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# Optimization of freeze-drying process

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# Optimization of freeze-drying process

The thesis work was conducted in collaboration with HyTest Ltd which develops and manufactures immunological reagents for the in vitro diagnostics industry and research community. The goal of the thesis was to optimize the freeze-drying process for the immunological reagents with a SP VirTis Advantage Pro freeze dryer.

In a freeze-drying process, the frozen products are stabilized by removing water by sublimation and desorption. The goal is to achieve a moisture level which inhibits and decelerates chemical degradation reactions. Freeze drying improves the stability and shelf life of products and provides easy handling during shipping and storage.

Optimization of the freeze-drying process was carried out by performing test runs with the new freeze dryer. The test runs included freeze drying of sample solution in 3 ml and 10 ml glass vials with different process parameters. The process parameters were changed between the test runs based on the data recorded by the freeze dryer.

The goal of the thesis was achieved and a new procedure for freeze drying of 3 ml vials was created. More test runs must still be performed especially with 10 ml vials and with actual products.

Keywords:

freeze drying, supercooling, sublimation, desorption, stability

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# Kylmäkuivausprosessin optimointi

Opinnäytetyö tehtiin yhteistyössä HyTest Ltd:n kanssa, joka kehittää ja valmistaa immunologisia reagensseja IVD-teollisuudelle ja tutkimuskäyttöön. Opinnäytetyön tavoitteena oli optimoida immunologisten reagenssien kylmäkuivausprosessi SP VirTis Advantage Pro -kylmäkuivaimella.

Kylmäkuivauksessa jäädytetyt tuotteet stabiloidaan poistamalla niistä vettä sublimaatiolla ja desorptiolla. Tavoitteena on saavuttaa alhainen kosteuspitoisuus, joka estää ja hidastaa kemiallisia hajoamisreaktioita. Kylmäkuivaus parantaa tuotteiden stabiiliutta ja säilyvyyttä sekä helpottaa niiden käsittelyä kuljetuksen ja varastoinnin aikana.

Kylmäkuivausprosessi optimoitiin suorittamalla koeajoja, jotka sisälsivät näyteliuoksen kylmäkuivausta 3 ml:n ja 10 ml:n lasisissa injektiopulloissa erilaisilla prosessiparametreilla. Prosessiparametreja muutettiin koeajojen välillä kylmäkuivaimen tallentamien tietojen perusteella.

Opinnäytetyön tavoite saavutettiin ja uusi menetelmä luotiin 3 ml:n injektiopullojen kylmäkuivaukseen. Koeajoja on kuitenkin tehtävä vielä lisää etenkin 10 ml:n injektiopulloilla ja oikeilla tuotteilla.

Asiasanat:

kylmäkuivaus, alijäähdytys, sublimaatio, desorptio, stabiilius

# Content

**1 Introduction** **7**

**2 Freeze drying** **9**
2.1 Freezing 10
2.2 Primary drying 12
2.3 Secondary drying 15
2.4 Backfill and stoppering 16
2.5 Properties of freeze-dried products 17

**3 Equipment of freeze dryer** **19**
3.1 Refrigeration system 19
3.2 Product chamber 20
3.3 Condenser 20
3.4 Vacuum pumping system 20
3.5 Control system 21
3.6 Types of freeze dryers 21

**4 Optimization of freeze-drying process** **24**
4.1 SP VirTis AdVantage Pro freeze dryer 24
4.2 Background 25
4.3 Freeze drying of 3 ml vials. 29
4.4 Freeze drying of 10 ml vials. 38
4.5 Freezing on the shelves 43

**5 Results and conclusions** **48**

**References** **51**

# Figures

Figure 1. Phase diagram of water (More 2020). 9
Figure 2. Vapor pressure of ice (Barley 2021). 14
Figure 3. A general layout of a freeze dryer (Vikumer n.a.). 19
Figure 4 Manifold freeze dryer (ATO 2023). 22
Figure 5 Shelf freeze dryer (SciTech 2023). 23
Figure 6. SP VirTis Advantage Pro Freeze Dryer. 25
Figure 7. Expected and real behaviour of the shelf temperature. 27
Figure 8. Collapsed sample. 27
Figure 9. Shelf and average probe temperature during the manual freeze drying. 29
Figure 10. Shelf and average probe temperature during Program #1. 31
Figure 11. Freeze-dried 3 ml vials after Program #1. 31
Figure 12. Shelf and average probe temperature during Program #2. 33
Figure 13. Freeze-dried 3 ml vials after Program #2. 33
Figure 14. Behaviour of the shelf temperature during the modified recommended program. 34
Figure 15. Shelf and average probe temperature during Program #3. 36
Figure 16. Freeze-dried 3 ml vials after Program #3. 36
Figure 17. Shelf and average probe temperature during Program #4. 37
Figure 18. Freeze-dried 3 ml vials after Program #4. 38
Figure 19. Cracked 10 ml vials after freezing with liquid nitrogen. 38
Figure 20. Shelf and average probe temperature during Program #1. 39
Figure 21. Freeze-dried 10 ml vials on the tray A after Program #1. 40
Figure 22. Freeze-dried 10 ml vials on the tray B after Program #1. 40
Figure 23. Cracked 10 ml vials on the tray B after Program #1. 41
Figure 24. Shelf and average probe temperature during Program #5. 43
Figure 25. Freeze-dried 10 ml vials after Program #5. 43
Figure 26. Shelf and average probe temperature during freezing 8 pieces of 10 ml vials. 44

Figure 27. Shelf and average probe temperature during freezing 16 pieces of 10 ml vials. 45
Figure 28. Shelf and average probe temperature during freezing 14 pieces of 3 ml vials. 46
Figure 29. Shelf and average probe temperature during freezing 20 pieces of 3 ml vials. 47

## Tables

Table 1. Recommended program. 26
Table 2. Manual freeze drying. 28
Table 3. Program #1 for the freeze drying. 30
Table 4. Program #2 for the freeze drying. 32
Table 5. Recommended program after modifying. 34
Table 6. Program #3 for the freeze drying. 35
Table 7. Program #4 for the freeze drying. 37
Table 8. Program #5 for the freeze drying. 42
Table 9 Results of the test runs. 49

# 1 Introduction

The thesis work was conducted in collaboration with HyTest Ltd. HyTest has been developing and manufacturing immunological reagents for the IVD industry and research community over 25 years. HyTest provides and supplies products for several clinical and research areas including monoclonal and polyclonal antibodies, antigens, plasma and serum. (HyTest Ltd 2023). HyTest has been using freeze drying for its antigen products but because the company has grown over the past few years, the production capacity and the need for freeze-dried products have also increased. In order to freeze dry a larger number of products, a new freeze dryer was purchased. The main goal of the thesis was to find an optimal freeze-drying process with the new SP VirTis AdVantage Pro freeze dryer.

Freeze drying is used in various industries and applications. Well-known examples of freeze drying are freeze-dried food products such as berries or backpacking meals, but the most extensive use of freeze-drying process is in the healthcare industry. (Jennings 2008.) It is used as a part of the manufacturing process of pharmaceuticals and immunological reagents which are unstable in the solution state. In the freeze-drying process, water is removed from the products under a vacuum, which inhibits and decelerates chemical degradation reactions. Freeze drying improves the stability and shelf life of the products, but it also provides easy handling during shipping and storage. (Tang & Pikal 2004.)

The company has used an internal standard procedure for freeze drying. The products have first been frozen in glass vials submerged in liquid nitrogen, and then freeze dried with a manifold freeze dryer. Dried product vials have been closed manually and stored at -20 °C. A few test runs had been carried out on the new freeze dryer, but they had failed due to several problems with the performance of the device. In this study, new test runs were performed by following the standard procedure. The samples were freeze dried in glass vials of different volumes by using SP VirTis AdVantage Pro freeze dryer. Some of

the vials cracked because they did not tolerate freezing with liquid nitrogen so the vials were frozen on the shelves in some test runs. In this study, the problems with the performance of the device were solved and a new procedure for freeze drying was created.

## 2 Freeze drying

Freeze drying, also known as lyophilization, is a process to achieve products with long-term stability. Freeze drying is especially used as a method in pharmaceutical manufacturing processes. About half of all pharmaceuticals are freeze dried because it inhibits and decelerates chemical and physical degradation reactions. (Kasper & Friess 2011.) In freeze drying, the frozen product is stabilized by reducing the quantity of the solvent. The solvent is usually water and it is removed by sublimation and desorption to moisture levels which will not support biological growth or any chemical reactions. (Jennings 2008.)

Freeze drying consists of three steps: freezing, primary drying, and secondary drying. The product must first be frozen because the free water in the product is removed by sublimation during primary drying. Sublimation is a process where a frozen liquid transits directly from solid phase to the vapor phase without passing through the liquid phase (Figure 1). After sublimation the residual bound water in the product is removed by desorption during secondary drying. Desorption is a process where the adsorbed water is released from the surface of the product. (Labconco Corporation 2010.)

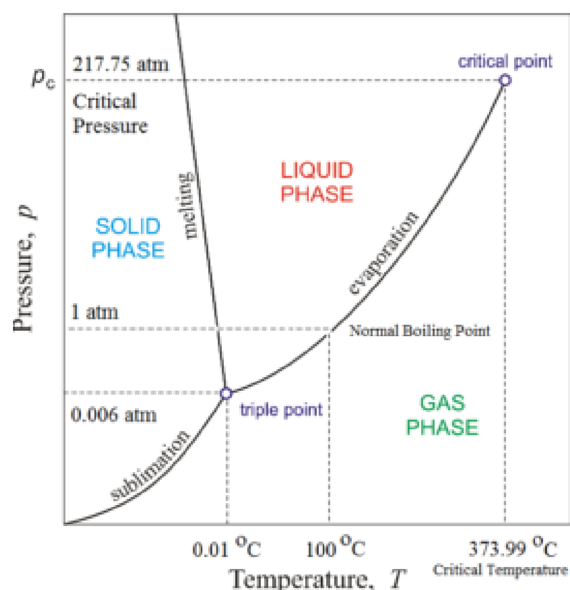


Figure 1. Phase diagram of water (More 2020).

## 2.1 Freezing

Freezing is the most complex and critical step in freeze drying and it affects both process performance and product quality. In freezing, water is separated from solutes when it changes from the liquid phase to the solid phase. The solutes arrange themselves in the space formed between the ice crystals, which leads to concentration of the solutes. Cooling and freezing rate have an effect on the ice nucleation and the size of ice crystals has an effect on the morphology of the frozen product. The morphology of the product is directly correlated with the efficiency of sublimation during primary and secondary drying, which further affects the final properties of freeze-dried product. The freezing method and rate may also have an effect on the biological activity of the active pharmaceutical ingredients. (Kasper & Friess 2011.)

Water does not freeze spontaneously at its freezing point at atmospheric pressure. Freezing is the process of ice crystallization from supercooled water. Supercooling means that water is cooled below its freezing point without it becoming a solid. The degree of supercooling is defined as the difference between the thermodynamic ice formation temperature and the actual temperature at which ice crystals first form. The degree of supercooling depends on the solution properties and process conditions. The higher the degree of supercooling, the higher the rate of nucleation and the faster the rate of freezing. A high degree of supercooling results in a high number of small ice crystals and a low degree of supercooling results in a low number of large ice crystals. The larger the ice crystals, the faster the drying. (Kasper & Friess 2011.)

Products can freeze in two ways depending on the composition of the products. Most of the products which are intended to be freeze dried consist primarily of water (solvent) and the other substances (solutes) are dissolved in it. It is common that the product is eutectic which means that the other substances have a lower freezing point than water. When the product is cooled, the water is separated from the solutes as it freezes, which creates concentrated areas of

solutes. These areas freeze at a lower temperature than the water, and the temperature depends on the concentration of the solutes. The temperature at which all the solutes are frozen the eutectic temperature and the product is completely frozen at this temperature. Eutectic products have a sharp transition between the liquid and solid phases. (Labconco Corporation 2010.) There are also products whose viscosity increases with decreasing temperature and the product becomes rigid at a certain temperature. These products are called noncrystalline or amorphous. Amorphous products undergo glass transition during the freezing and the temperature at which they will become rigid is termed the glass transition temperature. (Ó´Fagáin 2004.)

The freezing method depends on the type of freeze dryer. A manifold freeze dryer only consists of a vacuum pump unit attached with a manifold for the freeze drying of multiple flasks so the vials must be frozen in separate equipment. They can be frozen in a freezer or in a shell bath or even with liquid nitrogen. In a shell bath, the vials to be frozen are rotated so that the product freezes on the walls of the vials. Shell bath freezing maximizes the product surface area and minimizes its thickness. If a thick layer of product is frozen to the bottom of the vial, the efficiency of water removal decreases and there is a risk of vial breakage due to uneven stress. (Barley 2021.)

In a shelf freeze dryer, there is a capability to freeze the product on the shelves of freeze dryer. The product can be loaded in vials or in bulk form directly onto a tray. With a shelf freeze dryer, the cooling rates can be controlled precisely to achieve a certain freezing rate and crystal size. (Barley 2021.) There are two freezing methods that can be used with the shelf freeze dryer: shelf-ramped freezing and the pre-cooled shelf method. In shelf-ramped freezing the vials are placed on the shelf and the shelf temperature is decreased linearly (from 0.1 °C/min to 5 °C/min). In the pre-cooled shelf method, the vials are placed on the shelf which has been cooled to the wanted shelf temperature (e.g., -40 °C). The pre-cooled shelf method results in higher nucleation temperature but slower freezing rate than the shelf-ramped freezing. Freezing with liquid nitrogen is a quench freezing method in which the vials are immersed into liquid nitrogen (ca.

-200 °C) until they are completely frozen. Quench freezing results in a low degree of supercooling and a high freezing rate. (Kasper & Friess 2011.)

In general, freezing may cause stresses to proteins which may weaken their stability. During freezing, the amount of liquid water in the product decreases, which leads to an increase in the concentrations of the proteins. The increase in protein concentration may lead to aggregation of proteins and other harmful reactions. Crystallization of buffer salts may change the pH value of the product, which may cause protein denaturation. If the freezing is too slow, it may cause more damage to proteins due to phase separation. Phase separation may separate protein and stabilizer from each other, which has a direct effect on the stability of the product. Slow freezing may also increase degradation reactions of proteins because it prolongs the time the proteins are in concentrated areas. To minimize destabilizing stresses to proteins, the supercooling should be reduced but at the same time the freezing should be performed as quickly as possible. (Tang & Pikal 2004.)

Before starting the freeze-drying process, it is important to keep the product below its eutectic or the glass transition temperature long enough to ensure proper freezing. If the product is not completely frozen, it may expand outside of the container when the vacuum is applied. (Barley 2021.)

## 2.2 Primary drying

The main purpose of primary drying is to remove a major amount of water from a frozen product. When the product has completely frozen, the pressure in the freeze dryer is decreased and the temperature is increased to start the sublimation process. During sublimation the water evaporates from the product and the condenser collects the released water vapor. (Jennings 2008.)

Sublimation requires heat energy for the phase change from solid to gas. Method of heat transfer depends on the used freeze dryer. In a manifold freeze dryer the heat is transferred to the product through convection and radiation from the surrounding environment but in a shelf freeze dryer the heat is

transferred to the product through conduction. The vials on the edges of the shelf and near the chamber door are drying faster than the other vials in the centre because there are radiant heat coming from the inside walls of the product chamber. (Barley 2021.)

Primary drying should be performed below the critical collapse temperature of the product. Critical collapse temperature is the maximum temperature that the product can withstand without melting or collapsing. However, the product temperature should be as close as possible to the critical collapse temperature to decrease the time which primary drying will take. The temperature difference between the product temperature and the critical collapse temperature is called the temperature safety margin and it usually is 2-5 °C. (Barley 2021.)

The product temperature has a huge effect on the rate of sublimation because only one degree rise can increase the rate of sublimation by 13 %. The filling volume of the vials and configuration of the product also affects the rate of sublimation. Because the drying occurs from the top of the product, the drying becomes more difficult as it proceeds because the water molecules have to travel longer through the dried parts of the product. The greater the ratio of the surface area to the volume of the product, the faster the drying is. The ratio can be increased by rotating the vials during freezing so that the product would freeze on the walls of the vials. If a thick layer of product is frozen to the bottom of the vial, the ratio decreases (Labconco Corporation 2010.)

The rate of sublimation also depends on the difference in vapor pressure of the product and the condenser. The vapor pressure of liquid is the pressure at which the gas phase is in equilibrium with the liquid phase at a certain temperature and in a closed container. When the vapor pressure of liquid is reached, the molecules are evaporating and condensing at the same rate. (USGS Water Science School 2018.) The vapor pressure is related to temperature and the lower the temperature the lower the vapor pressure. Because water molecules have a natural affinity to migrate from the higher pressure area to the lower, it is important that the condenser temperature is significantly colder than the product temperature. (Labconco Corporation 2010.)

The system pressure should be set by following the vapor pressure of ice (Figure 2). An appropriate system pressure is 20-30 % of the vapor pressure of ice at the target product temperature. Typical system pressures for freeze-drying are between 50mTorr (0.0667 mbar) and 300mTorr (0.4000 mbar). When the system pressure has been set, the shelf temperature is increased until the target product temperature is reached. (Barley 2021.)

| Temp | Vapor Pressure | | | Temp | Vapor Pressure | | |
|---|---|---|---|---|---|---|---|
| [°C] | [milliTorr] | [milliBar] | [pascal] | [°C] | [milliTorr] | [milliBar] | [pascal] |
| 0 | 4,584.000 | 6.1115 | 611.148 | -46 | 48.000 | 0.0640 | 6.399 |
| -2 | 3,883.000 | 5.1769 | 517.689 | -48 | 37.700 | 0.0503 | 5.026 |
| -4 | 3,281.000 | 4.3743 | 437.429 | -50 | 29.500 | 0.0393 | 3.933 |
| -6 | 2,765.000 | 3.6864 | 368.635 | -52 | 23.000 | 0.0307 | 3.066 |
| -8 | 2,325.000 | 3.0997 | 309.974 | -54 | 17.900 | 0.0239 | 2.386 |
| -10 | 1,949.000 | 2.5984 | 259.845 | -56 | 13.800 | 0.0184 | 1.840 |
| -12 | 1,630.000 | 2.1731 | 217.315 | -58 | 10.600 | 0.0141 | 1.413 |
| -14 | 1,359.000 | 1.8118 | 181.185 | -60 | 8.100 | 0.0108 | 1.080 |
| -16 | 1,130.000 | 1.5065 | 150.654 | -62 | 6.160 | 0.0082 | 0.821 |
| -18 | 936.800 | 1.2490 | 124.896 | -64 | 4.660 | 0.0062 | 0.621 |
| -20 | 774.400 | 1.0324 | 103.245 | -66 | 3.510 | 0.0047 | 0.468 |
| -22 | 638.200 | 0.8509 | 85.086 | -68 | 2.630 | 0.0035 | 0.351 |
| -24 | 524.300 | 0.6990 | 69.901 | -70 | 1.960 | 0.0026 | 0.261 |
| -26 | 429.400 | 0.5725 | 57.248 | -72 | 1.450 | 0.0019 | 0.193 |
| -28 | 350.500 | 0.4673 | 46.729 | -74 | 1.060 | 0.0014 | 0.141 |
| -30 | 285.100 | 0.3801 | 38.010 | -76 | 0.780 | 0.0010 | 0.104 |
| -32 | 231.200 | 0.3082 | 30.824 | -78 | 0.570 | 0.0008 | 0.076 |
| -34 | 186.800 | 0.2490 | 24.905 | -80 | 0.410 | 0.0005 | 0.055 |
| -36 | 150.300 | 0.2004 | 20.038 | -82 | 0.290 | 0.0004 | 0.039 |
| -38 | 120.600 | 0.1608 | 16.079 | -84 | 0.210 | 0.0003 | 0.028 |
| -40 | 96.300 | 0.1284 | 12.839 | -86 | 0.150 | 0.0002 | 0.020 |
| -42 | 76.700 | 0.1023 | 10.226 | -88 | 0.100 | 0.0001 | 0.013 |
| -44 | 60.800 | 0.0811 | 8.106 | -90 | 0.072 | 0.0001 | 0.010 |

Figure 2. Vapor pressure of ice (Barley 2021).

There are many methods to determine the end of primary drying and the most common method is to monitor the product temperature with thermocouple probes. The product temperature is lower than the shelf temperature during primary drying because the heat from the shelf is consumed in sublimation phase change. When all the ice crystals have sublimated, the product temperature starts to increase. In the end of primary drying the product temperature increases steeply and reaches the shelf temperature, followed by a steady temperature phase. When the product temperature equals the shelf temperature, the primary drying is finished. There should be an additional time

at the end of primary drying when the product temperature is monitored with the thermocouple probes. The vials with the probes will dry faster than the other vials because the probes conduct more heat into the vial so when the temperature of the thermocouple probes equals the shelf temperature, it does not necessarily mean that all the ice has sublimed from all the vials. (Barley 2021.) The length of primary drying may also vary even under the same conditions so there should be an additional time at the end of primary drying to make sure that all the ice has sublimed. (Ó´Fagáin 2004.)

### 2.3 Secondary drying

The main purpose of secondary drying is to reduce the amount of residual water to moisture level which will not support biological growth or any chemical reactions. There will still be some bound water in the product after primary drying and the moisture content in the product is usually 5-10 %. Such moisture content is too high in most of the cases and the desired stability of final product can’t be achieved. The moisture content in the product is reduced by desorption of the bound water and it is performed by increasing the temperature of the product. (Jennings 2008.) The desorption is a surface phenomenon describing the transition between separate phases. During desorption the water molecules transfer from the drying solid into a gaseous phase. The gaseous phase consists of water vapor which is continuously removed by a condenser. The desorbing water must be transported from the inner parts of a drying product to the surface. In other words, the water molecules have to be transported from higher water concentration to the relatively dry surface in which the water concentration is lower. This transportation is called a diffusion which is due to concentration differences. When the solid and the gaseous phases reach an equilibrium state, it means that there are no more concentration differences. At this point, the diffusion of water stops, and the product has a homogeneous composition. (Spieles et al. 1995.)

The product temperature should not increase above the critical collapse temperature at the beginning of secondary drying. But when the more the

residual water is removed, the more the critical collapse temperature increases. Therefore, the product temperature can be significantly increased towards the end of the secondary drying. (Ó´Fagáin 2004.) However, the heating rate depends on the product type. If the product is amorphous, a lower heating rate should be used to avoid collapse but with crystalline products a higher heating rate may be used without a fear of collapse. (Tang & Pikal 2004.) When the product temperature is increased, the desorption of water molecules starts to occur. (Barley 2021.) System pressure may be the same that it was in primary drying because the desorption rate does not depend on the system pressure if it is less than 200 mTorr (0.2666 mbar) (Tang & Pikal 2004).

The secondary drying time depends on the shelf temperature, product type and solute concentration. The higher the shelf temperature, the faster the desorption of water. Crystalline products are easier to dry than amorphous products and they require lower temperatures and less time to dry. The higher the solute concentration in the product, the more difficult the removal of the absorbed water. An optimal secondary drying time can be determined by real-time residual moisture measurement. Samples are taken from the freeze dryer at certain intervals during secondary drying and their moisture content are measured. (Tang & Pikal 2004.) Secondary drying may be finished when the product has a suitable moisture content for a long term storage. An optimal moisture content for the final products depends on the application but it usually is between 0.5 % and 3 %. (Barley 2021.)

## 2.4 Backfill and stoppering

Freeze-dried products are very hydroscopic so they need to be sealed airtight in the vials after freeze drying to prevent rehydration from atmosphere. Some freeze dryers have a stoppering function to seal the vials under vacuum inside the freeze dryer. The vials usually have partially inserted stoppers and when the gap between the shelves is reduced, the stoppers are pushed down, and the vials are sealed. (Barley 2021.)

Stoppering of vials under a vacuum is the most optimal for the freeze-dried products. Moisture and oxygen are harmful to the freeze-dried products so the vacuum prevents them from coming in contact with the product. The stoppering also protects the products from chemical and biological contamination. In addition, the vials can be backfilled with an inert gas to protect the product. Suitable gasses for the backfilling are for example argon and nitrogen but they have to be ultrapure, and they cannot contain any oxygen or moisture. The containers used for freezing dried products must be impermeable to air and atmospheric moisture. (Labconco Corporation 2010.)

## 2.5 Properties of freeze-dried products

The conditions during each step of the freeze drying affect the appearance of the freeze-dried product. The ice structure which is formed during the freezing defines the cake structure of the freeze-dried product. An ideal cake structure is a spongelike and its volume is equivalent to the volume of the frozen product. (Jennings 2008.) There are also many factors which affect the stability of freeze-dried products. The most harmful factors are moisture and oxygen. There are always residual moisture remaining in freeze-dried products but the amount of it depends on the product and the length of secondary drying. Freeze-dried products are very hydroscopic which means that they easily absorb moisture from the air. If the freeze-dried products are exposed to moisture during the storage, destabilization of the products may occur. The harmful effects of moisture and oxygen depend on storage temperature. The higher the storage temperature, the faster the degradation of the product is. Storing of freeze-dried products at low temperatures extends their shelf life and usually refrigerator temperatures are suitable for most of the products. (Labconco Corporation 2010.)

The purpose of freeze drying is to stabilize the products. The freeze-dried product is stable as long as its activity is within specified limits. The shell life of the product is the length of time that its activity remains within the defined limits. There are few methods to determine the stability of the freeze-dried product. In

real time stability testing the product is stored under the specified temperature and humidity. Samples are removed at a certain time intervals to determine the remaining activity of the freeze-dried product. As a result, the expiration date can safely be determined for the product. (Jennings 2008.) In accelerated stability testing the product is stored at elevated temperature. Samples are removed periodically and the activity and the rate of degradation of the product are measured. The relationship between the temperature and time at an elevated temperature can be used to calculate the rate of degradation of the product at lower storage temperatures and to predict the shelf life of the product. (Labconco Corporation 2010.)

To be able to use the freeze-dried product, it first need to be reconstituted. In reconstitution the product is restored to its original formulation by adding a defined quantity of diluent to the cake structure. Because the freeze-dried products are very hydroscopic, the dissolution of the dried product happens very quickly. The properties (e.g., activity, pH, and concentration) of the reconstituted product should be within the specified limits of the original solution. (Jennings 2008.)

# 3 Equipment of freeze dryer

Freeze drying requires controlling and monitoring of product temperature and chamber pressure. In freeze drying the products need to be cooled to below their freezing temperature and heated at a low pressure to sublime the ice from the products. The released water vapours need to be collected and condensed back to ice. (Franks & Auffret 2007.) The essential equipment for freeze dryer is a refrigeration system, a product chamber, a condenser and a vacuum pumping and a control system (Figure 3) (Barley 2021).

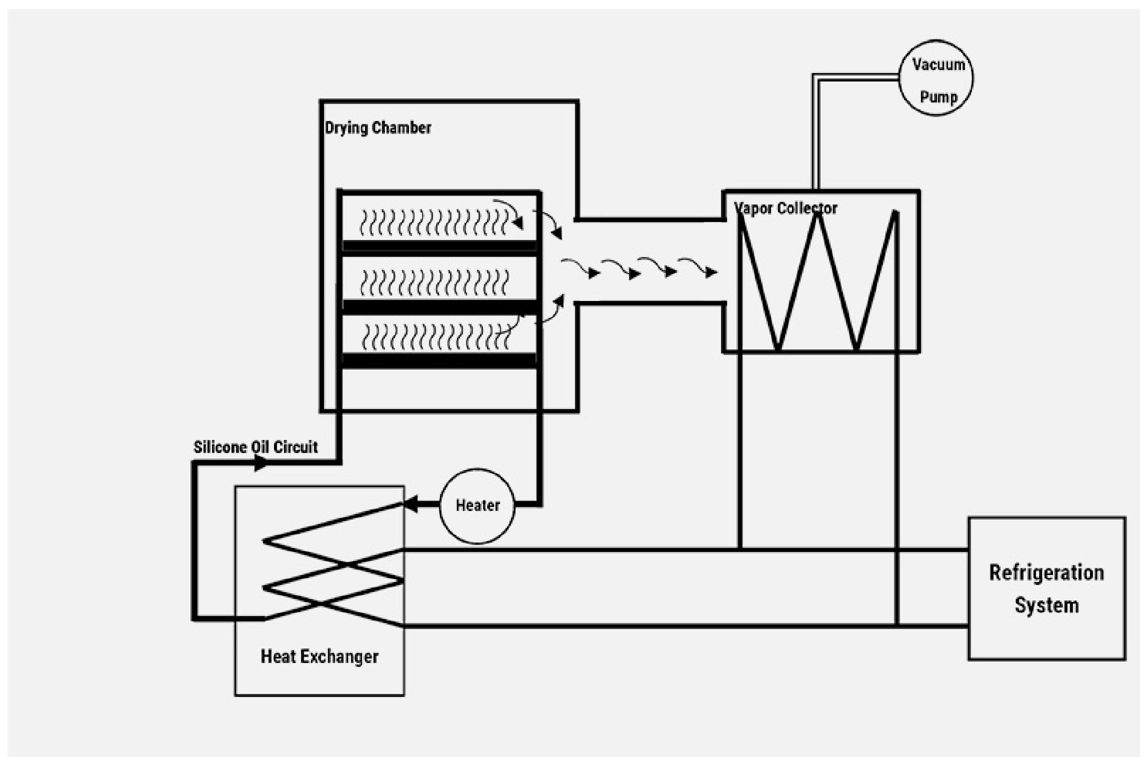


Figure 3. A general layout of a freeze dryer (Vikumer n.a.).

## 3.1 Refrigeration system

Refrigeration systems cools and heats the product chamber inside of the freeze dryer. It can be used to freeze the products or to provide the needed heat energy for the primary and secondary drying. (Jennings 2008.) The refrigeration system must be able to maintain the product chamber and the condenser at the

required temperatures (Barley 2021). In a shelf freeze dryer the refrigeration system circulates the fluid through the shelves so that it maintains the shelf temperature at the desired value (Franks & Auffret 2007).

### 3.2 Product chamber

There are different kinds of product chambers but usually they are either a manifold with attached flasks or a chamber with shelves (Barley 2021). Product chamber is an airtight space which provides an environment for freeze drying of the products. The product chamber in a shelf freeze dryer is usually constructed from stainless steel because of its cleanability, strength and corrosion resistance. The chamber door can also be manufactured from stainless steel but there are clear plastic chamber doors too. (Jennings 2008.)

### 3.3 Condenser

Condenser collects the water vapors coming from the product chamber which have been sublimed of the product and condenses them back to ice. The accumulated ice is removed by defrosting at the end of freeze drying. The condenser located inside the product chamber or in a separated chamber which is connected to the product chamber with a vapor port. The required condenser temperature depends on the eutectic and the critical collapse temperature of the product. (Barley 2021.) The condenser temperature must be at least 20 °C lower than the product temperature during primary drying for the effective condensation of water vapors (Jennings 2008). The condenser must also be able to reach high temperatures to defrost the accumulated ice. (Franks & Auffret 2007.)

### 3.4 Vacuum pumping system

Vacuum pumping system consists of a vacuum pump which is connected to an airtight product chamber and a condenser (Barley 2021). Vacuum pumping

system provides low pressure for the freeze drying and maintains it at the required level during the primary and secondary drying. Typically, the used vacuum pumps in freeze dryers are mechanical and oil lubricated. (Jennings 2008.) The vacuum pumping system must be able to maintain the product chamber pressure lower than the vapor pressure of ice at the target product temperature for long periods (Franks & Auffret 2007).

## 3.5 Control system

Control systems usually include the ability to measure temperature and pressure and the progression of freeze drying can be monitored with it. The suitable control system for the freeze dryer depends on the application and use. (Barley 2021). Modern freeze dryers contain computer-based control and monitoring systems with which the programs for freeze-drying process can be programmed (Franks & Auffret 2007).

## 3.6 Types of freeze dryers

Freeze dryers can be classified by the type of product chamber. The most common freeze dryers are manifold and shelf freeze dryers or a combination of them. Manifold freeze dryers are simpler, and they consist of a vacuum pump unit attached with a manifold for the freeze drying of multiple flasks (Figure 4). (Barley 2021.) In manifold drying the products in flasks are frozen in a separate freezing equipment and then they are individually attached to the ports of a drying chamber or manifold. The vacuum needs to be created quickly in the flasks and the low product temperature is maintained by evaporative cooling. The manifold drying is suitable for products with small volumes and high eutectic and critical collapse temperatures. In manifold drying each flask has its own path to the condenser and the short distance to it makes the drying more efficiency. The heat of the products is regulated by exposing them to the ambient temperature, so the precise temperature control is not possible. Because each flask can be individually attached and removed, freeze drying of

different products at the same time is possible. (Labconco Corporation 2010.) With manifold freeze dryer a precise control of the freeze-drying process and monitoring of the product temperatures is not possible (Barley 2021).

Figure 4 Manifold freeze dryer (ATO 2023).

More developed shelf freeze dryers are suitable for freeze drying products with larger volumes with a precise control of the whole process (Figure 5). In shelf freeze dryer the products are directly on the shelves which are temperature programmable, and the product temperatures can be monitored with temperature probes. (Ó´Fagáin 2004.) There are two methods to use the shelf freeze dryer: batch and bulk drying. In batch drying a large amount of similar sized vials are placed in the shelf freeze dryer and frozen on the shelves. During drying there may be small differences in heat transfer in different areas of the shelf but in general all the vials are under the same conditions. Only slight differences in residual moisture may be detected between the vials. In batch drying all the vials can be stoppered at the same time under the same conditions so there is an identical environment in each vial, which leads to a uniform product stability between the vials. Batch drying is suitable for freeze drying of a large number of vials of one product. In bulk drying the shelf freeze dryer is also used but the product mass is poured directly on the shelf as a single unit. Even though the thickness of the product layer may be the same as

in vials, the heat transfer is limited in bulk trying because there are only a few empty spaces within the product mass. Bulk drying is suitable for products which are stable and not too sensitive to moisture and oxygen because the sealing of products cannot be performed under controlled conditions. (Labconco Corporation 2010.)

Figure 5 Shelf freeze dryer (SciTech 2023).

Another way to classify freeze dryers is by a size and use. There are smaller laboratory bench-top freeze dryers for research and development, medium sized pilot freeze dryers for process development and scale-up and also larger freeze dryers for larger production. (Barley 2021.)

# 4 Optimization of freeze-drying process

Optimization of the freeze-drying process was carried out by performing test runs with the new SP VirTis AdVantage Pro freeze dryer. The test runs included freeze drying of the sample solution in 3 ml and 10 ml glass vials. Bovine serum albumin (BSA) solution in PBS buffer whose concentration was 1 mg/ml was used as a sample solution. The sample solution volumes were 1.5 ml in 3 ml vials and 5 ml in 10 ml vials. Vials with Milli-Q water were also used to control drying of water visually and the sample temperatures were monitored with four temperature probes. Stoppering of the vials was performed on the shelves or manually by hand but no backfilling was used.

## 4.1 SP VirTis AdVantage Pro freeze dryer

The SP VirTis AdVantage Pro freeze dryer is a combination of a manifold and a shelf freeze dryer. There are four manifold ports on the side of the freeze dryer and two shelves inside the freeze dryer. It was used as a shelf freeze dryer in the test runs, which offered new options for freeze drying (Figure 6). It was possible to freeze the vials on the shelves, control the shelf temperature and vacuum precisely during the freeze drying by creating different drying programmes, monitor the temperature of the products with temperature probes, and seal the vials on the shelves. There were four temperature probes in the freeze dryer which were placed at the bottom of vials during the test runs. The freeze dryer recorded the temperature of each probe and their average. In drying programmes, the shelf temperature could be programmed to maintain a certain temperature for a certain time (HOLD), or it could also be programmed to achieve a certain temperature during the defined time (RAMP). The vacuum was programmed to maintain a certain pressure during each step of the drying program. Before the drying program started, there was an evacuation step during which the target pressure was approached. There was also an option to control the freeze dryer manually.

Figure 6. SP VirTis Advantage Pro Freeze Dryer.

## 4.2 Background

Most of the antigen products are freeze dried inside the company. The ingredients of each product are different, which means that each product also has its own freezing and critical collapse temperature. Freeze drying of the products is challenging because these temperatures are not known for them so they must be freeze dried very carefully. The new freeze dryer also brought new challenges because there was no access to its internal memory at the beginning so the data from the test runs could not been examined. Many system alarms also appeared when the freeze dryer was used. These problems were caused by a programming error, but they could be fixed.

A recommended program for the freeze drying was received from within the company so the test runs were performed according to it with the new freeze dryer. (Table 1).

Table 1. Recommended program.

| **Step** | **Shelf temperature (°C)** | **Ramp (min)** | **Hold (min)** | **Vacuum (mTorr)** |
|---|---|---|---|---|
| Pre-freeze | -30 | N/A | N/A | N/A |
| 1 | -17 | 300 | 0 | 50 |
| 2 | -11 | 360 | 0 | 50 |
| 3 | -3 | 400 | 0 | 0 |
| 4 | 0 | 200 | 0 | 0 |
| Storage | 22 | N/A | N/A | 50 |

There were Ramp times in each step, which meant that the shelf temperature would rise from the shelf temperature of the previous step to the shelf temperature of the current step during the determined time. In the first step according to the recommended program the shelf temperature should rise from -30 °C to -17 °C in 300 minutes so the shelves were prefrozen to -30 °C. During the test run the shelf temperature did not behave as expected. Instead of a continuous increase in the shelf temperature, the temperature always first decreased when the drying step changed and only after that did it start to rise again (Figure 7).

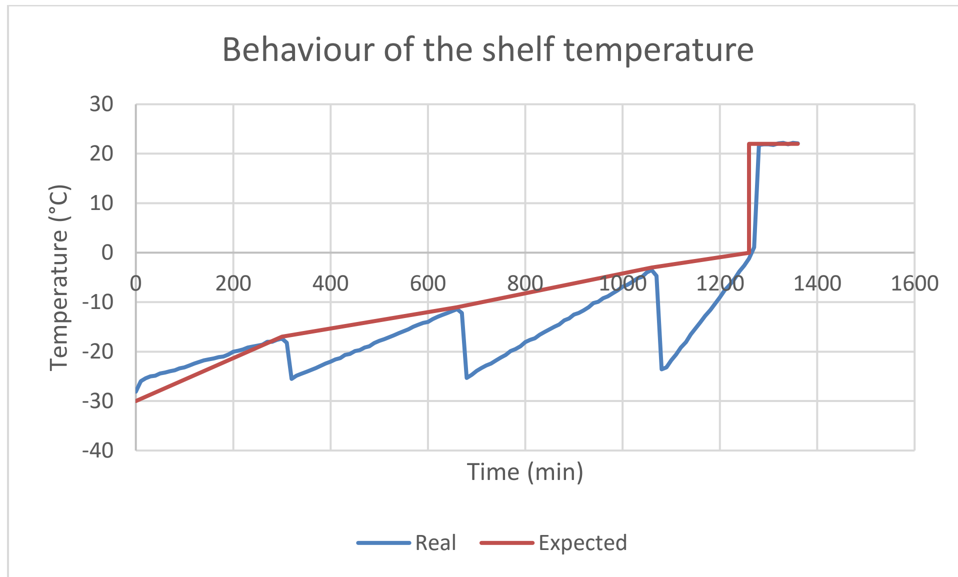


Figure 7. Expected and real behaviour of the shelf temperature.

The test run was not successful because of the behaviour of the shelf temperature and the collapse of the samples (Figure 8).

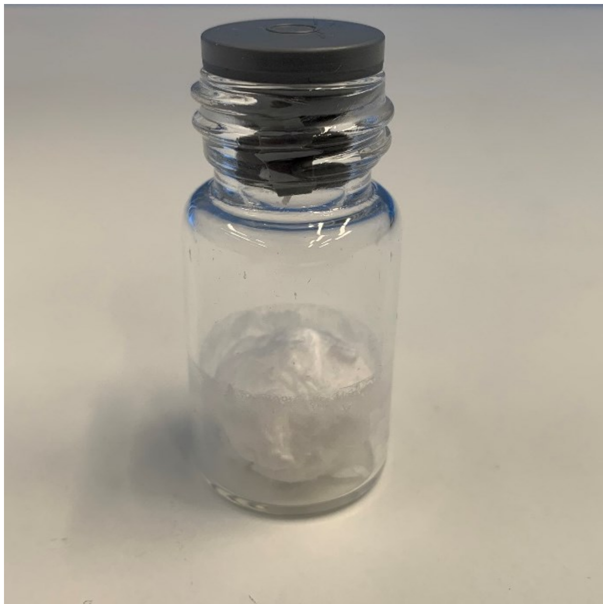

Figure 8. Collapsed sample.

Because the problem with the behaviour of the shelf temperature could not be solved, the recommended program could not be used. Freeze drying of the products was started by changing shelf temperatures manually. In each step the

shelf temperature was kept stable at least until the average temperature of the probes had exceeded the shelf temperature (Table 2).

Table 2. Manual freeze drying.

| **Step** | **Shelf temperature (°C)** | **Time (min)** | **Vacuum (mTorr)** |
|---|---|---|---|
| Pre-freeze | -30 | N/A | N/A |
| 1 | -25 | 966 | 50 |
| 2 | -17 | 43 | 50 |
| 3 | -15 | 154 | 50 |
| 4 | -10 | 219 | 50 |
| 5 | -5 | 153 | 50 |
| 6 | 0 | 798 | 50 |
| 7 | 5 | 66 | 50 |
| 8 | 10 | 20 | 50 |
| 9 | 22 | 277 | 50 |

The freeze drying was successful but the problem with this manual control of the shelf temperature was that in some steps the shelf temperature had to be kept the same overnight, which made some of the steps too long (Figure 9).

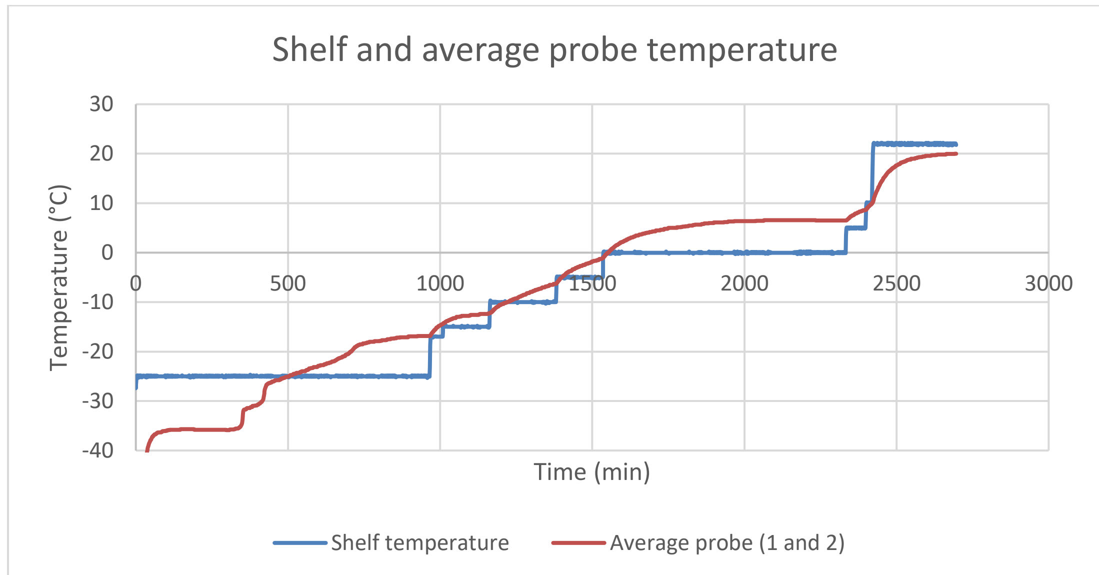


Figure 9. Shelf and average probe temperature during the manual freeze drying.

4.3 Freeze drying of 3 ml vials.

Freeze drying of 3 ml vials was performed by programming only Hold time for each drying step. 4 of 3 ml vials with the sample solution and 2 of 3 ml vials with 2 ml of Milli-Q water were freeze dried. All the temperature probes were inserted into the vials with the sample solution. The drying steps were almost the same as in the manual freeze drying of the products and a program (Program #1) was created with these steps (Table 3). The vials were sealed manually by hand after the freeze drying.

Table 3. Program #1 for the freeze drying.

| **Step** | **Shelf temperature (°C)** | **Ramp (Min)** | **Hold (Min)** | **Vacuum (mTorr)** |
|---|---|---|---|---|
| Pre-freeze | -30 | N/A | N/A | N/A |
| 1 | -25 | 0 | 965 | 50 |
| 2 | -17 | 0 | 45 | 50 |
| 3 | -15 | 0 | 155 | 50 |
| 4 | -10 | 0 | 220 | 50 |
| 5 | -5 | 0 | 155 | 50 |
| 6 | 0 | 0 | 800 | 50 |
| 7 | 5 | 0 | 65 | 50 |
| 8 | 10 | 0 | 20 | 50 |
| Storage | 22 | N/A | N/A | 50 |

The average temperature of the probes already exceeded the temperature of the shelves between 470-480 minutes and remained higher until the end of the program. During step 4 (step time 91/220 minutes) Milli-Q water had already evaporated from control vials. The total duration of the freeze drying was 2830 minutes (Figure 10).

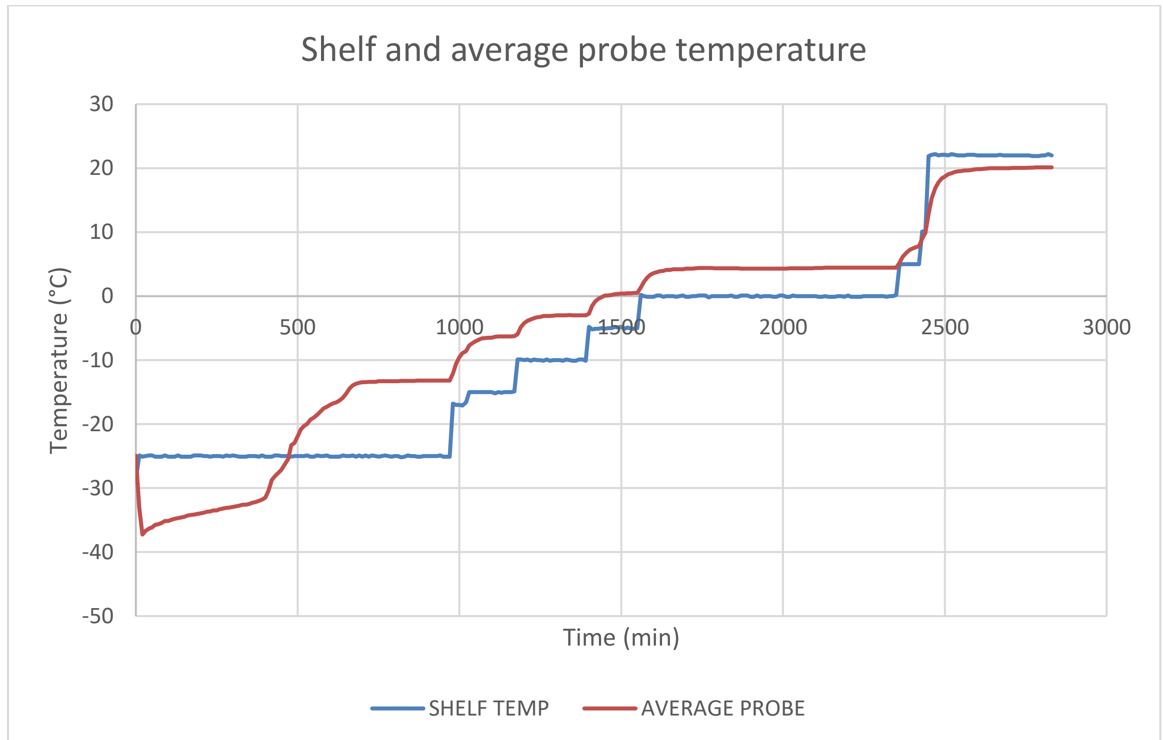


Figure 10. Shelf and average probe temperature during Program #1.

The freeze drying was successful, and there was no collapse observed in the vials. Milli-Q water had evaporated from the control vials. (Figure 11).

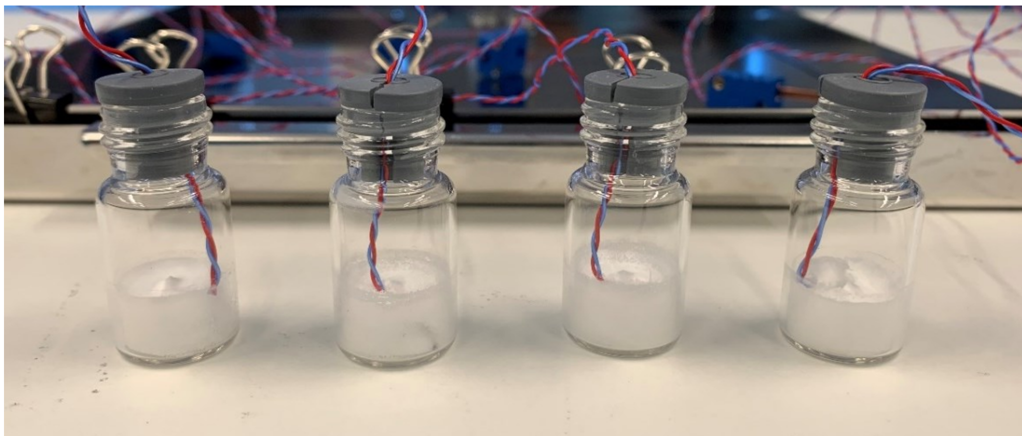

Figure 11. Freeze-dried 3 ml vials after Program #1.

For the next test run, Program #1 was modified so that the shelf temperature would increase faster and would be higher than the average temperature of the probes most of the time. A new program (Program #2) was created and the

steps with shelf temperatures -25 °C (Step 1) and 0 °C (Step 6) were shortened and the step with shelf temperature -17 °C (Step 2) was removed (Table 4). Steps 1 and 6 were unnecessarily long and step 6 was completely unnecessary. 15 pieces of 3 ml vials with the sample solution and 2 pieces of 3 ml vials with 2 ml of Milli-Q water were freeze dried. Three temperature probes were inserted into the vials with the sample solution. The vials were sealed manually by hand after the freeze drying.

Table 4. Program #2 for the freeze drying.

| **Step** | **Shelf temperature (°C)** | **Ramp (Min)** | **Hold (Min)** | **Vacuum (mTorr)** |
|---|---|---|---|---|
| Pre-freeze | -30 | N/A | N/A | N/A |
| 1 | -25 | 0 | 500 | 50 |
| 2 | -15 | 0 | 150 | 50 |
| 3 | -10 | 0 | 200 | 50 |
| 4 | -5 | 0 | 150 | 50 |
| 5 | 0 | 0 | 500 | 50 |
| 6 | 5 | 0 | 60 | 50 |
| 7 | 10 | 0 | 20 | 50 |
| Storage | 22 | N/A | N/A | 50 |

The primary drying was finished after 750 minutes. It took slightly longer now because there were more vials, but the total duration of the freeze drying could still be shortened to 1640 minutes (Figure 12).

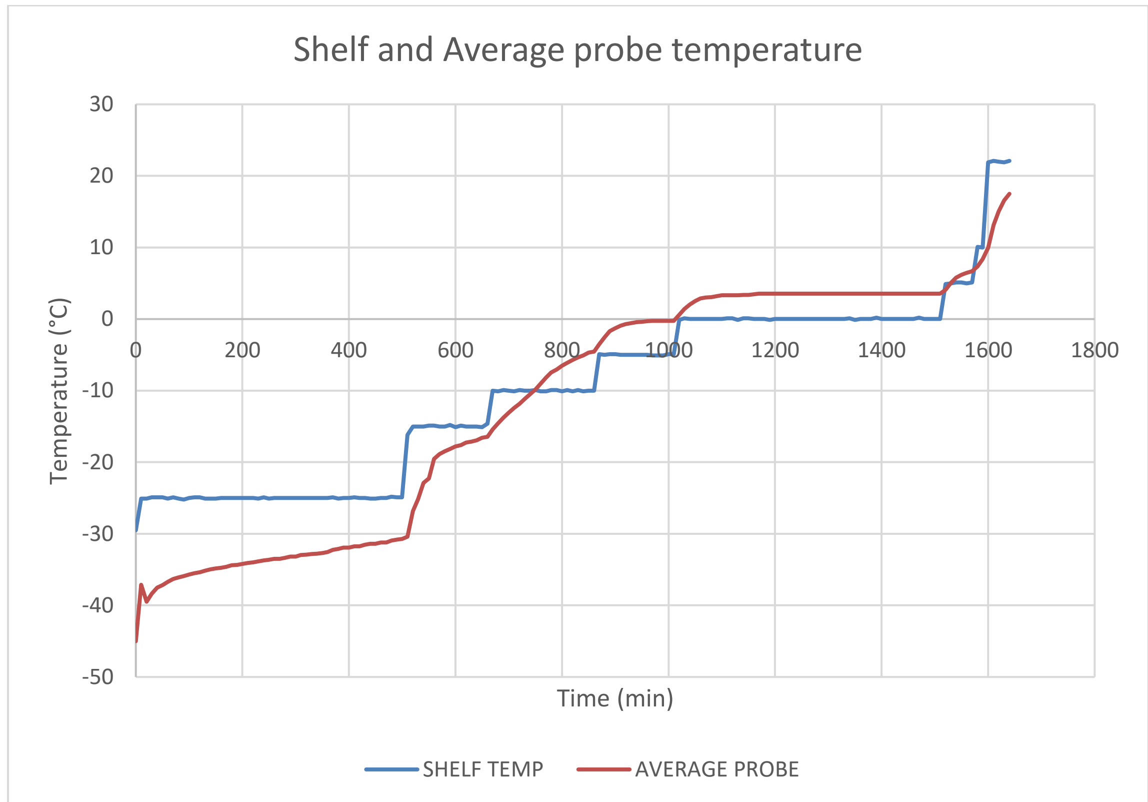


Figure 12. Shelf and average probe temperature during Program #2.

The freeze drying was successful, and there was no collapse observed in the vials. Milli-Q water had evaporated from the control vials (Figure 13).

Figure 13. Freeze-dried 3 ml vials after Program #2.

Although freeze drying was successful when programming only Hold time for each drying step, the goal was to make the recommended program with temperature ramping work. The manufacturer of the freeze dryer was contacted about the problem with the behaviour of the shelf temperature and a

recommendation was received to solve the problem. The recommended program was modified by adding Hold times to each step in addition to Ramp times but all the other parameters were kept the same (Table 5). The test run was performed without the samples.

Table 5. Recommended program after modifying.

| Step | Shelf temperature (°C) | Ramp (Min) | Hold (Min) | Vacuum (mTorr) |
|---|---|---|---|---|
| Pre-freeze | -30 | N/A | N/A | N/A |
| 1 | -17 | 300 | 2 | 50 |
| 2 | -11 | 360 | 2 | 50 |
| 3 | -3 | 400 | 2 | 50 |
| 4 | 0 | 200 | 2 | 50 |

After adding the Hold times in each step, the shelf temperature behaved as expected and it continuously increased during the program (Figure 14).

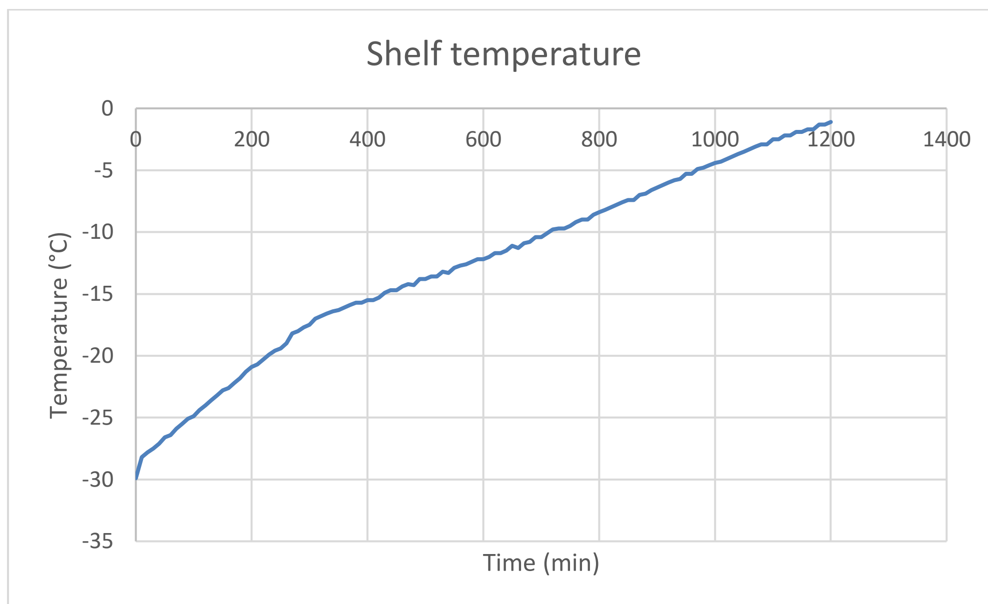


Figure 14. Behaviour of the shelf temperature during the modified recommended program.

After the problem with temperature ramping had been solved, 14 pieces of 3 ml vials with the sample solution and 2 pieces of 3 ml vials with 2 ml of Milli-Q water were freeze dried. A new program (Program #3) was created and the step with shelf temperature -30 °C and 5-minute Hold time was added because the vials were frozen on the shelves at -60 °C instead of liquid nitrogen (Table 6). Without this additional step, the shelf temperature would have risen from -60 °C to -17 °C in 300 minutes and it would no longer have been in accordance with the recommended program. All the temperature probes were inserted into the vials with the sample solution and the vials were sealed manually by hand after the freeze drying.

Table 6. Program #3 for the freeze drying.

| **Step** | **Shelf temperature (°C)** | **Ramp (Min)** | **Hold (Min)** | **Vacuum (mTorr)** |
|---|---|---|---|---|
| Pre-freeze | -60 | N/A | N/A | N/A |
| 1 | -30 | 0 | 5 | 50 |
| 2 | -17 | 300 | 2 | 50 |
| 3 | -11 | 360 | 2 | 50 |
| 4 | -13 | 400 | 2 | 50 |
| 5 | 0 | 200 | 2 | 50 |
| Storage | 22 | N/A | N/A | 50 |

The primary drying was finished after 740 minutes, and the total duration of the freeze drying was 1340 minutes. (Figure 15).

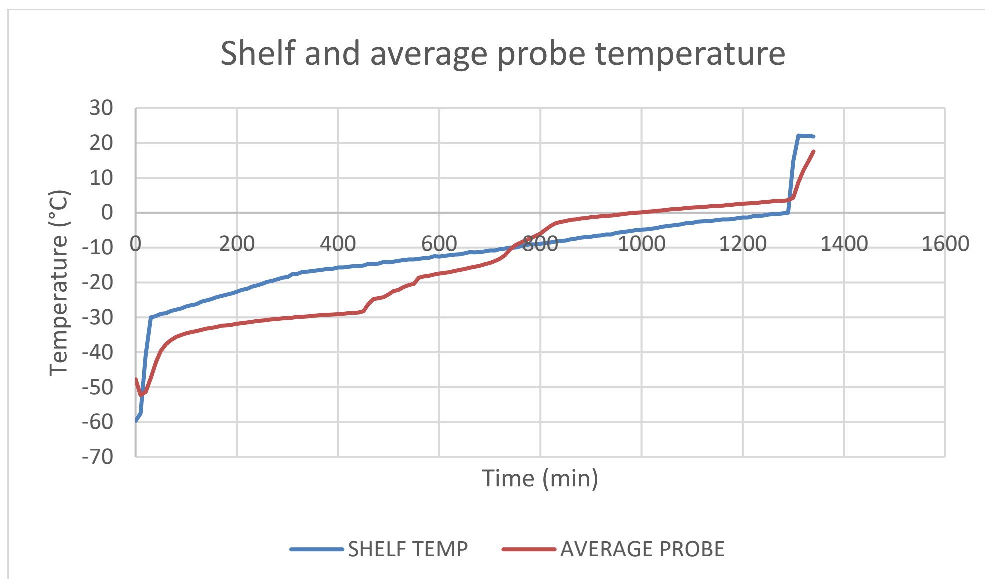


Figure 15. Shelf and average probe temperature during Program #3.

The freeze drying was successful, and there was no collapse observed in the vials. Milli-Q water had evaporated from the control vials (Figure 16).

Figure 16. Freeze-dried 3 ml vials after Program #3.

A new program (Program #4) was created by modifying Program #3. The shelf temperature was programmed to start from -20 °C and increase to -10 °C in 600 minutes. After that the program was programmed to stay at 0 °C for 600 minutes (Table 7). 20 pieces of 3 ml vials with the sample solution and 2 pieces of 3 ml vials with 2 ml of Milli-Q water were freeze dried with Program #4 after freezing the vials on the shelves at -60 °C. All the temperature probes were

inserted inside of the vials with the sample solution and the vials were sealed manually by hand after the freeze drying.

Table 7. Program #4 for the freeze drying.

| Step | Shelf temperature (°C) | Ramp (Min) | Hold (Min) | Vacuum (mTorr) |
|---|---|---|---|---|
| Pre-freeze | -60 | N/A | N/A | N/A |
| 1 | -20 | 0 | 2 | 50 |
| 2 | -10 | 600 | 2 | 50 |
| 3 | 0 | 0 | 600 | 50 |
| Storage | 22 | N/A | N/A | 50 |

The primary drying was finished after 680 minutes. The drying was a little faster than with Program #3 due to the higher shelf temperature. The total duration of the freeze drying was 1310 minutes (Figure 17).

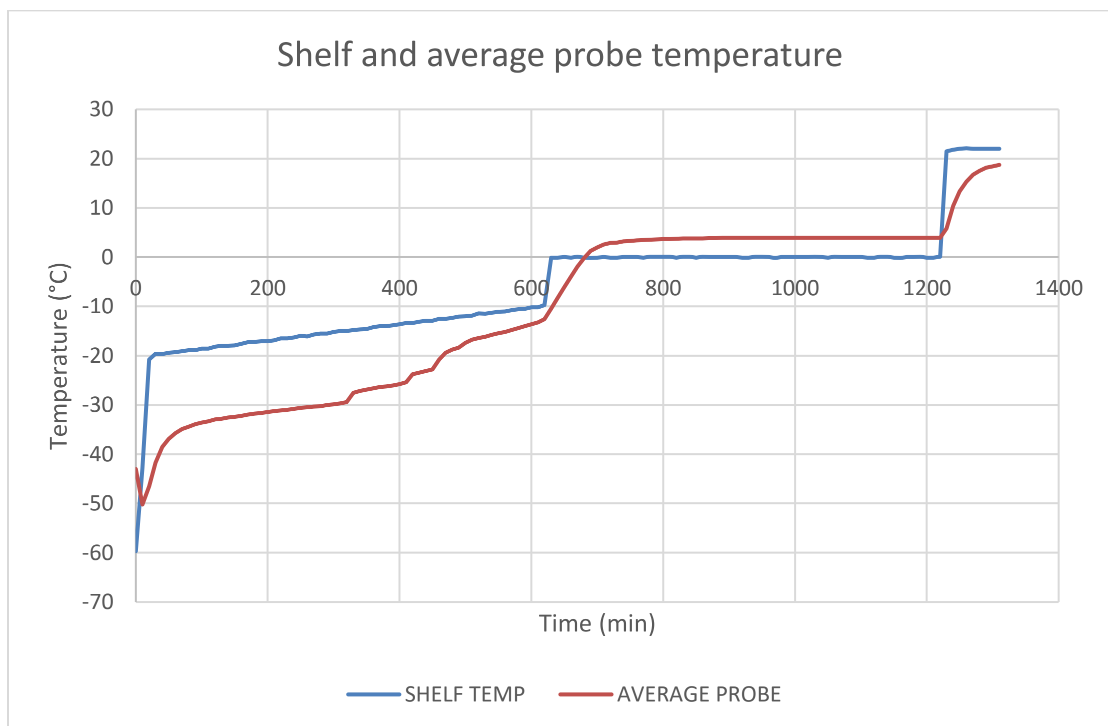


Figure 17. Shelf and average probe temperature during Program #4.

The freeze drying was successful, and there was no collapse observed in the vials. Milli-Q water had evaporated from the control vials (Figure 18).

Figure 18. Freeze-dried 3 ml vials after Program #4.

## 4.4 Freeze drying of 10 ml vials.

Freeze drying of 10 ml vials were tried four times with freezing the vials with liquid nitrogen, but all the test runs had to be aborted due to the cracking of the vials. Over half of the frozen vials cracked because they could not tolerate huge temperature differences (Figure 19).

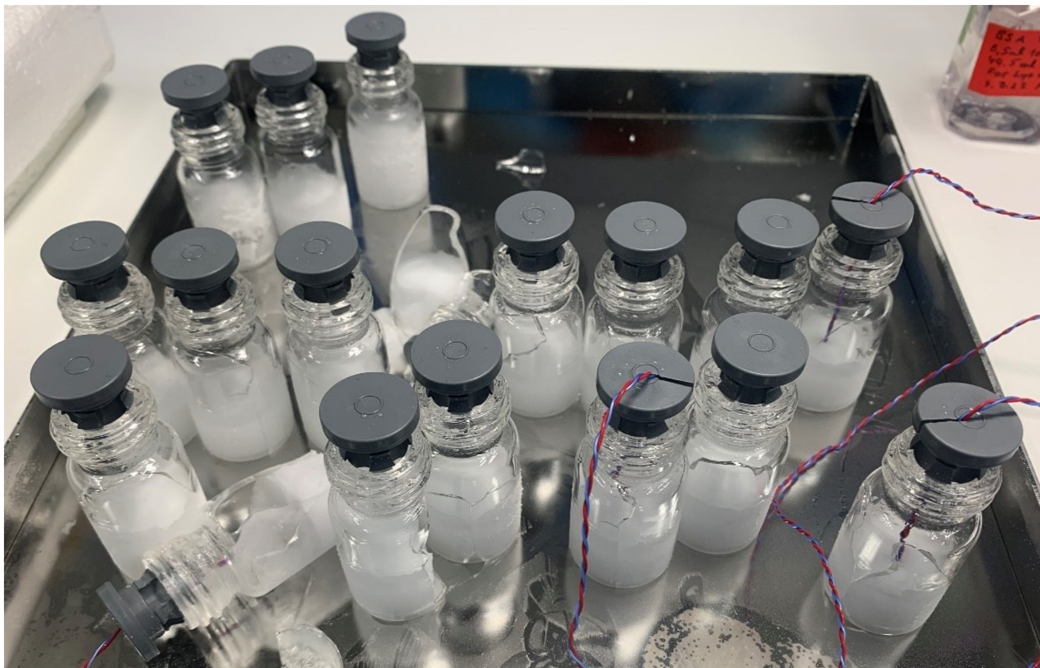

Figure 19. Cracked 10 ml vials after freezing with liquid nitrogen.

Because 10 ml vials did not tolerate the freezing with liquid nitrogen, other freezing methods had to be concerned. Freezing on the shelves was a natural option because with the new freeze dryer it was possible. However, freezing on the shelves was first tried with a deep freezer at -70 °C. 24 pieces of 10 ml vials with the sample solution and 4 pieces of 10 ml vials with 5 ml of Milli-Q water were divided in half. One half was frozen only in the deep freezer (Tray A) and the other was first frozen with liquid nitrogen and then quickly transferred to the deep freezer to reduce the temperature differences (Tray B). Both trays had the same number of vials, and they were freeze dried at the same time with Program #1 (Table 3). Two temperature probes were inserted inside of the vials with the sample solution on both trays. After the freeze drying the vials were stoppered on the shelves.

The primary drying was finished after 1200 minutes, and the total duration of the freeze drying was 2740 minutes. The average probe temperature increased steeply after the Step 1, which indicated that the samples were almost dried (Figure 20).

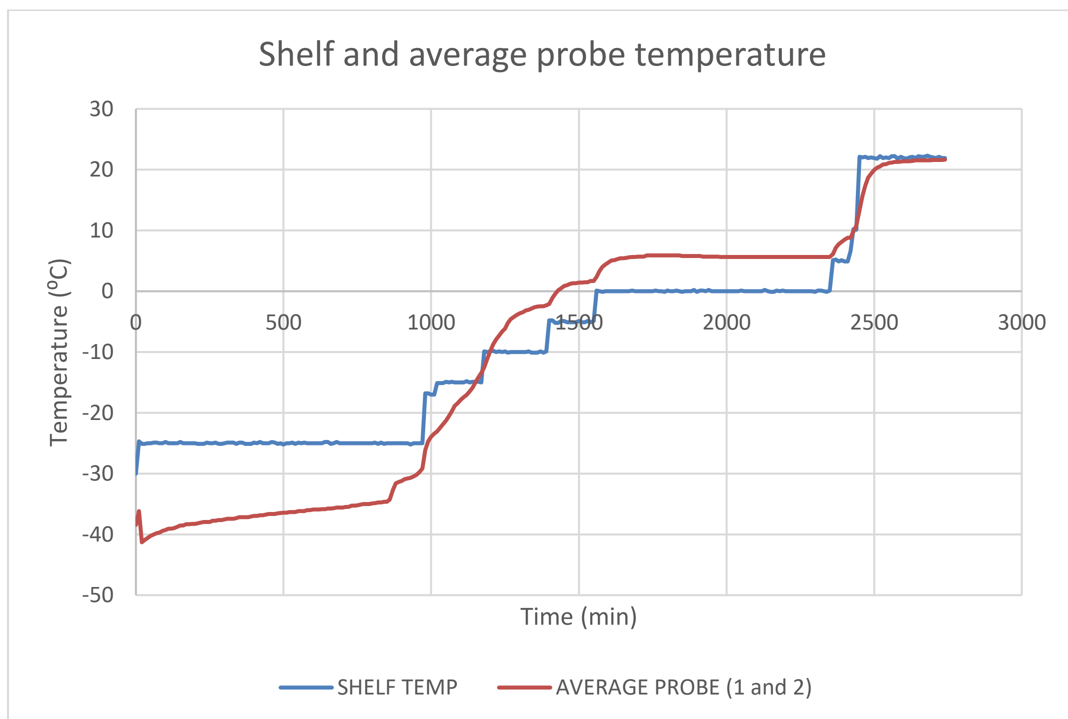


Figure 20. Shelf and average probe temperature during Program #1.

There was no collapse observed in the vials on the tray A and Milli-Q water had also evaporated from the control vials. All the vials on the tray A were unbroken (Figure 21).

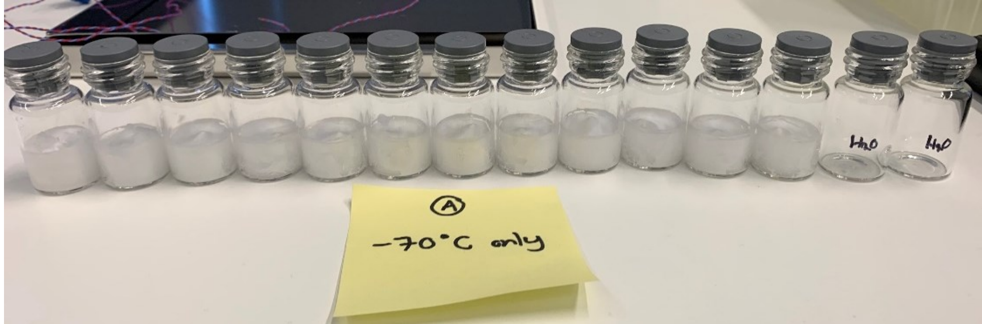


Figure 21. Freeze-dried 10 ml vials on the tray A after Program #1.

There was no collapse observed in the vials on the tray B and Milli-Q water had also evaporated from the control vials (Figure 22), but three vials were cracked during the freeze drying (Figure 23). Both temperature probes on the tray B gave different values than the probes on the tray A because they were inside of cracked values, so they were not taken into account when calculating the average temperature of the probes.

Figure 22. Freeze-dried 10 ml vials on the tray B after Program #1.

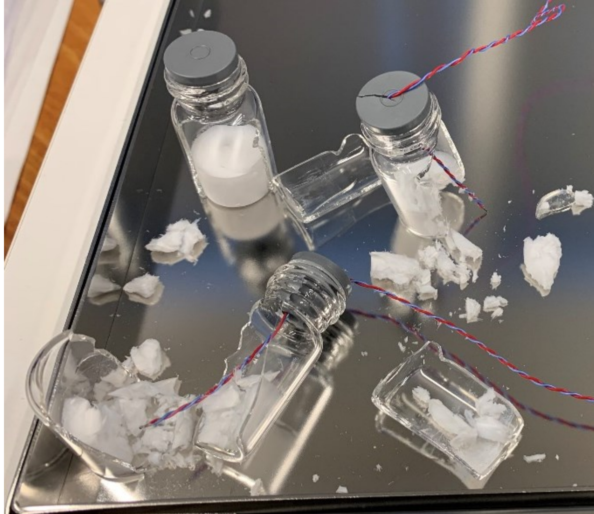

Figure 23. Cracked 10 ml vials on the tray B after Program #1.

A new program was created (Program #5) by modifying Program #1. The first step was extended and the second step with shelf temperature -17 °C was removed so that the average temperature of the probes would not rise higher than the temperature of the shelves so quickly. The step with shelf temperature 0 °C was also shortened (Table 8). 16 pieces of 10 ml vials with the sample solution and 2 pieces of 10 ml vials with 5 ml of Milli-Q water were freeze dried after freezing them on the shelves at -60 °C. All the temperature probes were inserted inside of the vials with the sample solution and the vials were sealed on the shelves after the freeze drying.

Table 8. Program #5 for the freeze drying.

| Step | Shelf temperature (°C) | Ramp (Min) | Hold (Min) | Vacuum (mTorr) |
|---|---|---|---|---|
| Pre-freeze | -60 | N/A | N/A | N/A |
| 1 | -25 | 0 | 999 | 50 |
| 2 | -25 | 0 | 200 | 50 |
| 3 | -15 | 0 | 150 | 50 |
| 4 | -10 | 0 | 150 | 50 |
| 5 | -5 | 0 | 150 | 50 |
| 6 | 0 | 0 | 300 | 50 |
| 7 | 10 | 0 | 60 | 50 |
| Storage | 22 | N/A | N/A | 50 |

The average temperature of the probes exceeded the temperature of the shelves for the first time after 980 minutes. After increasing the shelf temperature, the average temperature of the probes stayed lower during the next two steps. The primary drying was finished after 1400 minutes, and the total duration of freeze drying was 2650 minutes (Figure 24).

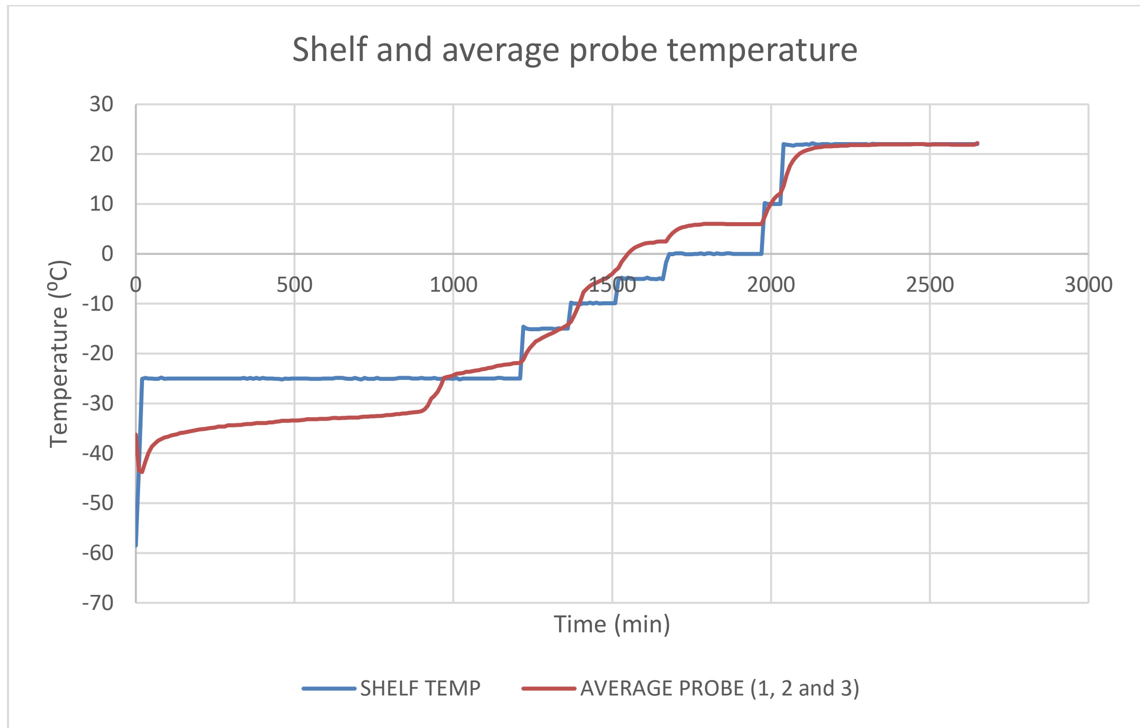


Figure 24. Shelf and average probe temperature during Program #5.

The freeze drying was successful, and there was no collapse observed in the vials. Milli-Q water had evaporated from the control vials (Figure 25).

Figure 25. Freeze-dried 10 ml vials after Program #5.

## 4.5 Freezing on the shelves

Because 10 ml vials did not tolerate the freezing with liquid nitrogen, freezing on the shelves had to be concerned. When the 10 ml vials were frozen in the deep freezer at -70 °C and freeze dried with Program #1, none of the vials on the tray A cracked. It was decided that freezing of the vials will be tried with the new

freeze dryer. The shelves were pre-cooled to -60 °C because it was the minimum temperature that the freeze dryer can reach in a reasonable time.

Freezing on the shelves was performed with 8 pieces of 10 ml vials with the sample solution. Four of the vials were placed on the upper shelf without a tray and the other four on the lower shelf with a tray. Two temperature probes were inserted inside of the vials on the lower shelf. The probes were not inserted inside of the vials on the upper shelf because the vials were without a tray, and they would have fallen. The vials started to freeze from bottom to top immediately after the chamber door was closed (run time 135 minutes). The shelf temperature increased a little after the vials had been placed on the shelves, but it quickly reached the shelf temperature -60 °C again. When a small cone appeared in the middle of the vial, it could be determined to be completely frozen. All the vials were completely frozen after 15 minutes when the temperature probes had reached the temperature -30 °C (Figure 26). There were no cracked vials after the freezing and no considerable differences between the shelves during the freezing.

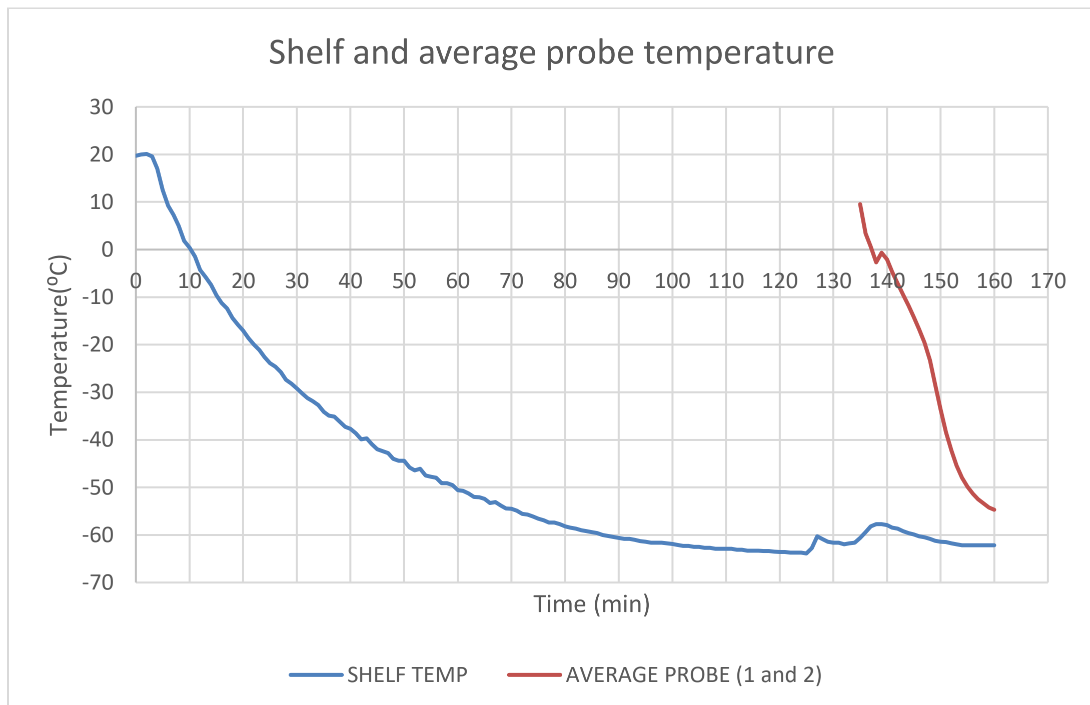


Figure 26. Shelf and average probe temperature during freezing 8 pieces of 10 ml vials.

The number of vials was increased. 16 pieces of 10 ml vials with the sample solution were frozen on the shelves at this time. All the temperature probes were inserted inside of the vials and the vials were placed on the upper shelf with a tray (run time 201 minutes). The shelf temperature increased again a little after the chamber door had been closed. The first vial started to freeze 3 minutes after the vials were placed on the shelf and the last vial started to freeze after 8 minutes. According to the previous freezing the vials were completely frozen at -30 °C. It took 25 minutes that all the probes had reached the temperature -30 °C (Figure 27). The freezing took longer due to the larger number of the vials. There were no cracked vials after the freezing.

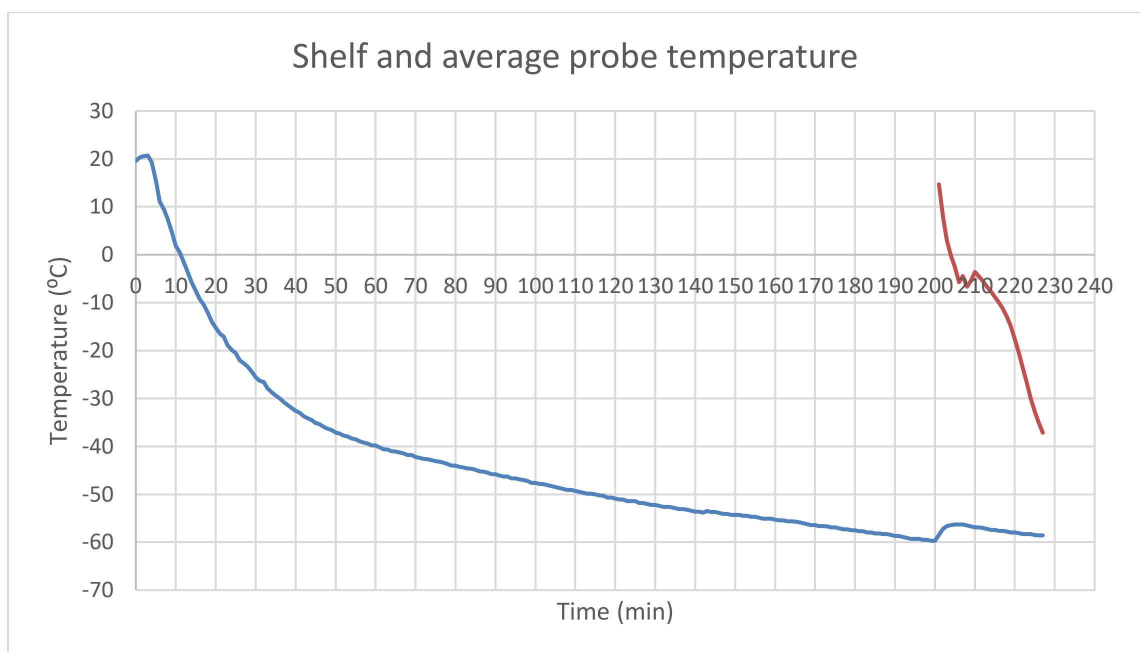


Figure 27. Shelf and average probe temperature during freezing 16 pieces of 10 ml vials.

Because freezing of the 10 ml vials was successful, freezing of the 3 ml vials was also performed on the shelves even if the cracking of the vials was not a problem with them. 14 pieces of 3 ml vials were placed on the lower shelf with a tray and all the temperature probes were inserted inside of them (run time 267 minutes). During the first 5 minutes all the vials had started to freeze and after 13 minutes all the vials were completely frozen (Figure 28).

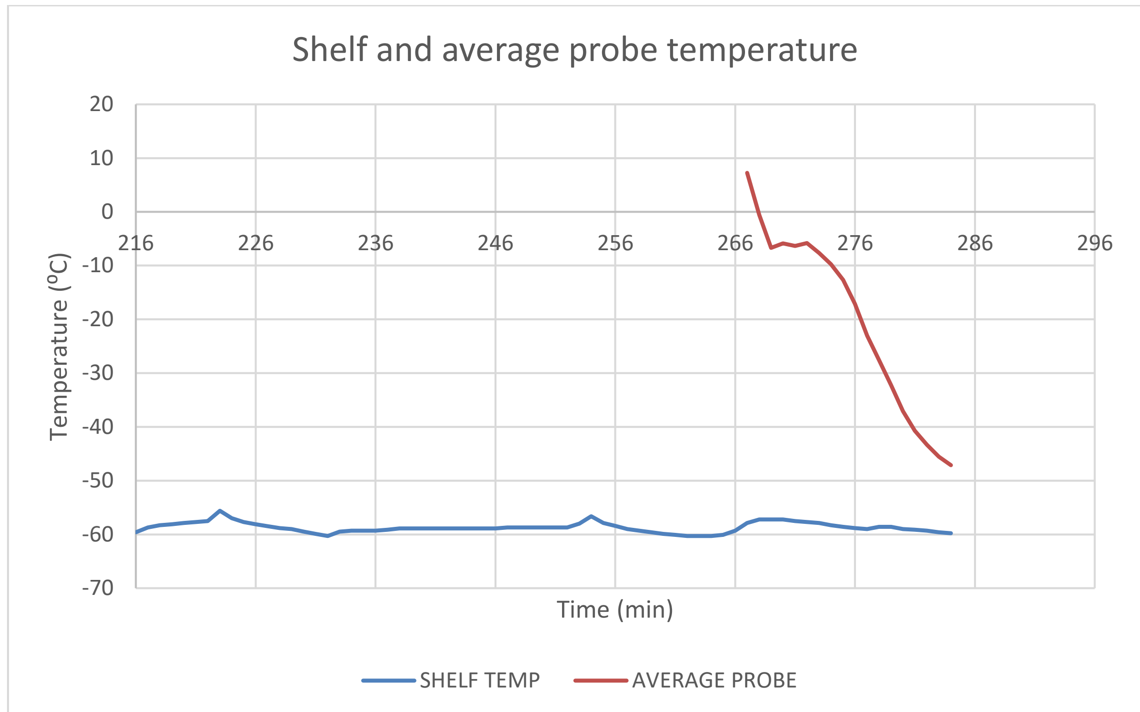


Figure 28. Shelf and average probe temperature during freezing 14 pieces of 3 ml vials.

The freezing of the 3 ml vials on the shelves was performed a second time. 20 of 3 ml vials were placed on the lower shelf with a tray and all the temperature probes were inserted inside of them (run time 135 minutes). The vials in the middle and at the back of the shelf started to freeze first and after 5 minutes all the vials had started to freeze. After 7 minutes the vials in the middle were fully frozen and after 15 minutes every probe had reached temperature -30 °C (Figure 29).

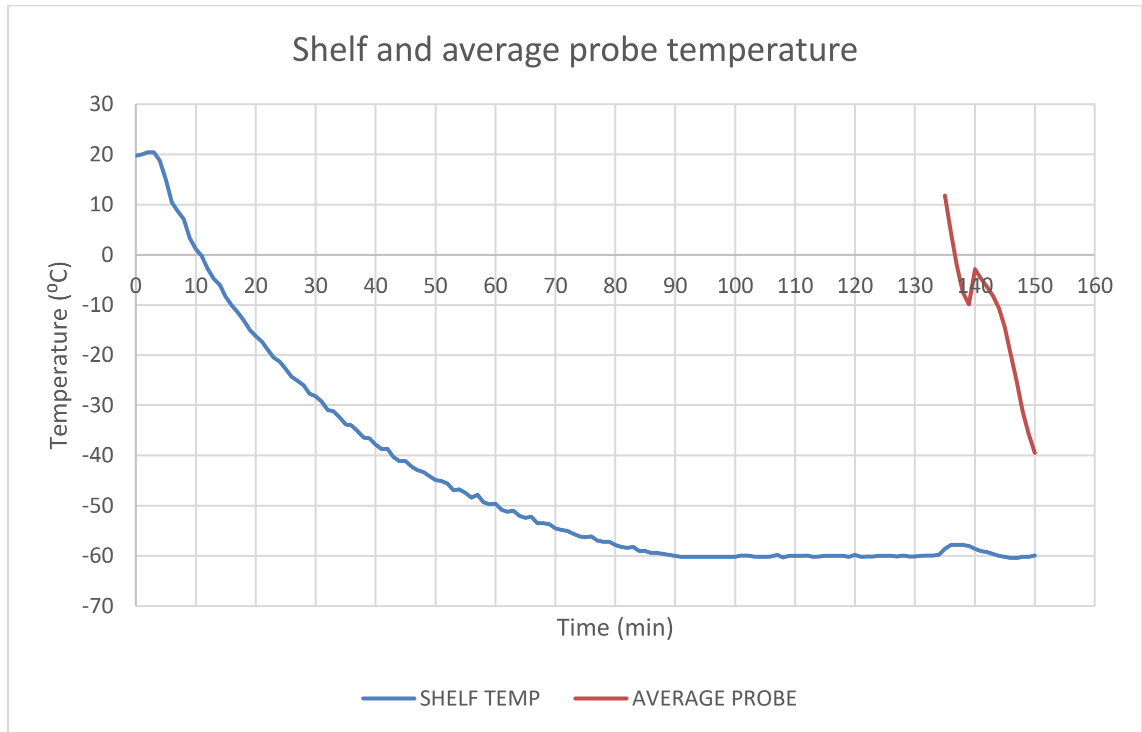


Figure 29. Shelf and average probe temperature during freezing 20 pieces of 3 ml vials.

# 5 Results and conclusions

The success of freeze-drying was evaluated only on the basis of the cake structures of the freeze-dried samples and the data provided by the freeze dryer. The stability and residual moisture content of the samples were not measured after drying so the primary drying could only really be compared between the test runs. All the programs used in the test runs were able to dry the samples. The cake structures were spongelike, and no collapse was observed during any of the test runs. As mentioned in Section 2.5, the cake structure of the freeze-dried product is an ideal when it is spongelike, and its volume is equivalent to the volume of the frozen product.

A successful freeze-drying process was created for 3 ml vials and its length could practically be reduced to half of its original length. The programs are not completely comparable due to the different process parameters, but according to the results, the higher the number of 3 ml vials, the longer the primary drying time (Table 9) because the number of vials affects the heat transfer. The vials in the center of the shelves dry more slowly than the vials on the perimeter of the shelves due to radiant heat from the inside walls of the product chamber. Of course, the length of the primary drying is also affected by many other factors, as mentioned in Section 2.2. Product temperature, filling volume of the vial, configuration of the product and the difference in vapor pressure between the product and the condenser have a huge effect on the rate of sublimation. The 3 ml vials were successfully freeze dried by freezing them both with liquid nitrogen and on the shelves of a freeze dryer. There were no significant visible differences in the cake structures of the freeze-dried samples between the freezing methods.

A successful freeze-drying process was also created for 10 ml vials but only by freezing them on the shelves. According to the results, the freeze-drying times with 10 ml vials were about two times longer than with 3 ml vials due to greater filling volume (Table 9). It was not possible to conduct as many test runs with the 10 ml vials as with the 3 ml vials due to the cracking of the vials. Freezing

with liquid nitrogen was tried several times before the freezing method was changed to shelf freezing. Freeze drying was a success with a shelf freezing but it needs to be studied more. Based on these test runs, only indicative durations for shelf freezing are known and no major conclusions can be drawn yet.

Table 9 Results of the test runs.

| **Program #** | **Volume of the vial (ml)** | **Number of vials** | **Freezing method** | **Primary drying time (min)** | **Total time (min)** |
|---|---|---|---|---|---|
| 1 | 3 | 4 | Liquid nitrogen | 480 | 2830 |
| 2 | 3 | 15 | Liquid nitrogen | 750 | 1640 |
| 3 | 3 | 14 | Shelf freezing | 740 | 1340 |
| 4 | 3 | 20 | Shelf freezing | 680 | 1310 |
| 1 | 10 | 24 | Shelf freezing | 1200 | 2740 |
| 5 | 10 | 16 | Shelf freezing | 1400 | 2650 |

Freezing with liquid nitrogen is challenging for larger batch sizes. Freezing on the shelves is a simpler and safer freezing method but it provides slower cooling and freezing rates. Cooling and freezing rates affect the efficiency of the drying and slower freezing may also cause destabilizing stresses to the products. The effect of the freezing on the stability of the different products needs to be determined with different cooling and freezing rates. Furthermore, a shelf-ramped freezing method and a pre-cooled shelf method must be compared.

In addition to freezing, the entire freeze-drying process needs to be tested with the products. Every product has its own physical properties, so all the products do not dry the same way. The effect of the product type and especially freezing and critical collapse temperatures of the products should be determined to achieve more optimal freeze drying. The stability and residual moisture content of the products should also be determined after freeze drying to evaluate the

safety and efficiency of the process. Especially the determination of the residual moisture content is important in the optimization of secondary drying. In freeze drying of products, the used volumes are much lower than the volumes of the samples in the test runs so the freezing and drying should be quicker with the products.

The goal of this thesis was to find an optimal freeze-drying process which would be suitable for most of the antigen products. This goal was achieved and a new procedure for freeze drying was created. 3 ml vials can be freeze dried with Program #4 (Table 7) and 10 ml vials can be freeze dried with Program #5 (Table 8). Freezing with liquid nitrogen and shelf freezing are both suitable freezing methods for 3 ml vials but 10 ml vials must be frozen on the shelves. More test runs must be performed with both vials but especially with 10 ml vials and shelf freezing.

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