The Influence of Moisture Content and Temperature on Storage Stability of Freeze-Dried Biologics

By

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Copyright declaration

Declaration of Originality

I hereby declare that this thesis and the work presented herein is entirely my own work. Information presented and derived from the published and unpublished work of others has been acknowledged in the text and references as given.

Acknowledgments

“Water is the driving force in nature.”

— Leonardo da Vinci

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Abstract

Moisture and temperature are both critical factors that affect the long term storage stability of Freeze-dried (FD) biologics. Both physical structure and biological activity can be affected by the conditions that the FD material is subject to over a prolonged storage period. This project aimed to investigate how these key factors affected the long term storage stability of FD biologics. The relationship between moisture content, cake structure and the physical/biological stability for model proteins/antigen standards during long term storage was investigated. Novel techniques and procedures were developed to measure the effects of moisture and storage temperature in FD material. Dynamic vapour sorption (DVS) instrumentation, in conjunction with a real time video imaging, was used to measured visible collapse/shrinkage of FD materials. DVS data used in conjunction with video images for a series of temperatures were analysed to provide stability maps. These provided critical moisture content levels that should not be exceeded in order to retain structural cake stability. In addition, other novel techniques were utilised to measure morphological or physical changes with regards to moisture and temperature. Mechanical properties of the FD materials was measured with a flat punch indenter, while inverse gas chromatography (IGC) was used to measure the cake specific surface area (SSA). Mechanical indention data showed that increasing moisture and storage temperature lead to a reduction of mechanical properties and specifically Young's modulus. IGC was shown to be suitable alternative to measure SSA of FD biologics, providing comparable SSA values to standard volumetric gas adsorption techniques. Advantages of IGC included being able to show and measure changes to SSA of FD materials conditioned at different relative humidities.

A series of long term stability trials were also conducted for high protein concentration formulations (IgG) with a range of 10 - 200 mg/mL to further investigate mechanisms of protein stabilisation in regards to optimum moisture and temperature. Higher concentration proteins

had lower SSA's with larger Young's Modulus but suffered from longer reconstitution times. IgG stability during 12 month storage trials showed evidence for both vitrification and water replacement theories. The data also provided further evidence for the bell shaped distribution theory of optimum moisture content for some materials and that over-drying with a low moisture cycle might not necessarily be the best option for long term storage stability of IgG. High moisture contents of up to 5% w/w did not seem to have any impact on stability until storage above 45°C. With high concertation FD proteins above 50 mg/mL, there was low of risk of structural collapse with increasing moisture content compared to lower concentration materials. Water ingress into vials during their long term storage is of huge concern especially for all FD materials, but especially so for low mass products such as FD Influenza antigens. Comparison of different closure storage formats for FD antigens was explored and it was found that vials with vacuum-oven dried stoppers had less moisture ingress than vials with unprocessed stoppers (straight out of manufacturers packaging). Vials with vacuum-oven dried stoppers were shown to give comparable potency and moisture content ingress as glass ampoules for reference standard influenza antigens over a 1 year period from -20°C to 45°C. Thus not only can vials with vacuum oven dried stoppers reduce moisture ingress compared to unprocessed stopper vials, but can also result in retaining greater potency and stability during long term storage.

In summary, this thesis via use of prolonged stability trials, expanded and further consolidated knowledge on current theories of mechanisms of stability in context to moisture content and temperature during long term storage for real world commercial FD biological standards. This thesis also promoted and endorsed the adoption of novel techniques or practices (such as vacuum oven drying stoppers) to provide further aid and insight in optimising the long term storage stability of FD biologics for future use in industry.

Publications

Journal Papers

Duralliu, A., Matejtschuk, P., and Williams, D.R. Humidity induced collapse in freeze dried cakes: A direct visualization study using DVS. European Journal of Pharmaceutics and Biopharmaceutics, (2018). 127: p. 29-36**.** https://doi.org/10.1016/j.ejpb.2018.02.003 (Adapted from Chapter 3)

Duralliu, A., Matejtschuk, P., and. Williams, D.R. Measuring the specific surface area (SSA) of freeze-dried biologics using inverse gas chromatography. European Journal of Pharmaceutics and Biopharmaceutics, (2019). 142: p. 216-221.https://doi.org/10.1016/j.ejpb.2019.06.026 (Adapted from Chapter 4)

Duralliu, A., Matejtschuk, P., Dubey, S., Koroma, H., Gubinelli, F. and Williams, D.R. The influence of the closure format on the storage stability and moisture content of freeze-dried influenza antigen. Vaccine, (2019). 37(32): p. 4485-4490. https://doi.org/10.1016/j.vaccine.2019.06.070 (Adapted from Chapter 6)

Book Chapters

Hedberg S.H.M., Devi S., **Duralliu A**., Williams D.R. Mechanical Behaviour and Structure of Freeze-Dried Cakes. In: Ward K., Matejtschuk P. (eds) Lyophilization of Pharmaceuticals and Biologicals. Methods in Pharmacology and Toxicology. Humana Press, New York, pp. 327-351. https://doi.org/10.1007/978-1-4939-8928-7_13 (Adapted from Chapter 4)

Manuscripts in Preparation

Duralliu, A., Matejtschuk, P., and Williams, D. R. (2018). "The Long Term Storage Stability of High Concentration Freeze-dried Immunoglobulin G". *In preparation*. (Adapted from Chapter 5)

Duralliu, A., Matejtschuk, P., and Williams, D. R. (2018). "Effect of moisture on mechanical properties of FD biologics". *In preparation*. (Adapted from Chapter 4)

Conference Papers / Oral Presentations

Duralliu, A., P. Matejtschuk and D. R. Williams. Measuring Collapse of FD Materials using DVS. ***Advances in Biophysical Methods for Protein Characterisation Conference***, **Sicily, ITALY, 2017**.

Duralliu, A., P. Matejtschuk and D. R. Williams. Humidity Induced Collapse in Freeze Dried Cakes: A Direct Visualization Study using DVS. ***8th International Society for Lyophilization and Freeze-Drying Conference***, **Havana, CUBA, April 2017**.

Duralliu, A., P. Matejtschuk and D. R. Williams. Storage Stability of High Concentration Formulations of Immunoglobulin G. ***NIBSC PhD Symposium***, **Hertfordshire, UK, 2018**.

Duralliu, A., P. Matejtschuk and D. R. Williams. Investigating the Long-Term Storage Stability and Effect of Moisture Content on Freeze-Dried Immunoglobulin G (IgG). ***Annual BioProccess UK***. **Edinburgh, UK, 2018**.

Duralliu, A., P. Matejtschuk and D. R. Williams. Effect of Stopper Treatment on Moisture Ingress of Influenza Antigens. ***NIBSC PhD Symposium***, **Hertfordshire, UK, 2019**.

Duralliu, A., P. Matejtschuk and D. R. Williams. The influence of moisture and temperature on the long term storage stability of Freeze-dried Biologics**, *IC PhD Symposium,* London, UK, 2019**

Table of Contents

Abbreviations

API	Active Pharmaceutical Ingredient
BSA	Bovine Serum Albumin
BET	Brunauer–Emmett–Teller Theory
CFR	Code of Federal Regulations
CQA	Critical Quality Attribute
cGMP	Current Good Manufacturing Practice
DNA	Deoxyribonucleic Acid
DSC	Differential Scanning Calorimetry
DTA	Differential Thermal Analysis
DMTA	Dynamic Mechanical Thermal Analysis
DVS	Dynamic Vapour Sorption
ELISA	Enzyme-Linked Immunosorbent Assay
FD	Freeze Dried
FDA	Food and Drug Administration (USA)
FDM	Freeze Drying Microscopy
FMS	Frequency Modulation Spectroscopy
FTIR	Fourier Transform Infrared
GC	Gas Chromatography
GLC	Gas Liquid Chromatography
HPLC	High Performance Liquid Chromatography
IgG	Immunoglobulin G
IR	Infrared
K	Kelvin
KF	Karl Fischer

LN_2	Liquid Nitrogen
LOD	Loss on Drying
LP	Low Pressure
Mcp	Critical moisture content at onset of structural collapse at specific temperature
M_{Tg}	Critical moisture content when glass transition occurs at specific temperature
MHRA	Medicines and Healthcare Products Regulation Agency (UK)
MS	Mass Spectrometry
MTDSC	Modulated Temperature Differential Scanning Calorimetry
MTM	Manometric Temperature Measurement
NIBSC	National Institute Of Biological Standards And Control
NMR	Nuclear Magnetic Resonance
PAT	Process Analytical Technology
pH	Potential of Hydrogen
PPM	Parts per Million
SSA	Specific Surface Area
SEM	Scanning Electron Microscopy
RH	Relative Humidity
T_c	Collapse Temperature
TDLAS	Tuneable Diode Laser Absorption Spectroscopy
T_{eu}	Eutectic Temperature
T_g	Glass Transition Temperature Dried Product
TG	Thermogravimetric
T_g'	Glass Transition Temperature in Freeze-concentrated Matrix
TGA	Thermogravimetric Analysis
XRPD	X-ray Powder Diffraction

Chapter 1. Introduction

1.1 Preface

Water is an essential component from which most known biological life originates from. Almost 71% of our planet is covered by water. It surrounds us everywhere we go and has a predominant function to play in almost all-natural biological activity [1]. However, somewhat perversely and ironically the excess presence of this very simple molecule can sometimes have detrimental effect on stability of many biologics. Throughout human history, man has been trying to exclude the presence of too much water for a variety of reasons. Early on our ancestors realised that an overabundance of water in food can lead to rapid spoilage. Hence crude drying procedures and methods were put in place to dehydrate materials (see Section 1.2). Skip a few millennia forward and you find today's modern biopharmaceuticals industry grasping at the same problems, albeit in a very different fashion. Many therapeutic proteins are inherently unstable in liquid formulation [2]. This brings uncertainty and stability problems, which end up reducing overall profits. One solution to this problem is freeze-drying, otherwise known as lyophilisation, which removes frozen water via sublimation at sub-zero temperatures (see Section 2.1). Since the 20th Century, the number of publications and applications relating to freeze-drying has soared (Figure 1.1) and the technique has been applied in variety of applications including food and biological product stability.

The current worldwide pharmaceutical market for biologicals and biopharmaceuticals is projected at above $50bn [3]. Of the top 15 bestselling pharmaceutical drugs in 2018, 9 of those were mAb or mAb fragment and of those, 3 were freeze-dried (FD) [4]. Most biologics are unstable in liquid solutions therefore freeze-drying is able to offer a proven way to provide greater solid state product stability for long term storage [5]. The aim is to preserve the material by removal of water whilst maintaining the original chemical structure and biological activity. Additionally FD products are able to offer longer shelf lives, more accurate dosing under sterile

conditions and are viewed as being usually easy to reconstitute back to an aqueous solution form, thus being suitable for clinical usage. However, disadvantages include the capital costs of equipment/electricity, long cycle process times and uncertainty over storage and long term stability issues (see Section 2.6).

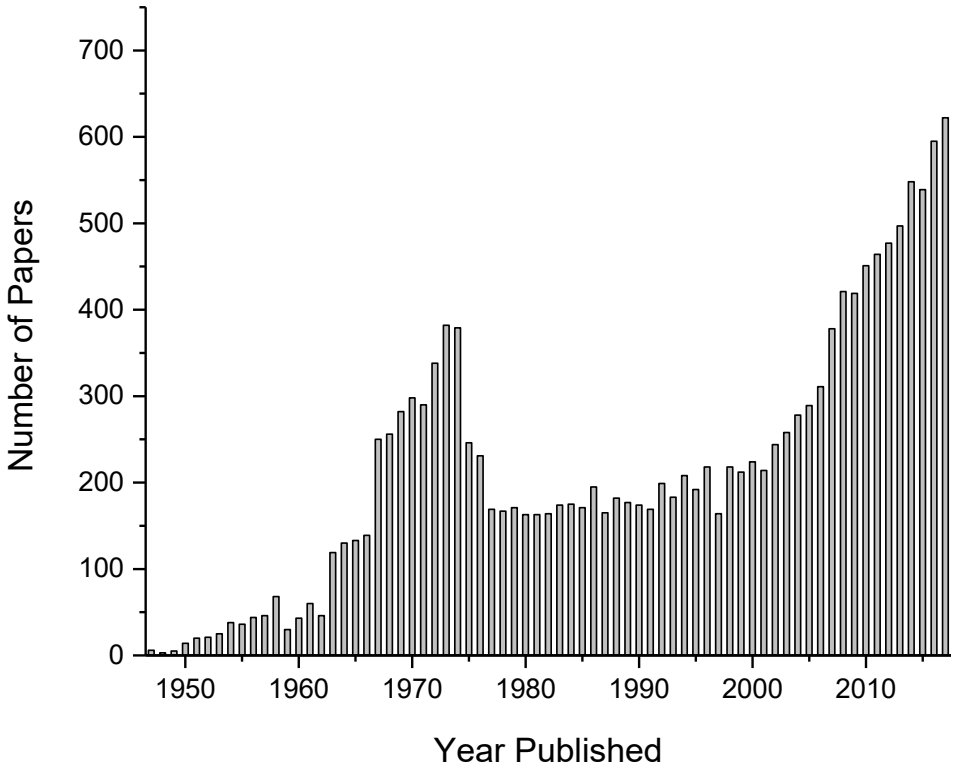


Figure 1.1 Increasing trend in number of papers published per year from 1946 - 2018 containing the search term "Freeze-Drying". Data sourced from the PubMed database from NCBI [6].

Moisture content and storage temperature have been seen as crucial factors to maintaining the long term storage stability of FD biopharmaceuticals [7-9]. Low moisture content has been shown to correlate with prolonged stability for most FD biologics, although there are inconsistent opinions on whether this is always the case and if an optimum or acceptable moisture region may exist for certain products (see Section 2.6.2 for further details). The way in which water is absorbed and interacts with a FD biologic is principally determined by a number of factors such as product formulation and storage conditions. The effect of moisture on product stability is not only a functional concern but also a structural physical one. The hygroscopic nature of FD materials means that the structural stability is also effected and subject to change during long term storage. The emergence of new commercial characterisation techniques, allows the moisture and temperature dependent physical changes to be studied in more detail than ever before (see Section 2.9). In the next few Chapters, this thesis will investigate to provide a clearer picture of how changes in moisture during different storage conditions influences the activity and structural properties of FD material over time. The use of newer characterisation techniques will provide complimentary characterisation data to current traditional long term stability trials for real world FD biological standards. Understanding, characterising and minimising the effects of moisture and storage conditions is crucial to achieving long term storage stability of FD biologics. A better knowledge of how these factors affect solid state stability is essential in order to further build upon suggested general framework of guidelines for future optimisation.

1.2 Freeze Drying History and Current State

1.2.1 Natural Freeze-drying preservation

Throughout early human history, freeze-drying has been deployed (albeit in a crude arrangement) to maintain the integral quality and preservation of certain foods. As early as 1200 BC – 800 BC Peruvian Incas noticed that placing of their crops above the high altitudes of Machu Picchu would result in their produce being FD [10]. Similarly in the Andes Mountains of South America, the indigenous locals would place their potatoes to be dried onto the high altitude mountain tops where the temperature ranges would be found to go below freezing point [11]. Unbeknownst to them was that a combination of these cold conditions aided by the low pressure of the high altitudes would vaporise any internal water and in essence dry their produce, hence extending product shelf life. Comparable cases where dehydration would happen under natural environmental conditions have been noted through early society from Buddhist monks placing Tofu inside snow dug up in Koya to Vikings utilising suitable environmental conditions for drying their fish [12].

1.2.2 Development and Understanding of Process: Post 20th Century

It wasn't until the arrival of the 20th century that a real understanding of the fundamental principles and applications behind freeze-drying began to be understood [13]. In 1890, Altmann successfully produced the first dry tissue by subjecting it to below atmospheric pressure and temperature at - 20°C [14]. In 1905, Benedict and Manning used a chemical pump at pressures less than 1 atm to dry animal tissue samples [15]. Significantly, Bordas and d'Arsonval demonstrated in their 1906 paper that a fragile material which had been frozen could be dried under a vacuum, thus birthing the start of modern freeze-drying [16]. In 1910, Shackell further expanded on the work of Benedict and Manning by creating a vacuum from

an electronically driven pump rather than displacement of air with ethyl ether [17]. He also understood and confirmed that for it to be dried the material must first be frozen before undergoing the process. By the 1920s and onwards, the potential applications of FD was beginning to take a hold for stabilization of certain heat sensitive materials [18]. In 1927, the US patent office granted a submission by Tival [19] that vaguely mentioned the drying of frozen material under vacuumed conditions. In 1934, Elser was also issued with a patent but for a more detailed description of a drying based on dry ice refrigerated cold trap and a vacuum chamber [18, 20]. Earl W. Flosdorf and his colleagues published a paper in 1935 regarding research into what they termed as “lyophilisation” emanating from Greek word *lyophile* meaning “likes the solvent” [21].

Post 1940s saw the first notable industrial and commercial uses of conventional Freeze-Drying come into play (Figure 1.2). During World War 2, freeze-drying apparatus and procedures were advanced (pioneered by Greaves at Cambridge University) to supply FD blood plasma and penicillin to save the lives of thousands of wounded Allied soldiers [13, 17]. The 40s and 50s were deemed as the golden years of FD, with Flosdorf [22] and Harris[17] also publishing the first books on the new subject. In 1951, the first ever symposium on FD was held in London [13], and in later years forerunners such as Professor Louis Rey introduced a series of academic courses focusing on the new topic. The late 1960s saw dried coffee automation developed by Nescafé and Whirlpool Corporation contracted by NASA , helping to create FD ice-cream to reduce weight for the Apollo space missions [23]. Post millennium, FD is employed and has become a common staple technique in billion dollar industries all the way from food preservation to pharmaceutical drug stabilisation [10].

Figure 1.2 Freeze-drying uses throughout history. **A:** Natural drying of potatoes crops high altitudes of Machu Picchu, **B:** WW2 FD Plasma and Insulin, **C:** NASA Dried Ice-Cream, **D:** Large Scale FD in Biopharmaceutical Industry.

Currently it is estimated that almost 50% biologics are routinely FD to provide greater solid state stability, and this share is expected to continue rising [24]. Table 1.1 shows examples of some commercially available antibodies that are commonly FD. For instance the FD antibody, Trastuzumab (also commonly known under the brand name Herceptin), is a major cancer drug that is used for different types of breast, oesophageal and stomach cancers [25]. FD is used to improve protein stability which, when in the liquid state, is susceptible to a host of degradations including hydrolysis mediated loss of its tertiary structures [26, 27]. However, the removal of water can also sometimes destabilise proteins as it is necessary for maintenance of proteins tertiary structure. There is also a current trend towards ever increasing need for high concentration proteins, however there are added complications with these in regards to long term stability, increasing protein-protein interactions and long reconstitution times [28, 29]. Optimisation of each FD material is also of great of importance and is considered on a case by case basis in regards to not only formulation but also moisture content and storage conditions (see Section 2.6 for more details on how these factors might affect stability). Typical thermal degradation studies involve stability trials over 6 - 12 months (or longer in some cases) and are conducted to assess the long term stability of different FD material and to deduce the effects of different storage conditions. These FD biologic stability trials usually involve a range of temperatures including specific stress conditions, in which the information gathered can also be used to develop Arrhenius relationship models to predict the storage at other desired temperatures [30, 31]. These stability trials are also required for documentation and regulatory purposes when it comes to supervisory agencies such as the Food and Drug Administration (FDA) or World Health Organisation (WHO). Being able to prove that the product can have wide storage temperature/moisture range to operate in over a set storage period is vital also for customer expectations and confidence in the product. However, the disadvantages of performing such real-time stability trial assessments is that they are clearly time consuming, expensive and can often be very laborious to perform, especially when testing or assaying for more than one indicator of stability.

Table 1.1 Examples of commercially available FD antibodies [27].

Product Name	mAbs	Company	Indication	Concentration	Formulation
RAPTIVA (efalizumab)	Humanised IgG1K	Xoma & Genetech (2003)	Psoriasis	150 mg/vial 150 mg/mL after reconstitution	123.2 mg sucrose, 6.8 mg L-histidine HCl, 4.3 mg L-histidine, 3 mg polysorbate 20
Xolair (omalizumab)	Humanised IgG1K	Genetech, Novartis	Asthma	202.5 mg/vial 150 mg/1.5 mL after reconstitution	145.5 mg sucrose, 2.8 mg L-histidine HCl, 1.8 mg L-histidine, 0.5 mg polysorbate 20
Synagis (palivizumab)	Humanised IgG1K	MedImmune	Respiratory syncytial virus	100 mg/vial 100 mg/mL after reconstitution	40.5 mg mannitol (5.6% w/v), 5.2 mg histidine (47 mM), 0.16 mg glycine (3.0 mM)
HERCEPTIN (trastuzumab)	Humanised IgG_1K	Genentech (1998)	Metastatic breast cancer	150 mg/vial 21 mg/mL after reconstitution	136.2 mg trehalose, 3.36 mg L-histidine HCl, 2.16 mg L-histidine, 0.6 mg polysorbate 20

Chapter 2. Literature Review

2.1 Freeze Drying Process

Freeze-drying is the removal of water via sublimation at low temperatures. Many therapeutic proteins within liquid formulations can lose their tertiary structure and are susceptible to degradation reactions occurring during prolonged storage and transportation [26, 27]. Freeze-drying is able to enhance long term stability of these therapeutic protein formulations via conversion to a solid state final product (see Section 2.5). Other advantages of freeze drying include reduction in sample weight, elimination of cold chain transportation requirements, being (mostly) easy to reconstitute back and more accurate dosing under sterile conditions. Negatives of freeze-drying can include high cost of power required to run the equipment and processes. Additionally, long cycle times and the need to optimise and characterise each individual formulation for scale up may be considered as a drawback. The freeze-drying process results in solid FD 'cakes' (named due to their porous cake like appearance) with a bright white appearance (Figure 2.1). Typically the process results in extremely hygroscopic cakes with small moisture contents (as low as 1% w/w). How much water is acceptable or optimum for long term product stability can often be a matter of debate, but generally the lower the moisture content is then the lower the chance of degradation occurring, although there may be other factors to consider and this is not always the case for all (see Section 2.5).

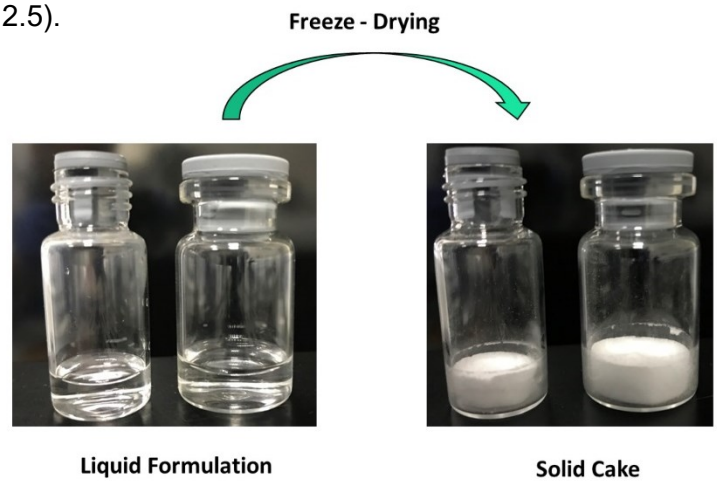


Figure 2.1 Freeze-drying to turn a liquid formulation into a solid state product.

The freeze-drying process is comprised of three principal stages (Figure 2.2):

i) Freezing

ii) Primary drying

iii) Secondary drying

For each of these stages there are a set of controllable process and formulation parameters that can be adjusted and need to be considered to fully optimize the freeze-drying cycle process in order to achieve the targeted and desired product qualities (see following sections).

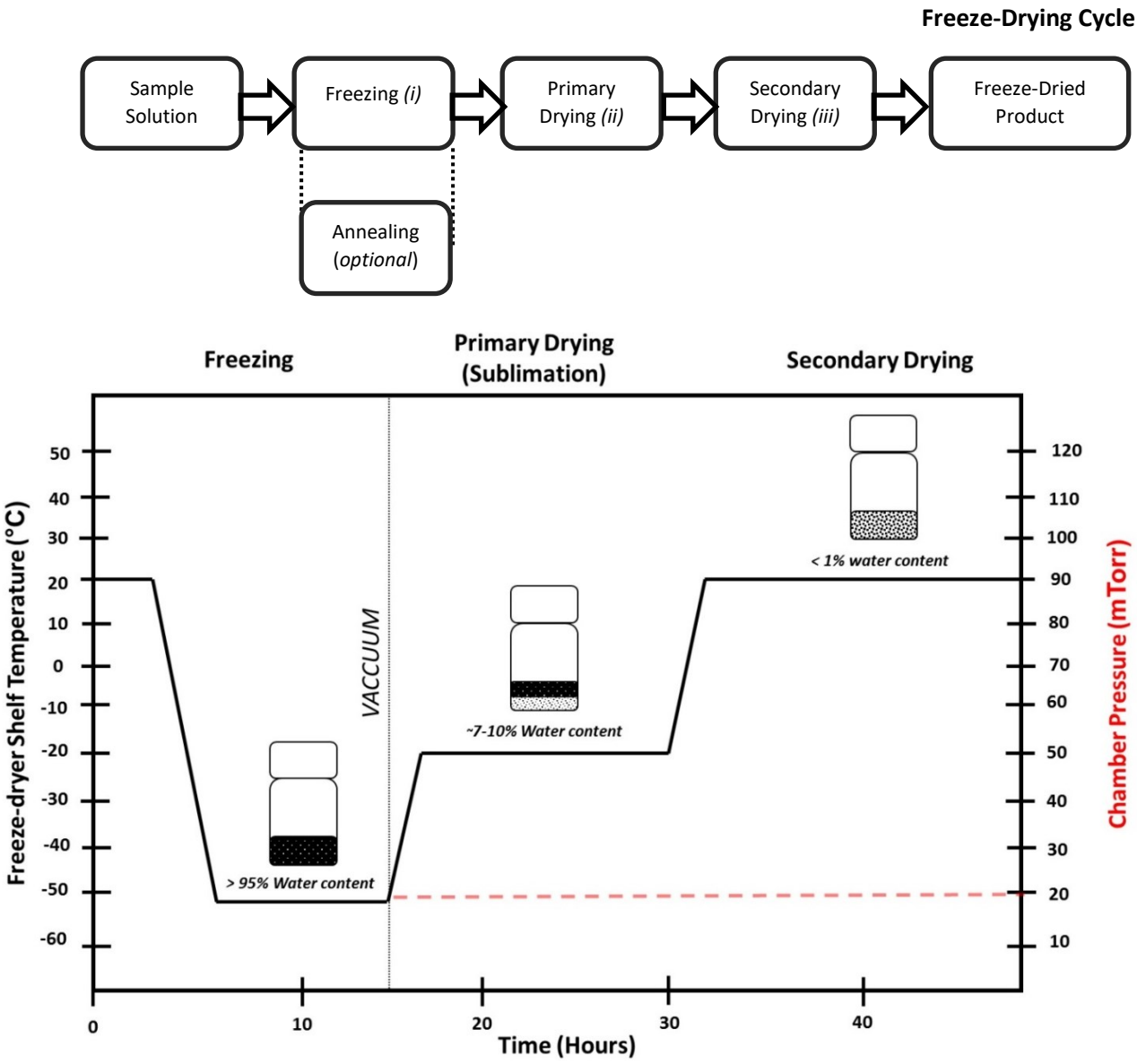


Figure 2.2 Process diagram of typical lyophilisation stage cycles in biopharmaceutical industry.

2.1.1 Critical Temperatures

Solid state materials can be categorised as amorphous or crystalline (Figure 2.3). Amorphous materials can be formed through rapid cooling of liquid melt so that molecules do not have time to rearrange [32]. They also lack the long range order that is characteristic of crystalline materials [33]. Amorphous materials are often described as been “glassy” due to being a solid with a liquid like structure that has extremely high viscosity of $>10^{12}$ Pa•s [32, 34]. Amorphous materials can undergo second order phase transitions, which also include an accompanying change in heat capacity. Crystalline materials on the other hand are defined by lattice and organised structures. Crystalline materials undergo first order transitions where there is a transfer of heat between systems in the surroundings with an abrupt discontinuous change in volume. In the freeze-drying industry, it is common for amorphous or crystalline formulations to be cited as having either a characteristic collapse (T_c) or eutectic (T_{eu}) temperature. The collapse temperature (T_c) refers specifically to when an amorphous material goes past its glass transition point and softens to a point of not being able to support its own weight and collapses. The eutectic (T_{eu}) temperature refers to when a solute material (crystalline) melts which prevents any structure from forming after solvent has been removed. The glass transition temperature in a freeze-concentrated matrix (T_g') and the glass transition temperature of a dried product (T_g) are used to label the reversible transition event where only amorphous material go from a brittle “glassy” state to one of a more rubber like state before melting.

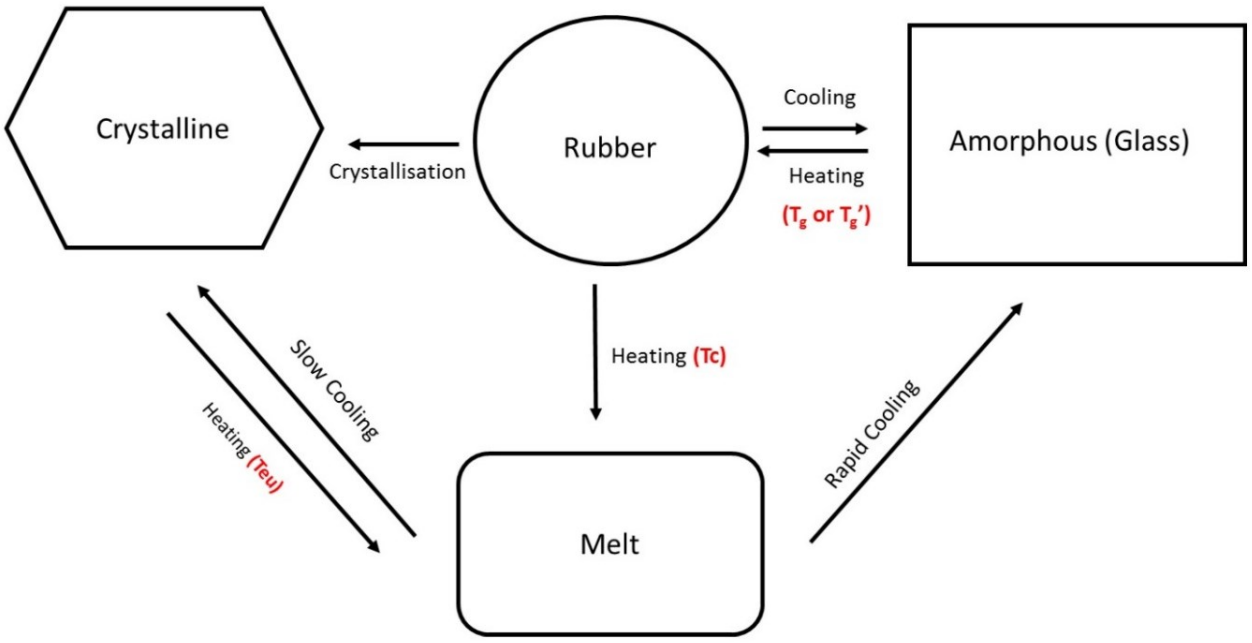


Figure 2.3 Typical states of materials. Adapted from [32].

When undergoing a freeze-drying cycle run, one must be able to characterize the formulation in order to find out these critical temperatures. Differential Scanning Colorimetry (DSC) and Freeze Drying Microscopy (FDM) are two common techniques used to determine such critical temperatures (see Section 2.9 for more details). Knowledge of the critical temperatures can help make an efficient cycle recipe. By not exceeding these critical temperatures the material can be preserved from being of poor product quality and potentially avoiding full or partial collapse during the drying stages.

2.1.2 Equipment and Process Parameters

Liquid samples for lyophilisation are loaded into a receptacle and then attached via a manifold or placed on temperature-controlled shelves within the freeze-drier. In manifold drying, flasks or vials are attached individually onto ports of the drying chamber (relying on external freezing and then using ambient conditions to provide heat for sublimation). Advanced shelf-dryers are more commonly used by industry for greater control of batches. In these, glass vials containing the formulated solution are placed onto a metal tray whereby the temperature is controlled via the shelf (both freezing and heating controlled internally). Freeze-drying machines with shelf dryers can be found at the large scale, able to carry many thousand vials, or in the small lab-scale being able to hold around 200 vials (Figure 2.4). Process parameters and recipes can all be inputted via the computer software on PC and then be directly downloaded onto machines. Parameters that can be changed in the cycle recipe software include: temperature, pressure, time of each step and whether to ramp or hold.

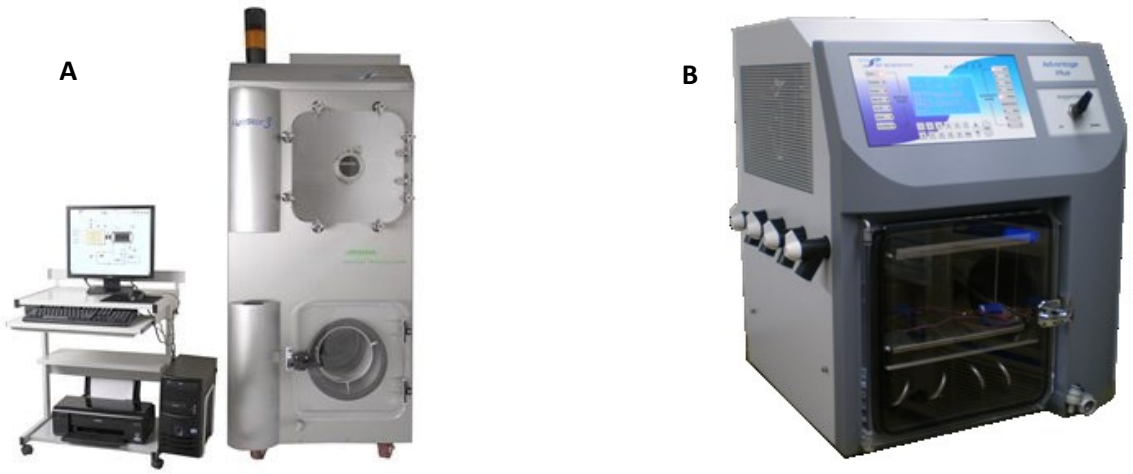


Figure 2.4 Typical Freeze-drying equipment. **A:** Large scale SP Scientific LyoStar Freeze Drying System, **B:** Virtis AdVantage Plus laboratory shelf freeze dryer used in the current study.

2.2 Freezing Stage

Freezing is the first stage of freeze-drying which whereby the solvent (water) is separated from the solution and solutes to form ice. It is at this point that the interfaces between the systems separates into multiple phases to create a periphery between the ice and protein/drug. Freezing has been known to induce many destabilizing stresses in protein drugs. It can lead to an increase of protein concentration where eventually the number of protein to protein interactions increases likely leading to aggregation [35]. Pikal also highlighted an localised increase in ionic strength for some excipients during this stage [36]. Shifts in pH (mostly from crystallization of buffer salts) can also occur but can be minimized by reducing buffer concentration, using lyoprotectants or picking optimum choice of buffer - avoiding phosphate, succinate and tartate [37-39].

2.2.1 Cooling Rate and Freezing Temperature

A solution does not immediately freeze spontaneously at below the thermodynamic freezing point and can still remain as a liquid until random ice nucleation forms. The nucleation temperature can be controlled through a number of ways including electro-freezing [40], gap-freezing [41], ice-fog technique [42], ultrasound induced ice nucleation [43] and more recently with improvements made via the vacuum-induced surface freezing method [44]. The degree of difference between the temperature when the first ice crystal forms (nucleation temperature) and that of the freezing temperature is termed as supercooling. Different freezing methods and solution compositions have different supercooling effects (Table 2.1). Usually liquid nitrogen vial dipping (quenching) produces the highest supercooling, whilst ramped shelf cooling or pre-cooled shelf methods give moderate to low supercooling, respectively. One way to achieve greater protein stabilization is to minimize the surface area of the ice. Higher supercooling rates produces smaller ice crystals with a larger surface area. On the other hand

lower supercooling rates produces larger ice crystals but a smaller surface area. However since cooling rates are limited to about 2°C/min in controlled ramping, there is limited opportunity to change the degree of supercooling within such small ranges [45]. Heller et al. contends that slow freezing results in greater potential for proteins to be damaged in systems with phase separations. Slow freezing also extends the time that the protein exits in a concentrated fluid state, where degradation reactions are amplified [46]. Tang and Pikal recommend that picking a cooling rate of about 1°C/min is a good compromise that gives moderate supercooling, moderate surface area, fast freezing rate and uniform ice structure [35]. Upon the end of freezing stage the product is typically set below the T_g' or T_{eu}. The shelf temperature for freezing should be set below the T_g' if amorphous or T_{eu} if crystalline and be kept long enough at this temperature so as to ensure all the solution has been solidified. Freezing time can be dependent on the fill volume. Usually the larger the volume in the vial then the longer it will take to fully freeze [47]. A safety margin of about 2-5°C below the T_g' or T_{eu} can be used for the final shelf temperature to ensure product integrity.

Table 2.1 Effects of cooling rate on FD product. Adapted from [27].

Cooling rate	Ice nucleation rate	Size of Ice-crystals	Ice sublimation time	Specific Surface Area
High	High	Small	Longer	Large
Low	Low	Large	Shorter	Small

2.2.2 Annealing

Annealing is an optional step by which the product is held for a set period above the final freezing temperature to allow crystallization to occur if crystalline components are present in formulation e.g bulking agents present in the formulation such as mannitol or glycine. Failure to crystallize the bulking agent could potentially have the risk of reducing the T_g' and it crystallising from the dry state solid during storage. This would mean that the storage stability could be possibly compromised [48]. Additionally another risk is vial breakage occurring during primary drying as is commonly seen with high fill depths of mannitol solutions [49]. The ideal annealing temperature should be set between the T_g' of amorphous phase and T_{eu} of crystalline agent to ensure optimum crystallization rate. The best annealing time should be one that allows for complete crystallization to occur. This is largely dependent on the properties and ratio of the crystalline bulking agent used. Higher mass ratios of the bulking agent compared to solute crystallizes at faster rates than lower ratio formulations. Annealing above the T_g' can result in growth of ice crystals which reduces the flow resistance of water vapour in the product. This gives a shorter primary drying time [50, 51]. However the product specific area is reduced which leads to a decrease in the desorption rate in secondary drying. The consequence of this is that not only does it lead to less efficient and longer secondary drying times but also an increase in residual moisture for the final product which can affect overall stability [52].

2.3 Primary Drying (Sublimation)

Primary drying is the process in which pressure is reduced below the triple point (Figure 2.5) and enough heat is supplied to provide a shift in energy (latent heat of sublimation) to allow frozen water to undergo sublimation (transition directly from solid to gas phase). The aim is to bring the product temperature up to the target temperature as soon as possible and hold it there for the constant duration of primary drying. As the water vapour migrates it is captured by the cold condenser in the freeze-dryer which acts as a surface for the water to adhere to (as ice), whilst at the same time also simultaneously shielding the vacuum pump from high levels of water. Usually primary drying takes the longest time of all the stages in freeze-drying cycle due to requiring most of ice to be sublimated off before moving to secondary drying [53]. The primary drying process can be a delicate process step to balance as too much heat can modify the properties and structure of the material. About 95% of water from the solid solution material is removed during this stage [54].

In standard protein formulations, the current guideline practice is to freeze-dry amorphous material at temperatures below the T_g' and T_c [47]. The target product temperature can be put very close to the T_c, as higher product temperatures produce a faster primary drying process. It is stated that for every 1°C increase in product temperature the overall primary drying time is reduced by almost 13% [55]. Trying to optimize the process by increasing the temperature of the product can however run the risk of final product collapse occurring if straying too close to the T_c. As such the policy of having a 'temperature safety margin' is often seen a compromising act between safety, prudence and trying to get faster drying times. A safety margin of 2°C to 5°C below the T_c or T_{eu} is recommended in order to ensure integrity and that the product does not collapse. Overloading can cause loss in chamber pressure control and fluctuating product temperatures termed choked flow [35].

It is argued that drying some FD products above the T_g' temperature could cause instability due to having faster rates of unfolding and increased molecular flexibility. As such when drying below the T_g' usually the target temperature of the product will inevitably be very low (especially when formulations contain stabilizers such as sucrose or salts), which results in longer process time. However, other evidence has shown that drying about the T_g' had no effect on physical or chemical stability and vastly improved cycle times for high concentration proteins [56, 57]. Bjelošević et al. employed such aggressive cycle conditions and showed that the primary drying time could be reduced by up to 54% [57]. For increasing high concentration proteins the T_c is often raised, though T_g' may remain lower [28]. This would depend on a case by case basis depending on each material, tested though individual stability trials.

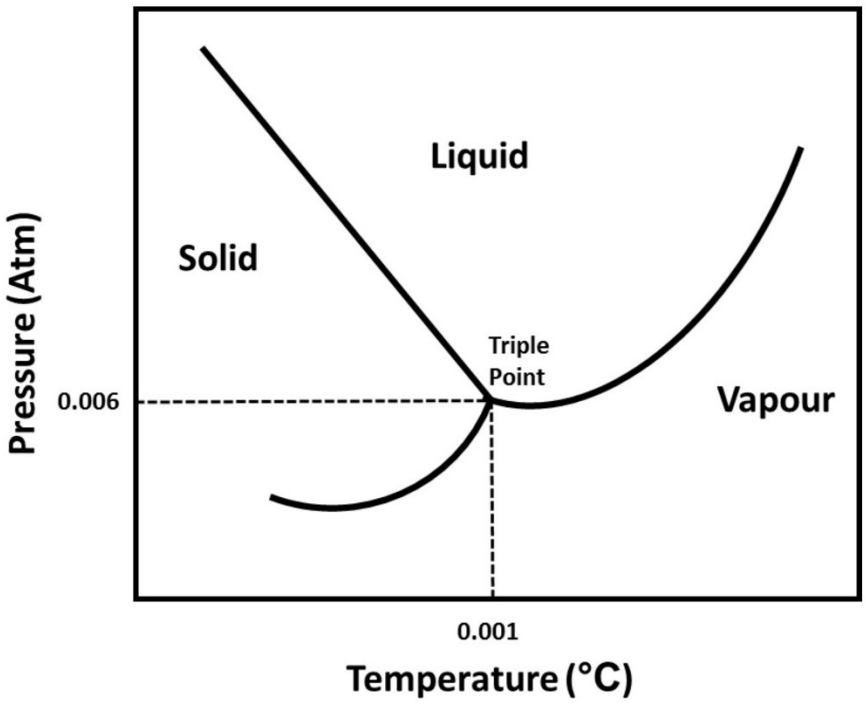


Figure 2.5 Phase diagram of water.

2.3.1 Gas Chamber Pressure and Mass Transfer

The chamber pressure (P_c) is set between 20 to 200 mTorr through application of a vacuum. Anything lower than 20 mTorr is hard to consistently maintain and above 200 mTorr serves little purpose to improving the rates of sublimation. Pikal et al. put forward that for vials, the optimum heat transfer consistency would be given from average chamber pressures of between 100 to 150 mTorr [58]. Chamber pressure is a very important parameter when it comes to affecting both the rate of mass transfer and temperature in freeze drying. In order to create high sublimation rates, the P_c must be lower than the ice vapour pressure at the target product temperature. The sublimation rate is proportional to the pressure difference between vapour ice and that of the partial pressure of water present in chamber. The difference in this pressure is the driving force for ice sublimation to occur.

2.3.2 Shelf Temperature and Heat Transfer

Shelf temperature is another crucial parameter that affects the time of the process. The product temperature is affected by a number of factors such as chamber pressure, shelf-temperature, heat transfer coefficient of vials, thermal history of formulation and the freeze dryer equipment used. Shelf temperature is often measured in the recirculating thermal fluid in the shelf on larger dryers though the surface temperature of the shelf can be monitored by thermocouples. The temperature inside selected vials can also be measured and monitored with thermocouples (Type-T). Vials placed around the perimeter of the sample and aluminium foil on the inside of perspex chamber door can act as thermal and radiation shields in order to minimize/prevent temperature heterogeneity occurring [59]. It is found that the product temperature in vials at the front and back of a shelf of vials are higher than those in the interior, as such having those shields in place can lower the chance of temperature heterogeneity.

2.3.3 Determining End-Point of Primary Drying

The primary drying endpoint time can be challenging to accurately determine. One approach is to usually presume the endpoint to be once product temperature in the vial measured by a thermocouple reaches the same as that of shelf temperature, due to all the ice having been removed [60, 61]. However, this method can be somewhat unreliable due to vials containing thermocouples maybe not being representative of the entire batch on shelf because those vials might have shorter primary drying times due to less supercooling, larger ice crystals and faster ice sublimation [62]. Patel et al. [60] conducted a study, which compared various techniques to establish best choice for endpoint determination, which included: comparative pressure measurement (Pirani gauge vs. capacitance manometer), Dew point monitor, Lyotrack (gas plasma spectroscopy), Process H_2O from TDLAS and Pressure Rise Test (Figure 2.6). The limitations on a single vial method (be it a thermocouple, a wireless thermocouple or a microbalance) are that they are all potentially atypical of the batch whereas the whole batch methods (TDLAS, pressure rise or comparative barometric pressure) give averages for the whole batch and as drying will be distributed over a wider range.

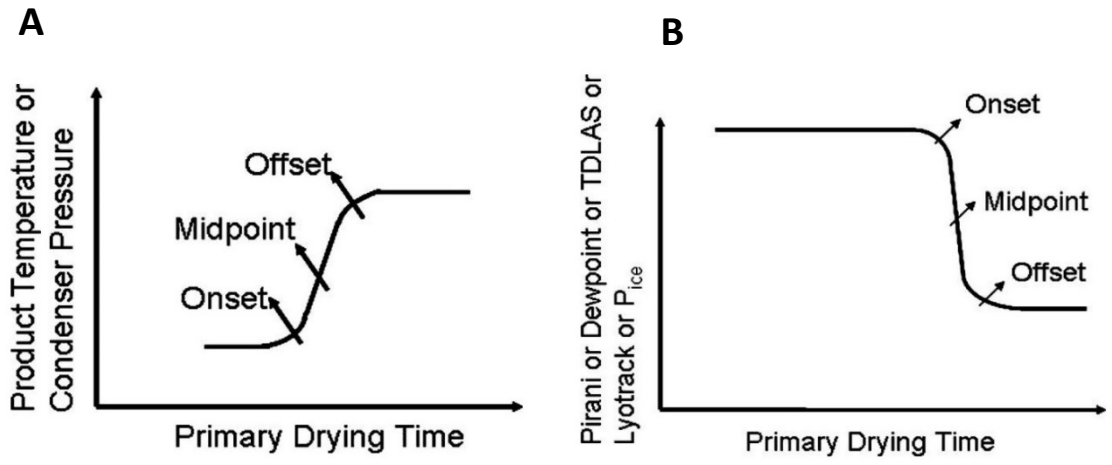


Figure 2.6 Schematic of endpoint determination indicators. **A:** Increase in product temperature or condenser pressure. **B:** Drops in Pirani pressure, dew point, TDLAS (Process H2O), Lyotrack gas composition or P_{ice} (vapour pressure of ice) to indicate end of primary drying. Adapted from [60].

The Pirani pressure gauge measures the thermal conductivity of air. When this occurs, the vapour composition also changes from being all water vapour present to that of air or nitrogen [58]. Higher vapour pressure is observed when there are large amounts of water vapour in the air compared to other gases. The end of primary drying is determined when the measured pressure in the Pirani gauge drops because there is no more water vapour and matches that of the capacitance pressure gauge (which measures actual normal air vapour pressure) [35]. Tunable diode laser absorption spectroscopy (TDLAS) is a spectroscopic technique which measures the water vapour concentration in the chamber and condenser [63]. Laser beams are shined at a gas mixture with targeted wavelengths to measure absorption of water vapour. TDLAS can be used to also measure the sublimation rate from the gas velocity and concentration of water vapour. The point when water vapour concentration decreases is taken as the endpoint of sublimation and hence primary drying. Dew point measurement works by using an electric moisture sensor to measure frost point or dew point. Adsorption of water at a given partial pressure changes the capacitance of a thin film (typically aluminium oxide). When gas composition changes from water vapour to nitrogen it results in the dew point dropping and hence the end of sublimation and primary drying. Lyotrack (gas plasma spectroscopy) is a recent new addition to measuring the end point of primary drying, consisting of a plasma generator and optical spectrometer [64]. A certain radio frequency wave (440 MHz) is transmitted to gases inside a plasma tube, and after electron de-excitation a light is emitted corresponding to a characteristic signature of an atom or molecules. When water vapour concentration decreases it corresponds with a change in the gas composition indicating the end of primary drying. However, one disadvantage is that protein damage can occur due to free radicals [60]. Finally, the pressure rise test is where the interconnecting valve between chamber and condenser is periodically closed and the rate of pressure rise is monitored for short time, typically in 30 second periods. If primary drying has reached the end, there should be a drop-in pressure rise as most of ice has been removed. Of these, the Pirani gauge vs. capacitance manometer was concluded as the best choice for

end point determination. Other advantages of this technique is that it is easy to install without any modification, is cheap and can withstand steam sterilization (although it requires a steam-sterilisable Pirani gauge to be used in GMP operations) [60].

2.4 Secondary Drying Stage (Desorption/Diffusion)

Secondary drying is the final process stage and aims to remove 'bound' unfrozen water molecules from the FD product through the use of elevated temperatures (above those used for primary drying). This allows the bonds to be broken between the FD material and water molecules. Typically, after primary drying the product might still contain 5 - 10 % w/w residual moisture content. The objective of the secondary drying stage is to typically reduce moisture content levels to near or below 1% w/w which has been shown to offer optimal stability in most cases of FD biological based products [65]. These unfrozen water molecules are commonly dissolved in an amorphous solid (our FD cake) to form a solid solution, or it could be reacted in the hydrated content form of a crystalline product [66]. As secondary drying process occurs, the FD material is able to retain its porous like internal structure. The important factor at this stage of the process is making sure that all the ice has been allowed to sublimate (hence the importance of determining the correct endpoint in primary drying) before commencing with increasing the shelf temperature. This is a careful balancing act so that the probability of melt-back or collapse occurring is decreased (Figure 2.7).

2.4.1 Heating Ramp Rate and Chamber Pressure

The chance of product collapse occurring is greatest at the start of secondary freeze drying. High water moisture content present at the beginning means that it produces a

plasticising effect on the product thus lowering its T_g value to give a greater chance of collapse occurring by exceeding the critical temperatures [35]. As the water present is removed, the plasticisation effect is reduced which results in the steady increase of the T_g over time. The shelf temperature, T_s, is increased gradually as faster ramp rates can give rise to collapse for the amorphous material. For amorphous products it is recommended that a slow ramp rate of between 0.1 to 0.15°C/min is maintained to be safe and cautious, at least until the product reaches ambient temperature, which then it can be increased when necessary. On the other hand, for crystalline products you can have a much higher ramp rate as there is almost no risk of collapse during secondary drying. This can be set between 0.3 to 0.4°C/min to ensure optimum efficiency. The desorption rate is not dependant on pressure as long as it is maintained at low levels at below 200 mTorr. In most instances it would be sufficient to continue using the same chamber pressure as that from primary drying.

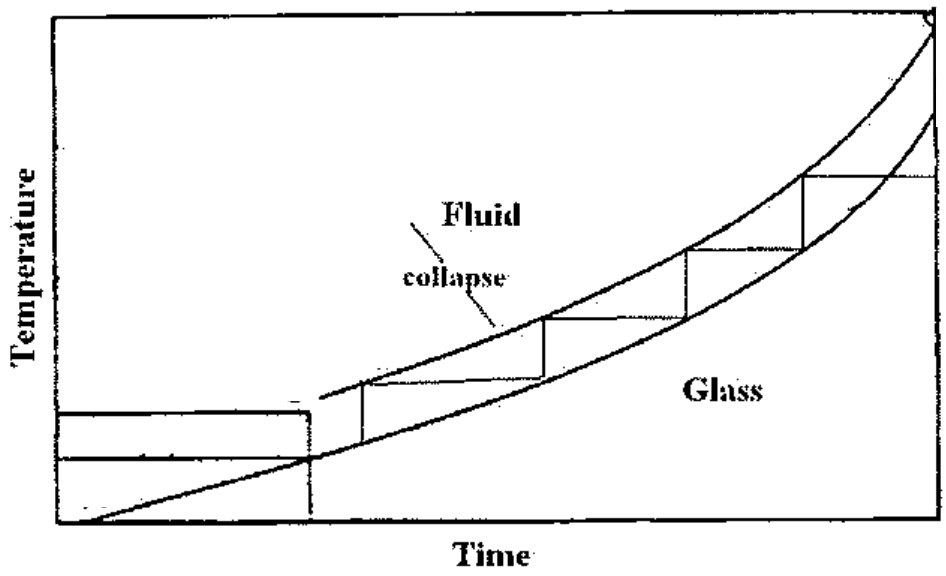


Figure 2.7 Schematic representation of practical secondary drying process based on stepwise temperature ramping, between limits of T_g and T_s. As sufficient water is removed, the T_g increases and thus so can the shelf temperature be gradually increased in time. Adapted from [67].

2.4.2 Shelf Temperature and Process Time

Secondary drying uses the shelf temperature in the region typically between 20 - 60°C for a sufficient time for complete water desorption to occur. The higher the shelf temperature in secondary drying, the lower the residual moisture will be. The higher the temperature is then the faster the drying rate will be and hence a shorter process time. It is beneficial to set it at the higher temperature range (40 - 50°C) but this needs to be balanced with considerations such as product collapse and protein denaturation. Pikal et al. [66] notes that it is better to have a process in which you have high temperature and short times rather than that of one with low temperatures with longer times. This is because the water desorption rates drop significantly with time at a given temperature and past the 3 - 6 hour mark has little effect in reducing the water moisture content. Amorphous products require higher temperatures and longer times compared to crystalline products. Drying conditions can also be dependent on factors such as solute concentration. At high concentrations, there is a smaller specific surface area present and it is harder to remove the water so higher temperatures and longer times are required. Once completed the vials are usually stoppered with rubber elastomer closures under vacuum conditions or backfilled with inert gas such as nitrogen.

2.5 Factors Effecting Stability in Solid State

A successful freeze-drying run is able to produce a solid state material which can significantly enhance the stability of the product. However, this conferred stability is limited to an extent and is conditional on a number of factors such as temperature and moisture content in regards to long term storage [68].

2.5.1 Storage Temperature

One crucial factor for long term stability of FD materials is their storage temperature. In general the higher the storage temperature, the more product degradation is observed, with reductions in both physical and chemical stability of proteins. Ford and Dawson noted that fluctuating temperatures can sometimes produce worse effects rather than a single high temperature [69]. The complex nature of proteins means that it is hard to pinpoint on exact mechanism of instability caused by temperature. However, high temperature has been observed to significantly increase the physical aggregation of FD materials due to increase in protein to protein interactions [68]. The increase in temperature results in higher molecular mobility of proteins thus facilitating aggregation.

Figure 2.8 Schematic of relationship between glass transition (T_g) and storage temperature (T) on molecular mobility of FD amorphous material.

Of course, the effects of temperature on stability is often related to the glass transition temperature (T_g) of the material in dry solid state. The T_g is often one of the most important consideration when attributing stability of a material [70]. As stated previously, this is the temperature at which an amorphous material transitions from that of a glassy brittle state to that of a softer rubbery state (Figure 2.8). Even when an amorphous material is stored below its T_g it can spontaneously approach a more stable state, which is known as relaxation enthalpy [32, 33, 71]. Above the T_g value, materials exhibit a change in physical, mechanical, electrical, thermal and other properties with increased molecular flexibility due to greater free volume and translational/rotational freedom [32, 72]. Amorphous FD materials are considered to be in a more thermodynamically unfavourable state and may be prone to crystallisation. Storage below T_g means that the chances of crystallisation happening is lower compared to when above T_g [73]. Techniques such as Differential Scanning Calorimetry (DSC) are commonly used to determine these glass transitions (see Section 2.9.3 for more detail), though the reported values in literature vary for many materials due to a number of factors such as ramping rate, thermal history of samples, presence of multiple glass transitions and amount of moisture content present [74-76]. The presence of water in particular is of crucial importance due to it being a plasticising agent which reduces the T_g of materials (see Section 2.5.2 for more details). Generally, some consider that the higher the T_g value is for an amorphous FD material then the more stable a product will be during storage. It has been suggested that the T_g of a product be at minimum 20°C higher than the storage temperature during storage and shipment [74, 77]. However, Hancock et al. have the view that the storage temperature should be at least between 50°C below the T_g value in order to have negligible molecular movement occurring [78]. These points of views are very much informed by the theories of mechanisms of stabilisation in the solid state (see Section 2.7 for more detail). In most reported cases, proteins stored above the T_g have usually exhibited greater extents of degradation [79].

However there have also been variable research results in terms of correlating storage stability and T_g. Strickley and Anderson showed that FD human insulin still degraded with the formation of cyclic anhydride intermediates even though it was stored below the T_g value [80]. Chang et al. also warned that even some FD protein formulations stored below T_g can still experience deamidation and aggregation during long term storage [79]. At the opposite end of the research spectrum, there are studies which have described FD materials with lower T_g values being in fact more stable than those with higher ones. Cardona et al. showed that while FD invertase containing trehalose had lower T_g than with maltodextrin, it still managed to retain better activity after 6 hours storage at 90°C [81]. Similarly other studies have shown the inferiority of polymers compared to sugars such as trehalose and sucrose, even though they have higher T_g values probably due to reduced hydrogen bonding with proteins for polymeric stabilisers [82]. Increasing sucrose excipient concentration from 1 to 10% w/w in a rhIL-1ra formulation showed reduced T_g, but actually also had lower rates of aggregation and deamidation during storage at 50°C [79]. Such variable conclusions on T_g and stability could stem from the fact that a host of combined factors including moisture content, individual degradation pathways and different stabilisation mechanisms are all playing a complex and interactive behaviour.

2.5.2 Moisture Content

Moisture content has a significant impact on the solid state stability of many FD biologics. Proteins generally have reduced stability in aqueous solutions irrespective of any preservatives added to improve them [2, 27]. Freeze-drying is seen as the solution to reducing the rates of decomposition in biopharmaceutical proteins and extending shelf life through the solid state, but even in solid state it too can be prone to physical and chemical degradation in regards to long-term storage stability [83]. Freeze-drying removes water based upon sublimation of ice. During the process, the bulk water residing in the ice matrices of frozen

solution is the first to sublimate. Following that, the multilayer water surrounding the protein is removed until it is left with the residual monolayer on the protein surface. Effectively this allows for heat labile biologics to be dried under low residual moisture content and modest temperatures. The final water content varies depending on the parameters of the freeze-drying process and in particular secondary drying [10]. It has also been reported that moisture ingress can change over time under storage due to rubber closures [84] (see Section 2.8 for more details). Water plays a key role in both the conformation and chemical stability of a protein. More complexities arise as water can be present in many forms: free, absorbed, chemically bound and hydration shells which may or may not be always directly linked to stability [85]. Moisture content determination of FD material is usually performed by a variety of techniques including the most commonly used one being Karl Fischer titration (see Section 2.9.1).

For many biopharmaceutical companies, moisture content in FD products presents both an economical and regulatory challenge as they always need to adhere to strict requirements controlling safety before they can be allowed to trade the products where water content is seen as critically important, for instance. The World Health Organisation (WHO) guidelines stipulates less than 1% w/w should be present in FD biological reference standards, although in some rare cases it may be permissible to have higher percentages providing you can show acceptable stability in trials [31]. Residual moisture can be described as being between 1% to 3% residing in the final biological product after FD [85]. The optimum level of residual moisture content is up for debate as there is not one specific all-encompassing rule for every biological product (unless stipulated in specific pharmacopoeial product monographs). However, the general opinion has been that lower moisture content is linked with retaining better protein stability. At higher residual moisture content there is a greater chance of degradation, phase/structural changes and chemical reactions occurring such deamidation, aggregation, oxidation, denaturation and oxidation (see Section 2.6). This is a result of: a) larger availability of water for hydrolytic reactions b) the increased conformational mobility of the protein and c) increased mobility of reactants. An optimum moisture level is

largely dependent on the individual protein physiochemical makeup and its degradation pathway routes as well as its effect on glass transition temperature [2, 86-88].

Many studies have shown a correlation between high moisture content and lower stability. Towns et al. described how increasing water in FD amorphous material leads to increasing mobility due to an expanding of the free volume [89]. Hsu et al. showed that the activity of FD tissue type plasminogen activator (tPA) decreased with higher moisture content at elevated temperatures [2]. FD enzyme invertase formulated with trehalose, showed a reduction in activity within increasing moisture content ranging from 2.1% to 10.9% at 90°C [81]. Costantino et al. found that increasing relative humidity exposure of samples (and hence moisture content) led to greater moisture induced aggregation of FD insulin [90].

Adding to the complexity is the property of water to act as plasticizer which reduces the T_g of amorphous material [91]. As described in previous section, how water sorption affects T_g is an important factor in long term stability in regards to the storage temperature and increasing molecular flexibility (see Section 2.9.2). If a material has a high moisture content or absorbs enough moisture during long term storage, then you could have a situation where the T_g is close to or below the storage temperature to cause instability and even product collapse [92-94]. Bell et al. suggested that greater destabilisation would be seen with progressively decreasing excipient T_g [95]. Increasing moisture content and reducing the T_g means there is also a greater chance that an amorphous materials might crystallise [68]. The crystalline state is more thermodynamically stable compared to amorphous state [34].This would in theory be more desirable for stability but there is a fundamental difference between small molecule API which are often optimally stable when crystalline and the biologics where they naturally are amorphous in structure. A reduction in the T_g can also occur due to the relative amount of remaining amorphous material decreasing, although in some cases it has been observed that if a material crystallises to form a hydrate (such as trehalose di-hydrate) then the T_g can actually see an rise [96]. However, in most cases crystallisation is often negative as it often leads to protein destabilisation due to reduction in amorphous content of a stabilizing excipient.

Costantino et al. attributed mannitol and sorbitol crystallization (at high molar ratios) as the primary cause for the decreases in storage stability recombinant human growth hormone (rhGH) [97]. According to Wu et al. crystallisation of sucrose was responsible for possible shifts in pH of FD fibroblast growth factor (BFGF) [98]. Schmitt et al. also stated that the rate of crystallisation increases with both temperature and or humidity [99] . Moreira pointed out that amorphous sucrose in FD lactate dehydrogenase (LDH) formulations, often crystallised with increasing moisture content and elevated storage temperature [100]. Increase in moisture content was also linearly correlated with that of temperature of crystallisation of sucrose during long terms storage [101]. MP te Booy et al. demonstrated that a sucrose containing FD formulation could change to a crystalline form in just under 1 month at 60°C storage [94]. The amount of amorphous material present can also affect the rate of crystallisation [97]. Polymers such as PVP have been shown to reduce moisture induced crystallisation for carbohydrate based excipients such as sucrose [76].

Table 2.2 Effect of varying moisture contents on approximate glass transition temperatures (T_g) of FD amorphous sugars 10% w/w. Adapted from [102, 103].

Moisture Content (% w/w)	Sucrose T_g (°C)	Lactose T_g (°C)	Trehalose T_g (°C)	Raffinose T_g (°C)
0	74	112	115	103
1	60	102	101	92
2	50	94	90	83
3	32	85	80	75
4	< ambient	80	70	67
5	<ambient	71	60	58

Most of these negative consequences can be avoided and reduced by having a low residual moisture content final product. However, reducing the moisture content level too low can sometimes also lead to potential unwanted side effects. Over drying is a symptom that can often arise, which for many FD products also reduces stability and activity. This is possibly due to loss of hydrogen bonding with the protein and or overexposure of protein surface that creates an environment for undesirable reactions to occur. Wang acknowledged in one of their FD formulations that over drying did cause higher insoluble aggregates to form and exhibit a loss in protein activity [104]. Chang et al. showed FD Recombinant Human Interleukin-1 Receptor Antagonist (rhIL-1ra) with relatively low moisture contents at 0.8% had lower stability than formulations with high moisture content of >3% w/w [105]. Matejtschuk et al. described how over drying of insulin caused damage with increased levels of degradation products detected [30]. Indeed for a reference plasma used to measure the labile coagulation factors Factor V and Factor VIII, Hubbard et al. also showed that a more modest moisture content with optimised formulation demonstrated better long term stability than one with extremely low final moisture content [106].

Since both too high and too low moisture can be detrimental to the final stability of the FD material, it is natural to assume that an acceptable or optimum “goldilocks” moisture region may exist for each product in order to have optimal storage stability. Some have postulated that a bell distribution relationship might exit. Greiff et al. hypothesised that the thermostability of FD proteins may even correspond to that of a bell-shaped distribution with moisture content [86]. However, others have suggested that a combination of different relationships existing between moisture and stability at different storage temperatures. Pikal et al. saw that for FD human growth hormone at 40°C the relationship between moisture and aggregation was linear while at 25°C it took a bell shaped curve [107]. Of course these acceptable moisture contents ranges or relationships would need to be worked out on a case by case basis in context for each individual formulation and be based on long term stability trials.

2.5.3 Formulation and Excipients

The choice of excipients (classified as stabilizers, bulking agents, preservatives, buffers or surfactants) are also an important factor to consider for stability purposes for a given formulation [108]. As mentioned previously, sugar based excipients can maintain stability during the FD process and also enhance the long term stability of post FD in the solid state [27]. In addition to stability, excipients can also be added to increase cake stiffness and strength in order to improve mechanical properties of FD materials, especially during transportation [109, 110]. Sucrose and trehalose are two of the most commonly used disaccharide excipients utilised to aid the long term storage stability of products. It is interesting to note that in nature how something similar is observed in anhydrobiotic organisms, which rely on synthesis and accumulation of trehalose and sucrose during prolonged periods of droughts for survival [111]. Similarly trehalose and sucrose have been shown to protect bacterial membranes and proteins during drying [112]. It is theorised that one possible mechanism for this is hydrogen bonding, which help stabilise surface proteins native structure, and replaces the bonds lost due to water dehydration (see Section 2.7 for more on theories and mechanisms of stability). In some cases, addition of excipients can sometimes prove detrimental to product stability and also have an effect of FD cycle optimisation. Duru et al. showed that increasing the sucrose concentration for their FD influenza antigen reference standards from 1% w/w to 2% w/w caused structural collapse of FD cake, reducing its visual elegance [113]. Chang et al. showed that the activity of porcine pancreatic elastase was reduced significantly when adding increased concentrations of ascorbic acid to the formulation at 40°C storage and 75% relative humidity [114]. However, in several studies by Schersch et al. they also concluded that the physically collapsing of FD IgG1 during the freeze-drying process to different extents by using a combination of different amounts of stabilizers/bulking agents, still produced a product with good protein stability post FD [115-117]. It is worth noting that the moisture content of these collapsed cakes was similar to the non-collapsed, usually collapsed cakes have raised moisture contents.

2.6 Types of Instability Pathways in Solid State

During storage, FD solid state materials can undergo a variety of physical (e.g non-covalent aggregation or denaturation) or chemical (e.g covalent aggregation, deamidation, oxidation and Maillard reactions) degradations [68, 83]. Physical degradation can involve changes in high order structure such a secondary or above, while chemical degradation typically involves covalent modification of primary structures through bond formation/cleavage [83].

2.6.1 Denaturation

Denaturation often occurs when the resulting folded globular proteins unfolds and there is a loss off tertiary and sometimes secondary structure. Many physical stresses can induce denaturation effects including the FD process at low temperatures such as the ice-surface interface [118]. Surface adsorption can also cause physical and loss of native protein structure at the solid-liquid-air interfaces [119]. The consequences of this is that amino acid residues might be exposed due to unfolding of the protein further facilitating instabilities such as aggregation and chemical modifications.

2.6.2 Aggregation

Protein aggregation is the process in which mis-folded proteins clump and accumulate together and is one of the most common routes of instability with potential to also trigger immunogenic responses in patients. Aggregation can occur as physical (non-covalent) or chemical (covalent) processes in solid state materials. In non-covalent aggregation denatured proteins might associate with each other through hydrophobic interactions in order to shield

any hydrophobic amino acid residues away from water [120]. Covalent-aggregation (chemical) occurs when bonds are formed. This can be through disulphide bond formation or exchange. Constantino et al. stated that moisture induced both covalent and non-covalent aggregation in FD insulin [121]. The rate of aggregation has been linked to high storage temperature conditions [122]. Moisture content has also been linked with aggregation due to increased mobility of water molecules around protein. Jordan et al. showed for FD BSA, that the rate of aggregation was largely dependent on the moisture content [123]. The FD processing conditions can also lead to insoluble aggregates in FD IgG formulation due to denaturation at the ice/freeze concentrate interface [118].

2.6.3 Hydrolysis

Hydrolysis refers to the chemical breakdown of a compound due to reaction with water molecules. Some peptide bonds are particularly prone to hydrolysis and so hydrolytic potential may be related to amino acid sequence for a given protein. Even though moisture content may be low in FD product, hydrolysis can still occur during prolonged storage in solid state. Wu et al. reported the occurrence of hydrolysis in FD fibroblast growth factor (BFGF) [98]. Other studies have shown that some FD materials with certain excipients can be more prone to hydrolysis. Li et al. proposed that FD Human Relaxin formulations with glucose hydrolysis occured (elimination of serine at the C - terminal of the B chain) which resulted in greater losses of activity when compared to either mannitol or trehalose formulations [124].

2.6.4 Deamidation

Deamidation can occur as a one of the degradation pathways observed for FD material during storage. It is a chemical reaction which the functional amide group in side chains of either asparagine (Asn) or glutamine (Gln) are converted to produce a carboxylic acid such as aspartic acid or glutamic acid. Pikal et al. reported that deamidation occurred for FD rHGH [107]. At elevated temperatures of 50°C, Chang et al. observed that FD interleukin-1 receptor antagonist (IL-1ra) underwent both aggregation and deamidation [79].

2.6.5 Oxidation

Oxidation is a type of chemical reaction in which electrons are transferred from one chemical species to another. Typically side chains of amino acids such as cysteine (Cys), methionine (Met), tryptophan (Trp), histidine (His) and tyrosine (Tyr) can be damaged by reaction with a number of reactive oxygen species [5]. Regardless of stoppering under vacuum or nitrogen gas, it has been observed that oxygen as low as 1% in the headspace can still have drastic effect on stability [83, 107]. Pikal et al. showed that methionine oxidation occurred inside FD hGH vials due to the presence of only 0.4% oxygen from the atmosphere at room temperature [107]. Methionine oxidation can also be independent of how much moisture there is present in the samples [68].

2.6.6 Maillard reaction

The Maillard reaction (or browning reaction) refers to when an amino group in proteins, reacts with a carbonyl groups of reducing sugars to form a product with a distinctively brown visual appearance [125]. Maillard reactions are often observed in the food industry but can also occur for a variety of FD biological materials if reducing sugars were present in formulations. Browning and discolouration was noticed in IgG1 formulated with lactose [126]. At accelerated storage temperatures, it was found that sucrose-dextran IgG probably also had undergone Maillard reaction [127]. The rate and colour change of Maillard reactions can also be influenced by amount of moisture and water activity [128]. Typically the degradation rate can increase with increasing moisture content and when the RH% is at or above 25% [129]. As a consequence of unwanted Maillard reactions occurring, non-reducing sugars should be used for excipient stability and moisture content should be kept at a minimum especially at higher storage temperatures. However, in some rare cases it has been suggested that it might be possible for non-reducing sugars such as sucrose to break down at high temperatures into reducing sugars such as glucose or fructose and for Maillard reactions to subsequently occur [130, 131].

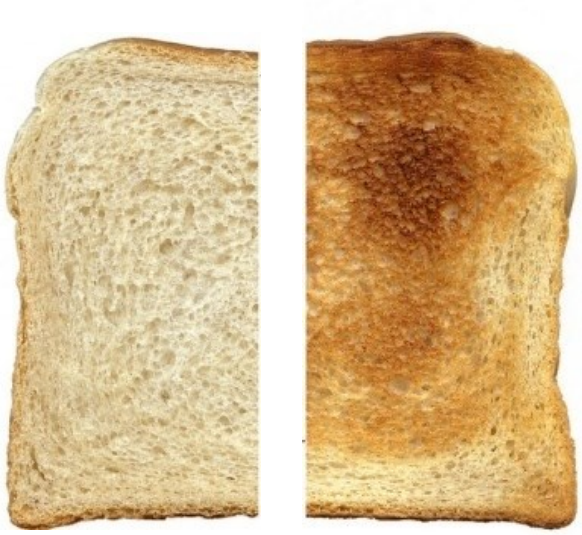


Figure 2.9 Maillard reaction occurring in bread during heating causing amino acids and reducing sugars to react forming distinct browning discolouration.

2.7 Theoretical Mechanisms of Protein Stability by Excipients

The two most common theoretical mechanisms for protein stabilisation in FD solid state is that of i) the water replacement theory or ii) the vitrification theory otherwise also known as the glass dynamics hypothesis (Figure 2.10) [27, 132]. However, as Cicerone pointed out, it can be often difficult to discriminate between these two hypothesis due to the fact that that they are not mutually exclusive [133]. Mensink et al. also agrees that that there is no single unifying theory or hypothesis on stabilisation due to multiple potential degradation routes for each protein, which in addition are effected by various stress conditions and factors [128]. Both theories describe how the resulting preservation of structure is due to preventing molecular mobility and structural changes from occurring. Interestingly, Grasmeijer et al. contends that water replacement is the dominant mechanism for stabilisation as long as there is sufficient vitrification (i.e the glass transition is higher than storage by 10 to 20 °C). However, this deviates when the T_g reaches at or above the storage temperature with the protein becoming immobile and water replacement mechanisms dominating stability [134].

2.7.1 Water Replacement theory

This theory states that protein confirmation is maintained by water interactions (in the form of hydrogen bonds) in solution [83]. When undergoing drying, these hydrogen bonds can be broken and thus need other molecules to replace these in order to retain stability. Sugar excipients can be used as a substitute to form these new hydrogen bonds and maintain protein integrity and structure upon drying. Several spectroscopic studies have indicated that replacement of water with excipients allows for the preservation of native structures of proteins both in freezing stage and post FD [135, 136].

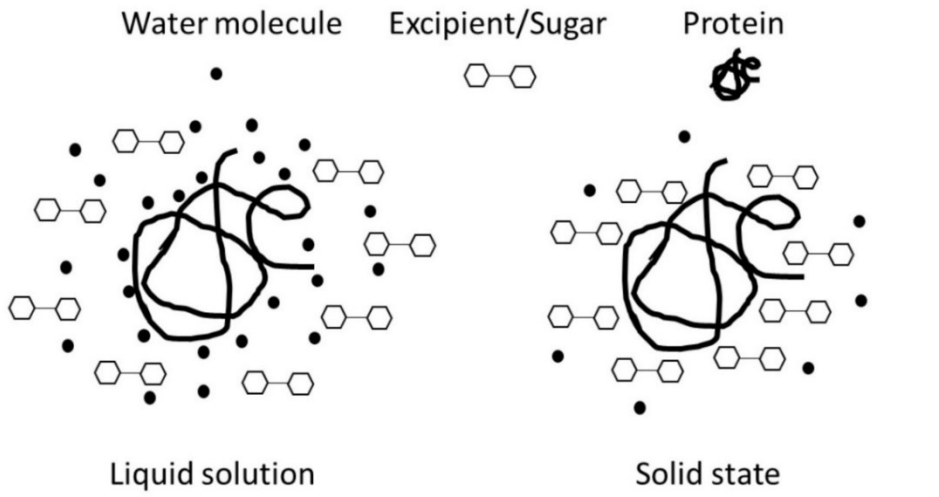
2.7.2 The Vitrification theory or (Glass Dynamics Hypothesis)

The alternative theory positions that the excipient sugar immobilizes the protein inside a sugar matrix thus preventing any translational molecular movement occurring and hence any degradation. It is stated that the thermodynamic state of the sugar is responsible for the translational molecular mobility of the sample. For example, in an amorphous sugar the direct difference of the T_g and the storage temperature (T_g - T) will determine the translational molecular mobility [89].

2.7.3 Local versus global mobility

Another refinement of theories of stabilisation is the hypothesis that local mobility (β-relaxation) can be a better predictor of stability than global mobility (α-relaxation), which vitrification is based on. β-relaxation processes are often difficult to measure requiring facilities with neutron backscattering for example for testing. Via experimentation with plasticisers and anti-plasticisers, Cicerone and Douglas found that the high frequency of β-relaxation processes correlated with protein degradation in glasses (relaxations coupling to local motions and to diffusion of small molecule reactive species) [137]. It is hypothesised that global mobility (α- relaxation) could correlate with physical degradation, whilst local mobility (β-relaxation) links to chemical degradation [128]. However, this again may not be a general rule for all. Yoshioka and Aso found evidence for chemical stability of amorphous products being affected by both global mobility and or local mobility, subject to different length scale of molecular mobility [138].

A



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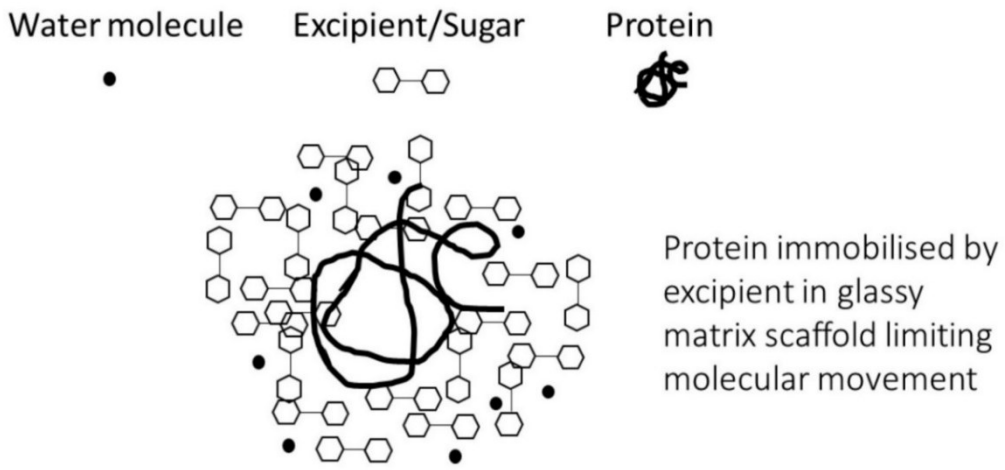


Figure 2.10 Schematic representation (not to scale) of **A**: water replacement theory and **B**: vitrification theory in FD materials. Adapted from [139].

2.8 Stability Concerns from Vials and Stoppers

During prolonged storage, moisture content has been observed to rise for hygroscopic lyophilized materials in rubber closure-stoppered vials [69, 84, 140]. As stated in the previous sections, water can facilitate a host of reactions and is a plasticising agent that can significantly alter and reduce the T_g of a material, thereby affecting storage stability and likelihood of phase transitions occurring. Moisture ingress in vials during storage may arise from a number of places such as either due to trapped moisture in vial headspace, moisture directly from the rubber stopper or from micro-leaks at the vial rubber-seal [141]. There is debate as to efficacy of vials vs. ampoules. Glass ampoules are generally considered to be the gold standard for FD sample storage, but the more complex manufacture and higher costs are disincentives. Some studies showed that stoppered vials could be just as good as ampoules in some conditions however in a recent study it has been shown that for ampoules, oxygen and moisture contents did not detectably change even under stress conditions compared to vials [84].

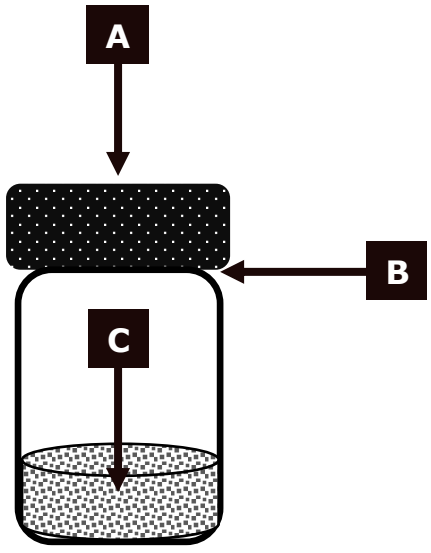


Figure 2.11 Mechanisms of moisture ingress into freeze-dried cake mass through vials with rubber stoppers. **A:** Moisture diffusion from outside environment through the stopper, **B:** Moisture from micro-leaks or improper sealing at stopper vial interface and **C:** Moisture directly from the stopper itself.

Moisture adsorption and desorption in the rubber stoppers depends on factors such stopper polymer composition, aeration procedures and storage conditions [142]. Past studies have shown that drying via heat treatment of stoppers can effectively remove most of the moisture present and reduce the potential for moisture ingress into the product. As a result there even have been cases of where if the rubber elastomer is dry enough, there could be a reversal in the opposite direction whereby the moisture content of the cake is reduced [104].

There is danger in particular for low mass FD material that are susceptible to moisture ingress, and any developments in reducing moisture content would be beneficial for stability. It is easy to underestimate the impact that moisture ingress from stoppers can have on moisture content in such FD cakes. This is especially critical for extremely low mass FD materials - where for example a dry weight 10 mg of FD antigen, would only require about 100 µg in moisture from a stopper to raise the FD moisture content by just 1% w/w. However, heat drying procedures for rubber stoppers pre-lyophilisation are underutilised at the present in the industry. There is further need to demonstrate the benefits of such procedures for real world FD standards and spread more awareness in the industry. Utilising these largely overlooked information to reduce moisture could further enhance stability of moisture sensitive FD products over prolonged storage.

2.9 Freeze Drying Product Characterization

Product characterization is at the core of all preliminary work regarding formulation and process improvement in freeze-drying. Additionally, there is a need for post process analysis to monitor the long-term effects needed to be characterised for further stability and regulatory purposes. Common FD product characterisation techniques are presented in the following sub-sections below.

2.9.1 Moisture Content Determination

2.9.1.1 Gravimetric (Loss on drying)

The gravimetric method measures the total change in weight of a material before and after drying to determine the moisture in a sample. The initial weight is subtracted by the final weight and then divided by the initial weight and multiplied by 100 to give a percentage (% w/w). May et al. [143] describes the approved test method (Code of Federal Regulations 21 CFR 610.13) involving measuring the maximum loss of weight of a sample equilibrated over a desiccant such as anhydrous phosphorus pentoxide (< 1mmHg at 20-30°C) until a constant mass has been reached. Sample preparation would need to be handled in a controlled low humidity box in order to minimise outside interference from the environment effecting the results. One of the disadvantages of this method is that it may only remove the surface moisture of samples and not the more firmly bound moisture to protein or excipient thus giving an underestimation. Past studies have shown that gravimetric method seems to slightly underestimate the moisture content when compared to other methods. Another disadvantage of the gravimetric method is that it requires large sample masses (which mean multiple vial contents might have to be pooled) of between 100-200 mg for each analysis. Although the method is rather straightforward in concept, it can be relatively more time consuming and fiddly to perform when compared to other methods for water determination.

2.9.1.2 Karl Fischer Titration

Karl Fischer (KF) titration is one of the most widely adopted methods for accurate water content determination. The German chemist Karl Fischer first described the method in 1935, based upon the modified Bunsen reaction between iodine and sulphur dioxide to detect trace amounts of water [144]. The Karl Fischer reaction can be written as follows:

$$ROH + SO_2 + R'N \rightarrow [R'NH]SO_3R + H_2O + I_2 + 2R'N \rightarrow 2[R'NH]I + [R'NH]SO_4R \quad (2.1)$$

The base (R'N) is typically imidazole although in past pyridine has also been used but abandoned due its carcinogenic properties. The alcohol (ROH) typically methanol, reacts with the sulphur dioxide (SO_2) to form the intermediate alkylesulfite salt (R' NH]SO_3R). This intermediate is then oxidised by the iodine (I_2) to from alkylsulfate salt ([R'NH]SO_4R), whilst in the process also consuming water (H_2O). Both water and iodine are consumed in a 1:1 mole ratio, with the reaction occurring until all the water present has reacted and been consumed. The method can be operated in two modes: either volumetric or coulometric. In the volumetric method the iodine is added mechanically during the titration and the amount of water determined is based upon the amount volume of reagent consumed in the burette to get an end point (stoichiometric). In coulometric approach, the iodine is generated electrochemically from titration in the cell where water is quantified by how much total charge has passed up to the end point, where 1 mg H_2O is equivalent to almost 10.72 coulomb. The coulometric method usually has higher precision and is best used to determine samples below 2% moisture, while volumetric is best for samples with moisture contents above that. The adoption of automated KF systems has meant easier and faster experimental times in the number of samples being able to be run. KF is widely used to measure moisture content of samples after the freeze-drying process to determine if the cycle has been sufficient to produce low moisture contents desired. Another common use for the method is to determine if any moisture ingress during long term storage of FD vials has occurred. The consequences of high moisture can lead to a deal of unwanted reactions and degradations and thus being able to monitor any changes is

of vital importance. Usually these long term trials are accompanied with highly stressed temperature storage conditions. Another variation of the vapouriser coulometric KF titration has also been adopted to measure the moisture content in rubber stoppers using a vapouriser oven. The stopper/sample is placed inside a heating tube with a controlled set temperature of between 150-250°C. As the sample is heated up, the moisture is released and the vapour is sweapt through by a carrier gas (usually dry nitrogen) down to the titration cell where the gas is bubbled through the KF liquid, where then the normal KF reaction occurs. Past studies have shown how rubber processing conditions and storage have an effect in the long term moisture of the stoppers. Moisture transmission through rubber stoppers can in turn can have the consequence for the sample cake in the vial experiencing an increase in moisture content over time. The KF method is a destructive method and the sample cannot be reused. Other problems that can arise from the method is that not all sample might be wholly soluble in the KF medium or unwanted cross-reactions with iodine/reagent components might occur, thus effecting the accuracy of the water determination. Careful sample preparation is also required as any contact with the outside atmosphere will mean an uptake in moisture of the sample, especially if it is highly hygroscopic. Usually samples have to be prepared in a dry-environment box or a dry-air bag purged with nitrogen.

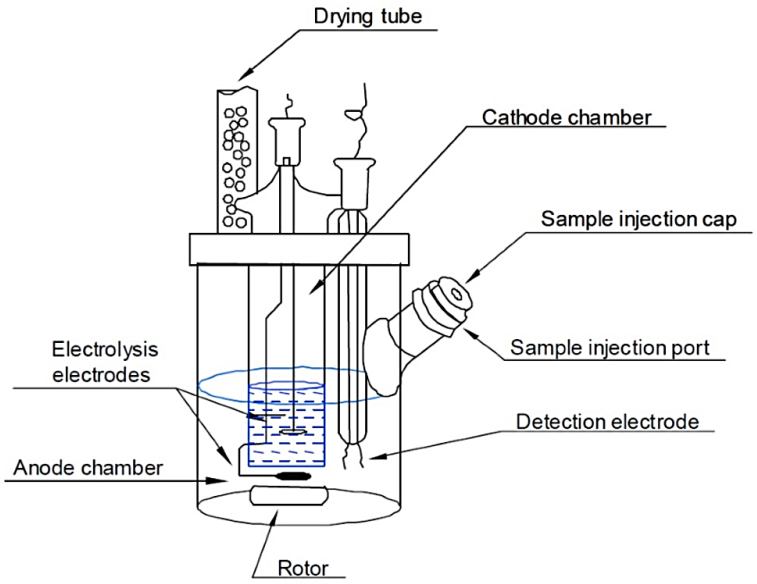


Figure 2.12 Schematic diagram of electrolysis cell for coulometric Karl Fischer titration. Adapted from [145].

2.9.1.3 Thermogravimetric analysis (TGA)

Thermogravimetric analysis measures the continuous weight loss of a sample as the temperature is increased. The temperature is increased at a constant heating rate over or a function of time. Changes in the sample mass can be related to thermal events. This technique can be used to measure the moisture that is driven out of the sample. It can also measure any other volatiles that are given off as heating continues approaching sample decomposition. Thus one of the drawbacks of the technique is that some knowledge of elemental composition of your sample must be known in order to understand the profiles that are given. This method is destructive and you are unable to perform any additional tests on the sample. Like other moisture determining techniques, TGA is susceptible to outside environmental moisture effecting results. Some studies have showed that panning (inserting sample in metal pan) in a dry box gives significantly lower moisture content results when compared to panning in ambient conditions. Additionally, TGA gives slightly higher moisture content values that that of coulometric Karl Fischer. However, the advantage of TGA is that is more sensitive to measuring moisture determinations of very low dry weight samples (< 5mg) compared to Karl Fischer which requires about 10 mg or higher for accurate determinations. The specificity of the TGA method is greatly increased by hyphenation with a mass spectrometer - termed evolved gas analysis. The difference between bound water and water resulting from thermal decomposition are thus clearly distinguished [146].

2.9.1.3 Near-infrared spectroscopy (NIR)

Near-infrared spectroscopy is a non-intrusive and non-destructive method of determining moisture content in FD materials in vials. It utilizes the near-infrared region in electromagnetic radiation (EM), with a fibre optic probe being used to measure reflectance from 1100 to 2500 nm [89]. Kamat et al. showed that NIR could be used to measure residual moisture lyophilised sucrose with good correlation to that of Karl Fischer (R^2=0.97) results [147]. Lin et al. was able to determine the moisture content of lyophilised protein pharmaceuticals with NIR to the similar precision as that of Karl Fischer method [148]. Multipoint NIR has even been used to measure the moisture content of batches in-line during the freeze-drying process [149]. One of the advantages of NIR is that is considered a rapid technique with usually a 20 second analysis time between samples. Another advantage is that there is very little preparation required and since the container remains closed during testing, there is no risk of outside environment posing a risk and introducing error [89, 147]. NIR is particularly useful when trying to determine the moisture content of infectious biological reference materials, which might otherwise be a hazard risk when dealing with other techniques [146]. However, NIR requires a construction of a standard curve with same lyophilised product which is cross-referenced to Karl Fischer analysis in order to deduce correlation. A calibration curve would be needed for each container format and or fill depth used.

2.9.2 Water Sorption Isotherms

For highly hygroscopic material such as FD products, water can be detrimental to both their physical and chemical stability. When a FD material vial is exposed to the atmosphere, it will naturally absorb moisture and eventually equilibrate if the relative humidity and temperature is constant. The resulting uptake in mass corresponds to the uptake of water present by the FD sample. A series of water sorption experiments can be carried out a specific temperature to produce water sorption isotherms of different types plotting the resultant change in mass (equilibrating moisture content) at that particular water activity (ranging from 0 to 1) or labelled in terms of relative humidity (%). In the past, typical water sorption isotherms have been constructed from static based methods using saturated salt slurry (Figure 2.13).

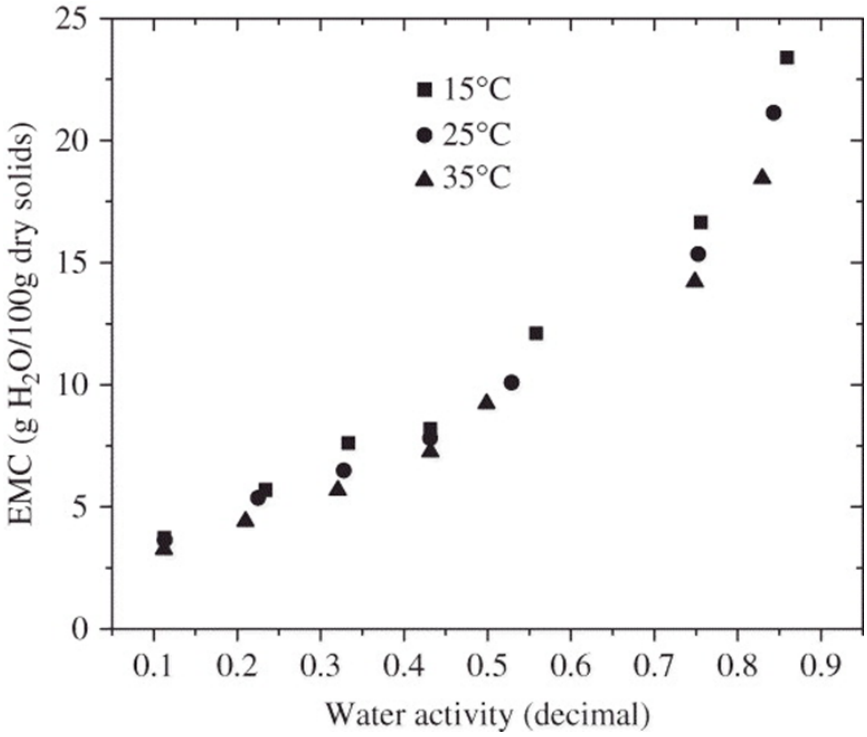


Figure 2.13 Typical example of water sorption isotherm for FD Bovine Colostrum. Seven saturated salt solutions were prepared give different relative levels of humidity in the range of 0.11–0.86 at 15°C, 25° and 35°C. Adapted from [150].

Usually at the start of water sorption isotherms, for an amorphous glassy material, there is low uptake and a small change in mass, due to water adsorbing primarily at surface. When the monolayer of water molecules has completely formed at the surface then you have a transition into bulk absorption occurring into the material. This is characterised by an increase in mass uptake rate in water isotherm graphs. This also corresponds with a phase transition from a glassy phase to a rubber phase due to increased molecular flexibility (Figure 2.14).

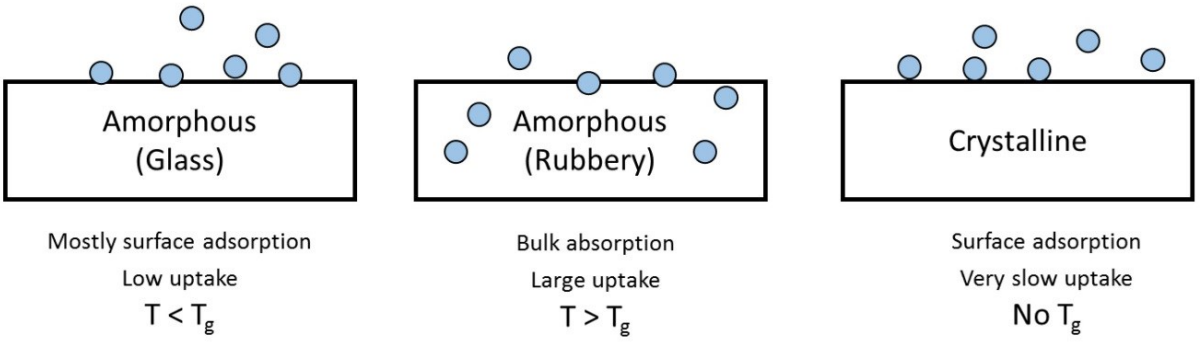


Figure 2.14 Schematic of typical material state change during water sorption.

Water can act as a plasticizer that reduces the T_g for an amorphous polymer where thereby an increase in temperature would result in phase transition from one of dynamically constrained to relaxed [151]. The binding and interacting of water with proteins is a direct result of intermolecular forces such as hydrophilic/hydrophobic and ionized groups on protein working in tandem. Sorption isotherms can be used to describe the equilibrium of the water sorption of a material at constant temperature. It has been stated by some that water sorption isotherms for a protein can be split into three distinct regions [152]. The first region shows the binding of proteins to highly charged regions such as for the charged or highly polarised groups. The second region conveys the transition point from monolayer to multilayer. Finally the last region describes the multilayer region with water condensing at very weak binding sites [153]. Modelling fits for sorption isotherms and or finding the monolayer capacity can be worked out with pre-existing theories such as Brunauer-Emmett-Teller (BET) or Gugenheim-

Anderson-de Boer (GAB) equations [150, 154]. It has been suggested that movement of the absorbed water and flexibility of the protein are restricted when below the Brunauer-Emmet-Teller (BET) monolayer level [89]. This usually comprises somewhere in the region of 5-9% water content [153]. Close to this level of hydration there might be an observed secondary relaxation of the amorphous system and decomposition pathways will also occur over a range of time. In turn as you increase the hydration level then the greater dynamic mobility that will result. The water that has been adsorbed would be acting as a plasticiser giving rise to an increase in free volume. Internal protein flexibility has been shown to increase as you reach the BET monolayer of water [155]. The greatest amounts of internal movements from proteins occurs when you pass this BET threshold. In proteins, the monolayer accounts for the amount of water required to sufficiently cover the entire active heterogeneous sites. Since water has an unequal affinity to strong or weak binding sites, there is a worry that the use of the BET equation to calculate the monolayer water content for FD proteins could be deemed to be inadequate compared to FD food [153]. However at low relative humidity's, moisture absorbed by solid state protein samples associates itself with protein molecules, and in doing so confers additional molecular mobility to these species. This may be be erroneously associated with the BET monolayers of water in the sample. This may not be true as the FD samples often have very low surface areas on which the true BET water surface monolayer data (as correctly established via the true N_2 BET surface area data) is much lower than the claimed data for BET monolayer of water. The cause of this faulty data analysis easy to understand - water sorption data for solid state proteins materials can be fitted using the BET model. Such good fits of the experimental data to these models does not mean the physics of the BET model is true for these experimental data sets. That is, the fact that the BET model fits the isotherms of foods and solid state proteins does not make it correct.

Nevertheless, the direct effect of water plasticisation is that it lowers the T_g of a FD sample below its surrounding temperature. That means that phase transition from a brittle "glassy" state to a more dynamically relaxed viscoelastic rubbery state occurs at that applied temperature. Reactions which otherwise would have not been able to occur because of limited water or protein mobility can now happen freely [74, 156]. In essence you have domino effect whereby as more water moisture sorption occurs. more rapid increase in plasticisation will happen which then leads to lowering of T_g [157]. Often the relationship between the T_g and mass fraction of water can be fitted via the Gordon-Taylor equation or other similar semi-empirical models [158, 159]. This can often be time consuming experimentally as you would need to set up equilibrating experiments with desiccators at different water activities and then measure the T_g with DSC.

Hancock and Dalton measured the effects of temperature and humidity of several FD amorphous excipients for 3 months between 0 - 90% RH using saturated salt solutions [103]. They showed significant water was sorbed even at modest RH % and that amorphous materials can undergo changes from glassy to rubber and sometimes crystalline form (Figure 2.15). Once again, there is a limit on how much data can be obtained from saturated salt solution water isotherms at fixed RH points. It is also a labour intensive method in order to equilibrate the samples at different water activates in order to plot these isotherms. New commercial equipment shifting from static to dynamic equilibrium sorption experiments, have been successfully used to measure water sorption at greater resolutions (± 1% RH steps) and in faster time frames. The application of these techniques has the benefit of providing crucial information for establishing optimum storage conditions to not exceed [160, 161]. These instruments can be fitted with an optical camera below the sample chambers to record images of the solid state sample during water sorption. This has potential opportunities for to tracking visually physical changes of material that might not otherwise show up in isotherm data.

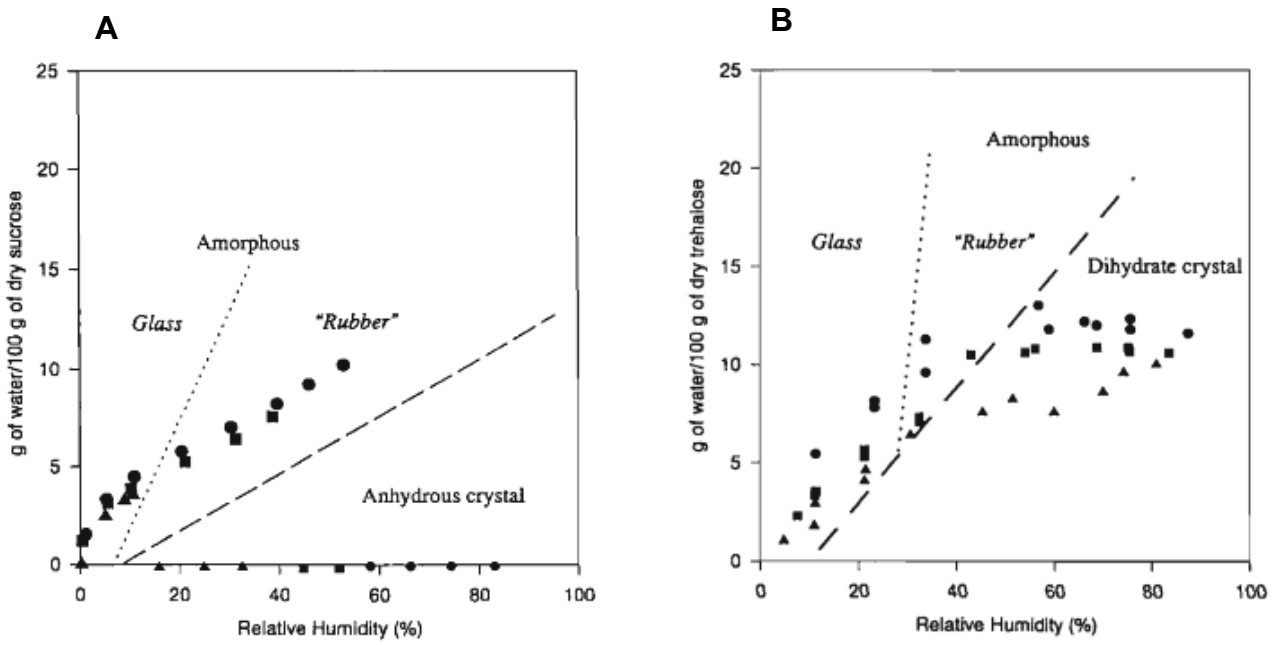


Figure 2.15 Water sorption isotherms for pharmaceutical amorphous **A:** sucrose and **B:** trehalose 10 % w/w (•: 5°C; ■: 30°C; ▲: 50°C). Adapted from [103].

2.9.2.1 Automated Dynamic Instruments

As stated previously, there are two main methods for water sorption a) static and b) dynamic (Figure 2.16). Commercial humidity generating instruments such as Dynamic vapour sorption (DVS) can measure gravimetrically the change in mass of a sample by being able to change to a particular RH% (water activity) from 0 - 98 % RH. It is a faster technique and less labour intensive compared to standard saturated salt slurry methods for measuring water sorption isotherms. With techniques such as DVS, there are potentially newer approaches and descriptor to measure the impacts of moisture content on structural and physical stability. A solids moisture content or % RH when T_g occurs at a fixed isotherm temperature could be determined whilst ramping the humidity. This method has the benefit of being a much faster approach while also having a less abstract value with a descriptors to a real world phenomena in terms what RH% or moisture content is required for structural stability at a particular relevant storage temperature.

Prior efforts and attempts had tried to look at using moisture sorption isotherms as a means to work out structural changes in matrix when T_g would occur [162]. Oksanen and Zografi [92] used saturated slurry method to produce water sorption isotherms of poly(vinylpyrrolidone), however due to limited number of data points, it required application of an empirical model for interpolation. They noticed an upward inflection in the isotherm curve which they also measured glass transition with DSC, but did not link this to working out the critical glass transition RH. Other reported studies such as Bell using the saturated salt slurry method (and hence limited data resolution). They did not see any significant in isotherms with PCP materials with different glass transitions. However, they concluded incorrectly that such moisture water sorption isotherms could not be used to determine the T_g [95]. All these past investigations had one common limitation being hampered by poor resolution (interpolation based on only a few fixed data points based due to limitations saturated salts method). The introduction of dynamic isotherm techniques made it possible for higher resolution data points

between (e.g a data resolution of 1% RH), eliminating the need for interpolating basic fitting models. Burnett et al. showed that with increased resolution using the ramping method on DVS, they could measure the critical RH% when T_g or onset of crystallisation occurs at a fixed experimental temperature [160, 161]. For T_g determinations this transition was taken as the inflection point in change in mass versus relative humidity, signifying that surface adsorption ends and bulk absorption begins in a sample. Whilst for crystallisation there is a characteristic drop in mass due to exclusion of water from crystal that form instead of amorphous solid which contained dissolved water initially. A ramping based % RH method allows for greater resolution to measure when T_g occurs by examining the transition from surface adsorption of water to bulk water absorption. They also compared the DVS results with IGC and DSC experiments and showed similar values. However, some variability may occur due to either measurement depending on definitions on DSC curves e.g onset , midpoint and endpoints as well as water activity not being able to catch up to the required RH before moving onto the next step in dynamic ramped RH experiments.

Others like, Ubbink et al. saw promise and proposed that moisture sorption isotherms of amorphous material could be used to determine critical water activities at T_g based on the point of intersection of two state isotherm models [163]. In the glassy state, they theorised and described sorption using the Freundlich model, while in the rubbery state they described sorption using the Florry-Huggins model. Since water sorption characteristics would be different based on whether the material was in glassy state or rubbery state, the intersection between these two regimes (where you have rapid change in mass uptake rate due to transition from surface adsorption to bulk absorption) would be where the critical water activity would reside. In their experiments they found a good correlation between water activity they determined at 25°C with T_g data from DSC, with a $R^2 = 0.95$. Others like Carter and Campbell, investigated the T_g of spray dried milk powder and saw an inflection point occurring at the same critical RH% every time, signifying the repeatability [162]. Similarly, Yuan et al. were able to show that the critical relative humidity could be determined when a glassy to rubber

transition occurs in polydextrose using water sorption isotherms using an automatic water vapour sorption instrument, [164]. They noticed a distinct inflection point occurring which corresponded to the glass transition at each temperature. They also found the change in flow rate shifted the critical RH, and that there was a linear correlation, which they could use to extrapolate to zero flow rate. There was good agreement between when comparing water sorption values obtained at critical RH and with those measured by DSC and MDSC.

Additionally, another unique advantage with dynamic instruments like DVS is that there is great untapped potential to visually observe and measure when any structural collapse changes might occur during water sorption. The DVS instrument can be potentially modified by fitting a camera attachment below the sample holder chamber. Thus not only can you measure critical moisture or % RH at T_g but also when structural collapse and shrinkage occurs. Thus providing a visual real-time recording of the events for a range of FD material. This further adds the potential to then construct critical moisture stability maps at a range of different storage temperatures. Unfortunately, dynamic based sorption instruments have been underutilised in the field of freeze-drying and thus there is a great potential applications such as visual experimentation to pursue further.

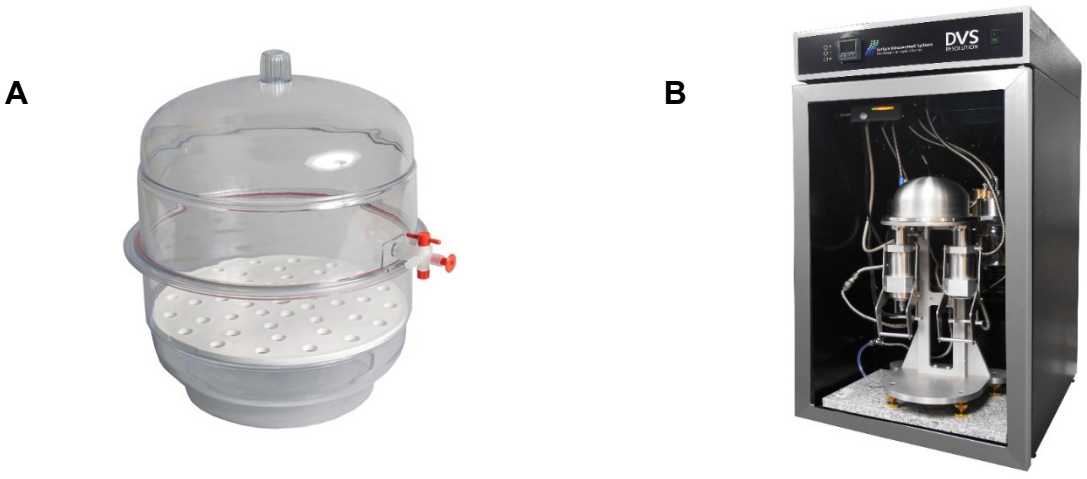


Figure 2.16 Water sorption experiments conducted either in **A:** saturated salt slurry (static) or **B:** dynamic humidity generating vapour sorption instruments (DVS).

2.9.3 Thermal Analysis

2.9.3.1 Differential Scanning Calorimetry (DSC)

Differential scanning colorimetry (DSC) is thermal analysis method used to commonly measure transition temperatures and heat flow. The principle mechanism behind DSC is that the heat flow difference is measured from a sample pan (either absorbed or released) relative to a reference pan (usually empty) as the temperature is changed [165]. The heat flow difference is measured by thermocouples located beneath the sample and reference pans [166]. DSC can be operated into two modes: conventional and modulated. In conventional mode DSC, the total heat flow signal is measured. Whilst in modulated mode (MDSC), the reversing and non-reversing signals can be displayed separately (the linear heating rate is superimposed with the sinusoidal temperature modulations). MDSC can be more sensitive and provide higher resolutions compared to conventional DSC for some transition events [165, 167]. DSC has been widely used to determine the glass transition temperature of maximally freeze concentrated solutions (T_g') and the glass transition temperature for solid amorphous samples after freeze-drying (T_g). A characteristic endothermic drop in heat flow is observed and the midpoint is taken using analysis software to measure the T_g value (Figure 2.17). DSC has been employed in many studies to monitor changes to the T_g of FD materials at different water activities or formulations [94, 107, 168-170]. Typically when measuring the T_g of a FD material, samples are usually loaded into hermetically sealed pans under low humidity dry conditions (< 5% RH) to avoid any moisture uptake from the atmosphere effecting the data.

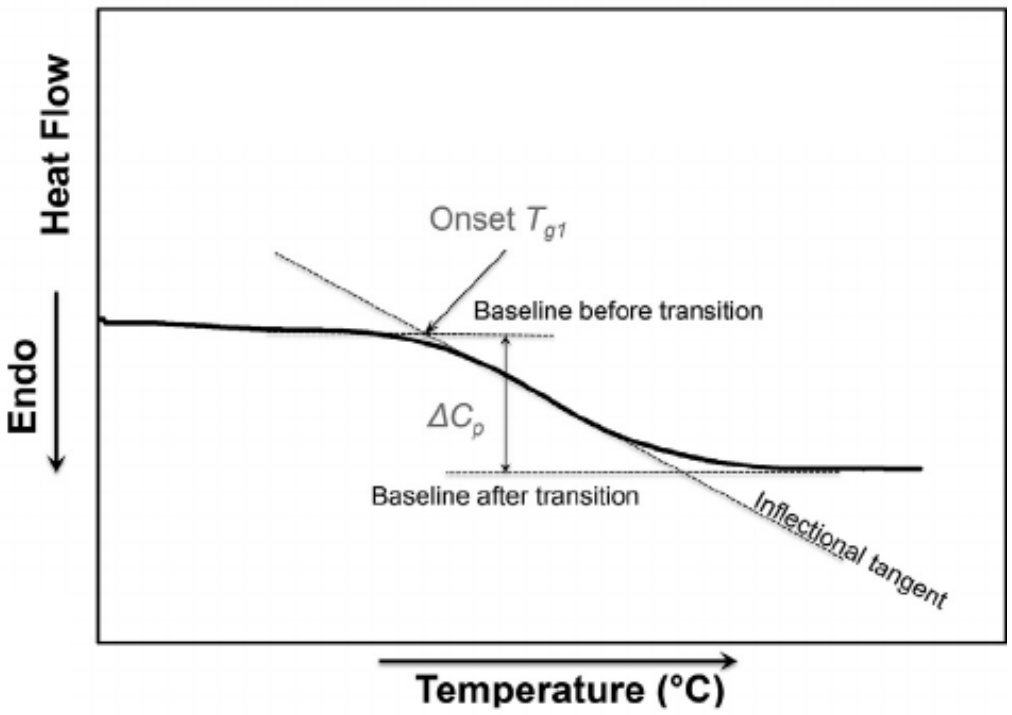


Figure 2.17 Schematic diagram of DSC endotherm measuring the glass transition temperature. Adapted from [171].

2.9.3.2 Freeze drying microscopy

Freeze drying microscopy (FDM) is a technique used to measure critical collapse temperatures of formulations in order to best determine the freeze-drying process parameters. Essentially, it is a miniaturised freeze-drying apparatus in which you can visually observe and record changes in real-time [172]. The sample is loaded onto a glass plate or crucible, frozen with liquid nitrogen and a vacuum is applied. This simulates the freezing step in an actual lyophilisation cycle. A sublimation front is formed and the temperature is then ramped up by a certain rate per min (°C/min). The critical collapse temperature is determined when the front begins to collapse visually (Figure 2.18). Meister et al. used FDM to observe the impact of nucleation temperature and excipient concentration on collapse temperature for FD material [173]. This technique is useful and important for formulation and process development for manufacturers in FD.

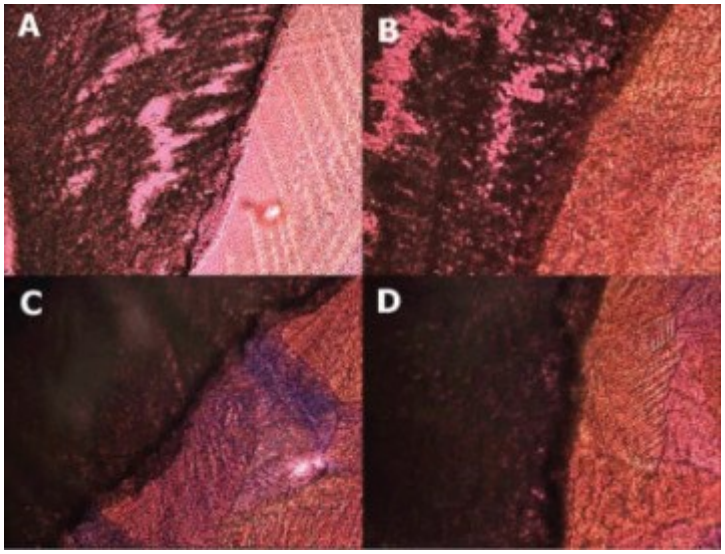


Figure 2.18 Collapse temperature for FD BSA with different mole ratio of sugar : protein measured by FDM [174]. (A) 50 mg/g pure BSA, onset of collapse –4.9 °. (B) 50 mg/g BSA/sucrose (90/10), onset of collapse: –6.2 °C. (C) 50 mg/g BSA/ sucrose (50/50), onset of collapse: –24.0 °C. (D) 50 mg/g BSA/sucrose (10/90), onset of collapse: –29.5 °C.

2.9.4 Structural and Surface Properties

2.9.4.1 Specific Surface Area (SSA)

2.9.4.1.1 BET Volumetric Gas Adsorption

One of the most common methods for determining the Specific Surface Area (SSA) is that of the gas adsorption method using the Brunauer–Emmett–Teller (BET) theory to calculate the value from isotherms [175]. BET theory is often used to determine the SSA with gas adsorption using adsorbates such Nitrogen and Krypton [175]. However it can also be applicable to other inert gasses and vapour adsorbates. The International Union of Pure and Applied Chemistry (IUPAC) officially recognises six types of adsorption isotherms (Figure 2.19) [176]. The BET equation can be applied to isotherms of Type II and Type IV over an adsorbate partial pressure range between 0.05 - 0.35 P/P_0.

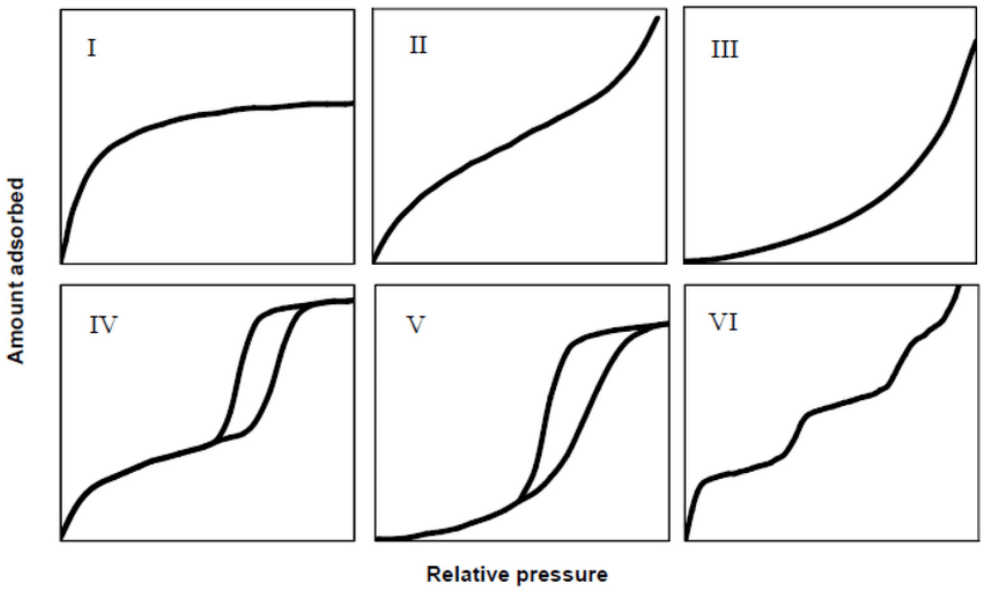


Figure 2.19 Six common types of adsorption isotherms according to IUPAC [176].

Typical gases used for adsorption are either nitrogen or krypton [115, 177-180]. These methods usually have a fairly low relative standard deviation and high degree of reproducibility for a sample in a vial. The SSA of FD material is an important parameter to measure since it has potential to be used as a critical quality attribute. The degree of supercooling can have a dramatic effect on the surface area of a formulation. Higher degrees of supercooling produce smaller ice crystals which can result in smaller pores in the dried matrix. Alternatively, lower degrees of supercooling produce larger ice crystals which in turn produce larger pores in the dried matrix.

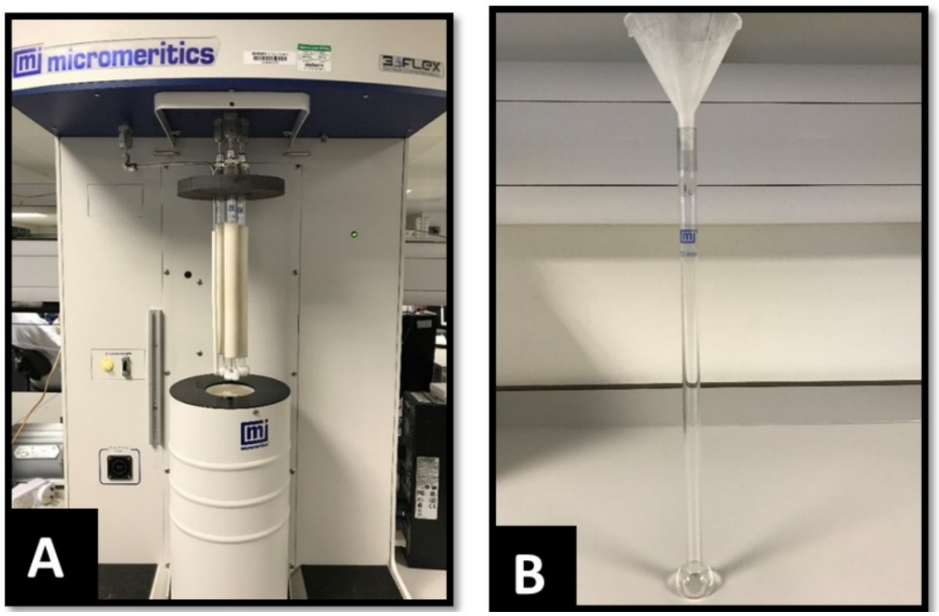


Figure 2.20 Nitrogen gas adsorption instrument used to measure specific surface area (SSA). **A:** Micromeritics 3Flex (N_2 adsorption), **B**: Round bottom glass tubes for 3Flex (12mm)

In a study by Beech et al., nitrogen adsorption BET was used to measure the effect of the cooling profile/annealing on the SSA of BSA and mAB1 formulations [181]. Quench cooled (dipped in liquid nitrogen for 2 min then placed in pre-cooled shelfs at -40°C) BSA samples

were found to have the largest SSA, whilst samples that had an annealing step in the cooling profile had the lowest SSA. Krypton adsorption (with can provide greater sensitivity) can be used to measure any cake shrinkage that might occur during freeze-drying process and consequences of your chosen parameters on product integrity [182]. There are however several disadvantages associated with nitrogen gas adsorption determinations in regards to requiring large amounts of samples for accurate determinations, long experimental time and having accuracy limitations for areas below 0.5 m^2/g [177, 183]. FD samples would need to be degassed before running the experiment to remove moisture. Typically for nitrogen adsorption experiments, measurements are conducted at around 77 K which could compromise the properties of the FD sample when compared to room temperature. Another point to consider is that for the more moisture sensitive FD material (such as dry weight flu antigens), sample loading from the vials into the BET sample tube could potentially introduce moisture from the environment which could cause physical changes of the sample before the degassing process. One potential solution is to prepare samples in a dry humidity environment but this might not be very practical for large sample tube holders.

2.9.4.1.2 Inverse Gas Chromatography (IGC)

Inverse gas chromatography (IGC) is a chromatographic technique that can be used as a potential alternative to measure the SSA of many materials such as fibres, films, or powders, including FD products [183-185]. IGC can determine BET isotherms using alkane adsorption at room temperature and humidity. In 1941, Nobel Prize winners, Martin and Synge, were the first to describe and introduce the concept of physical chemistry chromatography, the precursors to IGC, to measure partition coefficients between two liquids [186]. Pioneering work done by Wicke, Glueckauf, Cremer and Prior, and James and Philips during the start of mid-20th century, built the foundation upon gas chromatography (GC) as a tool for physiochemical studies [184]. The term “inverse gas chromatography” was coined by Kiselev from Moscow University, who led work in helping develop fundamental understating of chromatographic gas adsorption processes especially for adsorbents and catalysists [187].

IGC can be run in either infinite or finite concentration dilutions modes. In infinite dilutions a very small vapour adsorbate concentration is used (less than 3% partial pressure usually) where there is very small surface coverage on the adsorbent [188]. They follow Henry’s Law, where the amount of molecules that are absorbed is proportionally linearly dependent on the amount of adsorbate injection concentration. In finite dilution, probe injections are above Henry’s law region. The chromatographic peaks in finite concentrations can be tailed which relates to the rate change of the amount absorbed decreasing as a function of partial pressure [188]. IGC is an inversion on traditional gas chromatography (GC) methods. The roles of the stationary (solid) and mobile phase (gas/vapour) are reversed. Typically in GC, a known stationary solid is used to separate different unknown gas species or mixtures into separate components. However with IGC, a packed glass column with an unknown test sample material is subjected to injection with a known organic gas/vapour probe molecule. The retention time of this probe adsorbate through the column is measured via a flame ionisation detector (FID), which detects the ions formed during combustion of the organic

solute (Figure 2.21). The formation of the monolayer between of the adsorbate on the adsorbent can be characterised and plotted with sorption isotherms at different surface coverages (to produce typically Type II or Type IV graphs). The BET equation can be applied just like the volumetric gas adsorption method to calculate the SSA. IGC has gained increasing popularity over the years in part due to requiring small sample mass, being highly sensitive and relatively fast, easy to use, and the availability of commercial instruments to purchase [183]. To date there has been little work on of measuring the SSA for FD material using this technique. This method however also has the potential to be used for a variety of purposes including measuring the effects of relative humidity on the SSA of a sample.

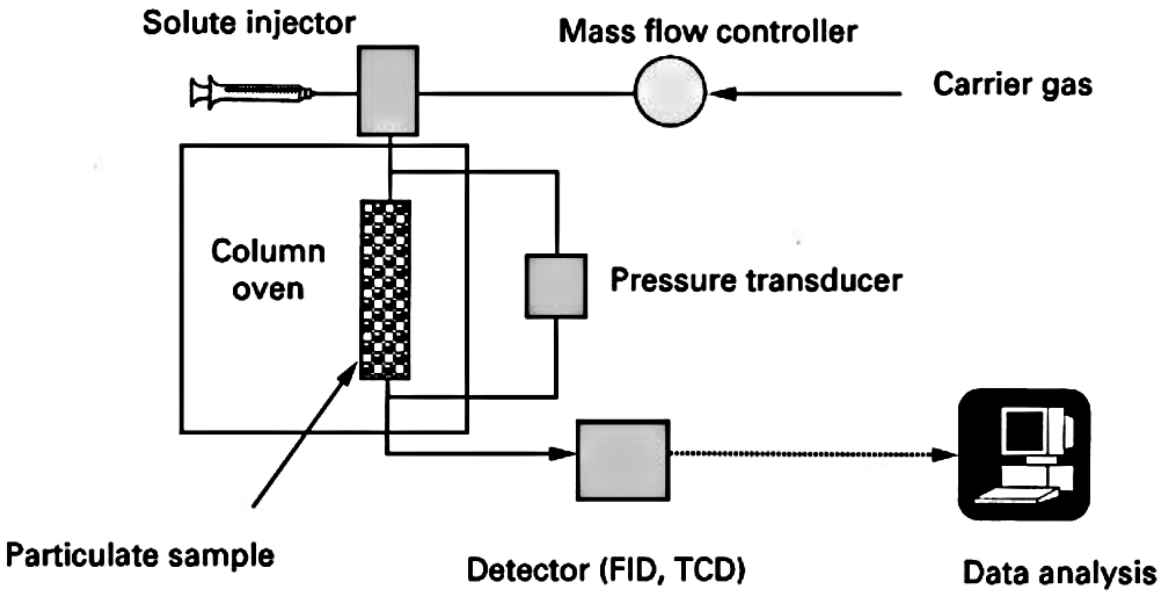


Figure 2.21 Schematic diagram of Inverse Gas Chromatography instrument. Adapted from [185].

2.9.4.2 Mechanical properties

2.9.4.2.1 Flat Punch Indentation

Although the FD process results in a solid-state material, they often can be mechanically fragile and even damaged by transportation [189]. The mechanical properties of a FD material can be dependent on a host of factors such as the cycle processes and parameters, types of excipients and protein concentrations used. FD cakes can often be categorised as example of cellular materials (often referred to as foams) which are composed of different units of cells formed by solid struts and plates [190]. Structures within cellular materials and foams can be considered either to be isotropic (no directionality) or anisotropic (axisymmetric or orthotropic direction) [191]. When undergoing stress, materials will exhibit deformation dependent upon the material behaviour and structure. Brittle materials will deform elastically up until a critical stress is reached and fracturing occurs. Although some past studies have tried to use texture analysers for force output data, it was only recently that Devi and Williams introduced and described a flat punch indentation based instrument specifically designed for FD materials in vials [110]. The technique is based on deformation/compression to measure the mechanical properties of FD material. A flat-faced probe attached to a load cell (250 g) was used to measure the resulting force and displacement inside FD vials. The resulting load produces a force versus displacement graph which can then be plotted as classical stress versus strain graphs.

The compressive strain (ε) can be calculated from the following equation:

$$\varepsilon = \frac{h_0 - h}{h_0} \tag{2.2}$$

Where, h_0 is the measured cake height (measured as height of indenter when in contact with cake subtracted by the height of indenter from bottom of empty vial). The compressed height, h

is the measured distance the indenter moved into the cake. It is assumed that no lateral sample spreading occurred due to the test sample being inside a vial.

The compressive stress (σ) can be calculated from the following equation:

$$\sigma = \frac{F}{A_0} \tag{2.3}$$

Where, F is the measured force (N) and A_0 is the contact surface area (m^2) of the flat circular indenter.

Alternatively, the Young's modulus (E_f), modelled after a material that is described as a foam with a flat punch [192], can be found directly from the raw indentation data graphs at the linear region and is given as:

$$E_f = \mathrm{F}\frac{1 - v^2}{h2r} \tag{2.4}$$

Where F is the force applied by indenter (N) during linear region, r is the radius of indenter (m), h is the displacement (m) at linear region and *v* being the Poisson's ratio, here assumed as 0.2 for brittle FD material based on past literature values such as sucrose and trehalose [109, 193].

The mechanical flat punch indentation technique, can be used to determine the effects of moisture and storage temperature conditions over long term storage. Additionally

formulation design and specific cake strength qualities can be determined using this instrument for rational design. It is also proposed that for mechanical properties there is a potential to be also used as a descriptor for critical quality attribute (CQA) as well as being directly or indirectly linked to stability.

2.9.4.2.2 Dynamic Mechanical Analysis (DMA)

Dynamic mechanical analysis (DMA) measures the mechanical (viscoelastic) properties which occur as the temperature is changed for a frozen or FD material [194]. FD samples are typically packed into a wick metal mesh under low humidity air bag in order to reduce moisture interference from the outside environment. Once loaded into the instrument, the material is vibrated (sinusoidal) at a fixed frequency measuring any changes to stiffness/dampness in relation to a change in the temperature. DMA has been used to measure the T_g in many pharmaceuticals [195]. Additionally, the T_g' can be also measured using DMA as alternative to DSC method (which for some complex formulations might be difficult to detect). Although a relatively new technique, it could potentially offer viable alternative at measuring FD material characteristics based on mechanical properties.

2.9.5 Visual Appearance

The question of cake appearance and what is acceptable can often be a subjective and ambiguous subject [196]. Cake appearance may or may not be critical in respect to stability and individual manufactures will have different criteria to what they deem as acceptable. In general, a FD cake should be “uniform and elegant” with the same size and shape as the liquid pre-drying. There should also be a uniform colour, classically a bright white appearance. Typical cake irregularities and non-uniformity cake appearances can be categorised as follows: i) collapsed cake, ii) meltback, iii) product ejection, iv) product at vial-stopper interface, v) slanted cake, vi) puffing , vii) lifted cakes, viii) splashing, ix) cracked cakes, x) dusting , xi) skin formation and xii) lyo ring [196].

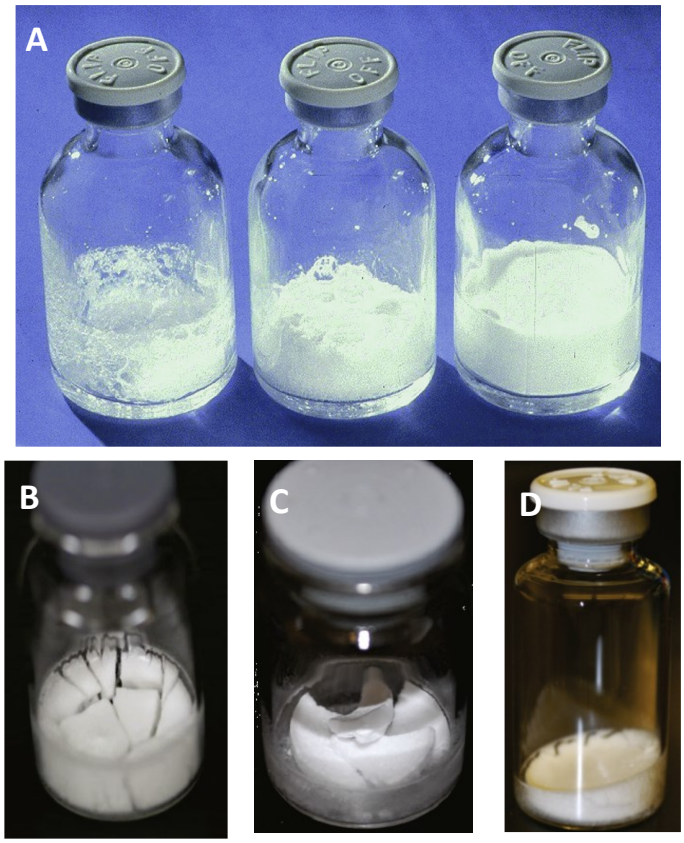


Figure 2.22 Examples of various FD cake appearance irregularities. **A:** Collapsed cake (from left to right; fully collapsed, partial collapse, no collapse), **B:** Cracked cake, **C:** Broken cake and **D:** Slanted cake. Adapted from [196].

As stated previously, collapsed cakes can occur when drying above critical product temperatures during primary or secondary drying (T_c, T_g' or T_g). Collapse is also linked to a reduction in SSA which can also lead to increase in final moisture content of the product [117]. The effect of product collapse on stability is a matter of debate, with some studies showing that it has no overall effect in terms of protein stability (albeit at carefully controlled moisture content). Of course collapse can also occur post FD during storage. If the storage temperature matches or exceeds, the T_g then you could have viscous flow occur and loss of structural matrix. Increasing moisture content results in plasticising effect during storage which can also expedite collapse or shrinkage to occur. Excellent visual appearance does not only apply to the solid state but can also influence to the rapid attainment of a liquid state post reconstitution. After reconstitution, the liquid in the vial should be virtually free from any visible particulate matter floating around or attached round the edges. However, for some high concentration FD materials, there is greater chance of reconstitution problems in terms of longer reconstitution times and full dissolution issues. Browning/yellow discolouration of the cakes can also occur if reducing sugars are present in the formulation. This is typically as a result of Maillard reactions occurring which are facilitated by increase of moisture. Any possible discolouration will inevitably effect the commercial aspects of the product with the risk of losing money and having to reformulate due to customer rejection of such FD materials.

2.9.6 Reconstitution Time

The reconstitution time of FD materials is an important parameter especially when administering to patients in a clinical setting. Commonly most FD materials are able to be reconstituted with water back to its prior liquid state in a matter of minutes. However, in some cases the reconstitution time can take a significant amount of time [197]. This is especially observed for FD materials with increasing high protein concentration above 100 mg/mL. This can have a significant impact on the practical and commercial usage. FD cycle process parameters and many factors have been linked with reconstitution time. Geidobler et al. showed how that controlled ice-nucleation was able to reduce the reconstitution time for a high concentration FD bovine serum albumin formulation [198]. Cao et al. meanwhile have described a number of novel strategies to reduce reconstitution time such as adding wetting agents, including an annealing step or reconstituting the vials under vacuum [197].

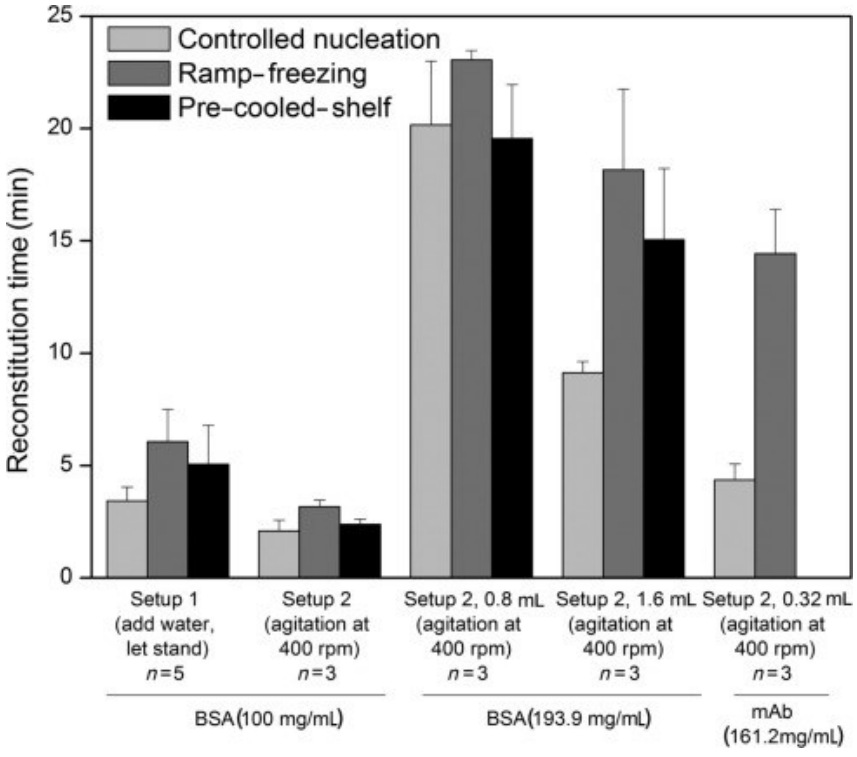


Figure 2.23 Effect of different freezing protocols on reconstitution time [198].

Another complex factor to consider is that reconstitution time can often be subjective, depending on what protocol acceptance criteria is being used. Reconstitution can either be done by adding water and letting it stand until fully dissolved or by having some sort of agitation. Further to this, when the point of reconstitution occurs can be a matter of debate, especially if there may be tiny particulate matter still floating around. At what point is a liquid fully clear or still cloudy are things that need to be considered. Photographic evidence can be used to help in conjunction to measuring data based on some visual change software. Generally the trend is that with higher concentration proteins the reconstitution time increases. It can typically take from minutes to hours to dissolve depending on a particular formulation, which in clinical settings is not ideal. Garidel and Presser, encountered similar reconstitution problems with ever increasing concentrations mAb's from 40 - 160 mg/mL [28]. However, by changing the formulation from succinate to other excipients such as trehalsoe, they were able to show a reduction the reconstitution by around 50% (see Figure 2.24).

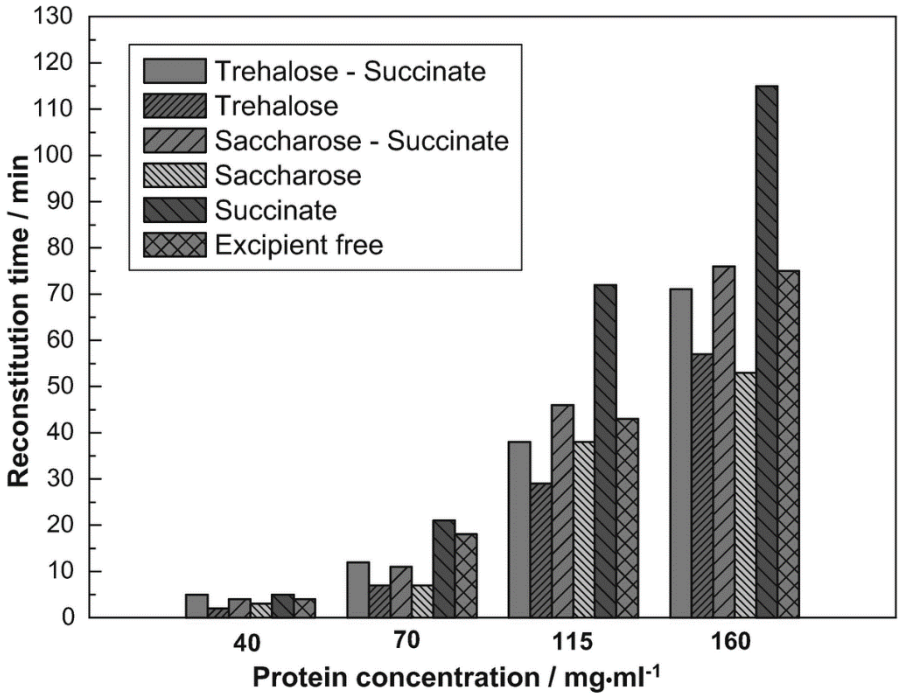


Figure 2.24 Effect of increasing mAb concentration and different excipient formulations on reconstitution time [28].

2.10 Summary and Research Gaps

Freeze-drying is commonly used to enhance the stability of liquid biologics to provide greater shelf-life and stability. However, physical and chemical stability can be affected by factors such as storage temperature conditions and most crucially moisture content in vials. The freeze-drying process itself results in a very hygroscopic cake with very little moisture content present. However, FD biologics can experience uptake of moisture which could lead to reduction in T_g, structural/mechanical/phase transitions as well as a decrease in biological activity.

Long term stability trials are usually carried out to assess the impact of these changes. In particular there has been limited investigation on the long term stability for high concentration mAb like proteins including the impact on moisture and storage conditions. Similarly at the other end of the spectrum, the impact of moisture for particularly low mass materials would benefit further investigation. A common theme in most stability trial studies is that there is always some sort of moisture ingress occurring during long term storage. How to reduce such moisture ingress over the long term storage is an important question and would benefit further additional investigations based on past studies looking at storage formats and heat treatments. Crucially, how much moisture may be permissible varies on a case by case basis and is a topic of debate in the freeze-drying industry. Further exploration of the role of critical moisture content and optimum stability (including physical stability) needs to be assessed. Hypothesis of optimum moisture in regards to theories with different mechanisms of stabilization would need further investigations to further add to current pre-existing knowledge.

Lastly, novel characterisation techniques relating to water sorption and mechanical-surface properties have been poorly utilised in FD applications at the present. Techniques such as Dynamic Vapour Sorption (DVS) have the potential to measure the critical moisture contents of collapse after storage and any phase transitions of FD biologics in order to map

out key stability maps. Similarly the use of flat punch mechanical indentation has been shown to successfully measure the mechanical properties of FD excipients, but there has been no further work exploring use of technique for assessing long term stability of real world biologics. The specific surface area of FD materials usually relies on gas adsorption methods (usually Nitrogen or Krypton adsorption), but there is potential to compare this with other novel techniques such as inverse gas chromatography, which has shown to be used successfully for a number of polymers, fibres and pharmaceutical powders. There is room here for application of this techniques to complement current characterisation methods during long term stability trials, which can provide more information on the impacts of moisture and storage temperature over time.

2.11 Thesis Objectives

The aim of this thesis is to investigate the effects of key fundamental factors, such as moisture and temperature storage conditions, on the long term stability in FD cakes. The relationship between residual moisture content, the structure of FD material and the stability of biologics in the FD state over long term storage will be explored with excipient and model proteins. Additionally application of novel techniques will be used to characterise and measure properties in relation to water sorption and physical behaviour of FD biologics. The outcome of this work will provide further insights that will cut across our core understanding of how to optimise the stability of FD biologic materials for long term storage, and provide direct industrially usable guidelines for future use.

The specific research objectives of this thesis are as follows:

1. To develop a standardised method for determining critical moisture content at which structural lyophilic cake shrinkage and collapse occurs. In addition to catalogue the maximum water capacity for a range of FD biologics exposed to different relative humidity.

2. To understand and measure how physical cake attributes such as mechanical properties and surface area of a range of FD biologics are affected by moisture content and temperature storage conditions using novel characterising techniques.

3. To establish stability trials to determine the effect of moisture and temperature on long term storage stability for model high concentration protein.

4. To minimise moisture emanating from rubber closure stoppers, especially in respects to establishing stability trials with low mass FD biological model proteins.

2.12 Thesis Outline

Chapter 1 and **2** introduces the background context and literature review of current knowledge and state of industry. Specifically, the effect of moisture and storage conditions on stability of FD biologics, different characterising techniques and gaps in research are all outlined.

Chapter 3 investigates the moisture sorption behaviour of FD material. A Dynamic Vapour Sorption instrument is used to measure the water sorption isotherms of FD biologics. Real-time imaging camera incorporated into the equipment was used to visibly measure cake shrinkage and collapse phenomena for various FD materials and construct stability maps.

Chapter 4 examines the way moisture effects the mechanical and surface area properties of FD material. Novel techniques such as mechanical indentation and Inverse Gas Chromatography are considered and assessed to determine the suitability for measuring such characteristics.

Chapter 5 investigates the effects of moisture and storage temperature on the stability of a real-world high concentration FD biological model, Immunoglobulin G (IgG). The effects of moisture and storage temperature are linked to functional activity and other characteristics such as reconstitution time, visual appearance, mechanical and surface properties and water sorption behaviour.

Chapter 6 explores one of the primary sources of moisture, that from rubber stoppers, and ways to minimise this during long term storage. Stability trials with low mass FD Flu Antigen standards were conducted with different storage formats and stopper drying treatments to measure their effectiveness at preventing water ingress.

Chapter 7 provides a concluding summary of all the chapters and how the work presented fits into current knowledge of long term FD stability. Potential lines of enquiry for future work are also deliberated upon.

Chapter 3. Moisture Induced Collapse in Freeze-Dried Material

3.1 Introduction

Most biologics are unstable as liquid formulations and freeze-drying can provide greater stability via a solid state format [5, 199]. FD materials by their nature are porous and highly hygroscopic (the substance is highly predisposed to absorb moisture from the environment). Most FD cakes contain amorphous excipients to enhance stability and mechanical properties [110]. Some of the most common excipients include sucrose and trehalose, which have been shown to improve product stability and enhance storage performance [90, 200]. Exposure to the outside environment or the presence of small amounts of moisture can have drastic effects on biological activity and also induce unwanted morphological structural changes. Increases in temperature and moisture content inside vials can have unwanted phase transitions and consequences for formulations containing amorphous materials. There is also a risk for some amorphous material reverting back to a crystalline state - due to increases in molecular mobility and free volume lowering of the activation energy barrier to crystallisation [161]. During storage if enough moisture is present at a given temperature, you could observe a resulting loss of cake structure/shrinkage and even complete lyophilic cake collapse occur [201]. FD products with small fill volumes are particularly susceptible and in danger of such collapse or shrinkage occurring due to only requiring a very small increase in moisture uptake to induce collapse and drive instability. Thus, the effects of moisture is critical factor for consideration over long term storage for such small FD cakes. Past studies have shown that over large periods of time in storage, moisture content can increase dramatically for FD materials in vials [202-208].

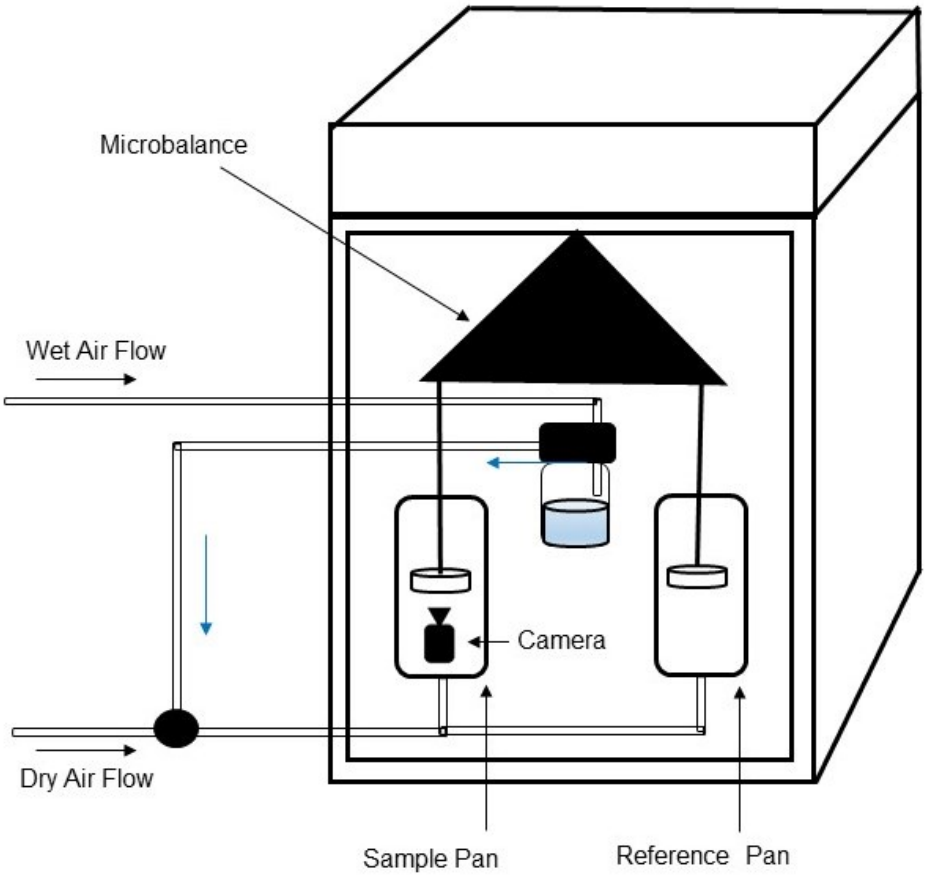
Changes in moisture also affect the glass transition temperature (T_g) - the transition temperature at which a material undergoes a transition from a hard and glassy brittle state to that of a soft rubbery state [61]. Water can act as a plasticising agent thereby reducing the T_g

of a FD material over time. Thus, increasing moisture content could eventually lead to the material transitioning from a glassy brittle state reverting to a flowing viscous fluid. These drastic T_g reductions in combination with the storage temperature facilitates the shrinkage or complete cake collapse in an amorphous FD material [91, 201]. The increase in moisture not only effects the structural stability of the product but also the biological activity. As the storage temperature approaches the T_g then increased molecular flexibility occurs which leads to greater product instability and chance of degradation occurring.

Past studies have tended to focus on measuring the structural collapse of FD materials in a dry state as a function of increasing temperature. This would typically involve taking a FD sample with a fixed moisture content and heating it until a loss in structure/radial shrinkage occurred at a specific collapse temperature, T_c. These experiments are not to be confused with collapse temperatures during FD in solid ice, but instead this refers to a dry state material post lyophilisation. The viscosity of the FD matrix becomes so reduced that it is unable to support its own weight and structure and thus results in a flow of the FD material. To and Fink postulate that while collapse and glass transition temperatures may be similar events, there is a difference in that the glass transitions are reversible whilst the collapse of FD matrixes is irreversible [209]. Tsourouflis et al. observed that with increasing moisture content, the collapse temperature of FD orange juice and carbohydrate solutions decreased [210]. The more water in the sample then the lower the temperature required to initiate collapse phenomena. Similarly Gerschenson et al., showed that a combination of high moisture and high temperatures resulted in structural collapse and loss of volatiles of FD tomato juice cake [211].

In past studies, the moisture content would remain fixed while the temperature was changed. The development of sophisticated dynamic vapour sorption instruments resulted in a shift towards measuring sample changes as a function of varying humidity (and hence moisture content) instead [212]. This involves ramping the water activity or relative humidity at a constant temperature around the sample, observing any resultant moisture induced

changes to the sample. Measuring moisture induced changes such as collapse or shrinkage phenomena has been a challenge for FD materials. As described in the previous Chapter, past water sorption studies have tended to be conducted using desiccators. This is a well-documented approach in the laboratory but is not very practical in terms of time and is also difficult in measuring quantifiable structural changes. The Dynamic vapour sorption (DVS) instrument was introduced in the early 1990s by Surface Measurement Systems (SMS). DVS is a gravimetric technique used to measure any changes in mass due to uptake of precise moisture/vapour concentrations. The sample in the pan is suspended from under an ultra-sensitive microbalance capable of recording changes in sample mass as low as 0.1 µg. The sample and microbalance are contained in a precise temperature regulated chamber controlled between 5 - 60°C. Typical output data sets are given as percentage change in starting sample mass (%) against time and relative humidity. The change in mass corresponds to equivalent water mass uptake (w/w %) of a sample and is measured by the ratio of the current mass, M, to reference samples mass at the start of experiment for t=0; M_0.



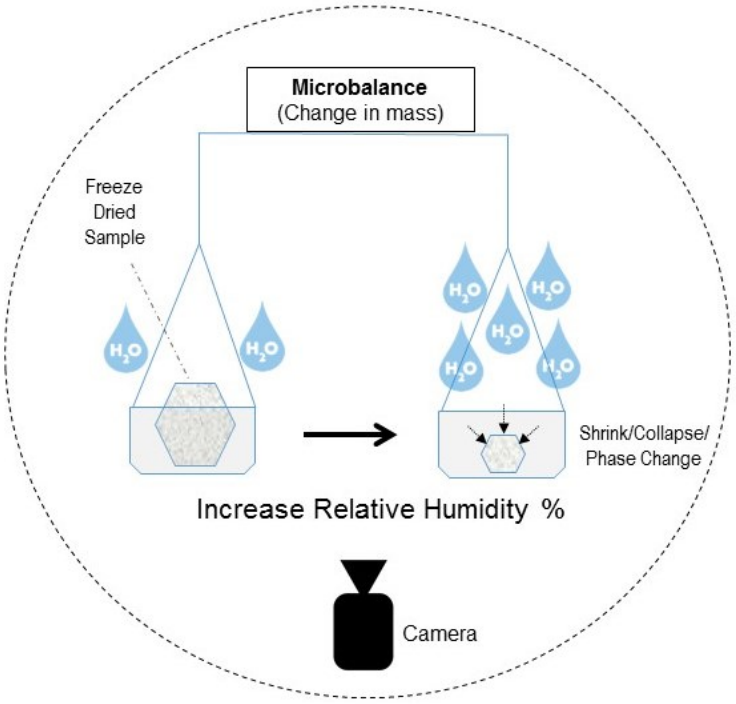


Figure 3.1 Schematic of DVS Advantage instrument.

The relative humidity is defined as the ratio between the actual amounts of water vapour in air relative to the maximum saturated water vapour that air can hold at a particular temperature (expressed as a percentage). It can be described by the following equation:

$$RH(\%) = \frac{p_{H_2O}}{P_{H_2O}{}^*} \times 100 \quad (4)$$

Where, p_{H_2O} is the partial pressure of water and $P_{H_2O}{}^*$ is the saturation equilibrium water vapour pressure at a given temperature. The required vapour pressure is generated using mass flow controllers attached to the DVS. This is achieved by mixing dry gas and saturated wet vapour gas flows in different ratios. An optical dew point sensor and PT100 temperature probes are situated close to the sample to get accurate verification of system performance and control.

DVS can run on two types of operational modes:

1) A % RH ramp mode (user specified RH% per hour)

2) A % RH step mode with a specific change in mass (% *dm/dt*) threshold for each step or fixed times for each step (or a series of steps)

In the time based % RH ramp mode, the relative humidity is usually increased by small incremental steps. In this method, one can set the time for how long each % RH step lasts before moving onto the next RH step. The alternate method is the % ramp step mode with a user specified change in mass *dm/dt* (%) threshold. The difference being that instead of a time based step change, it is instead decided by when the change in mass uptake of the sample reaches the set (% *dm/dt*) threshold value. This is to ensure that the mass change reaches near maximum level of water uptake equilibrium before moving to the next RH step. Both modes have their pros and cons which can be used for different applications. The time based

ramp mode produces faster experiments with higher data resolutions but at the cost of having to do repeats with different ramping rates. In order to see what specific % RH any phase changes or collapse might occur then a series of experiments with varying ramp rates would need to be done and then the data used to extrapolate the critical % RH at the theoretical ramp rate of drh/dt = 0. With the dm/dt (%) threshold mode it can take longer time per experiment and might need to do repeats if the value of dm/dt (%) was not sufficient for complete mass uptake equilibrium at each % RH step.

Burnett et al. have used the DVS technique to measure the onset critical humidity for glass transition and crystallisation for a range of a range of amorphous material [161]. Other studies have used DVS to measure and characterise the moisture sorption behaviour for amorphous sucrose [213] and amorphous trehalose [214]. However, there hasn't been any reported visual quantifiable ways to measure real-time moisture-induced collapse for FD material. The objective of this Chapter is through a series of experiments at different humidities and temperatures, to visually record what the critical change in mass (i.e moisture content % w/w) was when shrinkage or collapse occurs for a number of FD materials. The DVS instrument has been fitted with a 5 megapixel video camera and clear glass sample pans to be able to observe any changes in cake structure during each programmed humidity step in order to determine critical moisture content stability maps.

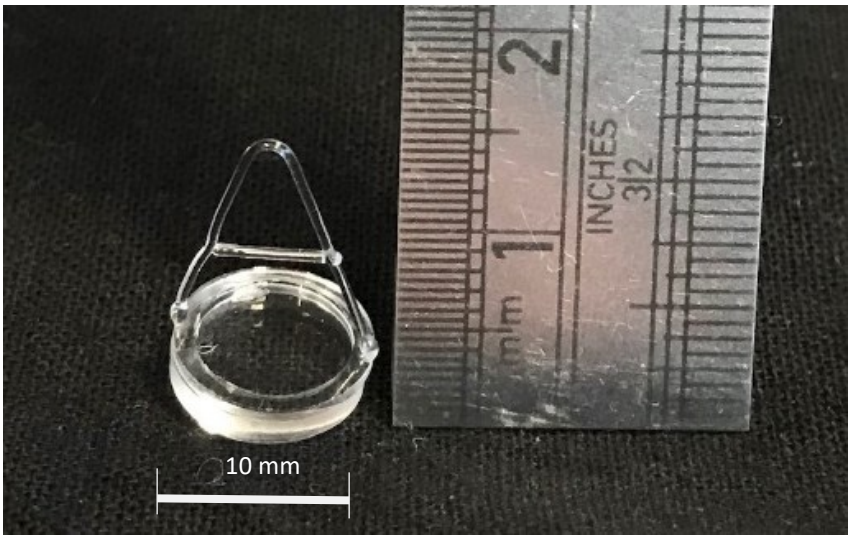


Figure 3.2 Glass sample pan holder used for DVS sorption experiments.

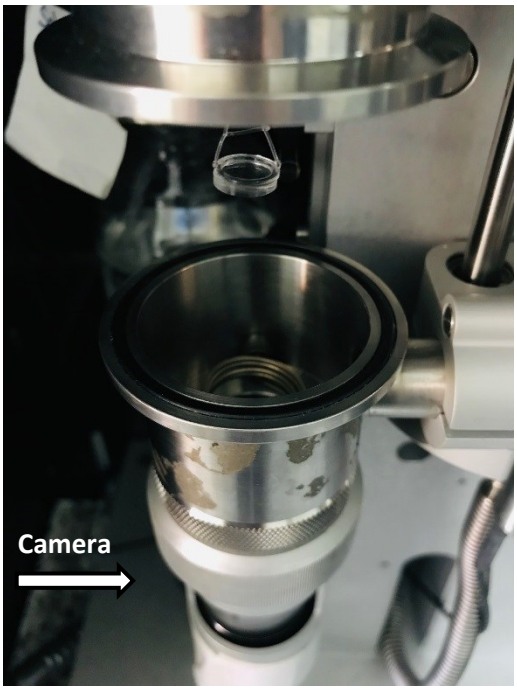


Figure 3.3 DVS Advantage sample holder in the open chamber with camera positioned below the glass pan.

3.2 Methods

3.2.1 Materials and Freeze-drying Cycles

Sucrose (S5016-1 Kg, Sigma, Dorset, UK) and trehalose (16400, Cargill, Surrey, UK) solutions in concentrations at 50 mg/mL solutions were prepared with distilled water. Biologicals such as Influenza B/Phuket antigen (60 µg/mL formulated with 1% or 20% w/w sucrose) and plasma derived Immunoglobulin G (formulated with 1% w/v sucrose, 10 mM citric acid adjusted at pH 6.6 with 5 M NaOH, 0.01% Tween 20) at 10-200 mg/mL, were obtained from the National Institute for Biological Standards and Control (NIBSC, Potters Bar, UK). All samples were filled in 5 mL screw capped vials (41.5 x 18 mm i.d., Adelphi Tubes, Haywards Heath, UK) to a fill volume of 1 mL using an automated multi-pipette (Eppendorf, UK). Sucrose and trehalose solutions were dried separately with a Virtis Advantage Plus (SP Scientific, USA). The influenza antigen and the IgG solutions were dried using the Telstar LyoBeta 15 (Azbil-Telstar SpA , Terrassa, Spain).

The freeze-drying cycles for all materials is outlined in Table 3.1. After drying cycles had completed, vials were backfilled with nitrogen gas under normal atmospheric pressure and stoppered down with the 14 mm halobutyl stoppers (Adelphi Tubes, Haywards Heath, UK).

Table 3.1 Freeze drying cycle for each material

Material	Freezing Temperature (°C)	Freezing Ramp Rate (°C/min)	Freezing Hold Time (min)	Primary Drying Ramp Rate (°C/min)	Primary Drying Temperature (°C)	Primary Drying Pressure (mTorr)	Primary Hold Time (min)	Secondary Drying Ramp Rate (°C/min)	Secondary Drying Temperature (°C)	Secondary Drying Pressure (mTorr)	Secondary Hold Time (min)
Sucrose (50 mg/mL)	-50	0.20	240	0.3	-35	70	2400	0.05	25	20	960
Trehalose (50 mg/mL)	-50	0.20	240	0.3	-35	70	2400	0.05	25	20	960
IgG (10 - 200 mg/mL)	-40	1.00	120	0.8	-15	150	1200	0.15	30	20	600
Influenza antigen B/Phuket 14/252 (1-20% w/w sucrose)	-50	0.30	240	0.45	-35	100	2400	0.07	25	20	1200

3.2.2 DVS Sorption Profiles

Sorption profiles were determined using the DVS Advantage instrument (Surface Measurement Systems, London, UK). Samples from FD vials, ranging from a mass between 5 - 20 mg, were placed on the sample pan using tweezers (Figure 3.2). A 5 megapixel video camera was retrofitted below the clear glass sample pan (Figure 3.3). A series of experiments were carried out using either the ramping step mode (Section 3.2.2.1) or % dm/dt threshold mode (Section 3.2.2.2). A flow rate of 200 standard cubic centimetres per minute (sccm) was used for all experiments and the temperature on DVS was set to either 10°C, 25°C or 45°C. Upon completion, raw data was exported into Microsoft Excel and analysis was generated using the DVS Macro Standard Analysis Suite v7.0.13 (Surface Measurement Systems, London, UK).

3.2.2.1 % *dm/dt* Mode

The method uses the percentage change of mass with time dm/dt to determine the end-stage time when the sample has reached equilibrium at a given RH step. The % dm/dt threshold was set to 0.0005 % for all experiments to ensure the sample had reached a necessary degree of equilibrium before moving on to next step. When the sample percentage change in mass is equal to or below this threshold for a given stability duration (10 min), the step stage is then ended and moved onto the next programmed RH% step. Methods were run in (0 - 90% RH) cycles with increments of 10% RH steps. The drying step at 0% RH was also set to the same % dm/dt threshold value.

3.2.2.2 Ramping Mode

Sorption studies were performed in either half (0%- 90% RH) or one full cycle (0% - 90% - 0%). An initial drying step of 6 hours at 0% RH was programmed to allow enough time to remove moisture from the sample before ramping. Humidity ramping steps were set to increase by an increment of 1% RH. At the end of each RH% step a photograph was taken by the camera below the sample pan that included metadata such as the time and RH% of current step to determine the collapse humidity and moisture content (see Section 3.3.3). Experiments were carried out at a ramp rate of either 6%, 12%, 24% or 30% RH / hour.

3.2.3 Residual Moisture Content

The moisture content of the cakes within the vials was measured using an automated coulometric Karl Fischer instrument (Mitsubishi CA-200, A1-Environsciences Ltd, Blyth, UK). Samples were transferred into HPLC autosampler vials within a pyramid dry bag (Captair pyramid, 2200A Cole Parmer, London, UK). Nitrogen gas was used to purge the pyramid air bag to ensure that a humidity of equal to or less than 5% RH was achieved. All samples were tested in triplicates (n=3).

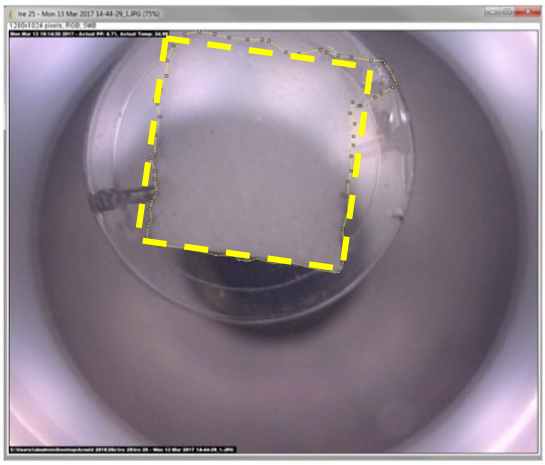
3.2.4 Measuring Collapse and Shrinkage in ImageJ

The structural change (collapse or shrinkage) was analysed using ImageJ 1.50i (National Institutes of Health, USA) on the obtained DVS images. For reference, a straight line was drawn across the known sample glass pan diameter (10 mm) to set a standardised global scale in pixels/mm. The outline of the FD cake was traced with the polygon selections tool and the 'analyse' > 'measure' tools was selected to give the relative area. Each image corresponds to the end of a specific % RH step and time it was taken. The measured change in area of the FD cake (%) was plotted against the sample % RH for each image. The % RH when the first decrease in area % occurred (within a ± 1% change) was taken as the critical % RH of collapse; RH_{cp}. Furthermore using the DVS sorption isotherms, the % change in mass at that specific % RH was used to determine what the critical water content % w/w, M_{cp}, was when that structural loss occurred.

The following assumptions are made with this procedure:

- The video camera image is from a 2-D cross sectional perspective of the cake, so it is assumed all collapse happens uniformly and simultaneously across the 3-D sample.
- Material have been sufficiently dried during the initial drying step at 0% RH.
- The selected sectioned FD test sample is representative of the entire FD cake in the vial.

A



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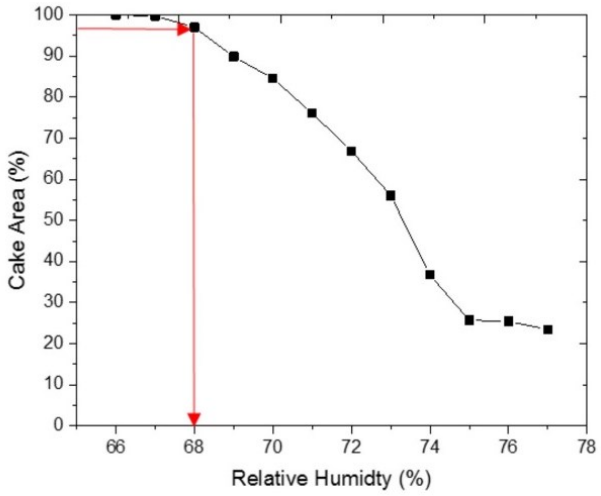

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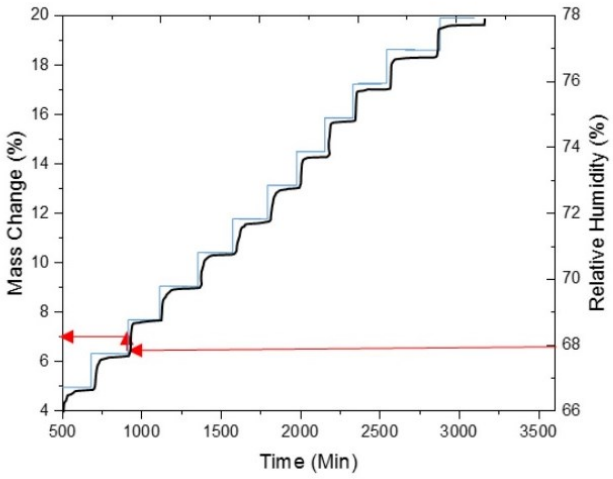


Figure 3.4: Procedure to measure the critical moisture content and % RH at collapse point. **A:** ImageJ software used to measure the change of area (%) in each image taken. **B:** Each image corresponds to a % RH at the time taken. Plot of FD sample cake area (%) versus % RH constructed. Agreed change in area % drops (1% loss in area), point is taken as the critical % RH point when collapse/shrinkage occurs. **C:** DVS sorption isotherms was used to determine critical moisture content % w/w when collapse occurred at that specific critical % RH and time point.

3.2.5 Determining the moisture content at RH_g

The critical relative humidity point, RH_g, was measured with same procedure as previously described by Burnett et al.[161]. In brief, the critical RH_g and its corresponding moisture content, M_{Tg}, was taken as the transition point when surface adsorption ends and bulk absorption begins in a sample. Figure 3.5 shows a schematic example in which intersecting the mid-point of the red lines between surface adsorption and bulk absorption is illustrated.

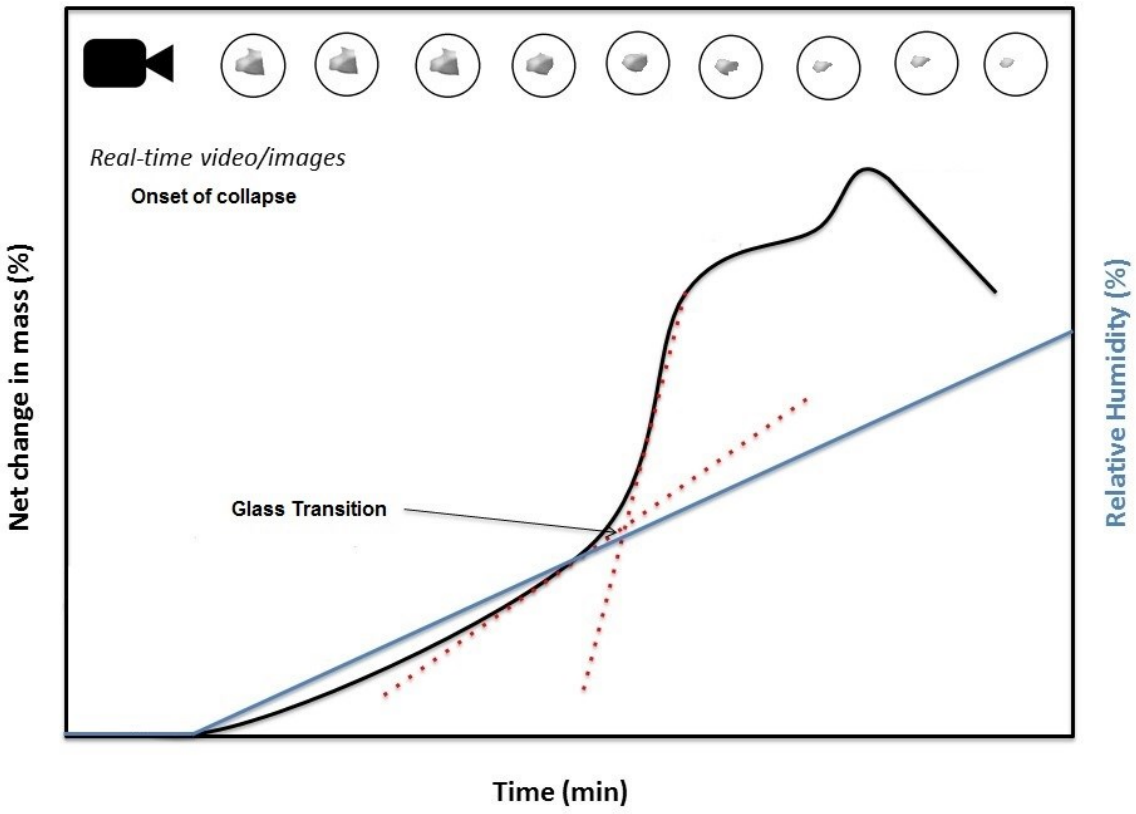


Figure 3.5: Schematic DVS plot and as well measuring the phase transitions and cake collapse.

3.2.6 Statistical Analysis

The statistical analysis used for the data with error bars was that of confidence intervals which is a range of values that are defined so that there is a specified probability that the value of a parameter lies within them. The following equation was used to calculate the 95% confidence intervals (C.I):

$$\mathrm{C.I} = \mathrm{X} \pm \frac{Z*SD}{\sqrt{n}} \tag{3.1}$$

Where, X is the sample mean, SD is standard deviation, n is the sample size and Z is the confidence coefficient (1.96) for 95% confidence. The advantage of confidence intervals is that statements are possible on the direction of the effects, including strength and the if present conclusion of a statistically significant result [215]. Many researchers argue confidence intervals, can be more appropriate to depending on type of study (considering that an overemphasis on hypothesis testing and the use of P values to regard or represent significant/non-significant results has detracted from more useful approaches to interpreting studies), and therefore should be used as alternative for findings in journals [215-217]. Important international journals of medical science, such as the Lancet and the British Medical Journal also recommend the use of confidence intervals especially in particular when interpreting the results of biologics clinical studies such as the products from NIBSC used in this study [217, 218]. Additionally, confidence intervals can be used to show indirectly conclusions of statistical significance for results without doing any other statistical approach (since significance level 0.05, corresponds with confidence level 95%) [215]. Therefore, for example if the 95% confidence interval comprises of all values less than 1.96 standard errors away from the sample value, testing against any population value in this interval will lead to p > 0.05 values and no significant result. Testing against values outside the 95% confidence interval more than 1.96 standard errors away (when no overlap with error bars) will lead to p-values < 0.05 values and significant result [217, 219]. Unless stated otherwise all 95% confidence intervals were used through all studies in this thesis.

3.3 Results and Discussion

3.3.1 Sample Moisture Content

In order to load samples onto the DVS sample pan, FD cakes in vials are cut into small pieces using tweezers. The sample mass varied depending on the material, but in general FD pieces were standardised to be between 5 - 20 mg. After freeze-drying cycles were completed the bound moisture content in the samples were measured in triplicate from the same batch (n=3) using coulometric Karl Fischer titration. Table 3.2 shows the initial bound moisture content of FD excipients and biologics used for the DVS experiments. The moisture content for the FD excipients sucrose and trehalose was below 1% w/w. IgG and B/Phuket (1% w/w sucrose) samples had moisture contents below 0.2% w/w , whilst the B/Phuket with increased sucrose concentration (20% w/w) had the highest moisture around 1.7% w/w. The initial moisture content in the samples can also change over time during storage, so all vials after completion of FD were stored at -20°C until required for further testing. In DVS, a drying step is often employed to remove the initial moisture content in the sample before running the main sorption experiment.

Table 3.2 Bound moisture measured in FD samples by automated coulometric Karl Fischer Titration (n=3). Error bars represent ± standard deviation.

FD Material	Moisture Content (% w/w)
Sucrose (50 mg/mL)	0.78 ± 0.07
Trehalose (50 mg/mL)	0.96 ± 0.19
IgG (50 mg/mL)	0.11 ± 0.05
Influenza antigen B/Phuket 14/252 (1% w/w sucrose)	0.13 ± 0.07
Influenza antigen B/Phuket 14/252 (20% w/w sucrose)	1.67 ± 0.18

The microbalance in the DVS with an empty sample pan in place was tared to zero before loading the sectioned FD sample. Between opening the vial, sectioning a small piece and loading onto the DVS, it took on average between 30 - 60 seconds. Although the cakes are only briefly exposed to the laboratory environment, for particularly highly hygroscopic materials there is an expectation that some moisture will be absorbed. All samples in DVS are dried at 0% RH with dry air flow going in before running the main sorption experiments. The initial mass is taken as M_0, represented as a percentage, 100%. Once the drying step is initiated a characteristic drop in mass (%) is seen during the drying stage, relative to initial 100% mass. Figure 3.6 shows an example of the typical drying curves for trehalose 50 mg/mL and B/Phuket (with 20% w/w sucrose). After a certain time the sample change in mass (%) plateaus indicating equilibration has been reached with no more moisture being desorbed. Trehalose reaches a faster drying equilibrium than the B/Phuket antigen, indicating less moisture present in the sample initially. The resulting total change in mass (%) drop for both cases was slightly higher than the initial moisture content measured by Karl Fischer, indicating that additional moisture had been gained during loading of samples into the pan. This highlights the importance of the drying step for the sample, as it is very difficult to be able to prepare samples in controlled RH% environment (such as pyramid bag purged with nitrogen) for DVS experiments. For ramping experiments (see Section 3.3.3 later on), the drying step was set for 6 hours in order for all samples to reach a drying equilibrium. Whilst admittedly water can exist in many forms (free, absorbed or bound) [85], it is the assumption here that for the remainder of these studies, most of the labile moisture has been removed and that if any bound moisture is present that can be assumed to be almost negligible in terms of overall impact. This follows the same precedent from previous studies, when conducting water sorption experiments via DVS, who have incorporated similar drying step before the main sorption sequence.

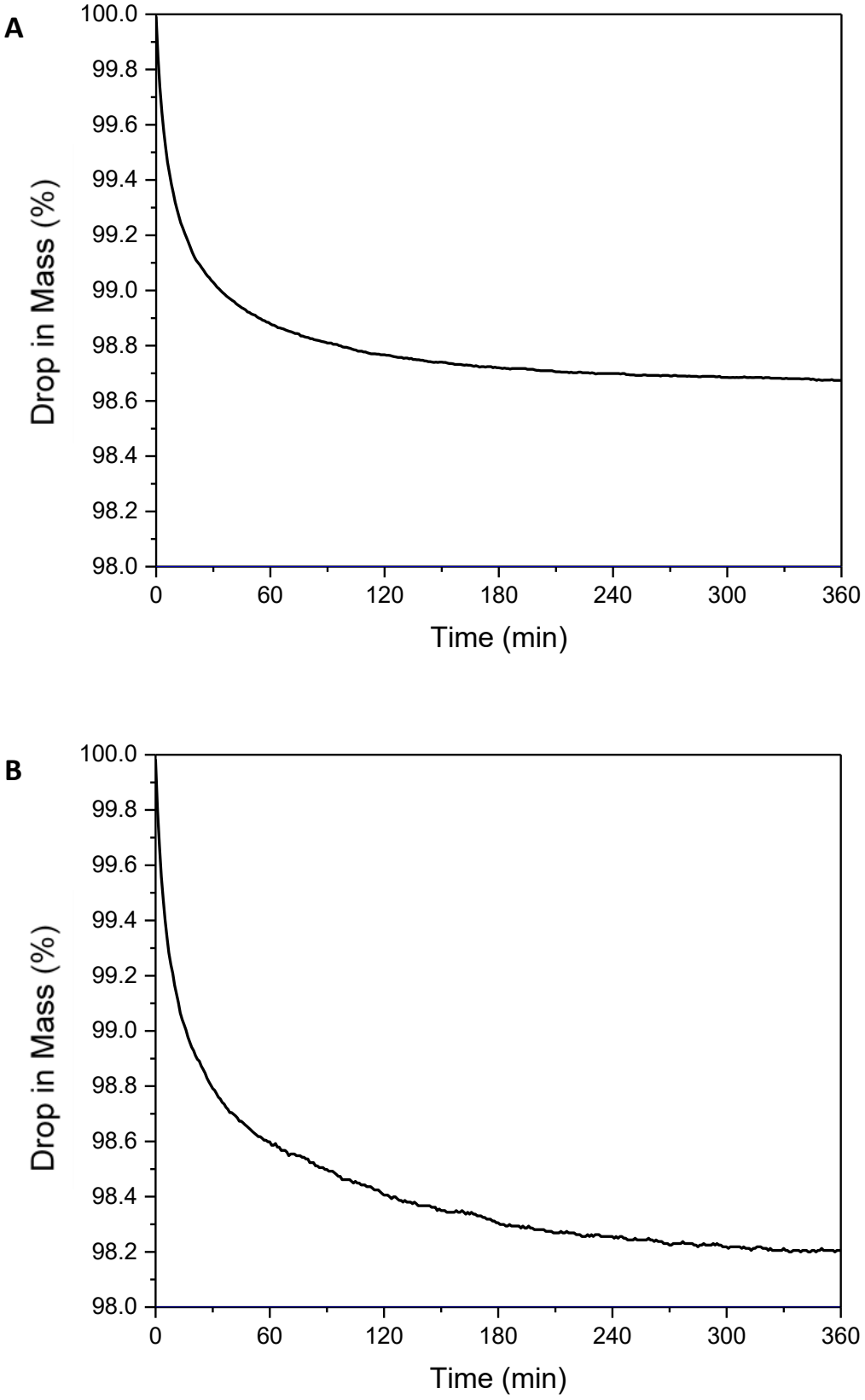


Figure 3.6 Examples of typical DVS drying curves at 0 % RH for freeze-dried **A:** Trehalose 50 mg/mL and **B:** B/Phuket flu antigen (formulated with sucrose 1% w/w) at 25˚C.

3.3.2 Moisture sorption isotherms (% dm/dt mode)

When a solid sample is exposed to a fixed humidity it gains moisture until it reaches an equilibrium change in mass. Typical water sorption isotherms in the past have been constructed with desiccators with saturated salt solutions to produce different % RH required. This is very time consuming and laborious to do. Instruments such as DVS allow for employment of a % dm/dt mode to measure water sorption isotherms in more dynamic and convenient fashion. The method involves inputting a certain change in mass (% dm/dt) value threshold at a specified stabilisation time window. In order to go to the next programmed RH% step, the sample change in mass over time % must stay below the % dm/dt value for that fixed stabilisation time. The % dm/dt value starts approaching zero when the sample reaches water uptake equilibrium at a particular % RH step hold. In practical terms, the value never reaches zero as it would take a very long time for a sample to reach true equilibrium. However if the % dm/dt value is set low enough, the resulting equilibrium mass uptake difference between that and the assumed zero value would be almost negligible difference. As a result the sorption isotherms can be constructed for maximum water adsorption at each % RH step. These are sometimes often referred to as “pseudo-isotherms” since the maximum adsorption at each % RH step is not taken at perfect true equilibrium but at a small enough point in time where the difference in change of mass is negligibly small. A series of % dm/dt experiments with DVS mimicking typical static (saturated salt) based water sorption experiments are shown in Figures 3.7 - 3.11 for a series of FD excipients and biologics. The change in mass represents at each % RH point represents the maximum water uptake capacity equilibrating for a % dm/dt value of 0.0005%.

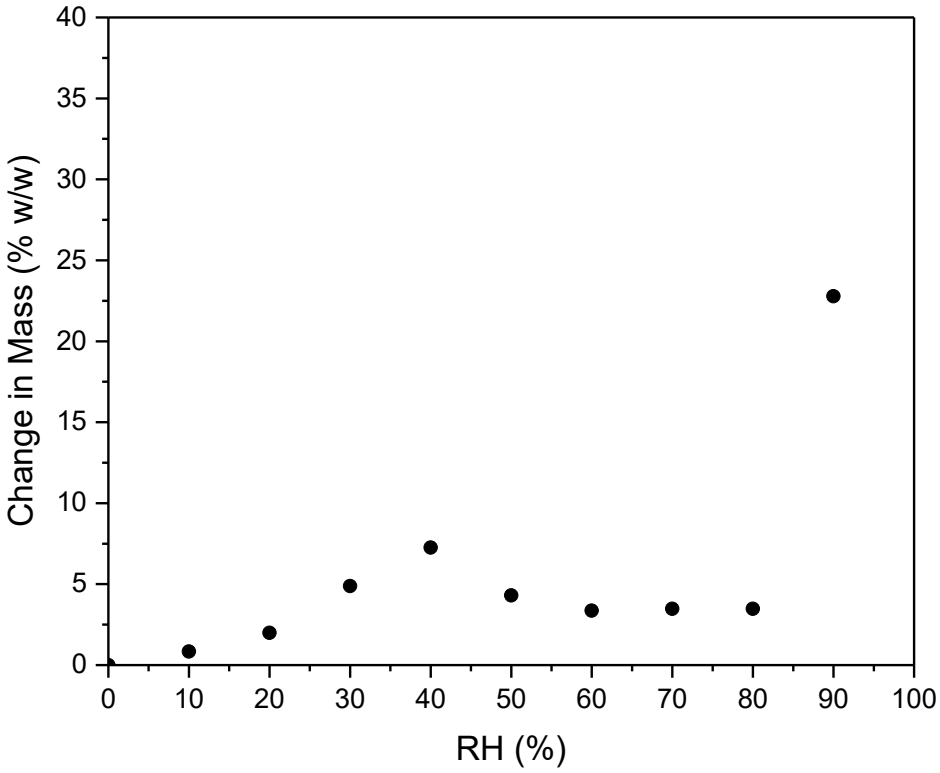


Figure 3.7 Moisture sorption isotherm of FD sucrose 50 mg/mL at 25°C.

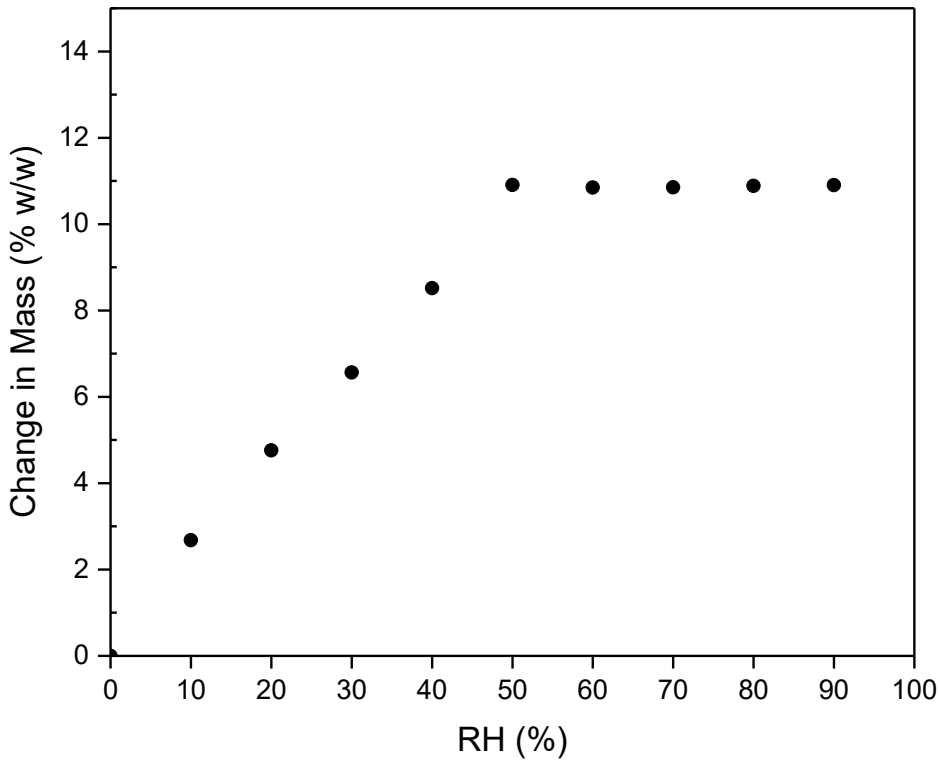


Figure 3.8 Moisture sorption isotherm of FD trehalose 50 mg/mL at 25°C.

The water sorption isotherm at 25°C for FD sucrose at 50 mg/mL is shown in Figure 3.7. The sorption isotherms show low uptake between 0 - 40 RH% up to 10% w/w moisture. After 40% RH, there is a drop in change in mass past. This indicates that crystallisation is occurring, which is then followed by deliquescence corresponding to the large uptake seen above 80% RH. Past water sorption studies with DVS have confirmed the minimum onset of crystallisation RH% to be 40% RH [213]. It has also been shown that deliquescence for sucrose can occur at around 83-85 % RH which corresponds to the uptake in the sorption isotherms after the initial drop in mass [213, 220]. Figure 3.8 shows the sorption isotherm for FD trehalose 50 mg/mL at 25°C. Between 0 - 10 % RH, it exhibits initial small uptake up to around 3% w/w. Past 20 % RH onwards, there is large uptake observed to a maximum of around 11% w/w. After 50% RH, the mass flat lines and does not change. This indicates that there is a probable phase transition occurring, in which the trehalose changes to the di-hydrate form. Hunter et al. saw a similar characteristic mass flat-line water uptake study on amorphous trehalose, which they too suggested was due to crystalline di-hydrate formation [214]. The results obtained from DVS matches with data seen from past water sorption studies involving saturated salt slurries for amorphous sugars in which they also observed changes from a glass to a rubbery morphology, - and sometimes followed by a crystalline form [221].

Figures 3.9 and 3.10 shows the water isotherms of commercial FD B/Phuket flu antigens formulated with either 1% or 20% sucrose, respectively. The B/Phuket with 1% sucrose has initially low uptake in moisture up till 40% RH, near 10% w/w moisture gain. After 50% RH, there is a sharp large water uptake going indicating that sample has completely reverted into liquid solution state. The B/Phuket with 20% sucrose, has very small uptake between 0 - 10% RH, which is then followed by a large increase past 20% RH. There is an initial shoulder plateau between 30 - 70% RH, indicating possibly that the sucrose might have crystallised. Significant mass uptake is only observed once again only above 80% RH, indicating that deliquescence most likely occurred.

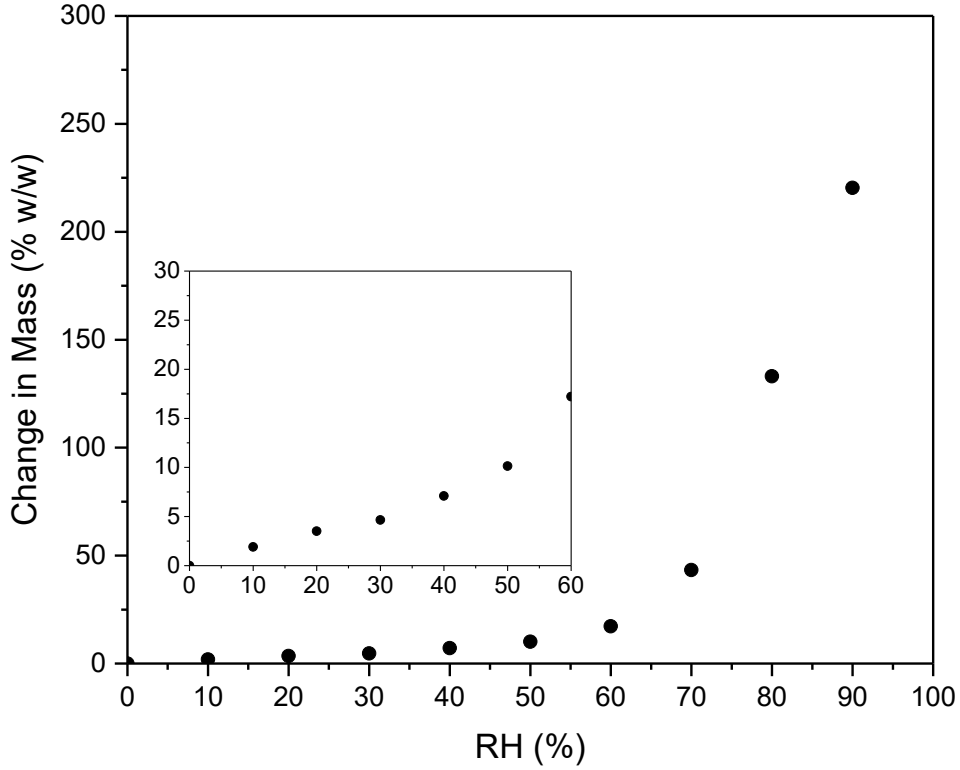


Figure 3.9 Moisture sorption isotherm of FD antigen B/Phuket (with 1% w/w sucrose) at 25°C.

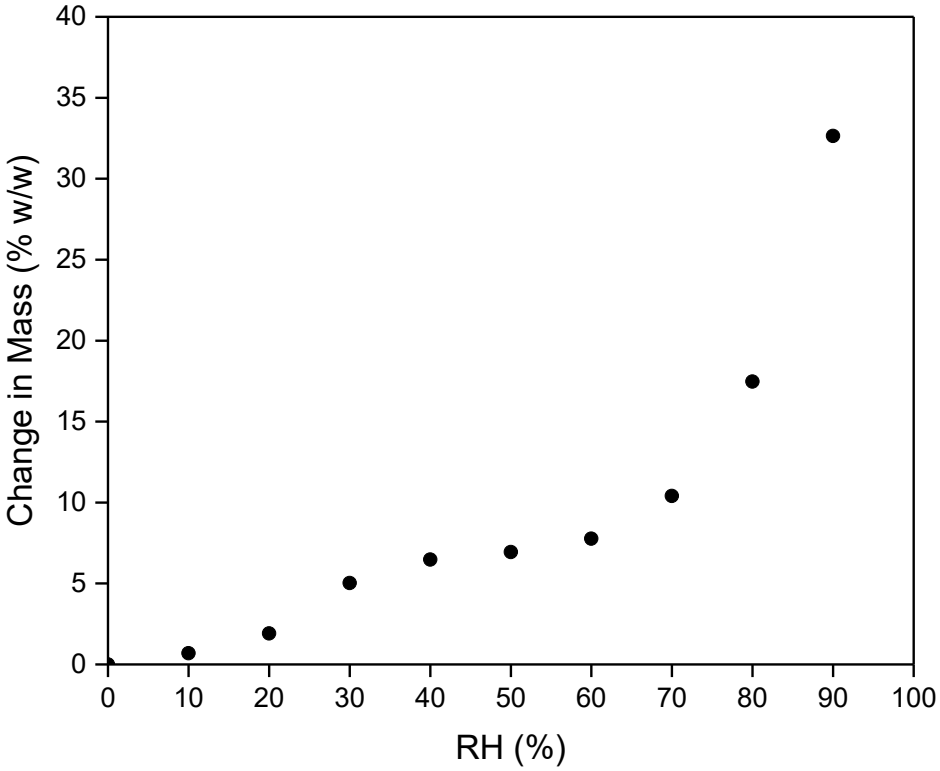


Figure 3.10 Moisture sorption isotherm of FD antigen B/Phuket (with 20% w/w sucrose) at 25°C.

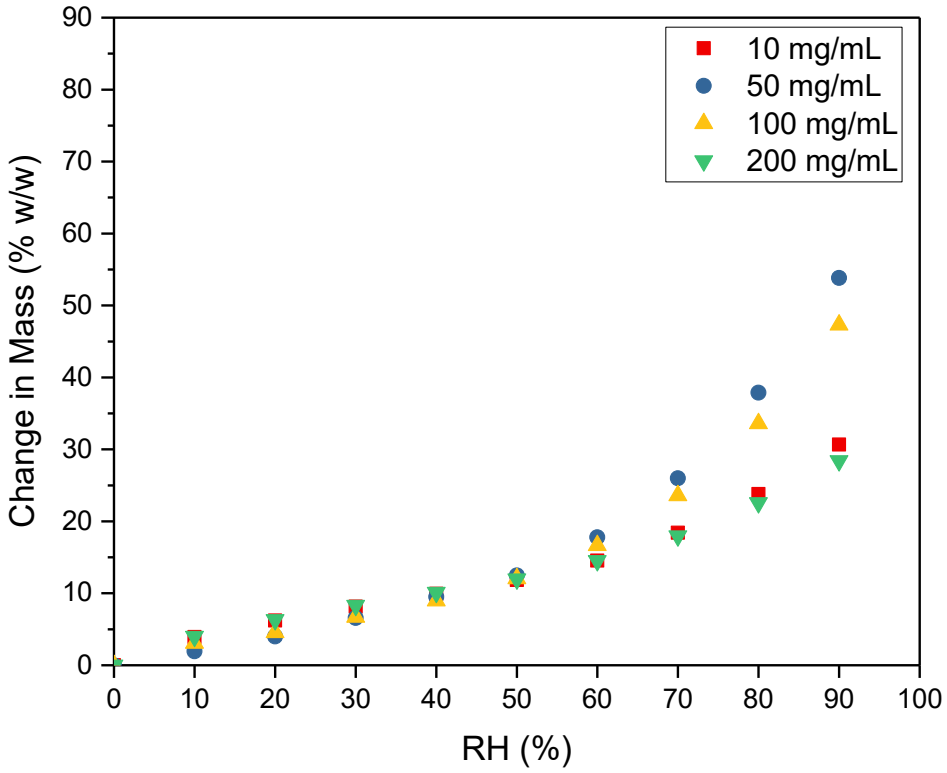


Figure 3.11 Moisture sorption isotherm of FD IgG 10-200 mg/mL at 25°C.

Figure 3.11 shows the water sorption isotherms of FD IgG from 10 - 200 mg/mL concentrations. Between 0 - 20% there is small water uptake below 10% w/w for all concentrations. Past 70% RH, there appears to be correlation with higher water capacity and the increase in IgG concentration from 10 to 100 mg/mL. However, the 200 mg/mL has lowest uptake at 90% RH. This could be because of slow uptake and it never reaching full equilibrium at 90% RH for the set % dm/dt threshold because the maximum stage time (6 hours) had been exceeded. Other reasons could be because cake collapse has occurred or the T_g has been crossed, indicating increased molecular flexibility and variable water uptake capacities at the higher end concentrations.

The limitations of the current and past water sorption studies is that that there is no visual data to inform these isotherm results. As such it is difficult to ascertain with confidence when glass transition, collapse or phase transition might be occurring. Much of the interpretations is based on logical extrapolation of data based on the isotherms. Hence the need to establish a visual and quantifiable methodology based on dynamic ramping to measure such changes.

3.3.3 Measuring Critical Moisture Contents (Ramping mode with Camera)

Typical static water sorption isotherms can be useful in terms of quantifying the water sorption capacity for a sample for a specific % RH's, but they are limited in measuring at what critical moisture when an induced collapse event might occur. A novel solution to this problem is to fit a camera below the sample chamber of a DVS and record visually what changes occurs in real time during a DVS ramping experiment. The ramping method ramp can be set to a rate of % RH per hour to provide increased resolution and data points compared to other modes or traditional static methods. This type of experimentation is not too dissimilar conceptually to FDM or DSC, but instead of ramping temperature at a fixed moisture content, you are instead ramping % RH (and hence moisture content) at a fixed temperature. Thus, in conjunction with the attached camera, you can find the critical moisture content (% change in mass) when induced collapse occurs at a given RH% step (see method Section 3.2.4). Additionally, the inflection point when moisture content reduces the T_g at a given temperature can be measured due to increased resolution of using ramping mode. The inflection point in change of mass (% w/w) occurs between the transition from surface adsorption and bulk absorption of water in material (see Section 2.9.2.1). Measuring such critical moisture contents at different experimental temperatures, thus allows for a 2-D stability maps to be constructed which are relevant to real world operating and storage conditions.

3.3.3.1 FD Excipients

DVS water sorption ramp data for FD excipients, sucrose 50 mg/mL and trehalose 50 mg/mL, are shown in Figure 3.12 and 3.13 respectively. The images were taken only from ramping upwards from 0 - 90 RH%. The humidity ramp rate was set to 12% RH per hour and the temperature was set to 25°C. The left y-axis represents the change is mass (%) of the sample as a function of time, where initial mass is 100%, and every % above that representing the moisture uptake in terms of % w/w (i.e 105% change in mass represents 5% w/w in

moisture content gained). The right y-axis represents the relative humidity (% RH) as a function of time. The % RH in the sample chamber was increased in 1% increments with a ramp rate of 12% RH per hour. Several images taken at the end of each RH% step (during adsorption 0 - 90% RH) are displayed in the below graphs, with their corresponding moisture content at that time point. Initially the samples show a decrease in mass as a drying step at 0% RH is set. For the FD trehalose 50 mg/mL sample, there is a fast and immediate uptake in water and subsequent change in mass after 10% RH. This indicates very quick transition from surface adsorption to bulk adsorption occurring. Onset of collapse and first sign of shrinkage occurs around 44% RH which corresponded to a moisture content of 9.32 % w/w. The mass increases to a maximum of about 12% w/w before a 2% mass loss indicating crystallisation into the di-hydrate form. The final mass stabilises around 10 - 11% w/w moisture, which is also close to the stoichiometric water content in trehalose di-hydrate; 10.4% w/w. Hunter et al, witnessed a similar characteristic drop in mass followed by a mass stabilisation with their water sorption studies with trehalose, which they also attributed to reversion to its di-hydrate form [214]. For the sucrose 50 mg/mL sample, as the RH% is ramped up, there is an initial low change in mass due to only surface adsorption occurring up till to the 25% RH point. Past that point, bulk adsorption begins to occur and you have a rapid increase in the change in mass up to 8% w/w of water. From then, images show the first sign of collapse which is observed at around 32% RH which corresponds to a value of 2.1% w/w moisture. There is possible crystallisation occurring between 40 - 50% RH, as reflected by inflection point in the isotherm accompanied by a visual change in opacity in the sample images. Sucrose has also been reported to crystallise at or above 40% RH in another DVS study [213]. Between 80 to 90% RH, the change in mass rapidly increases to again indicating deliquescence, which was also confirmed visually by images. Past studies have quoted the deliquescence point of sucrose to be near 85.7% RH at room temperature [220]. Roe and Labuza have argued that mixed formulations can also affect this point based on their DSC studies of trehalose-sucrose mixtures. Increasing dry weight fraction of trehalose to 20% w/w significantly hindered the crystallisation of pure amorphous sucrose [222].

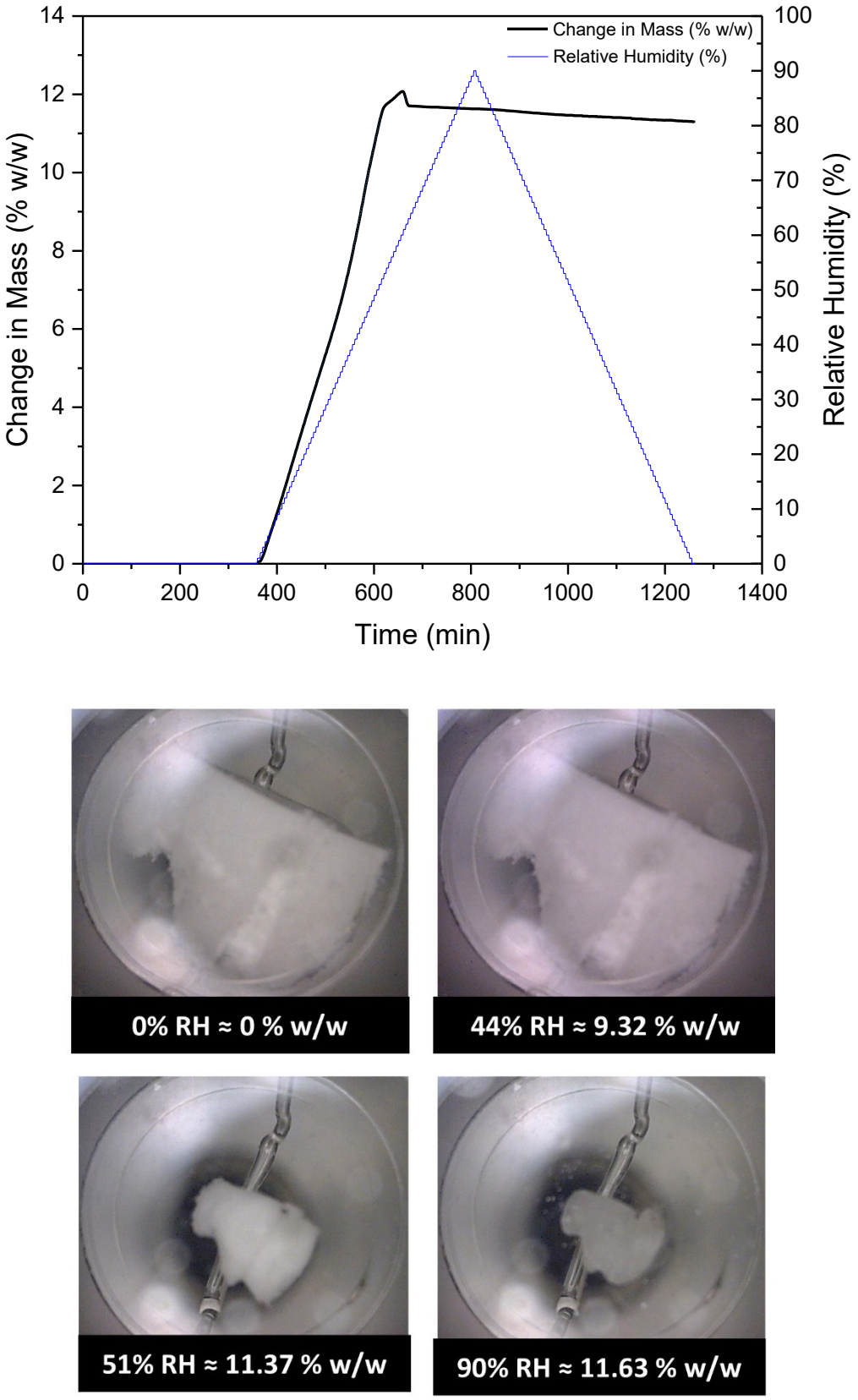


Figure 3.12 DVS sorption graph for freeze-dried trehalose 50 mg/mL at 25˚C. Images show the structure of the cake at the end of the RH% step and the corresponding change in mass (% w/w) at that time.

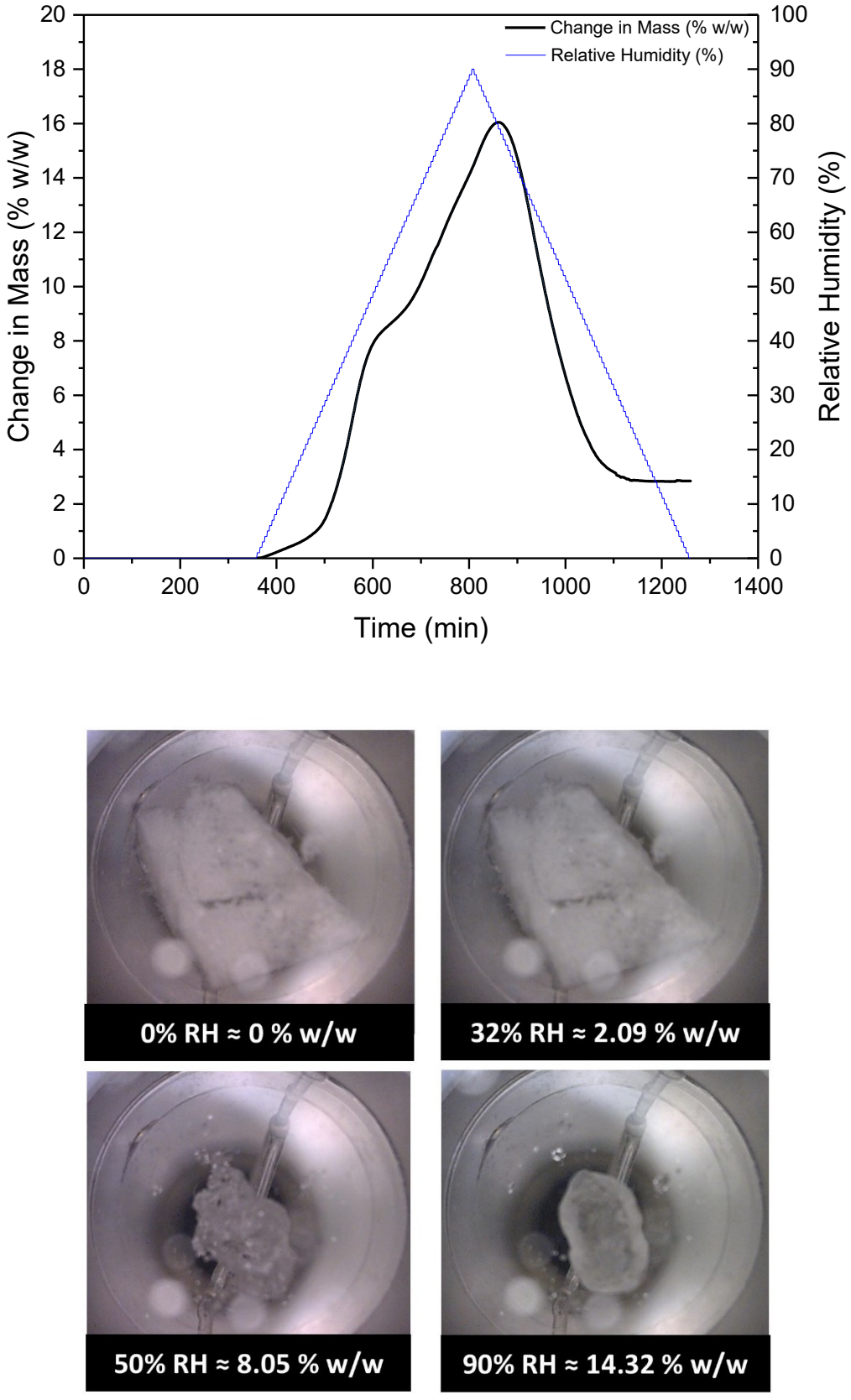


Figure 3.13 DVS sorption graph for freeze-dried sucrose 50 mg/mL at 25˚C. Images show the structure of the cake at the end of each RH% adsorption step and the corresponding change in mass (% w/w) at that time.

3.3.3.2 FD Biologics

Figures 3.14 - 3.16 show the dynamic sorption graphs for three representative FD biologics; IgG 10 mg/mL and B/Phuket flu antigen (60 µg/mL) formulated with either 1 or 20 % w/w sucrose. The accompanying DVS images were taken from the ascending ramp from 0 - 90 % RH. Figure 3.14 shows data for the IgG 10 mg/mL. Even after a 35% w/w moisture uptake, although slightly shrunken, it still retains most of its structure. The first sign of onset shrinkage occurs around 12 - 14 % w/w moisture content, although it does not result in full collapse. The IgG formulations were formulated with 1% w/w sucrose which is equivalent to a 1:1 w/w ratio with the protein. Due the compact and dense matrix of the IgG proteins in FD state, it is possible that there is resistance to full collapse with ever increasing concentration. Indeed for higher concentration mAb like FD proteins, as seen previously in Figure 3.10, they tend to take up more moisture at different partial pressures but there is little structural risk involved due to the highly compact and dense matrix. This most likely also parallels to the specific surface area (SSA), mechanical properties and reconstitution time, which will be explored in later Chapters. Figures 3.15 and 3.16, shows the dynamic sorption graphs of B/Phuket flu antigen (60 µg/mL formulated with either 1 or 20 % w/w sucrose). Immediately apparent is that the formulation with the lower concentration sucrose excipient, takes up larger amounts of water than the higher concentration formulation. More interestingly is that the high sucrose concentration formulation has quicker onset of collapse occurring than the 1% formulation. For the 20% sucrose formulation, onset of collapse occurs around 1.9 - 2 % w/w, while for the 1% formulation, it occurs at much higher moisture content of around 4.8 - 5 % w/w. The increase of the amorphous sucrose excipient in the formulation, whilst improving physical cake properties and possibly increasing stability, also increases the risk of structural collapse or shrinkage occurring at much lower moisture contents. This is also coupled with the fact that FD flu antigens are extremely hygroscopic, which will most likely also see moisture ingress during prolonged storage.

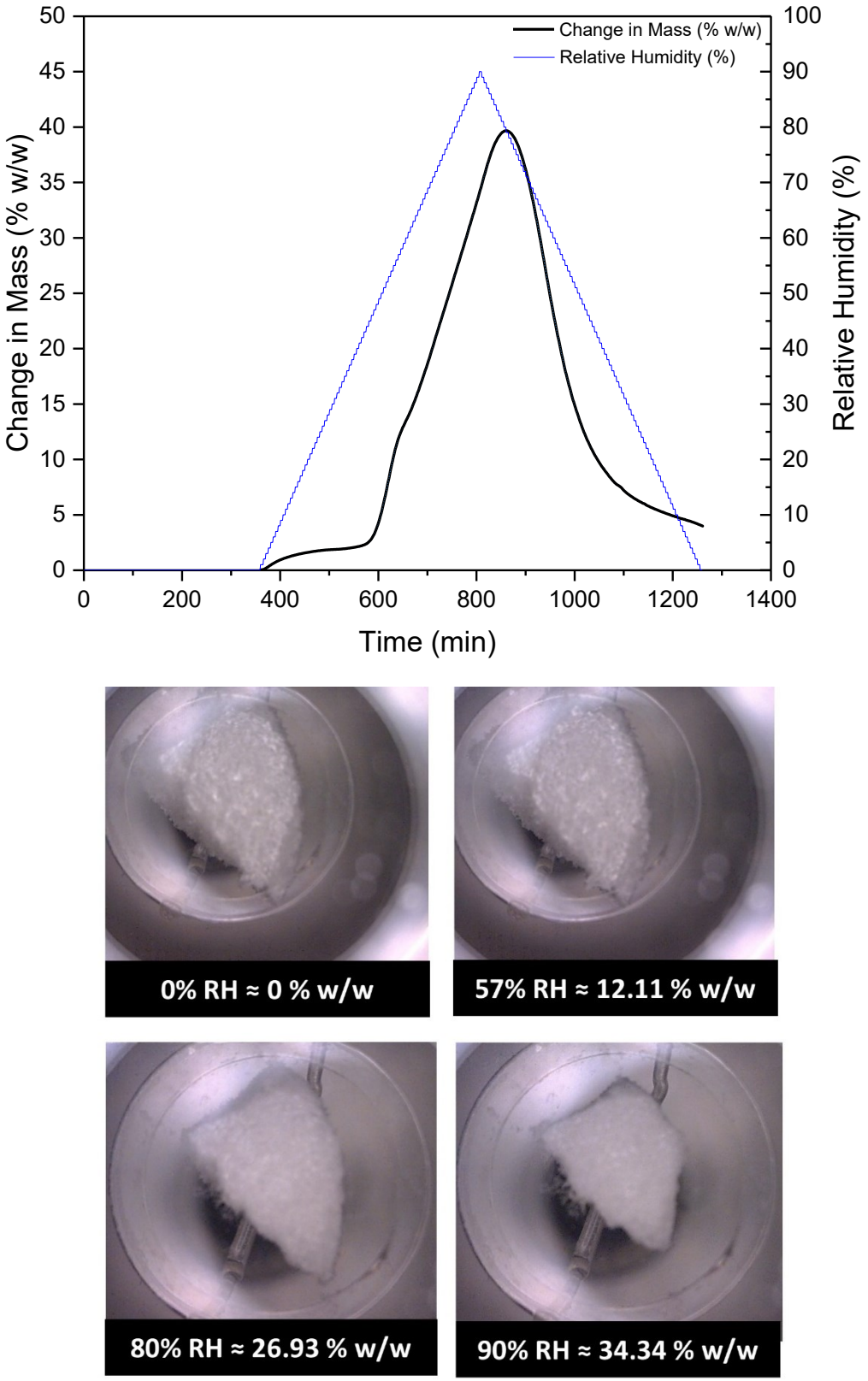


Figure 3.14 DVS sorption graph for freeze-dried IgG 10 mg/mL at 25˚C. Images show the structure of the cake at the end of the RH% step and the corresponding change in mass (% w/w) at that time.

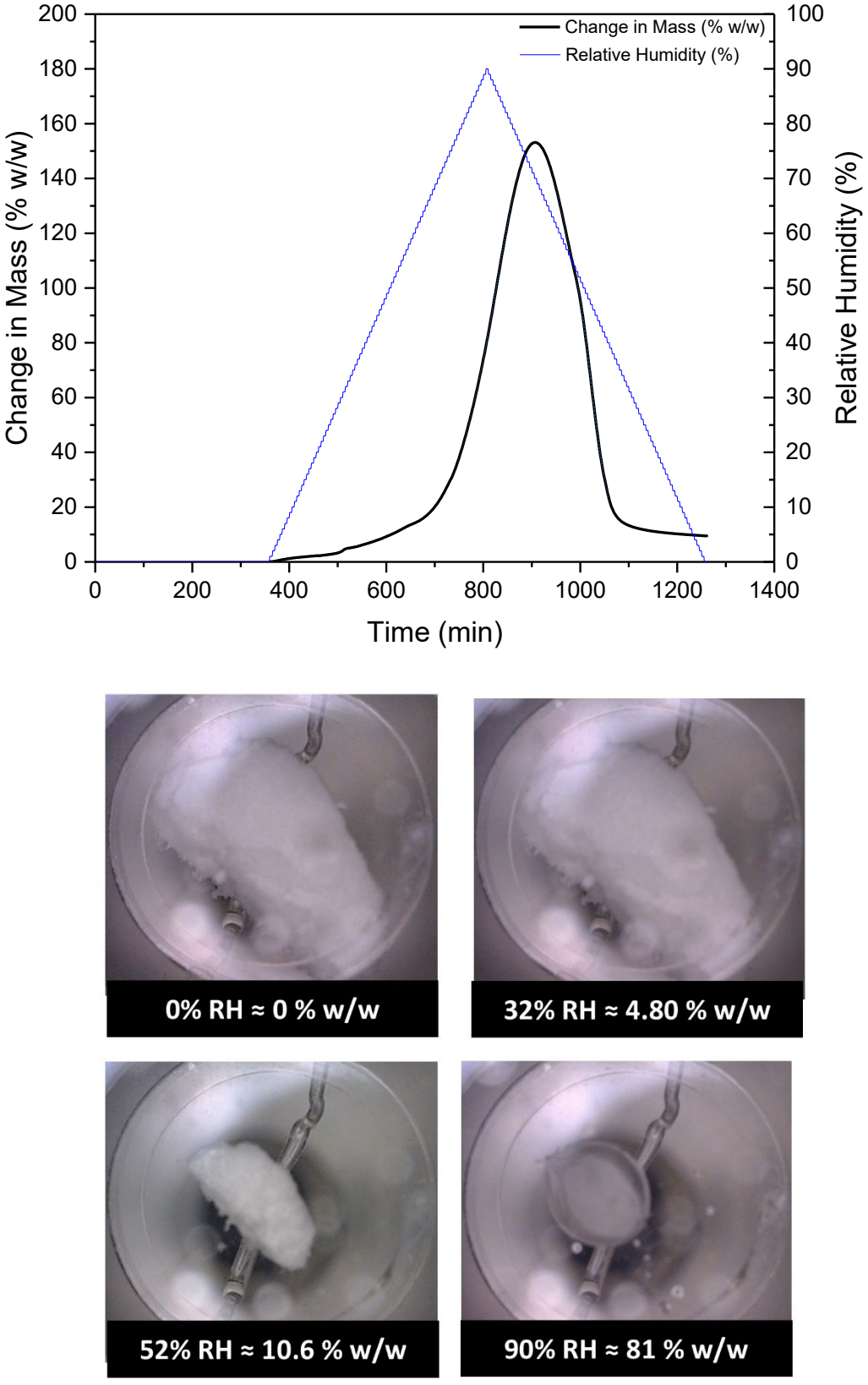
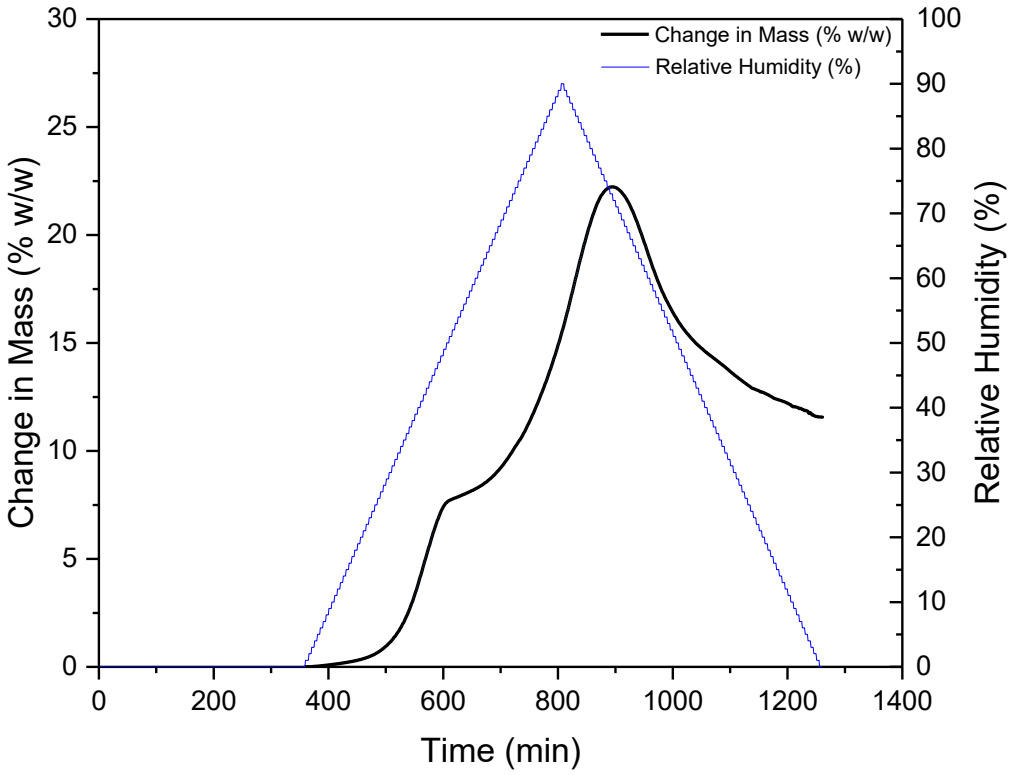


Figure 3.15 DVS sorption graph for freeze-dried B/Phuket flu antigen (formulated with sucrose 1% w/w) at 25˚C. Images show the structure of the cake at the end of the RH% step and the corresponding change in mass (% w/w) at that time.



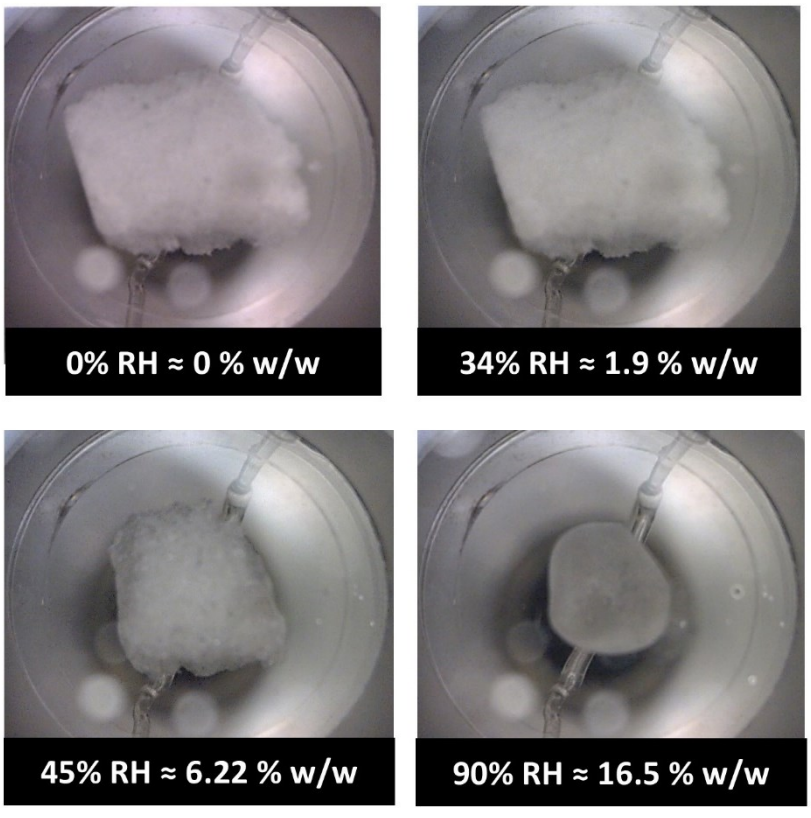


Figure 3.16 DVS sorption graph for freeze-dried B/Phuket flu antigen (formulated with sucrose 20% w/w) at 25°C. Images show the structure of the cake at the end of the RH% step and the corresponding change in mass (% w/w) at that time.

3.3.4 Critical Moisture Stability Maps

Amorphous materials can absorb a significantly large amount of water when compared to a crystalline material, partly due to increased free volume, free energy and surface area. Water acts a plasticiser thereby reducing the T_g of an amorphous material. At a critical moisture content point, the T_g is reduced below the experimental temperature and a loss of structural matrix occurs [91, 201]. Moisture stability maps were constructed by plotting the critical moisture content for onset cake collapse against the temperature of experiment. The critical moisture content was measured with ImageJ when a decrease of 1% in the area of FD cake from the image at the end of a particular RH% step. The corresponding change in mass (% w/w) at the critical % RH step image is equivalent to moisture content gained in the sample. One of the advantages to using DVS is being able to take high resolution measurements that are able to pick up inflection points in sorption graphs. When performing the ramping experiment at lower % RH, the initial water uptake is relatively low. This is due to surface adsorption initially occurring on the glassy material. Above a certain uptake level in moisture, usually a sharp inflection in moisture gain and rapid increase in change in mass (%) is observed. This corresponds to the glass transition humidity/temperature conditions having been reached for that sample. The material undergoes a change from surface adsorption to bulk absorption due to increased molecular flexibility and diffusion constants in rubbery versus glassy solids. Water adsorption characteristics in the glassy state have been described by Freundlich isotherm model, while the Flory-Huggins model has been used to describe during the rubbery phase [162, 163]. The intersection between surface adsorption and bulk absorption generates a critical % RH and thus a critical moisture content (% w/w) when T_g is reached for a material at a given temperature and humidity [160, 161, 164].

Moisture is a critical factor in terms of not only structural but functional stability of FD biologics. As described in the previous section, DVS is able to measure critical moisture contents at which the onset of cake collapse or glass transition occurs for a constant temperature using a relative humidity ramping method. Figures 3.17 - 3.21 display the

constructed stability maps of several FD materials, by plotting the moisture content (% w/w) against the temperature of experiment. The moisture content at which the onset glass transition or cake collapse occurred was measured between ranges of 10°C to 45°C with error bars representing 95% confidence intervals (n=3). The critical moisture was the moisture when first signs of onset of collapse and shrinkage occurred in the images taken with DVS, whilst the critical M_{Tg} was estimated by the intersection between bulk and surface sorption curves. All experiments at each temperature were conducted with the same ramp rate of 12% RH per hour.

As a general trend, with increasing temperature there is a decrease in the amount of moisture required for collapse to occur. At higher temperatures, there will be less moisture required to plasticise the material and hence lower the glass transition to eventually causing a collapse of the matrix under its own weight. When comparing FD excipients, sucrose has lower M_{cp} and M_{Tg} values than trehalose for all temperatures between 10 - 45°C. This indicates that FD trehalose would have less chance of collapse or T_g transition occurring even at high stress temperatures. IgG 10 mg/mL has the highest moisture content of collapse of around 10 - 14% w/w between 10 to 45°C. This indicates once again its relatively strong physical stability even at high moisture contents compared to the B/Phuket antigens. The B/Phuket formulation with 20% sucrose has the lowest moisture content of collapse of all materials tested between 10 - 45°C, between 3 to 1.9% w/w respectively. The increase in amorphous sucrose content has made the structural matrix more vulnerable to collapse and T_g transition happening at much lower water uptake compared to the lower concentration formulation at 1% sucrose.

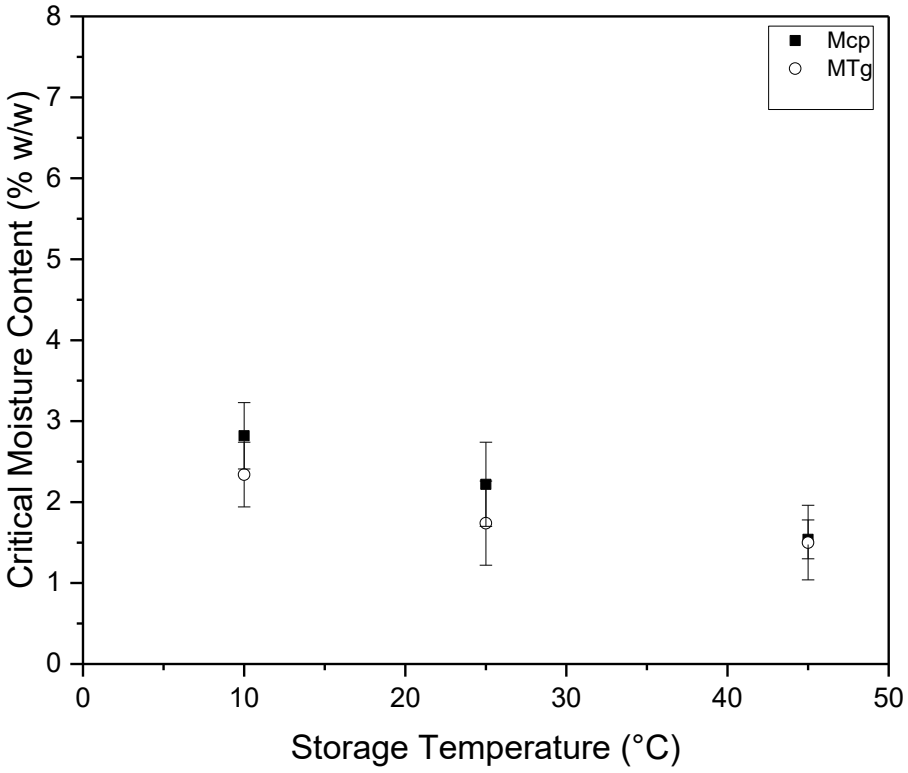


Figure 3.17 Critical moisture stability map for freeze-dried sucrose 50 mg/mL from between 10°C - 45°C. M_{cp} and M_{Tg} refers to the moisture content at onset of collapse point or glass transition, respectively.

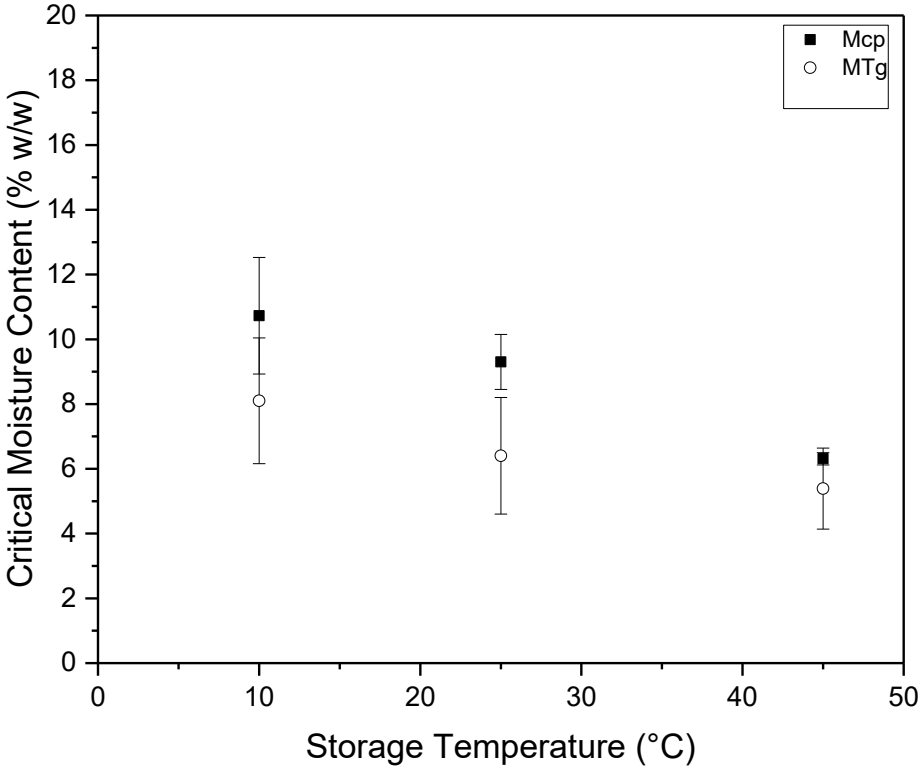


Figure 3.18 Critical moisture stability map for freeze-dried trehalose 50 mg/mL from between 10°C - 45°C. M_{cp} and M_{Tg} refers to the moisture content at onset of collapse point or glass transition, respectively.

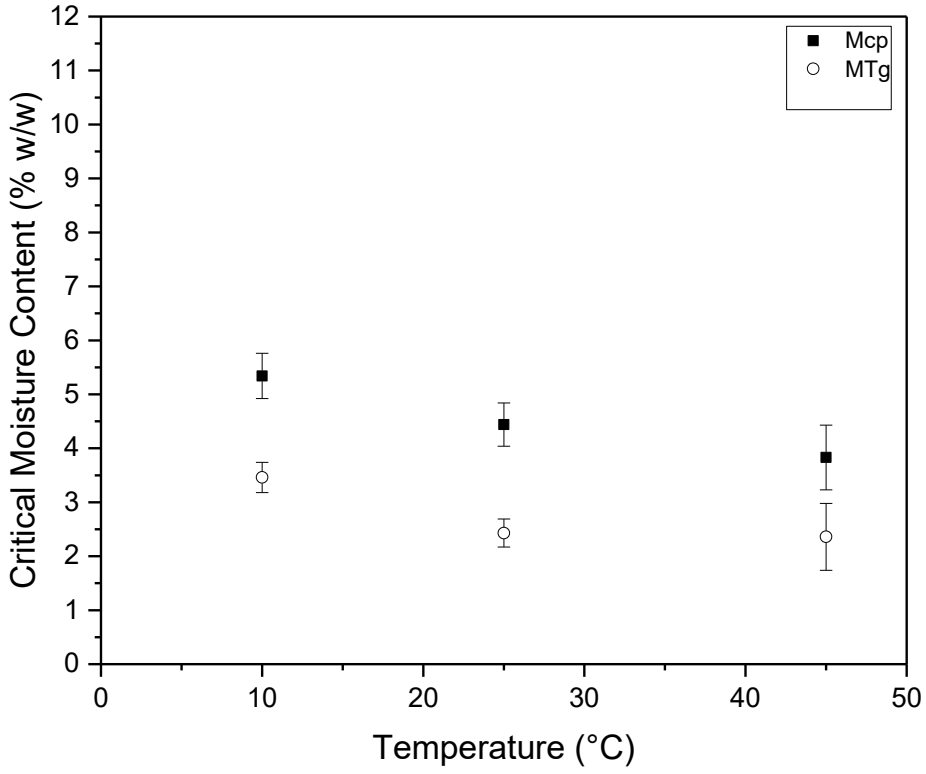


Figure 3.19 Critical moisture stability map for freeze-dried B/Phuket antigen with 1% w/w sucrose from between 10°C - 45°C. M_{cp} and M_{Tg} refers to the moisture content at onset of collapse point or glass transition, respectively.

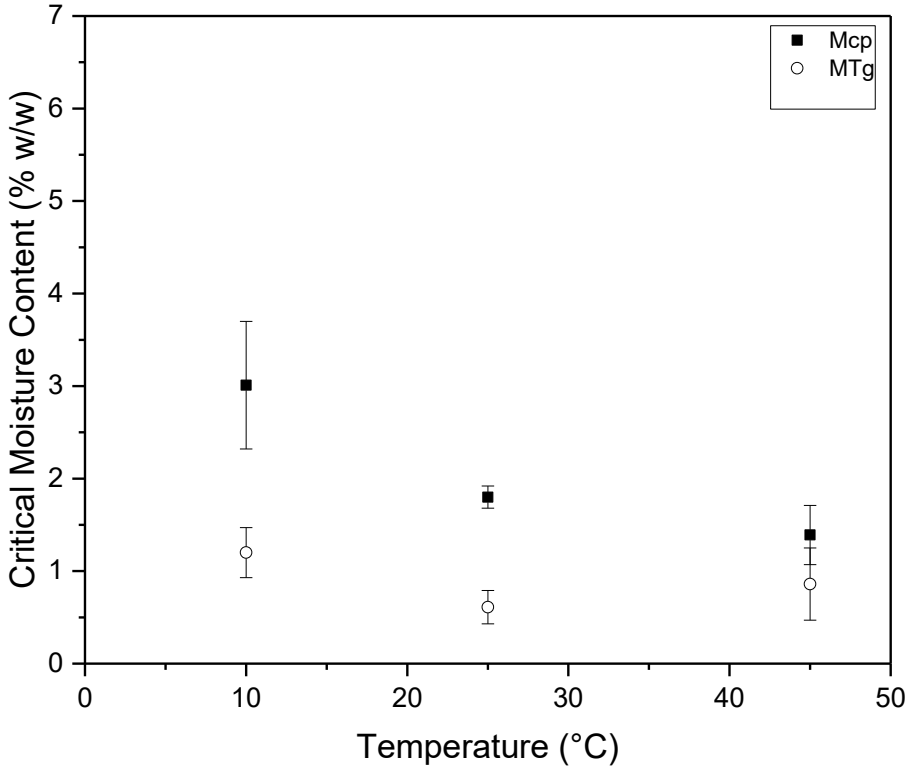


Figure 3.20 Critical moisture stability map for freeze-dried B/Phuket antigen with 20% w/w sucrose from between 10°C - 45°C. M_{cp} and M_{Tg} refers to the moisture content at onset of collapse point or glass transition, respectively.

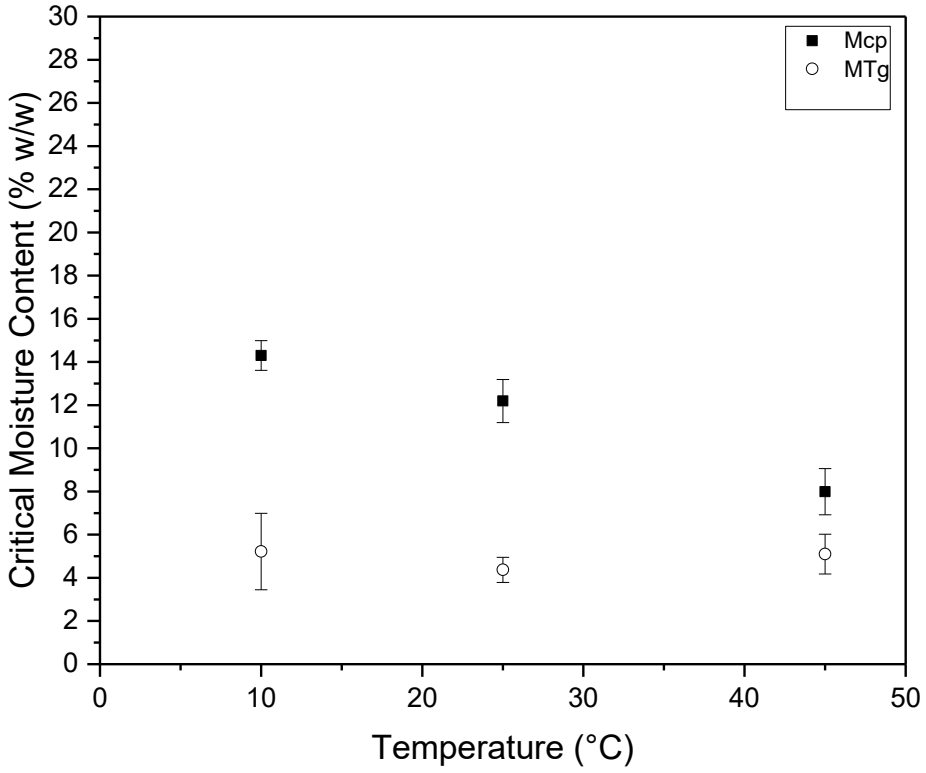


Figure 3.21 Critical moisture stability map for freeze-dried IgG 10 mg/mL from between 10°C - 45°C. M_{cp} and M_{Tg} refers to the moisture content at onset of collapse point or glass transition, respectively.

With increasing temperature, the moisture content at onset of collapse also decreases on average. When the sample is at higher temperatures, less moisture is required to exceed the T_g than at lower temperatures. During long term storage, samples stored at higher temperatures are therefore at greater risk of shrinkage or cake collapse than samples stored at lower temperatures. Not only could the reduction in T_g from moisture compromise structural stability but it could also affect biological activity. The glass vitrification hypothesis states that the closer the storage temperature value is to the products T_g value, then the more molecular flexibility and hence chance of degradation occurring.

In some cases, there is varying overlap between critical moisture when M_{Tg} is reached or M_{cp} collapse occurs. Whether there is a difference between the two in terms of structural collapse over a long enough time scale is up for debate. Although one can argue that T_g is reversible while the collapse of structure is not. However, one may theorise that the critical

moisture at M_{Tg} is a lower level condition (i.e minimum), whilst the M_{cp} is the upper level condition (i.e maximum). That is to say, that there is a chance that collapse could occur at any mass time between the two ranges (once the moisture content reaches between the M_{Tg} - M_{cp}). However, it is only when at or above the M_{cp} critical moisture content, that one can confidently predict that the onset of collapse will occur. This transition from glassy state to rubbery to matrix collapse, would also be affected by the storage temperature. The higher the storage temperature, than the greater degree of molecular flexibility occurs, which could also explain the blurring of the lines between T_g transition and collapse points. Of course, this point would require further exploration and research altogether. Nevertheless, these critical moisture contents given here in terms of stability maps could be used as stability guidelines during post storage. A proposed general rule could be that the moisture content of the samples in vials is not allowed to exceed 50% of the critical moisture content value (for example if M_{cp}/M_{Tg} for a material is around 2% w/w), then general guideline issued would therefore be not to exceed 1% w/w. The selection of 50% value is arbitrary choice used as an example and of course would be up to individual discretion.

Since moisture is the critical component in determining cake shrinkage and collapse, the critical relative humidity not to exceed would be the one where the equilibrating change in mass is below the critical moisture content. For example looking at the dm/dt equilibrium graph at 25°C for trehalose 50 mg/mL, at 40% RH the maximum water uptake is around 9% w/w, while at 50% RH the maximum is 11% w/w. On the collapse graphs, the moisture content of collapse is around 9% w/w therefore, the RH should always be kept below 40% to ensure it never reaches this critical moisture. At RH's below 40%, the moisture content would never be able reach the critical point, due to sample equilibrating below that moisture content.

In terms of cycle development and storage, these stability maps can be used as warning system, indicating at what point moisture in sample is getting too high. Of course, just because high moisture may result in collapse and loss in structure of FD cake, it does not mean to say this will be linked to loss in biological stability as well. Schersch et al. showed that

even though their FD samples had collapsed during storage they did not lose any biological activity [115]. High moisture might not necessarily be a threat for biological activity but is for structural stability, depending on a case by case basis for different FD biologics.

3.3.4.1 Effect of Ramp Rate

The experiments shown in Section 3.3.4, were all conducted at a ramp rate of 12% RH per hour. In Figures 3.22 and 3.23 the critical % RH and the critical change in mass (% w/w) when onset of collapse occurred is plotted versus different ramp rates for FD trehalose 50 mg/mL samples.

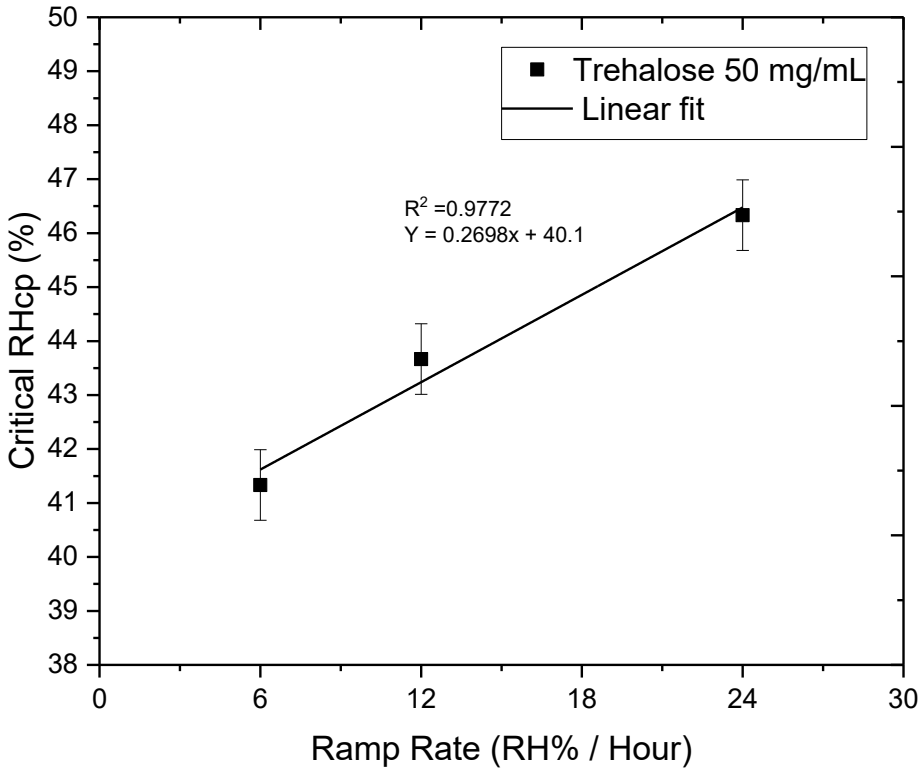


Figure 3.22 Ramp rate versus onset RH% and onset moisture content (%w/w) for trehalose 50 mg/mL at 25°C. Error bars represent 95% confidence intervals (n=3). Straight line represents the linear fit.

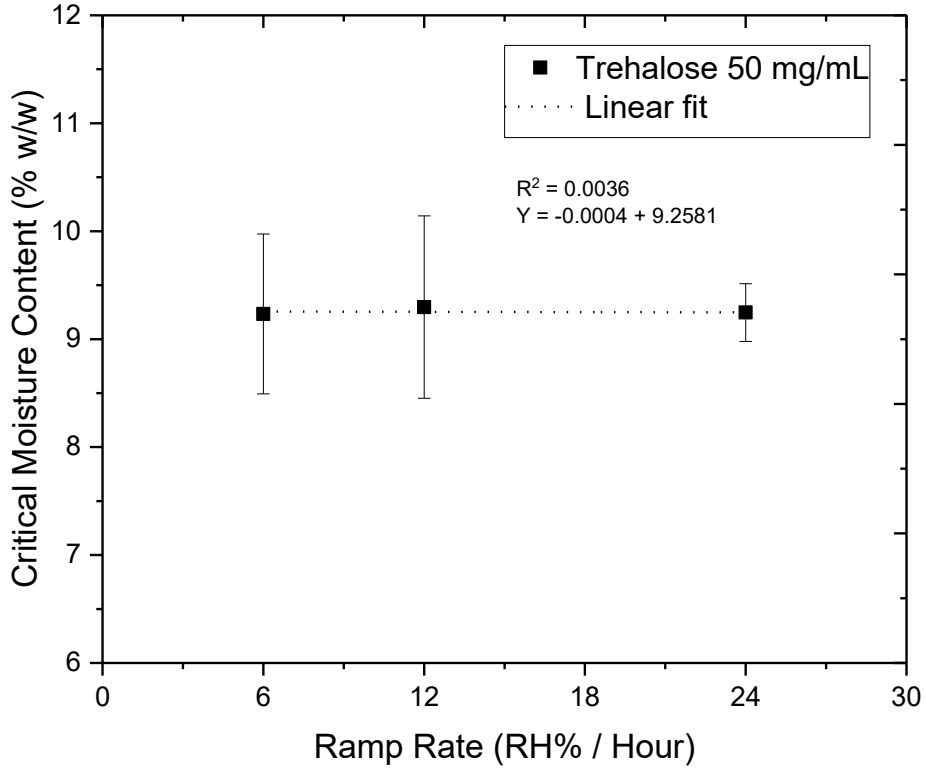


Figure 3.23 Ramp rate versus critical moisture content (%w/w) for FD trehalose 50 mg/mL at 25˚C. Error bars represent 95% confidence intervals (n=3). Dotted line represents the linear fit.

The higher ramp rates were correlated with higher collapse RH% point and vice versa with lower ramp rates in a linear trend (R^2 = 0.9772) . The critical RH is thus dependant on a linear relationship between ramp rates of a particular DVS experiment. However, in Figure 3.23, when plotting critical moisture content % w/w at collapse versus the ramp rates, no such linear correlation existed. Irrespective of ramp rate, the sample collapsed at the same critical moisture content value (within experimental error) for the same temperature, with a very low coefficient of determination (R^2 = 0.0036). This shows that once a critical moisture content is

reached then cake shrinkage and collapse will occur. This result typically signifies that moisture content has reduced the T_g of the sample sufficiently so that it is equal to or below the experimental storage temperature. In practice this means that one can select any ramp rate to find out the critical moisture content of collapse, but the critical % RH_{cp} this will vary. In order to find out the critical % RH_{cp} of collapse then a series of ramp rate experiments are required to extrapolate to zero ramp rate. The critical % RH of the sample would be when the maximum equilibrating water capacity is just below the critical moisture content for collapse.

Burnett et al. showed in their ramping method study that the critical % RH of glass transition correlated linearly with the ramp rate [161]. They used a linear extrapolation to find the RH_g by extrapolating to an infinitely slow ramp rate; a zero ramp rate. Additionally studies at higher temperatures lead to lowering of the threshold required. Likewise, Yuan et al. also determined the critical RH when glass transition occurred for polydextrose at different water activities [164]. They tested different flow rates instead of ramp rates and found that the critical RH was time/rate dependent. Their data also showed a linear correlation to the flow rate, which they used regression analysis to extrapolate to zero flow and work out the critical RH_0. However, in all these studies there was no data provided for what the critical moisture content in the sample was when the transition event occurred. From these results, the moisture content of the sample when collapse occurs is independent of the environmental ramp conditions it is exposed to.

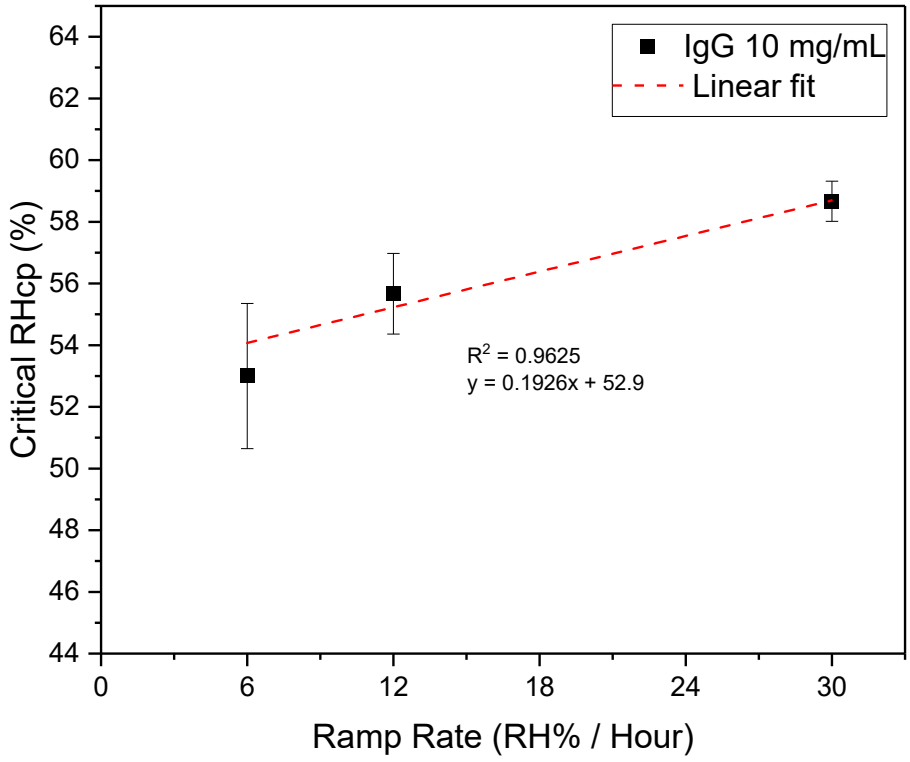


Figure 3.24 Ramp rate versus onset RH% and onset moisture content (%w/w) for IgG 10 mg/mL at 25˚C. Error bars represent 95% confidence intervals (n=3). Straight line represents the linear fit.

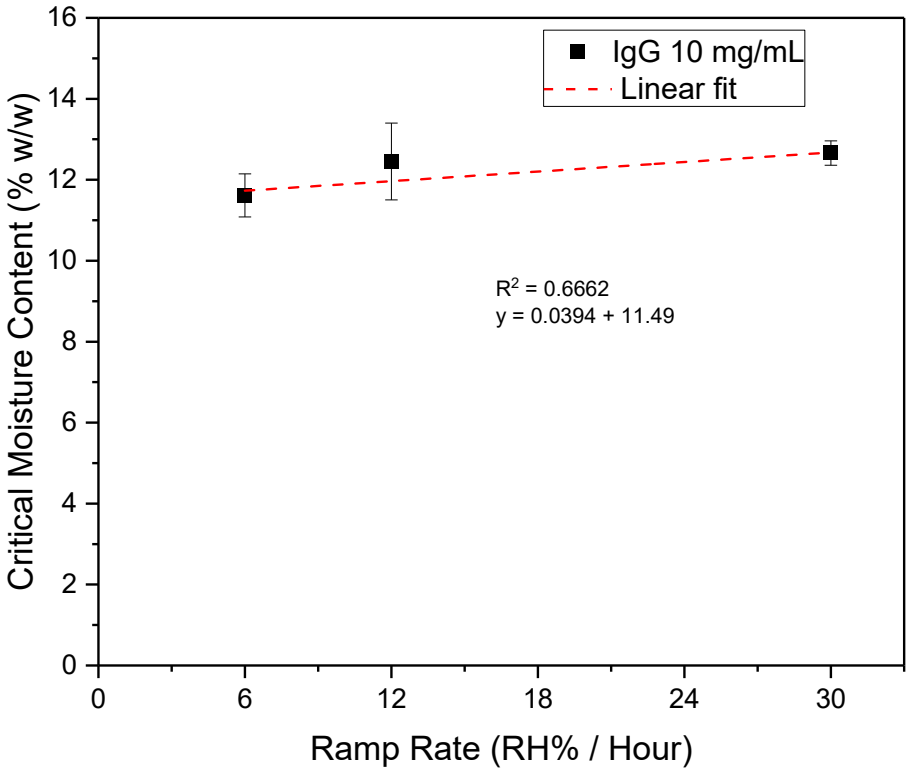


Figure 3.25 Ramp rate versus critical moisture content (%w/w) for FD IgG 10 mg/mL at 25˚C. Error bars represent 95% confidence intervals (n=3). Dotted line represents the linear fit.

Figure 3.24 and 3.25 shows the ramp rate versus the RH % or change in mass (M_{cp}) when onset of collapse occurs for 10 mg/mL IgG. Again as seen previously, there is a good correlation between ramp rate and critical % RH (R^2 = 0.9625), however not for when comparing to the change in mass (critical moisture content % w/w) of onset of collapse occurs (R^2 = 0.6662). This would once again indicate that critical humidity is time/rate dependant, whilst the critical moisture content % w/w is not. When doing a ramping experiment via dynamic sorption, the samples may not reach equilibrium before moving onto the next stage. Because of this, as the slower the ramp rate is closer to zero, then the closer the value will be to the true % RH of collapse. The % RH of collapse, is the point when the sample is able to equilibrate at or past the critical change in mass. The ramp rate does not affect this critical change in mass, as it only effects the speed of water uptake accumulation a particular % RH. Past studies have tended to quote particular critical % RH values for FD materials. However, future studies would be better to quote the actual change in mass when these changes occur; the critical moisture content % w/w. The critical RH would be ramp-dependant and would be time consuming requiring the need to extrapolate towards zero. Whilst knowing a critical % RH may be important for storage, a better descriptor for formulations would be in terms of critical moisture content.

3.4 Conclusion

The dynamic vapour sorption technique can be used to measure the water uptake of FD materials and create isotherms. This Chapter has described a ramping procedure with high data resolution for determining critical moisture content at collapse and shrinkage for amorphous FD materials through the use of real-time images with a fitted camera. Increasing experimental temperature lowered the overall critical moisture content % w/w required for collapse/shrinkage to occur which is independent of the ramp rate used in the experiment. It is anticipated that future researchers can use and further develop this standardised technique described here to measure and map out key moisture/humidity stability maps for a larger range of FD biologics. Overall, the process can be used to improve formulation and provide useful information for better cake stability in regard to critical moisture content and temperature over long term storage.

Chapter 4. Measuring the Mechanical and Surface Properties of Freeze-Dried Materials

4.1 Introduction

Freeze-drying produces solid state cakes with different morphological characteristics which are determined by a number of factors such as solution formulation and cycle process conditions. Many biopharmaceutical companies employ the freeze-drying to enhance the long term stability of their products, which can otherwise undergo degradation in the liquid state. Some of these FD cakes may be mechanically fragile and this could pose problems during transportation or shipping. Vial wall impacts and vibrations could result in cake breakage resulting in unsatisfactory vial cake appearance. During long term storage there might be physical changes occurring due to solid state degradation and aging which can affect the mechanical and the surface properties of FD cakes. In particular, water is a known plasticiser which reduces the T_g of FD biologics and in extreme cases if enough water is present, can cause structural collapse to occur as seen in the previous Chapter. The combination of moisture ingress over time into vials and the use of elevated storage temperature could lead to unacceptable changes to the physical properties of FD material in the vials. In industry, current characterization methods for FD materials have tended to focus more on thermal analysis properties, water content activity and cake appearance (as outlined in Section 2.10). The emergence of new instrumentation based on novel techniques, such as IGC and mechanical indentation, means there is opportunity for determining newer types of physical and surface properties. However, since being introduced these techniques have been under-utilised in terms of assessing long term mechanical and surface properties of FD materials.

Measuring and detecting changes in surface and mechanical properties of FD cakes during long term storage might have a number of other potential applications including:

i. Being able to potentially track batch to batch variations in final products and during storage

ii. Potential to correlate and compare structural changes with moisture, temperature and biological stability in the solid state

iii. Assisting in formulation and FD process design to produce cakes with improved properties as required by industry or regulators

As such determining the mechanical properties (Young's modulus) and specific surface area (SSA) more easily and quickly could help establish these characteristics as critical quality attributes (CQA's) for lyophilised biological products including during storage [223]. The most common methods for determining mechanical properties and SSA of FD biologics is the application of mechanical indentation and BET volume gas adsorption methods, respectively. IGC has successfully measured the SSA and surface energy for a wide range of materials such as polymers, fibres and pharmaceuticals [183, 188, 224]. However, there has been limited work focused on using IGC to measure the SSA of FD materials. Similarly, since the initial studies reporting the flat punch indentation instrument, there has been no long term work conducted focusing on moisture and storage conditions and their effects on mechanical properties of FD biologics. This Chapter aims to investigate the suitability of IGC and mechanical indentation technique at determining the SSA and mechanical properties, respectively, for a range of FD excipients and biologics.

4.2 Materials and Methods

4.2.1 Materials

A 1 mL solution was filled into 5 mL volume screw capped vials (41.5 X 18 mm i.d. Schott VC005 (Adelphi Tubes, Haywards Heath UK). IgG and B/Phuket were FD on the Telstar LyoBeta 15 (Azbil-Telstar SpA, Terrassa, Spain). The lysozyme, sucrose and trehalose were FD on a Virtis Advantage Plus (Biopharma Process Systems Ltd, Winchester, UK). 14 mm diameter igloo halobutyl stoppers (Adelphi Group, Haywards Heath, UK) were used to stopper the vials under backfill with nitrogen gas.

The following materials were freeze-dried:

- IgG 10 - 200 mg/mL (NIBSC, Potters Bar UK)
- Influenza antigen B/Phuket 14/252, 60 µg/mL, (NIBSC, Potters Bar UK)
- Sucrose (Sigma: S5016-1 kg, Dorset, UK)
- Trehalose (Cargill 16400, Minneapolis, MN, USA)
- Lysozyme (Sigma: L6876-5 g, Dorset, UK)
- Bovine serum albumin (Sigma-Aldrich: SLBX5725, Dorset UK)

4.2.2 Lyophilisation cycle

Same cycle as previously described in Chapter 3

4.2.3 Dynamic Vapour Sorption (DVS)

Same method as previously described in Chapter 3

4.2.4 Moisture Content

Same method as previously described in Chapter 3

4.2.5 Case Study 1: Mechanical Indentation of FD materials

The mechanical properties of FD materials can be characterised through the use of mechanical indentation based on work from Devi and Williams who used a flat-punch technique method to determine the mechanical properties for common FD excipients [110, 225]. In brief, a flat-punch face probe (5 mm diameter) constructed of polymethyl methacrylate was attached to a 250 g strain gauge load cell (FUTEK, Wyboston, Bedfordshire). Calibration of the load-cell was performed using calibration weights. Indentation was incremented in steps of 20 μm. The resulting data from the tests produces a force (g) versus displacement (μm) graph which can then be re-plotted as classical stress versus strain graphs (Equation 2.2). The Young's modulus (E_f), modelled after a material that is described as a foam, was found directly from the linear region of indentation data graphs (based on the Equation 2.3). As a result of limited available number of FD samples and experimental design, only 3 samples were tested for each experiment. Ideally in future scenario more repeats would have been useful. Confidence intervals were used to see differences between samples (see statistical analysis in chapter 3 for rationale) although in future work t-testing could also have been used in combination to further see more assured differences statistically.

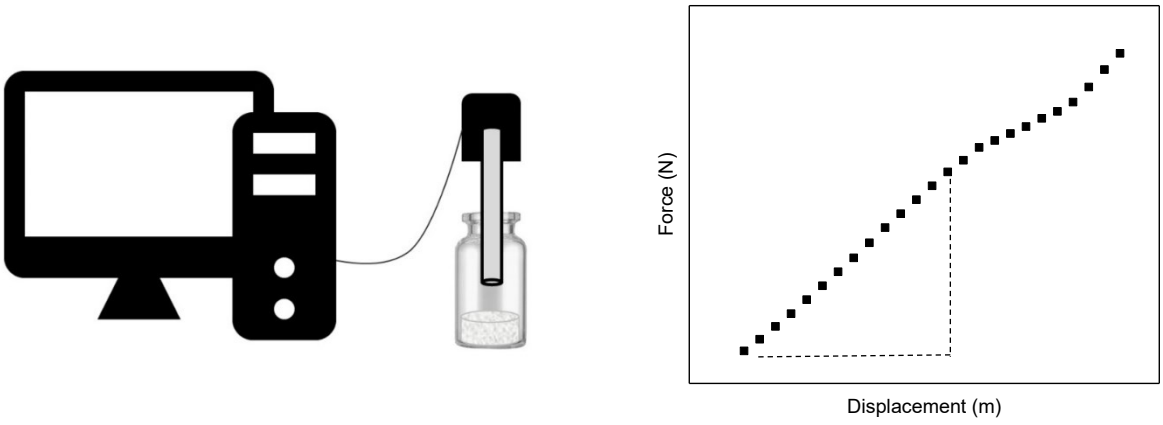


Figure 4.1 Schematic drawing of mechanical indentation instrument for determining mechanical properties.

4.2.5.1 Effect of humidity exposure

The mechanical properties of FD excipients (sucrose and trehalose) and biologics (IgG, B/Phuket antigen, BSA and Lysozyme) were measured after controlled exposure to room temperature humidity, as confirmed with a hygrometer (product code: 11536973, Fisherbrand, UK), at several time intervals (n=3). The moisture gained was measured gravimetrically via a balance (Ohaus Pioneer, NJ, USA) subtracting the initial weight from the new weight. A total of three repeat measurements for all samples were made and plotted (n=3).

4.2.5.2 Effect of storage temperature

FD lysozyme 10 mg/mL vials (10% w/w trehalose with 1.32% w/w dibasic sodium phosphate buffer with adjusted pH 9.13) was split into two different moisture content batches; high and low. Vials were exposed for 20 hours at room atmosphere to facilitate high moisture uptake, while the low moisture vials remained unopened after freeze-drying manufacture. Moisture content of the FD samples were confirmed via coloumetric Karl Fischer titration (Mitsubishi CA-200, A1-Envirosciences Ltd, Blyth, UK) in triplicate (n=3). High and low moisture content vials had average moisture contents of 9.16% and 1.94% w/w, respectively. Samples were stored either at -20°C, 25°C or 65°C over a period of 21 days. The Young's modulus was measured at days 0, 2, 7, 14 and 21 and in triplicate (n=3). FD BSA was made with either 1% and 5% w/w sucrose or trehalose (1:1 protein to excipient ratio) with 10 mM sodium phosphate buffer (pH 7.4).

FD IgG 50 mg/mL vials (with 1% w/w sucrose, 10 mM citric acid adjusted at pH 6.6 with 5 M NaOH, 0.01% Tween 20) was stored at -20°C, 25°C and 56°C for a storage period of 6 months. The moisture content at each time interval was measured with coloumetric Karl fischer titration (Mitsubishi CA-200, A1-Envirosciences Ltd, Blyth, UK) in triplicate (n=3). The monomer content (%) was measured via Size-exclusion high performance liquid chromatography (SEC-HPLC). Analysis was performed using the Thermo Scientific UltiMate 3000 HPLC System (Thermo Fisher Scientific, UK) with a TSKgel G3000SWXL HPLC column

(300 x 7.8 mm, Sigma-Aldrich, UK) and UV-detection selected at 280 nm (n=3). The mobile phase consisted of disodium hydrogen phosphate dihydrate (0.49% w/v), sodium dihydrogen phosphate monohydrate (0.17% w/v), sodium chloride (1.17% w/v) and sodium azide (0.01% w/v). The flow-rate on the apparatus was set at 0.5 mL/min and operating temperature of 25°C.

The FD cycle for excipients (sucrose and trehalose) and biologics (B/Phuket and IgG) was the same as the on Table 3.1 in Chapter 3. The FD cycles for BSA and Lysozyme are provided in Tables 4.1 and 4.2.

Table 4.1 Freeze drying cycle for BSA formulations. Vials were stoppered under normal atmospheric pressure.

Freezing Temperature (°C)	Freezing Ramp Rate (°C/min)	Freezing Hold Time (min)	Primary Drying Ramp Rate (°C/min)	Primary Drying Temperature (°C)	Primary Hold Time (min)	Secondary Drying Ramp Rate (°C/min)	Secondary Drying Temperature (°C)	Secondary Hold Time (min)
-50	0.50	120	0.50	-40	4500	0.50	20	900

Table 4.2 Freeze drying cycle for Lysozyme formulations. Vials were stoppered under normal atmospheric pressure.

Freezing Temperature (°C)	Freezing Ramp Rate (°C/min)	Freezing Hold Time (min)	Primary Drying Ramp Rate (°C/min)	Primary Drying Temperature (°C)	Primary Hold Time (min)	Secondary Drying Ramp Rate (°C/min)	Secondary Drying Temperature (°C)	Secondary Hold Time (min)
-50	0.78	300	0	-50	3765	0.12	15	560

4.2.5.3 Quantification of the extent of cake surface cracking

Vials were placed in each corner and middle of tray to map out what effect vial tray location had on cake cracking and to see if there was any edge vial effect occurring. Vials were examined by a camera positioned above the opened vials after FD. The distance from the camera to the vial was fixed at 20 cm. Photographs were taken of opened vials and images were then cropped prior to being processed with ImageJ (National Institutes of Health, USA). Images were converted to binary colour format, whereby cracked areas appeared in white and non-cracked black (see Figure 4.2). The histogram function on ImageJ was used to measure ratio of white pixels to the total number of pixels on the cake surface. This ratio was expressed as a fraction of the cracked surface (from 0 to 1) and then contour maps were plotted based on the location where the vials were taken from the freeze-drier tray location.

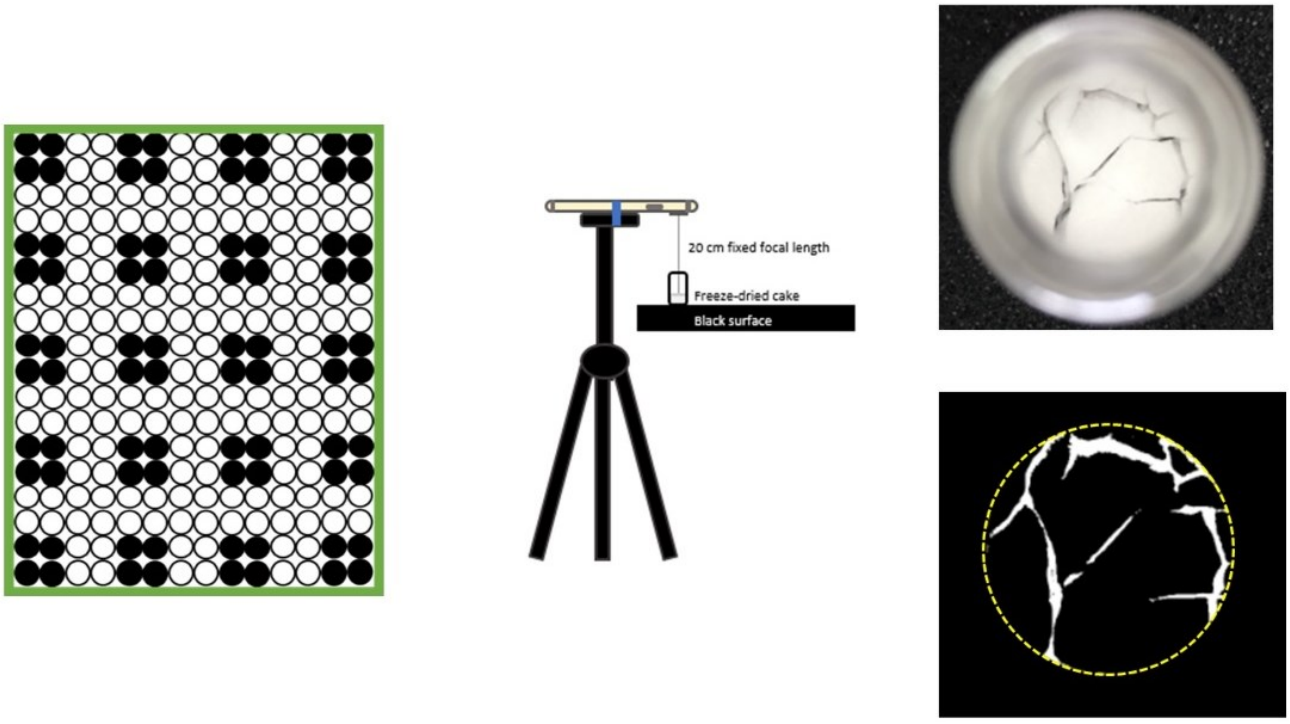


Figure 4.2 Schematic representation of methodology for measuring the extent of cake cracking in each cake vial.

4.2.6 Case Study 2: Specific Surface Area (SSA) of FD materials

The SSA of FD excipients and biologics were measured using via inverse gas chromatography and volumetric nitrogen gas adsorption.

The BET equations used for analysing isotherms obtained using both IGC and N_2 techniques [183] can be described as:

$$\frac{P}{n(P_0-P)} = \frac{C-1}{n_m C}\left(\frac{P}{P_0}\right) + \frac{1}{n_m C} \tag{4.1}$$

where P is the solvent or gas partial pressure in gas phase (Torr), P_0 is the saturated solvent vapour pressure (Torr), n_m is the monolayer capacity (Mol g^{-1}), n is the amount of gas absorbed (Mol g^{-1}) and C is the BET constant. The monolayer capacity n_m, can be determined from the slope and intercept of a linearised BET equation fitted to experimental data. The BET SSA is given by:

$$S_{BET} = aN_A n_m \tag{4.2}$$

where a is the cross-sectional area of the adsorbate, N_A is the Avogadro number and n_m is the monolayer capacity.

4.2.6.1 Inverse Gas Chromatography (IGC)

IGC (inverse gas chromatography) is a chromatographic technique that can be used to measure the SSA of many materials such as fibres, films, or powders, including FD products [183-185]. It can determine BET isotherms using alkane adsorption at room temperature and room pressure using user selected humidity's. In IGC, a known probe molecule (adsorbate) is injected via an inert carrier gas through an unknown solid sample (adsorbent) packed into a column. This approach is an inversion of traditional analytical gas chromatography. A flame ionisation detector (FID) is used to measure the alkane retention time [188].

An IGC-SEA instrument (Surface Measurement Systems, London, UK) was used to measure the SSA of FD material. FD samples were lightly crushed and packed into 30 cm x 6 mm x 4 mm id Glass Columns (Analytical Columns, Croydon, UK) with glass wool plugged at either end. A pool of between 4-8 vials were required to get sample mass up to 40 - 300 mg. The dead volume determination was worked out from methane and helium was used as the carrier gas. A flame ionisation detector (FID) was used to detect the retention time of the adsorbate, octane, and methane. The experiments were carried out at finite dilution concentrations at a column temperature of 293K with a flow rate set to 10 sccm (standard cubic centimetres per minute). The relative humidity (RH) inside the columns were set to 0% RH for normal experiments and between 0 - 80 % RH when the effects of moisture sorption were investigated. A % RH conditioning step of 2 hours was selected at 303K. A maximum of 5 minute elution time was chosen for each injection, at each specific total fractional surface coverage point. The peak maximum method was used for more determinations based on the time at maximum FID signal.

The BET equation was used to analyse the chromatogram data. As described in Equation (4.1), the linear region of the BET equation was fitted using equilibrium partial pressures P/P_0 between 0.05 – 0.35. The monolayer capacity n_m, can be determined from the slope and intercept of a linearized BET equation fitted to experimental data to determine the

BET SSA using Equation (4.2). Data points near 0.05 and 0.35 P/P_0 were excluded in some cases to decease non-linearity in the BET analysis. The resultant BET coefficients of determination was greater than $R^2 > 0.995$. The analysis of specific surface areas was calculated using the SMS-Surface Energy Analysis Software (v1.4.2.0).

4.2.6.2 Nitrogen (N_2) BET Adsorption

Typically, the most common ways to determine the SSA of FD material has been to use the Brunauer–Emmett–Teller (BET) analysis with volumetric gas adsorption [115, 177-180]. The adsorbate is usually krypton (Kr) or nitrogen (N_2) gases. In the current study N_2 adsorption was used to measure the SSA of FD material as a reference technique when comparing with the IGC values. FD samples in vials were gently broken up, placed inside BET analysis glass tubes and left to degas overnight at 303K under vacuum. Tubes were loaded in a 3Flex Physisorption Analyser (Micromeritics, Norcross, GA, U.S.A). Isotherms were generated at 77K using a P/P_0 range between of 0.01 - 0.99. Data analysis was performed using the 3Flex Software (Micromeritics, Norcross, GA, U.S.A) to determine the BET surface area using data in the 0.05 - 0.35 P/P_0 range.

4.2.7 Statistical Analysis

95% confidence interval analysis same as previously described in Chapter 3, section 3.2.6.

4.3 Results and Discussion

4.3.1 Case Study 1: Mechanical Properties of FD Material

FD materials are examples of materials that are classed as cellular or foams. These structures consist of a variety of cells with solid edges/faces. Previous work in regards to mechanical indentation on FD cakes has focused more on development of method and how the freeze-drying process parameters effected mechanical properties [110, 225, 226]. However, there is been limited focus on measuring and tracking mechanical properties of FD biologics in relation to moisture and temperature for real-world biologics. For example, mechanical indentation can be used to work out the effects of increasing concentration on cake mechanical properties. Figure 4.3 shows a typical stress versus strain with increasing concentrations of IgG from 10 - 200 mg/mL. Formulation is one important parameter for determining the physical properties of FD materials. Young's modulus can be measured to determine the stiffness of a cake relative to the effects of formulation and environmental factors such as humidity and storage temperature.

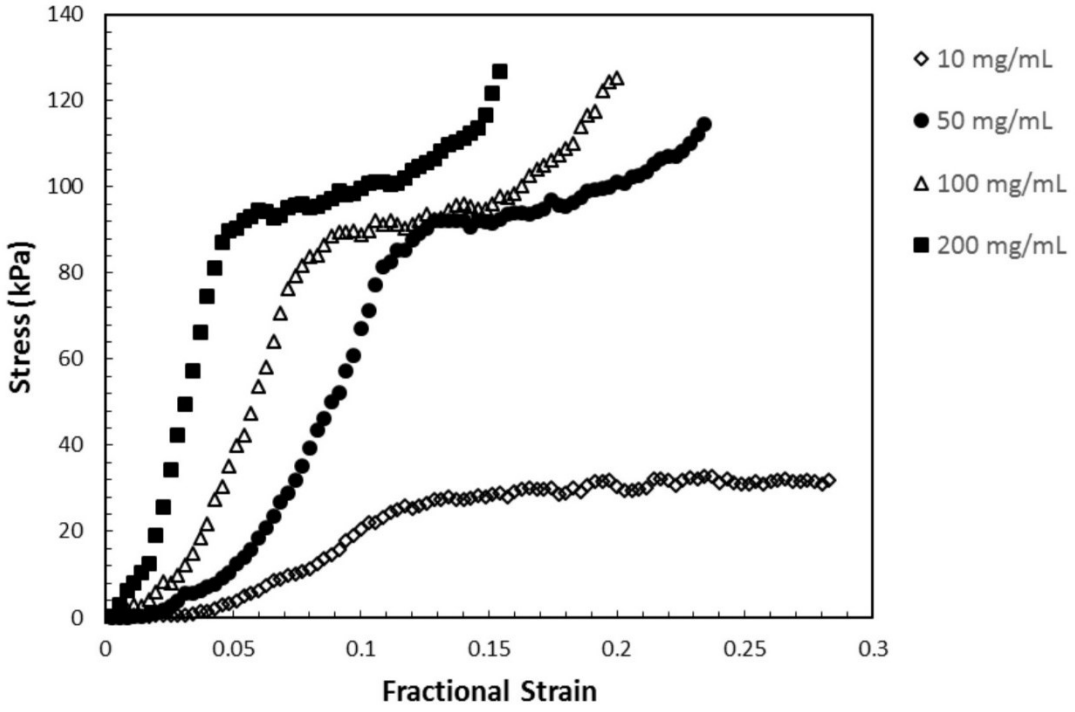
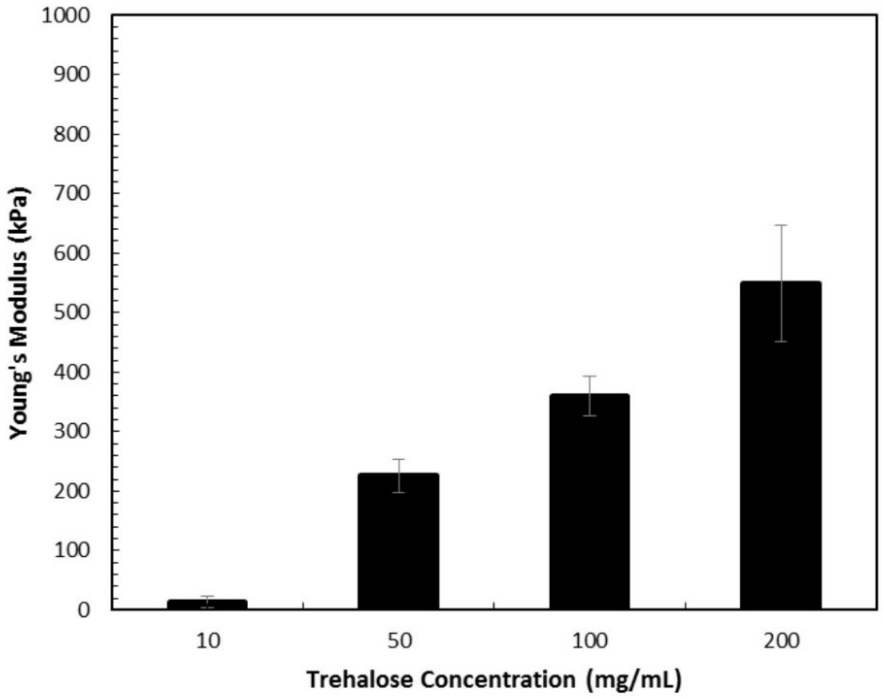


Figure 4.3 Typical Stress (KPa) versus Strain graphs from mechanical indentation tests for FD IgG.

4.3.1.1 Effect of Concentration on Young's Modulus

A



B

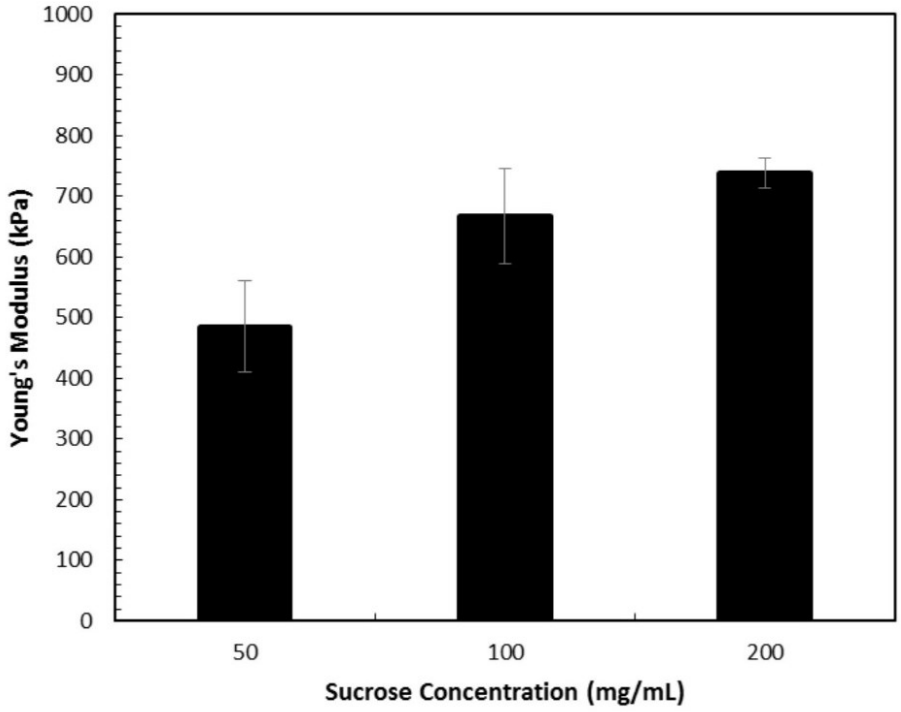


Figure 4.4 Young's Modulus of freeze-dried **A:** Trehalose and **B:** Sucrose at different concentrations Error bars represent 95% confidence intervals (n=3).

Excipients can often increase the physical stability of many FD products during storage [68]. Common excipients such as sucrose and trehalose are often used to enhance product formulation including bulking up cakes and improve visual appearance. Figure 4.4 shows the change in average Young's modulus for FD sucrose and trehalose between concentrations of 10 - 200 mg/mL tested at 25°C with a room RH of 30 %. The initial moisture content of the trehalose vials at 10, 50, 100 and 200 mg/mL was 0.65 ± 0.13, 0.96 ± 0.19, 0.85 ± 0.21 and 0.70 ± 0.14 % w/w respectively. While, the initial moisture content of the sucrose vials at 50, 100 and 200 mg/mL was 0.78 ± 0.07, 2.40 ± 0.22, and 2.40 ± 0.03 % w/w respectively. With increasing excipient concentration, the Young's modulus also increases. Trehalose has the higher Young's modulus than sucrose for all concentrations between 50 - 200 mg/mL. Devi et al. measured the Young's modulus for trehalose at 50 mg/mL at 207 ± 12 KPa [110] although they did not specify the moisture content of the cakes.

All excipient concentrations, underwent the same FD cycle, but as shown here, the sucrose samples with higher concentration had higher moisture contents than the lower concentrations after the cycle had ended. It is important to contextualise Young's modulus values will depend on the current moisture content of the samples in the vials. It is often difficult to ascertain the absolute zero moisture content Young's modulus values for materials due to the complexities involved with drying and non-feasibility of operating at some sort of almost zero RH environment when testing. All the samples reported here were immediately tested as soon as the stopper was removed and the total test took less than 15 seconds to perform. There is danger that if left open too long, the initial moisture content would have changed dramatically to what the actual moisture content in the vials is. Devi et al. showed that when FD sucrose samples were exposed at around 5 min room environment they gained around 1.3% w/w in moisture [110]. Qualitatively speaking, the samples with higher Young's modulus were hard and stiff, whilst the samples with lower Young's modulus were soft and fragile. This highlights how the formulation concentration selection is an important parameter for determining the physical cake attributes of FD material.

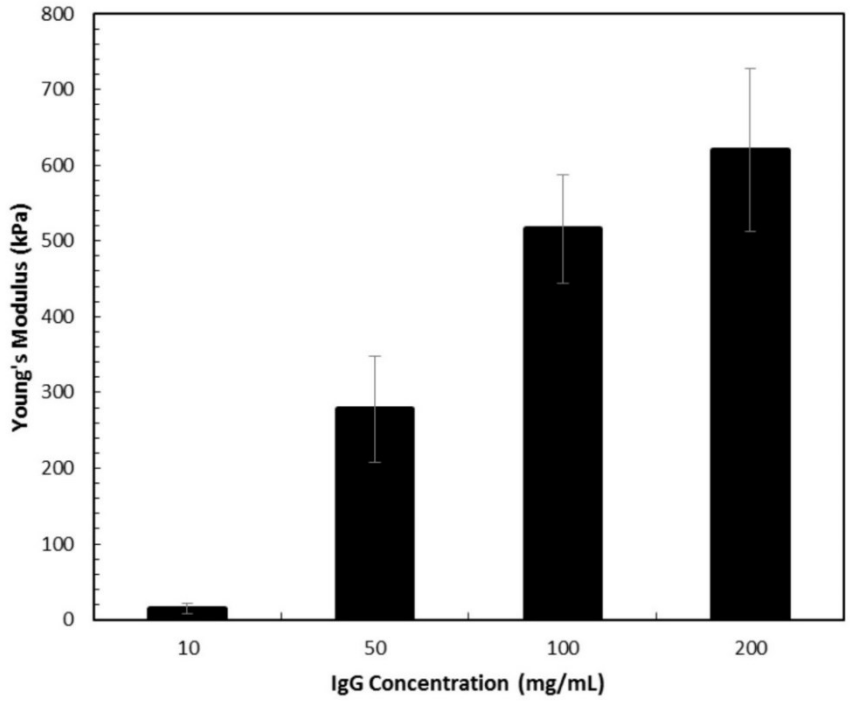


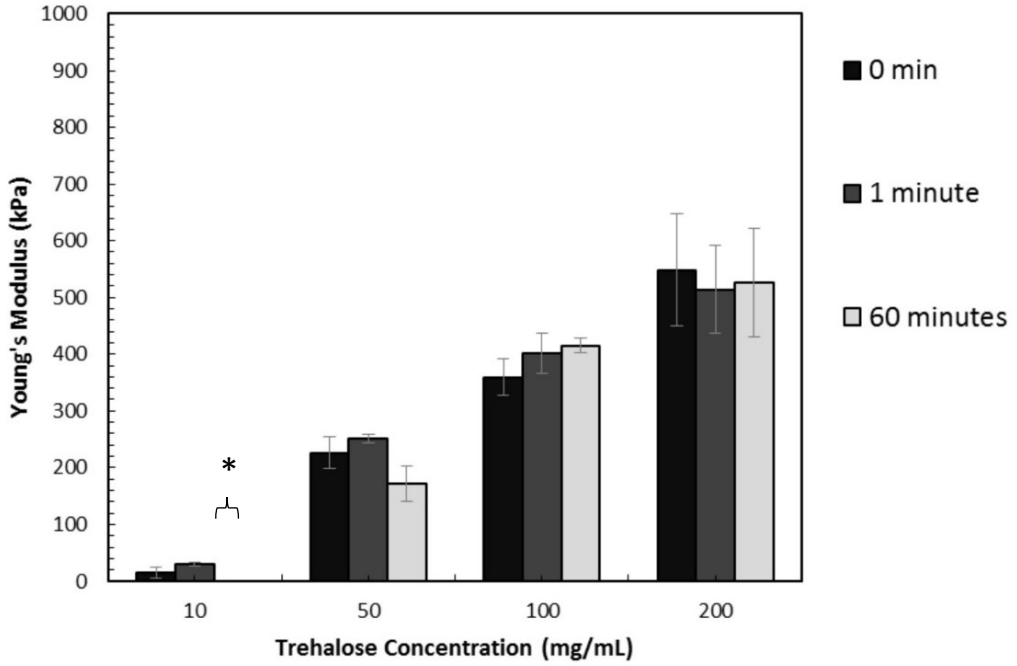
Figure 4.5 Young's Modulus (KPa) of freeze-dried IgG at various concentrations. Error bars represent 95% confidence intervals (n=3).

Increasing the concentration of most FD biological material often correlates with a change in cake morphology and physical properties. Figure 4.5, shows the mechanical stiffness Young's modulus (kPa) of different concentration IgG formulation (from 10 mg/mL to 200 mg/mL) after freeze drying. The mechanical properties were tested at 25°C with a RH 30 %. The initial moisture content of IgG vials at 10 mg/mL, 50 mg/mL, 100 mg/mL and 200 mg/mL was 0.82 ± 0.47, 0.11 ± 0.01, 0.16 ± 0.03 and 0.22 ± 0.05 % w/w respectively. Again as seen with FD excipients, increasing concentration of the API, the IgG, the cake stiffness in terms of Young's modulus also increased. The Young's modulus increases by a factor of around 30 times in going from 10 mg/mL to 200 mg/mL. Some FD cakes, which are particularly fragile, can experience cake breakage during transportation. This can also result in sub-optimal visual appearance which will hamper commercial viability. However, increasing the concentration of the API is not always practicable and can result in other problems such as longer reconstitution times [28]. Typically just increasing the concentration of API alone could cause stability issues. Increasing the excipient concentration (1:1 weight ratio of protein to sugar) in formulations is thought to lead to optimal stabilization due to limited molecular flexibility as a result of the API being trapped inside a glassy matrix.

4.3.1.2 Mechanical Properties of FD Cakes Exposed to Environment

4.3.1.2.1 Excipients

A



B

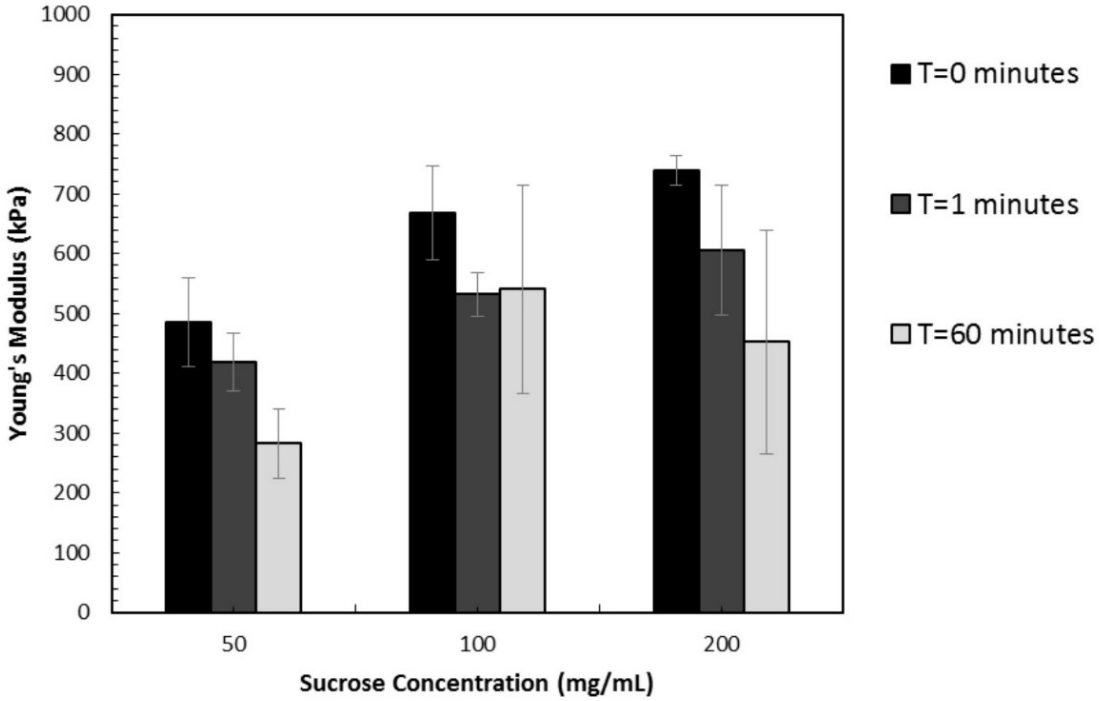


Figure 4.6 The effect of vial opening with exposure to moist atmospheric air (RH 40%) over time on the Young's modulus for different concentration cakes of freeze-dried **A:** trehalose and **B:** sucrose. (*) denotes collapse of cake. Error bars represent 95% confidence intervals (n=3).

Figure 4.6 shows the change in Young's modulus for excipients sucrose and trehalose based on how much time the vials were exposed to the environment. The FD vials were exposed to room atmosphere conditions 25°C, with RH measured as 40 %. Time point zero represents testing the vial immediately after removing the rubber stopper. For all trehalose concentrations, exposure of 1 minute did not have any major effect on Young's modulus. However, after 60 minutes the 10 mg/mL samples had partially collapsed and Young's modulus was unmeasurable. The 50 mg/mL samples had a small drop in Young's Modulus after 1 hour, but the 100 and 200 mg/mL samples remained unaffected. All concentrations of sucrose however, had a small drop in Young's modulus just after 1 minute (although still within error). After 1 hour, the 50 mg/mL samples had a significant drop in Young's modulus by about 40% compared to the start. The 100 and 200 mg/mL vials had large error bars, which meant that there were variations in the repeats (n=3). One reason for these error bars could be due to cake cracking having an influence (see Section later). Another possible reason is that the water sorption of FD cakes could be unevenly distributed. The distribution of moisture in the cake and complexities that might arise might give variations in the cake Young's modulus measured over time. These results also demonstrates how low concentration excipients are particularly vulnerable to moisture uptake. Any moisture increase or exposure to environment could have negative consequences on the cake stiffness.

4.3.1.2.2 Biologics

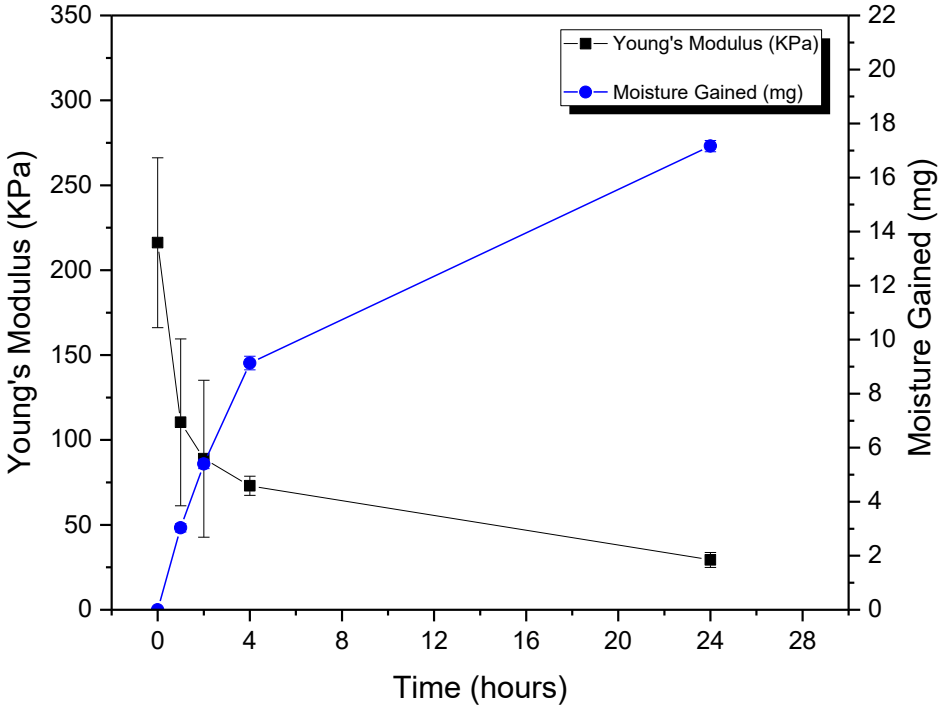


Figure 4.7 The effect of vial opening with exposure to moist atmospheric air (RH 40%) over time (hours) on the Young's modulus (KPa) for freeze-dried IgG 50 mg/mL. Error bars represent 95% confidence intervals (n=3).

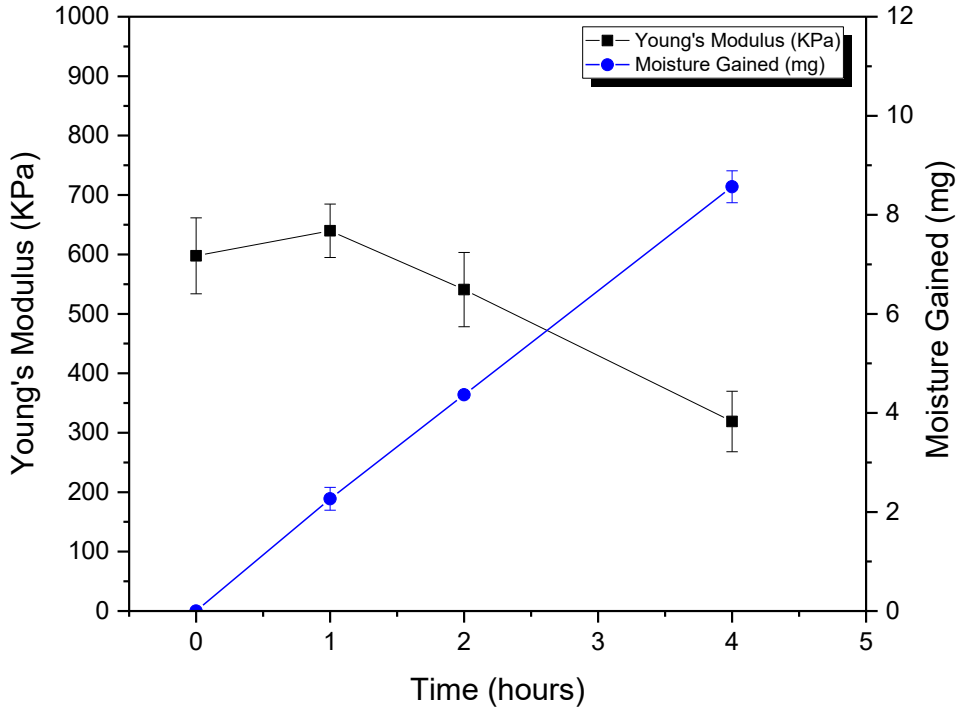


Figure 4.8 The effect of vial exposure to moist atmospheric air (RH 40%) over time (hours) on the Young's modulus (KPa) for freeze-dried B/Phuket (with 20% w/w sucrose). Error bars represent 95% confidence intervals (n=3).

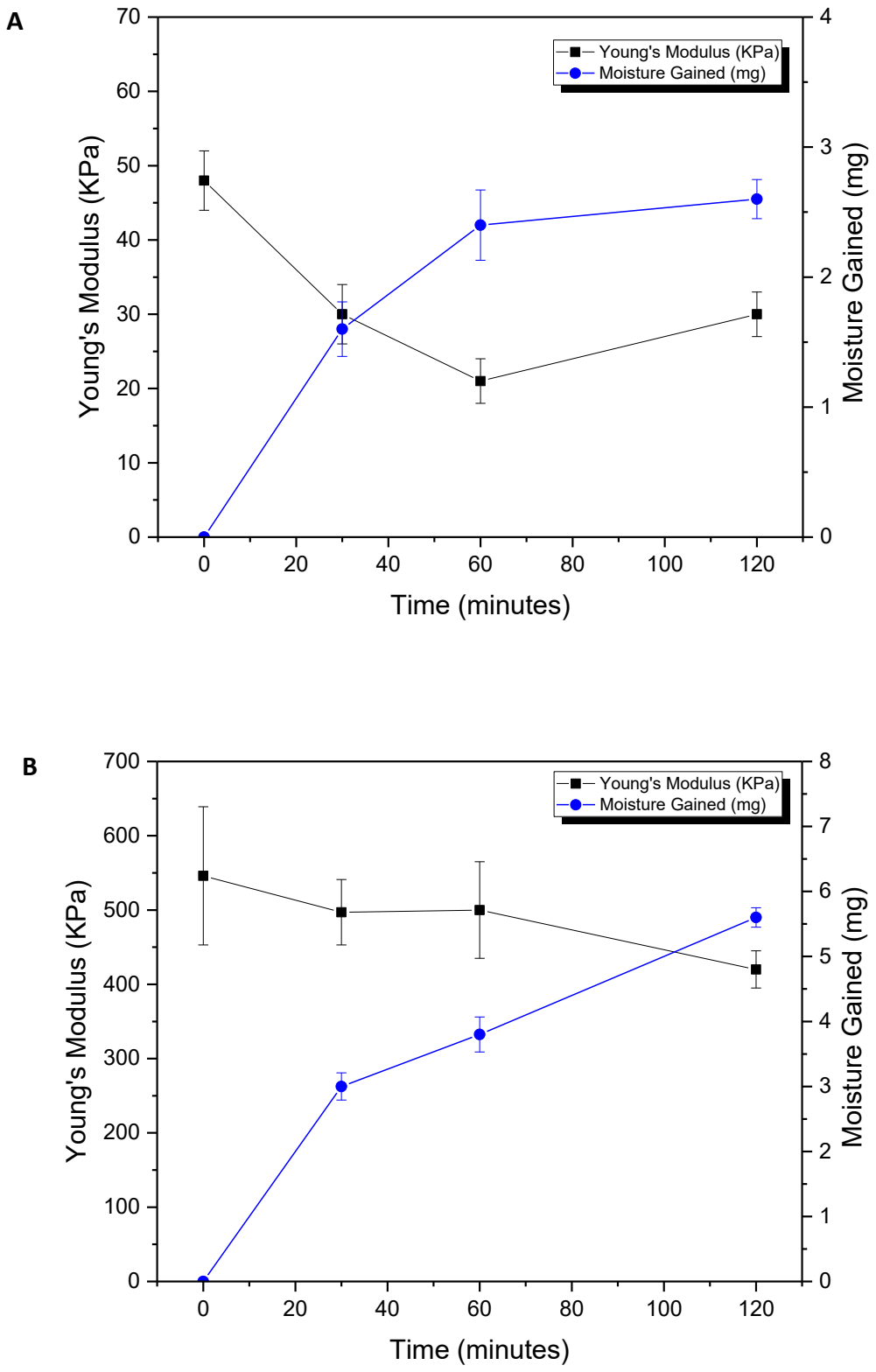


Figure 4.9 The effect of vial exposure to moist atmospheric air (RH 40%) over time (minutes) on the Young's modulus (KPa) for freeze-dried BSA (1:1 protein to sugar) formulated with **A:** 1% w/w sucrose and **B:** 5% w/w sucrose. Error bars represent 95% confidence intervals (n=3).

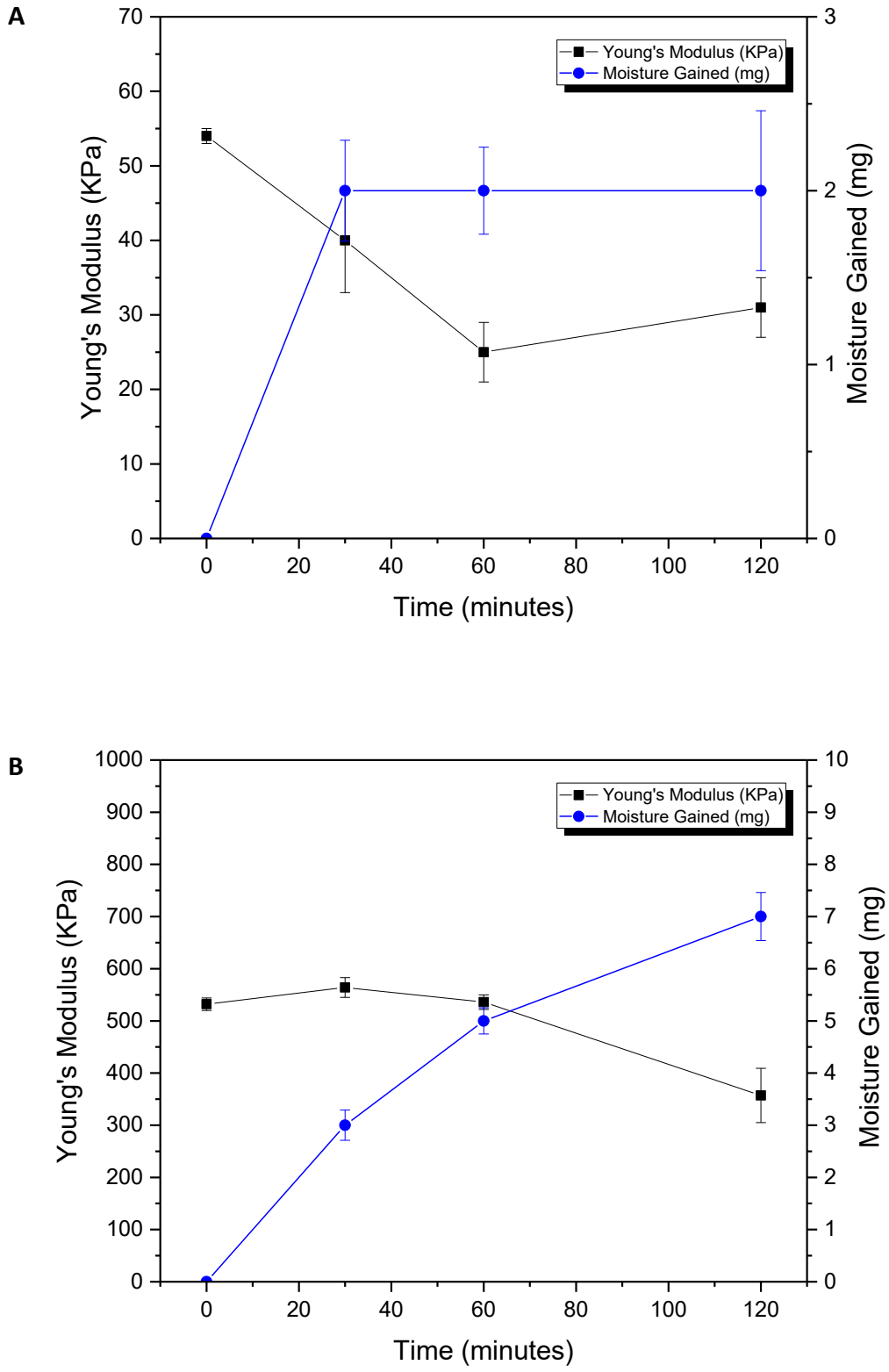


Figure 4.10 The effect of vial exposure to moist atmospheric air (RH 40%) over time (minutes) on the Young's modulus (KPa) for freeze-dried BSA (1:1 protein to sugar) formulated with **A:** 1% w/w trehalose and **B:** 5% w/w trehalose. Error bars represent 95% confidence intervals (n=3).

Figures 4.7 - 4.10[1] shows the Young's modulus (KPa) in relation the moisture gained (mg) over exposure time for FD biologics IgG, B/Phuket antigen and BSA (1:1 ratio of protein to sugar). The weight change was measured gravimetrically with a balance at each time point (n=3). Figure 4.7 shows that the Young's modulus for FD IgG 50 mg/mL has an inverse relationship with moisture. In just 24 hours, it took only 17 mg of moisture to effectively reduce the Young's modulus by a factor of 4. In Figure 4.8, the FD B/Phuket antigen (formulated with 20% w/w sucrose), does not have a significant drop in Young's modulus, until after 4 hours with just under 4 mg of moisture. The B/Phuket antigen loses almost 50% of its starting Young's modulus value.

Figure 4.9 shows the change in Young's modulus of FD BSA (1:1 protein to sugar ratio) formulated with either 1% or 5% sucrose. The BSA with 1% sucrose after 2 hours has had a ≈ 22% decrease in Young's modulus by gaining only 2.5 mg of moisture. However, the Young's modulus of the BSA with 5% sucrose, remains unaffected until the 2 hour time point, whereby only a small drop is observed even after up taking more moisture at 5.5 mg of water. The increased sucrose content resulted in the BSA with 5% sucrose being more robust in and retaining better Young's modulus due to exposure in environment then the BSA with 1% formulation.

Similarly, Figure 4.10 shows the BSA formulated with either 1% trehalose or 5% trehalose. Once again the higher concentration excipient formulation, has the least decrease in Young's modulus, even though it takes up more moisture during the 2 hours (around 7 mg). The BSA with 1% trehalose, sees a drop in Young's modulus in just 20 minutes. The moisture content uptake plateau's at 2 mg, whilst the Young's modulus also remains constant at around 30 KPa. This could indicate that mass uptake had reached equilibrium with the atmosphere or that trehalose might even have crystallised into its di-hydrate form.

[1] FD BSA laboratory indentation experiments were carried out by MSc Student Xinzu Zhang acting under supervision and planning by Arnold Duralliu.

4.3.1.3 Effect of Storage on Mechanical Properties

4.3.1.3.1 Comparison of storage for low vs high moisture vials of Lysozyme

A combination of different storage temperature conditions and the relative sample moisture content can have a number of effects on the structural stability of FD material. Figure 4.11[2] shows the Young's modulus of FD lysozyme between high and low moisture content vials of FD lysozyme, stored at -20°C, 25°C and 65°C over a period of 21 days. The higher moisture vials at time point zero have almost 30% lower Young's modulus value than the low moisture samples. However, from -20°C to 25°C, there is no significant change due in Young's modulus for the high moisture vials. The vials stored at 65°C just after storage had collapsed into a brown droplet indicating that the glass transition had been reduced lower than the storage temperature. For the low moisture vials there was no significant change in Young's modulus at -20°C (although there is an anomalous drop at day 14, but this is still within error). At 25°C and 65°C, the cakes had decreased in Young's modulus, reaching near same values as the high moisture samples. Due to experimental design constraints, the moisture content of the vials was not measured at each time point, but there would most certainly been moisture uptake occurring at those temperatures. It is unlikely that the high moisture vials would have gained any more moisture at 25°C. However, it is difficult to ascertain to what extent whether the factor of moisture and temperature is more important in the changes seen. It could be a mutual relationship in which both could have an effect in Young's modulus over storage time. As such minimising the moisture ingress in vials over time is important to consider as this could affect the Young's modulus. Both moisture and temperature are critical in terms of the T_g of the FD material. With increases in moisture you have a plasticising effect occurring thereby lowering the glass transition. With reduced T_g and increasing storage temperature

[2] FD Lysozyme indentation experiments were carried out by MENG students Hassan Azzan and Wei Ming Goh acting under supervision and planning by Arnold Duralliu.

then you have the higher chance of collapse occurring as you approach this value and collapse occurring, as observed here for the high moisture samples at 65°C.

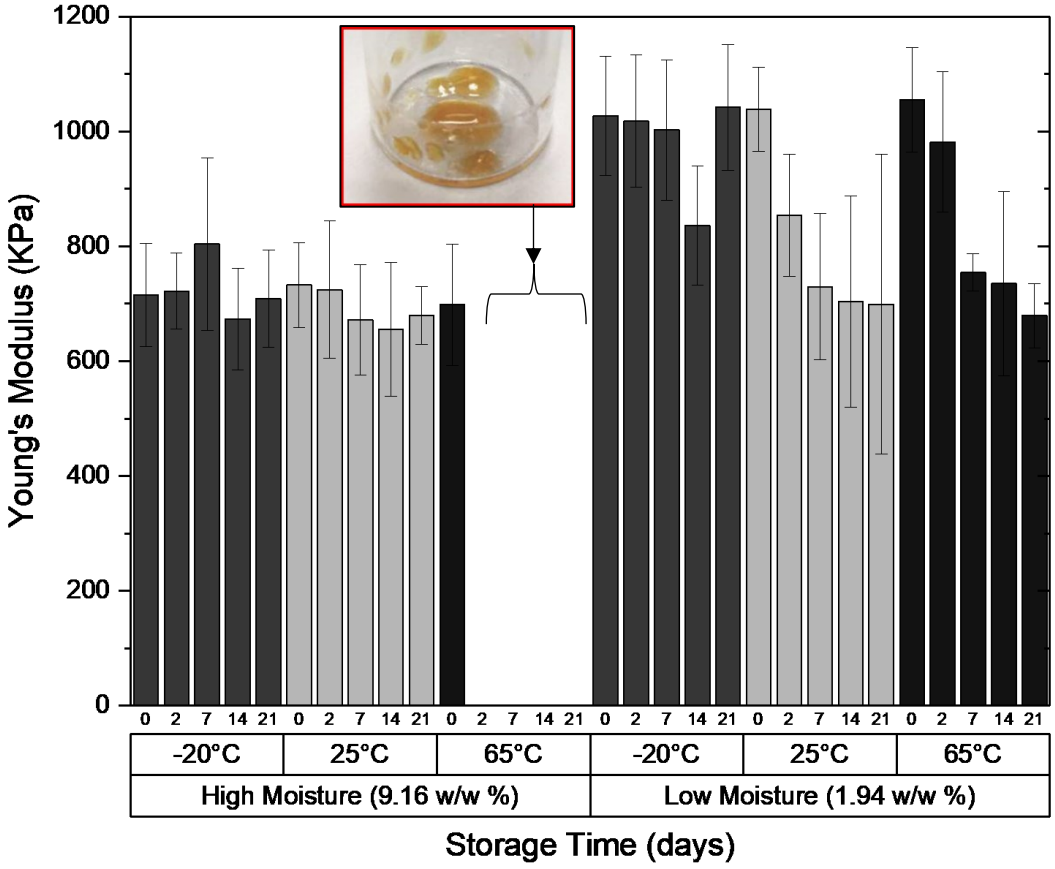


Figure 4.11 Young's modulus (kPa) against storage time (days) for high and low moisture vial FD lysozyme 10 mg/mL (formulated with 10% w/w trehalose). Samples were measured over a period of 21 days at -20°C, 20°C and 65°C. The photograph shows the appearance of high moisture FD cakes after 1 day storage at 65°C. Error bars represent 95% confidence intervals (n=3).

4.3.1.3.2 Effect of storage conditions on IgG

Some researchers have postulated that mechanical properties can be used as an indicator to correlate to product stability [223]. The correlation between Young's modulus (kPa in relation to moisture content (% w/w) or monomer (%) of FD IgG 50 mg/mL during 6 months storage is examined in Figures 4.12 - 4.14. Samples were stored in at either -20°C, 25°C and 56°C and samples every three months since the start of storage (n=3). At -20°C and 20°C, the Young's modulus decreases slightly over the 6 months storage but the monomer (%) stays relatively stable. It is only at 56°C storage temperature does the monomer (%) decrease correlate along with the drop in Young's modulus. However at all storage temperatures, there is an increase in moisture content over time, and this correlates better than the monomeric decreases and changes to Young's modulus. These results suggested that there is no direct correlation with mechanical properties and monomeric stability. The moisture content (%), temperature and storage time would be the factors that most effect the Young's modulus. However, indirectly a decrease in Young's modulus would mean that there is most likely due to come from the effects of moisture ingress, which would then in turn indirectly link to that of activity or monomer stability losses. The Young's modulus is more dependant and indicative of moisture content ingress and temperature over storage time. This links back to glass transition being lowered by moisture and at increased storage stress temperatures, there being greater molecular flexibility occurring. A shift in vapour sorption characteristics and mechanical properties would occur as the material passes the glass transition. As a result of increased molecular flexibility, the water absorption would occur in the bulk structure along with a major change in Young's modulus. The increased chance of moisture ingress and temperature raises the chance of degradation occurring such as aggregation, resulting in reduced monomer. As such it can be considered that Young's modulus could be used as an indirect measurement of stability as those factors will most likely have an influence on monomer loss. The Young's modulus can be possibly used to measure a change in

environmental conditions of the FD material, which would be symptomatic of some degradation occurring.

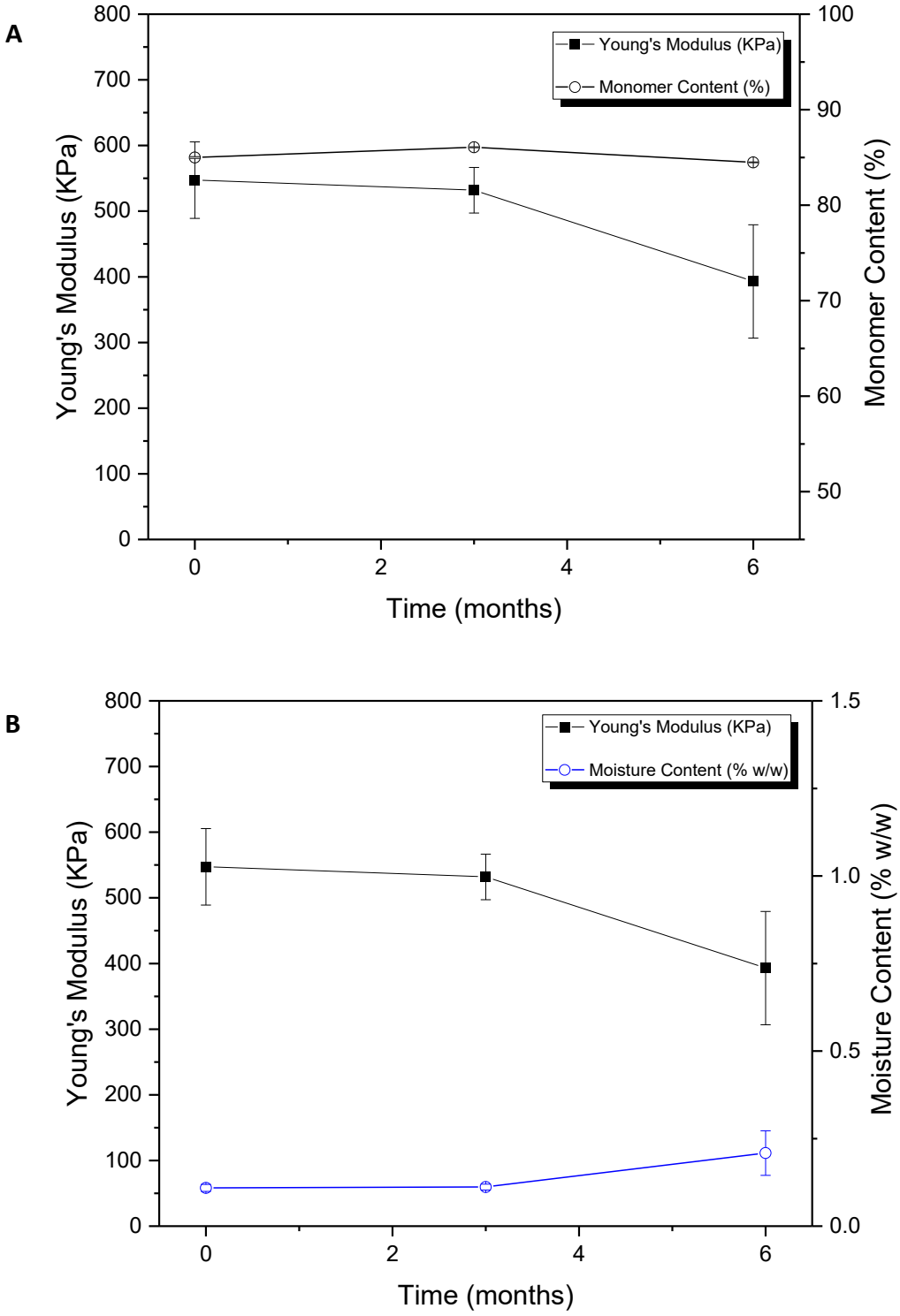


Figure 4.12 Comparison of Young's modulus (kPa) against **A:** monomer (%) or **B:** moisture content (% w/w) of IgG 50 mg/mL during 6 months storage at -20°C. Error bars represent 95% confidence intervals (n=3).

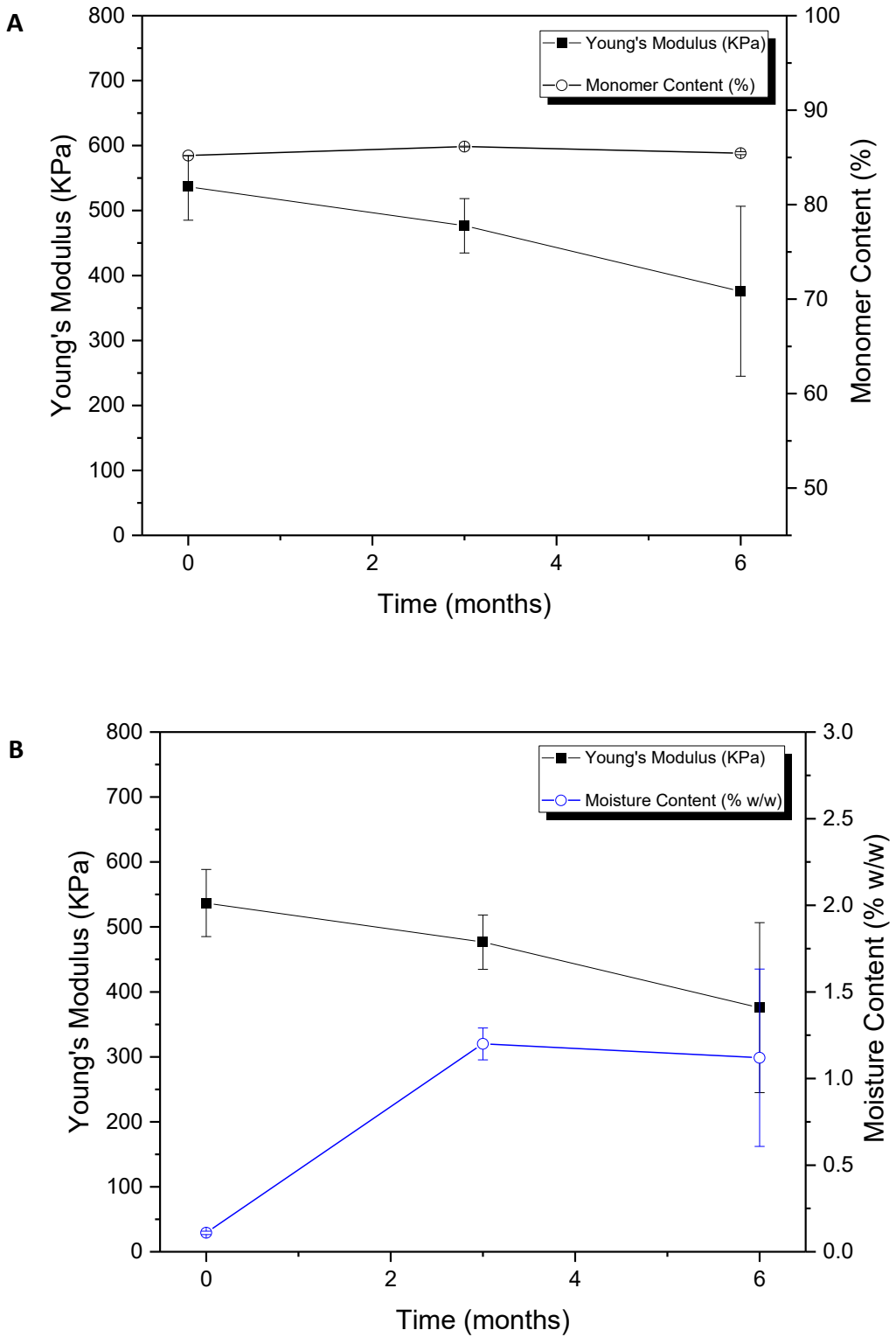


Figure 4.13 Comparison of Young's modulus (kPa) against **A:** monomer (%) or **B:** moisture content (% w/w) of IgG 50 mg/mL during 6 months storage at 25°C. Error bars represent 95% confidence intervals (n=3).

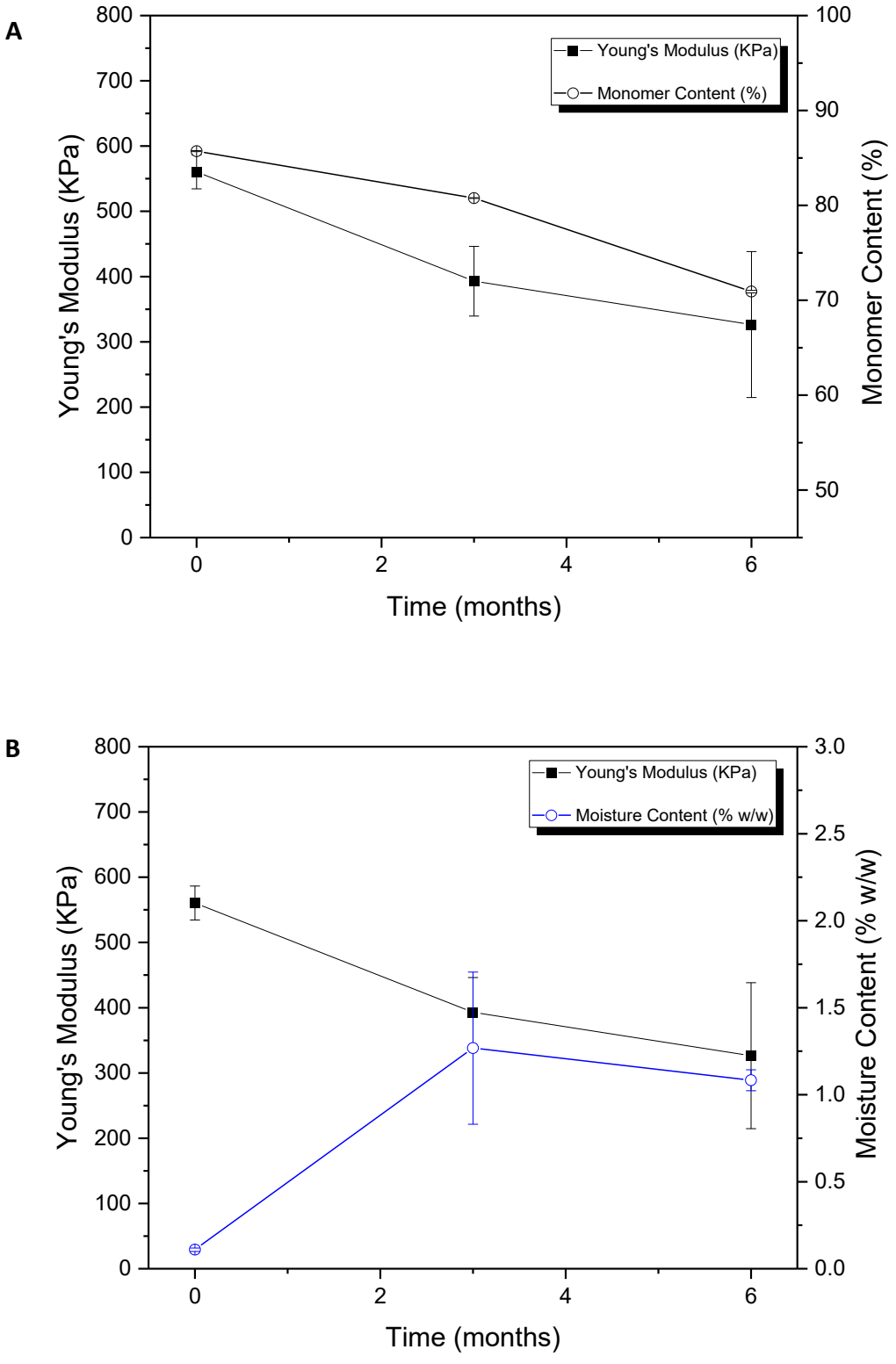


Figure 4.14 Comparison of Young's modulus (kPa) against A: monomer (%) or B: moisture content (% w/w) of IgG 50 mg/mL during 6 months storage at 56°C. Error bars represent 95% confidence intervals (n=3).

4.3.1.4 Variability in measurements

Large error bars from sample to sample with the flat punch technique can be associated to several factors including:

a) Variations due to moisture content in the vials

b) Cake heterogeneity differences in each vial

c) Cracking at the cake surface in each vial.

Water acts as a plasticiser, therefore moisture content is an important factor which can affect mechanical properties, especially if there are large differences from vial to vial. If one sample has more moisture than the others it could provide different Young's modulus and large deviations from the average. Karl Fischer results from a pooled batch showed at most around 10 % relative standard deviations in moisture content from vial samples taken from the batch (n=3). Another factor to consider is that heterogeneity differences in the cake varying between vials in the batch. One way to minimise this effect is to have controlled nucleation which would give more homogenous cakes morphology [44]. Lastly in particular, cracking/fracturing on the top of the cakes is another problem, especially with increasing concentration samples. Cracking (also linked to shrinkage) phenomena is a result of built up tensile ('drying') tension within a wet solid amorphous cakes during water removal [227]. Samples with more surface cracking than others could give rise to high variance when measuring using a flat punch indenter as the local mechanical properties are dependent on the local sample compliance. Figure 4.15 below shows the extent of cake cracking of FD lysozyme samples and how it can even be influenced by the vial location in the tray. For both runs, vials at the centre of the tray show more surface area cracking between 10 - 12 % of total surface area compared to vials on the outer edges of the tray. Interestingly a similar phenomenon occurs with vials located the tray periphery undergoing what's known as the "edge vial effect" whereby they have difference in temperature due to radiation losses from

the walls and door of the freeze-drier [228]. Although perhaps this may not be the main cause for the cracking at different locations, it does imply that when sampling vials, it might be worth in future to have a consistent location in which vials are tested in order to minimise sample variations. Also, to consider is that freeze-drying protocols could be optimised to ensure less cracking occurs. Ullrich et al. showed that changes to the shelf cooling rate from 0.4 to 0.2 °C/min resulted in substantial decreases in the degree of cracking for trehalose [229]. In another study it was shown how that hydrophobized vials can reduce cracking at trehalose concentrations of up to 15% [230].

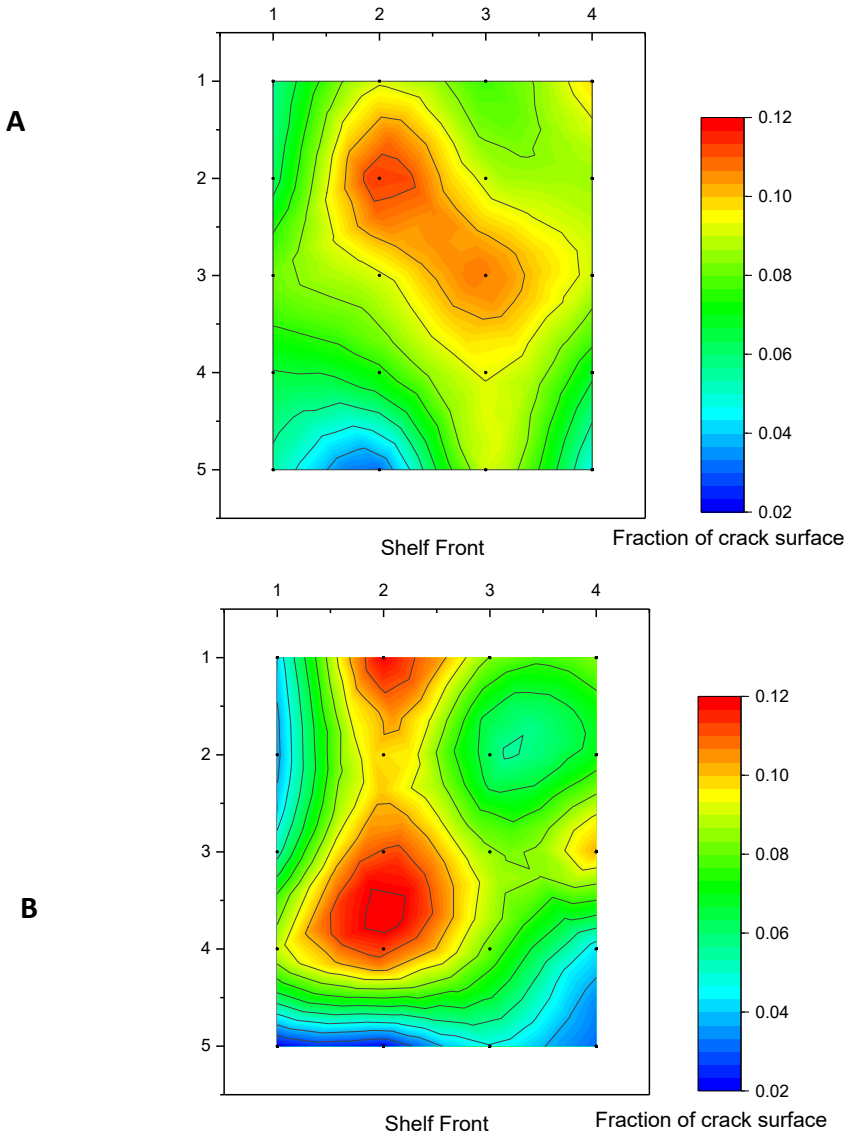


Figure 4.15 Effect vial location in tray and intensity of cracking on surface of FD lysozyme 10 mg/mL (with 10% w/w sucrose). **A:** Run 1 and **B:** Run 2. • Represents the point where vial measured was taken from relative to tray location (n=1).

4.3.1.5 Summary

Mechanical indentation is a vital tool for determining the physical and mechanical properties of FD materials. The effect of moisture and storage conditions can be monitored over time. Additionally, formulations can be enhanced in order to get particular cake characteristics and strengths. During long term storage the mechanical properties of materials can change. The increase in moisture content and temperature based ageing effects related to increased molecular flexibility of the amorphous excipients, can be linked a change in Young's modulus. Moisture content correlated with drops in mechanical properties over storage but did not directly link with activity and drop in monomer, although it can possible be indirect indicator on instability. Some potential drawbacks with mechanical indentation technique include large error variation of cakes due to vial to vial heterogeneity or different extent of cake cracking occurring. Also, the need to work in a low RH environment for more sensitive materials can add logistical complexities. Nevertheless, cake indentation and mechanical properties should be more routinely adopted and measured by the FD industry. Mechanical properties could also potentially be used a CQA's for individual production lines to monitor during prolonged storage.

4.3.2 Case Study 2: Specific Surface Area (SSA) of FD Materials

The freeze-drying process results in a solid-state final product being presented as 3-D porous solids. The SSA is defined as the total surface area per unit of mass and the key property descriptor for porous materials. BET nitrogen gas adsorption has been used to measure any changes in SSA depending on the cooling or annealing profile for FD mAb1 and BSA formulations [181]. Krypton gas is often deemed to have greater sensitivity, with Rambhatla et al. using it to measure cake shrinkage of FD materials, although this can be overshadowed by the high cost of adsorbate. [182]. In particular the volumetric BET nitrogen method has host of disadvantages associated with it. These include requiring large amounts of sample for accurate determination (with accuracy limitations for areas below 0.5 m^2/g), lengthy experimental times (including degassing) and unknown effects of exposure to extreme low temperatures (77 K) on sample integrity [177, 183]. IGC has been applied to a number of pharmaceutical products and powders over the years and has the potential to be used as an alternative to measure the SSA of FD materials.

The reproducibility of each instrument is shown in Tables 4.3 and 4.4. The same sample tube or glass column was repeated three times (n=3). The mean SSA values of the three repeats are displayed along with the relative standard deviation % (RSD). For all FD materials tested, IGC had lower instrument RSD % values than the nitrogen gas adsorption instrument method. IgG 50 mg/mL had a RSD value of 2.17% and 3.48% for IGC-SEA and Nitrogen 3Flex instruments, respectively. Sucrose 50 mg/mL had a RSD value of 1.58% and 4.89% for IGC-SEA and Nitrogen 3Flex instruments, respectively. This signifies a high level of reproducibility with the IGC instrument compared to nitrogen gas adsorption. The higher RSD's for the nitrogen determination could be likely due to the inherently large dead volumes present within the instrument, which is especially more critical for solids with relatively lower surface areas. Dead volumes are typically less critical for flow systems such as IGC-SEA, compared to volumetric systems. A study by Legras et al. investigating the SSA of natural fibres with IGC, also found similarly low RSD's raging from between 0.1 - 3.5% [183]. IGC is

able to provide better reproducible data based on the samples tested here compared to the 3 Flex nitrogen-based instrumentation.

Table 4.3 Reproducibility using IGC based octane adsorption at 293K and 0%RH to measure the BET SSA of FD biologics. The same sample column was repeated three times.

Material	Repeat 1 (m^2/g)	Repeat 2 (m^2/g)	Repeat 3 (m^2/g)	Mean SSA (m^2/g)	RSD (%)
IgG (50 mg/mL)	1.12	1.07	1..04	1.08	3.59
Sucrose (50 mg/mL)	0.47	0.46	0.45	0.46	2.88
B/Phuket 14/252 (20% w/w sucrose)	0.55	0.54	0.55	0.55	0.98

Table 4.4 Reproducibility using volumetric N_2 adsorption at 77K to measure the BET SSA of FD biologics. The same sample tube was repeated three times.

Material	Repeat 1 (m^2/g)	Repeat 2 (m^2/g)	Repeat 3 (m^2/g)	Mean SSA (m^2/g)	RSD (%)
IgG (50 mg/mL)	1.30	1.28	1.20	1.26	4.26
Sucrose (50 mg/mL)	0.86	0.86	0.97	0.90	7.48
B/Phuket 14/252 (20% w/w sucrose)	0.55	0.55	0.52	0.54	3.44

4.3.2.1 Comparison of SSA

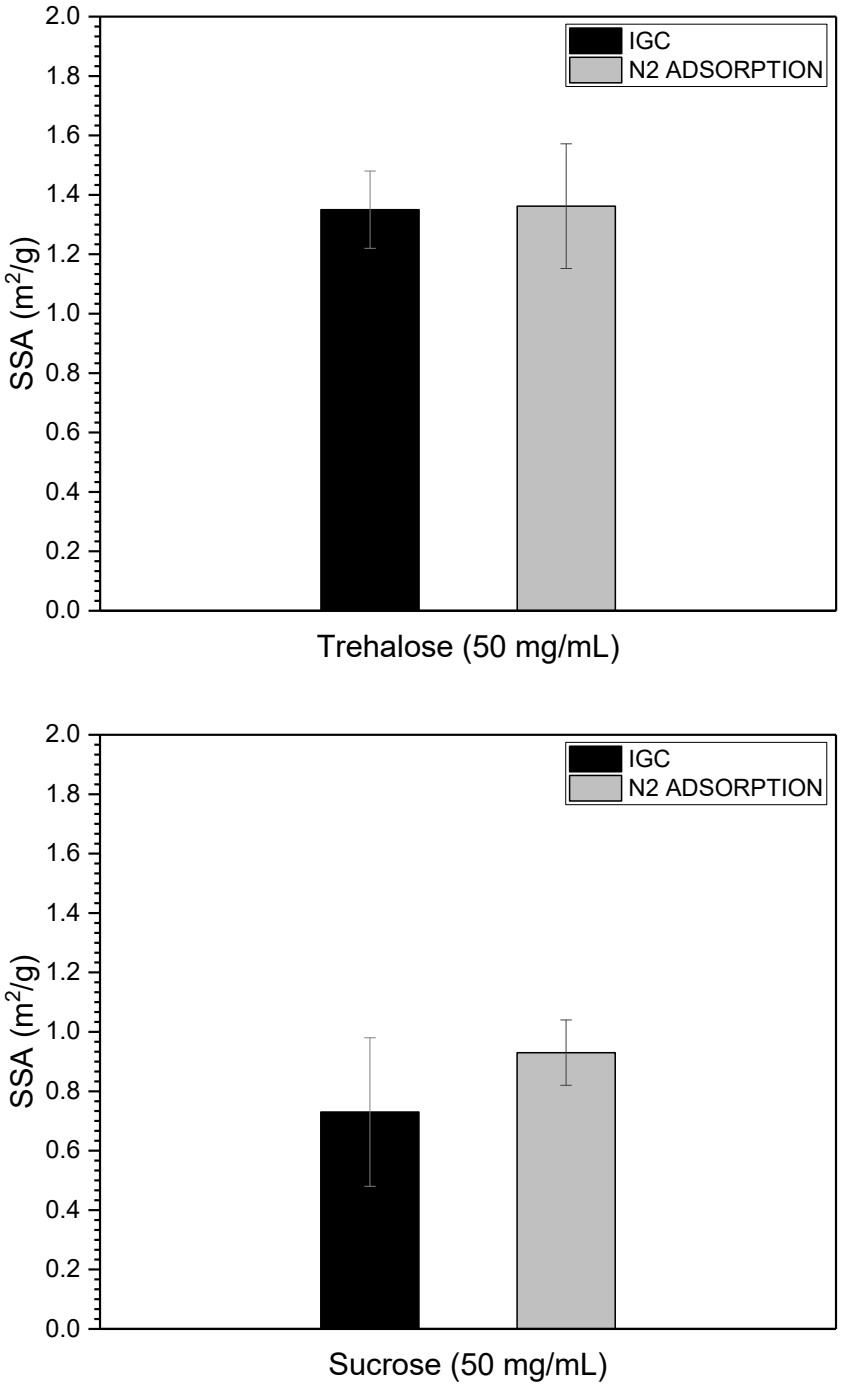


Figure 4.16 Comparison in SSA (m^2/g) of pooled vials from a batch of the FD excipients, trehalose and sucrose, as measured by octane IGC adsorption at 293 K and 0 % RH or N_2 adsorption at 77 K. A total of 3 columns or glass tubes were tested for each material (n=3). Error bars represent ±95 C.I.

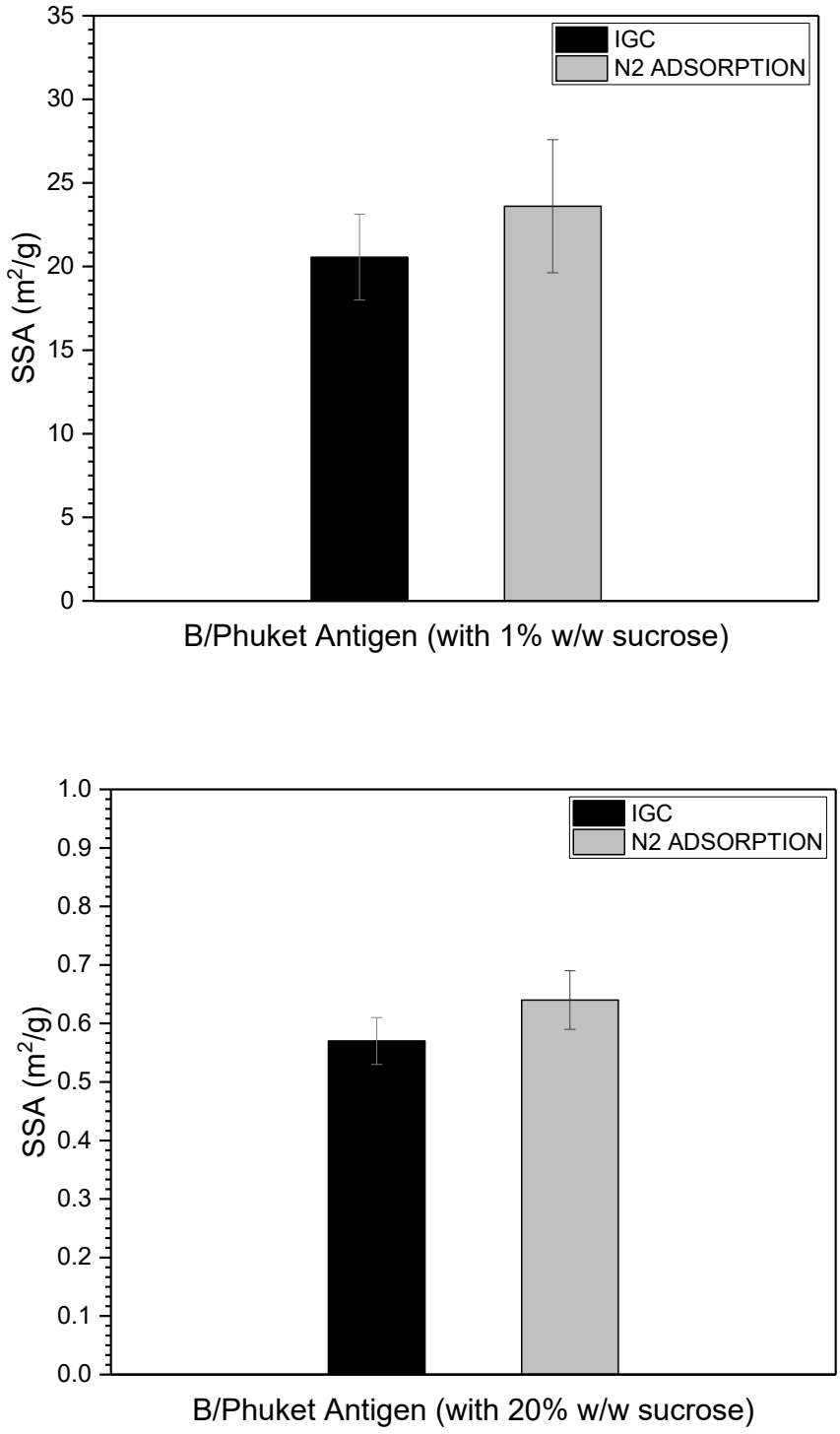


Figure 4.17 Comparison in SSA (m^2/g) of pooled vials from a batch of the FD B/Phuket antigen 60 µg/mL (formulated with either 1 or 20% w/w sucrose) as measured by octane IGC adsorption at 293 K and 0 % RH or N_2 adsorption at 77 K. A total of 3 columns or glass tubes were tested for each material (n=3). Error bars represent ±95 C.I.

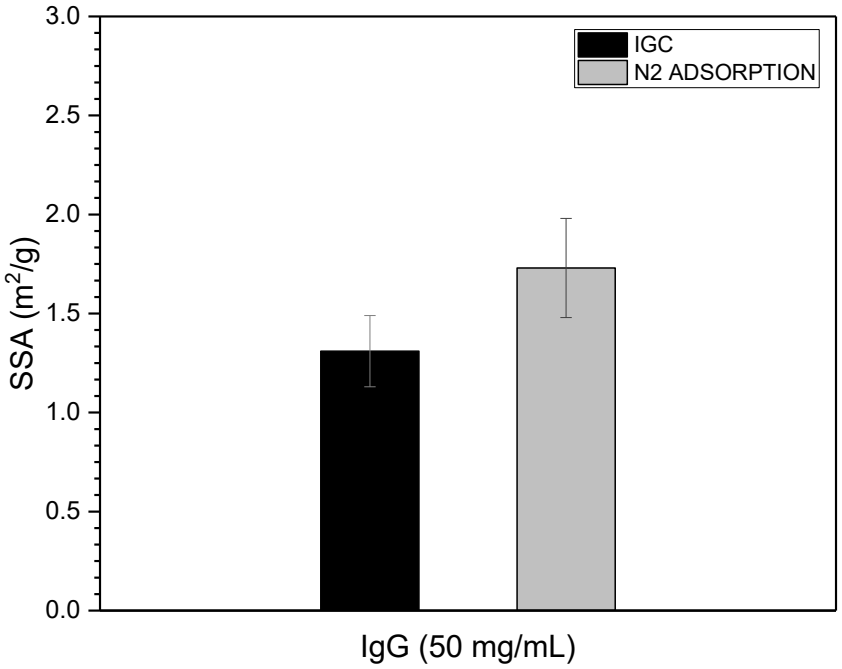


Figure 4.18 Comparison in SSA (m^2/g) of pooled vials from a batch of the FD IgG as measured by octane IGC adsorption at 293 K and 0 % RH or N_2 adsorption at 77 K. A total of 3 columns or glass tubes were tested for each material (n=3). Error bars represent ±95 C.I.

The SSA (m^2/g) of FD materials was measured with either N_2 volumetric adsorption or octane IGC method with BET analysis. In Figure 4.16 - 4.18 the comparative mean SSA values (± 95% C.I) for both methods are shown for an excipient and a FD biological material. In order to have enough material in the vials or columns, several vials were pooled together to be packed inside the columns. Vials were taken randomly from the same FD batch of materials. A total of three separate packed columns or vials were made to be tested (n=3). In general all IGC obtained mean SSA values were comparable with the reported nitrogen adsorption ones, (error bars within 95% confidence intervals). The FD excipients of trehalose 50 mg/mL and sucrose 50 mg/mL had IGC measured SSA values of 1.35 ± 0.13 m^2/g and 0.73 ± 0.25 m^2/g respectively. As reference, the nitrogen SSA values for trehalose and sucrose were 1.36 ± 0.21 m^2/g and 0.93 ± 0.11 m^2/g, respectively. In literature, past SSA values for FD sucrose measured via krypton gas adsorption have been quoted as being between 0.92 -

1.03 m^2/g [182]. Sucrose and trehalose has a freezing rate of 0.2°C/min. Higher cooling rates can result in smaller size crystals and higher surface area, whilst for lower cooling rates it is the opposite. Rambhatla et al. also showed that different nucleation temperatures (supercooling controlled an via ice fog technique) for sucrose produced as much as a 50% difference in SSA values [231]. SSA values should always be put in context to what its relative FD cycle parameters/supercooling techniques used, when comparing to other values based on literature.

IgG 50 mg/mL had IGC and N_2 adsorption mean SSA values of 1.31 ± 0.18 m^2/g and 1.73 ± 0.25 m^2/g, respectively. With increasing concentration FD proteins, there is lower SSA. Geidobler et al. showed that high a concentration FD BSA (100 mg/mL) had very low SSA values below 1 m^2/g when using ramped freezing [198]. On the other scale, FD influenza antigens are typically considered to have high SSA. FD B/Phuket antigens (60 µg/mL) were formulated with different concentrations of sucrose excipient. The increase in sucrose content from 1% to 20% w/w in the B/Phuket antigen formulation, decreased the SSA by roughly a factor of 20. B/Phuket 1% w/w and B/Phuket 20% w/w had an IGC measured SSA values of 20.56 ± 2.57 m^2/g and 0.57 ± 0.04 m^2/g, respectively. The N_2 adsorption method gave SSA values of 23.61 ± 3.98 m^2/g for 1% w/w B/Phuket and 0.64 ± 0.05 m^2/g for the 20% w/w B/Phuket. The B/Phuket antigen formulated with 1% w/w sucrose had the highest percentage variability of SSA error bars. The ‘fluffy’ and extremely porous nature of the cake meant there was great difficulty in column/tube packing. The variability of the mean SSA was higher for the FD pooled vials from the batch compared to the one sample repeated three times for reproducibility. This suggests that most of variability in SSA values arises from the vials in batch even when accounting for instrument error.

The extent of SSA variability within IGC or N_2 adsorption determinations could also range from a host of factors including [232-234]:

I. Physical changes to samples during extraction, breakage and loading into columns or test tubes

II. Differences from vial to vial due to cake heterogeneity and effects of tray location

III. Variations from different column packing and mass loaded

IV. Effect of moisture or storage in vials

In particular one of the limitations of investigating SSA, with this and other studies (N_2 or Kr gas adsorption), is that there is no standardised procedure for extracting and breaking apart the FD cake into small enough pieces to fit inside the glass column or glass tube. The extent of cake manipulation on SSA is an issue that has to be considered when assessing the variability in SSA data. Micromeretics, published an online application note '161', which compared the difference in SSA between fully intact lyophilised sucrose cakes (measured via a custom vial holder) and crushed samples. A special custom sample holder was designed to fit the FD vial head with the cake intact inside and measure isotherms with krypton adsorption. Data showed that fully intact FD cakes had between 36 - 53% smaller SSA than of the manipulated crushed samples [233]. This further highlights the difficulty in measuring SSA between FD materials, since the degree of sample extraction/manipulation could vary from person to person and could create variability upon repeat within same batch.

IGC columns were packed with the FD samples positioned near the centre of the glass columns, which was plugged consistently with small amounts of silanised glass wool at each end. The glass wool in IGC measurements could also possibly have an effect for samples with particularly low surface area. However ,as determined previously, the glass wool has a SSA of 0.29 m^2/g [235], and since only a very small mass (< 10 mg) is used to plug the ends of

each column, the resultant total surface area % difference would be relatively small and could be considered negligible.

Another point which must be considered is the environment when loading of the samples into the columns. For sensitive samples that are highly hygroscopic, such as FD influenza antigens, exposure at a high enough relative humidity's can induce high moisture uptake which can cause some cake shrinkage. One of the limitations of these experiments were that for both IGC and N_2 adsorption methods, samples were loaded in open laboratory environment. Ideally, the samples would be loaded in a dry air bag that has been purged, but due to logistics and time constrains this was not possible to do, which is also a problem in other studies measuring SSA. Samples were loaded at a laboratory room temperature of 23°C with an environment measured at 30 ± 5% RH. Samples on average would take about 1 min to load from opening the vial, breaking apart cake and putting the cake portions into the vials or glass columns via a funnel. For more extremely sensitive material, such exposure could have moisture induced structural changes occur before even reaching the degassing or pre-conditioning step to remove moisture. Lastly, another significant factor would be due to vial to vial variations from the FD manufacturing process including also the effect of vial tray location. It is possible that that techniques such as IGC can be used to detect lot-to-lot variations for different batches of FD material, in addition to currently available techniques at the present [232, 234]. Others like Hancock et al. have postulated about heterogeneity and the existence of possible various amorphous forms emerging out of complex FD processes which could also result in variability [236].

4.3.2.2 Effect of Relative Humidity on SSA

Table 4.5 Comparison of IGC and N_2 adsorption methods used for SSA Determinations in this study

	N_2 Adsorption Volumetric	*Octane Adsorption IGC*
Sample Mass (g)	0.1 - 0.4	0.1 - 0.4
RSD (%)	3 - 8	1 - 4
Temperature (K)	77	293
Pressure (Atm)	0.001 to 0.01	1
Relative Humidity (%)	N/A	0-90%

An additional advantage of using the IGC-SEA, is that it allows for pre-conditioning of samples in the glass column at different relative humidity's between 0 - 90% RH at ambient temperatures (Table 4.5). This is in contrast to most volumetric systems such as with N_2 adsorption, where all moisture must be removed which would otherwise compromise the volumetric approach. Varying the % RH conditioning step to which the column is exposed to can be used to monitor any possible changes in the SSA of packed FD material. By their very nature, FD material are highly hygroscopic and have been shown during long term storage to increase in moisture content. Water is a plasticiser that can reduce the T_g of FD materials and enhance molecular flexibility. As highlighted previously in Chapter 3, if enough critical moisture is gained in a sample there could be a phase transition or complete collapse occurring. A change of structure due to moisture would correspond with a change in SSA for the FD material. Thus, the moisture content is of critical importance in relation to context of SSA. As mentioned earlier, the IGC is able to condition a column with a packed sample for a set amount of time at different % RH's.

Figures 4.20 and 4.21 show the impact of the RH% conditioning step (held for 2 hours) on different FD excipients and biologics (n=1). The red dotted lines represents the last SSA determination after which BET sample analysis gave poorly fitted data (which was not plotted). From 0% to after 40% RH conditioning, sucrose and trehalose (both 50 mg/mL) have a drop in SSA by around 43% and 3% respectively. The sucrose SSA seems to be the more effected of the two after 2 hours exposure. After 40% RH step, both the sucrose and trehalose SSA values were not able to be obtained due to significant non-linearity in BET data. This would indicated that collapse or a phase transition had occurred for the packed samples in the column, which was confirmed visually on the column at the end of experiment for sucrose (Figure 4.19). Sucrose has been known to crystallise at or above 40% RH [213]. FD biologics such as IgG (10 mg/mL) are however more robust and do not exhibit significant change in SSA until after 80% RH conditioning. After 80% RH, the IgG had lost about 13% of its original SSA value was. FD lysozyme (10 mg/mL) has a large decrease in SSA just after 40% RH conditioning, losing about 50% of its original SSA. There is another decrease after 60% RH, where it loses 25% its previous SSA. After 80% RH, there had been a loss of around 2.5 m^2/g from the initial starting SSA. This highlights how sensitive the samples are to accumulating moisture and the resultant impact on SSA.

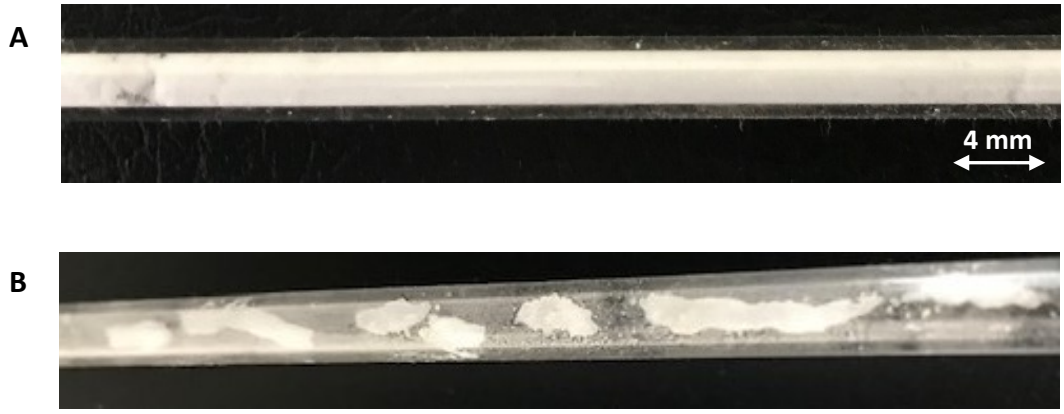


Figure 4.19 Photo of IGC column packed with FD sucrose 50 mg/mL. **A:** Before IGC and **B:** After finishing IGC conditioning steps consisting of 2 hours between 0 - 80 RH%.

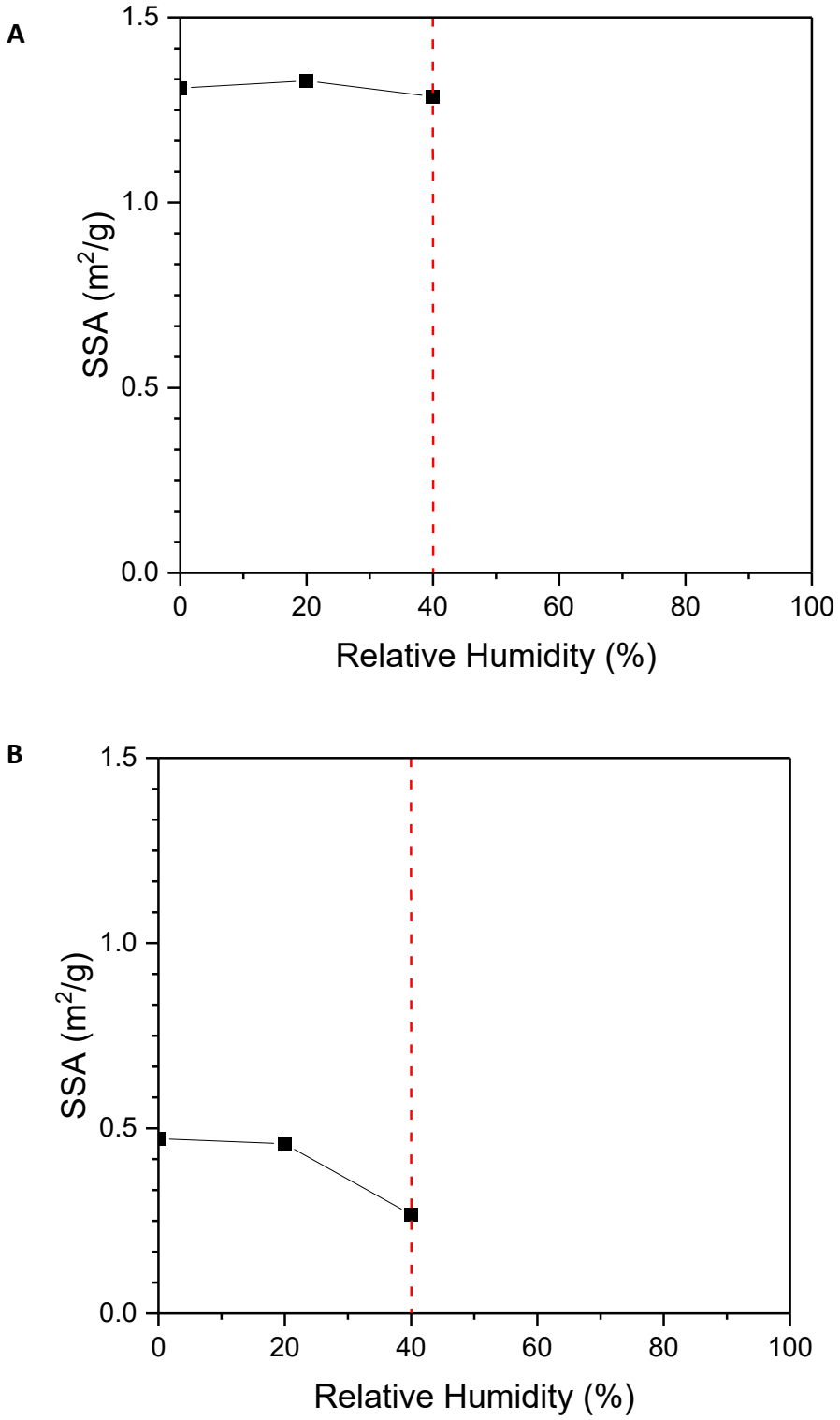


Figure 4.20 Effect of column conditioning for FD excipeints **A:** sucrose and **B:** trehalose at 50 mg/mL. Changes in SSA (m^2/g) of FD materials, conditioned at different RH (%) (held for 2 h at each step). The red dotted lines represents the last SSA determination after which BET analysis gave poor data. (n=1)

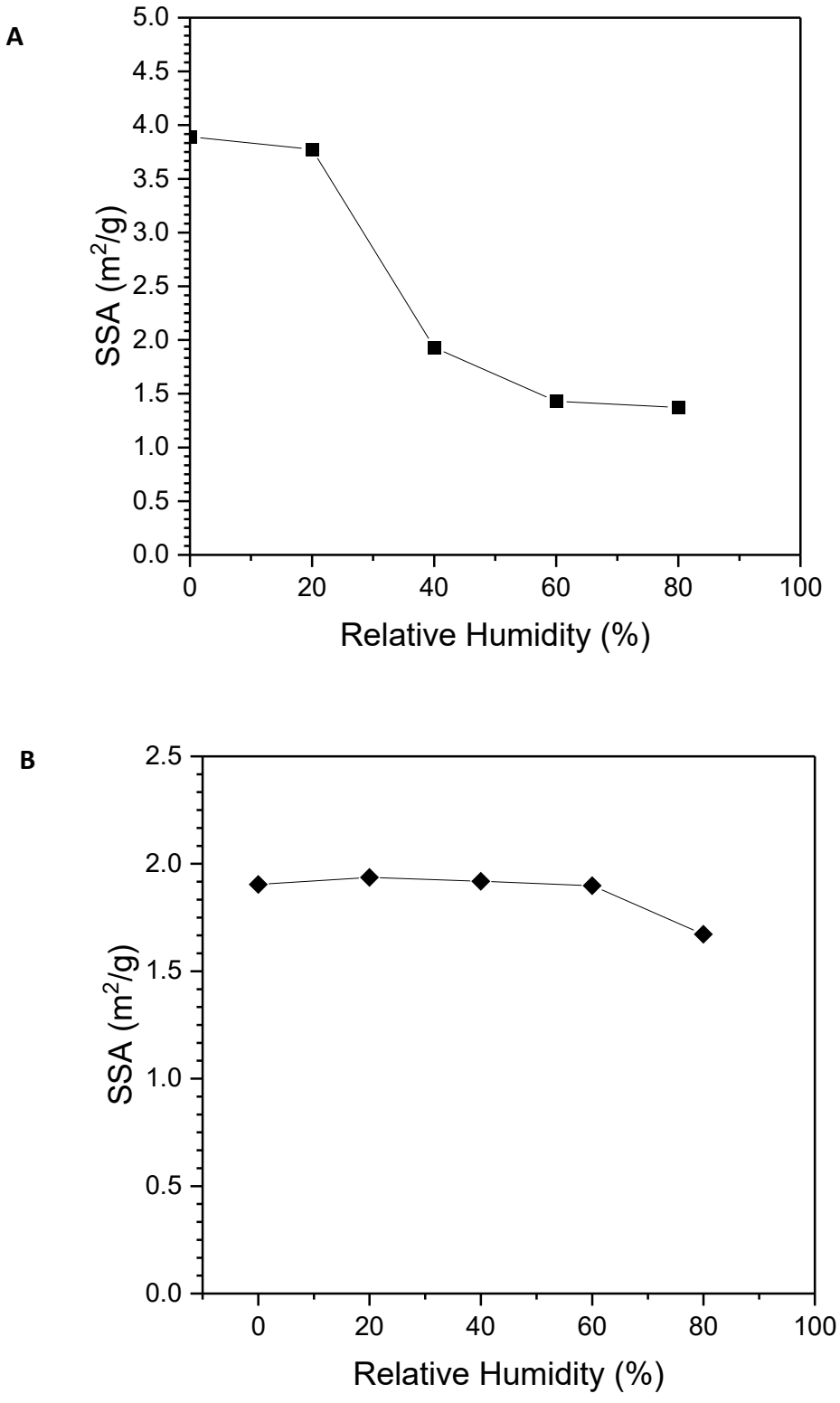


Figure 4.21 Effect of column conditioning for FD biologics **A:** Lysozyme (10 mg/mL) and **B:** IgG at 10 and 100 mg/mL. Changes in SSA (m^2/g) of FD materials, conditioned at different RH (%) (held for 2 h at each step). (n=1)

The potential application of IGC to be used in conjunction with DVS water isotherm plots, means that an approximate derivation of moisture content and SSA can be plotted together. In Figures 4.22 and 4.23 the impact of the RH% conditioning step (held for 2 hours) and moisture gain on SSA of FD flu antigens is shown. IGC with parallel conjunction with DVS have been used to measure and compare the change in moisture (% w/w) and the consequent change in SSA (m^2/g) due to different RH% conditioning. When fully dried, the B/phuket 20% w/w samples are able to uptake more moisture before seeing a change in SSA and collapse compared with the 1% w/w samples. A DVS run simulating the column conditioning process and thus water sorption (% w/w) at each RH% step was used to correlate with IGC SSA values. The limitation of this approach is that DVS and IGC RH conditioning are not strictly fully comparable in the sense that one is done in a packed sample column while the other in an open pan, nevertheless it provides a good approximation for comparative discussion. In both B/Phuket concentrations at 20% RH conditioning there is a slight increase in SSA compared to 0% RH. This corresponds to a slight swelling and expansion of the cake which is observed in the increase of outer diameter seen from DVS pictures. For B/Phuket Antigen 20% w/w formulation the SSA reduces by 30% from 20 to 60 RH% which corresponds to an increase in moisture by ≈ 7% w/w. After 60% RH, above ≈ 9% w/w, the SSA was not measurable indicating possible complete sample deterioration in column as seen in the DVS pictures. For the high surface area B/Phuket Antigen 1% w/w, the SSA was not measurable after 20% RH, above ≈ 4% w/w. These SSA values were not able to be measured past a certain point RH% conditioning step since there was non-linearity between 0.05 – 0.35 P/P_0 and the R^2 fell significantly below 0.995. The photos from the DVS show collapse occurring at similar RH% correlating to a drop in SSA measured by IGC. This indicates that sufficient partial collapse or possibly a phase transition of the sample inside the IGC columns had occurred, whereby, effecting the adsorption/desorption kinetics of octane adsorbate within the sample.

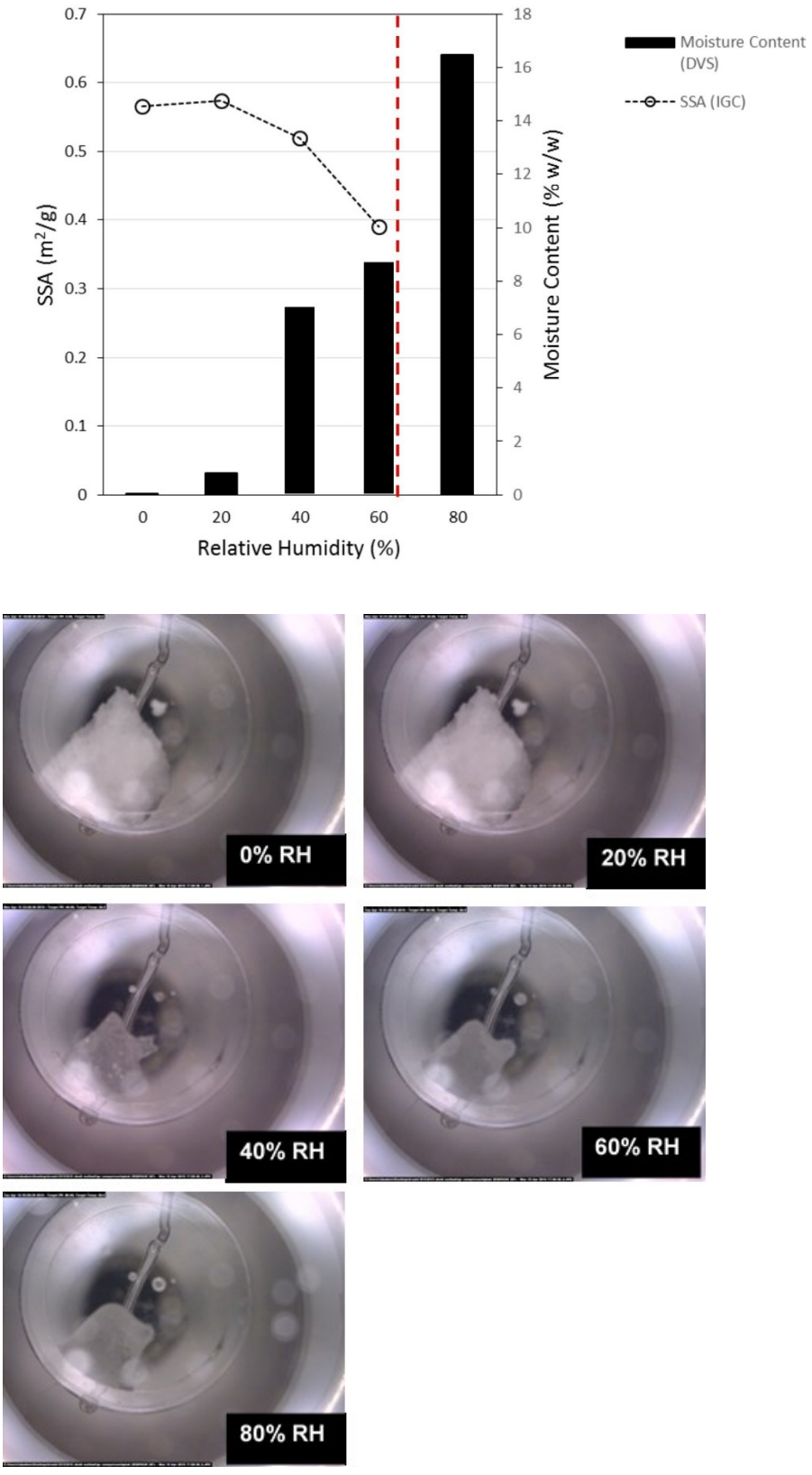


Figure 4.22 The change in SSA (m^2/g) of B/Phuket Antigen (60µg/mL with 20% w/w sucrose) at various RH% steps using IGC (column was conditioning and held for 2 hours at each RH % step). The sample conditioning was simulated on a DVS (held at each RH% step for 2 hours measuring moisture gained % w/w) with photos of sample pan taken at the end of each stage. (n=1)

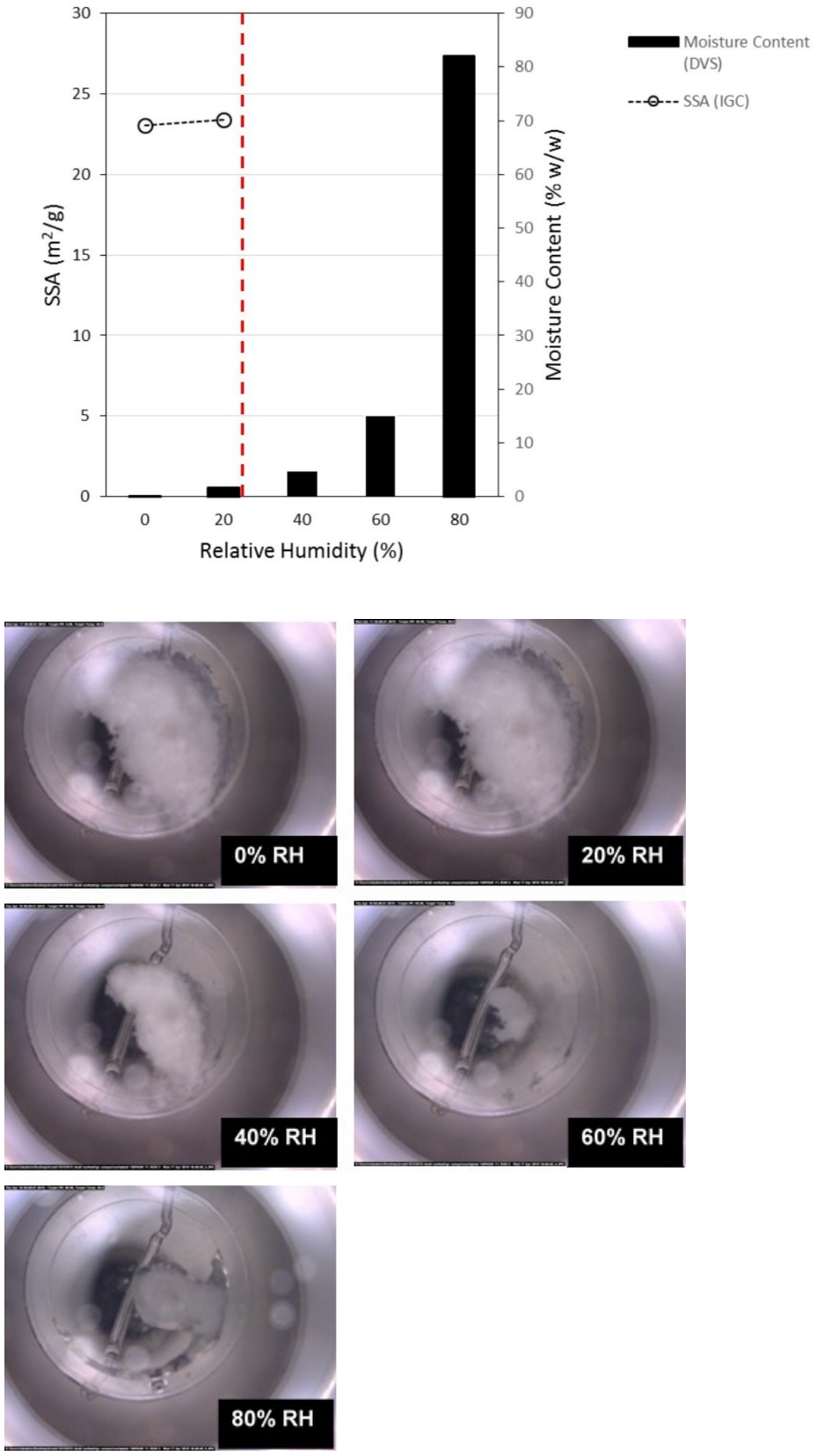


Figure 4.23 The change in SSA (m^2/g) of B/Phuket Antigen (60µg/mL with 1% w/w sucrose) at various RH% steps using IGC (column was conditioning and held for 2 hours at each RH % step). The sample conditioning was simulated on a DVS (held at each RH% step for 2 hours measuring moisture gained % w/w) with photos of sample pan taken at the end of each stage. (n=1)

4.3.1.1 Summary

IGC with an organic based adsorbate has been demonstrated to measure the SSA of a variety of FD excipients and biologics, within similar data reported to that obtained via typical gas adsorption methods (N_2 BET adsorption). Instrument reproducibility was shown to be better than the N_2 adsorption technique. SSA values had large variations in error with repeats due to a number of possible factors including vial to vial heterogeneity in batches and non-standardised approaches to sample extraction (in order to fit material inside tubes or columns). IGC also has the advantage (compared to traditional techniques) in being able to condition the samples inside the columns in situ as controlled temperatures and different relative humidity's (between 0 - 90% RH). As a result the SSA can be plotted between SSA as a function of RH (and in turn moisture content) for a wide range of FD material. This can be used to measure any changes to SSA at different conditions, where even phase transitions or complete collapse can occur.

4.4 Conclusion

Increasing moisture content decreases in mechanical stiffness of FD cakes of excipients and biologics. Over long term storage, the moisture content in the vials can increase and reduce the stiffness of the FD cakes and make them softer and more 'rubbery'. Increasing concentration of protein or excipients results in stiffer and more robust cakes, but the drop in mechanical properties due to moisture gain is often greater than lower concertation samples. IGC, was shown to be a suitable alternative to measuring the SSA of FD material. The reproducibility was just as good as standard nitrogen gas adsorption method with SSA values in general agreement with each other. Increasing concentration resulted in lowering of SSA but increasing Young's modulus. IGC, was used to measure the effects of humidity in SSA of materials. With increasing humidity and hence moisture content, the SSA of FD material decreases. If enough moisture is gained moisture past a critical moisture content the cake in columns will collapse. Overall, the mechanical and surface area properties of FD biologics can be successfully determined by mechanical indentation and IGC, respectively. The application of these techniques can help determine the mechanical and surface area properties of many FD material. They could be another vital tool to measure these properties as quality control (CQA's) in aiding to monitor the effects of ageing and moisture ingress during long term storage of FD biologics. In addition, these techniques can used as complimentary techniques in conjunction with long term stability trials as well as aid formulation of FD materials with regards to manufacturing specific cake attributes. This information further highlights the point that FD materials structural stability and properties such as SSA and mechanical properties are determined and affected in part largely to their moisture content.

Chapter 5. Long Term Storage Stability of High Concentration Plasma Derived Immunoglobulin G

5.1 Introduction

The increasing demand and development for high protein concentration formulations can lead to a host of challenges including an increase in instability, degradation, viscosity and of unwanted protein to protein interactions [237, 238]. Freeze-drying is commonly used to enhance the long term stability of these liquid formulations. An increasing amount of biopharmaceuticals today are FD and this is set to increase as manufacturers look to obtain better stability [24]. High concentration protein formulations are considered to be in the range of 50-150 mg/mL although there is no precise standardised definition [28]. Most monoclonal or polyclonal antibodies are administered by either subcutaneous injection, intra-muscular injections or intravenous infusions. Intramuscular injections are limited by volumes of below 1.5 mL. The highest currently approved FD monoclonal antibody drug products are formulated at concentrations between 100-200 mg/mL, with some hypothesising that this could go even higher with the maximum theoretical limit projected to be around 300 - 500 mg/mL [28, 29]. However, the continuing development of formulations with high protein concentrations brings many problems and challenges. Protein solubility is a key parameter in development of such high formulations. With increasing protein concentration the viscosity and opalescence also increases along with a host of challenges during sterile filtration. Liquid phase separation becomes more likely to occur as well. Besides the formulation aspect, challenges with regards to appropriate moisture and temperature can severely affect and complicate the long term storage stability

Plasma derived immunoglobulin has long been used as a therapeutic product and intravenous immunoglobulins at 50 - 100 mg/mL are common [239, 240]. Past studies have looked at the feasibility of drying high concentration IgG formulations. Dani et al. demonstrated that spray-drying along with glass stabilization technology produced suitable powders for

injectable delivery [241]. The monomer content was maintained with the preservation of native secondary and tertiary structures. However, they noted that additional studies were required with therapeutic mAbs to demonstrate long-term storage stability at high concentrations. Schüle et al. demonstrated how spray-dried IgG1 retained high levels of monomer over storage at 52 weeks at 2-8°C [126]. Other studies have also investigated the stability of high concentration proteins that have been FD. Garidel et al. showed the effect of formulation and storage time of monoclonal antibodies ranging from 40 mg/mL to 160 mg/mL at 40°C [242]. They concluded that stability of buffer-free formulations that contained a disaccharide stabilizer were comparable to that of buffer-based formulations for concentrations up to 115 mg/mL. Other studies have focused investigations into the slow reconstitution times of high concentration protein formulations and ways to improve them [181, 197].

Over long term storage, the moisture content and temperature can affect both physical and chemical stability. A comprehensive long term investigation into stability of FD IgG is assessed over a 12 month period in this Chapter. The monomer content, retention of anti-diphtheria/tetanus antibody titres, change in moisture content and physico-mechanical properties (using previously established techniques from preceding Chapters) are evaluated over a 1 year period under accelerated and real-time storage conditions (-20°C, 20°C and 45°C) to assess FD storage stability and which of the mechanisms of stabilisation might play a role.

5.2 Materials and methods

5.2.1 Materials and Formulation Characterisation

A bulk of 50 mg/mL IgG (2.2 L) was obtained for dialysis from time-expired clinical grade standard product (NIBSC, Potters Bar, UK) and maintained sterile at 2-8°C. 150 mL was dialysed into 1% w/v sucrose, 10 mM citric acid adjusted at pH 6.6 with 5 M NaOH, 0.01% Tween 20 against 3 x 5 L over 20 hours using a Spectrapor 8kDa cut off dialysis membrane. A portion from this bulk was diluted down to 10 mg/ mL. The remaining bulk pool was then ultrafiltered to get nominal target of 100 and 200 mg/mL IgG. All the preparations were then dialysed in dialysis membrane sacs against the citrate Tween 20 sucrose buffer. Target concentrations were achieved by diaflitration using the Vivaflow 200 crossflow system and with a 50kDa PES (polyethyl sulfone) membrane cartridge (Sartorius Stedim Biotech, Germany). Concentrations were confirmed by OD 280 nm (E1% for IgG = 13.5 AU) using triplicate 1:100 dilutions measured with a UV spectrophotometer (Pharmacia Biotech, GE Healthcare, UK) blanked on sucrose citrate buffer. Average readings were 0.98 ± 0.02 %, 5.43 ± 0.19 %, 9.86 ± 0.13 % and 20.4 ± 0.97 % w/v. These were approximated to 1, 5, 10 and 20% w/v which is equivalent to 10, 50, 100 and 200 mg/mL, respectively.

5.2.2 Freeze-drying microscopy (FDM)

FDM was used to work out the collapse temperature of the IgG samples to aid the cycle optimization. 5 µL samples were dispensed in quartz crucible with metal shim. A coverslip was added and sample was affixed on Linkam FDCS196 Cryostage (Linkam, Surrey, UK) and analysis performed using an Olympus BX51 microscope specifically fitted with plane-polarized light. Image capture was obtained using a CCD camcorder. Samples were cooled to -50°C in which after the vacuum was applied. Heating rate was set at 5°C/min until collapse was observed. Calibration of system was performed with 5% w/v trehalose (Sigma-Aldrich, UK) in deionised water.

5.2.3 Modulated DSC

Solid sample or liquid aliquots of IgG was panned into large volume hermetically sealed pans (part number 900825.902 TA Instruments, Elstree, UK). Samples were evaluated on Q2000 Standard DSC (TA Instruments, Surrey, UK) against an empty pan as reference. A heating ramp rate at 5°C/min up to 200°C. Refrigerator cooling accessory (RCS-90 cooler unit) was responsible for heating/cooling rates application. Instrument calibration is tested against indium, using a sample of known mass. Data analysis was performed on Universal Analysis Software.

5.2.4 Study 1: Freeze drying high concentration IgG

A “low” moisture cycle run batch included all the IgG concentrations (10 - 200 mg/mL). Sample filling was completed with automated multipette stream (Eppendorf, UK) in 5 ml volume screw capped vials (41.5 X 18 mm i.d. Schott VC005 (Adelphi Tubes, Haywards Heath UK). The fill volume for all concentrations was 1 mL. The vials were loaded onto the shelves of the Telstar LyoBeta 15 (Azbil-Telstar SpA , Terrassa, Spain). After the cycles had finished the vials were backfilled with dry nitrogen gas and were stoppered down with 14 mm diameter igloo halobutyl stoppers (Adelphi Group, Haywards Heath, UK). Afterwards the vials were screw-capped, labelled and stored at -20°C, 20°C and 45°C until further testing.

5.2.5 Study 2: Optimum moisture content of IgG

50 mg/mL IgG was lyophilised with the same conditions and same “low” FD cycle as described in Section 5.24. After FD, samples were placed inside the room at 20°C with a relative humidity of 57 ± 2 RH% measured with a TFH 620 Hygrothermometer (Ebro-Xylem, Germany). The stoppers were removed and vials were exposed to external atmosphere at different time intervals to provide different moisture contents. A total of 4 different moisture contents were made and confirmed using Karl Fischer titration in triplicate vials (n=3).

5.2.6 Study 3: Comparison of low and high moisture FD cycle

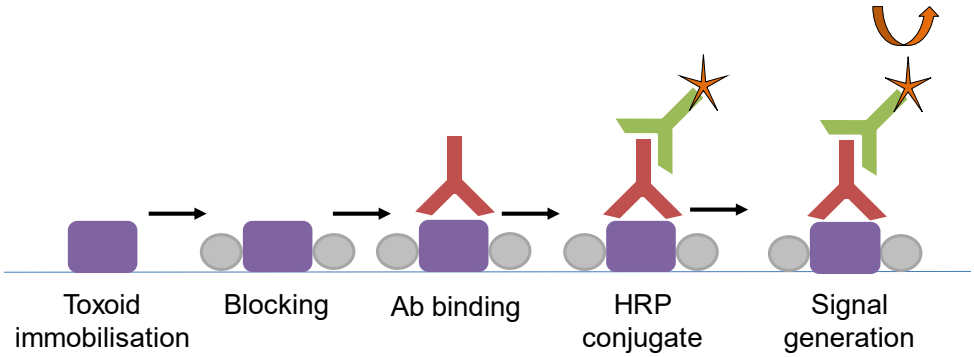
A separate “high” moisture cycle and “low” moisture cycle batch of FD IgG (50 mg/mL) was made to compare between cycle induced moisture. After FD cycles, samples were once again backfilled under vacuum with dry nitrogen gas and were stoppered down with 14 mm diameter igloo halobutyl stoppers (Adelphi Group, Haywards Heath, UK). Afterwards the vials were screw-capped, labelled and stored at -20°C, 20°C and 45°C until further testing

Table 5.1 Freeze drying cycle for IgG formulations.
Vials were stoppered under normal atmospheric pressure.

Cycle	Freezing Temperature (°C)	Freezing Ramp Rate (°C/min)	Freezing Hold Time (min)	Primary Drying Temperature (°C)	Primary Hold Time (min)	Secondary Drying Temperature (°C)	Secondary Drying Ramp Rate (°C/min)	Secondary Hold Time (min)
Low Moisture Cycle	-40	1.00	120	-15	1200	30	0.15	600
High Moisture Cycle	-40	1.00	120	-40	900	15	0.18	60

5.2.7 Diphtheria and Tetanus ELISA assay

ELISA (enzyme-linked immunosorbent assay) is plate based assay used for detecting and quantifying a variety of proteins or antibodies (Figure 5.1). Anti-diphtheria or anti-tetanus IgG binding activity levels are commonly determined using ELISA technique as alternative to *in vivo* toxin neutralization tests [243]. In brief, 96 well plates were coated with either diphtheria toxoid (NIBSC product code 13/212) or tetanus toxoid (code 02/126) as coating antigen, sealed and left to incubate overnight at 4°C. The next day plates were washed with PBS containing Tween-20 0.05% v/v (PBST) and then blocked with blocking buffer (PBST + 5% skimmed milk powder). Plates were incubated at 37 °C for 1 h and washed as before. Sample and reference antitoxin dilutions were prepared in sample buffer (PBST + 1% skimmed milk powder). WHO International Standard antitoxins for diphtheria (NIBSC product code 10/262) and tetanus (NIBSC product code TE-3) were included on each plate to allow specific IgG concentrations to be expressed in IU/ml. 200 µL of diluted sample or reference were added to the top wells and titrated down the plate using a manual multichannel pipette set to 100 µL. After serial dilutions, plates were once again incubated at 37 °C for 2 h and washed as before. Anti-human IgG HRP-conjugate (Sigma A8792, Sigma, Poole, UK) was diluted 1/2000 in sample buffer and 100 µL was added to each well before incubation at 37°C for 1 h. Substrate solution containing citric acid buffer and ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt]) tablets was added to all wells for color to develop. Absorbance was read at 405 nm on a Molecular Devices Vmax plate reader running Softmax Pro 6.5.1 (Molecular Devices, UK). Data analysis was performed in CombiStats (version 5.0, 2013, EDQM, Strasbourg, France).



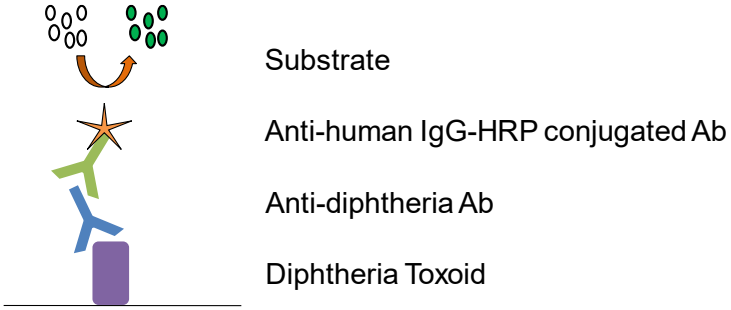


Figure 5.1 Schematic representation of ELISA assay used to determine the levels of Diphtheria or Tetanus binding activity for IgG.

5.2.8 SEC-HPLC

Size-exclusion high performance liquid chromatography (SEC-HPLC) is a commonly applied chromatographic method used for determining of aggregation or monomer content of biotherapeutic proteins in solutions via size or molecular weight differences [244]. SEC-HPLC was used to determine the monomer content of the FD IgG samples after reconstitution. Analysis was performed using the Thermo Scientific UltiMate 3000 HPLC System (Thermo Fisher Scientific, UK) with a TSKgel G3000SWXL HPLC column (300 x 7.8 mm, Sigma-Aldrich, UK). Ultraviolet (UV) detection was selected and measured at 280 nm in triplicate (n=3). The mobile phase was prepared from the formula as outlined in European Pharmacopeia 9.0: Human Normal Immunoglobulin for intravenous administration (01/2012:0918) [245], consisting of disodium hydrogen phosphate dihydrate (0.49% w/v), sodium dihydrogen phosphate monohydrate (0.17% w/v), sodium chloride (1.17% w/v) and sodium azide (0.01% w/v). The flow-rate on the apparatus was set at 0.5 mL/min. Peak analysis was performed using Chromeleon 7.2SR software (Thermo Fisher Scientific, UK).

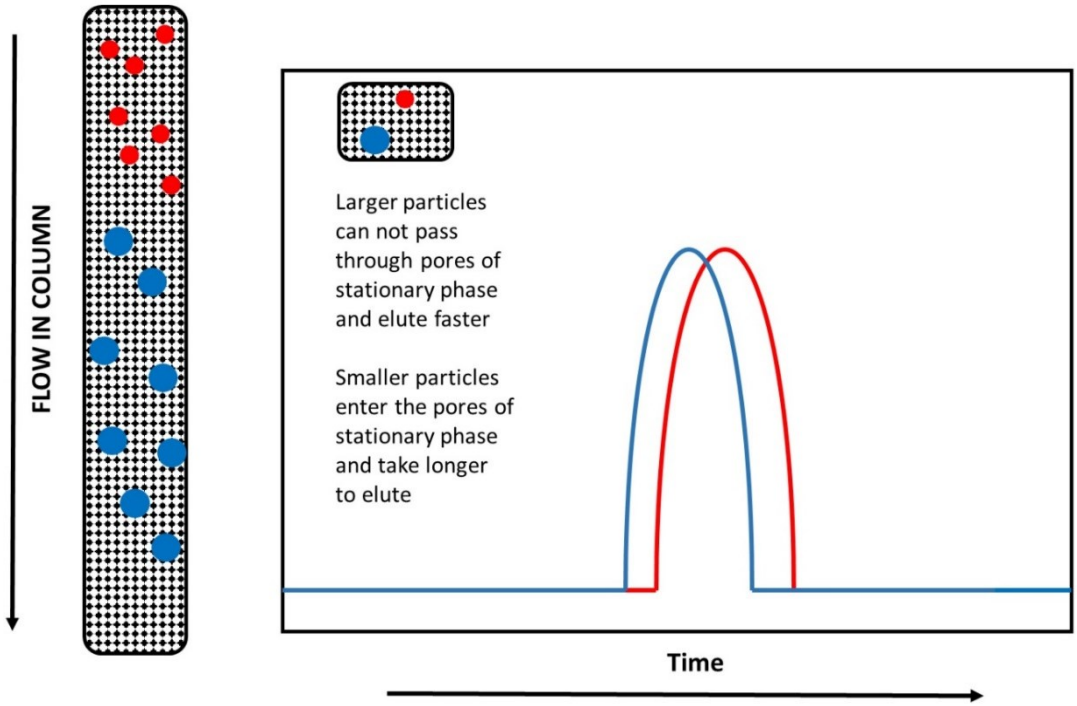


Figure 5.2 Schematic representation of Size-exclusion high performance liquid chromatography (SEC-HPLC).

5.2.9 Residual moisture content

Same method as previously described in Chapter 3

5.2.10 DVS

Same method as previously described in Chapter 3

5.2.11 Mechanical Indentation

Same method as previously described in Chapter 4

5.2.12 Specific Surface Area

Same method as previously described in Chapter 4

5.2.13 Statistical Analysis

95% confidence interval analysis same as previously described in Chapter 3, section 3.2.6.

5.3 Results and Discussion

5.3.1 Study 1: Stability of increasing high concentration IgG

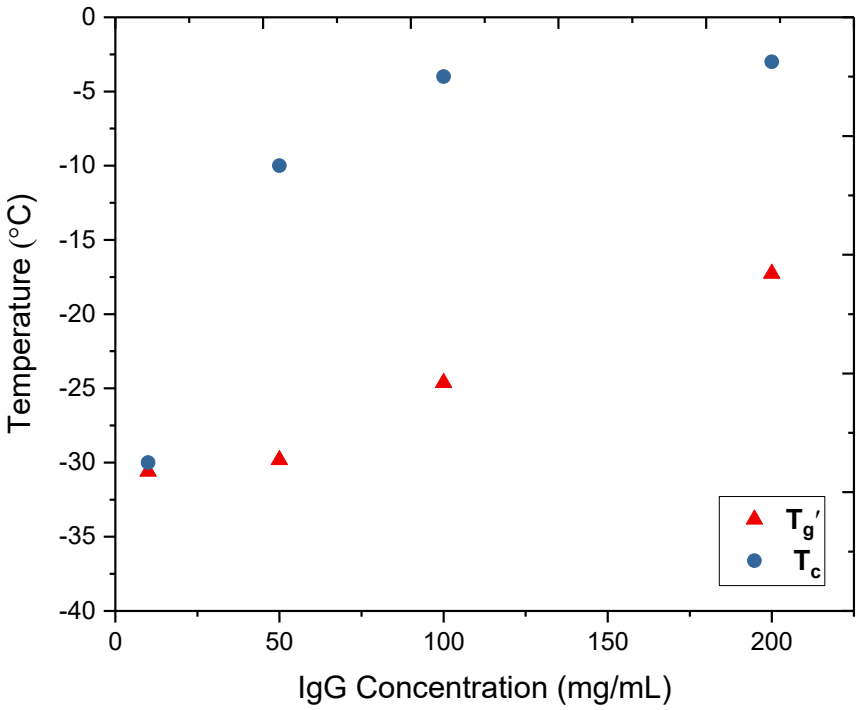


Figure 5.3 Comparison of the collapse temperatures (T_c) measured by FDM and glass transition temperatures in frozen state (Tg') measured by DSC (n=1) for different concentration of IgG (10 - 200 mg/mL).

Cycle development and characterisation are a crucial element in freeze-drying in order to maximise efficiency and maintain product quality. Critical parameters such as the collapse point and T_g' are essential for design and effective economical freeze-drying cycle. It has been shown that a primary drying temperature above the T_g' in some cases can lead to a loss in stability, while in others there is no effect [56]. In Figure 5.3, the FDM data showed that increasing IgG concentration increased the collapse temperature (T_c) - 10 mg/mL collapsed at -30°C, 50 mg/mL collapsed at -10°C, 100 mg/mL collapsed at -4°C and 200 mg/mL collapsed at -3°C. The data showed that while the T_g' also increased with increasing concentration so did the gap between the T_c also widen, and this was also observed by Garidel and Presser [28] with increasing concentration of saccharose-succinate formulation. As a

result of this a more developed aggressive lyophilization cycle can be implemented with shorter primary drying time.

Polyclonal human immunoglobulin is prepared from many thousand donors and a dimer content of around 8-10% occurs naturally and is of itself not an indicator of aggregation [246]. Proteins can undergo degradation and loss in stability during the various stages of the lyophilisation process. A number of problems such as cold denaturation, phase separation, pH shifts, and increasing solute concentration during ice formation could arise from freezing and influence the stability of the protein [247]. Figure 5.4 compares the monomer (%) content between liquid samples before freeze-drying and after, following immediate reconstitution (within standard deviation n=3). The freeze-drying process did not have any impact or loss of monomer (%) at 10 and 50 mg/mL as the values of both pre and post FD are almost unchanged. However, at the higher concentrations of 100 and 200 mg/mL there was a small drop of monomeric content by about 0.8% and 2.9% respectively.

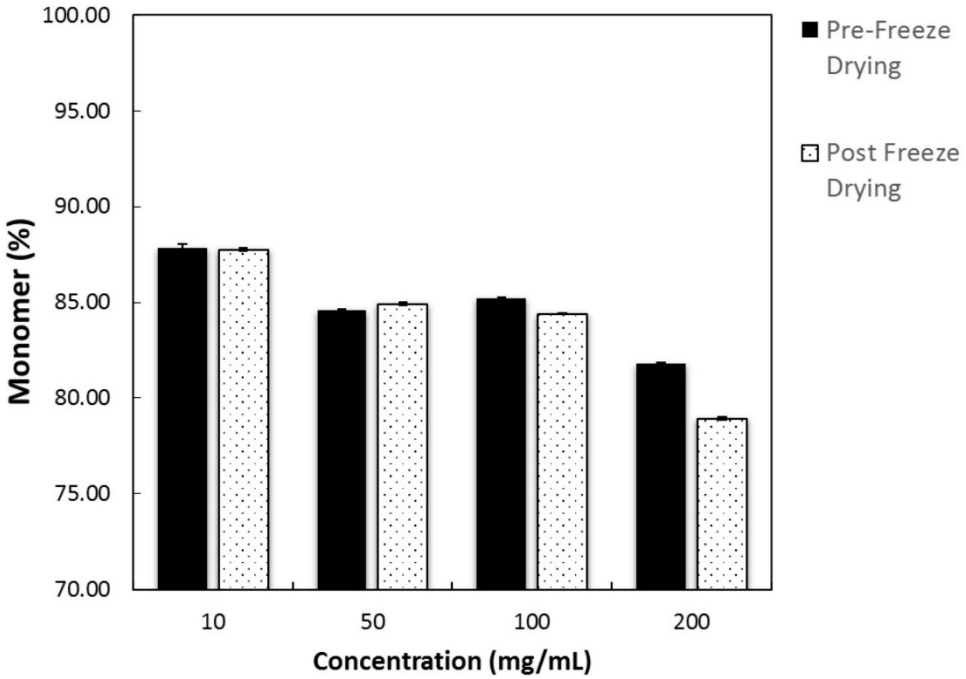


Figure 5.4 Monomer content (%) before and post freeze-drying of IgG. Error bars represent 95% confidence intervals (n=3). The molar ratios for IgG:sucrose - the 10 mg/mL IgG formulations has a molar ratio of 1:433. The 50 mg/mL IgG formulation has a ratio of 1:87, the 100 mg/mL has a ratio of 1:43 and finally 200 mg/mL has a ratio of 1:22.

It is also worth noting that the monomeric content of the pre-dried liquid samples decreases with increasing IgG concentration (mg/mL). This may be because of the impact of the ultrafiltration on the higher protein content formulations. In liquid formulations, as the protein concentration increases, the solution becomes more crowded and the protein to protein interactions increase, thereby inducing aggregation and affecting stability. During freezing the physical environment is altered which could affect the proteins. The higher concertation IgG might be more susceptible to cold denaturation (spontaneous unfolding of a protein at low temperature) compared to the lower concentration formulations. It is a possibility that the % monomer decreases with increasing concentration could also be a result of interfacial denaturation from interaction of the IgG at the ice/protein interface. This again would highlight the challenges of freeze-drying highly concentrated proteins as the freezing and drying could both have a detrimental effect on stability. Stability issues during processing can be addressed by addition of excipients. Tang and Pikal have demonstrated that stabilizers such as sugars or polyols can be used to stabilize against both cold and thermal denaturation [248].

Typically a 1:1 weight ratio of protein to sugar would be suitable for optimal stabilisation (suggesting that stabilisation was not at molecular level but trapping the protein in a glassy matrix). However for these IgG experiments, a lower molar/weight to weight ratio was intentionally used at a fixed excipient concentration of 1% w/w sucrose in the formulations in order to see the effect of temperature destabilisation and moisture ingress over time. As such, for example the 10 mg/mL would be 1:1 but at 200 mg/mL would be 20:1 (protein : sucrose, w:w ratio). IgG has a molar mass of 150,000 g/mol and sucrose 342.3 g/mol. In terms of the molar ratios for IgG:sucrose - the 10 mg/mL IgG formulations has a molar ratio of 1:433 (IgG is 67 µM and the 1% sucrose would be 29 mM). The 50 mg/mL IgG formulation has a ratio of 1:87, the 100 mg/mL has a ratio of 1:43 and finally 200 mg/mL has a ratio of 1:22. Post FD, each FD IgG cake concentration had different visual appearances (Figure 5.5). All cakes exhibited a sharp white colour at all concentrations. The 10 mg/mL IgG cakes were especially

fragile and had a “fluffier” texture compared to other concentrations. The 100 and 200 mg/mL cakes exhibited more cracking and fracturing across the diameter at the surface. Ullrich et al. also noted how the relationship between increasing concentration and higher cracking in cakes occurring [227, 229]. The significance of increasing concentration to the relationships with reconstitution time and physical mechanical properties is further discussed later in this Chapter.

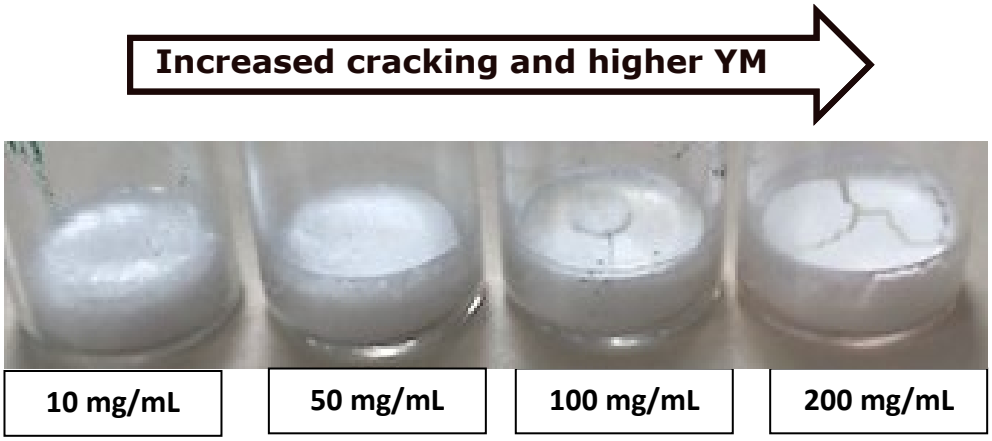


Figure 5.5 Visual appearances of FD cakes from 10 - 200 mg/mL concentration IgG.

5.3.1.1 Moisture Content After 12 months

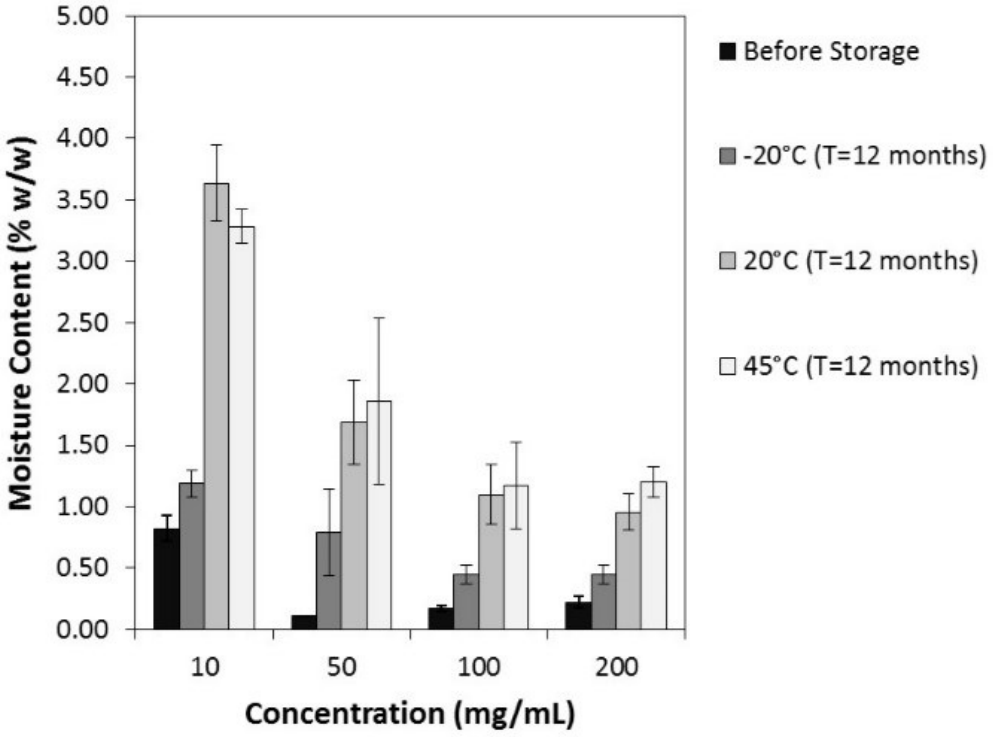


Figure 5.6 Average moisture content (%) for low to high concentration FD IgG before and after 12 months storage at -20, 20 and 45°C. Error bars on bar chart represent 95% confidence intervals (n=3).

The moisture content before and after 12 months storage is displayed in Figure 5.6. The moisture content in the vials does not stay static over the course of storage at all temperatures. The general trend was that vials with relatively lower IgG concentrations took up more moisture than vials with higher concentration samples. Before storage the moisture content in the vials from 10 - 200 mg/mL was less than 1% w/w. The largest moisture ingress happens at the higher storage temperatures of 20°C and 45°C – although there is some small increase at -20°C seen as well. Except for the 10 mg/mL concentration, which already had high moisture since before storage (0.82 ± 0.11 % w/w), all other concentrations between 50 - 200 mg/mL never exceeded more than 1% w/w moisture content at -20°C. At storage of 20°C and 45°C, the 10 mg/mL vials had a final moisture content after 12 months of 3.64 ± 0.31 and

3.28 ± 0.14 % w/w, respectively. At storage of 20°C the 50, 100 and 200 mg/mL vials had a final moisture content after 12 months of 1.69 ± 0.34, 1.10 ± 0.25 and 0.95 ± 0.11 % w/w, respectively. Likewise at storage of 45°C the 50, 100 and 200 mg/mL vials had a final moisture content after 12 months of 1.86 ± 0.68, 1.17 ± 0.36 and 1.20 ± 0.12 % w/w, respectively. In general, for all IgG concentrations there is very similar final moisture content at 20°C and 45°C after 12 months storage. As observed from water sorption isotherms in Chapter 3, the lower concentration IgG samples are more prone to water uptake than the higher concentrations. The most likely source of the moisture is from the rubber stoppers themselves. These results underlines the well-known point that the moisture over time increases inside vials and that the temperature plays a vital role in determining this ingress [69, 141]. The rising moisture content can sometimes be detrimental to long term stability [2, 121]. Jordan et al. showed that the rate of aggregation was linked to moisture content for FD BSA [123]. Additionally, water acts as a plasticiser and can reduce the glass transition (T_g) of the solids-state FD material. DSC was used to measure the T_g after 12 months storage (n=2). As shown in Figure 5.7, the T_g at 20°C and 45°C is different although they have roughly similar final moisture contents after 12 months. This could be due to aging effects and relaxation at higher temperatures, resulting in more molecular flexibility. Apart from moisture, other factors can influence the values of T_g such as such as ramping rate, thermal history of samples and presence of multiple glass transitions [74-76]. The impact on the storage conditions and moisture had on the monomeric (%) content and binding activity are shown in sections below.

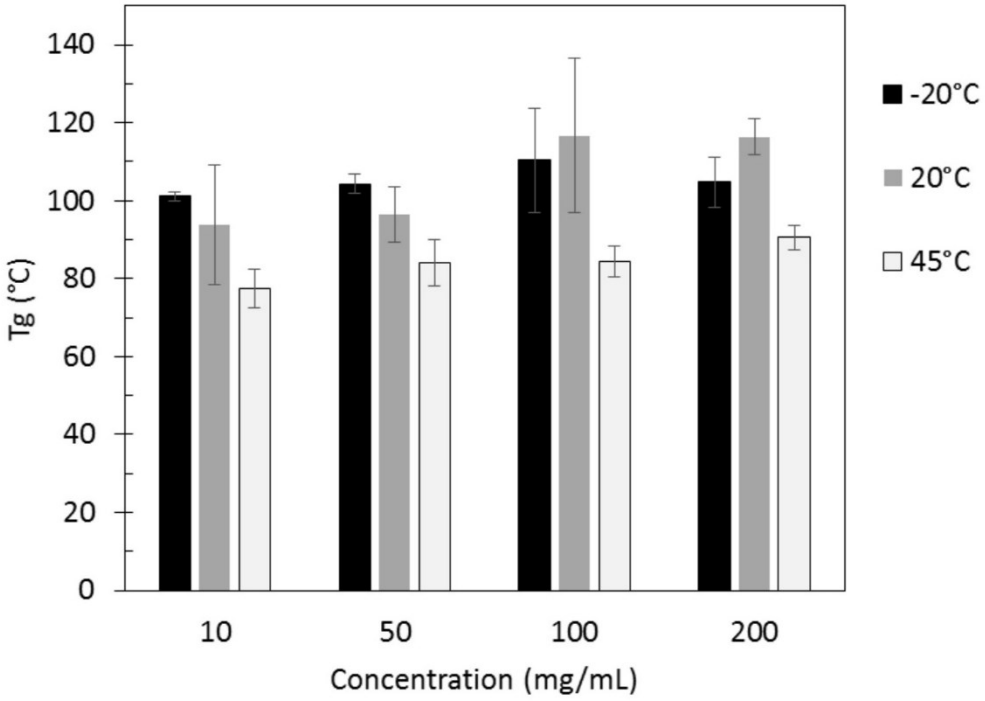


Figure 5.7 The average glass transition temperature measured by DSC after 12 months storage at -20, 20 and 45°C. Error bars represent standard deviation (n=2).

5.3.1.2 Monomer content over storage

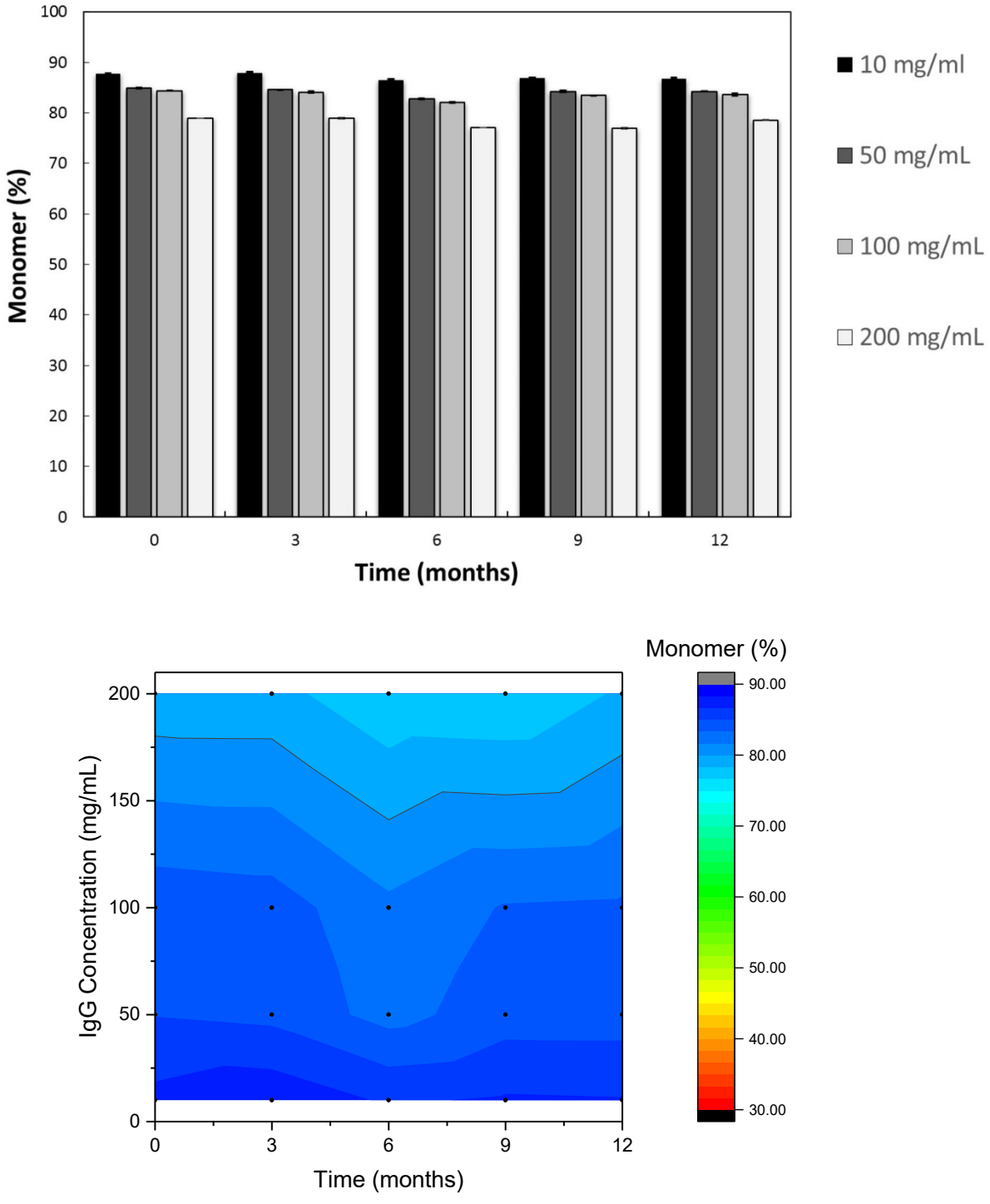


Figure 5.8 Average monomer content (%) over time for low to high concentration FD IgG with contour stability maps as measured by SEC-HPLC over 12 months storage at -20°C. Error bars on bar chart represent 95% confidence intervals (n=3). • Points on contour map represent average monomer content.

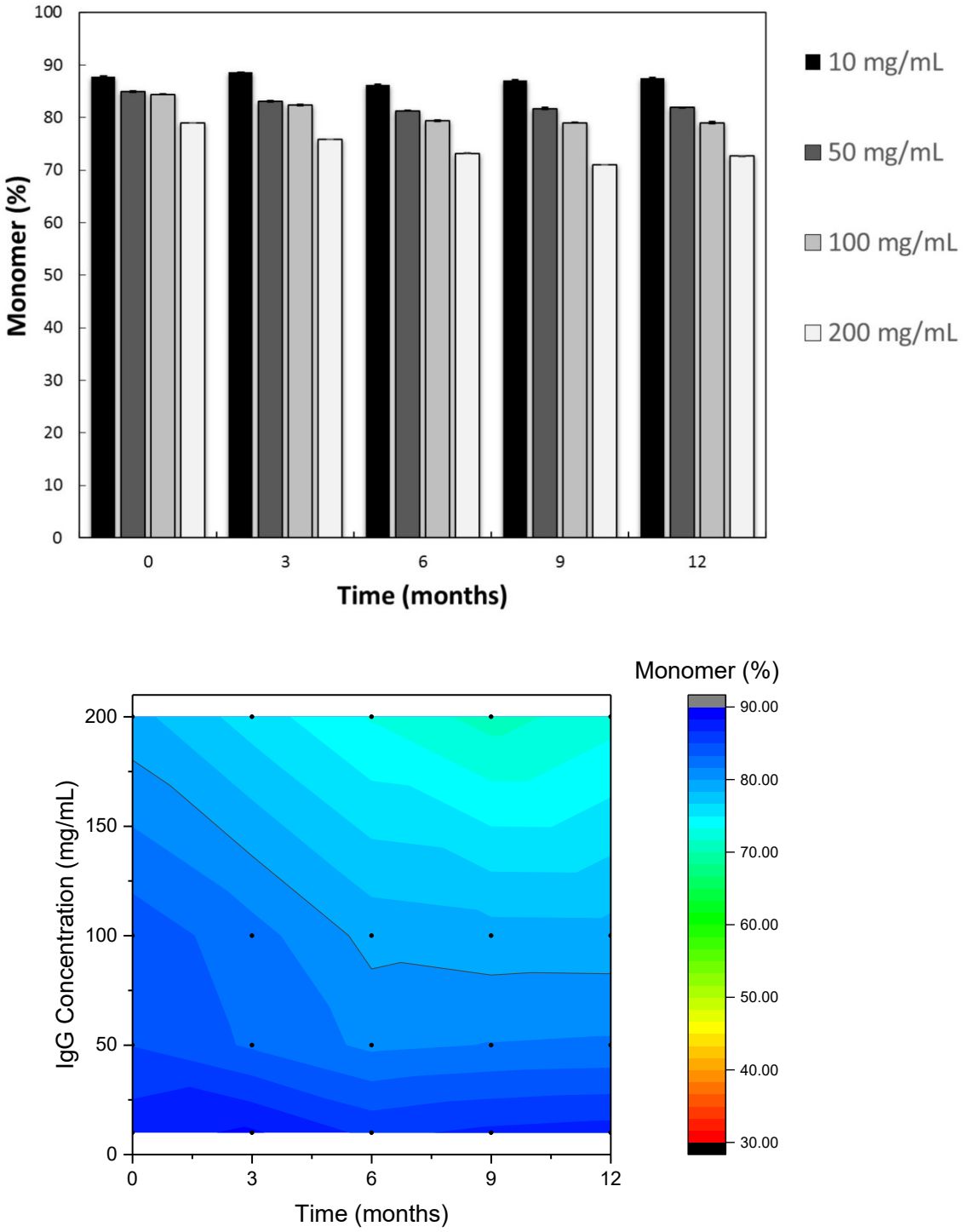


Figure 5.9 Average monomer content (%) for low to high concentration FD IgG with contour stability maps as measured by SEC-HPLC over 12 months storage at 20°C. Error bars on bar chart represent 95% confidence intervals (n=3). • Points on contour map represent average monomer content.

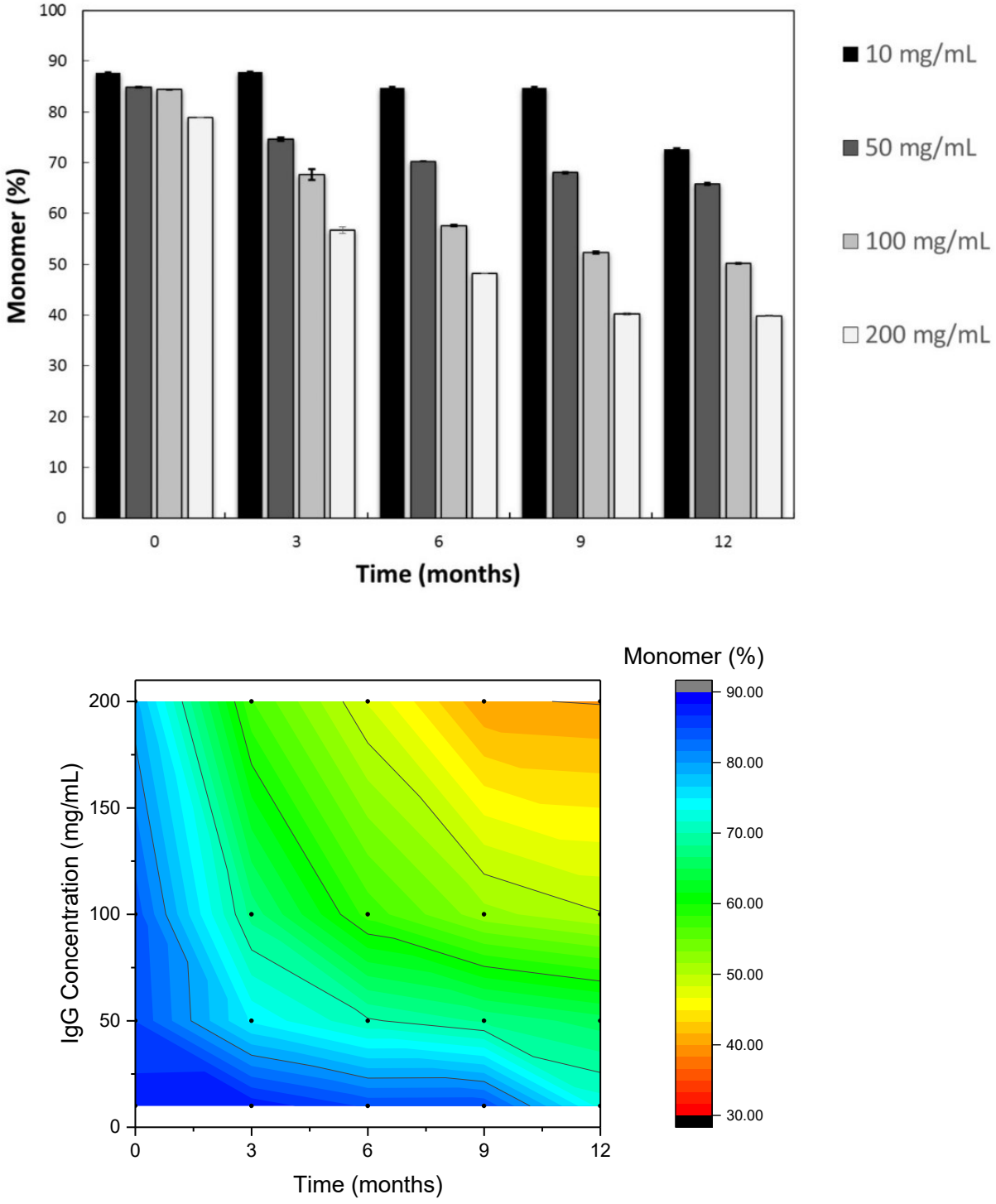


Figure 5.10 Average monomer content (%) for low to high concentration FD IgG with contour stability maps as measured by SEC-HPLC over 12 months storage at 45°C. Error bars on bar chart represent 95% confidence intervals (n=3). • Points on contour map represent average monomer content.

The monomer (%) content of IgG measured at various concentrations spanning over a 12 month storage period, are displayed in Figures 5.8 - 5.10. The total OD measured in an HPLC profile (total height mAU) showed that similar loaded total amounts of protein (between 800-900 mAU) at all storage conditions and samples. Vials were stored at -20°C, 20°C and 45°C. At -20°C, there was minimal losses occurring with less than 1% drop of monomer content observed for IgG samples at 10, 50, 100 and 200 mg/mL throughout all 12 months of storage (losses of 1.0%, 0.7%, 0.8% and 0.4% respectively). At 20°C once again the lower concentrations of 10 and 50 mg/mL remain stable throughout the entire 12 months with some minimal loss of monomeric content been seen (0.3% and 3.0% respectively). However, at 100 mg/mL a slight drop of monomer content was observed from T=6 months' time point by 3.6%, which then continued to cumulate to an overall total drop of 5.4% by the end of 1 years storage. The highest concentration sample at 200 mg/mL saw a total drop of about 6.2% monomer content after 1 year in storage at 20°C. The highest elevated stress temperature of 45°C produced the greatest drop in monomer content for all concentrations. Samples at 10, 50, 100 and 200 mg/mL saw a total drop of monomer by about 15.1%, 19.1%, 34.2% and 39.1% respectively after 12 months storage. The 200 mg/mL samples had almost 2.6 times higher monomer loss compared to the 10 mg/mL samples by the end of the year. At 20°C this difference in loss is almost 20 times higher, while at -20°C almost 0.3 times difference.

The results presented in this study are slightly unusual in that the highest concentration protein was shown to be the least stable. These results are counter to the general trend in some literature expecting higher concentrations tending to be more stable. It is the suggestion that very high concentrations of IgG are more temperature labile compared to lower concentrations. Another possibility is that there might be a lack of excipient protection from the formulation at high protein concentrations. The mobility of the protein in the solid state increases with temperature which may allow more degradation to occur. As observed in the liquid state, higher concentrations of IgG tend to have more protein-protein interaction than lower concentrations which could account for the lower thermostability. Table 5.2 shows that

the 10 mg/mL IgG had lower T_g -T gap values than the higher concentrations, yet was able to have less monomer loss at 45°C. Even though lowest concentration IgG had the highest moisture content and reduced T_g, it was shielded because it had enough excipient to protein ratio protection while the higher concentration didn't, thus supporting a case for water replacement theory. However, the moisture content was similar at both 20°C and 45°C, yet the largest drops were only observed at 45°C for samples 50 - 200 mg/mL. One possible explanation for this is that of the T_g being closer to high storage temperature and hence increasing molecular flexibility – supporting a role for the glass dynamics hypothesis (T_g -T). The question of optimum moisture content and mechanisms of stabilization (be it water substitution theory or glass vitrification hypothesis) has been a matter of debate [133]. Breen et al. have measured the effects of high moisture on the T_g of humanised monoclonal antibody formulations, where they found evidence for increasing moisture not effecting physical stability [7]. Higher moisture cakes saw higher aggregation only if stored above the T_g values, while intermediate moisture vials were more stable and had less aggregation compared to the driest samples.

Table 5.2. Comparison of difference in storage temperature and glass transition temperature after 12 months storage.

Storage Temperature (°C)	IgG Concentration (mg/mL)	T_g -T (°C)
-20	10	121
	50	124
	100	130
	200	125
20	10	74
	50	76
	100	97
	200	96
45	10	32
	50	39
	100	39
	200	45

5.3.1.3 Binding activity over storage

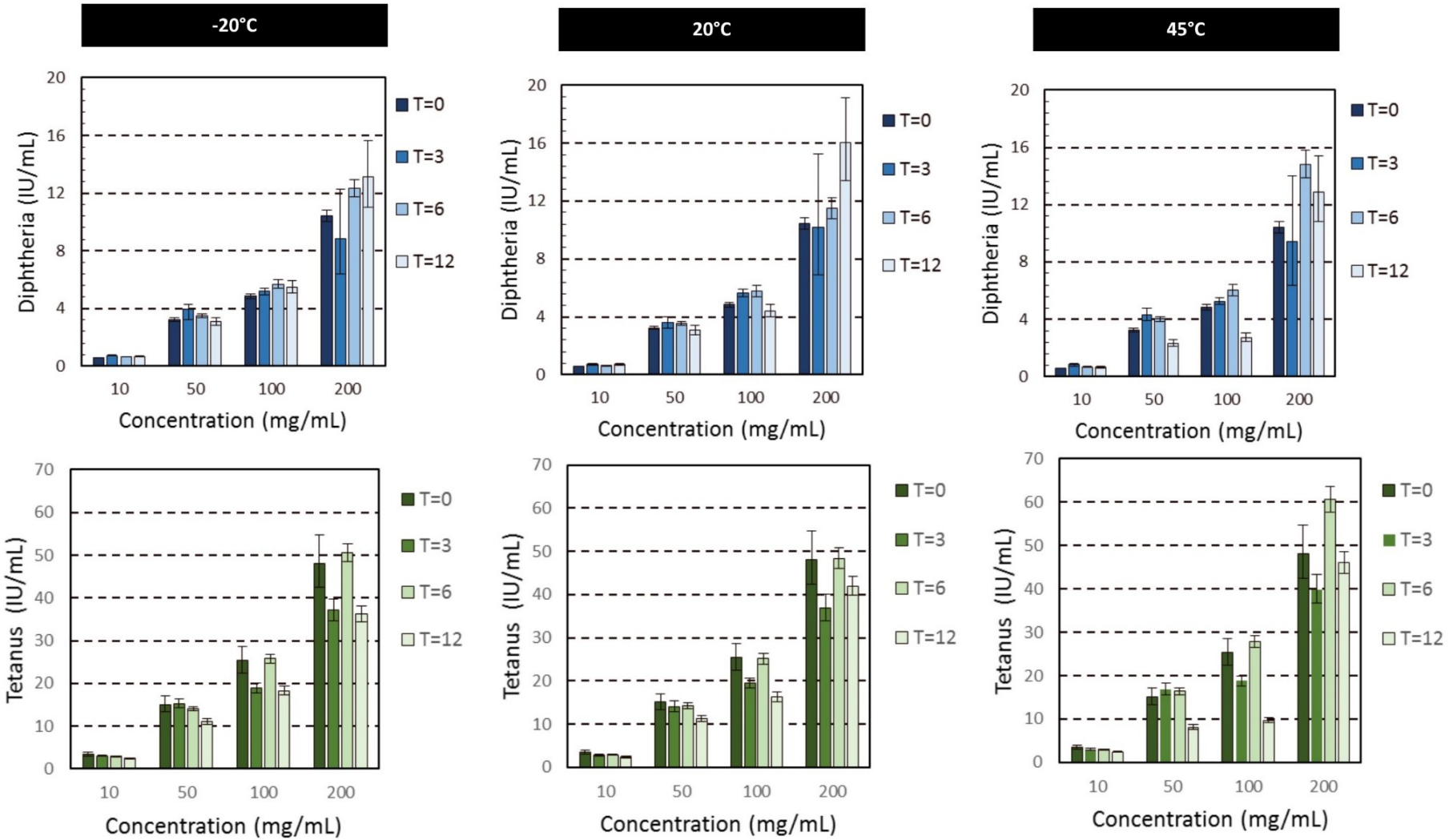


Figure 5.11 ELISA Diphtheria and Tetanus binding activity (IU/mL) for IgG over the course of 12 months stored at -20°C, 20°C and 45°C. Error bars represent ± 95% confidence intervals.

The binding activity of antibodies is an indicator of the effectiveness to bind the corresponding antigen in the body. Figure 5.11 shows the biding activity (IU/mL) as measured by ELISA, for specific IgG (diphtheria and tetanus) over the course of 12 months at the three storage temperatures of -20°C, 20°C and 45°C. Anti-tetanus and anti-diphtheria antibodies were chosen as markers for specific IgG since the majority of donors used in production of the IgG products will have received diphtheria and tetanus immunisation as part of routine immunisation programmes.

The results shown in Figure 5.11, at T=0 time point, confirm that diphtheria and tetanus antibodies were readily detectable in all formulations and, as expected, the antibody concentration increased with increasing total IgG concentration. The results at T=6 months show that there was no significant loss of diphtheria and tetanus binding activity for all concentrations (error bars representing ± 95% C.I). This corresponds with the relatively stable monomer activity (%) seen at 20°C and 45°C temperatures. At T=12 months, the data indicates that there was a loss in binding activity occurring for the concentrations at 50 and 100 mg/mL for the samples held at the high stress temperature (45°C). It is interesting to note that while the monomer content is decreasing throughout storage at elevated temperature, the binding activity remains relatively stable until the later time points. This might suggest it is a physical not immunological change occurring up until those time points and it is important to note that the specific antibodies being measured here represent only a small proportion of the total IgG in the formulation. For the 200 mg/mL samples, there was high variability in activity with difficulty in ascertaining any loss. One explanation for this variation could be due to fact that at the higher concentration, 200 mg/mL samples did not fully reconstitute (partially) thus giving variable ELISA results. The 10, 50 and 100 mg/mL IgG, had a clear visual appearance after being fully reconstituted. However the 200 mg/mL samples were slightly cloudy/viscous with tiny clumps of particulates still undissolved (Figure 5.13).

5.3.1.4 Reconstitution and Physical Properties

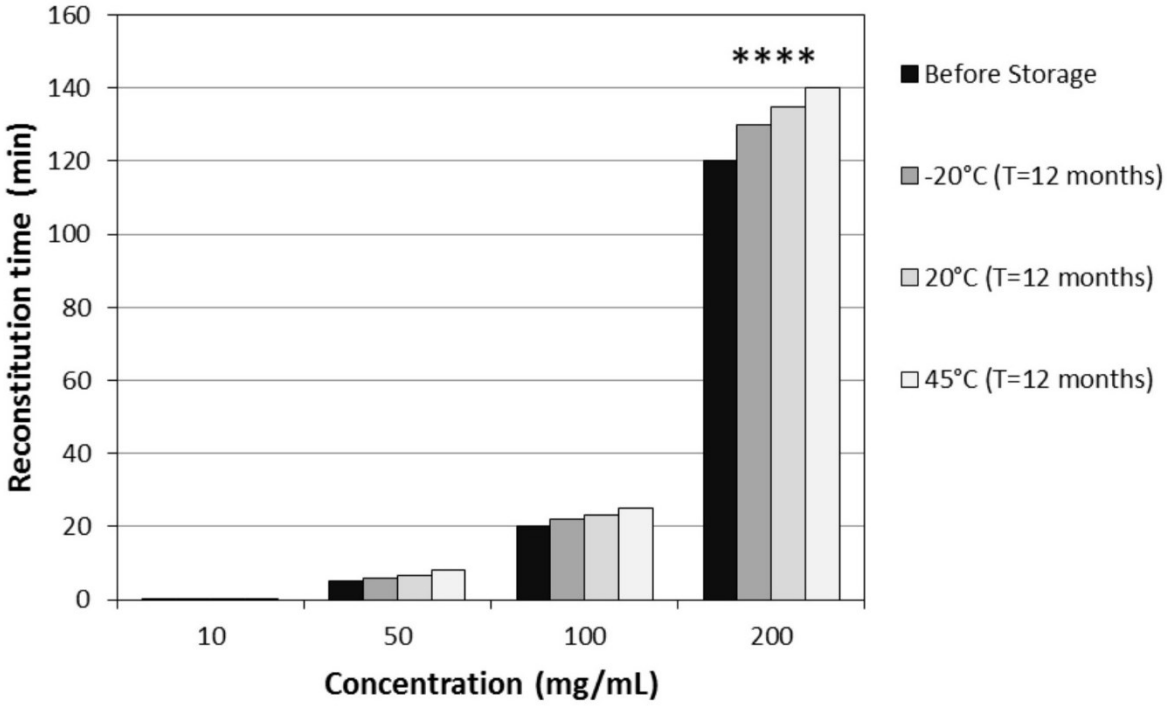


Figure 5.12 The reconstitution time (min) taken for FD IgG samples to fully dissolve with 1 mL of DI water. Symbols (*) denotes incomplete or partial reconstitution occurring.

Figure 5.12 shows the reconstitution times at before storage and after T=12 months for samples stored at -20°C, 20°C and 45°C. With increasing concentration, significantly longer times for reconstitution were observed even before storage. The time taken for samples to reconstitute at -20°C remains fairly constant from before storage to T=12 months' time with only slight increases for the higher concentrations at 100 and 200 mg/mL samples. Over 12 months the samples at 45°C were slower to reconstitute for all formulations. The reconstitution time can often be very subjective and there is no standardised methodology for determining at what point exactly full reconstitution has occurred. Although it is difficult to meaningfully correlate reconstitution time with stability, in general higher concentration samples that took

longer to reconstitute were also the samples that saw greater decreases in monomer or binding activity from pervious results.

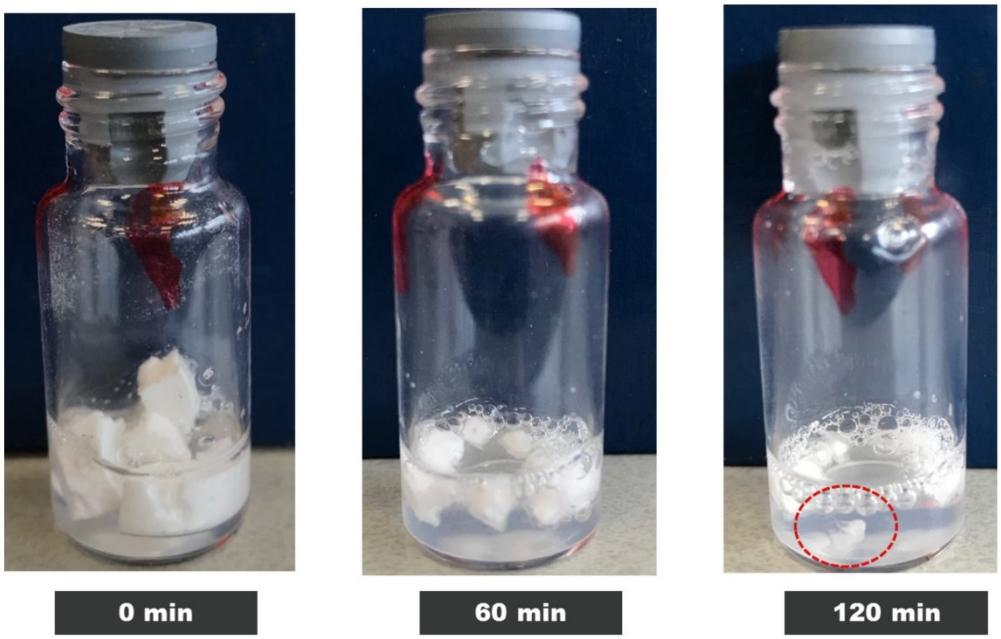


Figure 5.13 Visual appearance of IgG 200 mg/mL dissolved in water up to 2 hours (for the T=12 months sample).

Long reconstitution times have been commonly reported for high concentration protein solutions [29, 242]. Cao et al. [197] showed that the reconstitution time for high concentration protein formulations can be reduced by strategies such as adding wetting agents in diluents, introducing high annealing steps, and reconstituting under vacuum. It has been suggested that the lyophilisation cooling rate and not the annealing was what has the largest influence on the reconstitution time of a 50 mg/mL BSA sample [181]. Although, for the ultra-high concentrations of BSA at 200 mg/mL, altering the cooling profile (cooling at either 0.5 C/min, or quench cooling with liquid nitrogen with/without annealing) had no statistically different effect on reconstitution time. They also found that an increase in specific surface area of the formulation did not lower the reconstitution times, as also corroborated by other past studies

[197, 198]. Beech et al. [181] proposed that the difference in reconstitution time might be down to the lower concentration protein having an more open network of larger spherical pores. They also noted that one should consider the power-law relationships of fluids through porous networks and how there might be a cut-off point in pore size which reconstitution time is restricted.

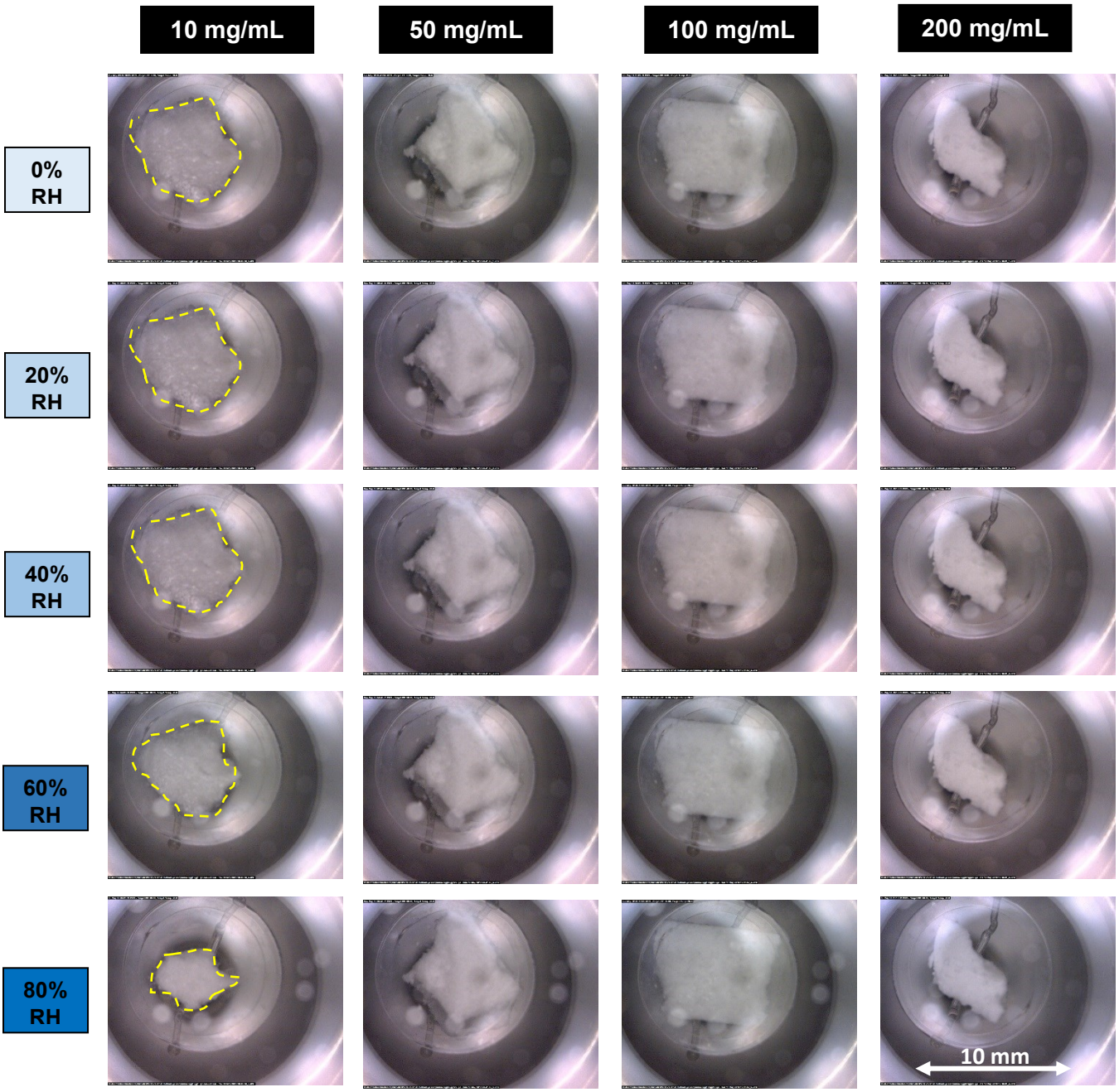


Figure 5.14 Images of FD IgG (10 - 200 mg/mL) samples at end of each RH% step (held for 2 hours) using DVS.

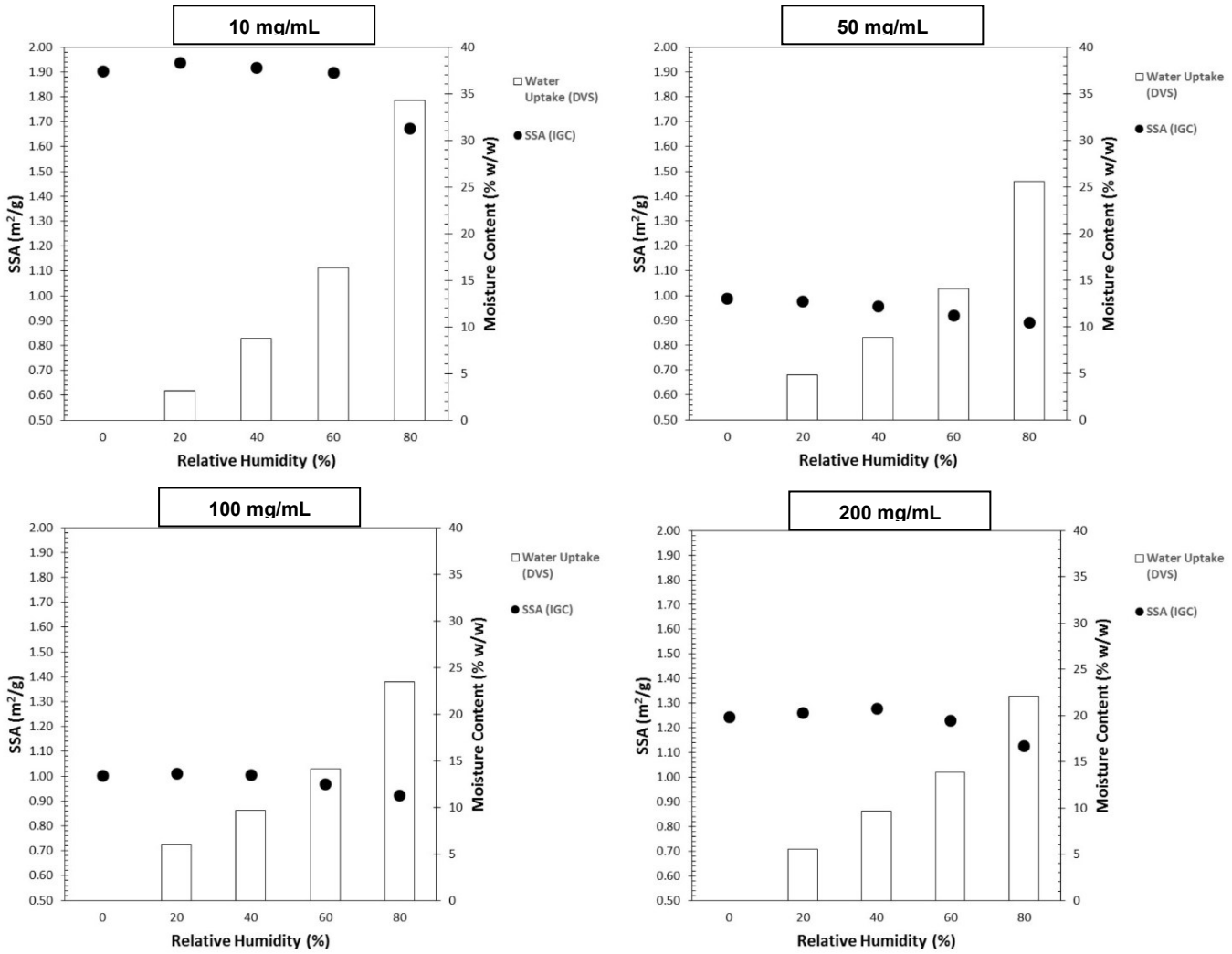


Figure 5.15 The change in SSA (m^2/g) of IgG at various RH% steps using IGC (column was conditioning and held for 2 hours at each RH % step). The sample conditioning was replicated on a DVS (held at each RH% step for 2 hours measuring change in mass % w/w).

The resistance and robustness to moisture induced changes for high concentration FD IgG cakes in regards to structural or SSA (m^2/g) changes are shown in Figures 5.14 and 5.15. Samples were conditioned at different RH% for a total of 2 hours before measuring the SSA with IGC or the moisture uptake change in mass % with DVS with accompanying images. Cake Collapse and shrinkage was only observed for 10 mg/mL samples only from between 60 - 80% RH (equivalent to 15 - 35 % w/w in moisture content). For ultra-high concentrations

of 50 – 200 mg/mL, no visible structural change was observed at all humidities (with uptake as high as 35% w/w in moisture content). All samples had very low specific surface area of less than 2 m^2/g. Although there was no link with increasing concentration and SSA for reconstitution problems suffered at these high concentrations.

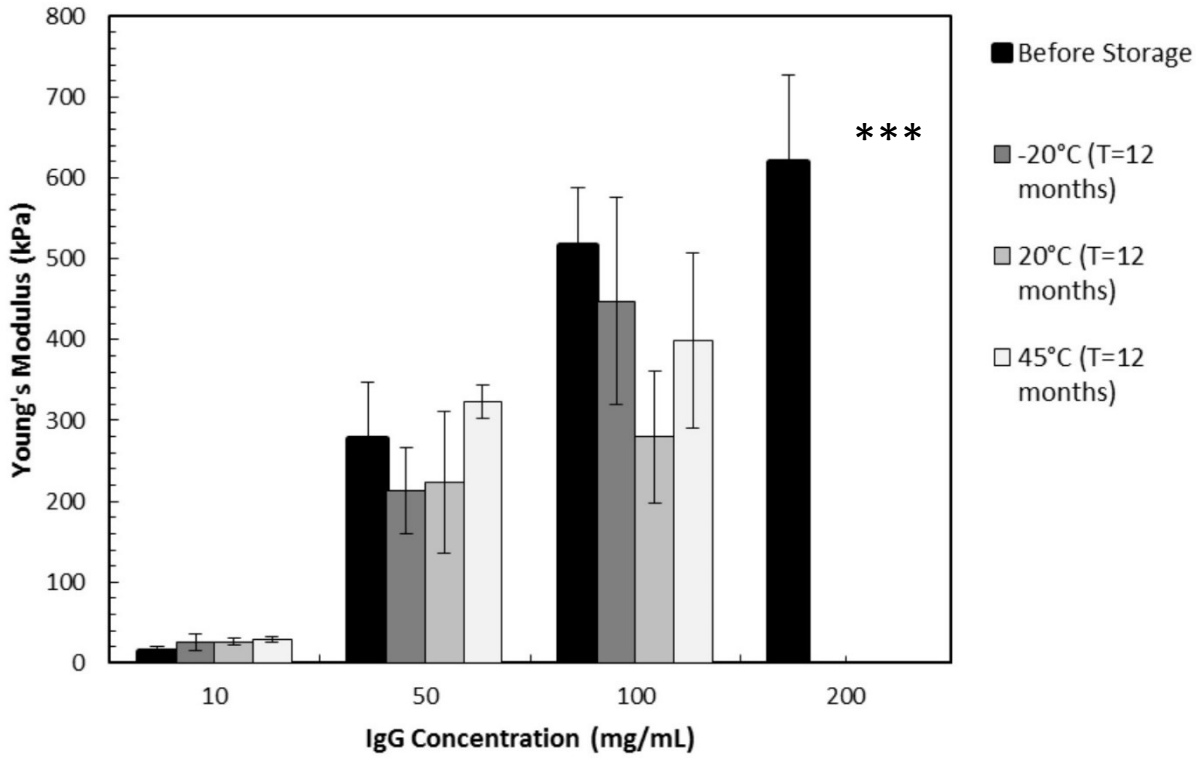


Figure 5.16 Young's Modulus (kPa) for low to high concentration FD IgG before and after 12 months storage at -20, 20 and 45°C. Error bars represent 95% confidence intervals (n=3). (*) denotes that indentation measurement unable to be taken due to extensive cake fracturing into pieces.

Figure 5.16 shows the mechanical properties in Young's modulus (kPa) before and after 12 months storage. With increasing concentration there was a corresponding increase in Young's modulus. From 100 and 200 mg/mL, the Young's modulus sees a small increase, indicting peak stiffness being reached past certain increase in concentration. The storage temperature (and subsequent small moisture ingress) seem to have minimal effect on the Young's modulus for all concentrations after 12 months, indicating the physical structural

robustness to these factors for such high concentration cakes. With increasing concentrations there was also an increase in the error bars. This is probably due to cracking in the cakes intensifying with increasing concentration, as also described in the previous Chapter.

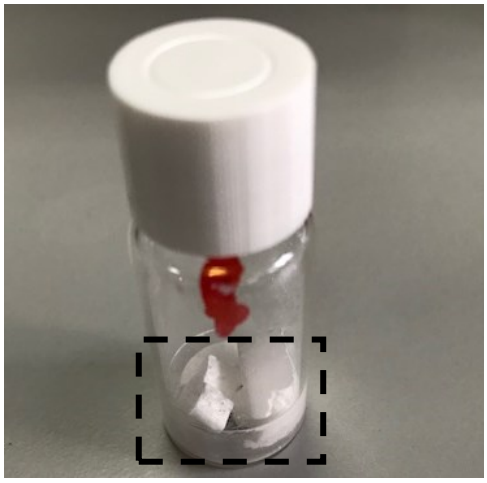


Figure 5.17 Typical FD IgG 200 mg/mL after 12 months storage. Brittle and very prone to fracturing into pieces.

The FD IgG samples (200 mg/mL) after 12 months had all been fractured into large pieces during the stresses of transportation between NIBSC and ICL inside the bags, highlighting the brittle nature of those samples. None of the other concentrations (from 10 - 100 mg/mL) experienced any cake fracturing into pieces in the vials during transportation. This indicates that ultra-high concertation are more prone to fracturing into pieces inside the vials as shown in Figure 5.17. Increasing API concentration often results in more shrinkage and cracking occurring. Those cracks if large enough can lead to eventual fracturing into pieces if enough stress is provided to vials. There also appeared to be no effect on reconstitution due to increased cracking of the cakes. When comparing the Young's modulus to the monomer data, the cakes with higher mechanical properties (and hence higher IgG concentration) seemed to correlate and suffer more in terms of monomer losses at 45°C than at -20°C or

20°C, indicating that physical properties might be related to stability and T_g whether directly or indirectly.

5.3.1.5 Summary

With higher FD IgG concentrations there are intensifications in cake robustness with increases in mechanical properties and decreases in SSA. However, the disadvantages are that reconstitution time gets significantly longer with ultra-high concentrations. Storage at -20°C showed that there was minimal monomeric loss (less than 1%) for IgG at all concentrations. Increasing temperature saw greater monomer drops – higher concentrations had more losses especially at 45°C. Higher concentration are usually expected to be more stable but suffered the greatest losses at elevated temperature – indicating possibly that maybe excipient too low (1% w/v sucrose) since the 10 mg/mL samples least losses. However, the moisture content was similar at 20°C and 45°C, yet only see biggest decrease at 45°C. Loss in binding activity was seen for 50 mg/mL and 100 mg/mL concentrations only after 12 months storage. No activity losses were observed for 10 mg/mL, while 200 mg/mL (20% w/w) has variable results due to partial reconstitution issues. The binding activity results for 200 mg/mL vials were variable and possibly unreliable throughout the whole 12 months most likely due to incomplete/partial reconstitution occurring. These results provides a case for both water replacement theory and vitrification hypothesis in terms of stabilisation. High concentrations IgG formulations > 50 mg/mL, have low chance of water induced cake structural changes occurring, as even with moisture content of up to 35% w/w there was no significant structural collapse or SSA change observed. Overall, moisture ingress during storage (from as high as 1 to 3.5% w/w) occurs for all FD IgG concentrations up to 200 mg/mL and needs to be taken into account, although for these high concentration proteins the moisture appeared to have minimal impact on the stability at -20°C and 20°C.

5.3.2 Study 2: Optimum Moisture Content of IgG (50 mg/mL)

An investigation further ascertaining the acceptable moisture limits for FD IgG (50 mg/mL) was conducted. Samples were made under a “low” moisture cycle and samples were then exposed to atmosphere at several different time intervals (57% RH at temperature of 20°C). A total of four significantly different moisture content batch vials ranging from “very low” to “moderately high moisture” cakes were made and assigned as letters A - D respectively (Figure 5.18). The moisture contents before storage were measured as (A) 0.05 ± 0.01 % w/w, (B) 0.67 ± 0.14 % w/w, (C) 3.02 ± 0.06 % w/w and (D) 4.95 ± 0.40 % w/w.

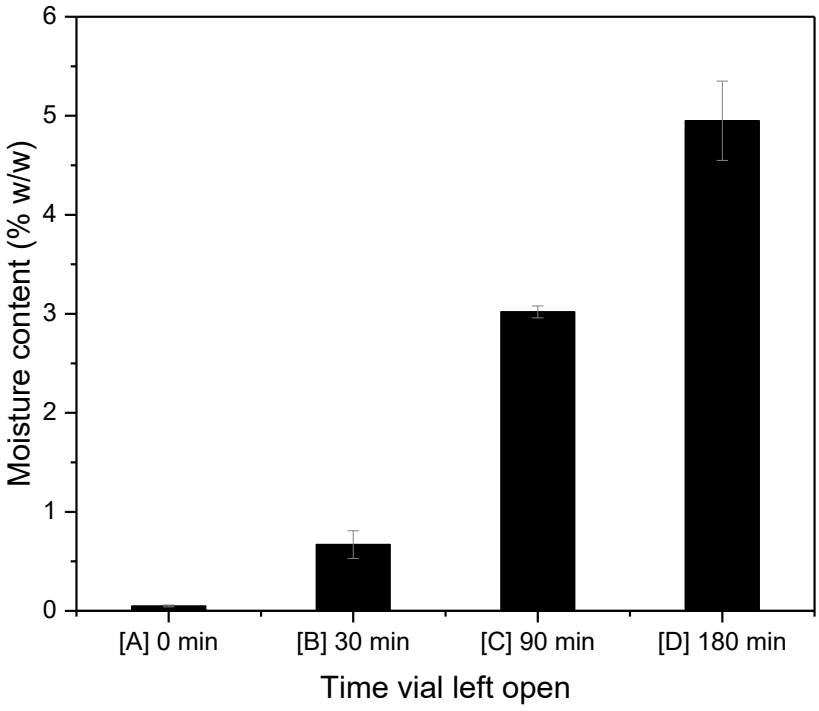


Figure 5.18 Average moisture content (% w/w) of IgG samples exposed to room environment (57% RH at 20°C) different time intervals (minutes). Samples are denoted as A - D to distinguish between the altered starting moisture contents resulting from exposure. Error bars represent the 95% confidence intervals (n=3).

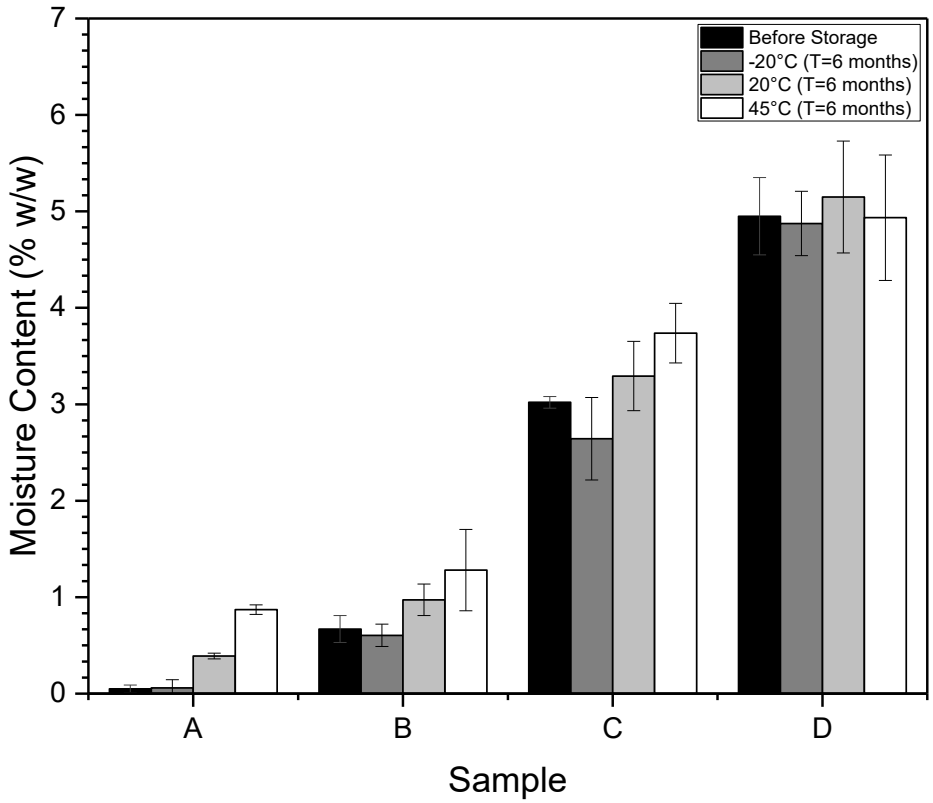


Figure 5.19 Change in moisture content (% w/w) after 6 months storage starting at different initial moisture samples of IgG (denoted as A - D) at -20°C, 20°C and 45°C. Error bars represent 95% confidence intervals (n=3).

Figure 5.19, shows the moisture content (% w/w) inside samples A - D over the course of 6 months storage at different temperatures of -20°C, 20°C and 45°C. For all vials (A - D), there was no change in moisture content after 6 months at -20°C compared to before storage. However for samples A, after 6 months storage at 20°C and 45°C there are significant increases in moisture observed. Samples B and C only saw slight increases at 45°C. In general the drier the sample started, then the more moisture it absorbed (e.g sample A having the largest increase at 20°C and 45°C). After 6 months storage at 20°C and 45°C, sample A vials had an increase in moisture of around 0.15% and 0.7% w/w respectively**.** For sample D vials (which began as the highest moisture cakes), there was no significant change in moisture content over 6 month's storage. Moisture had a significant effect on the mechanical properties of the materials. Figure 5.20, shows the change in mechanical properties in relation to moisture

content of the FD cakes. Before storage, the drier samples A and B (moisture content of 0.05% and 0.67 % w/w respectively) had larger Young's modulus compared to the higher moisture samples C and D (moisture content of >3% w/w). However, only samples A vials saw a significant decrease in Young's modulus over 6 months storage, but this is most likely due to change in moisture rather than of storage temperature. This is evident as well since, after 6 months storage, there was no significant change in mechanical properties for sample vials B - D at all temperatures. The initial moisture was the more influential factor on the mechanical properties than the storage temperature as also seen in the previously in Chapter 4. This indicates the importance of moisture for the physical mechanical properties of FD material.

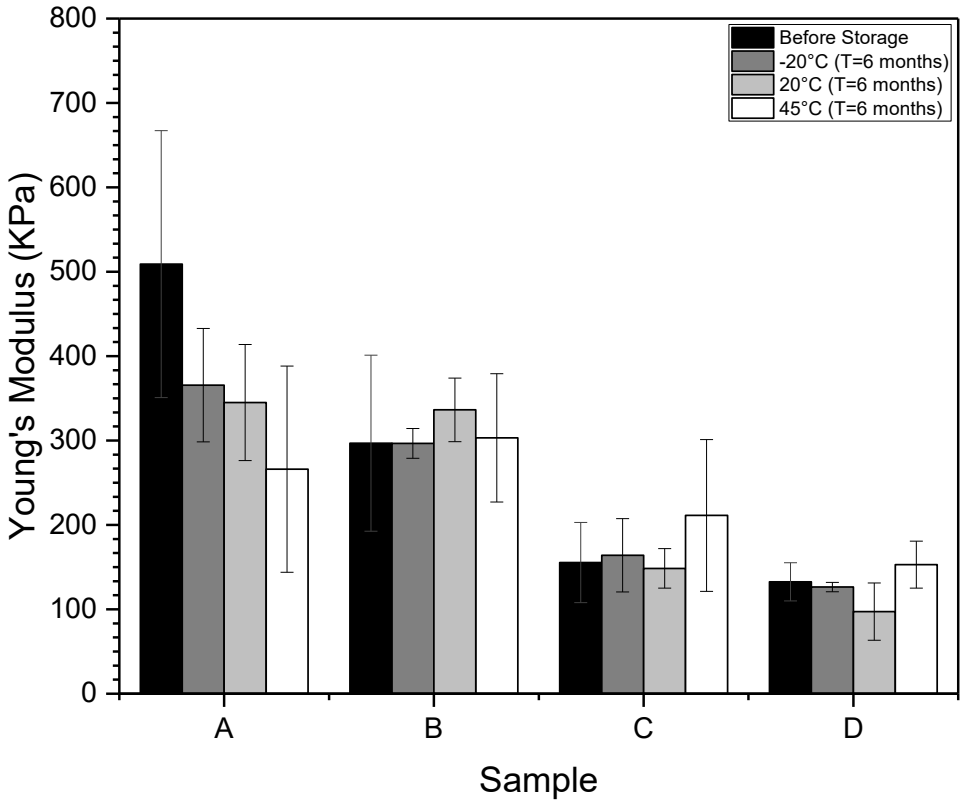


Figure 5.20 Change in Young's Modulus (kPa) before and after 6 months storage for different moisture content vials (denoted as A - D) at -20°C, 20°C and 45°C. Error bars represent 95% confidence intervals (n=3).

Qualitatively speaking, it was observed that the higher moisture samples > 3% w/w (C and D) physically felt much softer and rubbery than the lower moisture samples < 3% w/w (A and B). When trying to place FD contents with a scalpel into HPLC vials for Karl Fischer titration, the higher moisture samples were often difficult to break apart and had become "gum-like" like in texture, while the lower moisture vials were easier to break into powder form (Figure 5.21). FD IgG at high concentration can absorb large amounts of moisture with relatively little chance of collapse occurring. However, a decrease in Young's modulus and increase in caking/stickiness can occur as a result of ever increasing moisture. Caking and stickiness of amorphous materials can present problems in both food and pharmaceutical industries during production and during storage [249]. Stickiness refers to the tendency of the material to stick to other materials, while caking relates to the formation of permanent clumps. Caking can be effects by many factors including T_g, humidity, storage conditions and intrinsic properties of the material. The increasing tendency for the IgG samples to clump together with increasing moisture means a loss in a product characteristic as well as practical difficulties with some techniques such BET volumetric gas adsorption or IGC in attempting to fit samples in the columns/tubes to measure the SSA.

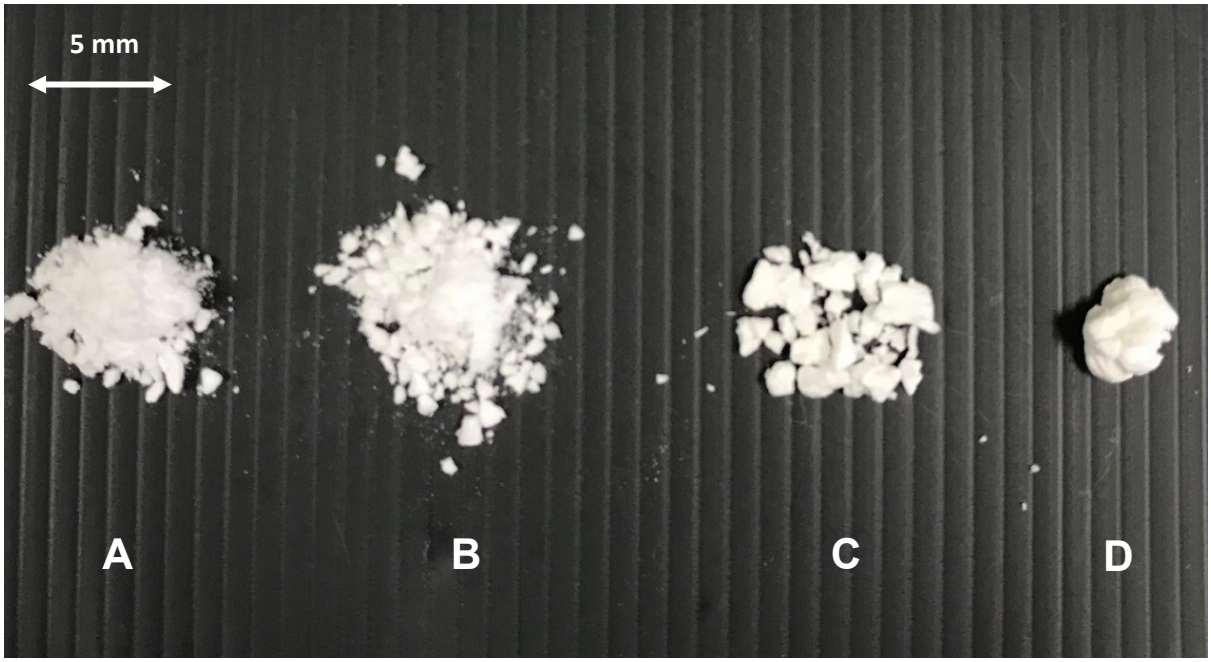


Figure 5.21 Increasing tendency of FD IgG material to clump together with increasing moisture content during extraction from vial.

5.3.2.1 Effect of moisture on visual appearance

A yellow discolouration had occurred on cakes with increasing higher moisture but only at the storage temperature of 45°C (Figure 5.22). The discolouration present in the higher moisture batch vials was probably due to Maillard browning (caused by protein free amino group reaction with sugars). Schüle et al. observed brown discoloration with lactose in their dried IgG1 stabilization study, which they attributed to Maillard reactions [126]. Kanojia et al., found that after accelerated thermal treatment a chemical modification had occurred in their dry sucrose-dextran IgG formulations which they too postulated was due to Maillard reaction products [127]. One explanation then might be that there was reducing sugar impurities present in the formulation. This study used sucrose in the formulation which is not a reducing sugar in itself. However, while sucrose is not a reducing sugar it does break down to fructose and glucose which are reducing sugars so this may explain why this discoloration was only observed for samples stored at higher temperature [131]. Matejtschuk et al., observed similar discolouration for formulations containing sucrose at high temperatures [130]. The presence of high moisture content further facilitates these reactions and results in a change in the appearance. It is also a possibility that at the formulated sucrose concentration, buffer crystallisation could have occurred during freezing thereby leading to a change to low pH at which sucrose could have broken down to fructose and glucose [250].

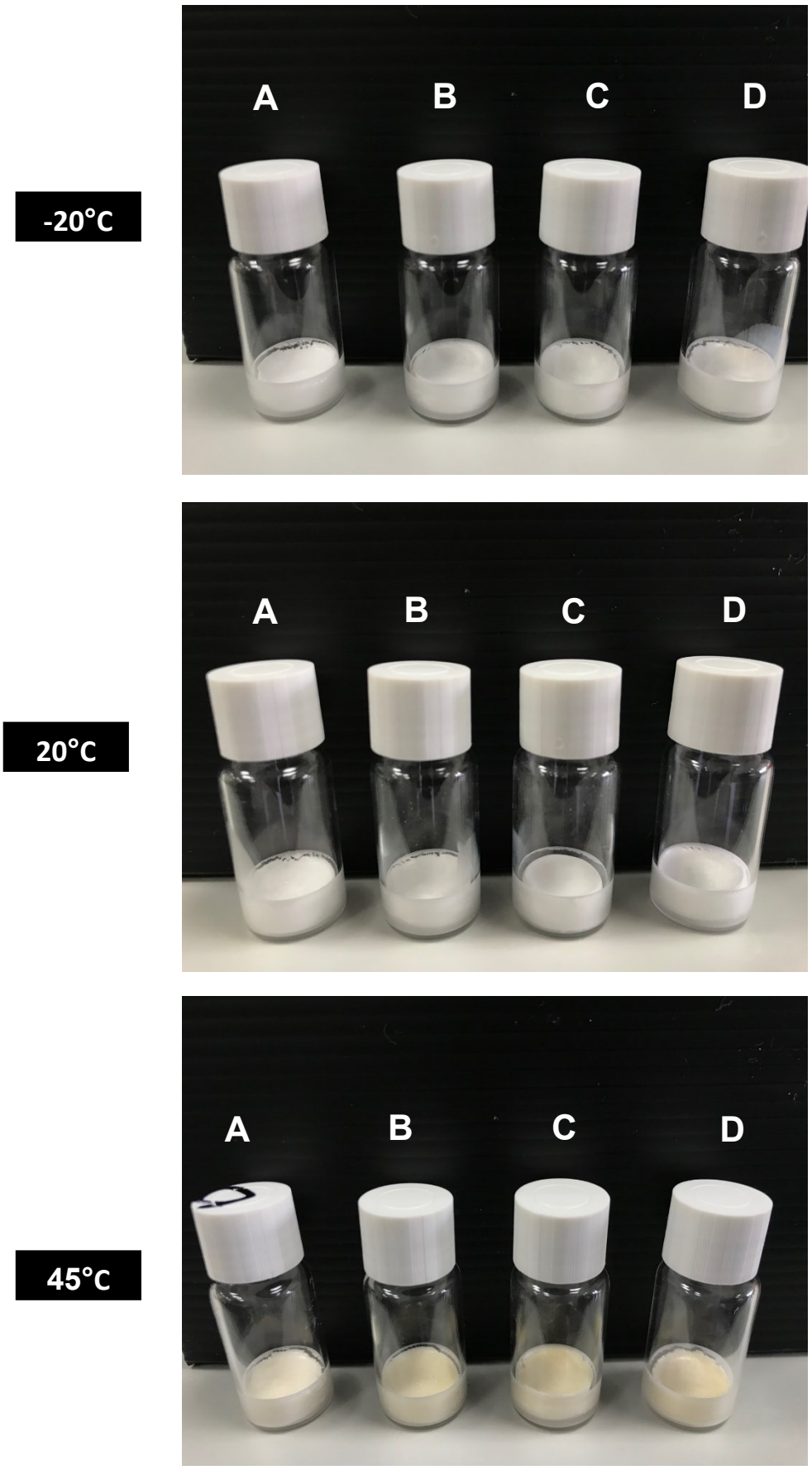


Figure 5.22 Visual appearance of cakes at different moisture contents after 6 months storage at 45°C.

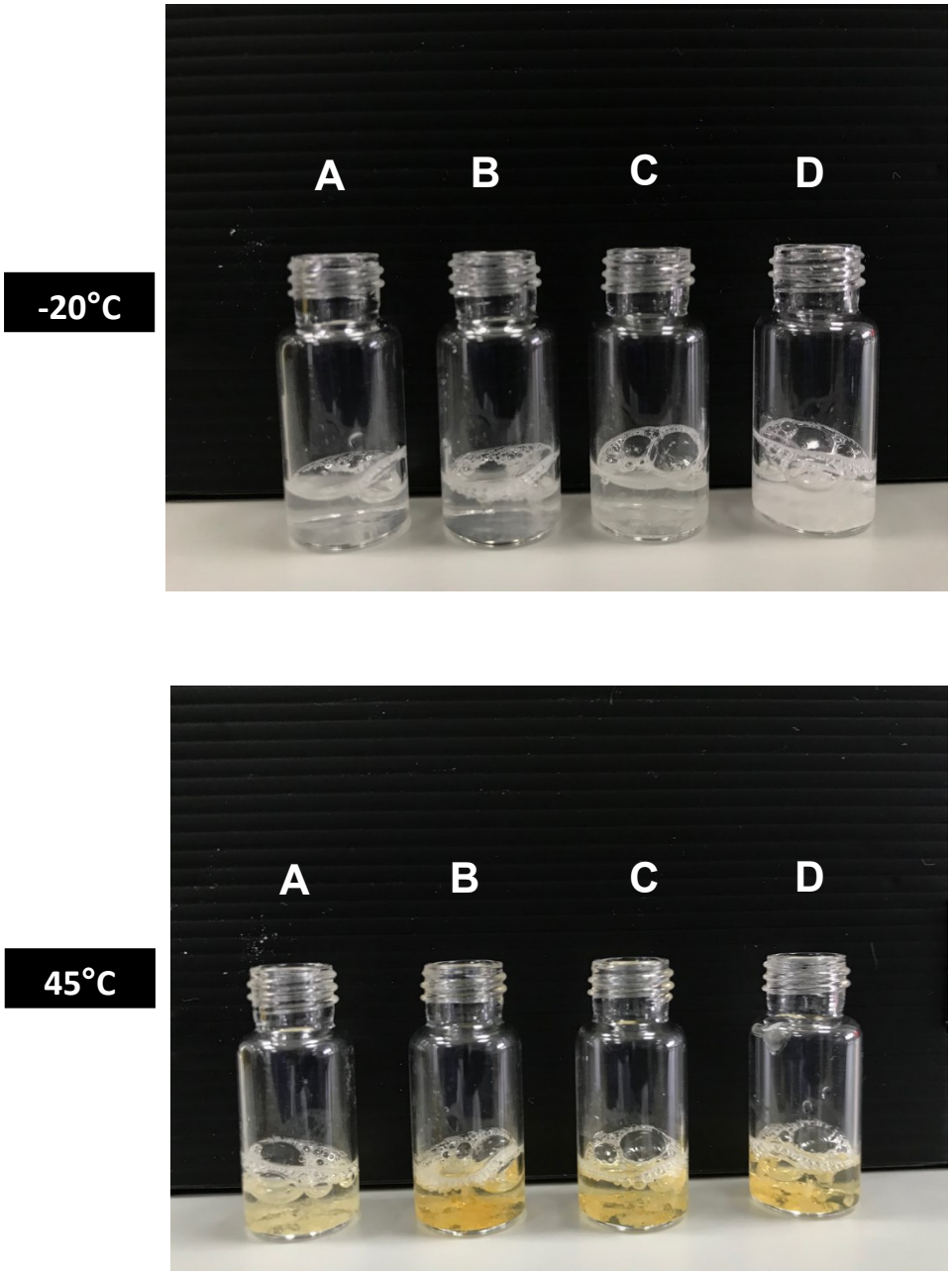


Figure 5.23 Visual appearance of different moisture contents vials, 2 minutes after reconstitution (T = 6 months).

Before storage, there was no significant difference in reconstitution time for all moisture samples (A - D) and all took an average of 5 minutes to fully dissolve. After 6 months storage, the reconstitution time remained unchanged and there was no significant difference for all samples at -20°C and 20°C. However at 45°C, samples took on average 1 minute longer than all the other samples to fully dissolve compared to the other storage temperatures (Figure 5.23). This corresponds with the discolouration and possible Maillard reaction occurring (protein amino acid group reacting with reducing sugars). The rate and colour change can also be influenced by the amount of water, as in general degradation rate due to Maillard reactions increases with moisture [128]. Although the formulations used sucrose, that in itself is not a reducing sugar, however at high temperatures it can possibly break down into fructose and glucose which are reducing sugars [130]. Samples stored at -20°C and 20°C saw no such discolouration, with only the samples at 45°C having a noticeable yellow discolouration (figure 5.23). At 45°C, sample A (with the last amount of moisture) had the least discolouration, whilst samples B - D had a clear yellow like appearance. The increased moisture might have facilitated greater rate of browning reactions and hence could also have had an effect on reconstitution time.

5.3.2.2 Effect of moisture on monomer

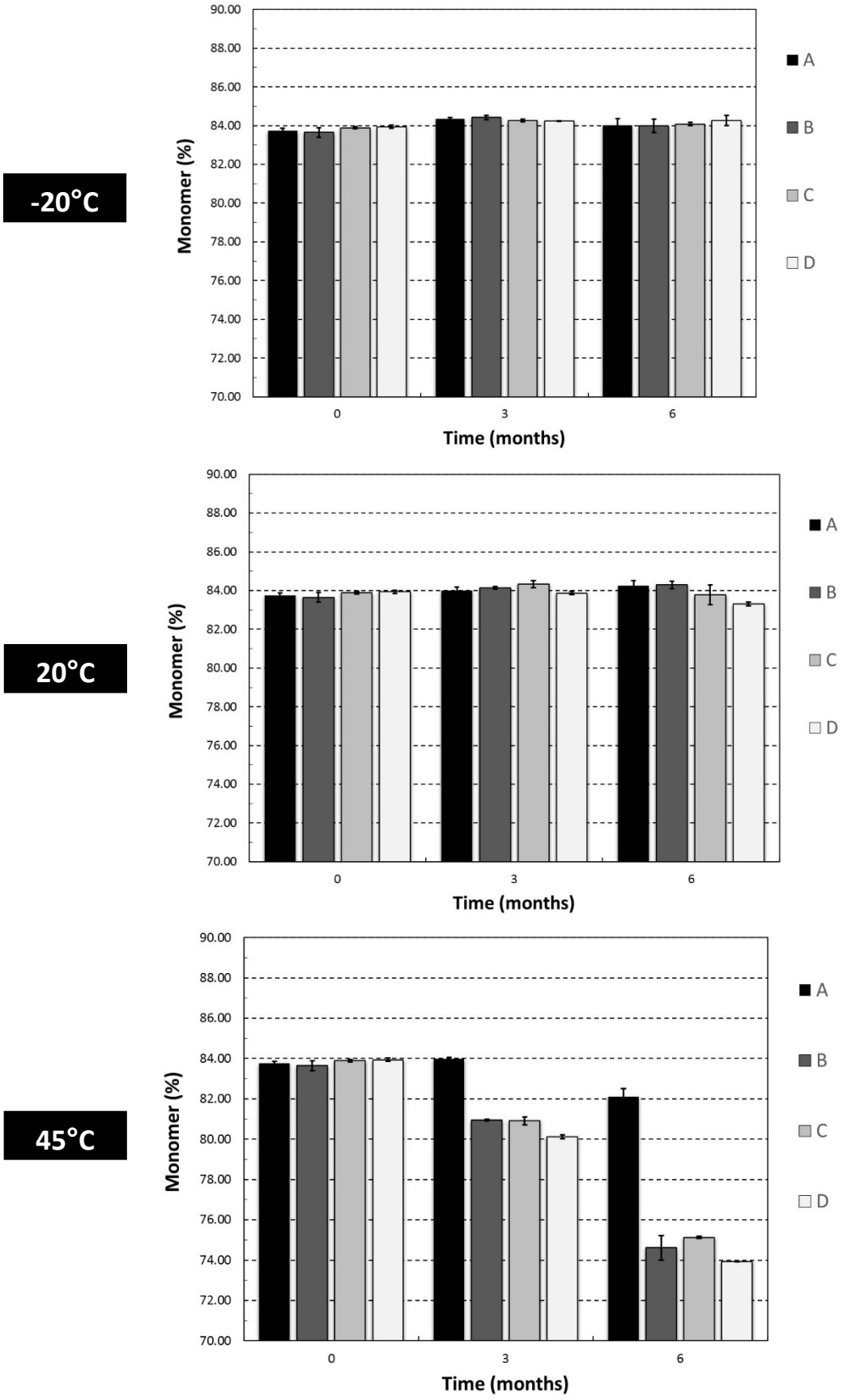


Figure 5.24 Change in monomer (%) after 6 months storage for different initial moisture samples of IgG (denoted as A - D) at -20°C, 20°C and 45°C. Error bars represent 95% confidence intervals (n=3).

5.3.2.3 Effect of moisture on Binding activity

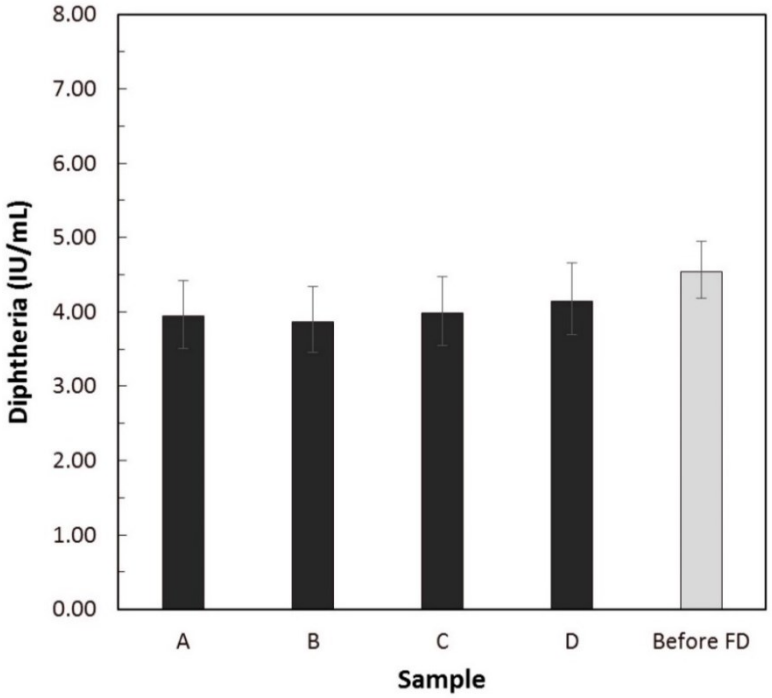


Figure 5.25 ELISA Diphtheria binding activity (IU/mL) for FD IgG at various moisture contents before storage. Grey bar shows the activity before FD in liquid state. Error bars represent ± 95% confidence.

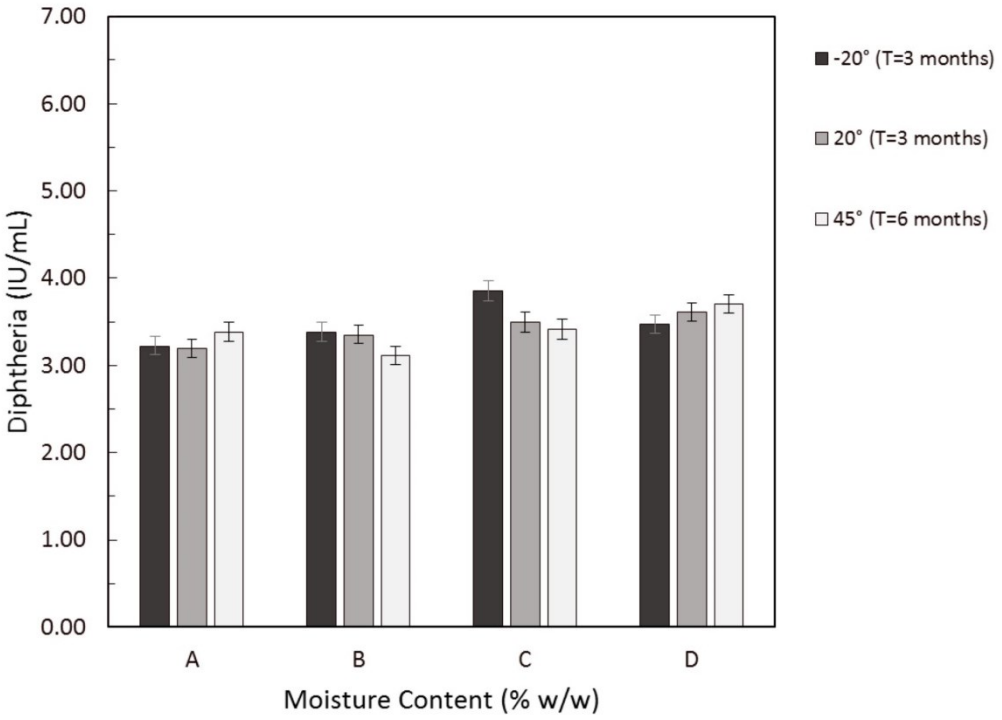


Figure 5.26 ELISA Diphtheria binding activity (IU/mL) after 6 months storage for different initial moisture samples of FD IgG (denoted as A - D) at -20°C, 20°C and 45°C. Error bars represent 95% confidence.

The monomer (%) for different moisture content samples are displayed in Figure 5.24. Thee monomer results suggested that moisture content (up till ≈ 3% w/w) had no effect on from storage at -20°C to 20°C over 6 months storage. It was only at storage temperature 45°C, that the high moisture samples saw a significant drop in monomer. One possible explanation for this is that the vials in this study were exposed to environment had oxygen trapped in headspace. Oxidation might have occurred in this study which would have induced pathways to lead to more protein-protein aggregation. Figure 5.25 and 5.26 shows the Diphtheria binding activity (IU/mL) results for IgG at various moisture content temperature before and after 6 months storage. The data before storage and after 6 months were not directly compared because after T=0 a new fresh aliquot of reference sample (10/262) was used for our ELISA tests. However, when looking at the Diphtheria binding activity (IU/mL) results at T=6 months only, there does not seem to be any significant difference for all temperatures (although there are some drops within error for sample B and C). The increasing moisture content also had no significant effect on the binding activity for all IgG storage conditions at T=6 months point, in contrast to monomer losses seen.

In a previous study by Breen et al., the effect of moisture content on lyophilised monoclonal antibodies was investigated. They found that higher moisture led to higher aggregation rates at storage above their T_g values. However when stored below T_g value, they found that stability was not compromised but might in fact even be enhanced [7]. The question of T_g and storage temperature is often a matter of debate. The results in this study showed that that only at elevated storage temperature does the high moisture content lead to losses in monomer. This would suggest further evidence for vitrification theory of stabilization since they all had the same sucrose content. The increased moisture content did not significantly reduce T_g but the increase in storage temperature reduced the (T_g-T). The gap is closer at higher storage temperatures than at lower storage temperatures (Table 5.3). The closer the two values are together then there might be increased molecular flexibility (which coupled with

oxygen ingress) could facilitate an increase in degradation processes and thus reduce in monomeric stability.

Table 5.3 Comparison of difference in storage temperature and glass transition temperature after 6 months storage.

Temperature (°C)	Sample	T_g-T (°C)
-20	A	135
	B	133
	C	133
	D	131
20	A	94
	B	91
	C	91
	D	90
45	A	67
	B	68
	C	65
	D	63

These monomer and binding activity results from the induced exposure moisture seemed to suggest that some moisture present (≈ 3% w/w) was perfectly acceptable for 50 mg/mL IgG up till 20°C. The question of how much moisture content in FD cakes is acceptable has long been a matter of debate. Hsu et al. described how an optimum moisture content range could exist, in which both over and under drying could be detrimental [2]. Others have postulated the existence of such optimum or acceptable moisture content ranges that could have steady stability. Greiff et al. have hypothesised that these moisture content could have a bell shaped distribution [86]. However, from the results in this study this was not observed, and a more linear correlation with moisture was observed for higher temperature. Similarly,

Pikal et al. noted that for FD Human Growth Hormone at 40°C, there was a linear relationship between moisture and aggregation rather than a bell shaped distribution [107].

5.3.2.4 Summary

Increasing the moisture content from up to 5% w/w in IgG (50 mg/mL) cakes results in more caking effect/stickiness and lowering of mechanical properties such as Young's modulus. However, samples with initially high moisture (upwards of 3% w/w) did not seem to exhibit any moisture ingress during storage compared to samples that started with low moisture's (< 3% w/w), which exhibited increased water ingress at or above room temperature storage. All samples (from low to high moisture) did not lose any significant monomer or binding activity at -20°C and 20°C storage over 6 months storage. It was only at elevated temperature of 45°C that samples with higher moisture saw the largest drops in monomer, however the binding activity remained unaffected. The lowering of the (T_g - T) gap meant increasing flexibility and degradation occurring at the higher temperature. Additionally some type of oxidation pathway might have had a role in reducing the monomer stability at higher temperatures due to the design of the experiment in exposing the vials to atmosphere. Overall, it was shown that there may be permissible moisture content (up to 5% w/w) from -20°C to room temperature without effecting either monomeric or binding activity of high concentration IgG (50 mg/mL).

5.3.3 Study 3: Stability of induced 'low' versus 'high' moisture FD Cycle

An alternative case study investigating the effects of moisture was set-up to monitor the stability between purposely prepared high and low moisture vials of IgG 50 mg/mL, but this difference in moisture content will be induced via the FD cycle. A "low" moisture FD cycle run produced cakes with an average moisture content of 0.11 ± 0.01 % w/w, and the "high" moisture cycle run produced cakes with an average moisture content of 3.02 ± 0.8 w/w %, measured at T=0 months (n=3). Previously it has been reported that much higher moisture contents (8% w/w) decreased the stability of a Mab's dried at 40mg/mL concentration [7]. However here, more representative moisture contents (0.1% w/w for low cycle and 3% w/w for high cycle) were engineered by control of the secondary drying temperature conditions. Figure 5.27 and 5.28, shows the moisture content change over 12 months storage period between vials that started with either made in low or high moisture cycles. Over the course of 12 months storage the high moisture cycle vials see no significant change and remain on similar moisture levels as they started (approximately ≈ 3 % w/w). For the low moisture run there is a significant change in moisture uptake at 20°C and 45°C from T=6 months point to about 1-1.5 % w/w moisture ingress but these then remain constant for the next 6 months. This may illustrate an equilibration of the moisture in the closure and the hygroscopic dry FD product. Similar equilibrating moisture effects in vials were seen by Matejtschuk et al. [84] in a model HSA formulation. Once again moisture increase over time are seen for vials at or above 20°C, especially for FD samples that initially have very low moisture content. Again, it was most likely that a significant portion of moisture ingress could be arising from the stoppers themselves.

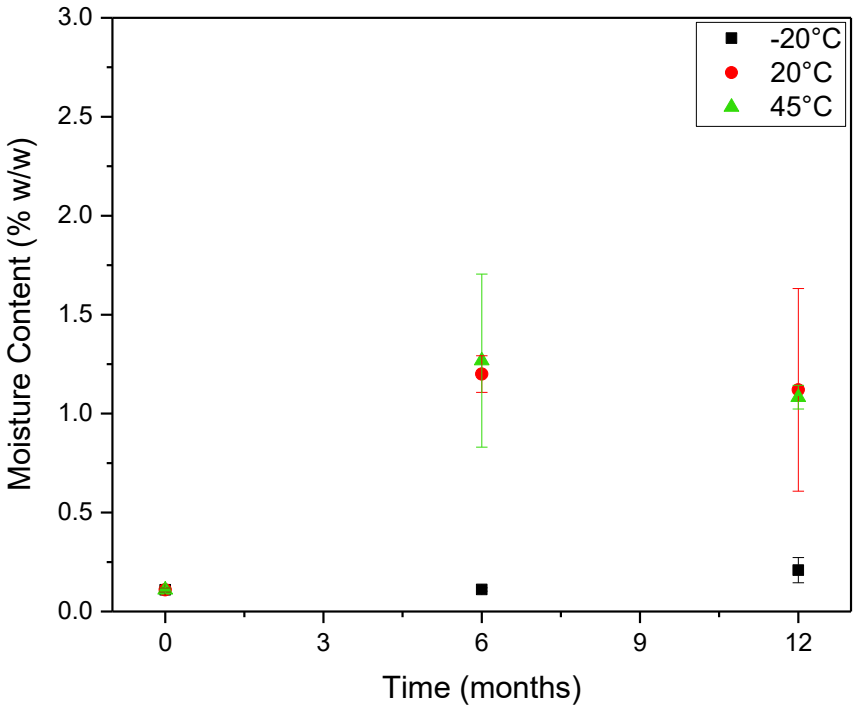


Figure 5.27 Average moisture content (%) for IgG 50 mg/mL FD in "low" moisture cycle recipe over 12 months storage at -20, 20 and 45°C. Error bars on bar chart represent 95% confidence intervals (n=3).

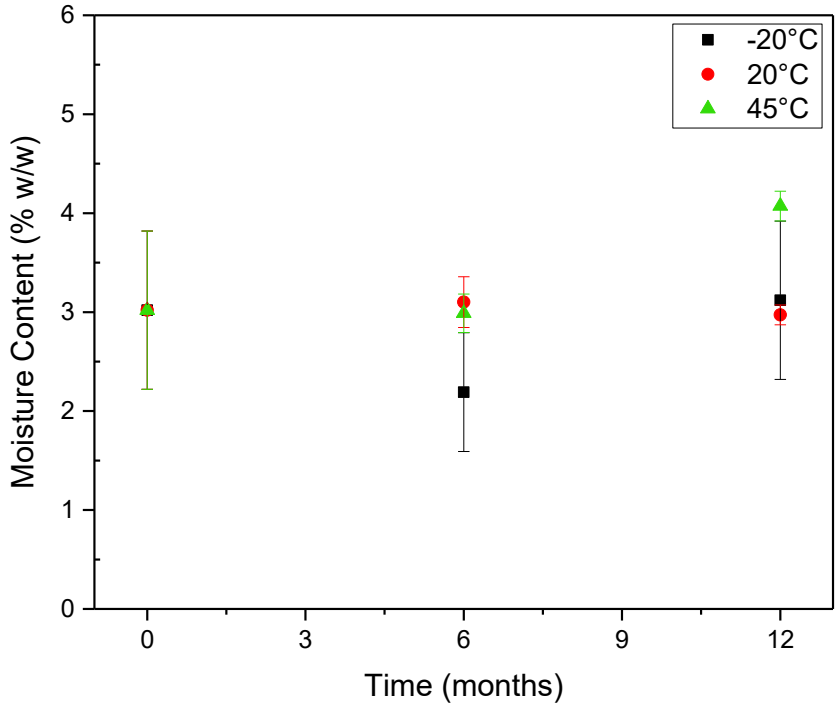


Figure 5.28 Average moisture content (%) for IgG 50 mg/mL FD in "high" moisture cycle recipe over 12 months storage at -20, 20 and 45°C. Error bars on bar chart represent 95% confidence intervals (n=3).

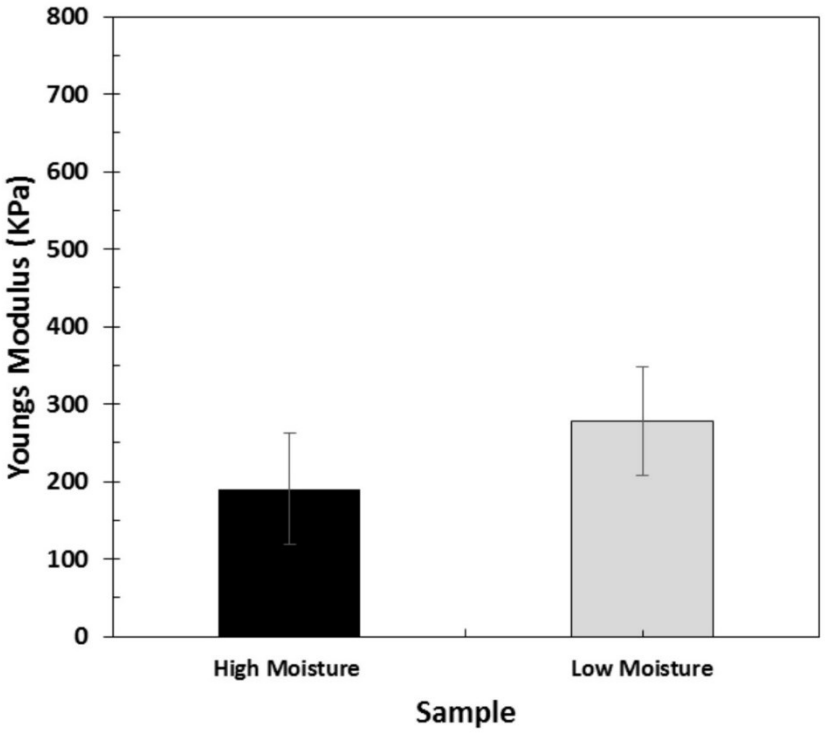


Figure 5.29 Comparison of mechanical properties of FD IgG 50 mg/mL FD in "high" moisture cycle recipe vs "low" moisture cycle recipe at T=0. Error bars on bar chart represent 95% confidence intervals (n=3).

The mechanical properties comparing the low and high cycle moisture cakes are shown in Figure 5.29. The high moisture vials on average had a difference of 33% in Young's modulus value compared to the lower moisture vials. However, the values were within the 95% Confidence Intervals, therefore no significant difference obtained between two moisture contents ($p>0.05$) The cycle chosen can influence the physical properties of FD material. The effect of moisture meant that the higher moisture cycle produced cakes with slightly softer and less stiff mechanical properties. This was also seen in the previous studies, whereby moisture past certain levels can begin to have a drastic impact on the physical properties of the cake and stickiness effects.

5.3.3.1 Monomer Content and binding activity

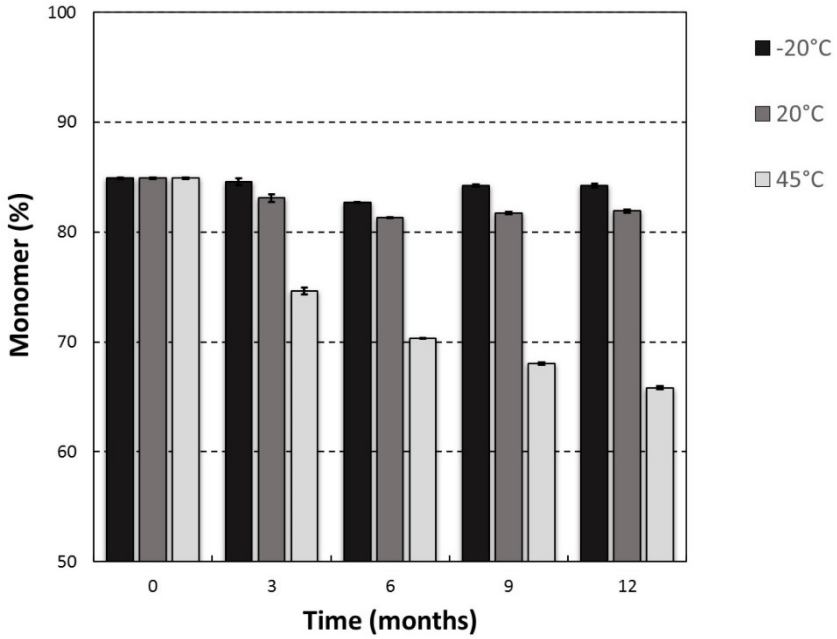


Figure 5.30 Change in monomer content (%) of FD IgG 50 mg/mL vials made under "low" moisture cycle over 12 months storage. Error bars represent 95% confidence intervals (n=3).

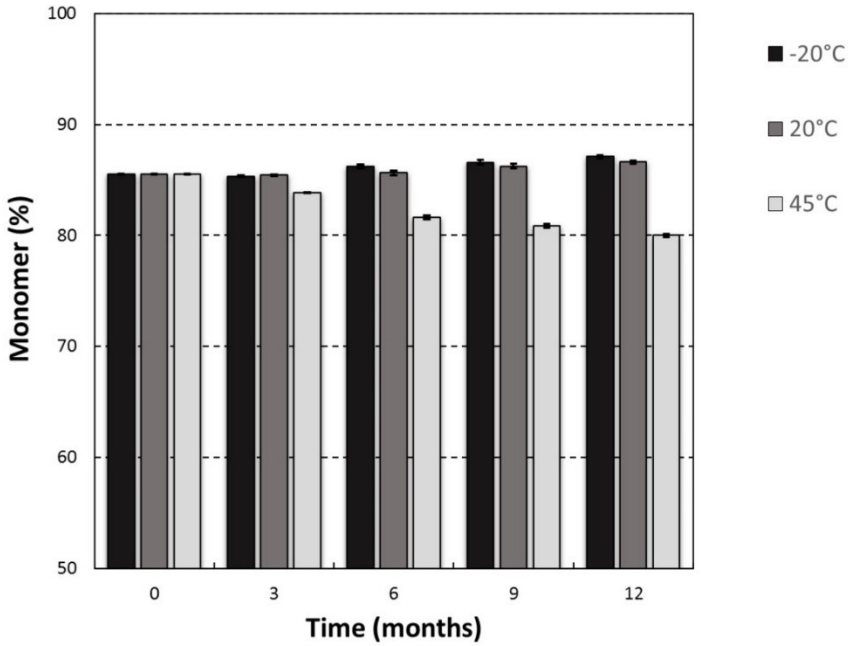


Figure 5.31 Change in monomer content (%) of FD IgG 50 mg/mL vials made under "high" moisture cycle over 12 months storage. Error bars represent 95% confidence intervals (n=3).

In Figures 5.30 and 5.31, the monomer (%) content is compared for IgG (5 % w/v) at both high and low moisture content stored at -20°C, 20°C and 45°C over a 12 month period. At -20°C and 20°C there appears to be a small drop in average monomer content from T=0 to T=3 months for the lower moisture vials (from 0.35% to 1.83% respectively). In the next 9 months the lower moisture vials continue losing monomer while the high moisture vials do not. At 45°C, there is large continuous drop in monomer (%) for the lower moisture content vials after T=3 months. Vials with higher moisture content only see a small loss in monomer over the 12 months compared to vials with lower moisture.

In the previous study it was shown that with higher moisture, larger monomer losses were observed than vials with low moisture at elevated temperature of 45°C. However in this trial, the high FD cycle vials with 3% w/w moisture had less drop in monomer the lower cycle moisture vials at 45°C. As stated previously, an explanation for this is that the vials in this study were stoppered under vacuum and nitrogen gas while in the other study the vials were exposed to environment and had oxygen trapped in headspace. Oxidation might have occurred in that case study, which would have induced pathways to lead to more protein-protein aggregation. This might also be further evidence for water replacement theory and some moisture being essential for the FD cake stability in allowing hydrogen bonds to form. Perhaps it might also be the case that no all moisture in cakes are equivalent and perhaps the way moisture is in the FD cake is distributed might make a difference (i.e whether moisture is induced via FD cycle or exposure). Residual moisture is controlled by shelf temperature in secondary drying. Exposure to controlled humidity using desiccants is well documented as an approach in the laboratory but not practical in manufacturing. Cycle induced moisture might provide different data results in regards to moisture content.

Studies from Wang [104], Liu et al.[251] and Greiff et al.[86] have all reported an increase in aggregation with higher moisture content (above the optimum level). Past studies have also shown that some acceptable moisture may be permissible in FD cakes without effecting stability. However, here is reported evidence to show that over drying can also have

a detrimental effect on stability. In another alternate study, Wang et al. demonstrated that with a different formulation, over-drying had greater loss in activity and increased aggregation. Over-drying of FD insulin has also showed to cause damage and increased product degradation [30]. Hubbard et al. showed that FD reference plasma with optimised formulation and a modest moisture content had better stability than one with very low moisture content [106]. These results shows some confirmation of the water replacement theory in action. When samples are over dried, the hydrogen bonds are lost between the protein and the water, hence you have a change in conformational structure. Some moisture may be crucial to maintaining, the stability of FD biologics. At higher storage temperatures, this effect might be even greater, hence the samples with higher moisture could have been less temperature labile.

5.3.3.2 Effect of moisture content in Binding activity

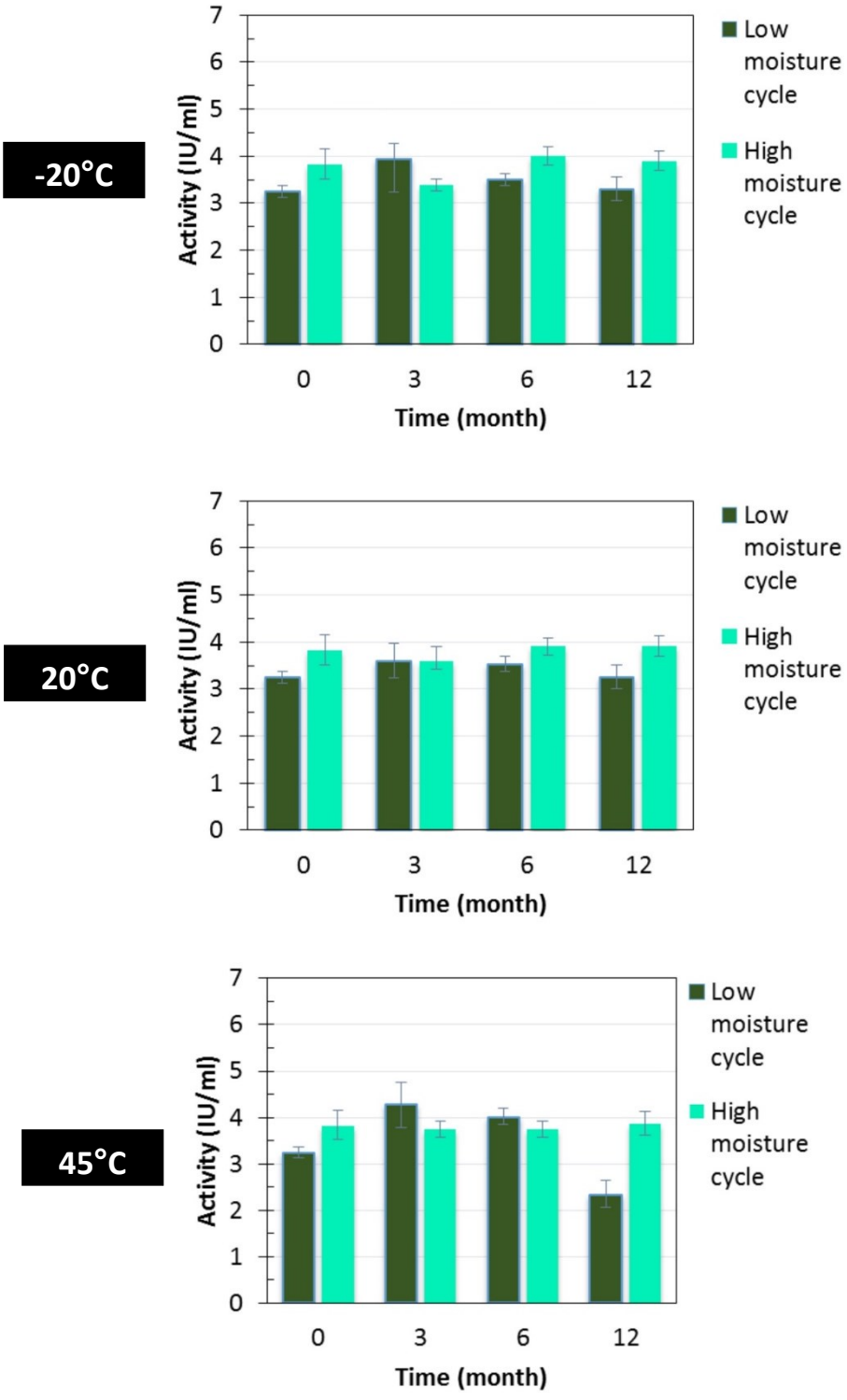


Figure 5.32 Diphtheria Binding Activity determined by ELISA for FD IgG 50 mg/mL in high and low moisture cycles batches over 12 months. Error bars represent 95% confidence intervals.

In Figure 5.32, ELISA binding activity data reveals that there is no significant difference between the high and low moisture vials at -20°C. However at 45°C there is a loss at T=12 months' time point. The drop in binding activity observed with the low moisture samples is consistent with the reduction in monomer % seen for the same sample. These results suggest that the high moisture (3% w/w) samples are as good as extremely low moisture samples (< 1% w/w) and might even have less monomer loss at elevated temperatures.

Of course when it comes to commercialisation, product appearance is a key factor and we did observe discolouration over time with the higher moisture samples (albeit at high temperature only). Upon visual inspection, there was no colour change between the high and low moisture vials at -20°C and 20°C for the entire storage duration. However at 45° storage, vials showed a yellow discolouration on cakes with increasing higher moisture (Figure 5.34).

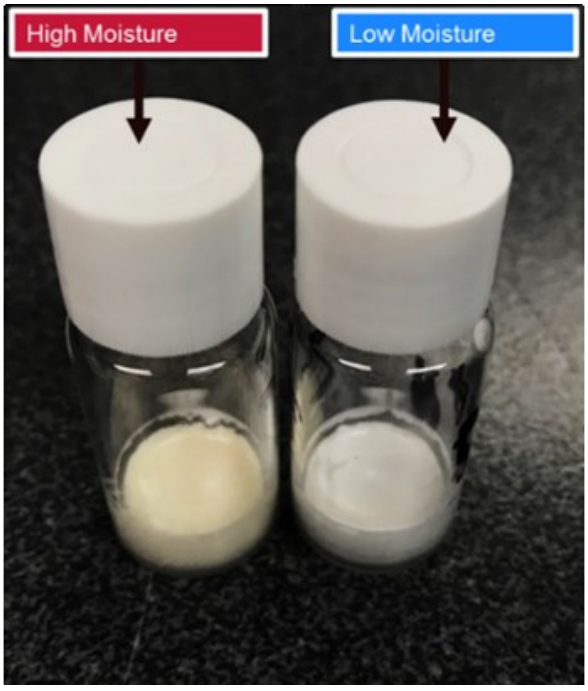


Figure 5.33 Comparison of the visual appearance between vials FD in either "low" or "high" moisture cycle for IgG 50 mg/mL after 12 months storage at 45°C.

Figure 5.33, once again shows yellow discoloration attributed to Maillard reaction only occurring at 45°C for the higher moisture samples, as also seen in the previous study. While the sucrose used in the formulation is not a reducing sugar, it still is the most likely to decompose. Although it may be possible to illustrate that a relatively higher moisture content is beneficial in terms of product stability, the risk of developing a yellowing appearance on storage would be a drawback for customers as most would be familiar with a white homogeneous product appearance. Additionally, since the change of appearance only occurred at high temperature it would be suitable to assume that is a product that requires low temperature storage.

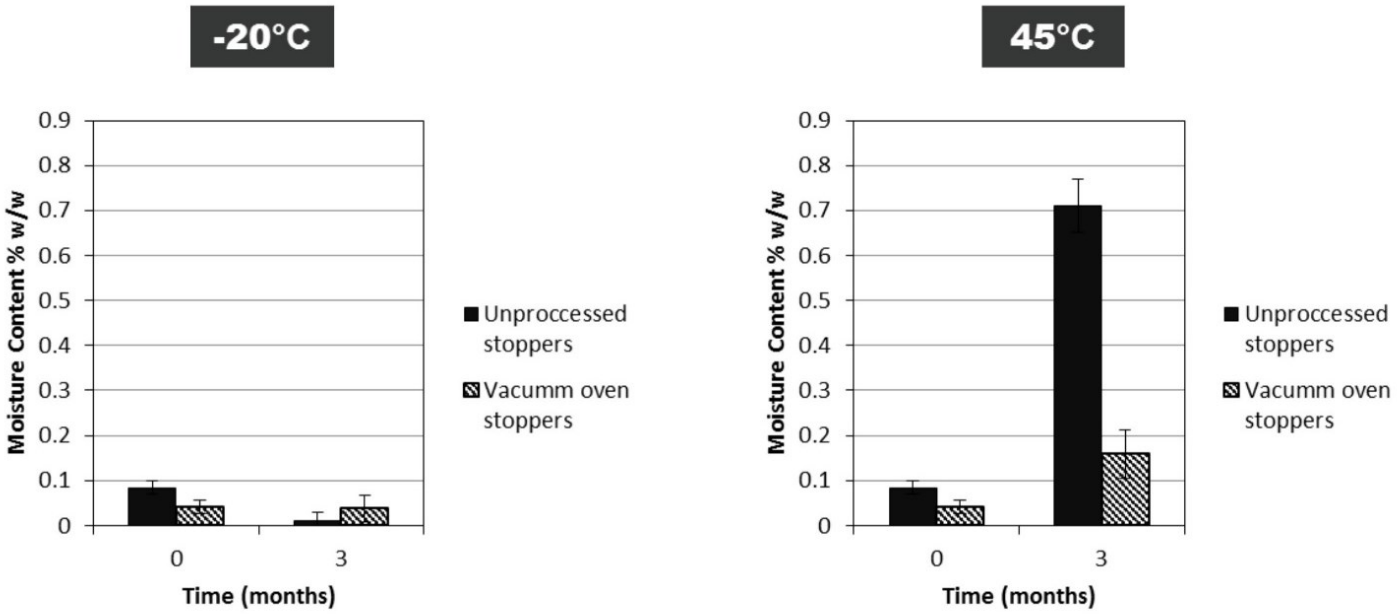


Figure 5.34 Difference in water uptake between vials with vacuum-oven dried stoppers and vials without vacuum oven dried stoppers. Error bars represent 95% confidence intervals (n=3).

It is therefore of much interest to maintain as low moisture as possible at least for elevated storage temperatures to reduce such product discolouration. Figure 5.34, shows the influence that the stopper in moisture content can have on the uptake of moisture during storage. Vials with stoppers that had been dried under a vacuum oven, significantly less moisture uptake than vials which did not at 45°C. Stopper drying has been effectively used in

the past to maintain low moisture content during storage, and is an underutilised procedure which can reduce chances of degradation at higher storage temperatures. However there would be concern whether the stoppers run smoothly on automated machinery, as baked stoppers might tend to be sticky and clump together and have unacceptable flow properties on the automated hoppers feeding such equipment.

5.3.3.3 Summary

Overall, the high moisture cycle vials (3% w/w) had similar stability compared to the lower moisture cycle vials (<1% w/w initially) at -20°C and 20°C over 12 month storage period. At 45°C, the 3 month time point, the higher moisture cycle vials were able to retain better monomeric content than the lower moisture vials. This further indicates evidence for water replacement theory and how some water present may be beneficial to retaining hydrogen bonding and stability. FD cycles that result in over-drying for some proteins could be detrimental to long term storage stability of high concentration IgG. However, whilst higher moisture content IgG samples might be acceptable and could provide greater stability compared to very low moisture vials, there might also be unwanted changes in visual appearance due to Maillard reaction occurring at high storage temperatures (facilitated by high moisture), which could damage commercial prospects. This may be resolved by changing formulation to that of a more stable non-reducing sugar excipient that does not break down to or maintaining slightly lower intermediate moisture contents.

5.4 Conclusion

Increasing protein concentration usually leads to more mechanically robust cakes but can cause issues with long reconstitution times. Freeze-drying can provide excellent long term stability for high concentration IgG proteins but needs to be considered in context with other factors such as such as moisture content and storage temperature. Cases for both water replacement theory and vitrification hypothesis have been found in these set of studies. There may be an optimum moisture content range for FD IgG, however this needs to be considered with context to how close the storage temperature is to the glass transition. From -20°C to 20°C moisture content up to 5% w/w does not seem to have any impact on monomer or binding activity during prolonged storage. It is only at elevated temperatures such as 45°C storage that high moisture seemed to effect the monomer for IgG 50 mg/mL although this might be because of oxygen ingress during exposure trial study. In a comparison between a “high” vs “low” FD cycle, the opposite was seen in that the higher moisture content vials performed better at 45°C than the extremely dry moisture samples. While some moisture content might be permissible and in fact even increase stability, there may be increased caking and clumping effects and also Maillard reactions occurring (discolouration), which could affect commercial prospects. Moisture ingress was observed to occur in every trial study and is especially higher at more elevated storage temperatures, although one potential way of counteracting this could be by vacuum heat treating rubber stoppers.

Chapter 6. Reducing Moisture Ingress from Stoppers during Long Term Storage of FD Influenza Antigens

6.1 Introduction

A sufficiently optimised freeze-drying cycle can result in producing very dry material with very little moisture content present. The primary drying stage removes most of the ice through sublimation, whilst the secondary drying stage removes most of the bound moisture present by desorption, to leave low moisture samples of usually less than 1% w/w. Current prevailing knowledge accepts that in some cases a negative association between high moisture content and long term storage stability exists. Past studies and research have indicated that higher moisture content in FD material is linked with a loss in biological activity and an increase in degradative chemical reactions, while other have stated that some moisture content may be permissible [86, 104, 251].

International reference material such as influenza (flu) antigens are of high importance for accuracy in assessment of the potency of materials with that of a known specificity. Reference materials are FD to provide long term storage stability and maintain viable potency over the prolonged periods of time. Single radial immunodiffusion (SRD) assay has been principally used since the 1970s to provide a tangible accurate way to measure the potency of inactivated influenza vaccines [252, 253]. The assay involves the use of standardised reagents such an antigen and antiserum reagent to provide accurate quantitative data for comparison. A slab of agarose containing antibodies is cut to make small wells. In these wells, serial dilutions of antigen or a known reference standard are added and precipitin rings are formed as the antigen diffuses into the gel. The diameter of the precipitin rings are measured and the unknown samples are compared with the reference standard to give a potency expressed in terms of micrograms of haemagglutinin (HA). SRD is widely used in virology research and as such is considered to be the gold standard method for potency determination. Alternative methods have been touted as alternative replacements with research focusing on

techniques such as SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and HPLC, however there are still issues in regard to suitability in terms of discriminating between degraded samples and active HA [254, 255].

Whilst, freeze-drying process can initially provide low moisture content samples, there is no guarantee that this will remain constant over time. During long term storage in vials with rubber stoppers, the moisture content of FD cakes can increase dramatically. For very low dry weight materials such as FD reference antigens, which are particularly hygroscopic and susceptible to moisture uptake, this can pose a high risk of problems including structural instability and loss of potency. WHO International Reference materials and seasonal typing reference materials such as influenza antigens are recommended to be stored in flame fused glass ampoules by the World Health Organisation in order to minimise the moisture and oxygen ingress into the vial space and ultimately the FD cake [65]. Flame sealed ampoules require adequate logistical space along the production lines and add to overall expenditure in energy costs. Research has shown that ampoules are able to reliably maintain low levels of moisture/oxygen and to minimise ingress over extended periods of storage at high and low temperatures. A previous study by Matejtschuk et al. concluded that the moisture or oxygen present when stored from -70°C to 56°C did not significantly differ for FD Albumin prepared in ampoules [208]. Standard FD vials with rubber stopper formulations are seen to be less reliable in terms of maintaining low moisture integrity over time.

A host of factors can influence how much moisture the FD cake sample absorbs from the stopper, which can include stopper processing treatment, stopper polymer formulation, stopper moisture transmission rate, the hygroscopicity of the sample, relative humidity and temperature storage conditions [69, 141, 202, 204, 256]. Moisture can come from either A) directly from the rubber stoppers themselves onto the FD cake, B) diffusion from the outside environment of vial storage or C) from micro-leaks from the rubber vial interface from improper sealing [68, 141, 142, 257].

This Chapter aims to investigate the role in which stopper moisture has effect on long term stability of low dry mass FD antigens. Vacuum oven-dried stoppers will be compared with untreated “straight from the pack” stoppers over a range of temperatures for a certain period of time. The moisture content and potency will be compared to those using ampoules which are considered to be the optimum gold standard storage format.

6.2 Materials and methods

6.2.1 Stoppers and Vacuum-oven Drying Treatment

Rubber stoppers were vacuum-oven dried for 24 hours with a Heraeus Vacutherm VT 6025 (Thermo Scientific, Loughborough, UK). The temperature was set to 120°C, while the pressure was kept to below 100 mBar. The temperature was selected as this was below the safe region before the T_g of rubber stoppers was crossed. The following different closure types were tested: 14 mm diameter stoppers (W1816 Bromobutyl grey, FDIA14WGBRTS, Adelphi Tubes, Haywards Heath, UK), 20mm diameter stoppers (4023/50 Bromobutyl grey, FDW20RTS, Adelphi Tubes, Haywards Heath, UK), 20 mm diameter stoppers (Flurotec D713 Butyl grey, FD20TBRtS, Adelphi Tubes, Haywards Heath, UK) and 13 mm diamter stoppers (Flurotec 4023/50 Bromobutyl grey, FD13TTB3WRS, Adelphi Tubes, Haywards Heath, UK).

6.2.2 Stability trials

6.2.2.1 Study 1: Influenza Antigen (A/Wisconsin/15/2009)

50 mL of antigen concentrate from bulk A: /Wisconsin/15/2009 x-183 FA376743VED, 190 µg HA/ml containing ~ 1.5% w/v sucrose (NIBSC, Potters Bar, UK) was added aseptically in laminar hood cupboard into 150 ml of PBS (to give a ratio of 1:3 and final overall concentration near 50 µg HA/ml). 1.4 g of Sucrose (Adelphi, UK) was added to the final mixture to dilute it down to ~ 1.1% w/v sucrose in PBS. Weights were measured using an analytical balance (Mettler AE50, Mettler Toledo, Leicester, UK) for all sets of experiments. Immediately afterwards, the vials were labelled, capped and placed inside sealed plastic bags and stored in temperature controlled incubators at either -20°C, 37°C and 45°C until further testing at each set time point.

6.2.2.2 Study 2: B/Phuket 14/252 antigen

Influenza antigen reagent 14/252 was sourced from a formalin-inactivated, partially purified B/Phuket/3073/2013 egg-derived virus, which was approximately 60 µg/ml HA antigen suspended in PBSA buffer containing 1% or 20 % w/v sucrose (NIBSC, Potters Bar, UK). The total fill volume for all vials was 1 mL, achieved using an automated multipette stream (Eppendorf, UK). 5 mL DIN glass ampoules (83 X 14.75 mm i.d. Adelphi tubes) were fitted with 13 mm diameter igloo stoppers (FDW13WRS, Adelphi Tubes, Haywards Heath, UK). Half of the 5 ml volume screw capped vials (41.5 X 18 mm i.d. Schott VC005. Adelphi Tubes) were loaded with 14 mm diameter cruciform rubber stoppers (FDIA14WGBRTS, Adelphi Tubes) used as received. The other half of the vials were loaded with stoppers that had been vacuum-oven dried. Immediately afterwards, the vials were labelled, capped and placed inside sealed plastic bags and stored at temperature-controlled incubators at either -20°C, 20°C and 45°C until further testing at each set time point.

6.2.3 Lyophilisation cycle

The freeze-drying cycles (Table 6.1 and 6.2) was run on the VirTis Genesis 25EL (SP Scientific, Biopharma Process Systems Ltd, Winchester, UK). Ampoules were loaded on the top shelf and 5 mL screw capped vials were loaded on the second shelf. Upon cycle completion, the ampoules and vials were backfilled with dry nitrogen gas to atmospheric pressure and stoppered in the dryer. Ampoules were flame sealed (stoppered end discarded) and all 5 mL vials were screw capped with an over sealing cap (Adelphi, Haywards Heath, UK).

Table 6.1 Freeze drying cycle for Influenza Antigen (A/Wisconsin/15/2009). Vials were stoppered under normal atmospheric pressure.

Freezing Temperature (°C)	Freezing Ramp Rate (°C/min)	Freezing Hold Time (min)	Primary Drying Ramp Rate (°C/min)	Primary Drying Temperature (°C)	Primary Hold Time (min)	Secondary Drying Ramp Rate (°C/min)	Secondary Drying Temperature (°C)	Secondary Hold Time (min)
-50	0.78	240	0.2	-40	1200	0.09	15	560

Table 6.2 Freeze drying cycle for B/Phuket Antigen. Vials were stoppered under normal atmospheric pressure.

Freezing Temperature (°C)	Freezing Ramp Rate (°C/min)	Freezing Hold Time (min)	Primary Drying Ramp Rate (°C/min)	Primary Drying Temperature (°C)	Primary Hold Time (min)	Secondary Drying Ramp Rate (°C/min)	Secondary Drying Temperature (°C)	Secondary Hold Time (min)
-50	0.45	240	0.30	-35	2400	0.07	25	1200

6.2.4 Residual moisture content of stoppers

The moisture content in rubber stoppers can fluctuate depending on time and storage conditions. Rubber stoppers were taken straight from manufacturer's packs and vacuum oven-dried to remove any moisture present. The moisture content of the rubber stoppers before and after was measured using a VA-100 Water vaporiser (Mitsubishi, A1-Environsciences Ltd, Blyth, UK) in triplicate (n=3). The stoppers were not cut into pieces but inserted as a whole into sample chamber. The instrument is based on Karl Fischer titration, with a titration cell volume reagents containing 150 mL of AX solution and two ampoules of CXU cathode solution. The VA-100 vaporiser consists of a heating tube in which the temperature is set (in this case 170°C). When the sample is ready to be tested, it is placed into the heating tube. As the moisture is released, the vapour is transported by a carrier gas (usually dry Nitrogen) to the titration cell where it is bubbled into the KF reagent (Figure 6.1). AQUAMICRON Solid water standard (Mitsubishi, A1-Environsciences Ltd, Blyth, UK) at 3.81% w/w was used to ensure accuracy and calibration at the start of each run.

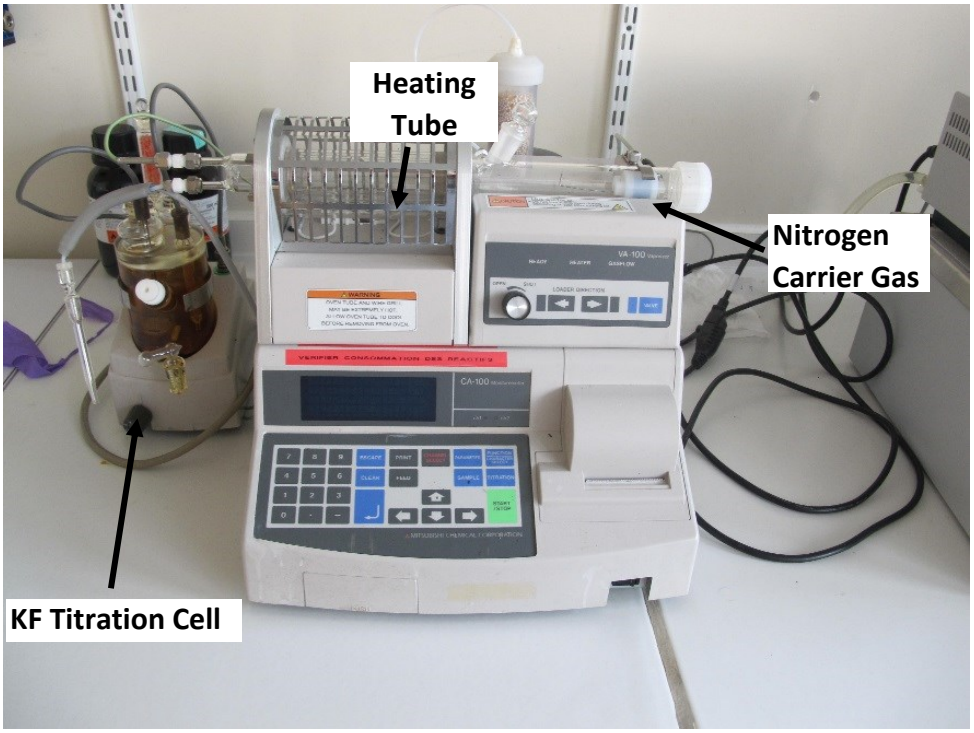


Figure 6.1 VA-100 Water vaporiser Karl Fischer oven used to measure moisture content of rubber stoppers.

6.2.5 Single Radial Immunodiffusion (SRD) Assay

SRD assay was used to measure the potency of the antigens as it is considered to be gold standard method with a high degree of reliability (Figure 6.2). The assays were conducted according to The National Institute for Biological Standards and Control (NIBSC), Division of Virology, operation procedure in accordance previously-described studies [258]. HA antiserum reference reagent for B/Phuket (Ref: 15/150, NIBSC, Potters Bar, UK) or Influenza A/Wisconsin/15/2009 (Ref: 10/182, NIBSC, Potters Bar, UK) was mixed with melted agarose (Reagent : Agarose, 35µL:1mL or 12µL:1mL, respectively) and poured in perspex moulds (90 mm circular or 103 x 103 mm). Once solidified, 4mm circular diameter wells were cut in the gel. FD test samples and antigen reagent standards for B/Phuket (Ref: 16/158, NIBSC, Potters Bar, UK) or for Influenza A/Wisconsin/15/2009 (Ref: 09/310, NIBSC, Potters Bar, UK) were reconstituted in ultra-pure water. Reconstituted samples (50µL) were treated and mixed with 10% w/v Zwittergent 3-14 detergent (450µL) (Catalogue number: 693017, Millipore, UK) into new tubes in duplicate. They were then left to incubate for 30 min at room temperature. Detergent-treated samples were then diluted with six salt phosphate-buffered saline on a dilution factor of 1.0 (200µL:0µL), 0.75 (150µL:50µL), 0.5 (100µL:100µL) and 0.25 (50µL:150µL). After mixing, a total of 20 µL of each sample or standard, were added to a well location in agar slab corresponding to number on the randomization scheme. The plates were closed with lids stored in a cooled incubator for 18 hours at 20°C. The following day, the plates were rinsed with water at the tap and filter paper was carefully placed over the top to remove any air bubbles. They were then pressed down with a 600g weight sandwiched between tissue paper for 30min. Plates were then dried with a fan and the filter paper peeled off before staining for 15 min with 0.3% (w/v) Coomassie brilliant blue R-250 stain. Plates were then dipped in a destain mixture (methanol, distilled water and acetic acid ratio 5:5:1) until the zones could be distinguished from the background. The zone diameters on the plate were measured with a Synoptics Image Analyser using the ProtoCol software (Synbiosis, Cambridge, UK). EDQM

CombiStats package software (version 5.0, 2013, EDQM, Strasbourg, France) was used for data analysis.

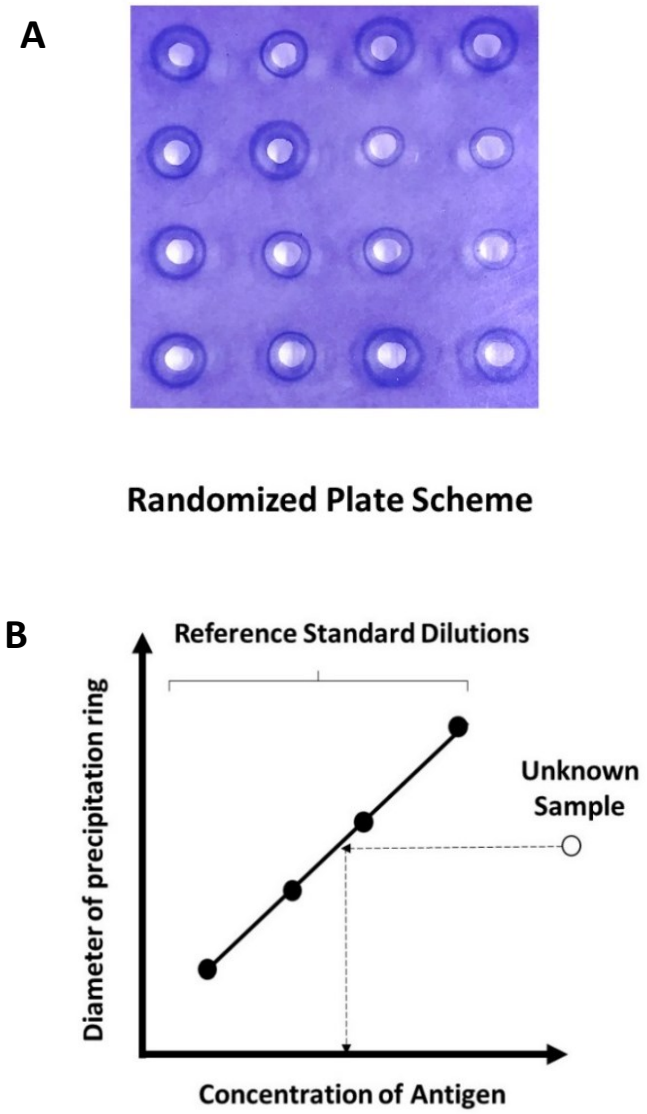


Figure 6.2 Schematic representation of single radial immunodiffusion (SRD) assay. **A:** Agarose gel slab with precipitin rings formed and **B:** Calibration curve of known standard with unknown sample diameter ring.

6.2.6 Residual moisture content of FD samples

Same method as previously described in Chapter 4

6.2.7 Modulated DSC

Same method as previously described in Chapter 5

6.2.8 DVS Sorption Collapse

Same method as previously described in Chapter 3

6.2.9 Mechanical Properties

Same method as previously described in Chapter 4

6.2.10 Specific Surface Area

Same method a previously described in Chapter 4

6.2.11 Statistical Analysis

95% confidence interval analysis same as previously described in Chapter 3, section 3.2.6.

6.3 Results and Discussion

6.3.1 Effect of Vacuum-oven drying

The rubber stoppers that come directly straight out of the pre-packaged bags from the manufacturer/supplier will have some moisture present. Usually these stoppers have had a host of pre-treatment such as steam sterilization for compatibility with aseptic applications. After transportation, these packs are usually placed in storage rooms at a facility awaiting until use for freeze-drying. Depending on the storage conditions and humidity of the rooms these stoppers might absorb even more water and increase in moisture content over time. The pre-treatment of rubber stoppers has been well known to remove moisture content. A selection of varying processing and drying treatments have been deployed in past studies in order to minimise the risk of moisture being present from the start of storage. These have included heat drying and steam sterilisation. Donovan et al. showed how stoppers (designated as either low or high moisture uptakers), absorbed large amounts of moisture during steam sterilization and that drying between 2-8 hours resulted in less than 0.5 mg/stopper [256]. During long term storage moisture transfer to product was dependant on initial stopper moisture content and storage temperature. To reduce moisture content, Ford and Dawson, used industrial methylated spirit to wash and dry rubber stoppers which was then followed by heat sterilisation at 116°C over 16 hours [69]. Corveleyn et al. similarly conveyed the notion that the treatment of stoppers such as sterilisation or heat drying are both critical factors to consider in addition to the closure formulation when comparing the significance in moisture levels inside the product [142]. Pikal and Shah, compared the moisture transfer of 13 mm rubber stoppers with no treatment with those that had been steam sterilized followed by vacuum drying, showing a reduced moisture ingress to product over time [141].

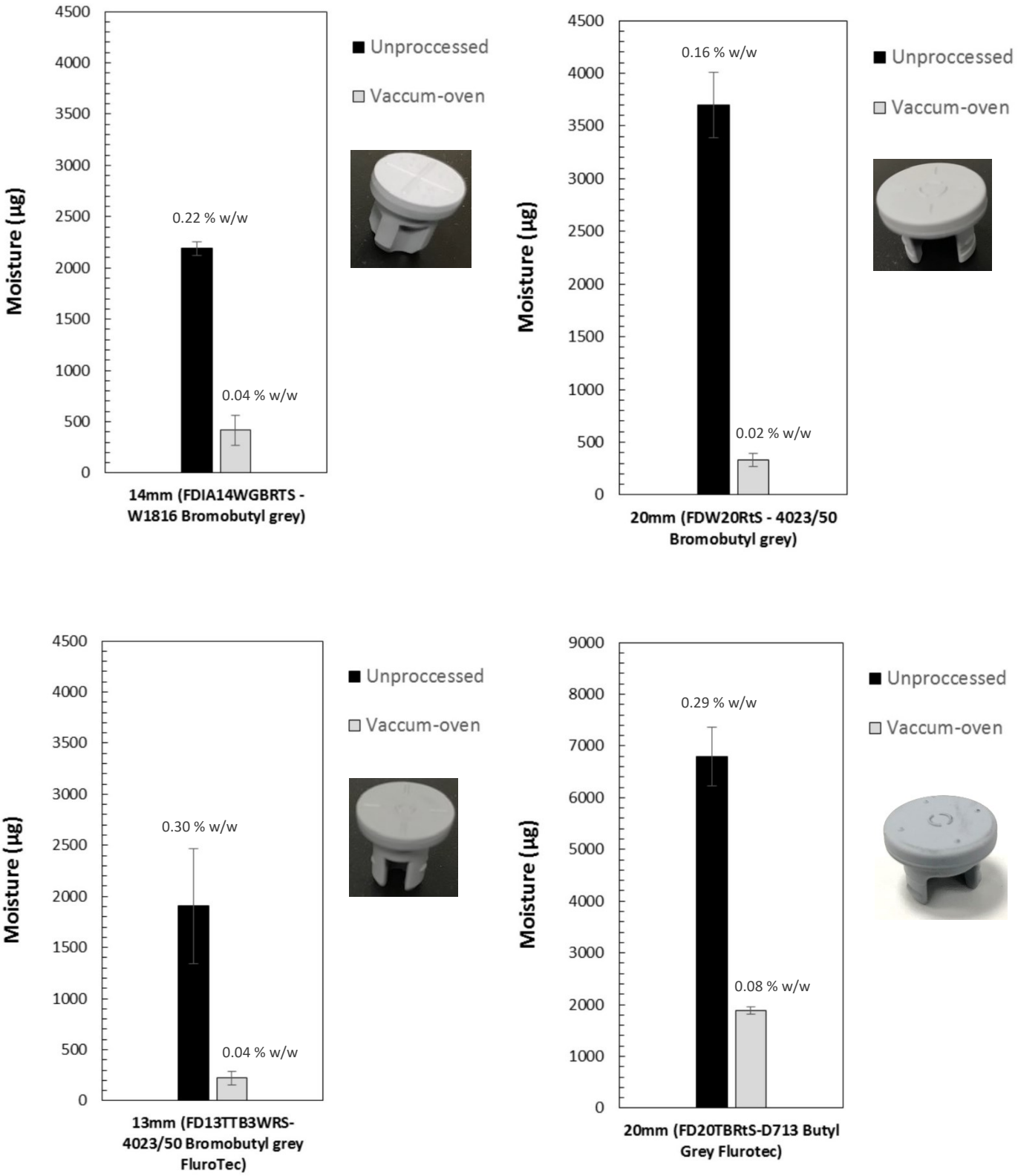


Figure 6.3 Comparison of moisture content between unprocessed and vacuum oven dried 14 mm rubber stoppers. Error bars represent ± standard deviation (n=3).

The vacuum-oven drying method was selected for its relative ease of use and for the fact that it can employ a combination of both high temperature and low pressure, meaning that there is a chance of more moisture in the stoppers being removed compared to just standard heating treatment. The difference in moisture content between vacuum-oven dried stoppers and unprocessed stoppers (straight from the pack) is shown in Figure 6.3. Different stoppers were tested and the average moisture content in terms of total water and % w/w is displayed with error bars representing ± standard deviation (n=3). The data shows that vacuum drying stoppers has a significant effect on the overall moisture content. Whilst the initial moisture present may vary because of a number of factors as described previously, what is clear here is that vacuum drying results in very little moisture remaining in the stoppers. For both the 14 mm and 20 mm size rubber stoppers, there is a clear and significant moisture loss as a result of being vacuum-over dried for 24 hours at a temperature of 120°C at less than 100 mBar. The average moisture content in the 14 mm (FDIA14WGBRTS) rubber stoppers straight from the pack stood at 0.22 % w/w and then dropped to about 0.04 % w/w. A similar significant change was also observed for the for the 20 mm (FDW20RtS) rubber stoppers, in which the moisture content dropped from 0.16 % w/w all the way down to 0.02% w/w as well. The prolonged exposure to high temperature and low pressures, for a period of about 1 day, had ensured that the stoppers were almost completely devoid of any moisture content. However, the 20 mm Flurotec coated rubber stoppers (FD20TBRTS), had the highest moisture content before and after vacuum drying. This indicates that the rubber formulation treatment, may inadvertently be impacting the amount of moisture being trapped inside. Corveleyn et al. showed that moisture desorption of stoppers after steam sterilisation, drying at 100°C, was also dependent on the type of treatment the stopper had, such as siliconisation [142]. Whilst, these moisture contents may appear to be low in terms of w/w %, they do have the capacity to inflict huge potential change in the cake moisture of FD samples. For particularly low dry weight samples such as flu antigens, which on average would weigh between 10 - 30 mg depending on the formulation, only a small amount of moisture would be required to cause dramatic shifts in moisture content. For example, a 10 mg dry weight antigen sample in the

vial would only require just 0.1 mg (or 100 µg) from the stopper to raise its moisture content by 1% w/w. All the stoppers tested here had at least 2000 µg or higher moisture content present straight out of the packs. The main roles of the vial and stoppers main is to act as a defence against external factors, and maintain the physical and chemical integrity of the FD product over storage. However, as seen here and described in other studies, the moisture in the stoppers can present an increased challenge for maintaining constant moisture for FD products.

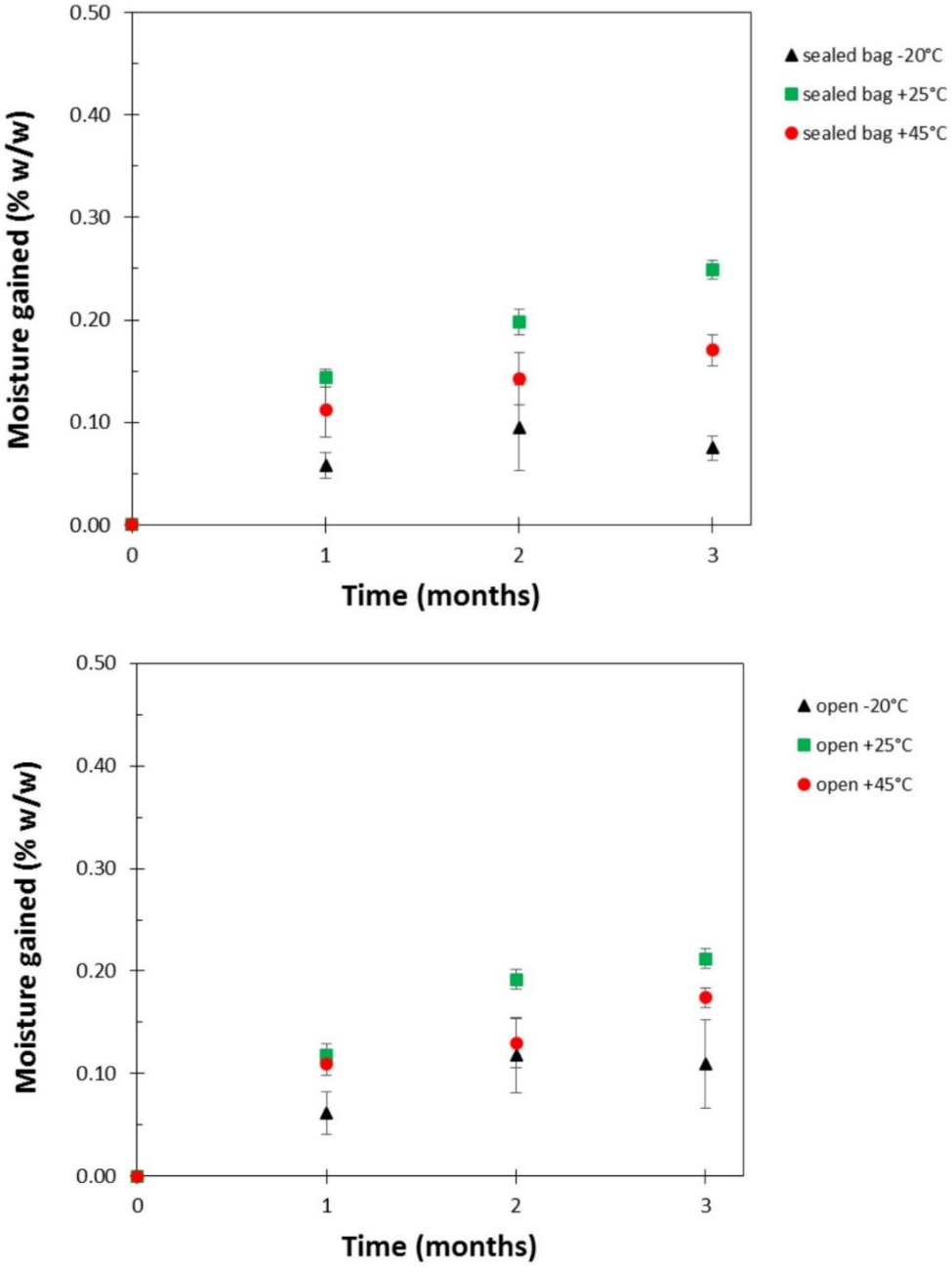


Figure 6.4 Moisture uptake (% w/w) over time at -20, 25 and 45°C for vacuum oven dried 14mm stoppers (FDIA14WGBRTS). Stoppers were stored in sealed polyethylene bags or exposed to room temperature and humidity. Error bars represent ± standard deviation (n=3).

Figure 6.4, shows an example of the moisture uptake experienced by 14 mm rubber stoppers, measured gravimetrically over the course of 3 months storage (n=3). All the stoppers tested were taken from straight out of the packaging and vacuum oven dried. 14 mm stoppers were then placed in temperature controlled ovens at -20°C, 25°C and 45°C. Stoppers were either left out in petri dish in the open conditions or placed inside resealable polyethylene plastic bags. The average relative humidity over 3 months at 25°C and 45°C was 34 ± 5 % and 32 ± 4 % RH, while for -20°C it was below 15 ± 7 % RH. These results showed that after vacuum-oven drying, stoppers gradually have an increase in moisture content over time at all storage temperatures. The moisture content in the 14 mm stoppers in just after 3 months, reaches close to the same % w/w levels that was present before vacuum oven-drying (Figure 6.3). There was little difference seen between samples stored in sealed bags and samples left out in the open exhibiting similar mass uptake over time % w/w, indicating water permeability occurring through them. Held and Landi, endorsed the claim that for their FD BCG vaccine dispensed in vials with grey butyl stoppers sealed in polyethylene bags, it could provide equivalent water ingress assurance as that of ampoules [259]. However, as seen by the results here there is doubt on the impact sealed bags could make. In terms of water uptake, at -20°C, there was the least amount of moisture gained, with up to 0.10 % w/w or less. The samples stored at 25°C had slightly higher uptake (≈ 0.20% w/w) than samples stored at 45°C (≈ 0.15 % w/w). This could be due to fluctuations in % RH inside the ovens between time points, as they were only temperature-controlled. Additionally, the sorption / desorption kinetics can vary depending on the temperature of storage and stopper type. The trend over time saw the stoppers initially display fast uptake but eventually slow down assuming that it reaches towards equilibrium on the following months, as also seen in previous studies [142].

The amount of moisture the stoppers can uptake and the vapour transmission rate is largely dependent on a number of factors such as size, elastomer formulation and time over storage conditions [260]. Moisture permeation through stoppers stored in humid conditions can also see an increase in FD cake moisture during late stages of long term storage [204,

207]. Natural rubber typically has the higher moisture vapour transmission rate of around 9 g.mm/m^2.day, while bromobutyl and chlorobutyl rubber have values of 0.3 and 0.1 g.mm/m2.day, respectively [260]. Held and Landi, found that Bromobutyl stoppers had lower moisture levels than chlorobutyl stoppers stored during 85 days at 95% RH–40°C. Additionally water uptake still occurred regardless of stopper used, but amongst the 12 stoppers compared the grey butyl stoppers and the silicone stoppers had the least uptake [259]. Sasaki et al. showed that the importance of elastomer formulation, with stoppers more prone to high moisture uptake corresponding with an increase in moisture inside the vials during early storage [204]. Stoppers with low moisture uptake saw lower moisture increase inside the vials. Different elastomer formulations may result in different initial starting moisture contents. Thus, heat treatment of some kind to remove the moisture after opening from the packaging should be used to minimise amount of moisture accumulating from the start.

6.3.2 Stability Study 1: Influenza Antigen (A/Wisconsin/15/2009)

6.3.2.1 Formulation Characterization

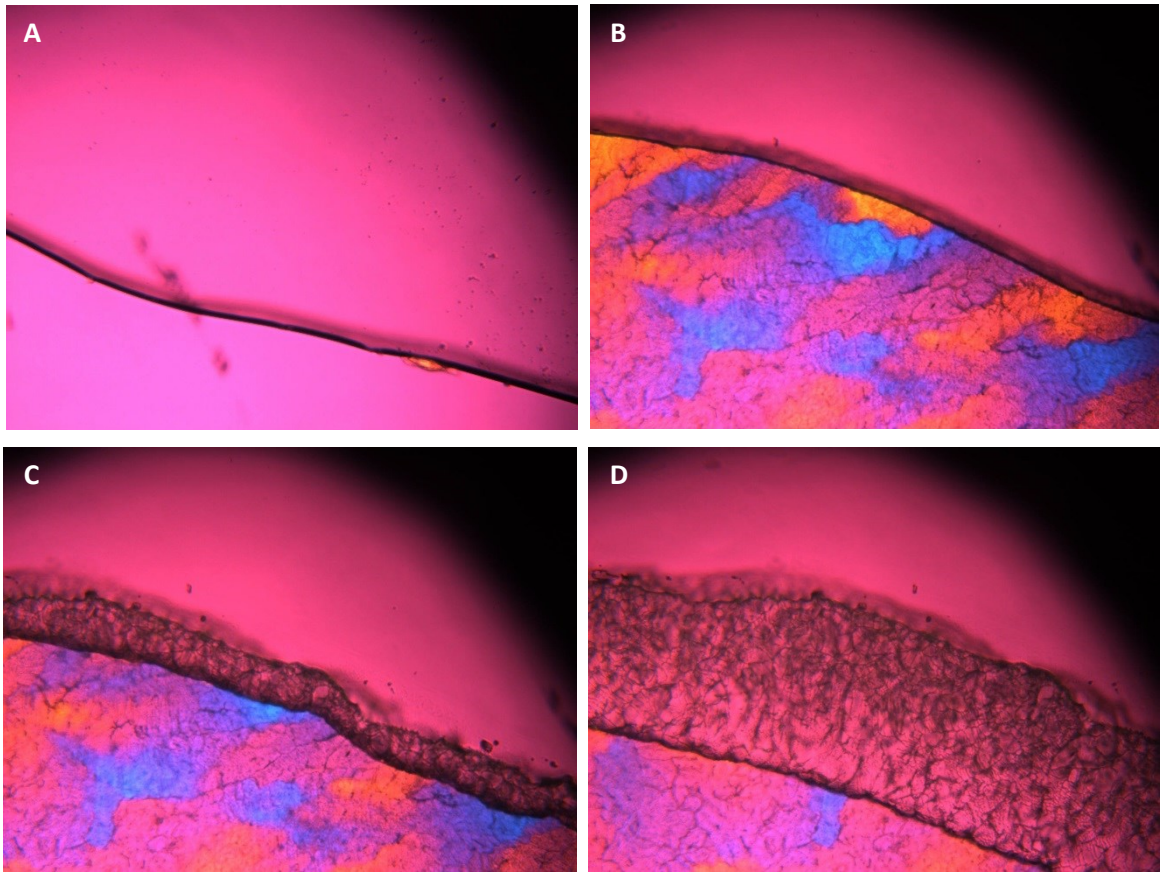


Figure 6.5 Freeze Drying Microscopy images of Influenza Antigen (A/Wisconsin/15/2009). **A:** Unfrozen liquid sample at 25.8°C, **B:** Frozen sample at -50°C, **C:** Sublimation front at -48.9°C, **D:** Collapse of structure around -37.3°C.

Exceeding the collapse temperature during freeze-drying can result in collapse of FD material due to insufficient sublimation of the ice occurring. FDM is a useful technique that has been adopted by the FD community to map out the critical temperatures in their lyophilisation cycles. In Figure 6.5, snapshot images of FDM at different temperature stages are shown. At image A, the sample is in the unfrozen liquid state at room temperature 25.8°C. When the temperature is taken down to -50°C, the ice formation can be seen by the change in colour due to the optical filter (image B). Once the pressure is lowered, a sublimation front emerges, highlighted by the grey porous looking facade. At image C, the sublimation front is seen being driven forward as the temperature is slowly ramped at -48.9°C. At the final image, D, the structure shows the first sign of collapse at -37.3°C, where the front begins to stretch apart and break. As a result of this the FD cycle primary drying temperature was to a conservative -40°C, which is well below the critical collapse temperature.

Table 6.3 Comparison of average dry weight, T_g, Young's modulus, specific surface area and moisture content at T=0 months (n=3).

Antigen Formulation	Average dry weight (mg)	Moisture Content (% w/w)	T_g (°C)	Young's Modulus (KPa)	Specific Surface Area (m^2/g)
(A/Wisconsin/15/2009)	23 ± 0.3	0.75 ± 0.02	62 ± 1	10 ± 1	20 ± 5

The FD cycle was designed to ensure a low moisture content run. Table 6.3 shows the moisture content before storage as measured by coulometric Karl Fischer and the dry T_g measured by DSC. The moisture content of the vials was below 1% w/w. The dry T_g of the antigens after FD was 62 ± 1°C. This is fairly low value even with such low moisture content. Typical storage recommendations have been suggested to be at least 40°C less than the T_g of the samples, which would equate around no more than room temperature. This of course would not be ideal if shipping or exposure to climates in hotter parts of the world. The dry weight mass of antigens are typically very low. Their cakes are extremely fragile and have a 'fluffy' like consistency.

The mechanical properties of the cake highlight the low stiffness in terms of Young's modulus. The cakes are extremely fragile and prone to crumbling. Table 6.3 also shows the dry weight immediately after FD of influenza antigen (A/Wisconsin/15/2009). What is clear here is the fact that the cakes dry weight is around 23 mg, highlighting once again the low mass of these influenza antigens. The moisture content before storage is under 0.8% w/w indicating an efficient cycle which has removed most water. As stated previously over long term storage, because of the low dry mass, only a small amount of moisture ingress would be needed to increase the chance of degradation or in extreme cases collapse to occur. This necessitates the need to maintain the moisture content and ingress as low as possible for long term storage stability.

6.3.2.2 Moisture ingress during storage

Vials with vacuum oven dried and unprocessed stoppers (14 mm diameter cruciform - FDIA14WGBRTS,) containing influenza antigen (A/Wisconsin/15/2009) were placed inside controlled temperature ovens for long term stability trials. Figures 6.6 - 6.8 shows the change in moisture content of the FD cakes over a 6 months period at -20°C, 37°C and 45°C respectively. At -20°C storage both the vials with unprocessed stoppers and dried stoppers, showed consistently low moisture content over the 6 months storage period. The moisture content remained consistently below 1% w/w. At low temperatures the amount of water that the air can hold is significantly reduced compared to higher temperatures. The stopper vapour transition rate will be severely limited, hence resulting in very little uptake over time regardless of how much moisture is present in them.

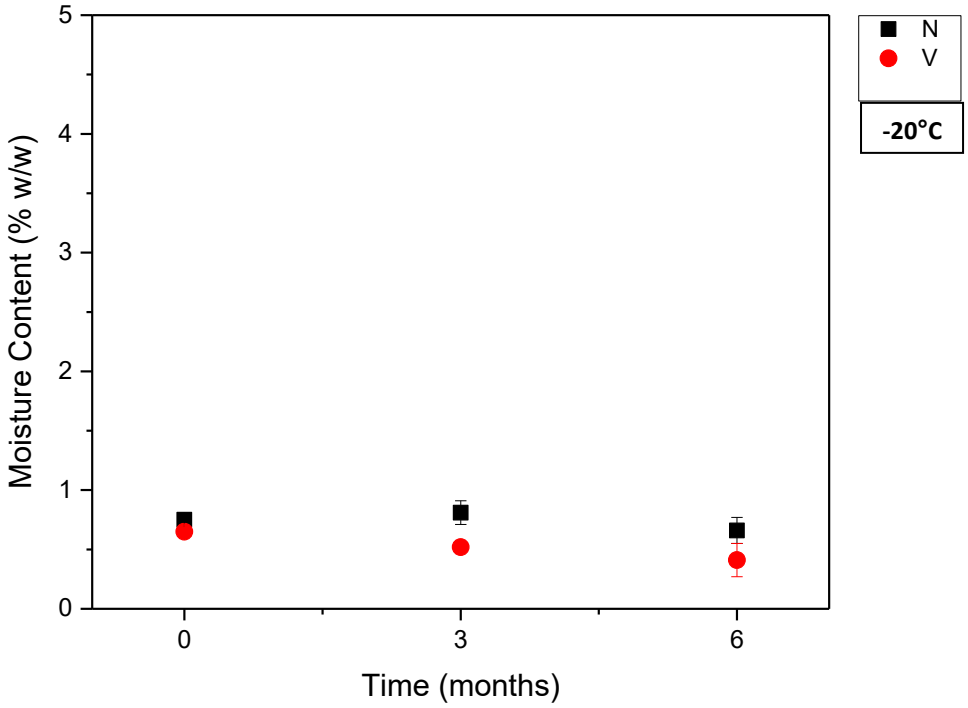


Figure 6.6 Comparison of the moisture content (% w/w) in product between vials with unprocessed stoppers (N) and vials with vacuum-oven dried stoppers (V) for influenza antigen (A/Wisconsin/15/2009). Samples were stored over a period of 6 months at -20°C. Error bars represent 95% confidence intervals (n=3).

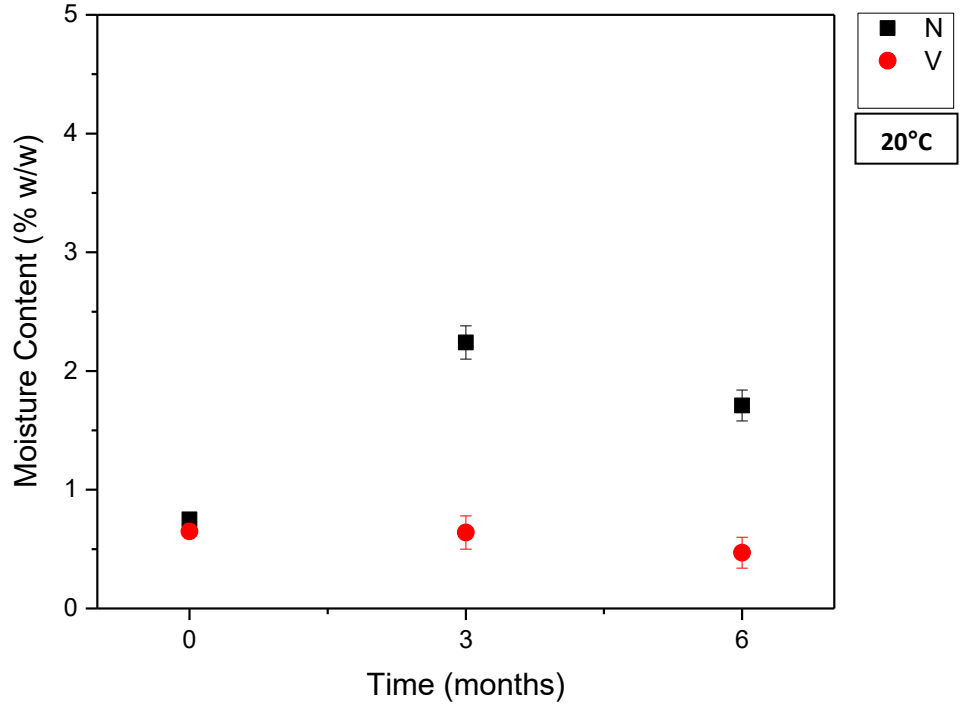


Figure 6.7 Comparison of the moisture content (% w/w) in product between vials with unprocessed stoppers (N) and vials with vacuum-oven dried stoppers (V) for influenza antigen (A/Wisconsin/15/2009). Samples were stored over a period of 12 months at 37°C. Error bars represent 95% confidence intervals (n=3).

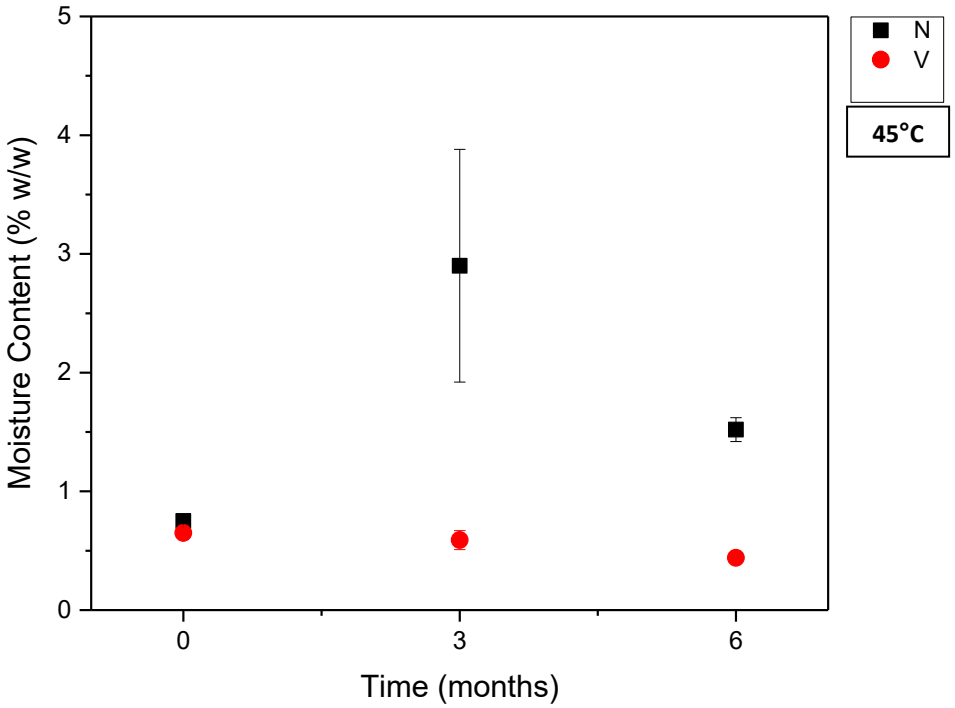


Figure 6.8 Comparison of the moisture content (% w/w) between vials with unprocessed stoppers (N) and vials with vacuum-oven dried stoppers (V) for influenza antigen (A/Wisconsin/15/2009). Samples were stored over a period of 12 months at 45°C. Error bars represent 95% confidence intervals (n=3).

However at 37°C and 45°C storage, there is a clear difference in moisture between vials with stoppers that had been oven-dried and those without. At both elevated stress temperatures, the vials with vacuum dried stoppers did not exhibit any increases in moisture above 1% w/w, unlike the vials with unprocessed stoppers. At 37°C it appears that after the initial first 3 months jump in moisture, that the unprocessed stopper vials have equilibrated to around 2 % w/w moisture content. Similar to water adsorption studies, the cakes will equilibrate depending on the amount of available water that is present in the stopper and on the storage temperature. Pikal and Shah, noted that product moisture increases over time and equilibrates at a certain value depending on the amount of product and stopper treatment method [141]. The same trend can be described for the vials with unprocessed stoppers at 45°C, although there was a drop around 1% w/w from between T=3 months and T= 6 months. The most likely reason was the occurrence of an outlying result in the average of three samples (n=3) taken for Karl Fischer water content determination at that point. Another possibility is that there may have been less moisture in one of the unprocessed stoppers compared to the rest. Alternatively it could have been that not all the stoppers having the same vapour transmission rates or that one sample might have absorbed slightly more or less moisture than the other cakes. All 14 mm unprocessed stoppers came from the same pack, containing 2190 ± 67 µg (Figure 6.4) of moisture with low CV% (n=3). Since the dry mass of the antigens was around 23 mg, it means at least 0.23 mg (or 230 µg) must have been transferred from the stoppers to the cake to raise its moisture content by 1% w/w. The overall general trend showed that vials with vacuum oven-dried stoppers were able to provide better lower moisture content compared to vials with unprocessed stoppers over a 6 month period.

The data suggests that most of the moisture did originate from the stoppers and not attributed to any previous moisture trapped inside the vial before storage. Otherwise, an increase in moisture at 20°C and 45°C would have been observed for the vials with vacuum dried stoppers, which was not seen. Similarly, since all vials regardless of the stopper treatment, had been screw capped and sealed in polyethylene re-sealable bags inside the incubators during storage, there is an assumption that there had been minimal moisture from the outside environment absorbing into the stoppers. A limitation of the study was that the moisture of the stoppers were not concurrently measured at each time point except for at before storage (T=0 months).

Previous studies have shown the unreliability of rubber stopper vials in regards to moisture content. Held and Landi, found the moisture content of their FD Bacillus Calmette–Guérin (BCG) vaccine increased during prolonged storage with rubber stopper vials [259]. Moisture transfer from the stopper to the product could have long term stability consequences. In some cases where the rubber stopper may be excessively over-dried, the phenomena of the reversal of moisture from the FD cake to the stoppers has been reported. In these cases, the moisture content in cakes dropped initially because the moisture was absorbed out by rubber stopper itself [104]. However this was not observed for any of the samples in this study.

6.3.2.3 Change in Potency

The presence of high enough moisture content may facilitate instability which can cause degradation and losses in potency. With increasing moisture content and temperature, there is also a correlated increase in molecular flexibility. The potency of the influenza antigen was measured simultaneously with the moisture content of the cake at each time point. Figures 6.9 - 6.11, shows the potency results from the SRD assay at storage in -20°C, 37°C and 45°C. The y-axis represents the potency in terms of µg HA/mL and the x-axis represent the time in months.

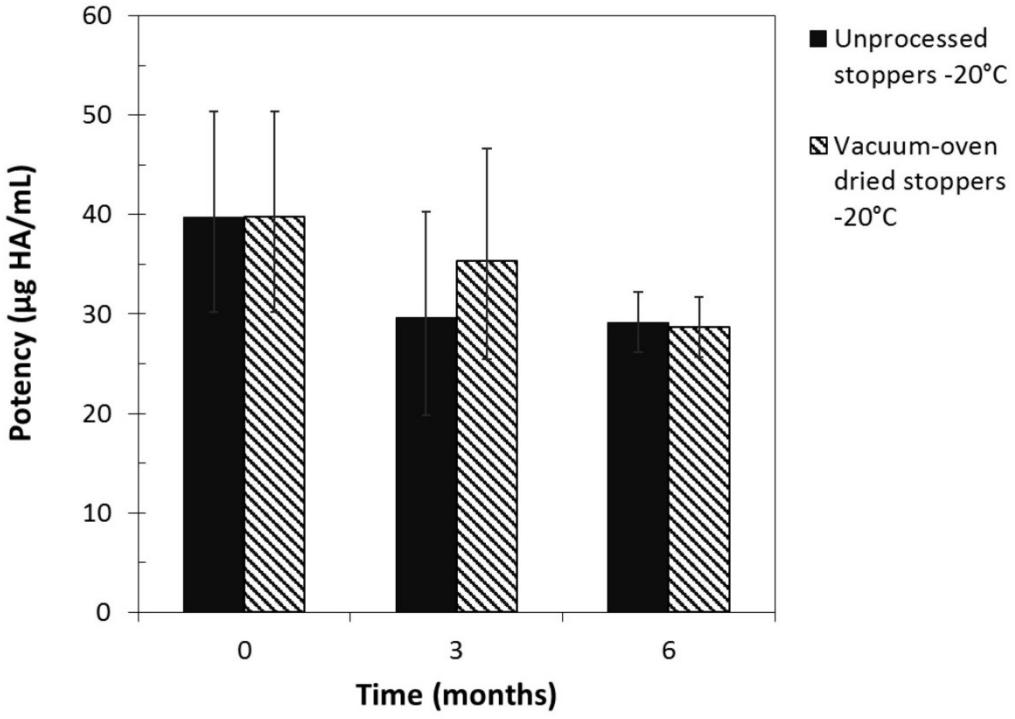


Figure 6.9 Comparison of the moisture content (% w/w) between vials with unprocessed stoppers (N), vials with vacuum-oven dried stoppers (V) over a period of 6 months stored at -20°C. Error bars represent 95% confidence intervals (n=3).

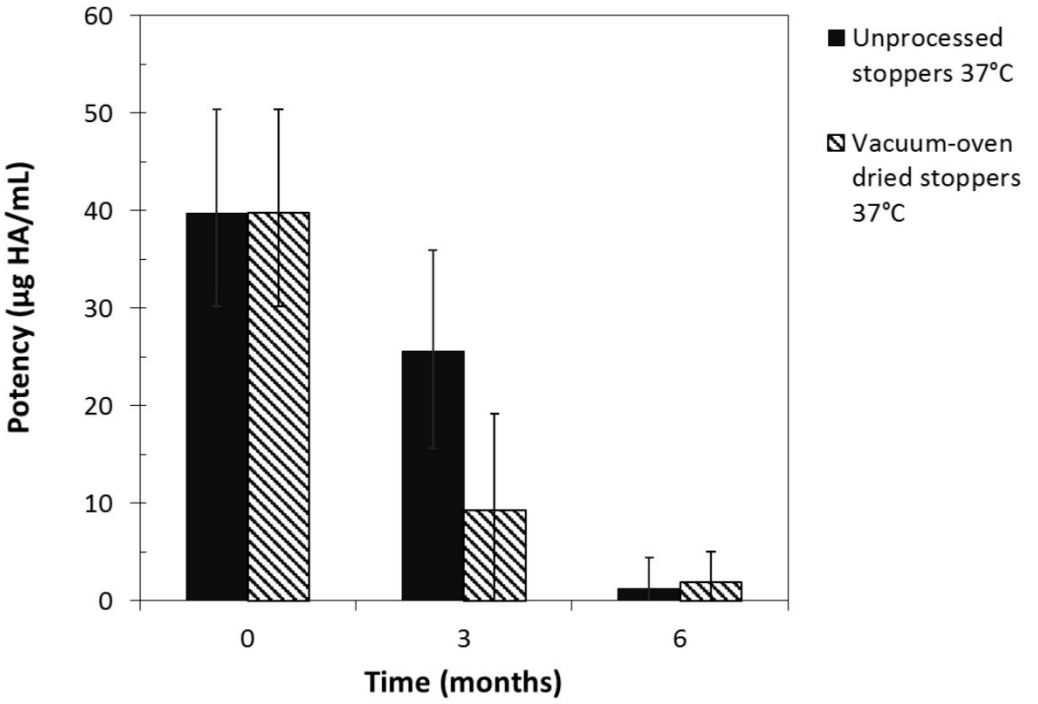


Figure 6.10 Comparison of the moisture content (% w/w) between vials with unprocessed stoppers (N), vials with vacuum-oven dried stoppers (V) over a period of 6 months stored at 37°C. Error bars represent 95% confidence intervals (n=3).

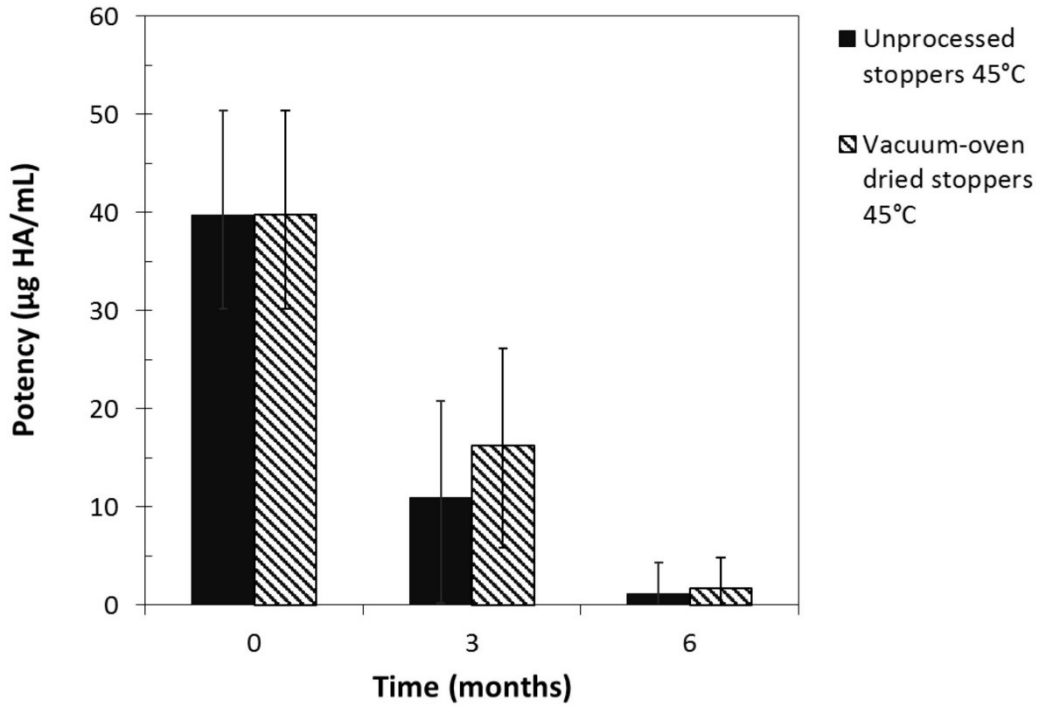


Figure 6.11 Comparison of the moisture content (% w/w) between vials with unprocessed stoppers (N), vials with vacuum-oven dried stoppers (V) over a period of 6 months stored at 45°C. Error bars represent 95% confidence intervals (n=3).

At -20°C, there was no significant difference in potency between vials with vacuum-oven dried stoppers and vials with untreated stoppers. The initial potency for both starts at an average of 40 µg HA/mL. After 6 months storage, both end up with an average of 30 µg HA/mL, representing almost a 25% loss. However, this is not significant due to the large overlapping confidence intervals. After 6 months storage, both vial formats have retained similar potency level, comparing similarly to that of the low moisture content ingress. Since there was very little moisture present and at such low temperature, there was not much risk of major degradation occurring. After 6 months storage at 37°C and 45°C storage, the potency of the antigen has been completely destroyed with an average of no higher than 2 µg HA/mL remaining. The effect of drying the stopper had no effect in potency retention at 37°C and 45°C. The moisture in the product after the first 3 months of storage for the vials with untreated stoppers was at around 2% w/w, while for vacuum-oven dried stoppers was no higher than 1% w/w. The vials with higher moisture would have been expected to have a greater drop in potency than those with lower moisture, however this was not seen. The antigen potency had been completely destroyed at the elevated temperatures, which was unexpected based on previous stability trials done internally by the virology division at NIBSC on sealed ampoules.

A significant limitation of this study was not being able to obtain fresh pool of liquid influenza antigen (A/Wisconsin/15/2009). The liquid pool of influenza antigen (A/Wisconsin/15/2009) obtained for this study, had been previously stored in the fridge at 4-8°C for several years before use. This meant that there had been natural ageing and instability from the start, which you would not expect to see with a fresh batch. Although at such high stress temperatures you would expect to see some losses occurring, one would not expect to see a complete loss in potency based on past internal data with previous reference antigens at NIBSC. The results from this trial were inconclusive and thus it was difficult to make any conclusions to correlate the potency, moisture content and effect from stoppers. While it is clear that vials with vacuum dried stoppers had low moisture content over all storage conditions, this did not seem to translate to any real impact on potency levels.

6.3.2.4 Summary

Hygroscopic material such as influenza antigen (A/Wisconsin/15/2009) are prone to moisture ingress during storage. Vials with vacuum-oven dried stoppers can significantly reduce moisture ingress at elevated storage temperatures compared to normal stoppers from the pack. To what extent the moisture ingress had on the potency at elevated temperatures was inconclusive. Both vials with dried and untreated stoppers had similar losses in potency at higher stress temperatures even though they had different moisture content suggesting perhaps that temperature was a greater factor in losses. However, another likely explanation is that age of the antigen flu batch might have contributed to the degradation effects and thus made the potency results reported here somewhat unreliable.

6.3.3 Stability Study 2: B/Phuket (1% w/w sucrose) Antigen

The SRD results from the previous flu antigen were inconclusive (Section 6.3.2.3) and the study adjourned. It was decided to repeat the same investigation but with an alternative antigen type supplied from a more recent batch. B/Phuket (15/252) antigen was selected and suspended in PBSA buffer containing 1% (w/v) sucrose. The total potency was confirmed to be 60 µg/ml HA from the start and the batch was sourced from a recent fresh supply which would rule out any corresponding aging effect lingering from previous study. The same experiment was repeated with vials with vacuum dried stoppers and vials without treatment. Additionally, it was decided to also introduce the storage format of flame sealed ampoules into this study as a way to assess how dried stoppers compared to the gold standard method for FD antigen storage (Figure 6.12). Flame sealed ampoules have been shown to be extremely good excluding moisture ingress over long storage periods. Apart from any moisture present in the headspace of the ampoule after sealing, there is almost no chance of moisture getting through the glass unless micro-cracks are present. Once again after FD, the vial samples were screw capped, sealed in re-sealable polyethylene bags and stored in controlled ovens at -20°C, +20°C and 45°C. It was decided to have a mid-point ambient room temperature instead of two highly stressed temperatures unlike the previous study. Following the same moisture and potency patterns, then the -20°C storage will effectively act as the controlled temperature to compare with.

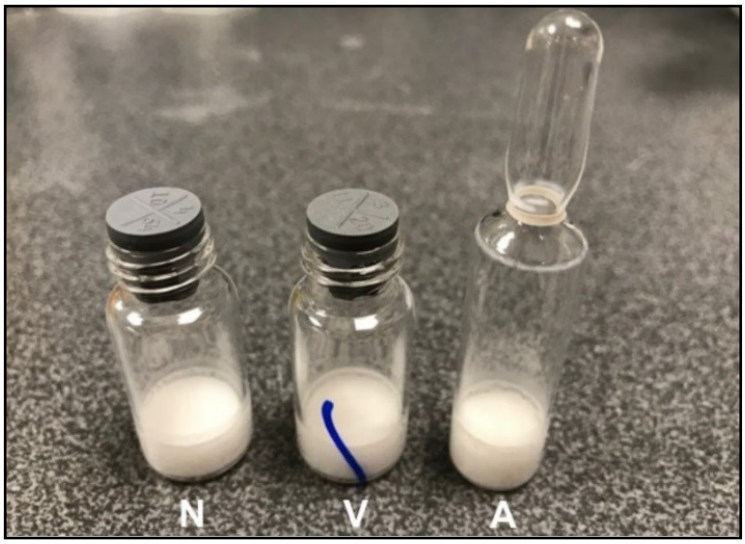


Figure 6.12 B/Phuket antigen trial storage format comparison. **N:** Vials with unprocessed 14mm stoppers. **V:** Vials with vacuum-oven dried 14mm stoppers. **A:** Flame sealed glass ampoules.

6.3.3.1 Change in Moisture Content

During long term storage, the moisture content in FD cakes can increase. Figures 6.13 - 6.15 shows the change in moisture content between different storage formats containing FD B/Phuket antigen over a 12 month storage period at -20°C, +20°C and +45°C. Vials had been sealed with either unprocessed 14 mm stoppers (N) from the pack or with vacuum oven dried 14 mm stoppers (V). The ampoules (A) had been flame sealed since the start of storage. A total of three vials were taken and opened under a dry air humidity bag at or below 5% relative humidity at each time point in order to assess the moisture content by coulometric Karl Fischer titration. The error bars represent the standard deviation (n=3).

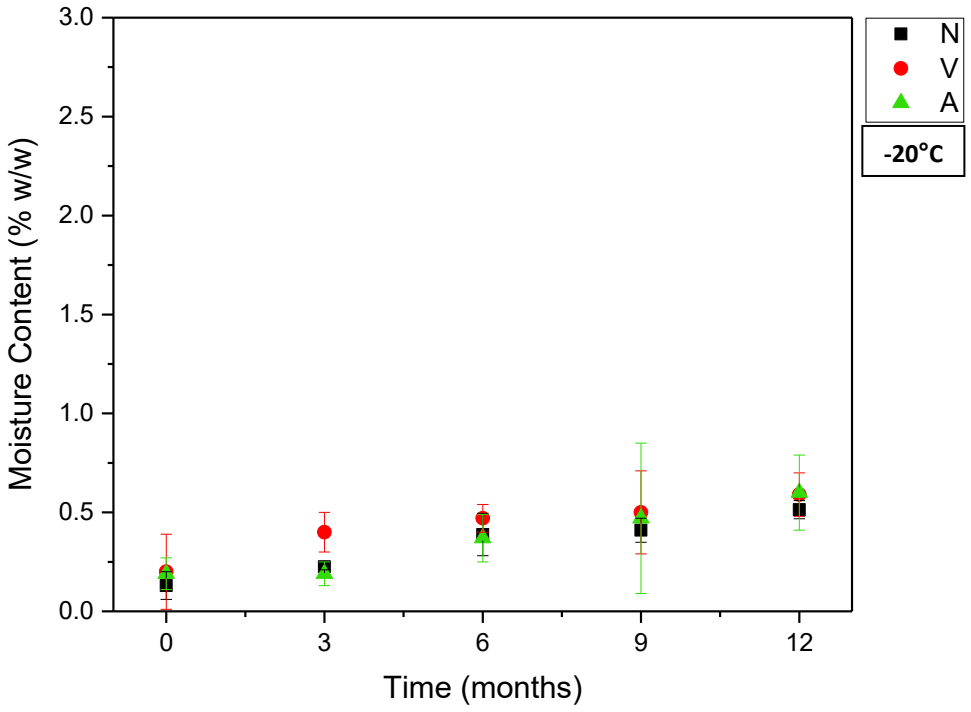


Figure 6.13: Comparison of the moisture content (% w/w) between vials with unprocessed stoppers (N), vials with vacuum-oven dried stoppers (V) and ampoules (A) over a period of 12 months stored at -20°C. Error bars represent the 95% confidence intervals (n=3).

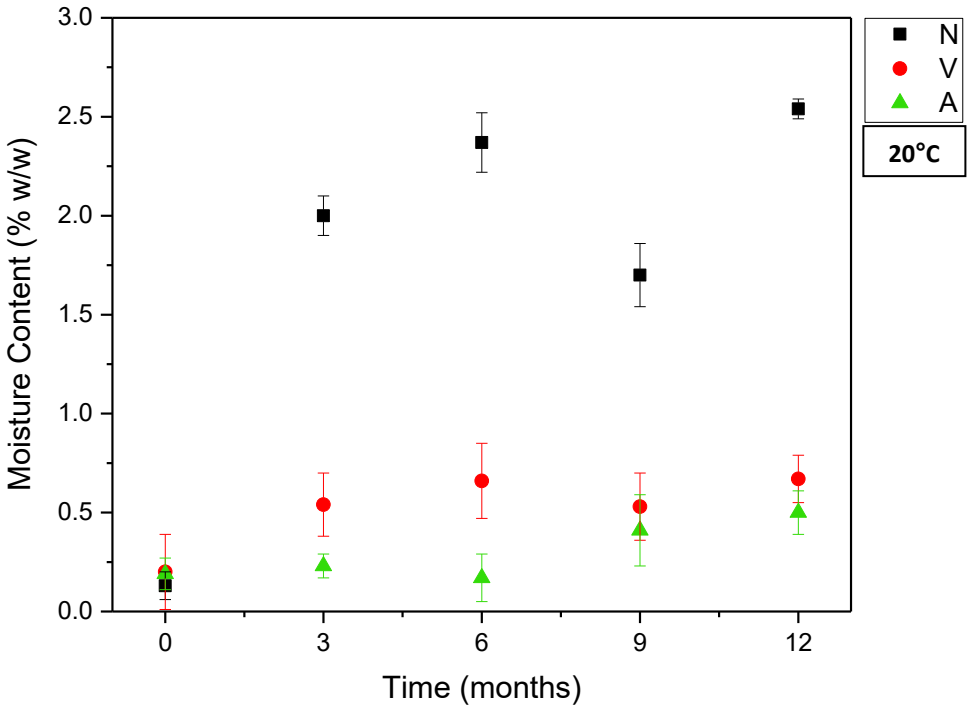


Figure 6.14: Comparison of the moisture content (% w/w) between vials with unprocessed stoppers (N), vials with vacuum-oven dried stoppers (V) and ampoules (A) over a period of 12 months stored at 20°C. Error bars represent the 95% confidence intervals (n=3).

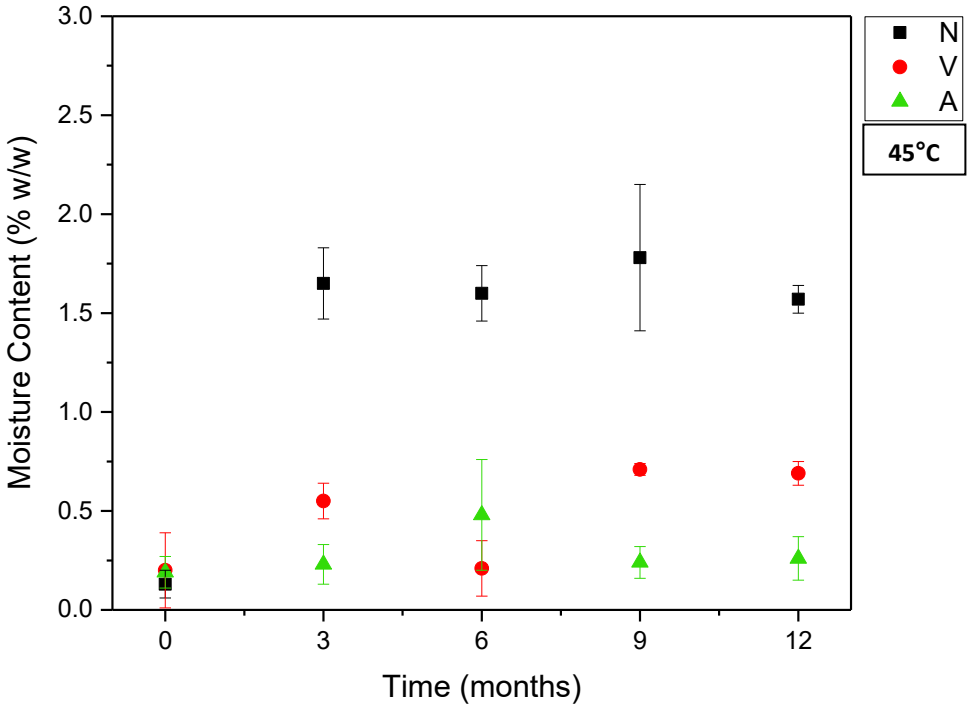


Figure 6.15: Comparison of the moisture content (% w/w) between vials with unprocessed stoppers (N), vials with vacuum-oven dried stoppers (V) and ampoules (A) over a period of 12 months stored at 45°C. Error bars represent the 95% confidence intervals (n=3).

Over the entire course of 12 months storage period at -20°C there was no significant difference in moisture between the vials with and without vacuum oven dried stoppers and the ampoules. The moisture content of the products remains consistently low, never exceeding above 0.60 % w/w for all storage formats after 12 months storage. This is in agreement with moisture content data from the previous study with the influenza antigen (A/Wisconsin/15/2009). At temperatures below zero there appears to be minimal moisture transfer from the stoppers to the product. With decreasing temperature, the air in the headspace can only permit a tiny amount of moisture compared to higher temperatures.

The increase in product moisture content, is once again observed at elevated temperatures for the normal unprocessed stoppers. At 20°C storage, following the first three months, the product moisture content rose from 0.13 ± 0.07 % w/w to about 2.00 ± 0.10 % w/w. This was then followed by a further increase to about 2.4% w/w at T=6 months, followed by a slight drop at T=9 months, before climbing back up to 2.54 ± 0.05 % w/w at T= 12 months. Like the previous data, it seems that as a general trend it began to plateau around the region of 2.6 % w/w equilibrium point. In comparison, vials with vacuum dried stoppers and ampoules show no such large increase in moisture. Similar to -20°C storage, the moisture content in the vials with the vacuum oven dried stoppers, saw very little change. Again it is only at the T=3 months that the moisture content jumps from 0.20 % w/w to 0.54% w/w. After that there is no significant change throughout the entire year's storage. The final moisture content at T=12 months was 0.67 ± 0.12 % w/w. The same similar low moisture content trend applied for the ampoules. The initial moisture content saw no significant change from T=0 to T=3 months and only rose to 0.41% w/w approaching T=9 months. The final moisture content at T=12 months was 0.50 ± 0.11 % w/w. The final moisture content between vials with vacuum oven dried stoppers and ampoules is within error of each other at 20°C storage. This shows that the treated stopper vials were able to maintain similar moisture product levels to the ampoules. The very slight increase moisture for the ampoules could be attributed to some moisture remaining in the headspace after flame sealing or possibly batch to batch variability in cake

moisture. The difference in moisture content in the product between vials with vacuum dried stoppers and vials without, compares once again with those results seen at elevated temperatures for influenza antigen (A/Wisconsin/15/2009).

Again at the highest storage temperature of 45°C, the vials with normal unprocessed stoppers have the highest product moisture content. After the initial increase at T=3 months 0.13 ± 0.07 % w/w to 1.65 ± 0.18 % w/w, the moisture content equilibrates between 1.5-2.0% w/w. This is slightly lower than at 20°C, which would indicate perhaps that the higher temperature lowered the transmission rate of the stoppers. After 12 months storage the vials with vacuum oven dried stoppers and ampoules have 0.69% w/w and 0.26% w/w moisture content respectively. At 45°C ampoules were the best format at keeping low product moisture content followed closely by vials with vacuum oven dried stoppers and finally worse performing are the vials with untreated stoppers.

A study by Matejtschuk et al. showed that ampoules containing lyophilised albumin did not exhibit any change in gas or moisture contents even at high stress conditions over 12 months, unlike that of vials with stoppers which saw a substantial increase [208]. Because of the flame sealed format of ampoules, there is very little chance of moisture content increasing significantly over time. This is of course assuming that there is only very small amounts of headspace moisture trapped in the ampoule and that there are no micro-cracks in the glass. As a result, the ampoules will always have the advantage over rubber stopper vials in regards to maintaining the lowest possible moisture over long term storage at temperatures above 20°C. However as shown here, vacuum-oven dried stoppers are able to provide comparable moisture integrity during long term moisture at -20°C and 20°C storage and near comparable low moisture levels at 45°C to that of ampoules. As a result of the vacuum oven drying process, the rubber stoppers had sufficiently less moisture present than untreated stoppers as seen previously by the data from the KF oven in Figure 6.4. Since most of the moisture present at the surface and in bulk was removed, there was very little moisture vapour transmission to the product as evidenced by the small increases at 20°C and 45°C compared to vials with

untreated stoppers. A study by Ford and Dawson, which had pre-treated their stoppers with methylated spirit and air dried, also concluded that they were just as effective as DIN ampoules at maintaining a low moisture content of FD alkaline phosphatase [69]. At 20°C and 45°C after 12 months storage for the vials with untreated stoppers, they appear to be reaching an equilibrium as there is no major increases past the 3 month time point. This also suggests that the critical moisture transfer from stopper to product happens early on during storage, within the first few months. In both the B/Phuket and the A/Wisconsin/15/2009 influenza antigen stability trials, the moisture content of the product sealed in vials with the vacuum oven dried stoppers never went above 1% w/w at all storage temperatures between -20°C to 45°C. Hence, further setting forward the strong case for all FD antigen products to be sealed and stored in vials with vacuum dried stoppers.

6.3.3.2 SRD Potency

The potency of the three storage formats was once again tested by SRD assay in order to determine what effects the moisture and temperature had. Figures 6.16 - 6.18 show the comparison between the three storage formats over the course of 12 months storage at either -20°C, 20°C or 45°C. At -20°C storage there is no significant difference for all three storage formats over the entire year. The potency levels consistently stay between the ranges of 50 - 60 µg HA/mL. This also corresponds with the low moisture in the vial and ampoules at that storage temperature (Figure 6.13). The same potency result was also observed for influenza antigen (A/Wisconsin/15/2009) at -20°C (Figure 6.16). This confirms that at such low temperatures the storage format and stopper treatment makes little difference to the overall potency for these two antigens tested.

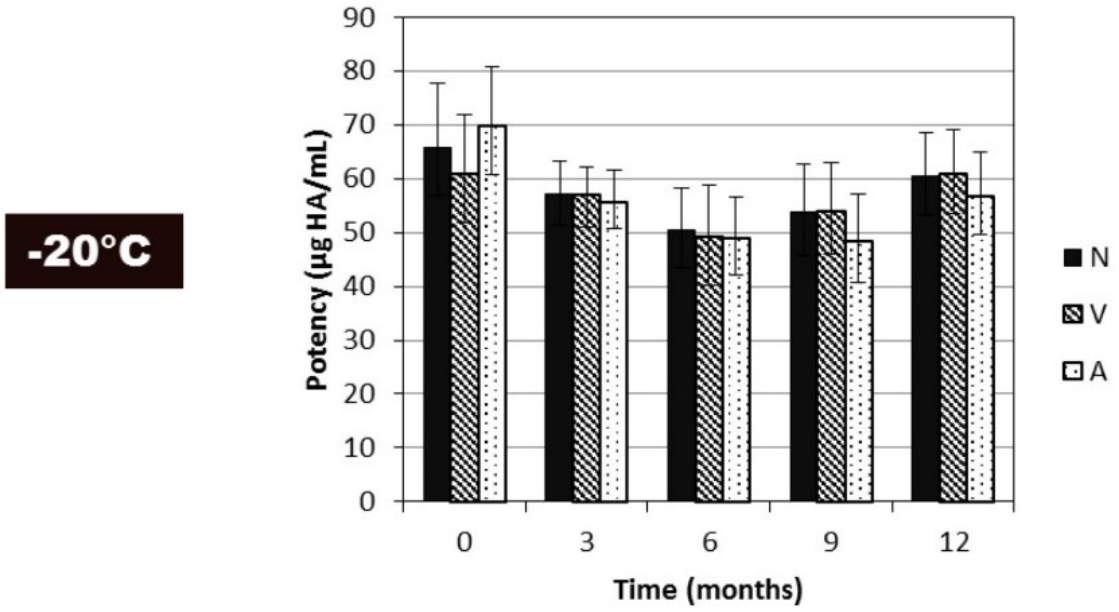


Figure 6.16 Comparison of the potency (µg HA/mL) between vials with unprocessed stoppers (N), vials with vacuum-oven dried stoppers (V) and ampoules (A) over a period of 12 months stored at -20°C. Error bars represent ±95% Confidence Intervals.

Interestingly, at 20°C (Figure 6.17), the same pattern is repeated with no significant difference between the storage formats once again through the course of the year. This is somewhat surprising especially for the vials with unprocessed stoppers, which after 3 months had a moisture content above 1.5 - 2.0% w/w (Figure 6.14). This increase in moisture would have been thought to cause some loss in potency but as shown here from the data, it did not seem to have any impact. The ampoules and vials with vacuum dried stoppers, had low moisture below 1% w/w throughout the year storage and were not expected to see any huge losses.

However, it is only at 45°C storage (Figure 6.18) that we start to observe losses in potency. Ampoules and vials with vacuum dried stoppers were able to retain better overall potency than vials with unprocessed stoppers. After T= 3 months, ampoule and vials with vacuum dried stoppers maintained potency levels between 40 - 50 µg HA/mL, whereas vials with unprocessed stoppers ranged between 20 - 40 µg HA/mL. The high moisture in the vials with unprocessed stoppers had resulted in significantly lower potency levels than the ampoules and vials with vacuum oven-dried stoppers which had less than 1% w/w moisture (Figure 6.15). This shows that vials with vacuum oven dried stoppers are able to retain higher potency than vials with unprocessed stoppers for B/Phuket antigen. This is in contrast to which the previous influenza antigen trial (A/Wisconsin/15/2009) at 45°C (Figure 6.11), which had almost complete destruction in potency after 6 months regardless of stopper drying.

20°C

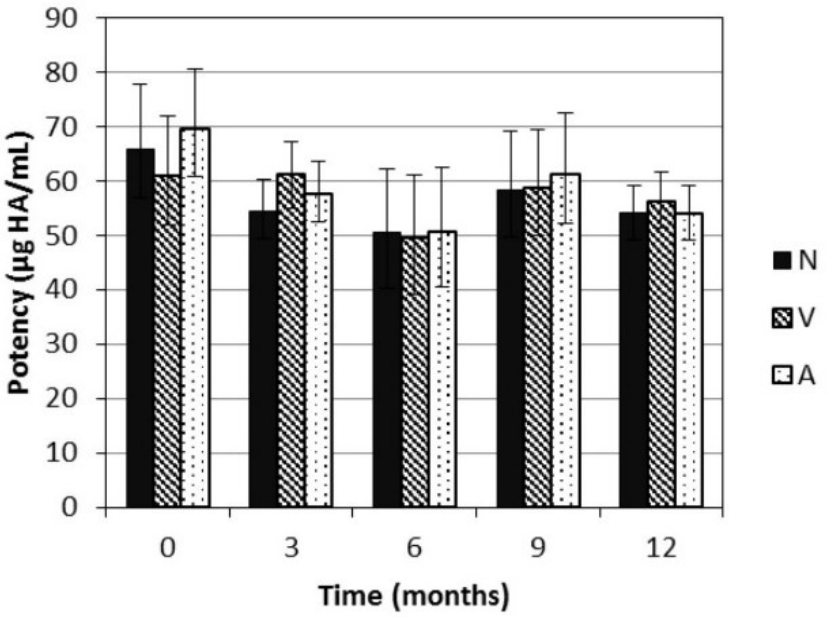


Figure 6.17 Comparison of the potency (µg HA/mL) between vials with unprocessed stoppers (N), vials with vacuum-oven dried stoppers (V) and ampoules (A) over a period of 12 months stored at 20°C. Error bars represent ±95% Confidence Intervals.

45°C

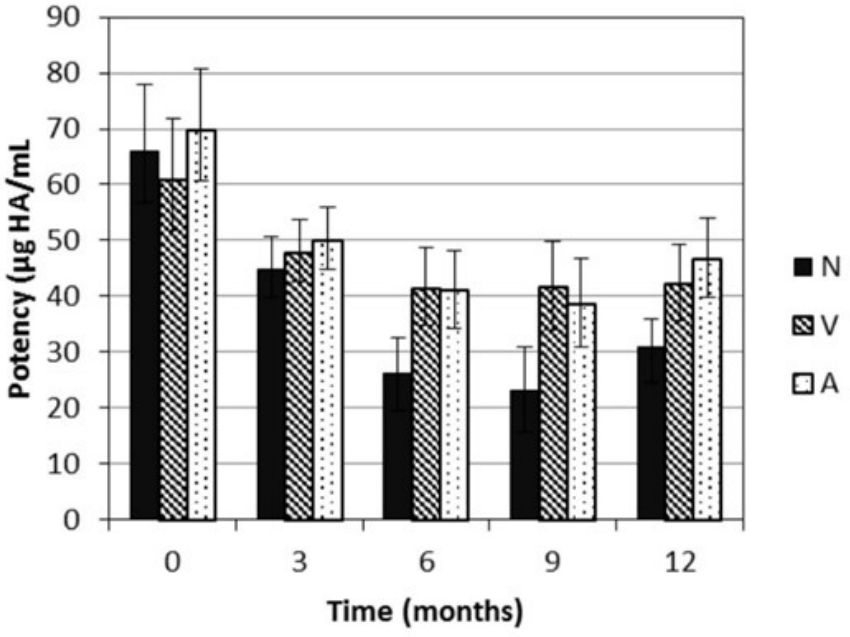


Figure 6.18 Comparison of the potency (µg HA/mL) between vials with unprocessed stoppers (N), vials with vacuum-oven dried stoppers (V) and ampoules (A) over a period of 12 months stored at 45°C. Error bars represent ±95% Confidence Intervals.

One possibility for the moisture content in vials with unprocessed stoppers not having any immediate effect at 20°C was that there could be lag in potency drop for samples stored. The stability trials were limited to only 12 months storage and there could have been a chance that at later point in storage time we might have seen the effects of the high moisture content on the potency as for the 45°C samples. A combination of both high storage temperature and moisture content was required for noticeable loss of potency to occur within 6 months at 45°C. In a previous study by Ford and Dawson, they also observed that only at high stress temperatures did vials with high moisture content have a significant impact of the enzymatic activity of FD alkaline phosphatase [69]. With increasing moisture content there is a reduction in the glass transition temperature. As the T_g value approaches your storage temperature then you have increased molecular ability and the chance of degradation occurring. The rise in moisture content at 20°C and 45°C for vials that they both had reduced T_g values. The samples at 45°C had higher molecular flexibility and were closer to this reduced T_g value than the samples at 20°C. This could explain why losses were observed at 45°C and not at ambient temperature.

Table 6.4 Comparison of storage temperature, moisture content (n=3) and glass transition temperature (n=1) after storage time of 12 months.

Format	T, Storage Temperature (°C)	Moisture Content (% w/w)	T_g (°C)	T_g - T (°C)
N	-20	0.51 ± 0.05	71	91
V	-20	0.59 ± 0.11	79	99
A	-20	0.60 ± 0.19	78	98
N	+20	2.54 ± 0.05	54	34
V	+20	0.67 ± 0.12	76	56
A	+20	0.50 ± 0.11	84	64
N	+45	1.57 ± 0.07	66	21
V	+45	0.69 ± 0.06	79	34
A	+45	0.26 ± 0.11	84	39

Past studies have shown that increasing moisture content was only a concern in context of the T_g and storage temperature. Breen et al. described that for their FD IgG that increased moisture content did not compromise stability, as long as the storage temperature was sufficiently below the T_g [7]. With water being a plasticiser, a subsequent reduction in T_g is often seen for materials with higher moisture content. Over storage, varying increase in moisture at 20°C and 45°C for different vial formats would have most certainly reduced the T_g values. Table 6.4 shows the difference between storage temperature, T_g and moisture content at the final time point of 12 months storage. All storage formats at 45°C, were closer to the reduced T_g -T values that at 20°C storage, which correlated with a drop in potency. Again, these set of results corresponds to the theories of stabilisation using the glass vitrification hypothesis. As the storage temperature approaches the T_g value of the material, increased molecular flexibility occurs and thus greater chance of degradation. Moisture content and ingress is a critical influencer on the T_g value. Past studies have indicated that storage should be below as far as 50°C below the Tg to completely ensure that there is negligible molecular movement occurring [78]. At 20°C, the vacuum-oven dried stoppers and ampoules were above this threshold, however the vials with normal stoppers were not. However, there was no difference in potency for all formats same at 20°C. This suggests that there may be a permissible moisture content range in context to the storage temperature and that some molecular movement does not always result in noticeable degradation.

6.3.3.3 Summary

Significant moisture ingress was once again observed for vials with rubber stoppers during prolonged storage. However, vials with vacuum-oven dried stoppers had less uptake than vials with untreated stoppers from the packs, which was almost comparable to that of ampoules at 20°C and 45°C. Vials with vacuum-oven dried stoppers and ampoules never exceed more than 1% w/w moisture at all storage temperatures. The moisture content for vials with normal unprocessed stoppers experienced increases above 1.5% w/w but this increase had no effect at 20°C. It was only at 45°C that moisture content in the normal stopper vials had any impact in the loss in potency (being lower than ampoules or vials with vacuum-oven dried stopper). This is consistent with vitrification hypothesis, with normal stopper vials having slightly more reduced (T_g - T) and thus seeing more degradation occur.

6.3.4.1 Stability Study 3: B/Phuket (20% w/w sucrose) Formulation

6.3.4.1.1 Change in characteristics

The previous B/Phuket study trial was based on a formulation with antigen suspended in PBSA buffer containing 1% (w/v) sucrose. The sucrose concentration was increased to 20% w/v in the formulation, in order to determine what effect increasing the excipient mass fraction had on moisture uptake and potency. Table 6.5, highlights the physical attribute changes to such increasing sucrose content. The average dry weight, from 1% w/w sucrose to 20% w/w sucrose formulation, increased by a factor of 6.5 times from 23 mg to 148 mg, respectively. The Young's modulus (i.e the cakes stiffness) increasing by a factor of ≈ 200 times difference from 3 ± 1 KPa to 604 ± 62 KPa. While, the 1% w/w sucrose formulation was described as fragile and "fluffy" like consistency, the 20% w/w formulation on the other hand had a hard and stiff cake. The moisture content after FD for the formulation with 20% sucrose, was dramatically higher than the 1% sucrose formulation. The exact same FD cycle was used for both formulations in order to maintain strict reproducibility. The increased sucrose content resulted in more moisture being trapped in the matrix and thus less moisture being removed in the secondary drying cycle, resulting in higher moisture content values before storage.

Table 6.5 Comparison of average dry weight, Young's modulus, specific surface area and moisture content between different concentration of excipients in antigen formulation (n=3) at T=0 months.

B/Phuket Excipient Formulation	Average dry weight (mg)	Moisture Content (% w/w)	Young's Modulus (KPa)	Specific Surface Area (m^2/g)
1% w/w sucrose	23 ± 0.3	0.13 ± 0.07	3 ± 1	20.56 ± 2.57
20% w/w sucrose	148 ± 0.4	1.67 ± 0.18	604 ± 62	0.57 ± 0.04

Whilst the 1% formulation may be mechanically weaker than the 20% formulation, the fact that it has less moisture present at the start of storage is a major advantage. Table 6.6, shows the critical moisture content at when onset of collapse occurs from data acquired via DVS ramping experiments. Both the 1% and 20% formulation have induced moisture contents of collapse around 1.5-2.0 % w/w at 25°C and from 0.8-1.6% w/w at 45°C. This would mean that the 20% sucrose vials, because of the initial high moisture content, at high storage temperature have a greater chance of T_g transition and induced collapse occurring than the 1% w/w sucrose vials during long term storage.

B/Phuket with 1% w/w sucrose has the higher SSA of ≈ 20 m^2/g. The 1% w/w sucrose formulation has the greatest porosity and appeared as a more 'fluffy' brittle cake. Increasing the sucrose content to 20% w/w decreased the resulting surface area dramatically as would be expected to about ≈ 0.5 m^2/g. FD biologic cakes with lower SSA's could exhibit superior long term stability given that that higher SSA's can contribute to greater chemical degradation [133]. Indeed, Costantino et al. observed a linear relationship between monomer loss and high surface area of spray-dried BSA with trehalose and mannitol formulations [261]. Abdul-Fattah et al. presented how the drying method and formulation not only determined the morphology, SSA, surface composition and protein secondary but also the storage stability [179]. Schersch et al. was able to show that the SSA reduced dramatically for lyophilised sucrose that was initially non-collapsed after 2 weeks of storage at 50°C [115].

Table 6.6 Summary of critical moisture content when induced onset collapse (M_{cp}) occurs for each FD biological material at different temperatures measured with DVS (n=3).

Material	M_{Cp} at 25°C	M_{Cp} at 45°C
Influenza antigen B/Phuket 14/252 (1% w/w sucrose)	4.44 ± 0.40	3.83 ± 0.60
Influenza antigen B/Phuket 14/252 (20% w/w sucrose)	1.80 ± 0.12	1.39 ± 0.32

6.3.4.1.2 Change in moisture content and potency

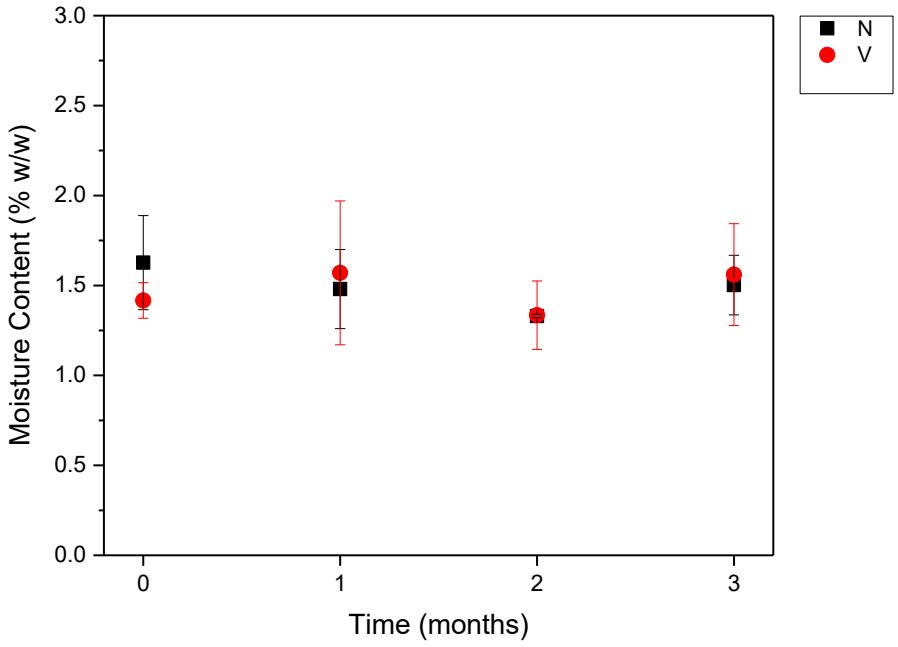


Figure 6.19 Comparison of the moisture content (% w/w) between vials with unprocessed stoppers (N), vials with vacuum-oven dried stoppers (V) and ampoules (A) for B/Phuket (20% w/w sucrose) over a period of 3 months stored at -20°C. Error bars represent ±95% Confidence Intervals (n=3).

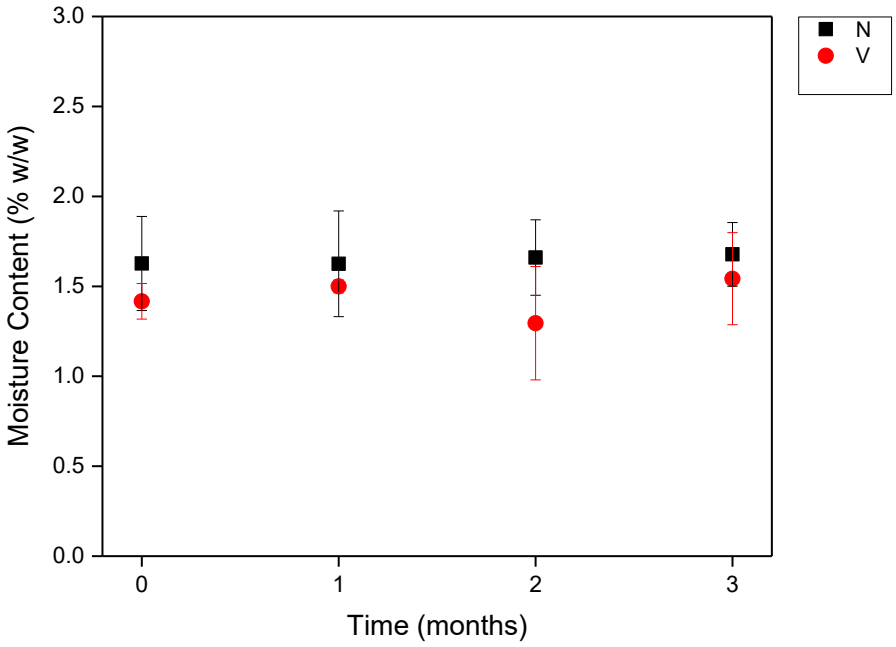


Figure 6.20 Comparison of the moisture content (% w/w) between vials with unprocessed stoppers (N), vials with vacuum-oven dried stoppers (V) and ampoules (A) for B/Phuket (20% w/w sucrose) over a period of 3 months stored at 20°C. Error bars represent ±95% Confidence Intervals (n=3).

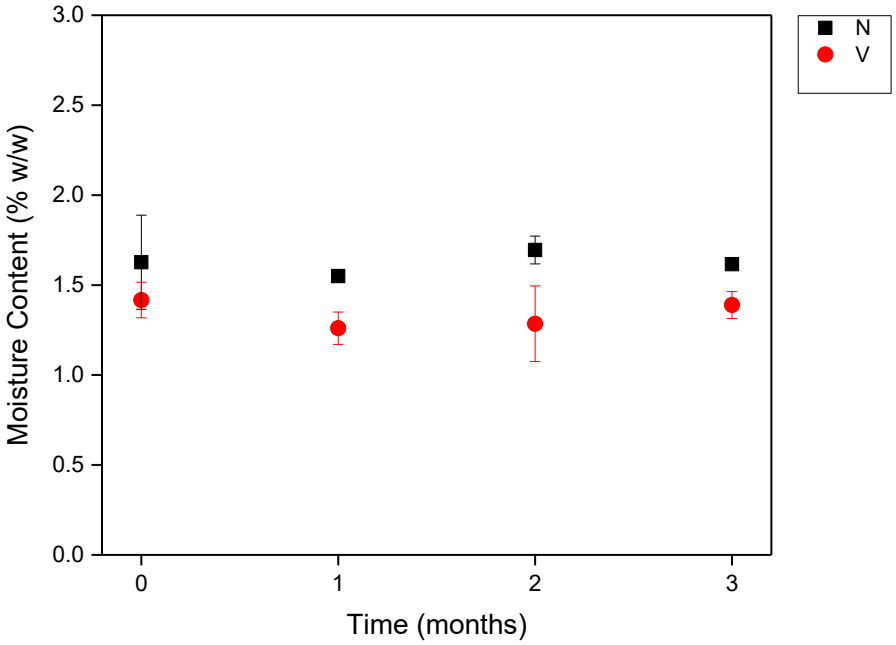


Figure 6.21 Comparison of the moisture content (% w/w) between vials with unprocessed stoppers (N), vials with vacuum-oven dried stoppers (V) and ampoules (A) for B/Phuket (20% w/w sucrose) over a period of 3 months stored at 45°C. Error bars represent ±95% Confidence Intervals (n=3).

Figures 6.19 - 6.21 shows the moisture content of the FD B/Phuket cakes (20% w/w sucrose) over a three month storage period at -20°C, 20°C and 45°C. Since the moisture content of the vials was already at high to begin with, there does not appear to be any considerable moisture increase over the 3 months for both stopper types. This is in stark contrast to the previous case study where the initially dry low moisture cakes saw a large uptake after 3 months at elevated storage temperatures. There is a chance that these vials have reached close to maximum equilibrium moisture content and as a result there is very little uptake as a result from the stoppers.

Figure 6.22 Partial cake collapse observed in for vials with both vacuum and unprocessed stoppers after 3 month's storage at 45°C for both vials with treated and untreated stoppers.

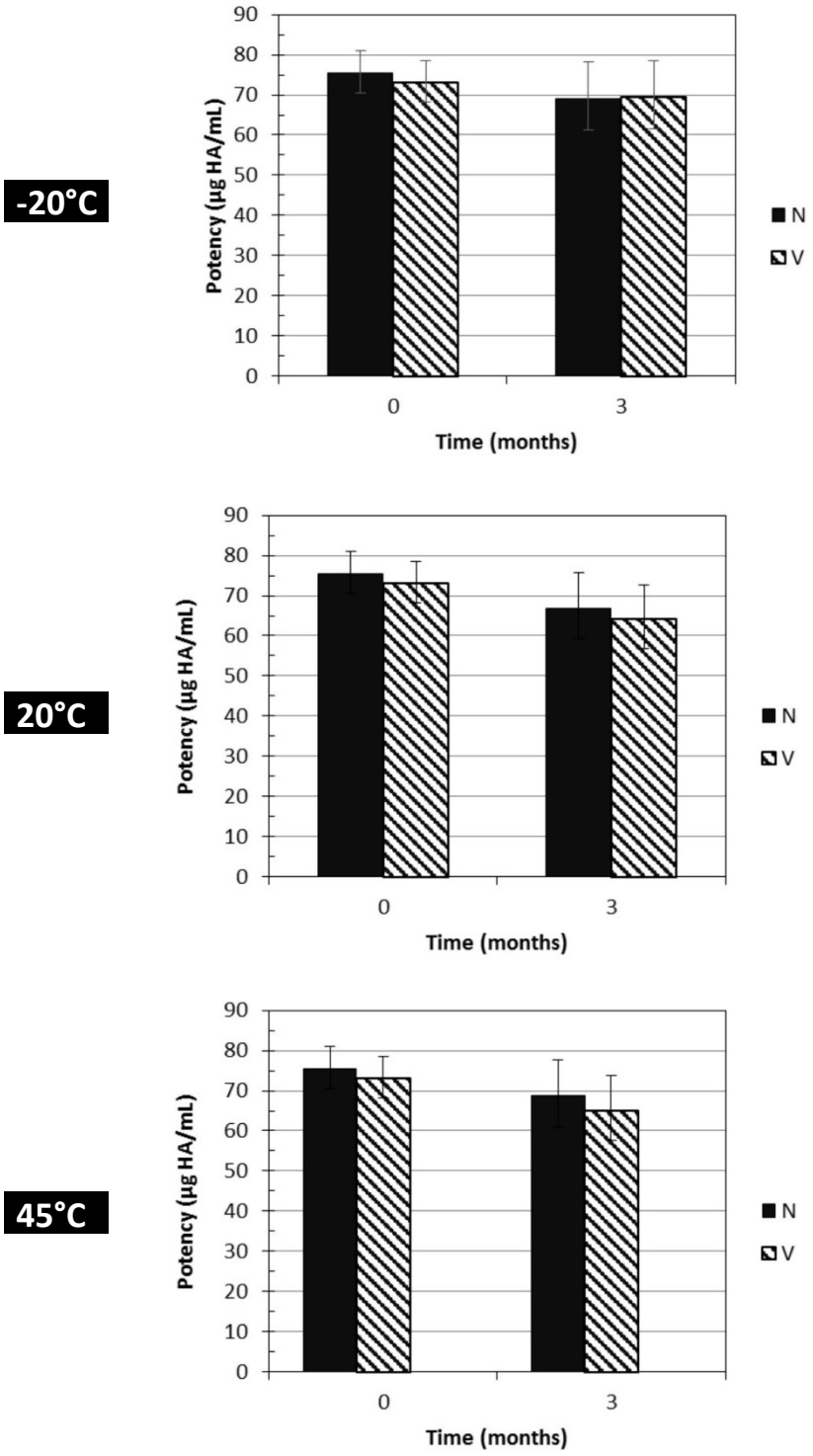


Figure 6.23 Comparison of the potency (µg HA/mL) of B/Phuket 20%w/w sucrose, between vials with unprocessed stoppers (N) and vials with vacuum-oven dried stoppers (V) over a period of 3 months stored at -20˚C, 20˚C and 45°C. Error bars represent ±95% Confidence Intervals.

Figure 6.22 shows images of partial collapse of FD B/Phuket 20% w/w cakes occurring at 45°C for both vials with unprocessed and vacuum-oven dried stoppers. As evidenced from the DVS data, the moisture content of the B/Phuket 20% w/w vials was sufficiently high enough for some vials to experience partial collapse at 45°C storage. The moisture content remained stable over the storage months. Only the shift in storage temperature at 45°C was enough to induce collapse, as it was not observed at lower storage temperatures. DSC results showed that the average T_g was close to the storage temperature (Table 6.7). However, when comparing the potency there appeared to be no significant difference at all storage temperatures after 3 months (Figure 6.23). This is especially surprising for the partially collapsed samples at 45°C, as it appeared to have no effect on stability. This is in contrast to the previous study with 1% sucrose formulation, where vials with higher moisture at elevated storage temperature experienced a loss in potency. The vials retain stability even in the face of such structural collapse. Similarly, a study by Schersch et al. showed that not only did full protein stability get retained for FD proteins that had collapsed, but in some cases they also showed greater stability than non-collapsed samples [116]. They speculated that the reason might be down to collapsed lyophilizates showing increased structural relaxation times, which translated into a greatly reduced global molecular mobility. These explanations for the results seen here could be related to theory of stability in dry state (combined glass dynamics and water replacement theories). The increase in sucrose excipient from 1% to 20% meant that there was greater hydrogen bonds occurring and more rigid glassy matrix limiting molecular mobility, even though the (T_g - T) gap had been reduced so low, which according to vitrification hypotheses should have resulted in potency losses. Such elevated moisture content at above 1.5% w/w, did not seem to have any direct impact on the potency but rather only on the visual and structural appearance at elevated temperatures. This shows how a change in excipient concentration formulation could result in unforeseen consequences but could also provide positives as well. The vials with increased moisture do not seem to suffer from moisture ingress and appear as stable as a very dry sample would be.

Table 6.7 Comparison of storage temperature, moisture content (n=3) and glass transition temperature (n=2) after storage time of 3 months.

Format	T, Storage Temperature (°C)	Moisture Content (% w/w)	T_g (°C)	T_g -T (°C)
N	-20	1.50 ± 0.17	51	71
V	-20	1.56 ± 0.28	50	70
N	+20	1.68 ± 0.18	51	31
V	+20	1.54 ± 0.25	46	26
N	+45	1.62 ± 0.01	46	1
V	+45	1.39 ± 0.07	49	4

6.3.4.1.3 Summary

The change in sucrose excipient concentration from 1% to 20% w/w in flu antigen formulation resulted an in increase to Young's modulus and moisture content but also decrease in SSA and critical moisture content for collapse. All samples had initially high moisture content above 1.5% w/w and remained which steady with no ingress even after 3 months storage at -20°C, 20°C and 45°C regardless of stopper type vial. Samples at 45°C experienced partial cake collapse and shrinkage. However, potency data indicated no significant difference between all samples regardless of collapsed state, storage temperature or moisture content. This would imply that the increased sucrose excipient was sufficient to provide some sort of protective coverage to hinder any instability. However, for commercial reasons the visual appearance of collapsed cake in vials could be unmarketable due to customer expectations, even though arguably there was no potency difference between collapsed and non-collapsed lyophilizates.

6.4 Conclusion

The Chapter has demonstrated the efficacy of vacuum-oven drying stoppers in order to reduce long term moisture ingress during storage for low mass samples. Vacuum drying stoppers results in significantly reduced moisture in final product compared to vials with unprocessed stoppers up to a 12 month storage period. Whilst ampoules were found to be the overall best format in maintaining the least amount of moisture during long term storage, vials with vacuum dried stopper performed comparatively as good and can be considered a suitable alternative for storing FD antigens. Evidence was found to further support the vitrification hypothesis to describe the stabilization mechanisms observed. For B/Phuket 1% at -20°C and 20°C, moisture content had no effect on potency, whilst as elevated temperatures it was associated with greater drops in potency. This correlated with a drop in T_g close to the elevated storage temperature and suggests that critical importance of the role of water in stability. Increasing concentration of the sucrose excipient for B/Phuket (20% w/w) resulted in significantly stiffer cakes and lower SSA, but resulted in a higher moisture content from FD cycle. The increased moisture content at higher storage temperatures resulted in collapse of some cakes at 45°C, suggesting that the T_g threshold value had been crossed as corroborated by DVS collapse data. However resulting high moisture content in high concentration excipient cakes had no impact in the potency at all temperatures, suggesting the increasing excipient concertation provided sufficient protection for stability. Overall, in particularly for small mass FD products such as flu antigen standards, formulation excipient concentration, storage temperature, and ultimately moisture content are critical factors to reflect on when considering the long terms stability of a product.

Chapter 7. Conclusions and Future Work

7.1 Summary

The project aimed to investigate how key factors such as moisture content and storage temperature affected the long term storage stability of FD biologics. The relationship between moisture content, morphology and the stability of model proteins/antigens during long term storage (12 months) was investigated. Novel techniques and procedures were used to measure the effects of moisture and storage temperature in FD material. In Chapter 3, a Dynamic Vapour Sorption (DVS) instrument was used to measure the water uptake of FD materials at different relative humidities. In conjunction with a real time video-camera attached to the DVS, visible collapse/shrinkage of materials was able to be recorded and analysed to provide stability maps with critical moisture content levels not to exceed in order to prevent collapse and retain structural integrity. In Chapter 4, additional novel techniques were introduced to measure morphological changes in regards to changes with moisture and temperature. Mechanical properties of the FD materials were measured with a flat punch indenter as well as using inverse gas chromatography (IGC) to measure the specific surface area (SSA) of materials, respectively. Increases in moisture content resulted in loss of Young's modulus and decreases in SSA. Typically increasing the protein/excipient concentration resulted in stiffer cakes and a decrease in SSA. FD material such as high concentration IgG's had high Young's modulus but low SSA's (<below $2m^2/g$), whilst other FD material such influenza antigens had higher SSA's (up to 20 - 30 m^2/g) but with low Young's modulus and were more fragile. Increases in moisture content resulted in reduction of SSA of FD materials, which could be measured with IGC-SEA instrument only. In Chapter 5, typical long term stability trials were conducted with high concentration IgG using typical assay methods such as HPLC or ELISA, as well as utilising novel techniques to provide greater information on stability over storage. The investigation also focused on the bell-shaped curve hypothesis with regards to whether an optimum moisture content applies and how this might be influenced by formulation. For high concentration immunoglobulin (50 mg/mL), moisture content up to 5% w/w was found not

to have any significant effect on stability from -20°C to 20°C. Additionally, over-drying due to a conservative FD cycle, could lead to reduced stability at higher temperatures compared to vials with moderate moisture of around 3% w/w. Only at higher stress temperatures of 45°C, did moisture content possibly play a role in stability in regards to vitrification hypothesis and how close the storage temperature is to the glass transition of the material (T_g - T). Increased flexibility results in more degradation occurring. Lastly, moisture ingress is another concern for FD material in rubber stoppered vials, and ingress was also observed in both FD IgG and influenza antigen studies over prolonged storage. In particular for low weight mass material such as FD antigens, moisture ingress can have impact on storage stability, especially at elevated temperatures. In Chapter 6, the closure storage format of FD material was explored and found that vials with vacuum-oven dried stoppers had less moisture ingress than vials with unprocessed stoppers. Vacuum-oven drying stoppers can be an effective procedure for minimising moisture ingress during long term storage. Vials with vacuum-oven dried stoppers had less moisture content then vials with normal “straight from the pack” stoppers at 20°C and 45°C. Overall, both moisture and storage temperature can have an effect on long term stability. There may be permissible moisture content at each storage temperature on a case by case basis for FD material. To summarise, this study promoted the adoption of novel analytical techniques as well as building on upon knowledge of how moisture effects activity for a range of real world relevant of FD biologics to inform industry and improve long term storage stability**.**

The observations summarised in this thesis complement and extend significantly current knowledge on long term stability in regards to moisture and temperature during storage. The ongoing debate in regards to how much water is permissible in FD products is one that still seems to polarise the community. Again we have seen evidence for both postulated mechanisms of stabilisation processes. There was no one unifying theory that could explain all the different stabilisations observed. Moisture content should be thought of in the context for each FD material at each storage temperature, rather than one all-encompassing rule. Of course, one could play it safe and try to have as low moisture as possible but that can sometimes also not result in the

optimum stability during storage as shown for FD IgG in Chapter 5 with over-drying FD cycle. Acceptable moisture to allow ambient temperature storage and still maintain long term stability will depend on case by case basis. At high storage temperatures, which may be experienced when shipping for global distribution, one should be wary of even small amounts of moisture content which could reduce the T_g by enough to cause structural stability problems. However even in some cases, although high moisture could result in collapse it might not affect activity as seen with the high sucrose concentration flu antigens that collapsed but still retained similar potency levels to non-collapsed cakes in Chapter 6. As shown, one way to help maintain low moisture content at such high storage temperatures, is via the employment of simple procedure of vacuum drying stoppers before FD, which could significantly reduce the long term moisture uptake for all FD materials. Novel techniques introduced and described in this thesis in Chapters 3 and 4 allows for also monitoring of physical and structural changes due to moisture and storage conditions. Figure 7.1 shows an example schematic outlining the view of how they can be incorporated help become commonly adapted techniques when undertaking stability trials. These complimentary techniques can add vital new information that can also possibly be used for quality control and formulation design.

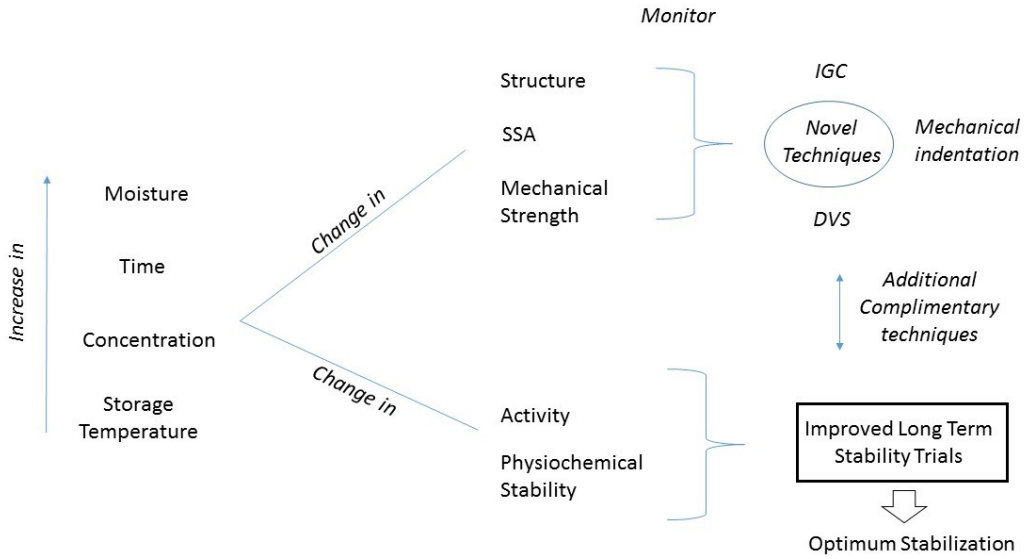


Figure 7.1 Schematic representation of using complimentary novel techniques to enhance long term stability trials in addition to traditional characteristics in order to improve product stability for FD biologics.

7.3 Future Work

In addition to the work presented in this thesis, there remains several key areas for much further exploration that could yield further interesting information.

7.3.1 Water sorption with DVS

- There still remains avenues to pursue in regard to mapping out moisture stability maps for a wider range of FD biologics.
- Additional work and perhaps modelling can be applied in regards to moisture uptake and changes in physical structure
- The question of whether at the critical moisture of induced collapse, whether the collapse happens unilaterally or not. Since the technique used in this thesis was based on 2-D cross sectional area, from the camera viewpoint, there may be room to explore option if a camera can be fitted over head the sample pan and see from the top down view of collapse or shrinkage.
- Also the question whether onset of collapse is affected by sample history during prolonged time scales and long term storage, could be further explored.

7.3.2 Mechanical Indenter and IGC

- More studies should be done to try and link changes with mechanical properties with and SSA for a range of FD biologics during storage. Additional work and perhaps modelling can be applied in regard to moisture uptake and changes in physical structure with regards to SSA. In combination to 95% confidence intervals, t-testing and p-values could be used to better statistically assess the differences between samples.
- Efforts to establish some sort of standardisation with regards to measuring the SSA of FD material in regards to crushing of materials should be pursued.

- Different column conditioning steps and times could be used to further map out effects of humidity on SSA

7.3.3 Optimum Moisture in FD biologics

- A study for a range of proteins should be done in order to investigate the effects such as if a correlation between molecular weight/size and moisture content. This would involve using stability trials with set different moisture contents over 12-month period.
- Future studies could investigate other high concentration proteins biologics and investigate to see the effects of moisture content compared to lower concertation material. Monoclonal antibodies if available in sufficient quantities for study would be the logical extension of the approach adopted here.
- Similarly others could investigate the maximum moisture content permitted for different strains of flu antigens and whether there might be acceptable up to room temperature storage without sacrificing potency. This of course would not be a general rule for all and will be dependent on a case by case basis for each FD antigen material.
- Similarly other high concentration protein biologics should be investigated to see the effects of moisture content compared to lower concertation material.
- The effect of leachable and contaminations from rubber stoppers and vacuum oven effects should be investigated. It would also look at if there are any issues related to passing stringent regulatory bodies.
- Additional further studies could also look at the influence of different sizes, rubber formulations and comparison of different drying treatments of rubber stoppers mapping their corresponding effects on long term moisture uptake and stability.

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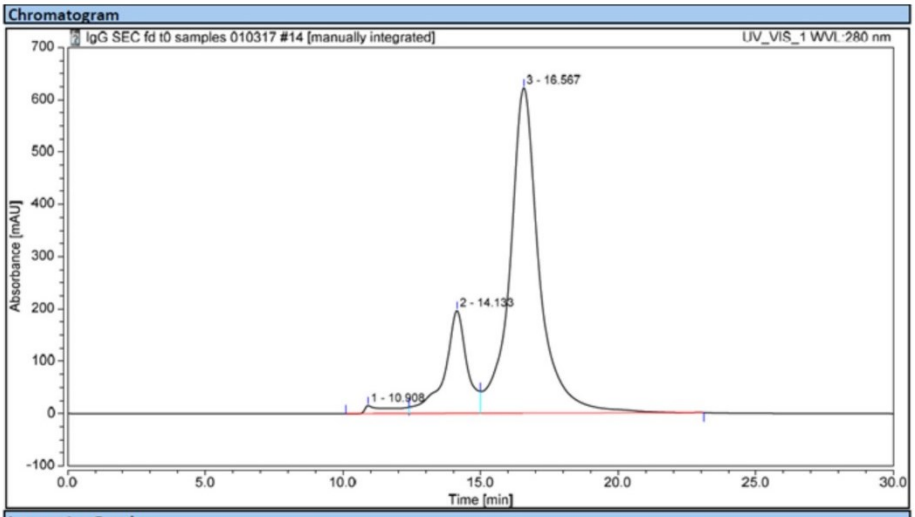
Appendix

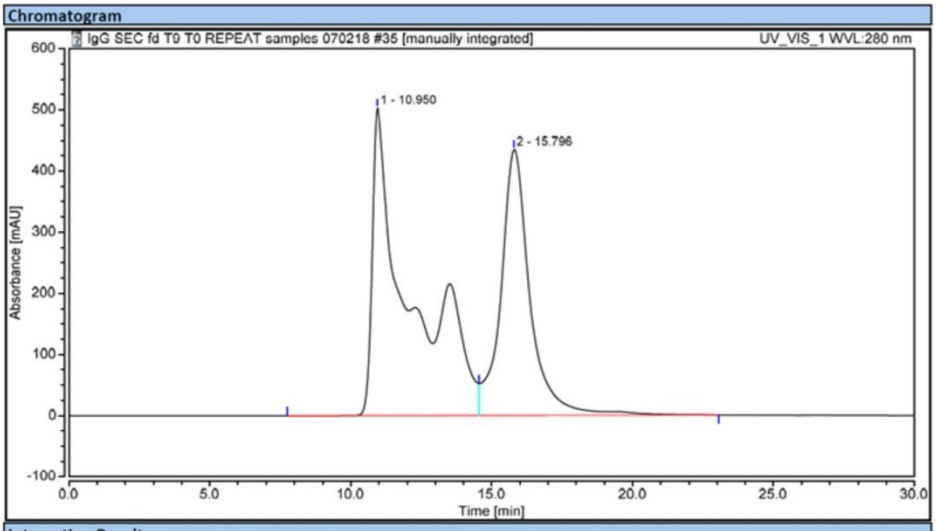
A



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount
1		10.908	17.964	15.345	1.84	1.84	n.a.
2		14.133	187.138	196.158	19.21	23.53	n.a.
3		16.567	769.226	622.221	78.95	74.63	n.a.
n.a.	Main peak	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Total:			**974.327**	**833.724**	**100.00**	**100.00**	

B



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount
1		10.950	766.720	503.175	59.55	53.63	n.a.
2		15.796	520.857	435.121	40.45	46.37	n.a.
n.a.	Main peak	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Total:			**1287.577**	**938.296**	**100.00**	**100.00**	

Figure A.1 Typical SEC-HPLC chromatograms of FD IgG 200 mg/mL at **A:** before FD and **B:** After 12 months storage.