).The difference in fill weight would have the effect that the bottle with the lower fill weight (bottle 15) would complete primary drying faster than the bottle with the higher fill weight (bottle 16) and, therefore, secondary drying would be experienced for a longer duration. Also, low shell thickness can result in lower product temperature during drying (at the same process conditions) causing lesser degree of collapse. This may explain why there is a difference in residual moisture between the two bottles.

Morphology differences are mostly due to temperature: the higher the temperature, the higher degree of collapse, resulting in higher moisture (discussed in Section 5.1.3, page 74). The difference in residual moisture between these bottles is, likely, not only due to the shell freezing process but also to other phenomena, such as stopper placement (discussed in Section 5.1.4, page 78). In order to achieve effective shell formation, the ethanol should be in contact with as much of the length of the bottle as possible. This is achieved by a combination of a maximizing ethanol bath depth (76 mm) and reducing the bottle angle. The amorphous/polysaccharide formulation inside the bottle will not freeze correctly unless it is at or below the level of ethanol in the shell freezer. The freezing parameters influence the ice morphology, which, in turn, influences the performance of the subsequent phases of freeze drying and also the quality attributes of the product (Jameel and Searles, 2010). It is therefore very important to standardize a shell freezing process to ensure bottle homogeneity during the freezing step.

5.1.2 Develop a cake morphology ranking system to assess degrees of collapse in a lyophilised serotype

A morphology ranking system was developed in order to rank and catalogue the appearance of bottles after lyophilisation, with the aim of correlating cake appearance to varying degrees of collapse/ micro collapse.

A total of two hundred and thirty four bottles of lyophilised serotype F were visually examined and ranked on a numerical scale from 1 to 4; with a ranking of 1 indicating a very good cake with little or no evidence of micro collapse and a ranking of 4 indicating a cake with significant micro collapse. The ranking system and criteria used to classify cake appearance is presented in Table 5-3, page 72.

Table 5-3: Morphology ranking system Rank 1-4

Morphology	Description
Rank 1: Very Good	1. No evidence of micro collapse 2. Good structure and cake cosmetics 3. Opaque to light
Rank 2: Good	1. Some evidence of micro collapse 2. Good structure and with some defects (cracking, shrinkage) 3. Opaque to light
Rank 3: Average	1. Intact structure with obvious micro collapse 2. Significant shrinkage and pinching (cake not in contact with bottle) 3. No glassy structure 4. Translucent to light
Rank 4: Very poor	1. Intact structure with significant micro collapse 2. Open pore structure 3. Significant shrinkage and pinching 4. Obvious signs of “wetness” 5. Glassy structure present 6. Translucent to light

An example of morphologies 1, 2, 3 and 4 is presented in Figure 5-3, page 73. From this analysis, it was determined that cake appearance could vary significantly within the same lyophilisation cycle for a given serotype. Clearly, the heterogeneous cake appearance indicates that conditions in individual 1 L bottles were not uniform during the lyophilisation process and this was investigated further in experiments 3 and 4.

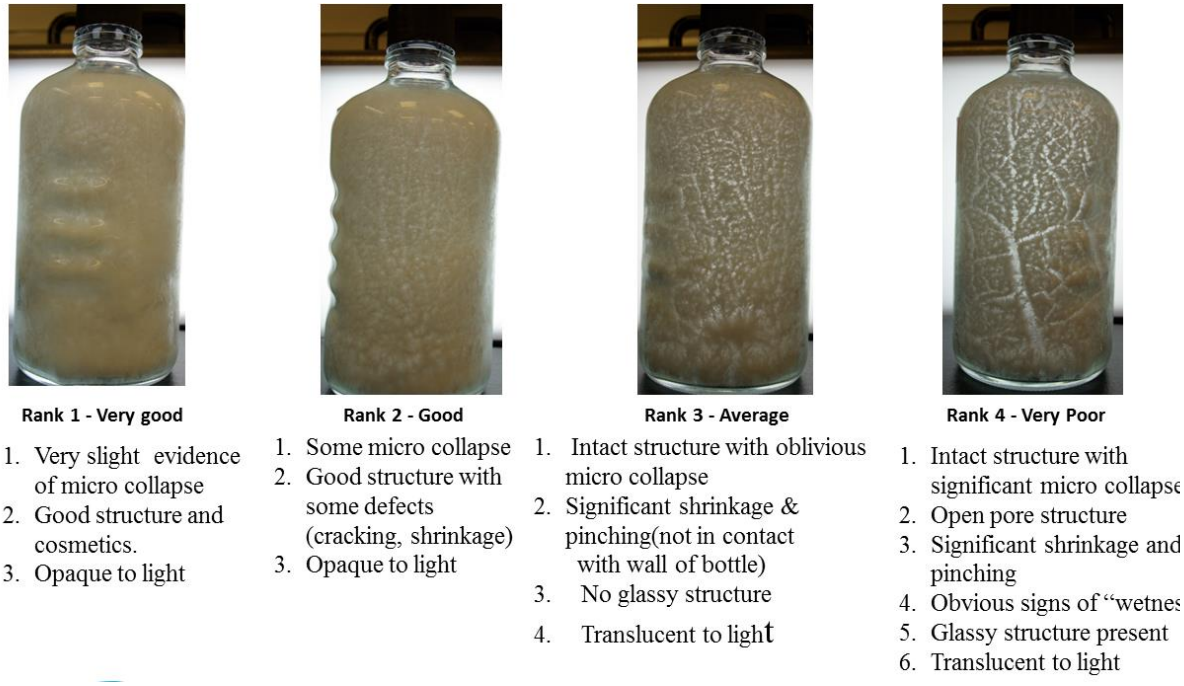


Figure 5-3: Bottle appearance/morphology ranking system. Morphology 1 to 4 in decreasing quality of cake appearance.

It is important to characterise all possible cake morphologies that may be observed after freeze drying. An excellent review by Patel and coworkers in 2017 provides a summary of current challenges with respect to visual appearance testing for lyophilised products (Patel et al., 2017). They provide a guide on how to critically judge cake appearance and provide an approach to develop a harmonized nomenclature system. While cake appearance is important, it may or may not be an indicator of product quality and striving for the "perfect cake" will only result in higher costs and potential delay in bringing treatments for life-threatening conditions to patients. The authors make the point that it is important to determine if cake appearance has any impact on other CQAs and that discarding product solely based on cake appearance (despite no evidence of the impact on other CQAs) would result in an unnecessary waste of product. Their most important point is that making a lyophilised drug product elegant should not be the primary focus, but rather making the drug product more affordable and bringing it faster to the patient with an acceptable safety and efficacy profile should be the main priority (Patel et al., 2017). However, this must be balanced with the expectations of medical professionals and patients that a parenteral product should have acceptable appearance. It must be remembered that lyophilised products will eventually be reconstituted and injected into human beings. Patients may be hesitant to use a product that "looks bad" and no amount of assurances that it meets all CQAs will change their opinion; this could result in product being sent back to the

supplier. Therefore while a “perfect cake” appearance isn’t the be all and end all, effort should be made to ensure that the product looks fit for human use.

5.1.3 The impact of product temperature during lyophilisation on moisture content and cake appearance

In a large scale freeze dryer, product temperature of serotype F during primary drying was measured and its impact on cake appearance and moisture content was assessed.

A total of 18 bottles of serotype F out of 58 were shell frozen with a thermocouple in place. After storage at -75 °C for a predefined time, bottles were loaded into to the Lyomax 1 freeze dryer and lyophilised. After lyophilisation, the bottles were visually inspected and the lyophilisation batch data were downloaded from the MCS. The data obtained demonstrate that the serotype F was freeze dried above its collapse temperature; see Figure 5-4, page 75. The temperature trends for individual bottles show how far above the collapse temperature those bottles were lyophilised; see Figure 5-5, page 76. It is clear from the temperature data that there is variation in the product temperatures achieved in individual bottles during the lyophilisation cycle.

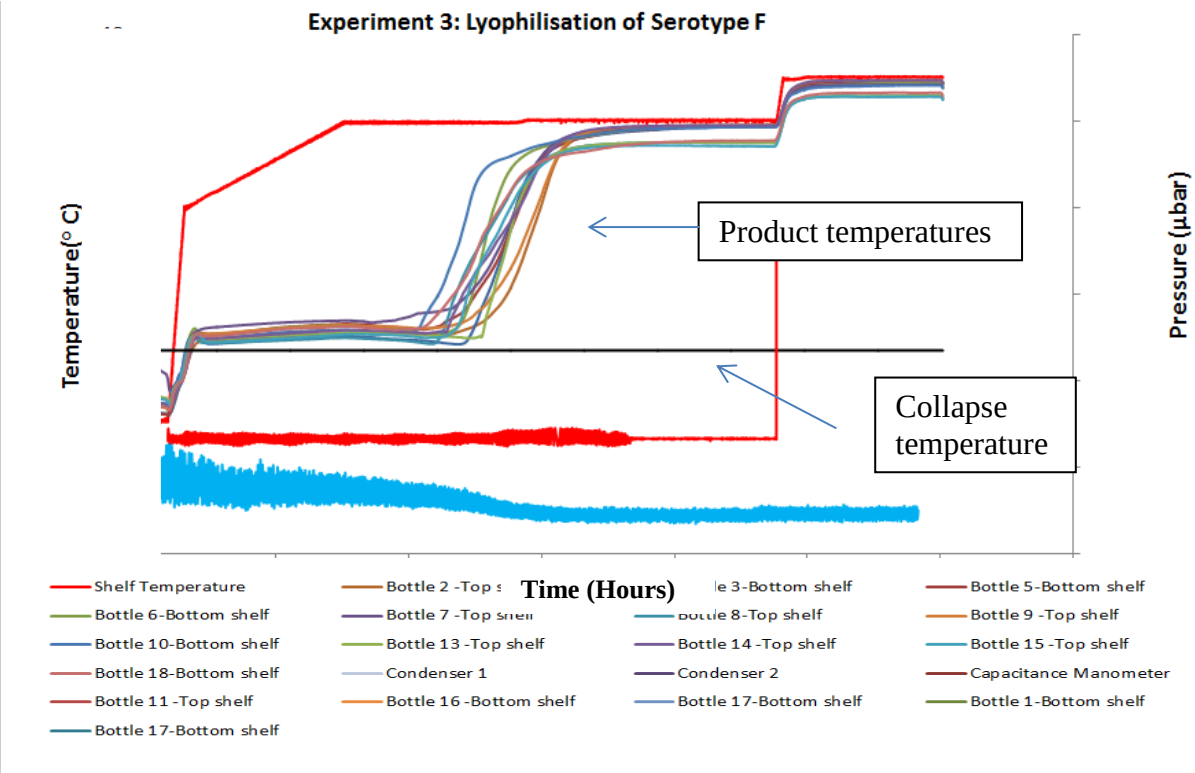


Figure 5-4: Lyophilisation cycle trend for Serotype F. The profile shows shelf temperature, capacitance manometer(absolute pressure) condenser temperature, and product temperature

The data indicated that as product temperature increased, cake morphology worsened; see Figure 5-5, page 76. A relationship between residual moisture and cake morphology was also established and is shown in Figure 5-6, page 76. As cake morphology worsened i.e. higher degree of micro collapse, residual moisture increased; see Table 5-4, page 77.

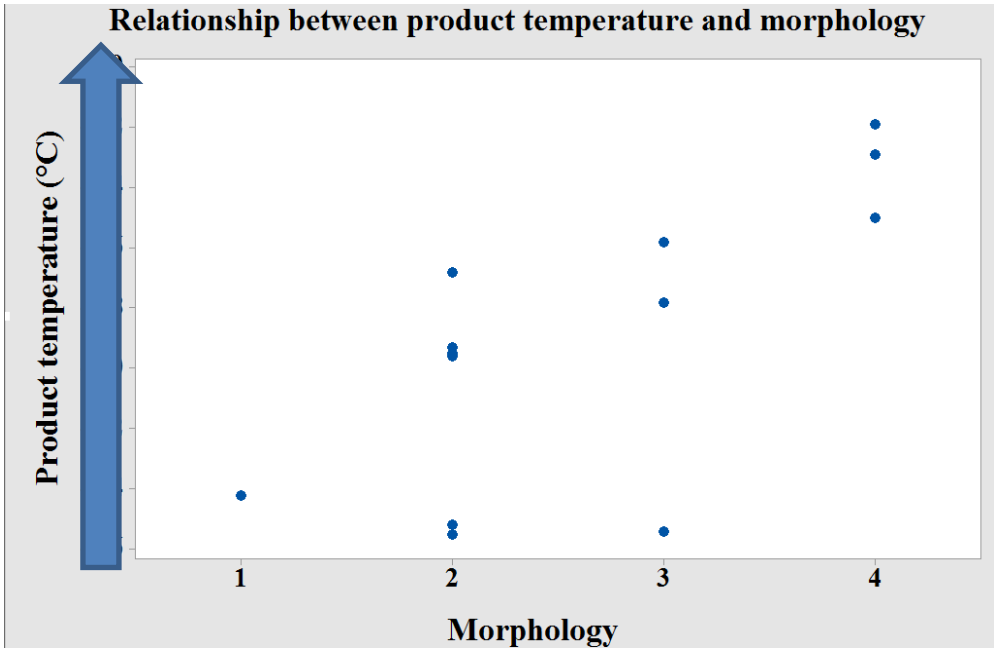


Figure 5-5: Influence of product temperature on cake morphology on serotype F

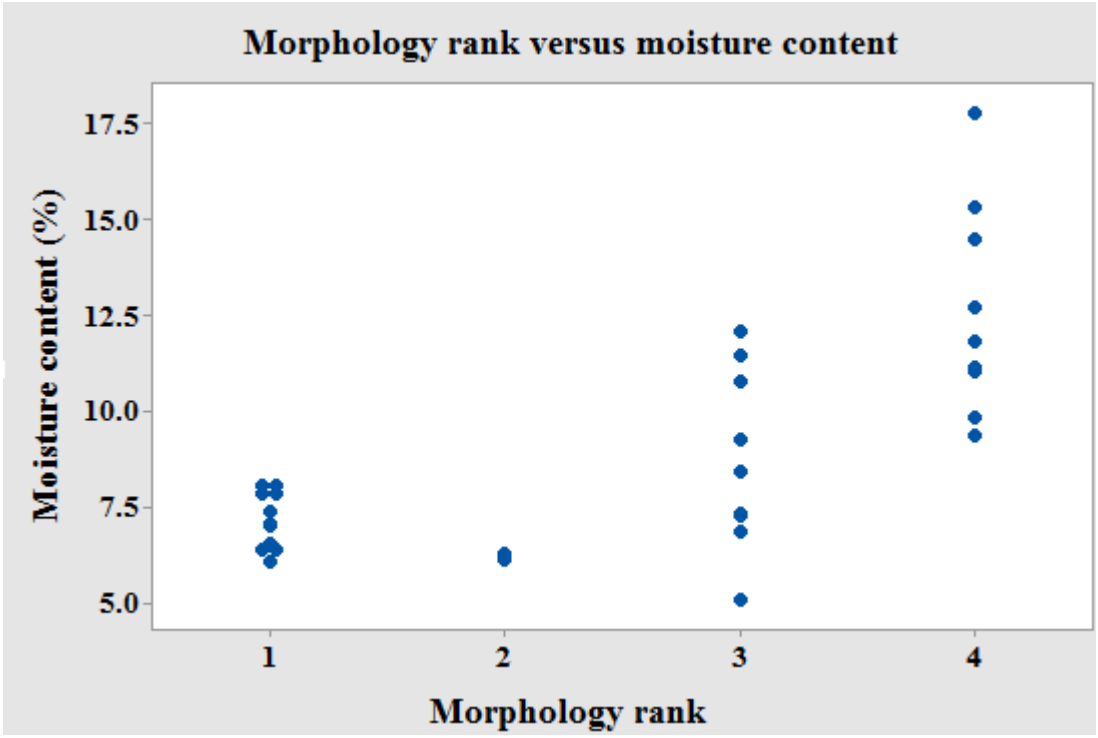


Figure 5-6: Relationship between residual moisture and morphology rank.

Table 5-4 Relationship between residual moisture and morphology

Bottle number	Morphology rank	Residual Moisture Mean ± St Dev (%)(n=3)
7	4	10.14 ± 0.91
22	1	6.86 ± 0.32
38	1	6.68 ± 0.68
49	3	6.58 ± 1.30
52	4	11.88 ± 0.85
54	3	8.20 ± 1.23
61	2	6.20 ± 0.08
93	1	7.44 ± 0.92
4	4	15.89±1.73
5	3	11.47±0.65
11	1	7.44±0.92

There was no significant effect of shelf position on cake appearance and there was no particular pattern to the bottles that exhibited higher product temperatures and poor morphology. However as discussed in Section 5.1.1, page 68, there is potential for shell freezing conditions to impact morphology and moisture content. Another potential source of variation was the stopper used for lyophilisation. During loading, the stopper is positioned on the top of the bottle. The main purpose of the stoppers is to hermetically seal the bottle after the drying process. The vents in the stopper allow water vapour to be removed during drying processes. After drying is complete, the stopper seals the bottle through the automated stoppering process. If the stopper is not correctly placed and "slipped" down into the mouth of the bottle during the lyophilisation cycle, the size of the aperture for sublimation would be reduced. If this were to occur, product temperature could increase, giving rise to the cakes with poor morphology and elevated moisture. This event could happen randomly and may explain why there was no pattern to the range of morphologies observed on the shelf.

5.1.4 The impact of stopper configuration on product temperature, appearance and moisture content

In this experiment, forty eight bottle of serotype F were freeze dried. The aperture port of five of these bottles was controlled using lids with different sized holes to restrict the flow of water vapour: 1.30 cm^2, 0.79 cm^2, 0.50 cm^2, 0.28 cm^2, 0.13 cm^2. Sixteen bottles were lyophilised without stoppers also (moisture content was determined in seven bottles only).

It was evident when aperture size was reduced to 0.28 cm^2, a Morphology Rank 4 bottle was observed with accompanying high moisture (11.18%); see Table 5-5, page 78. For bottles lyophilised with a 0.13 cm^2 aperture, the cake appearance was extremely poor and the moisture content was 13.50%. This observation gave rise to a new category for the morphology ranking system: Rank 5. This morphology is indicative of a cake appearance with visible melt-back, gross collapse and a complete loss of structure.

Table 5-5: Influence of bottle aperture on residual Moisture and Appearance

Bottle ID number	Aperture size (cm^2)	Residual Moisture Mean ± St Dev (%)	Morphology
1	1.30 cm^2	2.20 ± 0.88	2
2	0.79 cm^2	3.54 ± 0.34	2
3	0.50 cm^2	6.08 ± 0.74	2
4	0.28 cm^2	11.18 ± 1.10	4
5	0.13 cm^2	13.50 ± 1.36	5
6	No stopper 3.80 – 4.45 cm^2	2.18 ± 0.42	2
7		6.44 ± 0.12[6]	2
8		2.02 ± 0.74	2
9		5.98 ± 0.36[1]	2
10		2.52 ± 0.24	2
11		2.94 ± 0.22	2
12		3.20 ± 0.32	2

A higher level of residual moisture would be expected for collapsed material, since collapse results in decreased specific surface area of the freeze-dried solid compared to solids which retain the microstructure established by the freezing process (Wang et al., 2004). The bottles with wider stopper apertures displayed good morphology (Rank 2) and low residual moisture, indicating that control of product temperature by correct positioning

[1] The residual moisture results for two of the non-stoppered bottles are elevated (Bottles 21 and 22) for these two bottles the caps were observed to be loose which may have resulted in moisture absorption during storage prior to sampling and moisture testing

of the stopper was essential for a consistently performing process. Bottles with correctly positioned stoppers showed good performance (consistent appearance). It was observed that all bottles lyophilised with no stopper passed visual assessment (Morphology Rank 2) and had low residual moisture within specification (< 4%). It was also observed that two bottles exhibited elevated moisture (6.44% and 5.98% respectively), relative to the other bottles (approximately 3.0%). This appeared to be linked to a container closure issue: it was observed that upon sampling for moisture analysis, the bottle lids were loose, potentially allowing ingress of atmospheric moisture. Incorrectly closed or poorly sealed bottles of Morphology Rank 2 would have shown elevated moisture levels due to their hygroscopic properties.

For bottles lyophilised with a 0.13 cm^2 aperture, visible melt-back and gross collapse were observed. The utility of this experiment, along with experiment 3, shows the impact of aperture restriction on appearance and moisture content. A stopper inserted to the designed location provides a vapour port for the sublimed water to leave the container (bottle) and proceed to the condenser. If this port is restricted because the stopper is set too low in the container, then the rate at which the water vapour leaves the container is decreased while pressure inside the bottle increases. Potentially, at its most extreme, this can cause melt back or gross collapse. Inconsistent stopper placement may cause variability and creates concern for moisture content uniformity within a batch (Trappler, 2004b). It is essential, therefore, that the tolerances of the stopper dimensions and mouth of bottle are controlled tight enough to ensure that the stopper maintains the optimum vapour port size. The experiments described above aligned well with studies in the literature; i.e. when a product is freeze dried above the collapse temperature (albeit as a result of aperture size restriction and not with modification of cycle set points) it can lead to product appearance that is heterogeneous and relatively poor, and to higher residual moisture (Adams and Ramsay, 1996).

From examination of all available data from experiments 2, 3 and 4 (lyophilisation cycle trends, product temperature data and the visual inspection data) it was observed that (i) as **aperture size reduced**, (ii) **product temperature increased** (iii) **appearance worsened** and (iv) **residual moisture increased**. The impact of aperture size reduction on residual moisture content is presented in Figure 5-7, page 80 and the impact of product temperature on appearance is presented in Figure 5-8, page 80.

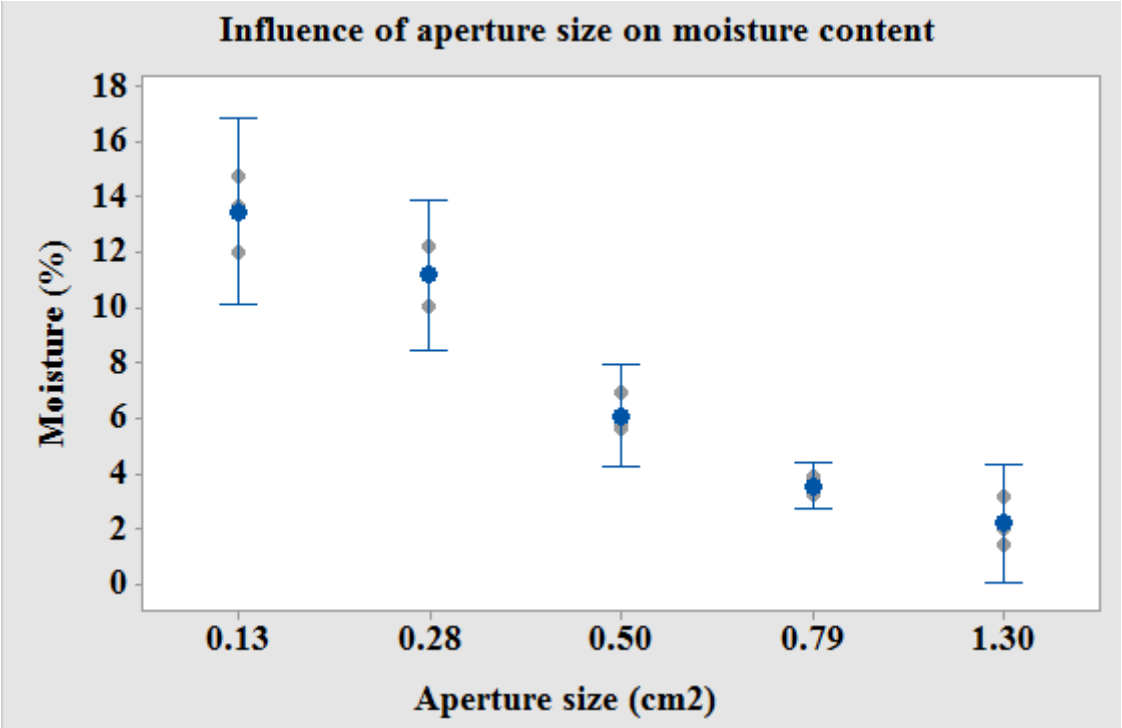


Figure 5-7: Influence of aperture size on residual moisture content for serotype F

Figure 5-8: Influence aperture size on cake morphology

5.2 Laboratory scale lyophilisation design of experiments

5.2.1 Influence of aperture restriction during lyophilisation of serotypes F and K in 1L bottles

In this experiment, bottles of serotype F and K were lyophilised in separate lyophilisation cycles in the Benchmark 500 Freeze Dryer. In each cycle, one of the bottles was lyophilised with a stopper placed in the normal configuration; this was the control bottle.

The other bottle was freeze dried with a restricted aperture cap of size 0.28 cm^2: this was the aperture bottle. Results for serotype F and K are presented in Table 5-6, page 81.

Table 5-6: Product quality of serotype F and K lyophilised with standard stopper and an aperture cap

Serotype	Test article	End of primary drying by PVG	Morphology rank	Degrees lyophilised above collapse temperature (ºC)	Moisture content n=3 (%)	Glass transition temperature n=3 (°C)
F	Control	Completed before transition to secondary drying	2	3	4.18 ± 0.42	56.53±1.58
	Aperture		4	8	10.56 ± 0.60	24.39±0.93
K	Control		2	4	3.41 ± 0.24	54.24±1.00
	Aperture		4	10	6.88 ± 0.57	24.89[7]

5.2.1.1 Influence of aperture restriction during lyophilisation of serotype F

(i) Visual inspection of serotype F

Upon completion of the lyophilisation cycle, the control bottle and aperture bottle were visually inspected using the morphology ranking system. The control bottle cake was opaque to light, with good structure and only slight evidence of micro collapse. This bottle was ranked as morphology 2, see Figure 5-9 , **A**, page 82. The aperture bottle was translucent to light, with evidence of micro collapse and an open pore structure. This bottle was ranked morphology 4; see Figure 5-9, **B**, page 82.

[7] Single testing.

Figure 5-9: Comparison of control bottle, morphology rank 2 (A) and bottle freeze dried with 0.28 cm^2 aperture, morphology rank 4 (B) for serotype F.

(ii) Product temperature and primary drying time of serotype F

From examination of the lyophilisation trend, no shelf temperature or pressure deviations were observed; see Figure 5-10, page 83. The measured product temperature for the control bottle indicated that it was freeze dried 3 ºC above the collapse temperature and the aperture bottle was freeze dried 5 ºC above the collapse temperature. The endpoint of primary drying was assessed by comparative pressure measurement of the Pirani vacuum gauge (PVG) and the capacitance manometer (CM) pressure gauge. The PVG measures the gas composition in the chamber during primary drying i.e. water vapour. The CM reads absolute pressure. The point where the PVG pressure starts to sharply decrease (i.e., onset) and eventually meets the CM indicates that the gas composition in the chamber is changing

from mostly water vapor to nitrogen indicating that sublimation is essentially over (Patel et al., 2010b). Primary drying completed before the transition to secondary drying.

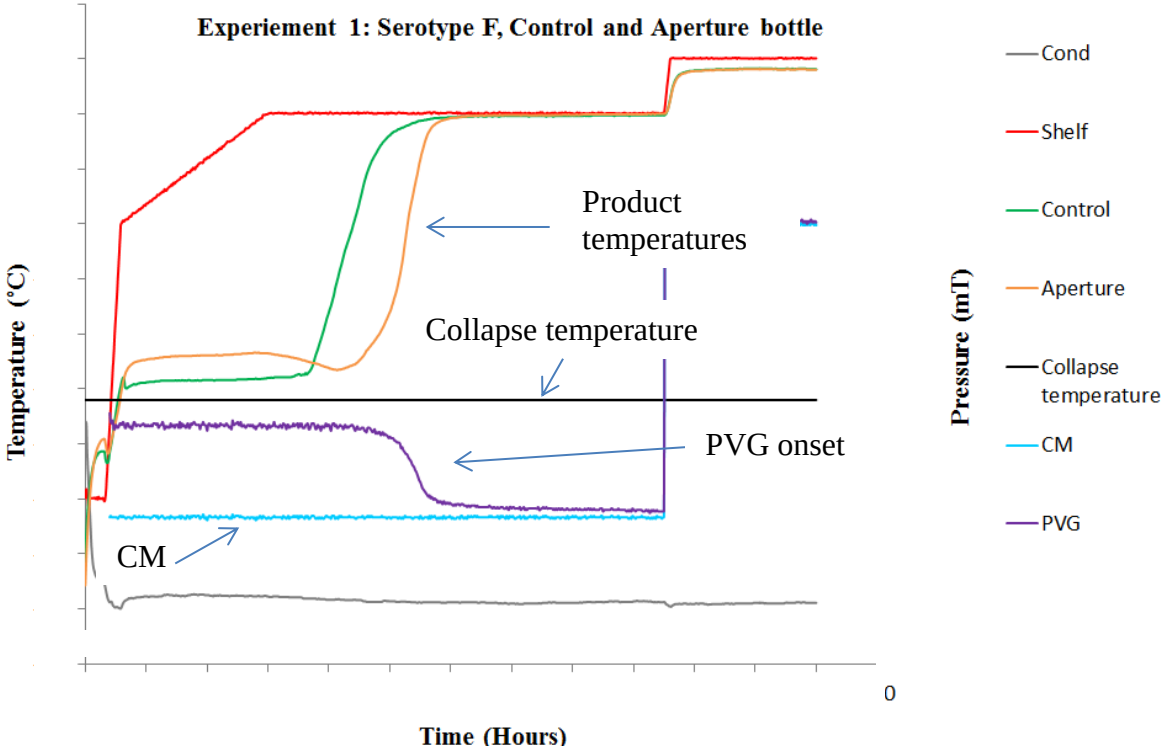


Figure 5-10: Serotype F, control (green trace) and the aperture bottle (orange trace) lyophilised 3 °C and 5 °C above the collapse temperature respectively.

(iii) Moisture content and glass transition temperature of serotype F

The moisture content of the control and aperture bottles was 4.18 ± 0.42% and 10.56± 0.60% respectively. The glass transition, Tg', was detected by analyzing the reversing heat flow (blue line on thermograph); see Figure 5-11, page 84 (the thermograph is representative of both serotype F and serotype K). The limits for measuring the glass transition region were estimated by using the boundaries of the endothermic event in the non-reversing heat flow (enthalpic recovery - brown line on thermograph). Glass transition values are normally calculated as a range, onset, midpoint and endpoint. The midpoint is the most important value, as it is more reproducible and will be used to define Tg in this report. The Tg for the control bottle and aperture bottle were 56.53±1.58 ºC and 24.39±0.93 ºC. The Tg is seen as a step in the heat flow due to the increase in the heat capacity of the sample through the glass transition.

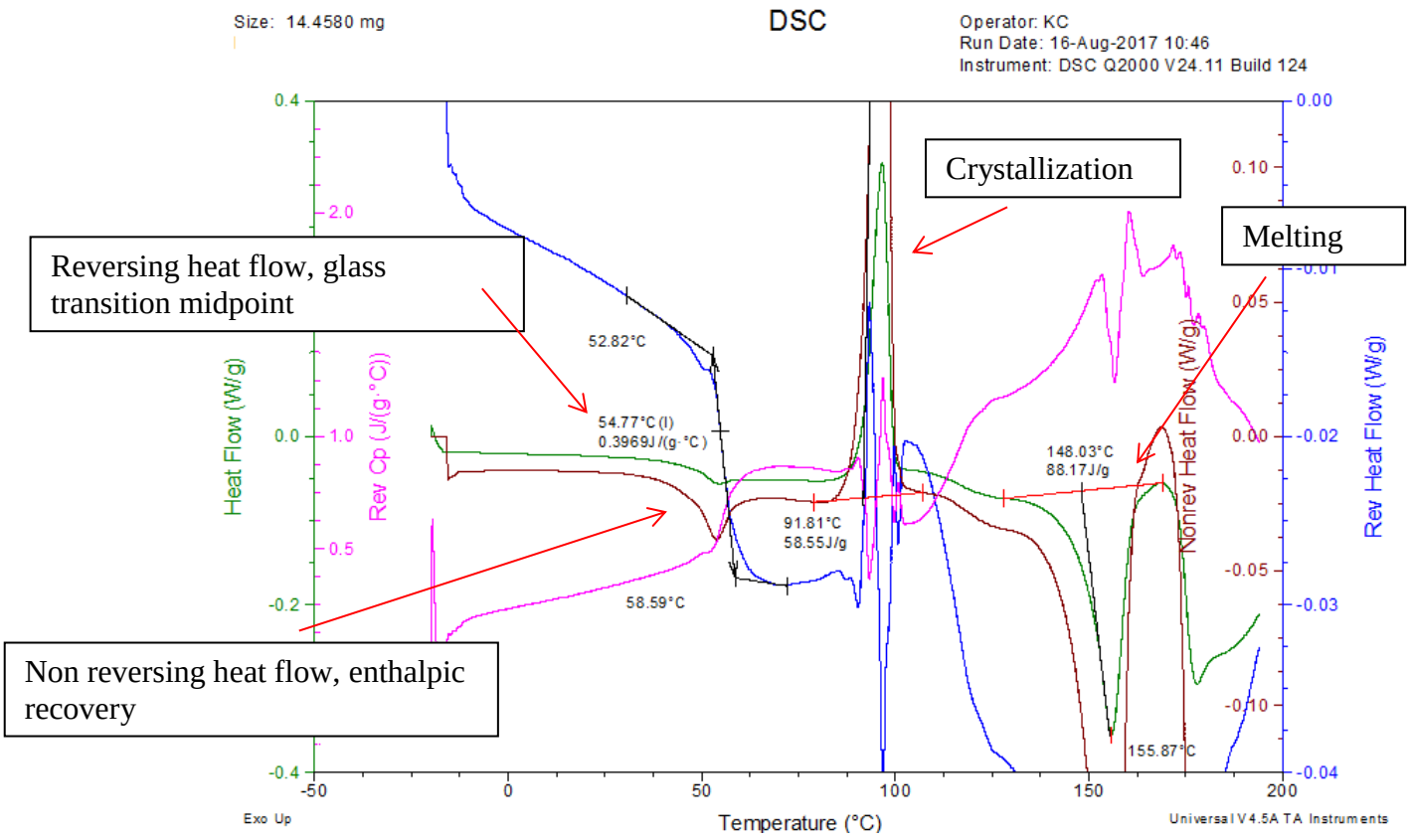


Figure 5-11: mDSC thermograph of serotype F control test article. Glass transition, enthalpic recovery, crystallization and melting events observed

5.2.1.2 Serotype K

(i) Visual inspection of serotype K

Upon completion of the lyophilisation cycle, the control bottle and aperture bottle were visually inspected using the morphology ranking system. The control bottle cake was opaque to light, with good structure and only slight evidence of micro collapse. This bottle was ranked as morphology 2, see Figure 5-12, page 85, **A**. The aperture bottle was translucent to light, showed evidence of micro collapse and had an open pore structure; it was ranked morphology ranking 4; see Figure 5-12, **B**, page 85.

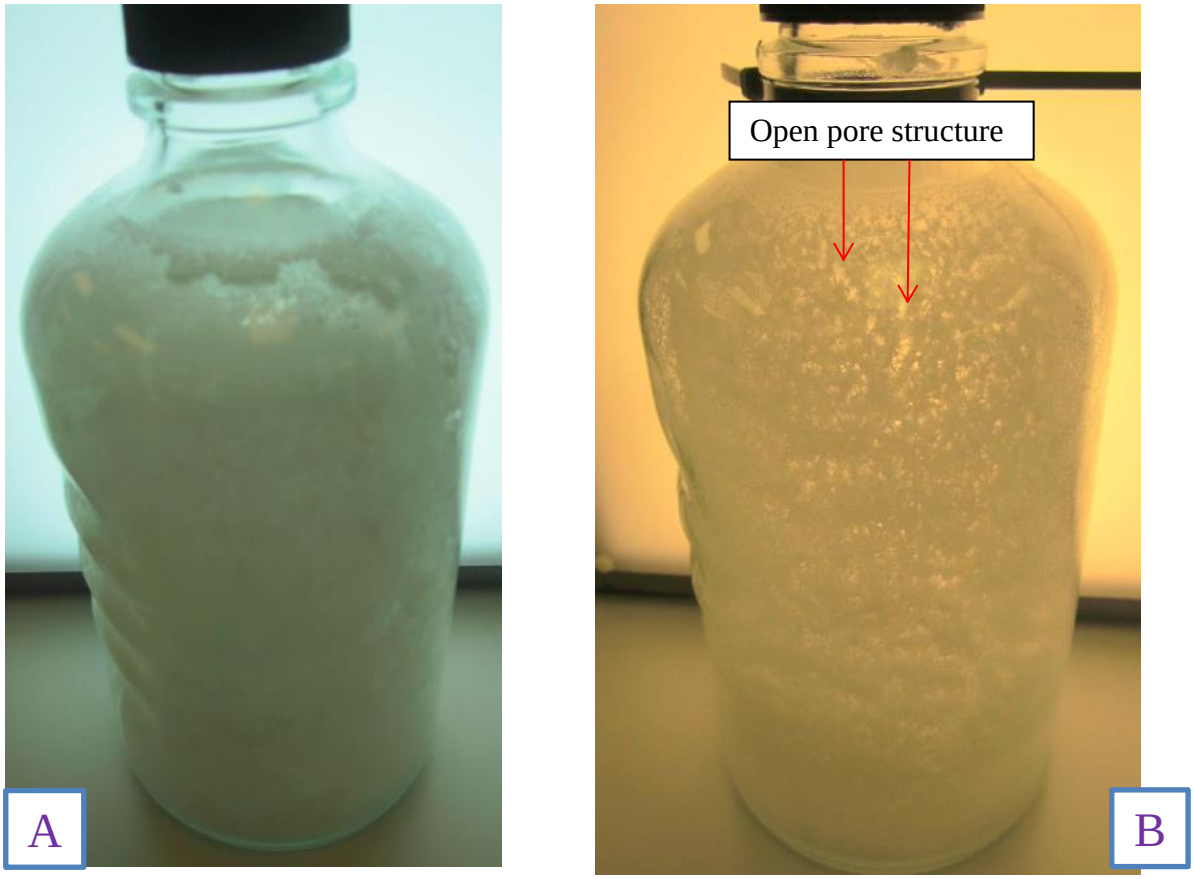


Figure 5-12: Serotype K control bottle, morphology rank 2 (A) and bottle freeze dried with 0.28 cm^2 aperture, morphology rank 4 (B)

(ii) Product temperature and primary drying time of serotype K

From examination of the lyophilisation trend, no shelf temperature or pressure deviations were observed; see Figure 5-13, page 86. The measured product temperature for the control bottle indicated that it was freeze dried 4 ºC above the collapse temperature and the aperture bottle was freeze dried 10 ºC above the collapse temperature. Primary drying had completed before the transition to secondary drying.

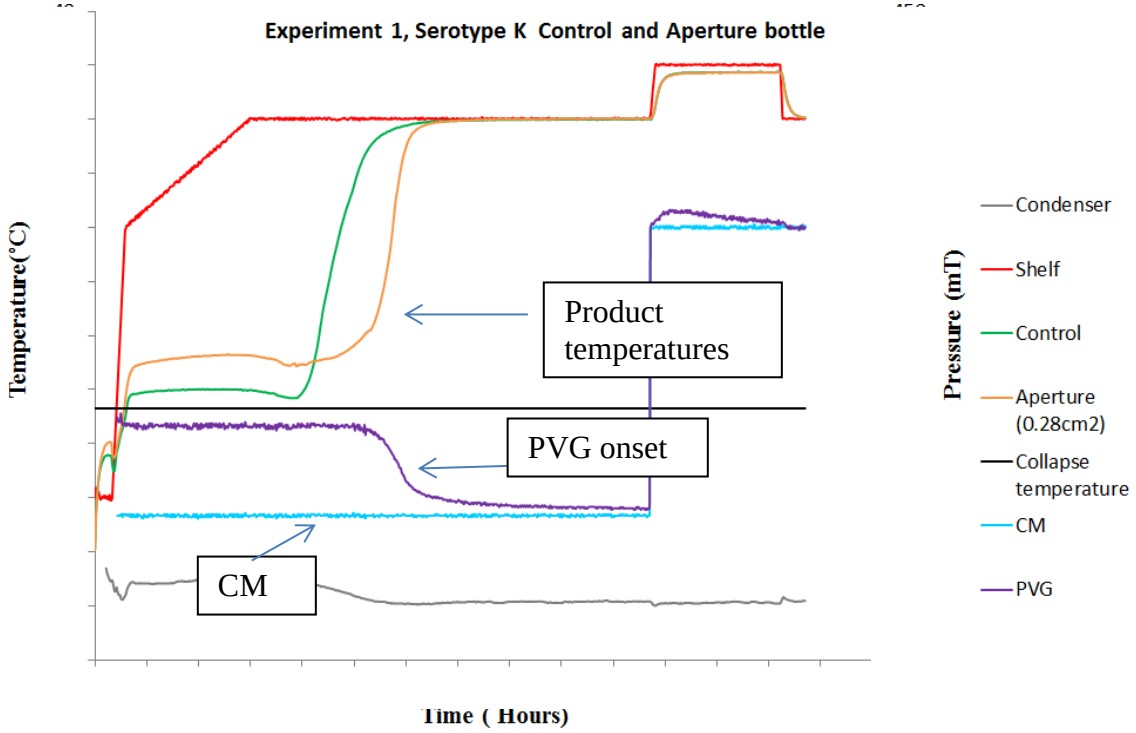


Figure 5-13 : Serotype K, control bottle (green trace) & aperture bottle (orange trace) lyophilised 4 °C and 10 °C above the collapse temperature respectively.

(iii) Moisture content and glass transition temperature of serotype K

The moisture content of the control and aperture bottles was 3.41 ± 0.24% and 6.88 ± 0.57% respectively. The Tg for the control bottle and aperture bottle were 56.53±1.58 ºC and 24.39 ± 0.93 ºC respectively.

It was observed that as product temperature increased due to aperture restriction, appearance worsened and residual moisture increased. The increase in moisture content for the aperture bottles demonstrates that when the product is freeze dried above the collapse temperature, the pores remaining after ice sublimation can collapse in on themselves. This blocks the path for water vapour to escape and the final product has a higher moisture content distributed unevenly through the sample (Adams and Ramsay, 1996).

The bottles of serotype F and K lyophilised with restricted apertures exhibited glass transition temperatures of approximately 24 ºC compared to a glass transition temperature of approximately 55 ºC when lyophilised with a standard stopper. The difference in Tg resulted romf the different levels of moisture in the bottles. Residual water in the cake can promote physical changes in an amorphous material because water acts as a plasticizer; i.e. it reduces the glass transition temperature (Shamblin, 2004). A high residual moisture content and high storage temperature may result in mobility of water which may damage proteins and impact the cosmetic properties of the lyophilised cake (Hancock and Zografi, 1994).

As the control bottles for both serotypes had glass transition temperatures above 50 ºC, a storage temperature of 2-8 ºC may be appropriate. Conversely, for both aperture bottles, the glass transitions were approximately 24º C; therefore, storage at sub ambient temperatures would be recommended, as it is good practice to store a lyophilised material 50 °C below its glass transition temperature (Hancock and Zografi, 1994).

5.2.2 Lyophilisation of serotypes F and K in 50 mL Schott vials above and below the collapse temperature

In these experiments, serotype F and K were lyophilised in 50 mL vials above and below their respective collapse temperatures. The lyophilisation cycles were designed using the primary drying modelling tool, which allowed product temperature and the cake resistance to be calculated as a function of chamber pressure. After lyophilisation, the cakes were inspected and moisture content and glass transition temperature measured.

The results for serotype F lyophilised below and above collapse are presented in Table 5-7, page 88 and

Table 5-8, page 88.

Table 5-7: Product quality of serotype F lyophilised below collapse

Cycle conditions	Appearance	Degrees lyophilised below collapse (°C)	Moisture content n=3 (%)	Glass transition temperature n=3 (°C)	Cake resistance coefficients
Primary drying T_{sh}: X ºC/P_{ch}: Z mT **Secondary drying** XºC for Y hours	Morphology 1	**Measured** Tc1: 5 Tc2: 5 **Calculated from model** 5 °C above the collapse temperature (Acceptance criteria ±1 ºC)	0.58±0.12	74.54±0.67	A:2.32 B:440.28 C:16.99

Table 5-8: Product quality of serotype F lyophilised above collapse

Cycle conditions	Appearance	Degrees lyophilised above collapse (°C)	Moisture content n=3 (%)	Glass transition temperature n=3 (°C)	Cake resistance coefficients
Primary drying T_{sh}: X ºC/P_{ch}: Z mT **Secondary drying** XºC for Y hours	Morphology 2	**Measured** Tc1: 4 Tc2: 4 **Calculated from model** 4 °C above the collapse temperature (Acceptance criteria ±1 ºC)	1.5±0.12	72.12±0.17	A:1.57 B:1.78 C:0.00

(i) Visual inspection of serotype F

Vials lyophilised in a below-collapse cycle exhibited elegant the cake appearance, with little or no evidence of micro collapse (Morphology rank 1); see Figure 5-14, page 89 (A). For vials lyophilised above collapse, the cake was quite elegant with only very slight evidence of micro collapse; see Figure 5-14 (B), page 89.

Figure 5-14: Cake appearance comparison serotype F freeze dried below collapse, (A) and freeze dried above collapse (B).

(ii) Product temperature and primary drying time of serotype F

The lyophilisation cycles executed below and above the collapse temperature were successfully completed; see Figure 5-15, page 90 and
Figure 5-16, page 91 respectively. For the below-collapse cycle, throughout primary drying the product temperature was approximately 5 ºC below the collapse temperature. For the above-collapse cycle, the product temperature was approximately 4 ºC above the collapse temperature. From examination of the PVG in both cycles, primary drying time in the above-collapse cycle was shorter (by a factor of 4.2) than in the below-collapse cycle.

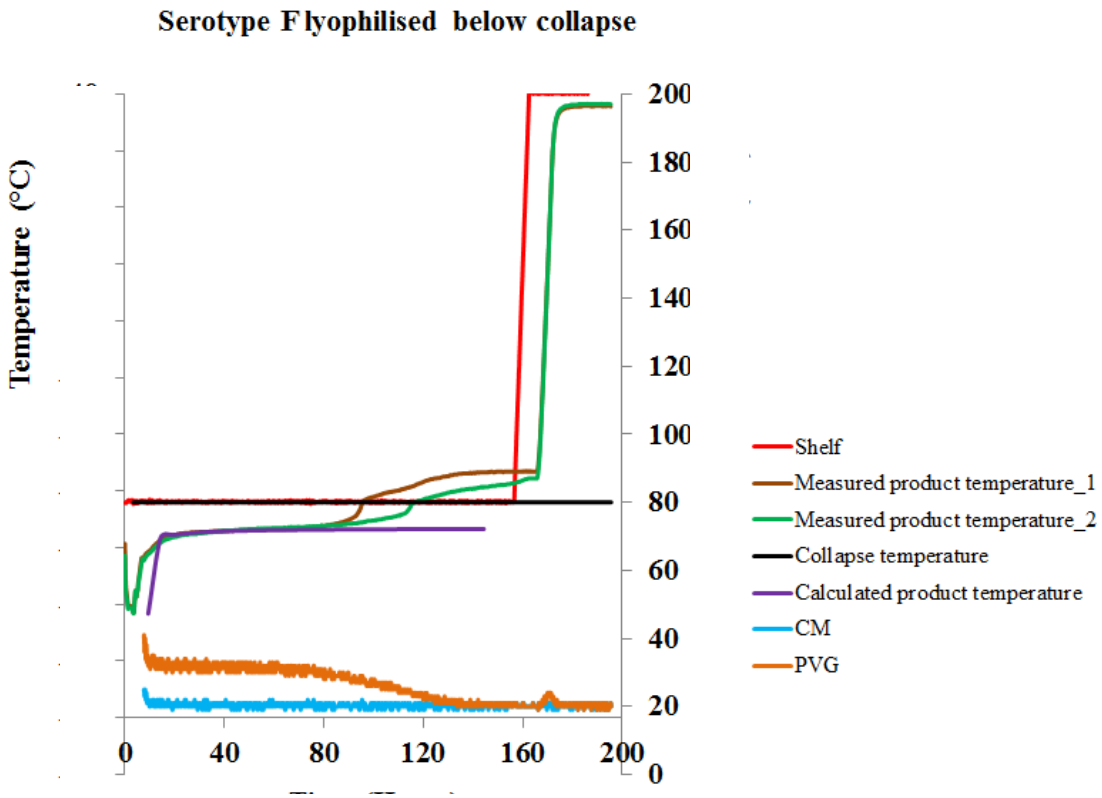


Figure 5-15: Serotype F lyophilised below collapse temperature. Product temperature was approximately 5°C below the collapse temperature during primary drying.

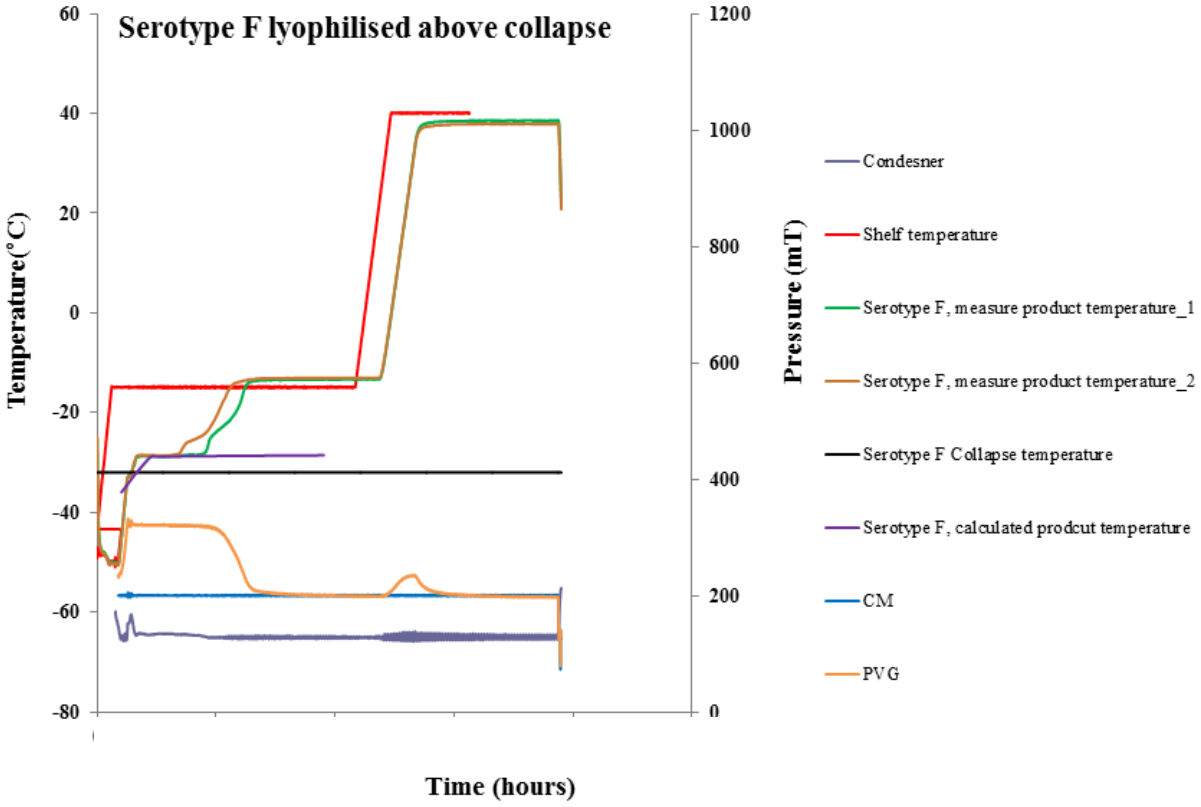


Figure 5-16: Serotype F lyophilised above collapse temperature. Product temperature was approximately 4°C above the collapse temperature during primary drying.

(iii) Moisture content and glass transition temperature of serotype F

The moisture content for the below-collapse cycle was 0.58±0.12% and the glass transition temperature was approximately 74 ºC. The moisture content of the above-collapse cycle was 1.5±0.16%, with a glass transition temperature of approximately 72 ºC.

(iv) Primary drying modelling of serotype F

It was observed in both below- and above-collapse cycles that the calculated and measured product temperatures were within 1 degree, thus validating the primary drying model; see Figure 5-15, page 90 and

Figure 5-16, page 91 respectively (purple trace on both graphs). Use of the model allowed calculation of cake resistance, R, in Torr*hr*cm^2/g as a function of dry layer thickness. The cake resistance measures the resistance of the dried product layer to the mass flow of water vapour formed during sublimation. Equation 5-1, page 92 was used to calculate cake resistance at a given cake layer height, h, in cm. The model outputs coefficients A, B, and C. For the below-collapse cycle, the cake resistance increased as a function of dryer layer

thickness; whereas, for the above-collapse cycle, cake resistance was independent of dry layer thickness; see Figure 5-17, page 92 and Figure 5-18, page 93.

$$R = A + \frac{Bh}{1 + Ch}$$

Equation 5-1: Calculated coefficients A,B and C to calculate cake resistance as a function dry layer thickness

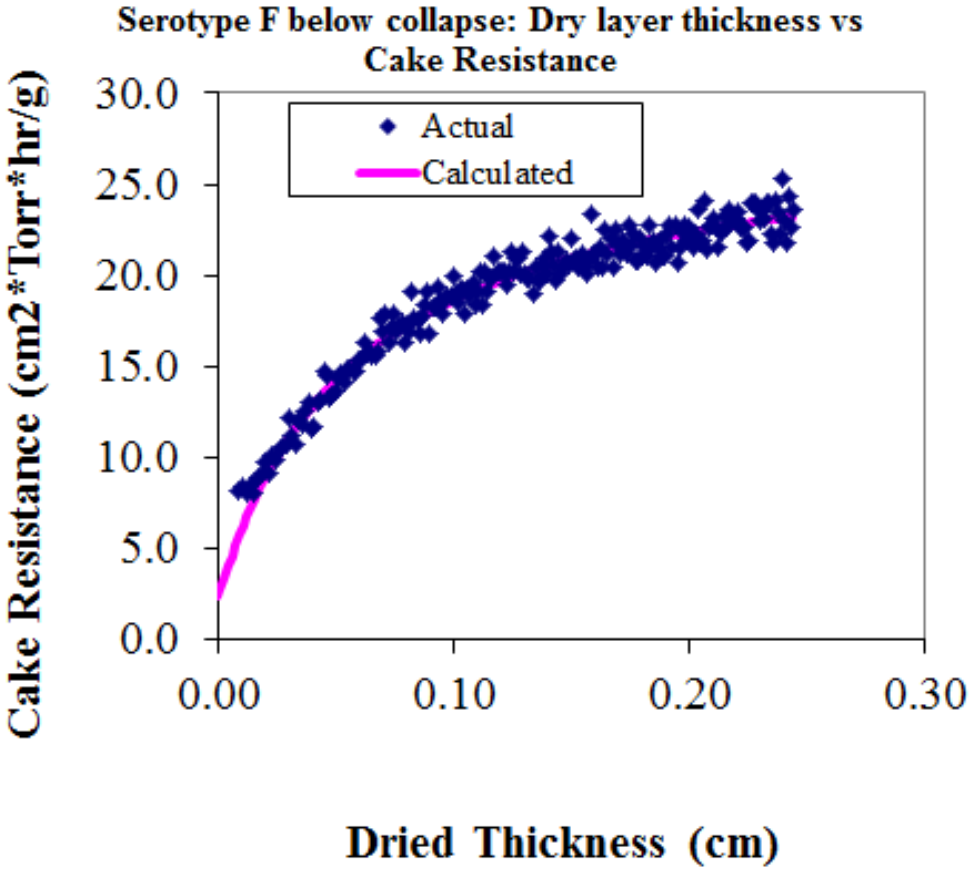


Figure 5-17: Relationship between calculated and actual cake resistance as a function of dried thickness for serotype F, freeze dried below collapse temperature.

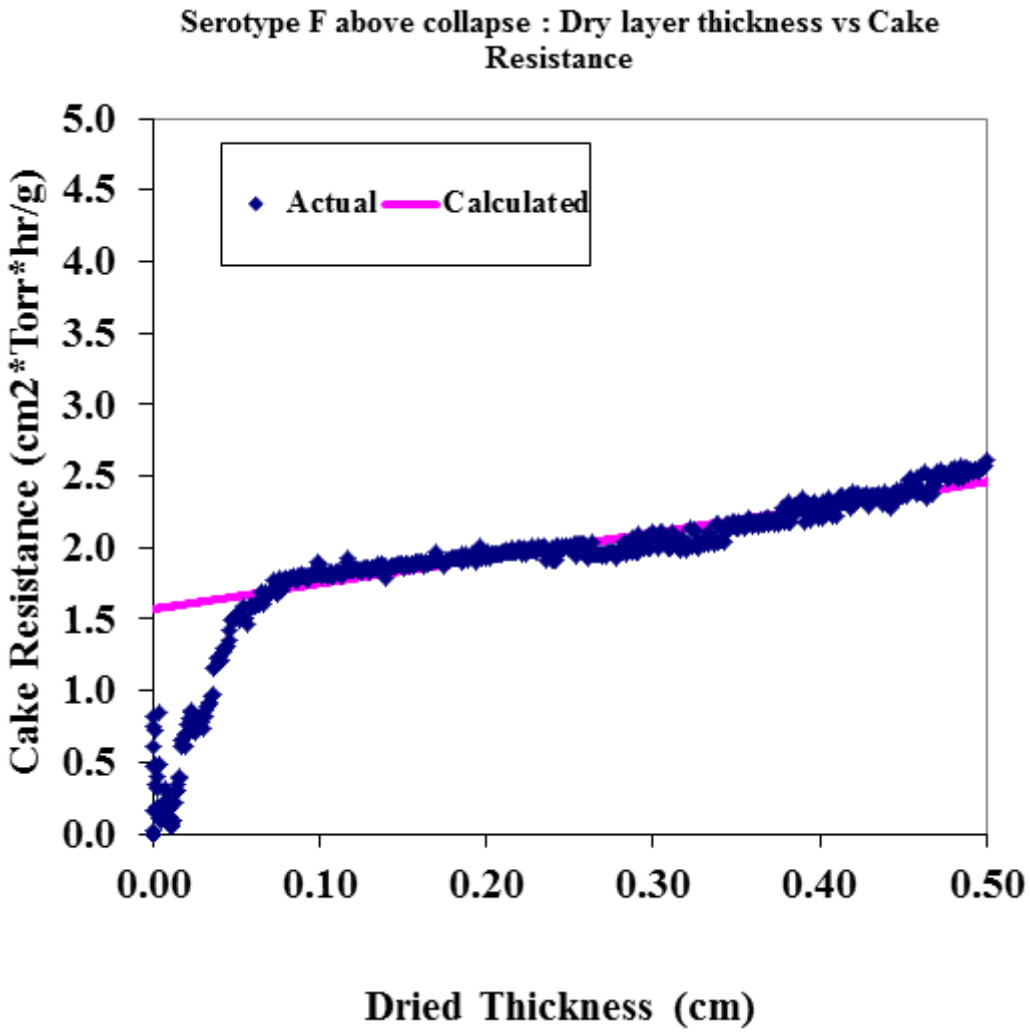


Figure 5-18: Relationship between calculated and actual cake resistance as a function of dried thickness for serotype F, freeze dried above collapse temperature.

5.2.2.1 Serotype K

The results for serotype K lyophilised below and above collapse are presented in Table 5-9, page 94 and Table 5-10, page 94.

Table 5-9: Product quality of serotype K lyophilised below collapse

Cycle conditions	Appearance	Degrees lyophilised below collapse (°C)	Moisture content n=3 (%)	Glass transition temperature n=3 (°C)	Cake resistance coefficients
Primary drying T_{sh}: X ºC/P_{ch}: Z mT **Secondary drying** XºC for Y hours	Morphology 1	**Measured** Tc1: 5 Tc2: 5 **Calculated from model** 5 °C above the collapse temperature (Acceptance criteria ±1 ºC)	0.64±0.04	68.70±0.74	A:5.54 B:63.35 C:8.54

Table 5-10: Product quality of serotype K lyophilised above collapse

Cycle conditions	Appearance	Degrees lyophilised above collapse (°C)	Moisture content n=3 (%)	Glass transition temperature n=3 (°C)	Cake resistance coefficients
Primary drying T_{sh}: X ºC/P_{ch}: Z mT **Secondary drying** XºC for Y hours	Morphology 3	**Measured** Tc1: 5 Tc2: 5 **Calculated from model** 5 °C above the collapse temperature (Acceptance criteria ±1 ºC)	3.36±0.27	60.95±0.35	A:0.74 B:4.34 C:0.00

(i) Visual inspection of serotype K

Vials lyophilised in a below-collapse cycle exhibited elegant cake appearance, with little or no evidence of micro collapse (Morphology rank 1); see Figure 5-19 (A), page 95. For vials lyophilised above collapse, evidence of micro-collapse was observed (Morphology rank 3) and a "bubbling" effect on the surface of the cake was observed; see Figure 5-19 (B), page 95.

Figure 5-19: Cake appearance comparison of serotype K freeze dried below collapse, (A) and freeze dried above collapse (B).

(i) Product temperature and primary drying time serotype K

The lyophilisation cycles executed below and above the collapse temperature were successfully completed, see Figure 5-20, page 97, and Figure 5-21, page 98 respectively. For the below-collapse cycle, the product temperature was approximately 5 ºC below the collapse temperature throughout primary drying. For the above-collapse cycle, the product temperature was approximately 4 ºC above the collapse temperature. From examination of the PVG in both cycles, primary drying time in the above-collapse cycle was 4-fold shorter than in the below-collapse cycle.

(ii) Moisture content and glass transition temperature of serotype K

The moisture content for the below-collapse cycle was 0.64±0.04% with a glass transition temperature of approximately 69 ºC. The moisture content of the above-collapse cycle was 3.36±0.27% and its glass transition temperature was approximately 61ºC.

(iii) Primary drying modelling of serotype K

It was observed in both below- and above-collapse cycles that the calculated and measured product temperatures were within 1 degree, thus validating the primary drying model; see Figure 5-20, page 97 and Figure 5-21, page 98 (calculated temperature is the purple trace on both graphs). Use of the model allowed calculation of cake resistance, R, in Torr*hr*cm^2/g as a function of dry layer thickness For the below-collapse cycle, the cake resistance increased as a function of dryer layer thickness; whereas, for the above-collapse-cycle, cake resistance was independent of dry layer thickness; see Figure 5-22, page 99 and Figure 5-23, page 100.

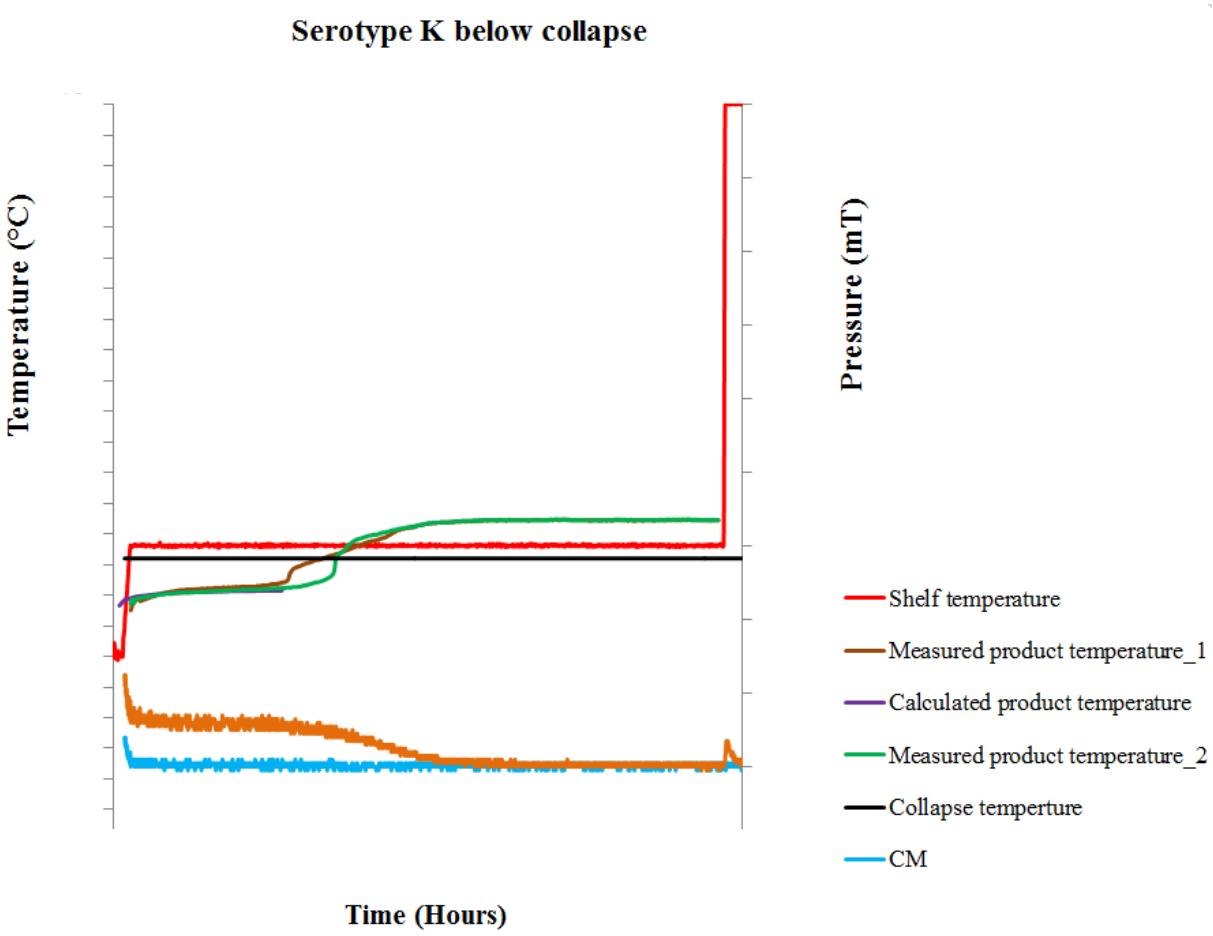


Figure 5-20: Serotype K lyophilised below collapse temperature. Product temperature was approximately 5 °C below the collapse temperature during primary drying.

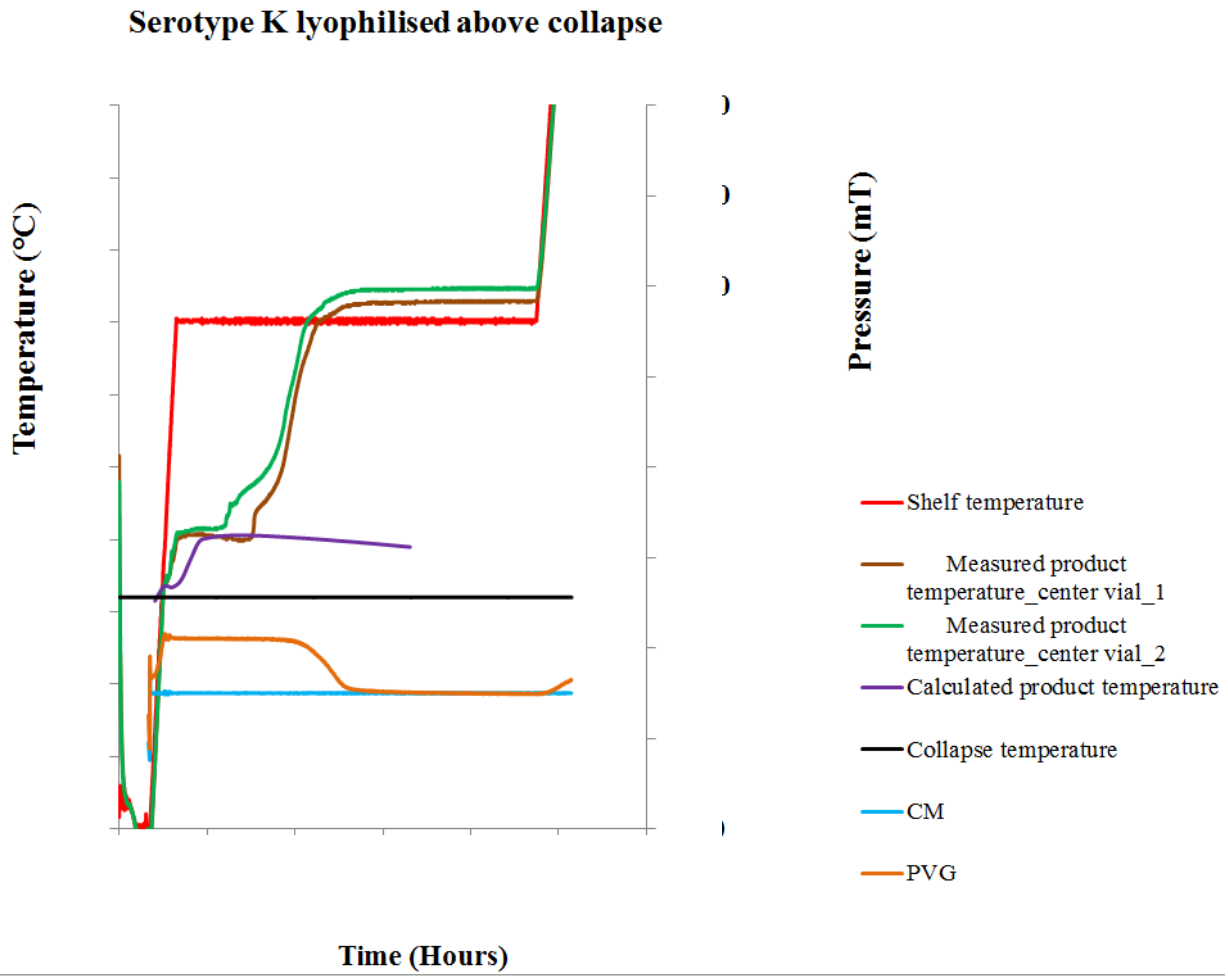


Figure 5-21: Serotype K lyophilised below collapse temperature. Product temperature was approximately 5 °C above the collapse temperature during primary drying.

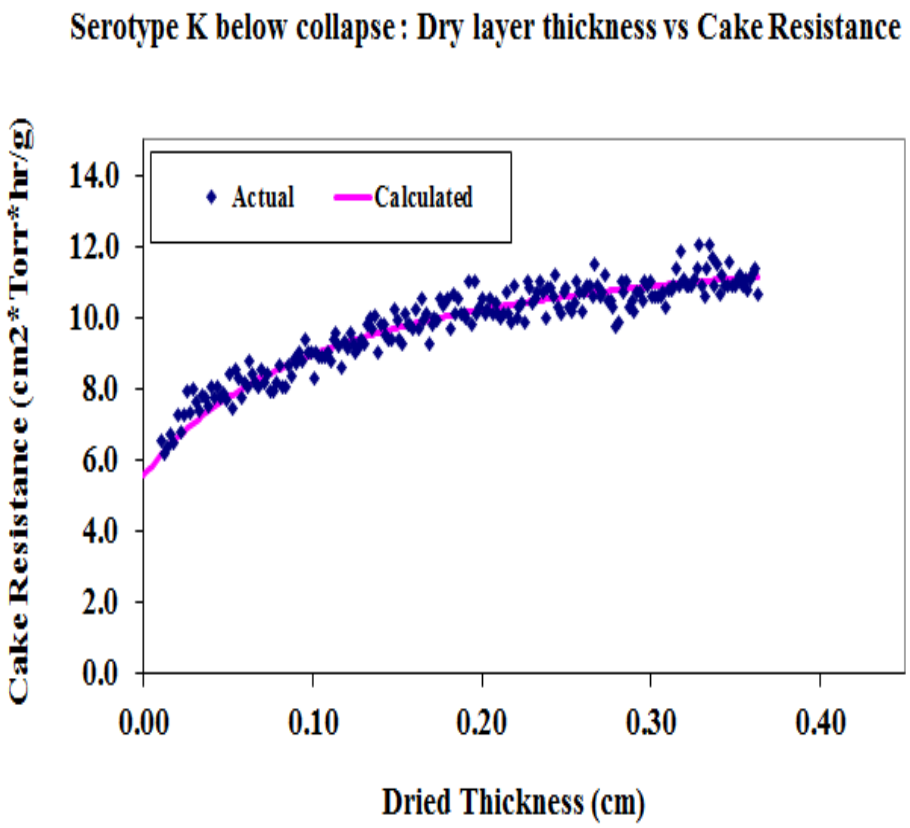


Figure 5-22: Relationship between calculated and actual cake resistance as a function of dried thickness for serotype K, freeze dried below collapse temperature

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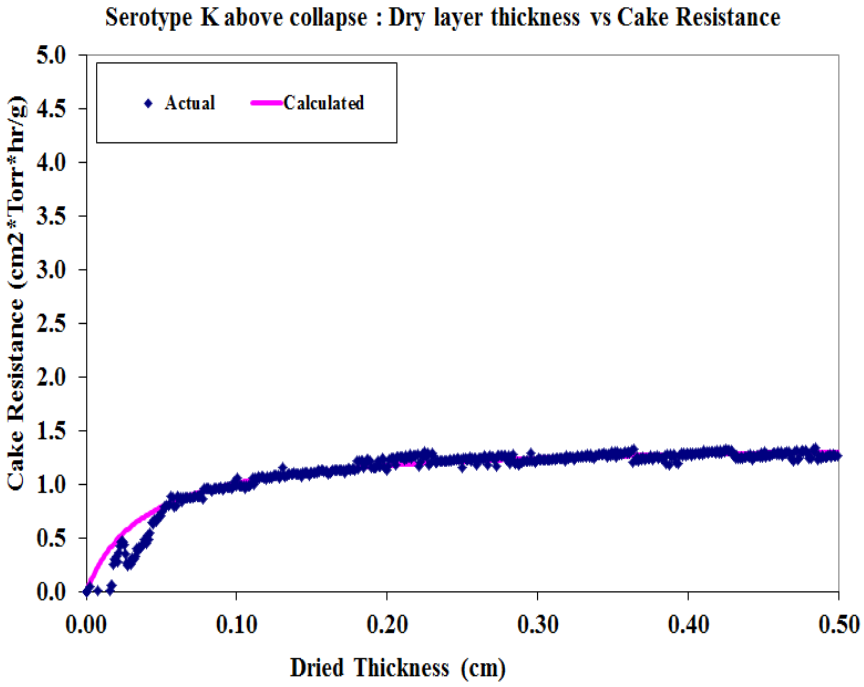

Figure 5-23: Relationship between calculated and actual cake resistance as a function of dried thickness for serotype K, freeze dried above collapse temperature

The two primary drying shelf temperatures and pressures employed to lyophilise serotypes F and K resulted in product temperatures being either above or below the collapse temperature. Sublimation rates were higher in the above-collapse cycle compared to the below-collapse cycle. For the below-collapse cycle, the resistance values increased monotonically as a function of dry layer thickness - but for the above-collapse cycle cake resistance was independent of dry layer thickness (Milton et al., 1997).

The morphology of the cakes lyophilised above collapse exhibited a more open pore structure compared to those lyophilised below collapse (Overcashier et al., 1999). This was most evident in serotype K, which was co-lyophilised with Protein X. Here, a “bubble” effect was observed on the cake. During freezing, concentration of the solutes can result in the formation of small air bubbles in the freeze concentrate and exceeding the temperature of the onset of collapse can result in expansion of these air bubbles. If the bubbles are physically stable enough to withstand further drying, the remnants of these bubbles are observed in the final product (Patel et al., 2017).

Primary drying time was significantly reduced when lyophilisation was conducted above collapse (approximately a 4-fold reduction in cycle time), as the open pore structure of the micro-collapsed state reduced the tortuosity of the path that the water vapour had to take in order to navigate through the dried material (Lewis et al., 2010, Parker et al., 2010). The residual moisture of the cakes lyophilised below collapse was lower than their above-collapse counterparts. For serotype F, which was discreetly lyophilised with the amorphous excipient, the moisture content of the above-collapse vials was 1.59 times higher compared to their below-collapse counterparts (0.58% and 1.50 %). The difference in moisture contents did not appear to have any impact on the glass transition temperature, as both conditions produced product with similar values (74 ºC and 72 ºC) and this Tg should allow storage at 2-8 ºC.

For serotype K, co-lyophilised with protein X, the moisture content of the above-collapse vials was 4.26 times higher compared to their below-collapse counterparts (0.64% and 3.36%) and the cake appearance was significantly worse than their below-collapse counterparts. The higher moisture in both above-collapse test articles is due to water vapour becoming trapped in the pores as they partially collapse, clogging the paths that allow vapour to escape (Adams and Ramsay, 1996) .

The impact of the plasticising effect of water within the above-collapse product was evident, as the glass transition temperature was lowered by 8 ºC compared to its below-collapse counterpart (61 ºC compared to 69 ºC) .It would seem logical that, if lyophilisation cycle conditions were more aggressive, the cake appearance would be worse (higher degree of micro collapse) and moisture content would be higher, reducing Tg even further. It is for this reason that it is very important to determine the glass transition temperature of a lyophilised product, as there could be stability implications if a product was stored at a temperature near or above Tg (te Booy et al., 1992).

The significant reduction in lyophilisation cycle times realized by drying at higher temperatures, can lead to substantial improvements in process efficiency and could result in reduced manufacturing costs at commercial scale (Depaz et al., 2016). Demonstrating proof of concept for freeze drying above the collapse temperature at laboratory scale is a pre-requisite before transferring a process to a commercial scale freeze dryer. As well as assessing appearance and moisture content, biochemical properties must be assessed and stability data at elevated temperatures for at least six months should be generated (Schersch et al., 2010, Schersch et al., 2012).

Another important factor to consider is the capability of the freeze dryer to withstand higher sublimation rates as a result of freeze drying above collapse. If the connection pathway between the freeze-drying chamber and the condenser is too small to handle the high rate of sublimation, overloading can occur (Tang and Pikal, 2004). High sublimation rates can lead to a phenomenon known as “choked flow”. This is where the required mass transfer through the duct connecting the drying and condenser chamber cannot be maintained, causing a loss of pressure control (Searles, 2004). If a more aggressive cycle is transferred to a commercial scale freeze dryer without considering these factors, product temperature could go out of control, resulting in complete collapse or melt back and ultimately could result in the loss of a batch (Patel et al., 2010a) .

5.3 Conjugation of lyophilised serotypes F and K above and below their collapse temperature

On three independent occasions, serotype F and serotype K were each conjugated to protein X. For each serotype, on each conjugation occasion, three test articles were conjugated: (i) a control bottle, (ii) an aperture bottle (both lyophilised at various degrees above the collapse temperature as described in 4.2.1, page 51) and (iii) vials lyophilised below collapse temperature (as described in section 4.2.2, page 52).

Once the conjugation reaction for both serotypes was completed, each test article was purified by NFF followed by TFF purification. These purification techniques are the validated methods used in the commercial manufacturing process. It is important to discuss the influence that TFF may have on product quality attributes. During processing in the lab, variability was observed during TFF compared to the manufacturing norms for pressure control. This may be attributed to the lack of a feed flow meter and variability in retentate volume, as the laboratory TFF system requires constant manual interventions. It must be noted that the filter membrane lot and specific porosimetry may also contribute to differences between scales. Factors such as drug adsorption on to the filter, shear stress, and purification equipment performance can also impact product quality attributes (Werner and Winter, 2015).

A separate side stream was also purified by dialysis filtration. The utility of performing dialysis is that it gives an indication of the success of the conjugation reaction independent of the NFF and TFF purification steps. The dialysis samples were taken at the very end of the conjugation process directly from the Chem glass reactor. In dialysis only molecules that are small enough to fit through the membrane pores are able move through the membrane, whilst the product of interest is retained and associated starting polysaccharides. It is important to state that for all quality attributes, there are no validated specifications in place for purification by dialysis. It would be expected that, because unreacted polysaccharide is retained in the dialysis membrane, the levels of SPR and unconjugated saccharide will be higher compared to their TFF counterparts.

Product quality was assessed using a range of assays; see Figure 5-24, page 104. A one way analysis of variance (ANOVA) was used to determine if there was a statistical difference between the means obtained from a given conjugation quality attribute as a function of lyophilisation conditions**.**

5.3.1 Conjugation product quality

Product quality of the serotype F and K test articles was assessed using a number of physiochemical assays. The most important quality attributes used to assess conjugation efficiency are (i) saccharide: protein ratio (SPR), (ii) the molecular weight of the conjugate (iii) the molecular size distribution of the conjugate and (iv) the level of free or unconjugated saccharide (Jones, 2005b). The aim was to determine if lyophilisation cycle conditions impacted on conjugation product quality attributes.

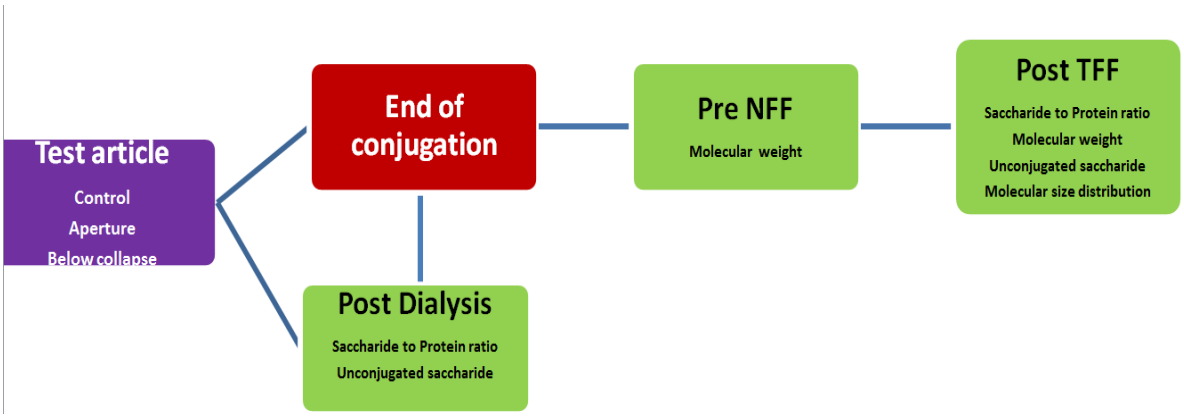

Figure 5-24: Sample points and assays used to assess product quality across the purification process

5.3.1.1 Serotype F Conjugate Product Quality

(i) Saccharide: Protein ratio (SPR) of serotype F

The success of the chemical conjugation of polysaccharide to the protein is measured by the saccharide: protein ratio (SPR). The SPR should be within the approved range for that particular conjugate as it can impact immunogenicity of a vaccine (Anderson et al., 1989). The SPR is calculated from the ratio the polysaccharide and protein content. The contents are measured using two independent assays and the ratio reflects the relative proportions of the polysaccharide and protein that reacted to create the conjugate (Ravenscroft et al., 2015). For serotype F, the SPR results for TFF and dialysis purification are presented in Table 5-11, page 105.

Table 5-11: Saccharide: Protein ratio ,(SPR) serotype F, post TFF purification and post dialysis purification

Conjugation Occasion	Test article	Post TFF SPR (Spec:10-22)	Post Dialysis SPR (Spec: No specification)
001	Control	11.16	15.31
	Aperture	12.32	15.88
	Below collapse	12.71	15.60
002	Control	10.66	15.81
	Aperture	11.42	16.71
	Below collapse	11.78	16.94
003	Control	11.31	16.91
	Aperture	10.88	16.30
	Below collapse	10.00	14.62
Average and standard deviation	Control	11.04±0.34	16.01±0.82
	Aperture	11.54±0.72	16.30±0.41
	Below collapse	11.45±1.45	15.72±1.16
ANOVA	P value: Test article	0.75	0.75
	P value: Conjugation occasion	0.53	0.23

The test articles purified by TFF were within specification. The means and confidence intervals calculated across the three conjugation occasions for each test article were similar and the p values of both conjugation occasion and the test articles were ≥0.05. There was no statistical difference between the SPR means of the test articles purified by TFF. The ratios obtained across each conjugation occasion were comparable to each other; with the

exception of the below-collapse test article in conjugation occasion 3. The SPR of this test article was just on the lower end of the specification range; see Figure 5-25, page 106.

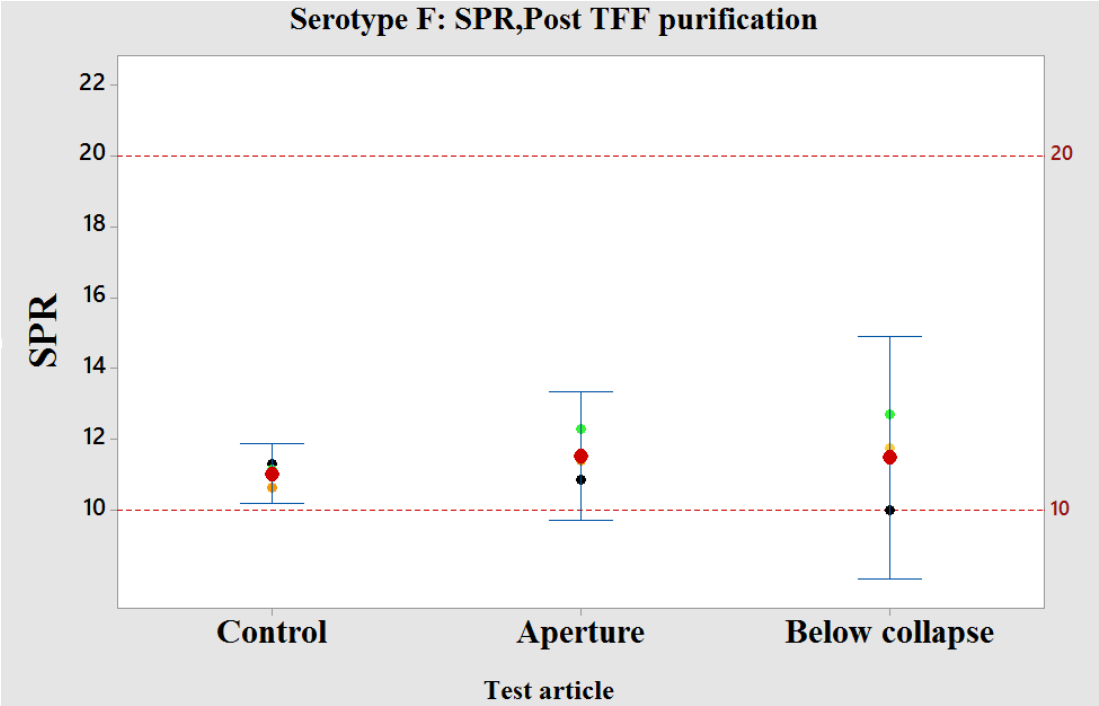

Figure 5-25: SPR Serotype F, TFF purification. Conjugation occasion 1 green dots, conjugation occasion 2 orange dots, and conjugation occasion 3 black dots. The mean is represented by red dots and the confidence intervals are represented by the blue lines.

For the test articles purified by dialysis, the means and confidence intervals calculated across the three conjugation occasions for each test article were similar and the p values of both conjugation occasion and the test articles were ≥0.05. There was no statistical difference between the SPR means of the test articles purified by dialysis. The SPRs of all dialysis test articles were comparable to each other with the exception of the below-collapse test article in conjugation 3. Similarly to the TFF purification, the SPR on this occasion below-collapse was lower compared to conjugation occasions 1 and 2; see Figure 5-26, page 107. The most likely explanation for this is variability associated with the conjugation process itself, or it could be due to assay variability. It must be remembered that the SPR result is a combination of the polysaccharide and protein contents measured using two independent assays and each assay has its own inherent variability.

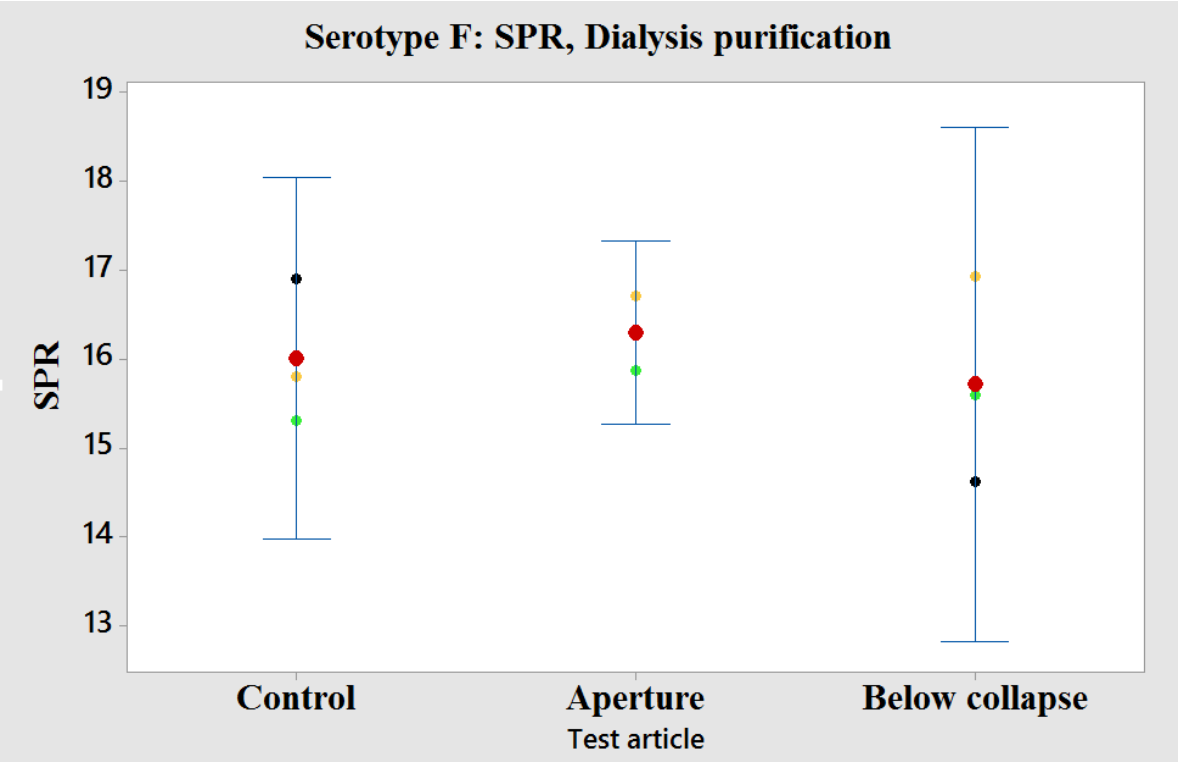

Figure 5-26: SPR Serotype F, Dialysis purification. Conjugation occasion 1 green dots, conjugation occasion 2 orange dots, and conjugation occasion 3 black dots. The mean is represented by red dots and the confidence intervals are represented by the blue lines.

(ii) Unconjugated Saccharide of serotype F

The level of unconjugated saccharide is the amount of saccharide that is not covalently linked to the protein carrier. Unconjugated saccharide can be present as a process residual after the purification of the glycoconjugate, but can also be formed during storage as a result of hydrolysis of the glycosidic linkages (Costantino et al., 2011). It is a very important release and stability quality attribute because unconjugated polysaccharide reduces the immunogenicity of the vaccine, possibly by competing for the antibody molecules (Peeters et al., 1992). On stability, degradation of the saccharide can be monitored by an increase in unconjugated saccharide levels (Jones, 2005a). For most conjugates, the amount of unconjugated saccharide will increase over time and is often the determining factor in setting shelf life limits (Frasch, 2009). There are a number of methods used to determine the level of unconjugated saccharide levels in conjugate vaccines, including but not limited to electrophoresis, ion exchange chromatography, hydrophobic interaction chromatography and colorimetric methods (Giannelli et al., 2017).

The unconjugated saccharide results for serotype F from TFF and dialysis purification are presented in Table 5-12, page 108.

Table 5-12: Unconjugated saccharide serotype F, post TFF purification and post dialysis purification

Conjugation run	Test article	Post TFF Unconjugated Saccharide (%) (Spec: ≤10%)	Post Dialysis Unconjugated Saccharide (%) (Spec: ≤10%)
001	Control	6.87	22.22
	Aperture	3.34	17.11
	Below collapse	3.57	21.16
002	Control	2.18	14.95
	Aperture	2.59	23.28
	Below collapse	3.13	22.95
003	Control	4.35	23.28
	Aperture	6.78	21.57
	Below collapse	6.67	10.50
Average and standard deviation	Control	4.46±2.35	20.15±4.53
	Aperture	4.23±2.24	20.65±3.19
	Below collapse	4.45±1.93	18.20±6.73
ANOVA	P value: Test article	0.98	0.88
	P value: Conjugation occasion	0.18	0.91

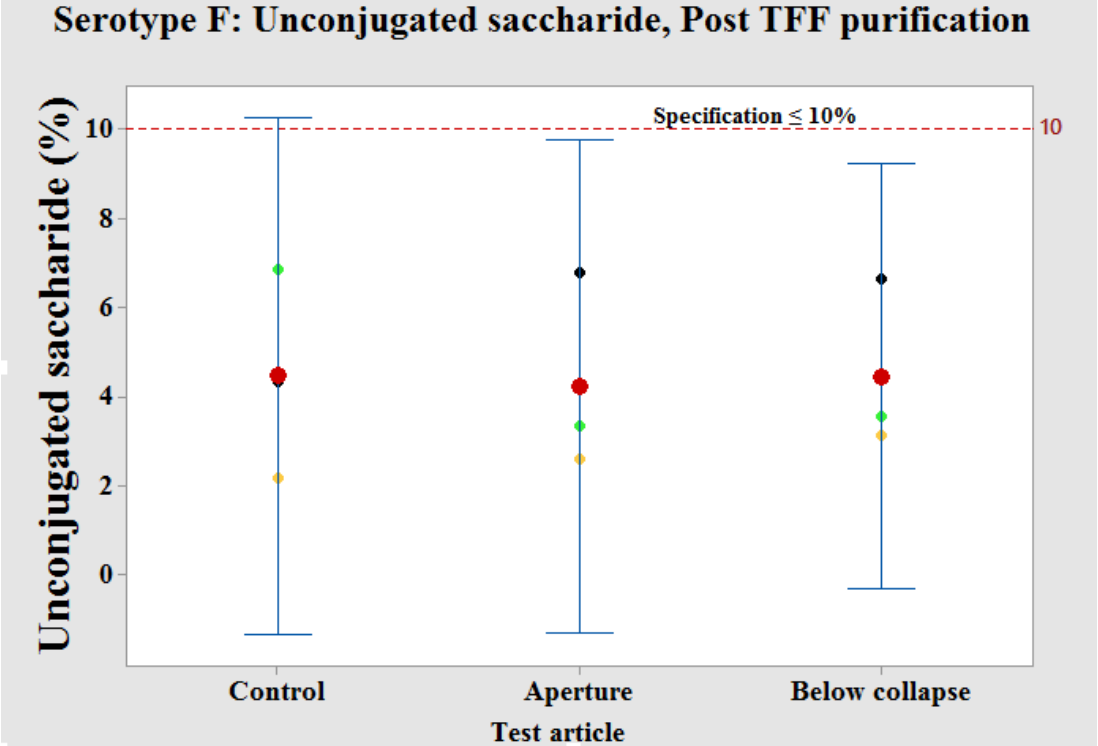

Figure 5-27: Unconjugated saccharide, serotype F, TFF purification. Conjugation occasion 1 green dots, conjugation occasion 2 orange dots, and conjugation occasion 3 black dots. The mean is represented by red dots and the confidence intervals are represented by the blue lines.

At the end of TFF purification, the unconjugated saccharide levels of all serotype F test articles were within specification and comparable to each other; see Figure 5-27, page 109. The means and confidence intervals calculated across the three conjugation occasions for each test article were similar and the p values of both conjugation occasion and the test articles were >0.05. There is no statistical difference between the unconjugated saccharide means of the test articles.

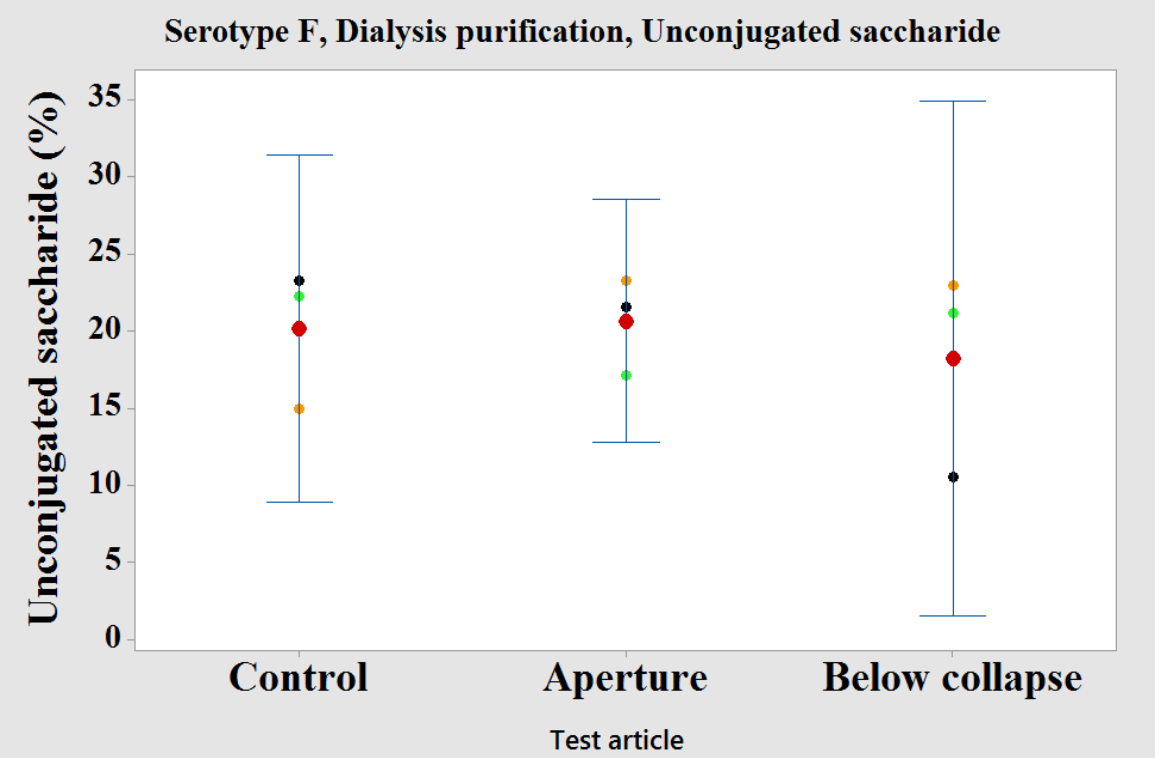

Figure 5-28: Unconjugated saccharide, serotype F, dialysis purification. Conjugation occasion 1 green dots, conjugation occasion 2 orange dots, and conjugation occasion 3 black dots. The mean is represented by red dots and the confidence intervals are represented by the blue lines.

For the test articles purified by dialysis, the means and confidence intervals calculated across the three conjugation occasions for each test article were similar and the p values of both conjugation occasion and the test articles were >0.05; see Figure 5-28, page 110. There was no statistical difference between the unconjugated saccharide means of the test articles. The higher unconjugated saccharide levels by dialysis purification is explained by the fact that unreacted polysaccharide, which is removed during the TFF purification, was retained in the dialysis tube, thus artificially inflating the level of free saccharide. However, similarly to purification by TFF, the unconjugated saccharide levels across all test articles and across all conjugation occasions were comparable. The dialysis purification results therefore confirmed the effectiveness of the conjugation reaction.

(iii) Molecular weight of serotype F

Determination of the molecular weight of conjugates is challenging because polysaccharides are composed of repeated monosaccharides linked together by glycosidic linkages of different chain lengths (Pelley, 2012). Therefore the polysaccharide is essentially a polymer, a large molecule which is formed using a repeating subunit called a monomer. Polymers do not a have a single molecular weight and in a given sample there may be polymer chains which contain different numbers of repeat units with different molecular weights (Pearce, 1992). The molecular weight of polymers is reported as averages and these averages are used to describe the distributions of polymer chain molecular weights (Pearce, 1992). The molecular weight of the conjugates was evaluated using a SEC liquid chromatography method with light scattering detection. Using this technique, a number of different molecular weight averages can be calculated. In this study the weight average molecular weight was used (Mw) (Wyatt, 1993). Mw is a weighted average which favours higher molecular weight molecules.

The molecular weight of both serotype F and serotype K conjugates was determined before NFF and after TFF purification; see Table 5-13, page 112. It was observed for both conjugates that the molecular weight of all test articles appeared to increase as they were purified. At first glance this makes little sense, because non target molecules such as unreacted polysaccharide, protein and residual solvents are removed during TFF, and it would be expected that the weight of the conjugate would be lower at the end of the purification process. The explanation for this is that the crude conjugate is composed of higher and lower molecular weight polysaccharide chains; i.e. it is polydisperse. Before TFF there are a greater number of smaller/ lower molecular weight chains which are quantified. As the TFF purification progresses, these lower molecular weight chains are removed, leaving higher molecular weight chains which are then quantified.

Table 5-13: Molecular weight serotype F, Pre NFF filtration and post TFF purification

Conjugation run	Test article	Pre NFF Mw (kDa)	Post TFF Mw (kDa)
001	Control	6.42	11.57
	Aperture	4.87	8.44
	Below collapse	4.44	8.67
002	Control	4.41	10.62
	Aperture	4.09	11.05
	Below collapse	4.28	12.77
003	Control	5.79	12.44
	Aperture	5.53	16.04
	Below collapse	5.50	11.64
Average and standard deviation	Control	5.54±1.03	11.54±0.91
	Aperture	4.83±0.72	11.84±3.86
	Below collapse	4.74±0.66	11.02±2.12
ANOVA	P value: Test article	0.24	0.90
	P value: Conjugation occasion	0.08	0.21

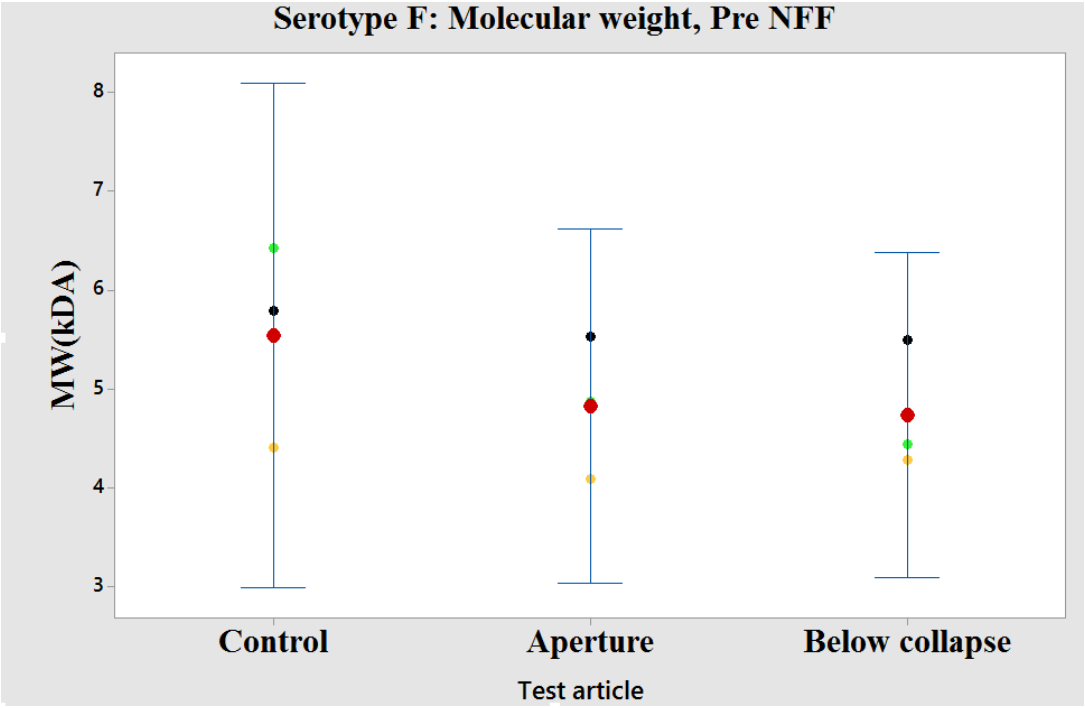

Figure 5-29: Molecular weight serotype F, pre NFF. Conjugation occasion 1 green dots, conjugation occasion 2 orange dots, and conjugation occasion 3 black dots. The mean is represented by red dots and the confidence intervals are represented by the blue lines.

The molecular weights at the pre NFF filtration stage across all conjugation occasions, regardless of the lyophilisation conditions used were comparable to each other; see Figure 5-29, page 113. The means and confidence intervals calculated across the three conjugation occasions for each test article were similar and the p values of both conjugation occasion and the test articles were >0.05. There was no statistical difference between the molecular weight means of the test articles.

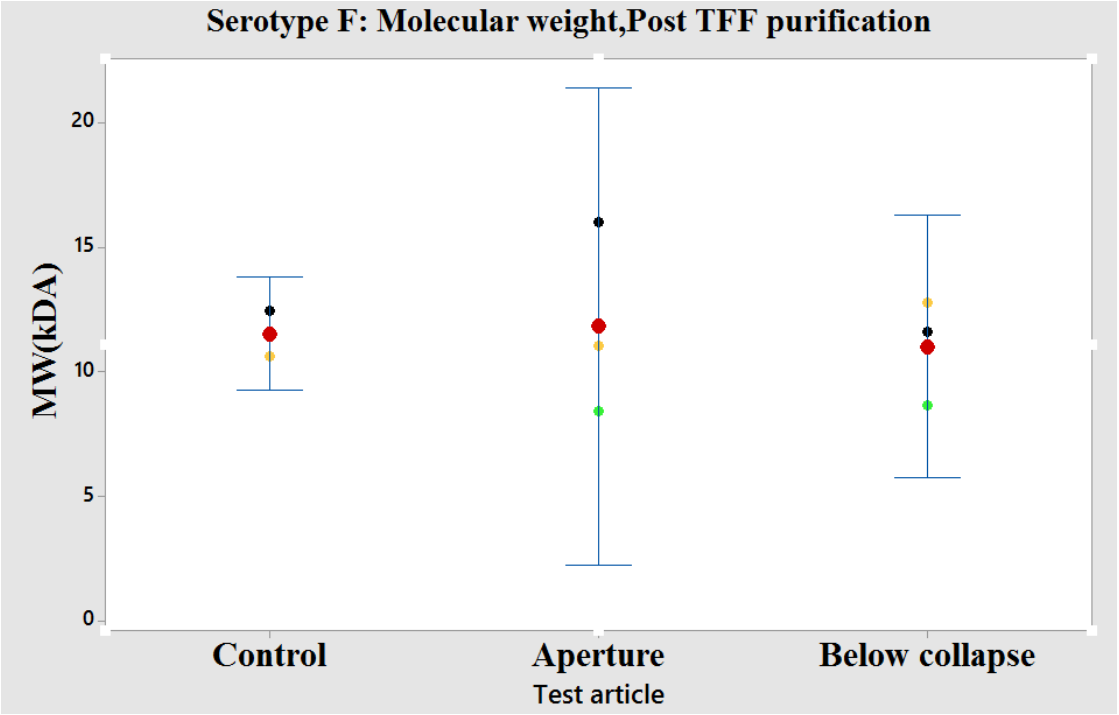

Figure 5-30: Molecular weight Serotype F. Conjugation occasion 1 green dots, conjugation occasion 2 orange dots, and conjugation occasion 3 black dots. The mean is represented by red dots and the confidence intervals are represented by the blue lines.

The molecular weights determined post TFF purification were also comparable to each other; see Figure 5-30, page 114. The means and confidence intervals calculated across the three conjugation occasions for each test article were similar, and the p values of both conjugation occasion and the test articles were ≥0.05. There was no statistical difference between the molecular weight means of the test articles.

(iv) Molecular size distribution of serotype F

One key parameter used to demonstrate manufacturing consistency and used as a stability indicator is the molecular size distribution of the conjugate. This is measured using a qualitative size exclusion method which separates macromolecules according to molecular size (Parisi and von Hunolstein, 1999). The sample concentration is measured as it elutes from a column. A specified percentage of the sample should elute by a specific elution time. Degradation is detected if the percentage of sample eluted has a longer retention time i.e. the size distribution is different. The molecular size distribution results for serotype F are presented in Table 5-14, page 115.

Table 5-14: Molecular size distribution serotype F, post TFF purification

Conjugation run	Test article	Post TFF Molecular size distribution (Spec ≥10%)
001	Control	14.52
	Aperture	15.71
	Below collapse	15.00
002	Control	18.33
	Aperture	17.38
	Below collapse	16.67
003	Control	15.48
	Aperture	16.67
	Below collapse	14.52
Average and standard deviation	Control	16.11±1.98
	Aperture	16.59±0.84
	Below collapse	15.40±1.13
ANOVA	P value: Test article	0.26
	P value: Conjugation occasion	0.04

All test articles were within specification and comparable to each other; see Figure 5-31, page 116. The means and confidence intervals calculated across the three conjugation occasions for each test article were similar and the p values of the test articles were >0.05. There was no statistical difference between the molecular size distribution means of the test articles. The p value for conjugation occasion was ≤0.05 (0.04) which indicates that conjugation occasion was a factor influencing the result.

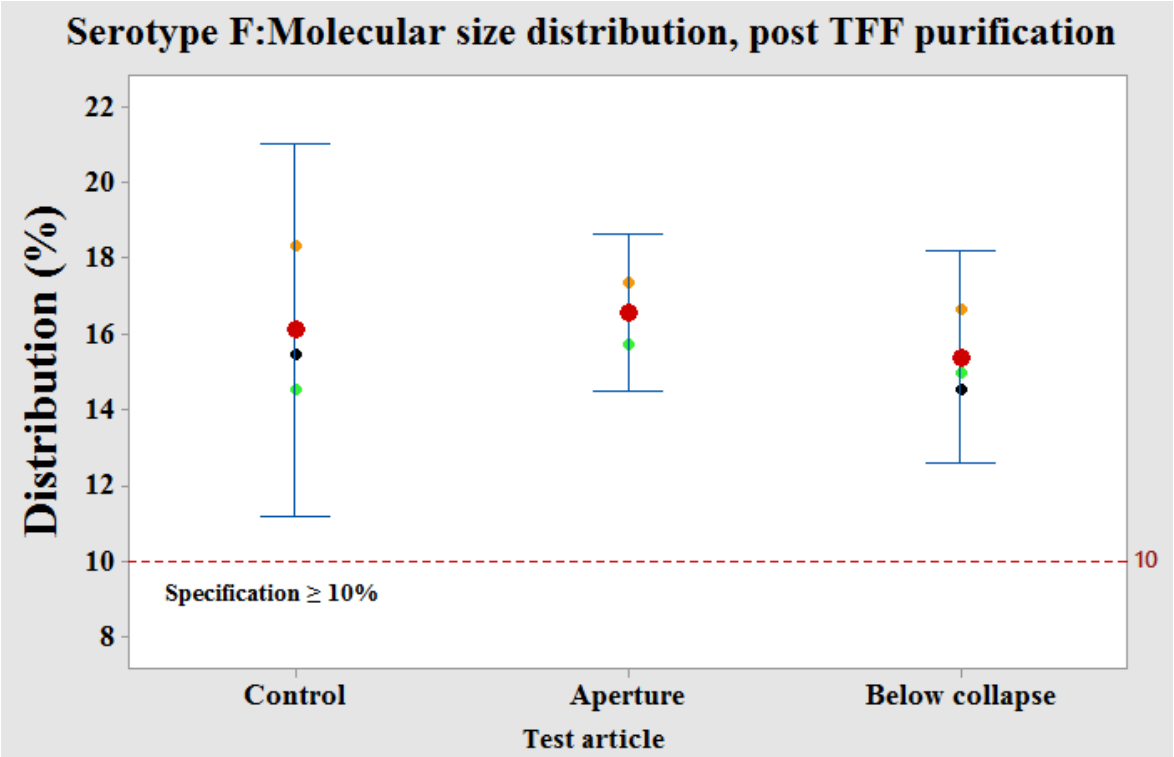

Figure 5-31: Molecular size distribution, serotype F. Conjugation occasion 1 green dots, conjugation occasion 2 orange dots, and conjugation occasion 3 black dots. The mean is represented by red dots and the confidence intervals are represented by the blue lines.

It was observed that the size distribution values obtained in conjugation occasion 2 were higher across all test articles compared to the other conjugation occasions. However they were within the calculated confidence limits and are not practically significant. This difference is most likely due to process and/or assay variability.

5.3.1.2 Serotype K Conjugate Product Quality

(i) Saccharide: Protein ratio (SPR) of serotype K

For serotype K, the SPR results for TFF and dialysis purification are presented in Table 5-15, page 117.

Table 5-15: Saccharide: Protein ratio serotype K, post TFF and post dialysis purification

Conjugation run	Test article	Post TFF SPR (Spec:10-22)	Post Dialysis SPR (Spec: No specification)
001	Control	15.54	21.58
	Aperture	13.10	22.01
	Below collapse	16.94	22.65
002	Control	16.56	21.58
	Aperture	14.53	22.01
	Below collapse	15.50	22.65
003	Control	14.95	21.58
	Aperture	13.82	22.01
	Below collapse	17.33	22.65
Average and standard deviation	Control	15.68±0.81	21.74±0.29
	Aperture	13.81±0.72	21.28±1.27
	Below collapse	16.59 ±0.96	22.44±0.23
ANOVA	P value: Test article	0.03	0.32
	P value: Conjugation occasion	0.72	0.55

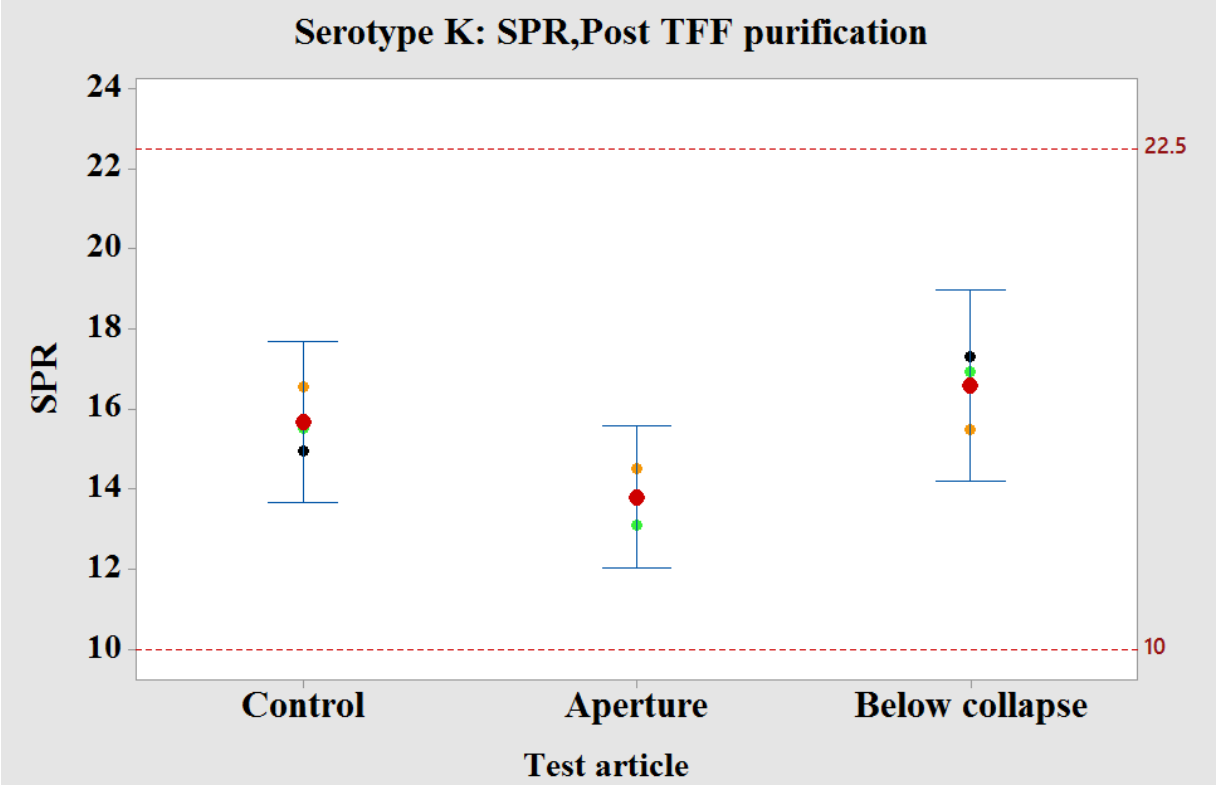

Figure 5-32: SPR Serotype K, TFF purification. Conjugation occasion 1 green dots, conjugation occasion 2 orange dots, and conjugation occasion 3 black dots. The mean is represented by red dots and the confidence intervals are represented by the blue lines.

All test articles purified by TFF were within specification; see Figure 5-32 , page 118. The means and confidence intervals calculated across the three conjugation occasions for each test article were similar. The p value of the test articles were ≤0.05 (0.03). This indicates that the test article was a significant factor. This result is driven by the aperture test articles which exhibited a lower mean across each conjugation occasion compared to the control and below-collapse test articles. The aperture test article was dried more aggressively than the other test articles (approximately 10 ºC above its collapse temperature, whereas the control was dried approximately 4 ºC above its collapse temperature). This result indicates that a less efficient conjugation was observed in the aperture test articles.

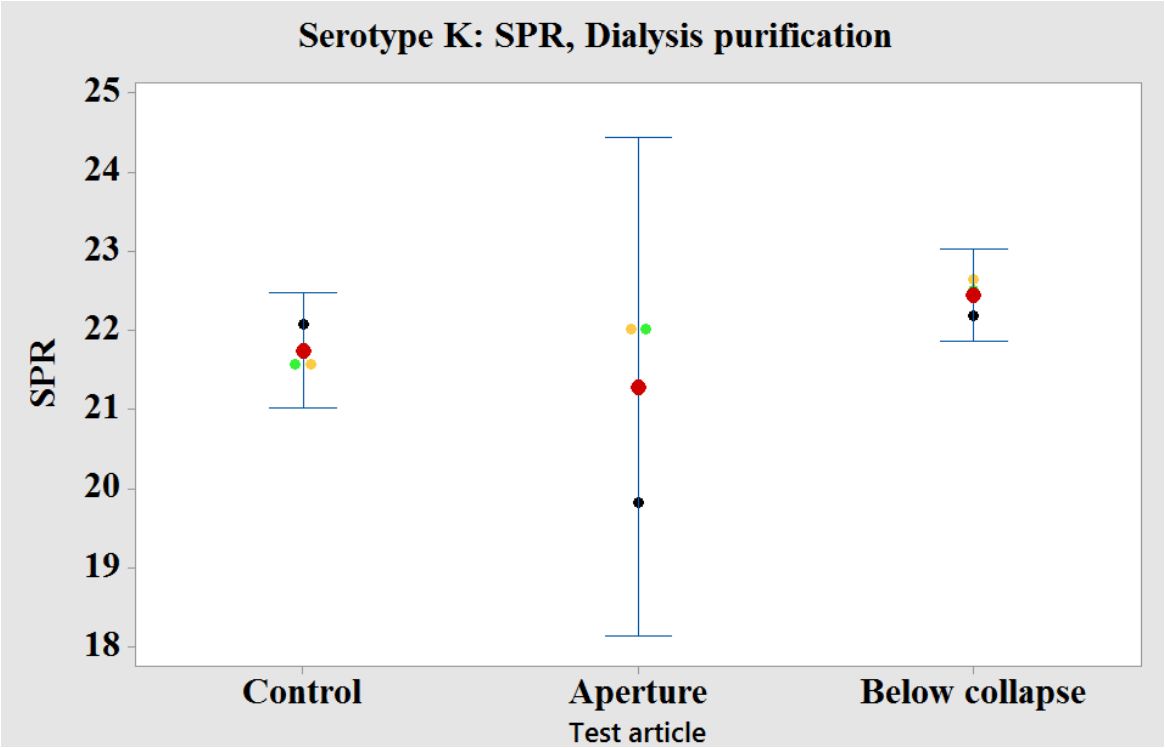

Figure 5-33: SPR Serotype K, Dialysis purification. Conjugation occasion 1 green dots, conjugation occasion 2 orange dots, and conjugation occasion 3 black dots. The mean is represented by red dots and the confidence intervals are represented by the blue lines.

For the dialysis purification the SPR was comparable across all test articles, with the exception of the aperture sample from conjugation occasion 3, which trended slightly lower; see Figure 5-33, page 119. This lower result could be as a result of assay variability. The p value was >0.05, indicating that SPR was not impacted by lyophilisation conditions. The SPR of the aperture test articles were lower when purified by TFF but not by dialysis. This would indicate that SPR was influenced by TFF processing conditions, as described in Section 5.3, page 103.

(ii) Unconjugated Saccharide of serotype K

The unconjugated saccharide results from TFF and dialysis purification are presented in Table 5-16, page 120.

Table 5-16: Unconjugated saccharide serotype K, post TFF and post dialysis purification

Conjugation run	Test article	Post TFF Unconjugated Saccharide (%) (Spec: ≤10%)	Post Dialysis Unconjugated Saccharide (%) (Spec: ≤10%)
001	Control	6.87	18.33
	Aperture	3.34	20.25
	Below collapse	3.57	13.87
002	Control	2.18	16.92
	Aperture	2.59	18.70
	Below collapse	3.13	15.06
003	Control	4.35	18.35
	Aperture	6.78	19.18
	Below collapse	6.67	14.71
Average and standard deviation	Control	4.46±2.35	17.87±0.82
	Aperture	4.23±2.24	19.38±0.79
	Below collapse	4.45±1.93	14.55±0.61
ANOVA	P value: Test article	0.08	0.01
	P value: Conjugation occasion	0.57	0.66

At the end of TFF purification, the unconjugated saccharide levels of all test articles were within specification; see Figure 5-34, page 121.

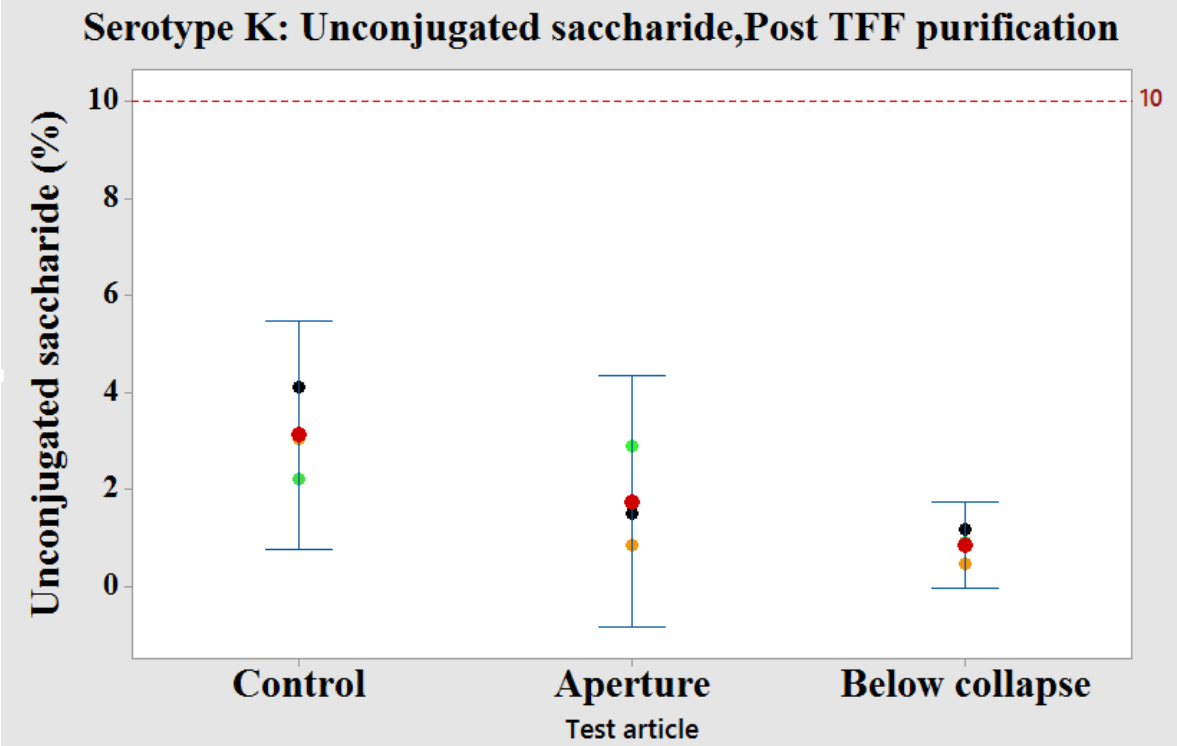

Figure 5-34: Unconjugated saccharide, serotype K, TFF purification. Conjugation occasion 1 green dots, conjugation occasion 2 orange dots, and conjugation occasion 3 black dots. The mean is represented by red dots and the confidence intervals are represented by the blue lines.

At the end of TFF purification, the unconjugated saccharide levels of all serotype K test articles were within specification; see Figure 5-34, page 121 . The means and confidence intervals calculated across the three conjugation occasions for each test article were similar and the p values of both conjugation occasion and the test articles were >0.05. There was no statistical difference between the unconjugated saccharide means of the test articles.

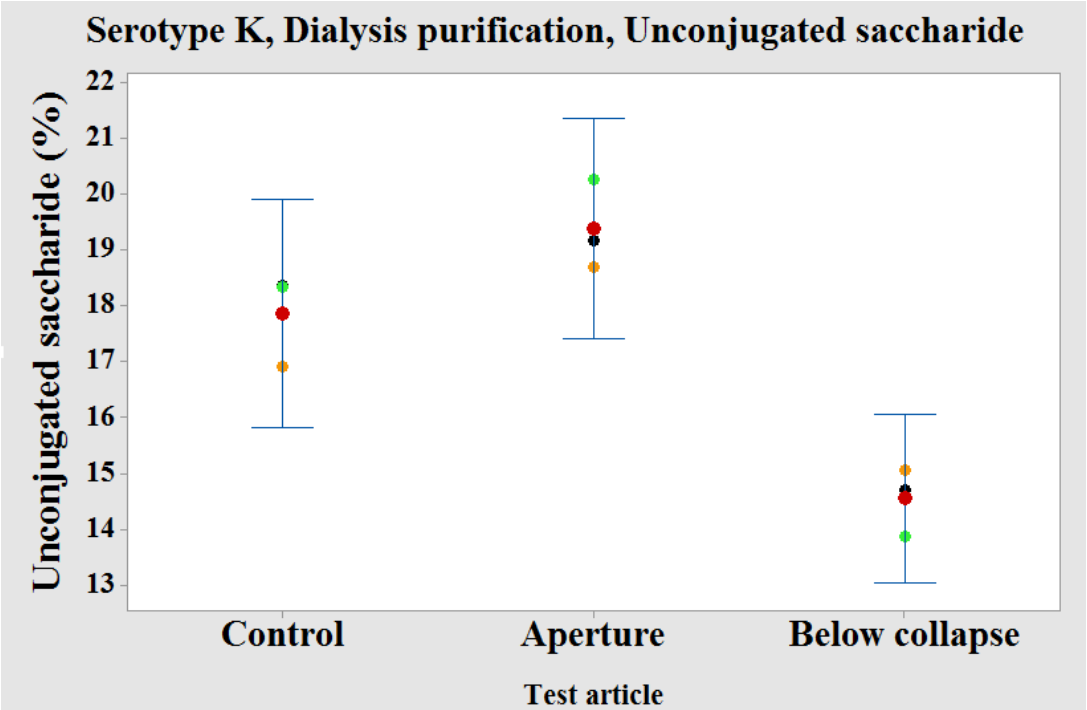

Figure 5-35: Unconjugated saccharide, serotype K, Dialysis purification. Conjugation occasion 1 green dots, conjugation occasion 2 orange dots, and conjugation occasion 3 black dots. The mean is represented by red dots and the confidence intervals are represented by the blue lines.

For the test articles purified by dialysis ,the means and confidence intervals calculated across the three conjugation occasions for each test article were different and the p values of the test articles was <0.05(0.01) ;see Figure 5-35, page 122. This indicates that there was a statistical difference between the unconjugated saccharide means of the test articles.

The levels of unconjugated saccharide measured in the dialysis samples provide insight into the success of the conjugation reaction, independent of TFF. It was observed across each conjugation occasion that the aperture test articles (most aggressive lyophilisation conditions) consistently exhibited higher unconjugated saccharide levels.

Conversely, the below-collapse test articles (least aggressive lyophilisation conditions) consistently exhibited lower levels of unconjugated saccharide. Lower levels of unconjugated saccharide in below-collapse test articles indicate that more efficient conjugation was achieved when freeze fried below the collapse temperature. The lower levels of unconjugated saccharide measured in the below-collapse test article may be due to the fact that as a result of less aggressive conditions, the protein was able to preserve its folded structure. This means that the lysine residues on protein X were still available for

covalent linkage to the activated polysaccharide K. This was not the case for the control and aperture test articles which were both freeze dried above collapse.

It is reasonable to infer that the lower levels of unconjugated saccharide were a function of the protein rather than the polysaccharide antigen itself. This supposition is supported by the fact that for the serotype F conjugates, there was no difference in unconjugated saccharide levels across all three test articles. Unlike serotype K, the protein X used in the serotype F conjugations was a liquid preparation and was not lyophilised. Only the serotype F (with an amorphous excipient) was lyophilised at different conditions and the unconjugated levels of saccharide were not impacted by lyophilisation conditions.

(iii) Molecular weight of serotype K

For serotype K, which was co lyophilised with Protein X, the molecular weights measured at the pre NFF and post TFF purification steps are presented in Table 5-17, page 123.

Table 5-17: Molecular weight serotype K: Pre NFF and post TFF purification

Conjugation run	Test article	Pre NFF Mw (kDa)	Post TFF Mw (kDa)
001	Control	4.47	6.40
	Aperture	4.62	8.12
	Below collapse	1.98	2.94
002	Control	3.68	6.02
	Aperture	3.45	7.04
	Below collapse	2.12	4.07
003	Control	4.14	7.30
	Aperture	3.47	5.93
	Below collapse	2.00	3.31
Average and standard deviation	Control	4.10±0.40	6.57± 0.66
	Aperture	3.85±0.67	7.03± 1.10
	Below collapse	2.03±0.08	3.44± 0.58
ANOVA	P value: Test article	0.01	0.02
	P value: Conjugation occasion	0.24	0.93

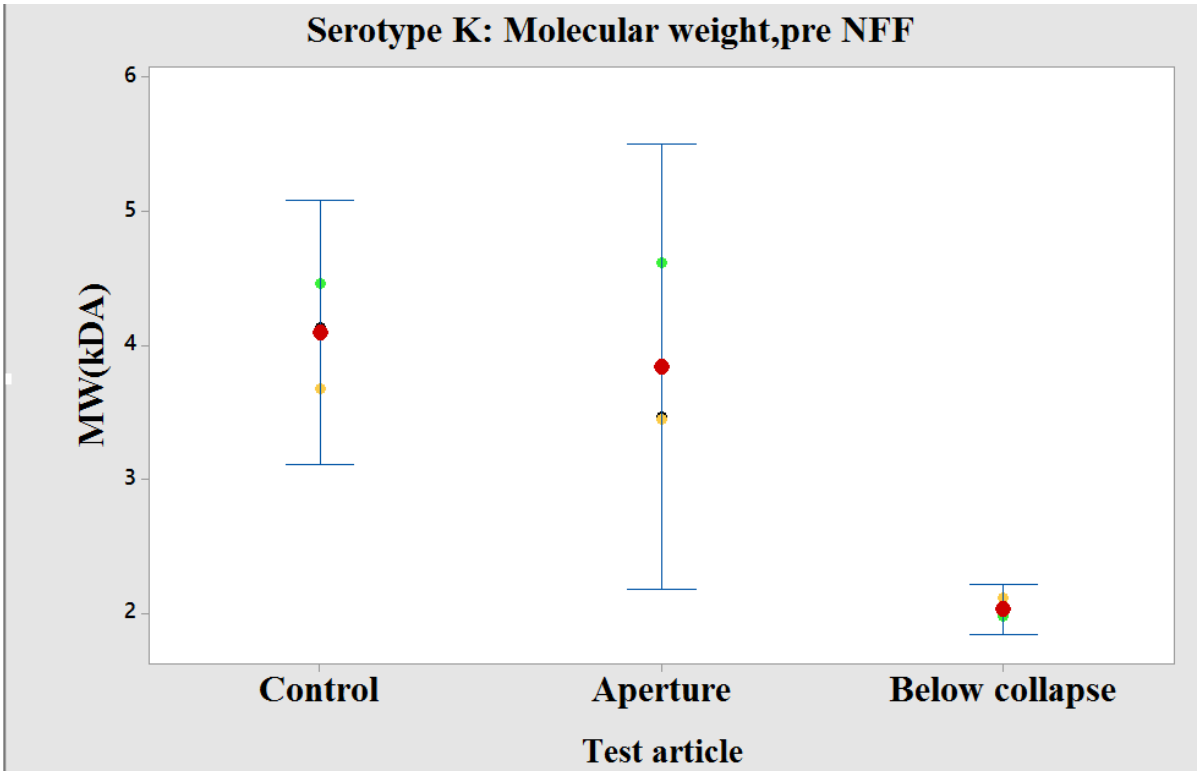

Figure 5-36: Molecular weight serotype K, pre NFF. Conjugation occasion 1 green dots, conjugation occasion 2 orange dots, and conjugation occasion 3 black dots. The mean is represented by red dots and the confidence intervals are represented by the blue lines.

For purification by NFF, the means and confidence intervals calculated across the three conjugation occasions for each test article were different; see Figure 5-36 , page 124. The control and aperture test articles had similar means but the below-collapse test article had a lower mean. The p value of the test articles were ≤0.05 (0.01). This indicates that the test article was a significant factor influencing molecular weight.

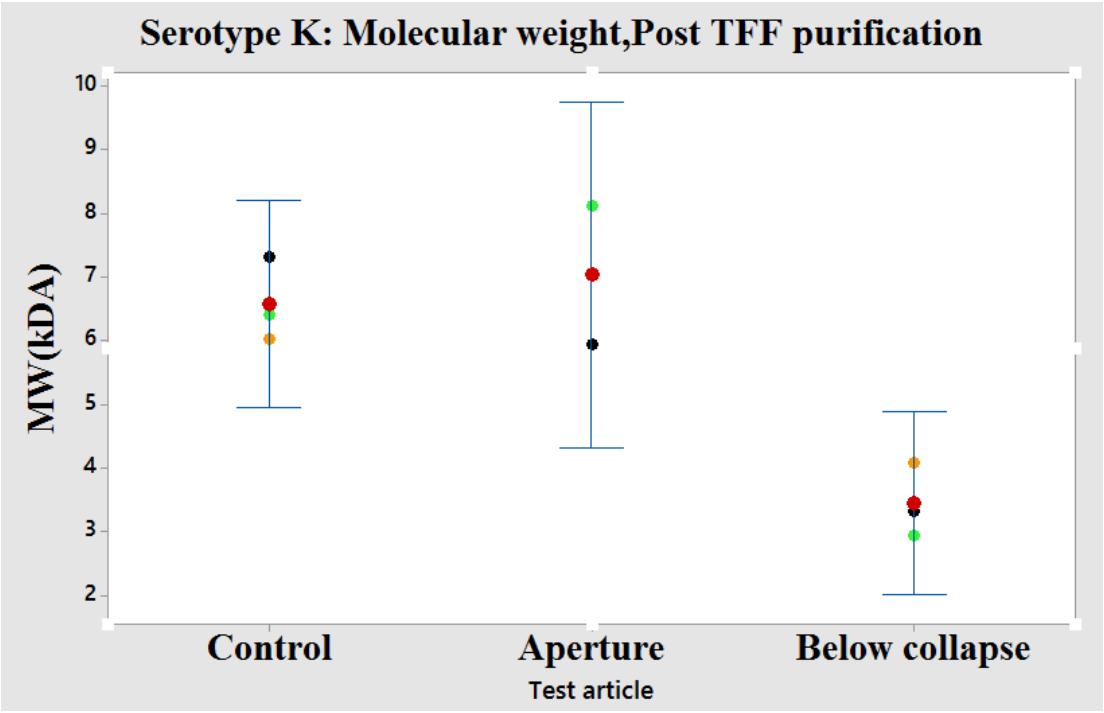

Figure 5-37: Molecular weight serotype K, post TFF. Conjugation occasion 1 green dots, conjugation occasion 2 orange dots, and conjugation occasion 3 black dots. The mean is represented by red dots and the confidence intervals are represented by the blue lines.

For purification by TFF, the means and confidence intervals calculated across the three conjugation occasions for each test article were also different; see Figure 5-37 , page 125. The control and aperture test articles had similar means but the below-collapse test article had a lower mean. The higher molecular weights of the control and aperture test articles indicate that as a result of heat stress from freeze drying above collapse, the protein unfolded from its three dimensional structure (Mensink et al., 2017b). When a protein unfolds, hydrophobic residues that are usually buried in the folded structure are exposed (Chang and Pikal, 2009). The reason why the test articles lyophilised above collapse (control and aperture) have higher molecular weights is because the unfolding meant that more polymeric chains were detected by the instrument, resulting in the determination of higher molecular weights.

The observed protein unfolding is the most likely reason why higher levels of unconjugated saccharide were observed in the control and aperture test articles. Lysine

residues are located on the surface of the protein (Yokota et al., 2006). When the protein unfolds, these residues are masked by the newly exposed hydrophobic residues and thus are not available for attachment to the polysaccharide. This results in higher amounts of unconjugated saccharide. It must be stressed that whilst higher levels of unconjugated saccharide did occur, the resulting conjugates were still well within specification. This supposition is supported by the fact that for the serotype F conjugates, there were no differences in molecular weight and unconjugated saccharide levels across all three test articles. The reason for this was because the liquid protein X in the serotype F conjugations was not exposed to the thermal stress of lyophilisation and, therefore, the protein did not unfold.

Protein unfolding, however, could be evidence of aggregation. Aggregation is a term used to describe protein species of higher molecular weight such as ‘‘oligomers’’ or ‘‘multimers’’ instead of the desired a monomer structure (Mahler et al., 2009). Aggregation can often be visually observed by the appearance of insoluble particulates; however, no such particles were observed in the control and aperture test articles. In addition, sub-visible aggregates can exist; however, it would be expected that the molecular weight method would detect these aggregates. In this study any aggregation that may have occurred did not result in specification failures. It is well established in the literature that proteins can undergo conformational changes during lyophilisation and different conformations can have different stability characteristics (Carpenter et al., 1997, Pikal, 1994). There is an important distinction made between pharmaceutical stability and thermodynamic stability. Irreversible degradation pathways are usually referred to as pharmaceutical instability whilst thermodynamic instability, as in the case of the above collapse test articles, refers to reversible protein unfolding (Pikal, 2006).

(iv) Molecular size distribution of serotype K

The molecular size distribution for serotype K was within specification for all test articles; see Table 5-18, page 127.

Table 5-18: Molecular size distribution serotype K, post TFF purification

Conjugation run	Test article	Post TFF Molecular size distribution (Spec ≥10%)
001	Control	12.94
	Aperture	11.76
	Below collapse	10.00
002	Control	11.57
	Aperture	12.16
	Below collapse	11.37
003	Control	11.18
	Aperture	10.00
	Below collapse	11.57
Average and standard deviation	Control	11.90± 0.93
	Aperture	11.18±1.37
	Below collapse	10.98± 0.85
ANOVA	P value: Test article	0.67
	P value: Conjugation occasion	0.62

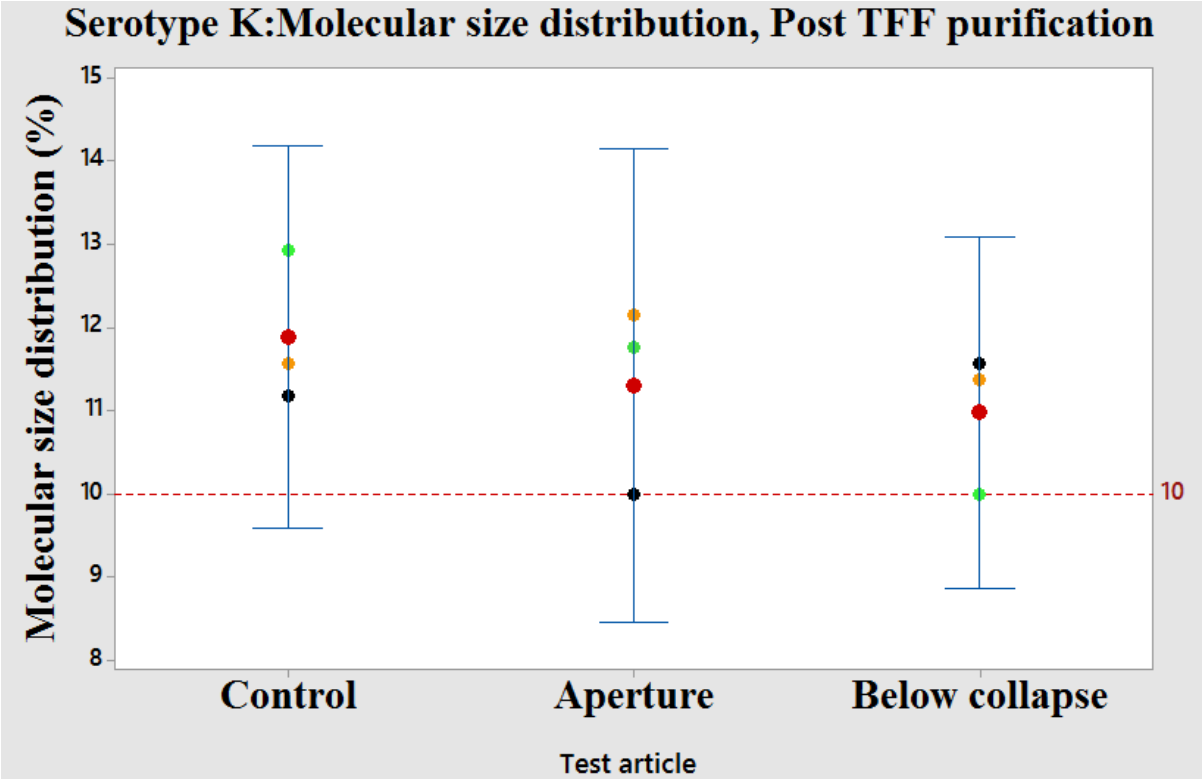

Figure 5-38: Molecular size distribution, serotype K, TFF purification. Conjugation occasion 1 green dots, conjugation occasion 2 orange dots, and conjugation occasion 3 black dots. The mean is represented by red dots and the confidence intervals are represented by the blue lines.

All test articles were within specification and comparable to each other; see Figure 5-38, page 128. The means and confidence intervals calculated across the three conjugation occasions for each test article were similar and the p values of the test articles were >0.05. There was no statistical difference between the molecular size distribution means of the test articles. This confirms that the lyophilisation conditions did not impact the molecular distribution achieved, as measured by this technique. It was observed that the aperture test article in conjugation occasion 3 was just on the specification. The most likely explanation for this is variability associated with the conjugation process or TFF conditions, or could be due to assay variability

5.3.2 Conjugation conclusion

Serotype F (discreetly lyophilised with an amorphous excipient) and serotype K (co-lyophilised with Protein X) were freeze dried above and below their respective collapse temperatures. They were then chemically conjugated to protein X followed by purification by NFF, TFF and dialysis. Conjugation efficiency was assessed using a number of assays with pre-defined specifications. Results for both serotypes met specification across all conjugation occasions. For all serotype F test articles, lyophilisation conditions had no impact on saccharide: protein ratio, molecular weight and levels of unconjugated saccharide. For the serotype K test articles lyophilised above collapse, the molecular weight was approximately 2 fold higher compared to the below-collapse test article. This was due to protein unfolding as a result of lyophilisation above the collapse temperature. In addition, purification by dialysis showed that the amount of unconjugated saccharide was lower for test articles lyophilised below collapse but was higher for test articles freeze dried above the collapse temperature. This would indicate a less efficient conjugation reaction as a result of freeze drying above collapse. The reason for this is because when the protein unfolds, lysine residues, which are essential for attachment of the polysaccharide to the protein, were masked by the newly exposed protein hydrophobic residues. Despite these observations, there was no practical product quality impact due to lyophilisation above the collapse temperature.

5.4 Impact of lyophilisation conditions on biological function of serotype F and serotype K conjugates

5.4.1 Experiment 1: Drug product model development

In order to assess the impact of lyophilisation conditions on total and adjuvant biological function a drug product model was developed. Over four discrete occasions, a number of 120 g formulations were prepared. These formulations comprised serotype F and K, along with a number of other conjugated, capsular polysaccharides serotypes. On formulation occasions 1, 2 and 3, three 120 g formulations were prepared. On formulation occasion 4, seven formulations were prepared, giving a total of sixteen formulations.

Immunochemical assays, such as nephelometry, are important tools for determination of antigen concentration in a vaccine (Salerno et al., 1984). Nephelometry is based on the principle that the antigen of interest reacts with a specific antibody forming a complex and will scatter light passed through the samples rather than absorbing it. The amount of light scattered at a certain concentration of antibody is proportional to the concentration of antigen (Johnson, 1987).

The total biological function is the antigenicity associated with the conjugated and non-conjugated polysaccharide present in vaccine and the adjuvant biological function is the antigenicity associated with the adjuvant. Both are critical product quality attributes.
The acceptance criteria for model qualification were as follows:

- Total biological function and adjuvant biological function results should be within specification.
- The %RSD of each 120 g formulation should be within the %RSD of total biological function and adjuvant biological function assays, $\leq 20\%$.

5.4.1.1 Total biological function model development

For total biological function of the polysaccharide, all sixteen formulations of serotypes F and K were within specification and the means and confidence intervals calculated across each formulation occasion were similar; see Table 5-19, page 131, Figure 5-39, page 132 and Figure 5-40, page 132 respectivly. The %RSD obtained for each formulation was < 20%. An apparent downward trend for serotypes F and K across formulations 1, 2 and 3 was observed. The same batch of buffer 3 and surfactant was used to prepare these formulations; therefore, fresh solutions were prepared for formulation 4. The downward trend in biological function was subsequently reversed for serotype F, but appeared to continue for serotype K. However as the total biological function model development criteria were met across each formulation occasion, the 120 g formulation model was qualified.

Table 5-19: Model development for total biological function for serotype F and K

Serotype	Formulation	Average biological function (µg/mL) F: Spec (20 -37 µg/mL) K: Spec (10 -18 µg/mL)	Total biological function % RSD
F (n=3 per formulation)	1	31.61±0.87	2.76
	2	29.61±0.12	0.39
	3	27.25±0.55	2.02
	4	28.76±0.44	1.52
K (n=3 per formulation)	1	14±0.00	0.00
	2	13.86±0.34	2.49
	3	12.92±0.068	0.63
	4	12.33±0.24	1.91

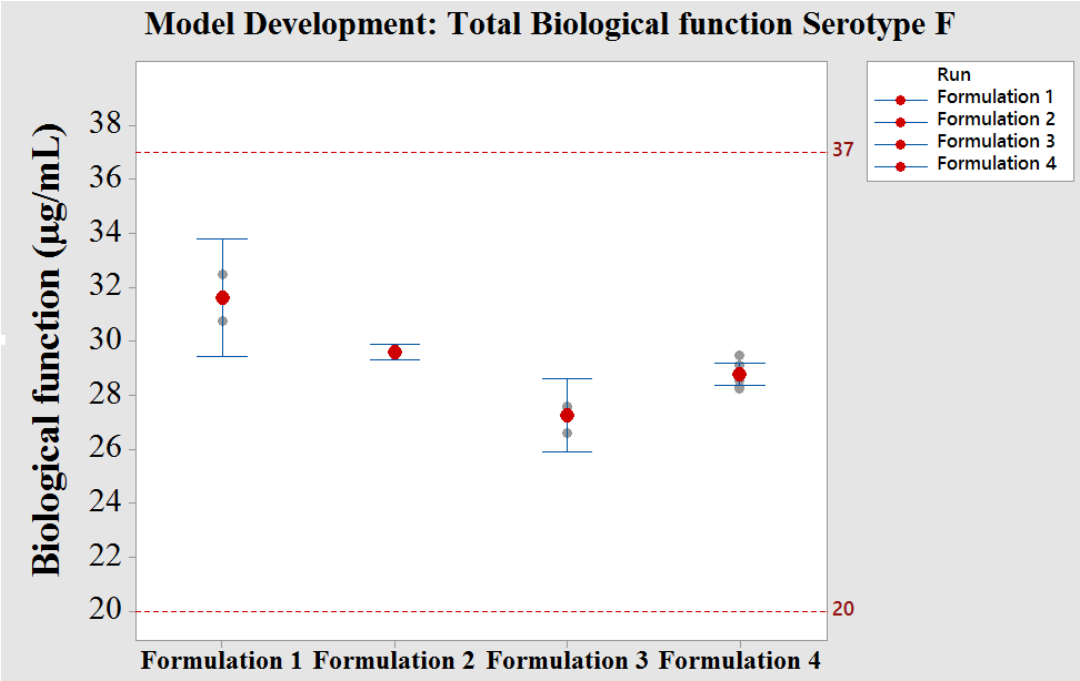

Figure 5-39: Model development for Total biological function for serotype F. The means of formulations 1 to 4 are represented by red dots and the confidence intervals are represented by the blue lines. All formulations within specification

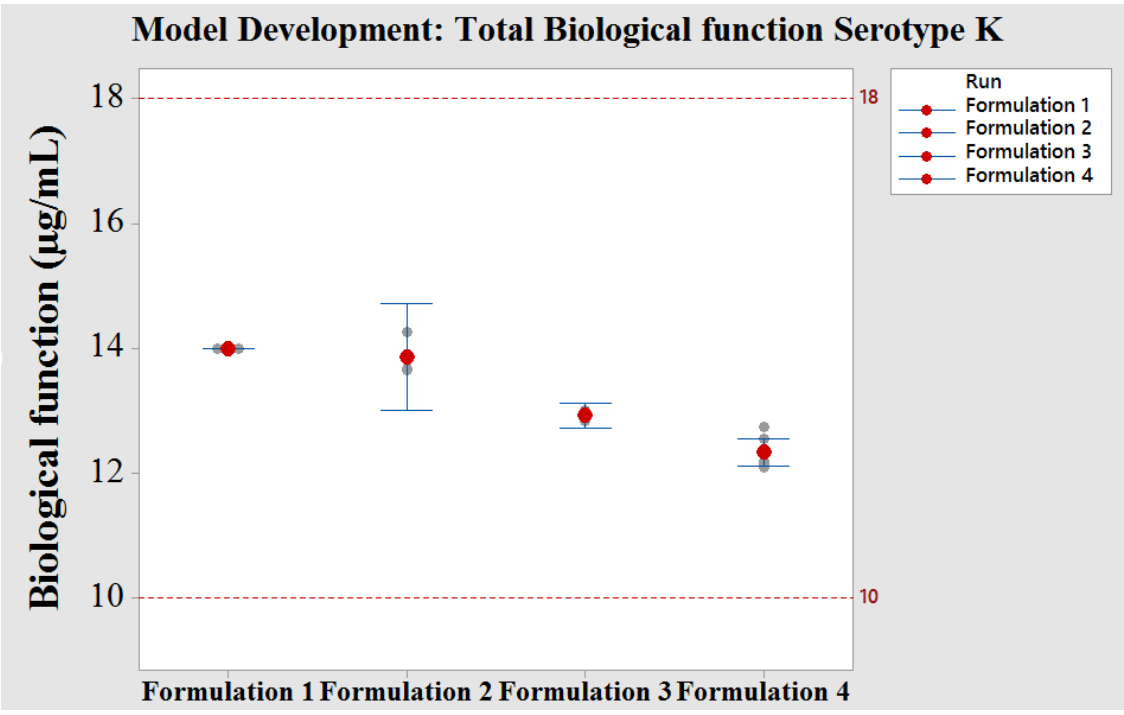

Figure 5-40: Model development for Total biological function for serotype K. The means of formulations 1 to 4 are represented by red dots and the confidence intervals are represented by the blue lines. All formulations within specification

5.4.1.2 Adjuvant biological function model development

For serotype F and K adjuvant biological function, all replicates from formulations 1, 2, 3, and 4 were within specification; see Table 5-20, page 133, Figure 5-41, page 134 and Figure 5-42, page 135 respectively. The %RSD obtained for each formulation was < 20%. The adjuvant biological function results obtained for each formulation were also comparable to manufacturing history. As the adjuvant biological function model development criteria were met across each formulation occasion, the 120 g formulation model was qualified.

Table 5-20: Model development for adjuvant biological function for serotype F and K

Serotype	Formulation	Adjuvant biological function (%) F: Spec (≥ 11 %) K: Spec (≥ 9 %)	Adjuvant biological function %RSD
F (n=3 per formulation)	1	21.63±0.12	0.57
	2	21.74±0.49	2.28
	3	18.01±0.36	2.02
	4	22.96±3.72	16.20
K (n=3 per formulation)	1	15.08±0.60	3.97
	2	15.57±0.70	4.49
	3	15.83±0.28	1.78
	4	18.11±0.42	2.34

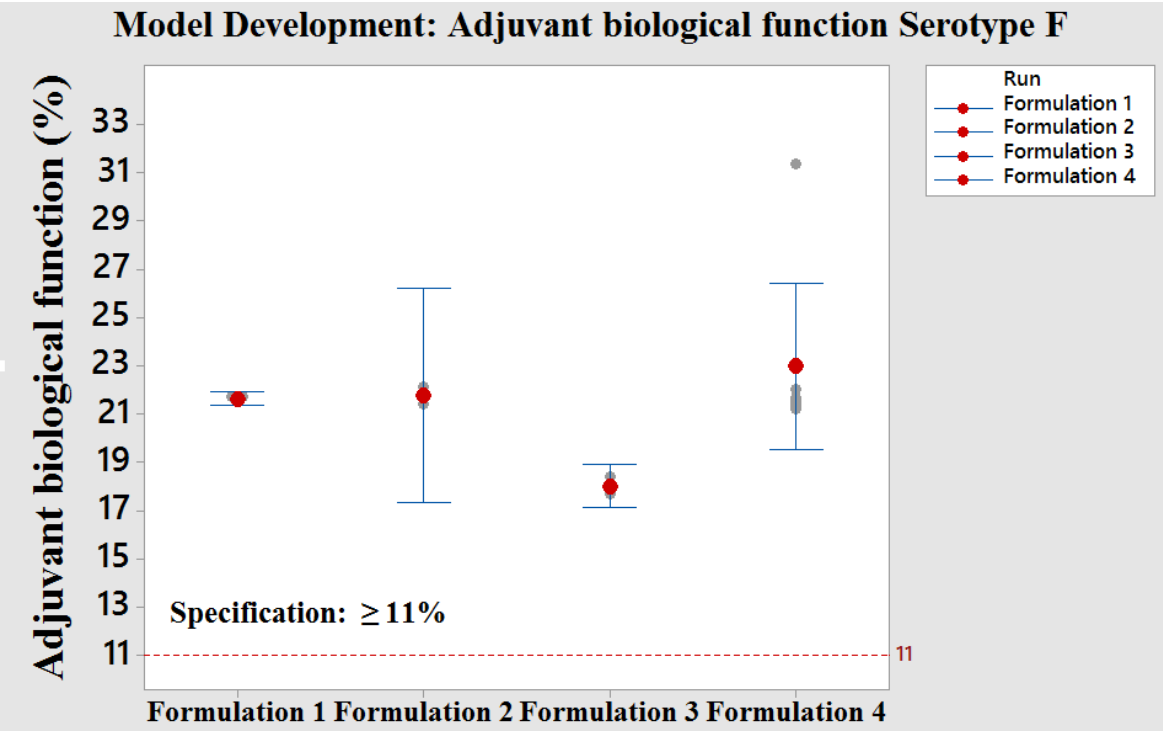

Figure 5-41: Model development for Adjuvant biological function for serotype F. The means of formulations 1 to 4 are represented by red dots and the confidence intervals are represented by the blue lines. All formulations within specification

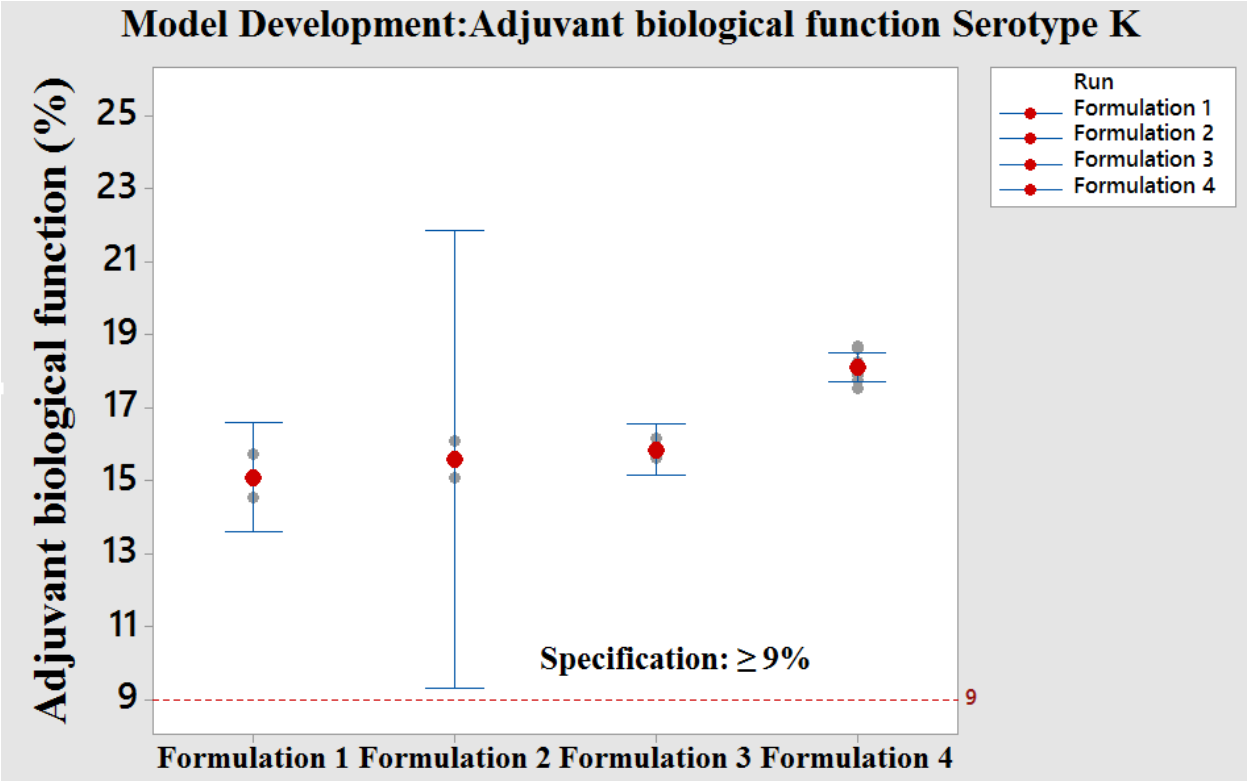

Figure 5-42: Model development for Adjuvant biological function for serotype F. The means of formulations 1 to 4 are represented by red dots and the confidence intervals are represented by the blue lines. All formulations within specification. Two replicates only were analysed for formulation 2.

5.4.2 Experiment 2: Drug product formulation of test articles Serotype F and K lyophilised above and below the collapse temperature.

Each serotype F and K test article (control, aperture and below collapse) from each conjugation occasion was further processed into individual multivalent 120 g drug product formulations. The purpose of doing this was to determine if lyophilisation conditions had any impact on the biological function of the conjugates.

5.4.2.1 Serotype F Total biological function

For serotype F, the total biological function and the adjuvant biological function of the control, aperture and below-collapse test articles, were within specification; see Table 5-21, page 136, Figure 5-43 and page 137 and Figure 5-44, page 137.

The means and confidence intervals calculated across the three conjugation occasions for each test article were similar. This indicates that lyophilisation conditions had no impact on total biological function and adjuvant biological function for serotype F.

Table 5-21: Serotype F biological function and adjuvant biological function

Test article (n=3 per test article)	Average total biological function (µg/mL): Spec (20 -37 µg/mL)	Adjuvant biological function (%) Spec (≥ 11 %)
Control	29.53±0.54	19.79±0.84
Aperture	29.99±2.56	20.44±0.79
Below Collapse	28.06±3.62	21.24±1.73

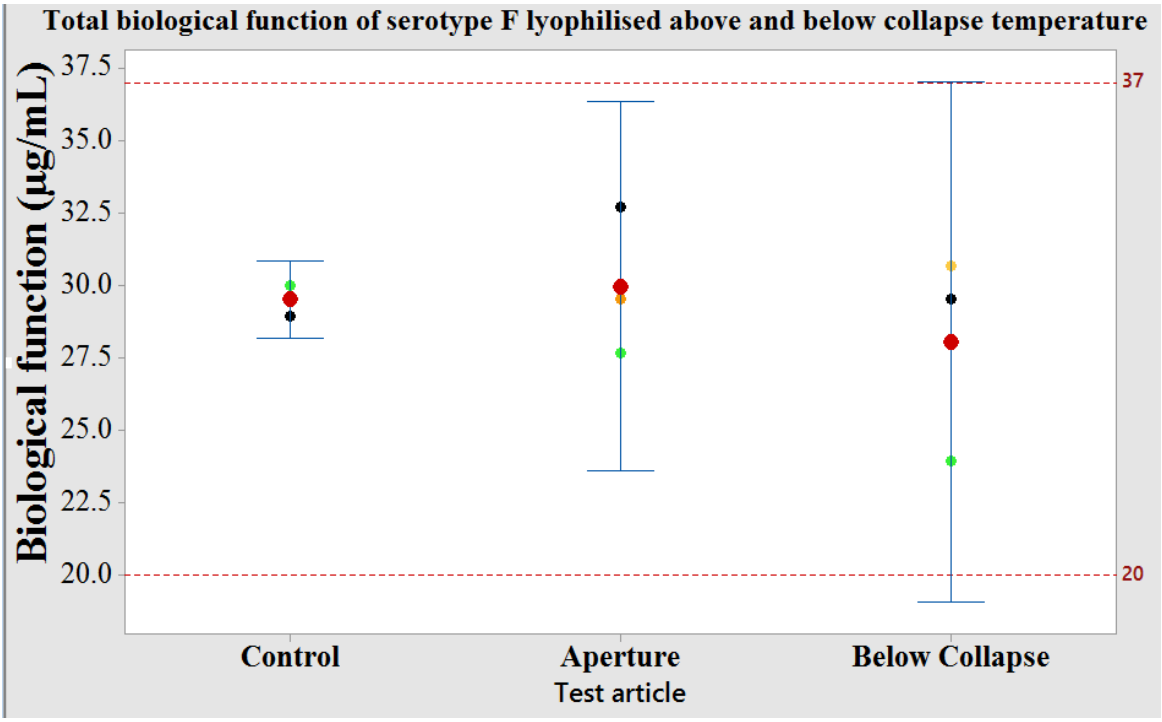

Figure 5-43: Serotype F, total biological function. Conjugation 1 green dots, conjugation 2 orange dots, conjugation 3 black dots. The mean are represented by red dots and the confidence intervals are represented by the blue lines. All test articles were within specification.

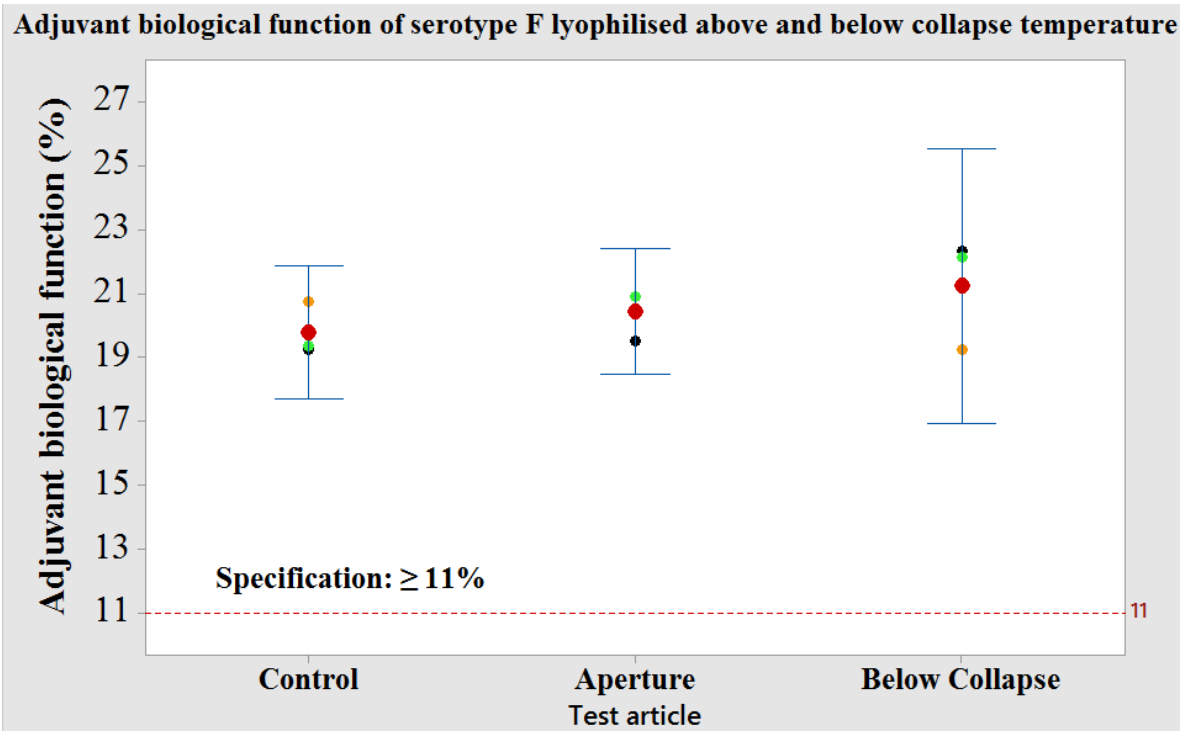

Figure 5-44: Serotype F, adjuvant biological function. Conjugation 1 green dots, conjugation 2 orange dots, conjugation 3 black dots. The means are represented by red dots and the confidence intervals are represented by the blue lines. All test articles were within specification.

5.4.2.2 Serotype K Biological function

For serotype K, the total biological function and the adjuvant biological function of the control, aperture and below-collapse test articles, were within specification; see Table 5-22, page 138, Figure 5-45, page 139 and

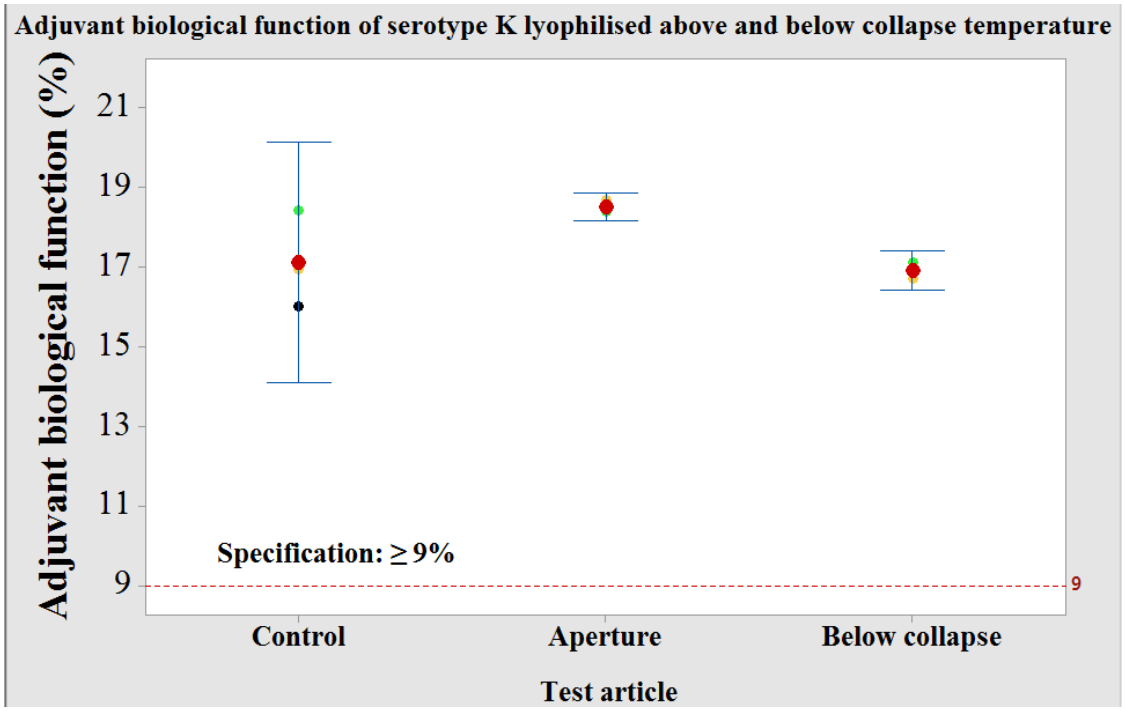

Figure 5-46, page 139. The means and confidence intervals calculated across the three conjugation occasions for each test article were similar. This indicates that lyophilisation conditions had no impact on total biological function and adjuvant biological function for serotype K.

Table 5-22: Serotype K total biological function and adjuvant biological function

Test article (n=3 per test article)	Average total biological function (µg/mL): Spec (10 -18 µg/mL)	Adjuvant biological function (%) Spec (≥ 9 %)
Control	13.29±0.43	17.14±1.22
Aperture	13.05±0.32	18.53±0.14
Below Collapse	13.43±0.73	16.93±0.20

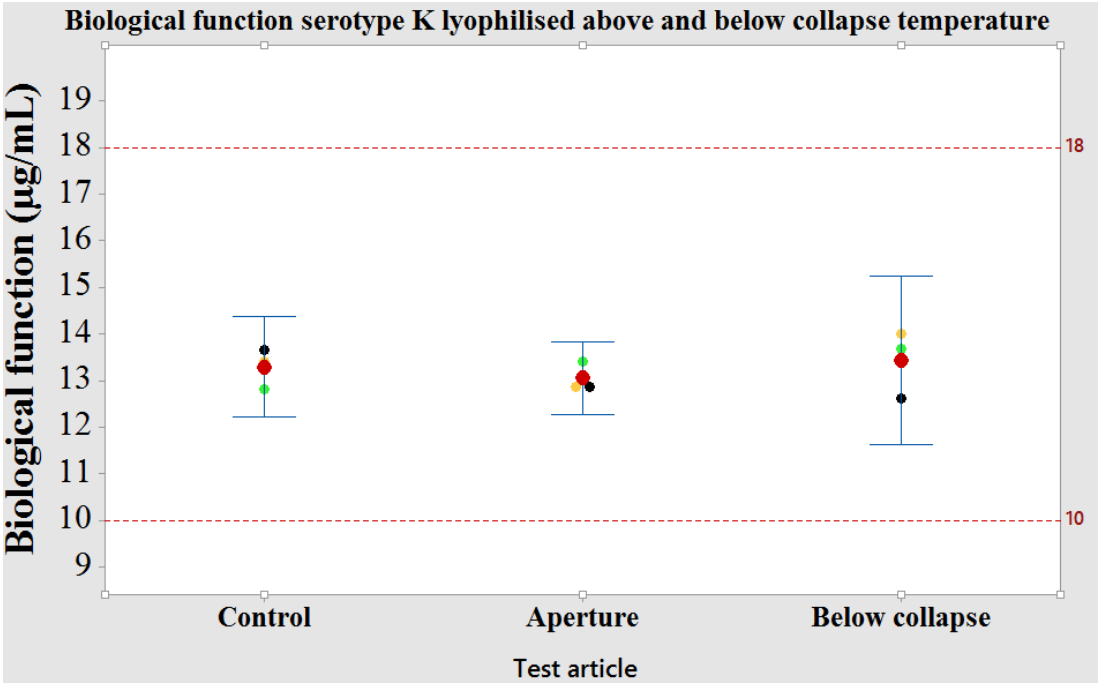

Figure 5-45: Serotype K, total biological function. Conjugation 1 green dots, conjugation 2 orange dots, conjugation 3 black dots. The mean are represented by red dots and the confidence intervals are represented by the blue lines. All test articles were within specification.

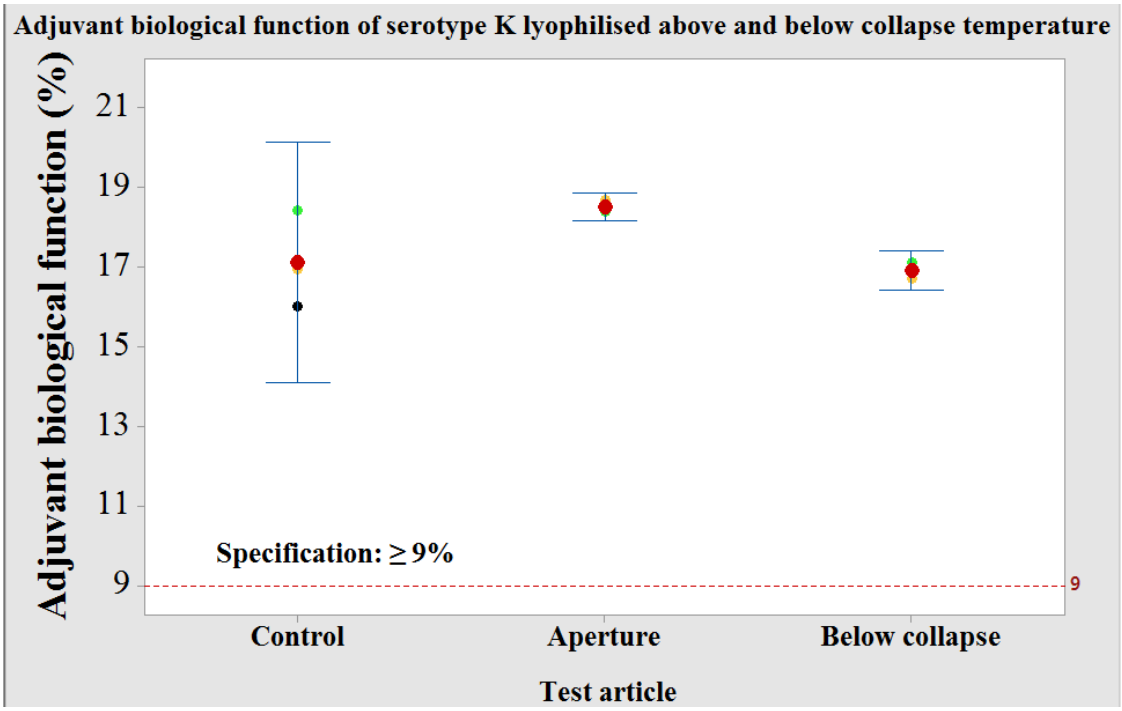

Figure 5-46: Serotype K, adjuvant biological function. Conjugation 1 green dots, conjugation 2 orange dots, conjugation 3 black dots. The means are represented by red dots and the confidence intervals are represented by the blue lines. All test articles were within specification.

6.0 CONCLUSION

Several studies were carried out to determine factors influencing cake appearance and moisture content of polysaccharide serotype F lyophilised in 1 L bottles. One element of this study examined the shell freezing process. The main factors that influence residual moisture and appearance during the shell freezing process were bottle angle, fill weight and ethanol level in the shell bath. In order to achieve effective shell formation, the ethanol should be in contact with as much of the length of the bottle as possible. This is achieved by a combination of a maximizing ethanol bath depth (76 mm) and reducing the bottle angle. The next study investigated aperture restriction during sublimation on cake morphology post lyophilisation at commercial scale. As a consequence of aperture restriction, the product can experience higher temperatures during primary drying resulting in collapse or melt back. This leads to poor cake morphology and increased residual moisture content. From the data generated, it was evident that bottles lyophilised with an increased aperture size displayed acceptable cake morphology and lower residual moisture contents (moisture < 6%) as a result of drying at a lower temperature. A relationship was established between product temperature during lyophilisation and its influence on cake morphology and residual moisture content. A classification system was then developed to correlate morphology of bottles post lyophilisation with different degrees of collapse and subsequent residual moisture results. Bottles that exhibited poor appearance with significant micro-collapse were designated as Morphology 4 and presented with higher moisture than bottles with good cake appearance (Morphology 1). Serotype F (discreetly lyophilised with an amorphous excipient) and serotype K (co-lyophilised with Protein X) were freeze dried above and below their respective collapse temperatures. Using the Excel primary drying modeling tool, two lyophilisation cycles were designed: one above, and one below the collapse temperature of serotype F and K. When the vials were lyophilised above collapse, the cakes produced had relatively poor appearance and higher moisture. However, as a result of the higher product temperature, primary drying in the above-collapse cycle was approximately 4-fold shorter than the below-collapse cycle. Freeze drying below the collapse temperature resulted in product with below-collapse elegant cake appearance, with moisture contents significantly lower than that of the above-collapse lyophilies. For the below-collapse cycle, the resistance increased linearly as function of dry layer thickness, while resistance was independent of dry layer thickness for above-collapse. The open pore structure of the above-collapse lyophilies facilitated the evolution of vapour though the dry layer, resulting in shorter lyophilisation times; however, residual

moisture content increased as a result of micro collapse and this impacts glass transition temperature.

Serotype F and serotype K were then chemically conjugated to protein X followed by purification by NFF, TFF and dialysis. Conjugation efficiency was assessed using a number of assays with pre-defined specifications. Results for both serotypes met specification across all conjugation occasions. For all serotype F test articles, lyophilisation conditions had no impact on saccharide: protein ratio, molecular weight and levels of unconjugated saccharide. For the serotype K test articles lyophilised above collapse, the molecular weight was approximately 2 fold higher compared to the below-collapse test article. This was due to protein unfolding as a result of lyophilisation above the collapse temperature. In addition, purification by dialysis showed that the amount of unconjugated saccharide was lower for test articles lyophilised below collapse but was higher for test articles freeze dried above the collapse temperature. This would indicate a less efficient conjugation reaction as a result of freeze drying above collapse. The reason for this is because when the protein unfolds, lysine residues, which are essential for attachment of the polysaccharide to the protein, were masked by the newly exposed protein hydrophobic residues. Despite these observations, there was no practical product quality impact due to lyophilisation above the collapse temperature

To assess the impact of lyophilisation conditions on total and adjuvant biological function, a drug product model was developed. Over four discrete occasions, a number of 120 g formulations were prepared. These formulations consisted of serotype F and K, along with a number of other conjugated, capsular polysaccharides serotypes.. Each conjugated test article lyophilised above and below collapse was then formulated into a 120 g multivalent formulation. It was determined that the total biological function of the polysaccharide and total adjuvant biological function of serotype F and K, lyophilised at different conditions, met specification and were comparable to each other. It can be concluded that lyophilisation above the collapse temperature had no practical influence on the biological function of the antigen.

7.0 FUTURE WORK

This work focused on the impact that lyophilisation above the collapse temperature had on lyophilisation quality attributes and conjugation quality attributes of activated polysaccharide-protein X conjugates. There are three focus areas that would be of interest to further explore.

(i) Aggressive lyophilisation of activated polysaccharides and impact on conjugation quality attributes

Using the primary drying modelling tool, additional lyophilisation cycles can be designed, ensuring that product temperature is at least 15 °C to 20 °C above the collapse temperature. The obvious advantage of this is that cycle time will be significantly reduced but it could result in product with extremely poor cake appearance, and higher moisture content, which may also have longer reconstitution times. These aggressively freeze dried serotypes could then proceed forward for conjugation to determine if there is any adverse impact on conjugation product quality attributes and biological function.

(ii) Stability and of product lyophilised above the collapse temperature

The aggressively lyophilised activated serotypes will generally have higher moisture contents and lower glass transition temperatures compared to products freeze dried below the collapse temperature. It would be prudent to determine if products lyophilised well above collapse have an acceptable stability profile in comparison to their below-collapse counterparts. Activated serotypes, lyophilised at various degrees above collapse, could be placed on stability test at three temperatures, 2-8 °C, 25 °C/60% RH and 40 °C /75% RH for a minimum of 6 months. Product quality could be assessed using a range of appropriate stability indicating assays. At the end of the stability study, the lyophilies could then be chemically conjugated to protein X to determine if conjugation product quality attributes were impacted.

(iii) Analytical characterization

A range of other analytical techniques can be used to assess the impact of freeze drying above the collapse temperature. Scanning electron microscopy (SEM) could be used to examine the structure of lyophilised cakes. This technique can produce magnified images allowing a comparison of pore size between product lyophilised above and below collapse. Fourier transform infrared spectroscopy (FTIR) and Circular Dichroism (CD) could be

used to assess the secondary and tertiary structure, respectively, of protein X as a function of lyophilisation conditions.

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