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The influence of the closure format on the storage stability and moisture content of freeze-dried influenza antigen

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ABSTRACT

Low moisture content is seen as crucial to achieving long term stability of freeze dried biologics and reference materials. Highly hygroscopic freeze-dried material are susceptible to moisture ingress over time which can lead to degradation and loss of biological potency. This study compared vials with unprocessed stoppers, vials with vacuum-oven dried stoppers and glass ampoules in order to determine the superior long term storage format in terms of moisture ingress and potency. B/Phuket influenza antigen was chosen as the model biological standard and the lyophilized antigen was stored at −20, 25 and 45 °C over a 1 year period. Ampoules had no significant moisture change across all storage temperatures as would be anticipated. Moisture content results at −20 °C showed no significant differences between ampoules, vials with vacuum-oven dried stoppers and vials with unprocessed stoppers over 12 months. Vials with vacuum-oven dried stoppers performed similarly to ampoules at −20 °C and 20 °C, but had a small increase in moisture content after 6 months at 45 °C. Vials with unprocessed stoppers preformed the worst and exhibited the largest moisture ingress after just 3 months at both 20 °C and 45 °C. Single radial immunodiffusion (SRD) potency assays showed at −20 °C and 20 °C there was no significant difference between all closure formats. At 45 °C there was a drop in potency for all closure formats, but ampoules and vials with vacuum-oven dried stoppers retained higher potency than vials with unprocessed stoppers. Thus, while ampoules are still considered to be the gold standard format for long term storage stability, using vials with vacuum-oven dried stoppers provides comparable stability and moisture integrity at −20 °C and 20 °C storage.

1. Introduction

Freeze-drying (FD) is a drying process that removes water through vacuum sublimation at low temperatures. It consists of three stages which includes freezing (solidification), primary drying (sublimation) and secondary drying (desorption). Most biologics are unstable as liquid formulations as they are prone to a host of chemical reactions which can lead to a loss of activity. Freeze drying is able to extend the shelf life of these materials and increase the stability by producing a solid state final product form with very low moisture content. However, high moisture content in freeze-dried cakes can lead to loss in biological potency and an increase in chemical reactions [1–3]. However, it is important to note that this not may be the case for all proteins. Over-drying might be also an issue and that there may even be an optimal range of residual moisture content for each specific freeze-dried product [1,4–6].

Since the 1970s, inactivated influenza vaccine potency has been measured using single radial immunodiffusion (SRD) assay [7,8]. Standardised reagents such as an antigen and antiserum reagent are required for quantitative results. Serial dilutions of antigen are added into wells of an agarose slab containing antibody and resultant precipitin rings are formed. The size of the ring of the unknown samples and reference standard are measured and compared to evaluate antigen potency relative to reference standard expressed in micrograms of haemagglutinin (HA). SRD is still currently considered to be the gold standard method for potency determination of inactivated influenza vaccines, with alternatives such as HPLC and SDS-PAGE potency assays not being sensitive enough to discriminate between active HA and degraded samples [9,10].

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The World Health Organisation (WHO) International Reference materials and seasonal typing reference materials, such as influenza antigens, are usually prepared and stored in ampoules. WHO recommends that flame-fused glass ampoules are used in order to minimise oxygen and moisture ingress [11]. Past evidence has shown that ampoules are reliably able to provide the best storage stability for a range of temperatures. Matejtschuk et al. showed that freeze-dried albumin stored in ampoules had no significant change in either moisture or oxygen ingress under low to high stress conditions from −70 °C to 56 °C [12]. Vials with rubber closures are seen as less suitable as they have been shown to have an increase in moisture content over time with the resulting percentage change in moisture content being influenced by many compounding factors including stopper formulation, stopper processing treatment, stopper moisture transmission rate, hygroscopicity, and storage temperature/humidity [13–17].

A study investigating water permeability of elastomers by Held and Landi, showed that the moisture content of freeze-dried BCG vaccine in vials with rubber stoppers increased during storage [18]. The mechanisms of this uptake can be either from a) direct transfer of moisture from rubber stopper to freeze-dried cake (with equilibration), b) diffusion/transmission through stopper from outside environment or c) microleaks from the stopper-to-vial interface [16,19–21]. A study by Corveleyn et al. investigating the moisture sorption and desorption of rubber closures, concluded that in addition to the rubber stopper formulation, the critical factor such as processing of the stoppers (e.g sterilisation and drying) and aeration procedure, all have an significant effect on the moisture control [20]. In order to minimise moisture ingress from stoppers some might employ certain drying/processing treatments of their stoppers, including oven heat drying and steam sterilisation [14,16,18,20]. Ford and Dawson showed that vials with treated stoppers (washed in industrial methylated spirit, dried and heat sterilised at 116 °C for 16 h) were as effective as DIN ampoules for freeze-dried alkaline phosphatase over a range of temperatures/humidity [13]. In some rare cases, it has been suggested that if the rubber stoppers are over dried one could initially see a reversal occur, whereby moisture is absorbed out of the lyophilised cake, resulting in a drop in moisture content over time [1].

In this study we evaluate how vials with vacuum-oven dried stoppers and vials with unprocessed stoppers compare to glass ampoules in terms of long term storage stability and moisture integrity for a real world biological reference standard. Lyophilized Influenza B/Phuket antigen was stored at −20 °C, 20 °C and 45 °C and was sampled over a 12 month period to measure moisture content and potency.

2. Materials and methods

2.1. Test materials and closure treatment

Influenza antigen reagent 14/252 was sourced from formalin-inactivated, partially purified B/Phuket/3073/2013 egg-derived virus, which contained approximately 60 μg/ml HA antigen suspended in PBSA buffer containing 1% (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 × 14.75 mm i.d. Adelphi tubes, Haywards Heath, UK) were placed with 13 mm diameter igloo halobutyl rubber stoppers (FDW13WRS, Adelphi Tubes). Half of the 5 mL volume screw capped vials (41.5 × 18 mm i.d. Schott VC005. Adelphi Tubes) were loaded with 14 mm diameter cruciform halobutyl rubber stoppers (FDIA14WGBRTS, Adelphi Tubes) used as received. The other half of the vials were loaded with stoppers that had been vacuum-oven dried for 24 h (<100 mBar at 120 °C) inside a Heraeus Vacutherm VT 6025 Vacuum Drying Oven (Thermo Scientific, Loughborough, UK).

2.2. Lyophilisation cycle

A 4-day freeze-drying cycle (Table 1) was run on the VirTis Genesis 25EL (SP Scientific, supplied by 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. After the cycle had finished 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 Tubes) (Fig. 1). Immediately afterwards, the vials were labelled and stored at either −20 °C, 20 °C and 45 °C until further testing at each set time point.

2.3. Residual moisture content

Moisture content determination of FD samples inside the vials was carried out with an automated Coulometric Karl Fischer CA-200 (Mitsubishi, A1-Envirosciences Ltd, Blyth, UK). Each sample was transferred into a HPLC auto sampler vial (15 × 45 mm screw thread clear c4015-1, Code: 10027364, Thermo Scientific, UK) under low humidity (less than 5% RH) in a pyramid dry bag (Captair pyramid, 2200A Cole Palmer, London, UK) that had been purged with dry nitrogen gas. The actual weight of the samples was determined by weighing the capped HPLC vials before and after addition of freeze dried material. A total of three samples for each closure type were tested at every time point (n = 3). The moisture content of rubber stoppers was determined using a Vapouriser Karl Fischer coulometer VA-100 (Mitsubishi, A1-Envirosciences Ltd). The titration cell volume reagents contained 150 mL of AX solution and the cathode two ampoules of CXU cathode solution. AQUAMICRON Solid water standard (Mitsubishi, A1-Envirosciences Ltd) with known water content was used to check calibration and accuracy. A total of three repeats for each stopper size was taken (n = 3).

2.4. Modulated DSC

Dry samples of B/Phuket was panned into large volume hermetically sealed pans (part number 900825.902 TA Instruments, Elstree, UK) under low humidity (less than 5% RH) in a pyramid dry bag that had been purged with dry nitrogen gas. Samples were evaluated on Q2000 Standard mDSC (TA Instruments) against an empty pan as reference. Sample equilibration was at 10 °C, then

Table 1
Freeze drying cycle for B/Phuket Antigen.

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

Vacuum set to 100μbar during primary and 30μbar in secondary drying.

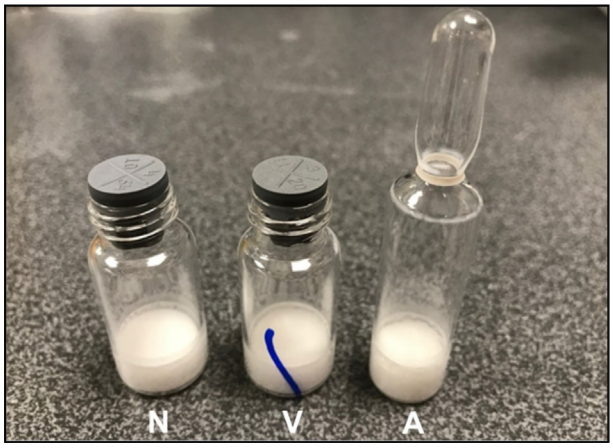

Fig. 1. Three types of vial closure container arrangements: vials with unprocessed 14 mm stoppers (N), vials with vacuum-oven dried 14 mm stoppers (V) and flame sealed glass ampoules (A).

isothermal for 5 min. A heating ramp rate at 5 °C/min up to 200 °C was then applied. 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 (TA Instruments).

2.5. Single Radial Immunodiffusion (SRD) assay

SRD assays were conducted according to Standard Operating Procedure (SOP) from NIBSC Virology Division as previously described [22]. In brief, agarose was melted and mixed with Influenza anti-B/Phuket/3073/2013 HA antiserum reagent (Ref: 15/150, NIBSC, Potters Bar, UK) on a basis of 35 μL of antiserum per mL of agarose. It was then poured into glass plates with perspex moulds (90 mm circular or 103 × 103 mm) and allowed to solidify. Wells were cut to a diameter of 4 mm. Freeze-dried samples and the antigen reagent (Ref: 16/158, NIBSC, Potters Bar, UK) were reconstituted with 1 mL of ultrapure water. Duplicates of all samples were treated with 10% (w/v) Zwittergent 3–14 detergent (Catalogue number: 693017, Millipore, UK) (9:1) and left to incubate for 30 min at room temperature. Dilutions of detergent-treated samples were prepared with six salt phosphate-buffered saline (1:1, 3:4, 1:2, 1:4). A total of 20 μL of each sample was then added to its corresponding well on the plates using a randomisation scheme. Plates with lids were incubated in 20–25 °C cooled incubator for a minimum of 18 h. Plates were then rinsed, overlayered with filter paper and then pressed with 600 g weight for 30 min. After plates were dried, the filter paper was removed before staining gels with 0.3% (w/v) Coomassie brilliant blue R-250 stain. Gels were then destained with a destain mixture (comprised methanol, distilled water and acetic acid (ratio 5:5:1)) until the zones were distinguishable from background. SRD zones were measured with a Synoptics Image Analyser (Synbiosis, Cambridge, UK) and ProtoCol software. Data analysis was performed using slope ratio analysis on the EDQM CombiStats package software (version 5.0, 2013, EDQM, Strasbourg, France).

3. Results and discussion

3.1. Moisture content

The moisture content (% w/w) of freeze-dried B/Phuket antigen in vials with unprocessed stoppers, vials with vacuum-oven dried stoppers and ampoules over a 12 month period at −20 °C, 20 °C and 45 °C is displayed in Fig. 2. The error bars represent the standard deviation between samples (n = 3). At −20 °C the moisture content for all storage formats remains consistently very low from the T = 0 months to T = 12 months' time points; and never rises above 0.60% w/w. After 12 months storage there is no significant difference in the moisture content between ampoules, vials with vacuum-oven dried stoppers and vials with unprocessed stoppers. In contrast at 20 °C, after 3 months the moisture content of the vials with unprocessed stoppers saw a large jump in moisture content from 0.13 ± 0.07 (% w/w) to 2.00 ± 0.10% w/w. This content remains fairly consistent and begins to plateau for the next 9 months between the moisture content regions of 1.70–2.60%

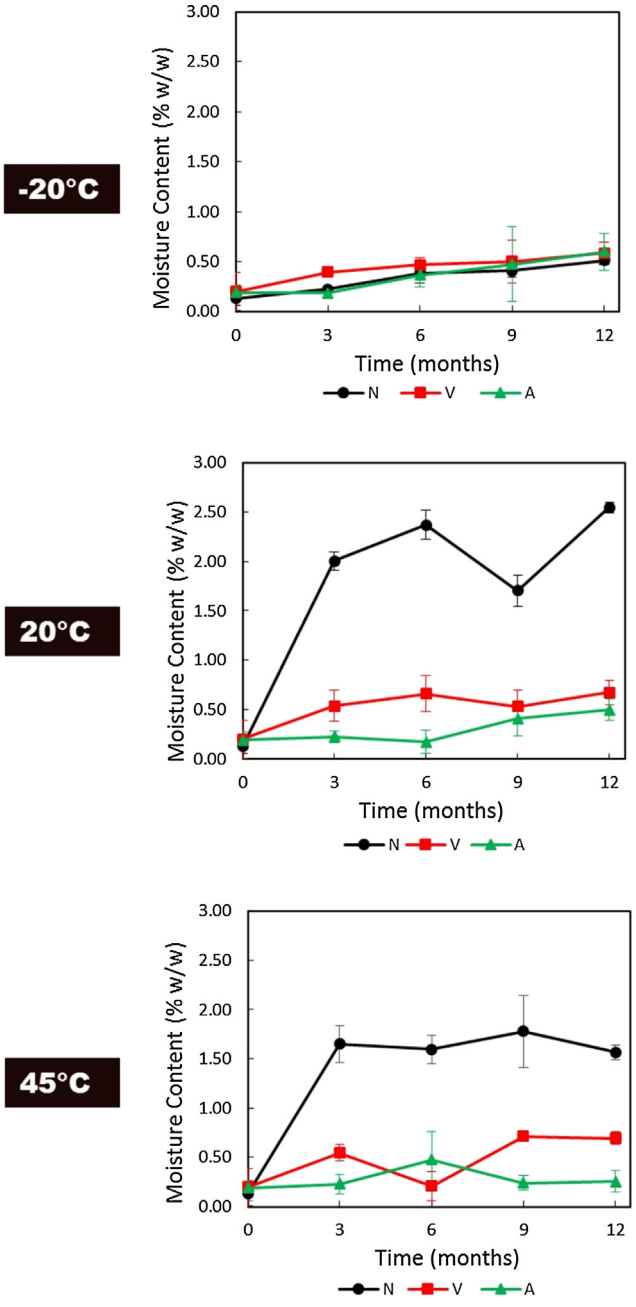

Fig. 2. Comparison of the moisture content (% w/w) between vials with unprocessed stoppers (N), vials with vacuum-oven dried stoppers (V) and ampoules (A) over a period of 12 months stored at −20 °C, 20 °C and 45 °C. Error bars represent the standard deviation (n = 3).

w/w. However, the vials with vacuum-oven dried stoppers and the ampoules do not exhibit such ingress of moisture. The vials with vacuum-oven dried stoppers also initially exhibit a small increase in moisture during the first 3 months from 0.20% w/w to 0.54% w/w. Ampoules saw no significant change between T = 0 to T = 6 months but had a small increase from T = 9 to T = 12 months. After 12 months of storage the vials with vacuum-oven dried stoppers presented similar low moisture content to that of the ampoules. At 45 °C, once again the vials with unprocessed stoppers saw a sharp rise in moisture content after the first 3 months from 0.13 ± 0.07% w/w to 1.65 ± 0.18% w/w. At longer time points the moisture content again plateaus around the region between 1.50 and 2.00 % w/w. Vials with vacuum-oven dried stoppers also saw a rise after 3 months from 0.20 ± 0.10% w/w to 0.55 ± 0.09% w/w. Moisture contents then plateaus between 0.50% and 0.70 % w/w in the next 9 months. At T = 6 months the vials with vacuum-oven dried stoppers saw a small average drop in moisture content based on the trend at other time points. This was probably due to error in Karl Fisher measurement or an anomalous sample (n = 3) lowering the average and increasing the standard deviation error bars. The ampoules performed the best, consistently retaining very low moisture content between T = 0 (0.19 ± 0.08% w/w) and T = 12 months (0.26 ± 0.11% w/w).

Maintaining low moisture has long been seen as crucial for the long term stability of freeze-dried products. Freeze-dried amorphous cakes are highly hygroscopic and especially so for influenza antigens where the dry mass is low. The results from Fig. 2 show that ampoules exhibited a low amount of moisture ingress throughout the whole 12 months storage, even at high stress conditions. Vials with vacuum-oven dried stoppers performed just as well at maintaining low moisture content at −20 °C and 20 °C, while at 45 °C they were able perform significantly better than vials with unprocessed stoppers and were very close to ampoule performance. Matejtschuk et al. saw comparable moisture content results when comparing ampoules and vials with rubber stoppers for human albumin [12]. The moisture content in their ampoules did not see any detectable change even at elevated conditions storage stresses, while the vials saw notable moisture ingress over time. Sasaki et al. observed that during the early stage of storage the moisture content increase in the cakes was due to moisture transfer from the stoppers to the cake, whilst at later stages it was caused by external permeation through the stoppers. Additionally low-moisture rubber stoppers corresponded with lower moisture cakes, whilst high-moisture rubber stoppers saw the reversal with larger moisture content [14].

The flame sealed and closed form factor of the ampoules naturally means that barring any trapped headspace moisture or cracks, it will have a significant advantage over vials with rubber stoppers when it comes to moisture ingress. In vials with rubber stoppers the moisture could come directly come from either the stopper itself or through transmission from the outside environment. The small intrinsic mass of these freeze-dried samples means that only a small amount of moisture exchange from the stoppers would be required to cause significant shifts in cake moisture content. For example a freeze-dried antigen sample of a weight of 10 mg, would only require 0.1 mg (or 100 μg) moisture from the rubber stopper to increase its moisture content by +1% w/w. Fig. 3 shows that the amount of moisture (μg) present in the 14 mm and 20 mm rubber stoppers before and after treatment in the vacuum-oven is between 10 and 22 times the required 100 μg which would result in 1% moisture uptake. Vials with vacuum-oven dried stoppers had significantly less moisture present than the unprocessed stoppers for both sizes. Unprocessed 20 mm stoppers had more moisture present (in terms of μg) than the unprocessed 14 mm stoppers but lower weight/weight moisture (% w/w) due to the size difference. After the vacuum-oven treatment both stopper sizes had a

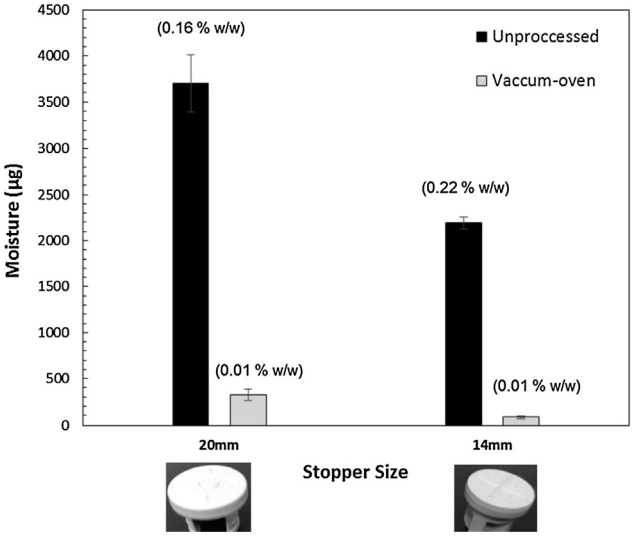

Fig. 3. Comparison of the average moisture (μg) difference between unprocessed stoppers (straight out of pack) and vacuum-oven dried stoppers (<100 mBar at 120 °C) for different sizes. Error bars represent the standard deviation (n = 3).

moisture content at or below 0.01% w/w. Thus, only a small percentage of moisture coming from the unprocessed stoppers would be sufficient enough to account for the significant shifts in moisture content as seen in Fig. 2, even when taking into consideration that not all moisture in the stoppers will be transmitted and absorbed by the cake.

The 14 mm rubber stoppers used for this study that were dried in the vacuum-oven (24 h at 120 °C under 100 mBar) had most of the moisture removed compared to the unprocessed stoppers straight out of the pack. Thus, over the 12 month storage period this could explain why the vials with vacuum-oven dried stopperes did not cause the same large moisture increases as the vials with unprocessed stoppers at temperatures above −20 °C. At −20 °C, all storage formats performed equally well and maintained low moisture contents. At temperatures below freezing point of water there is a very low ingress level due to the very low humidity in the surrounding external atmosphere. At 20 °C and 45 °C the moisture content of the vials with unprocessed stoppers appeared to plateau which infers that they achieved some type of equilibrium with the moisture that was present. The elastomer formulation of the rubber stopper will determine its moisture uptake behaviour, which of course will depend on the local relative humidity and temperature [20]. Held and Landi showed that water permeation occurred regardless of which rubber stopper they evaluated, although the grey butyl stoppers were the least hygroscopic [18], and that oven heat drying their stoppers resulted in rapid water release. The vials in this study that had vacuum-oven dried stoppers exhibited product moisture levels comparable to that of ampoules for the entire length of the storage trials.

3.2. SRD potency

The potency of the stored samples was measured by SRD assay. Fig. 4 shows the potency (μg HA/mL) between vials with unprocessed stoppers, vials with vacuum-oven dried stoppers and ampoules, stored at −20 °C, 20 °C and 45 °C over a 1 year period. The error bars represent ±95% confidence intervals. At −20 °C, there is no significant difference in potency between all container formats. There is a slight drop between T = 0 and T = 3 months but this still within overlapping confidence intervals. Throughout the year, potency levels remain within the region of 50–60 μg HA/mL

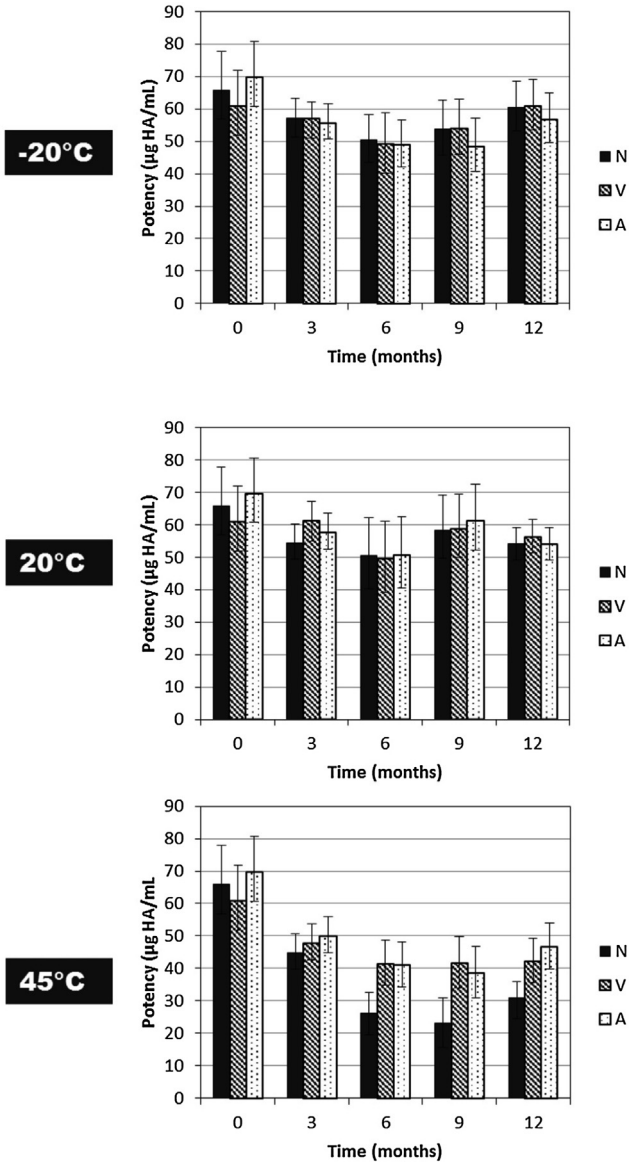

Fig. 4. Comparison of the potency (µg HA/mL) between vials with unprocessed stoppers (N), vials with vacuum-oven dried stoppers (V) and ampoules (A) over a period of 12 months stored at −20 °C, 20 °C and 45 °C. Error bars represent ±95% Confidence Intervals.

for all storage formats. At 20 °C, the same behaviour is observed, with no major difference between the vial formats throughout the whole year. However, at 45 °C, between T = 3 and T = 9 months, a loss is observed for all vials. The ampoules and the vials with vacuum-oven dried stoppers were able to better retain their estimated potency than the vials with unprocessed stoppers after T = 6 months. After 12 months at 45 °C, the final estimated potency for vials with unprocessed stoppers, vials with vacuum-oven dried stoppers and ampoules were 30.9, 42.2 and 46.6 µg HA/mL respectively.

Whilst high moisture was observed for vials with unprocessed stoppers (Fig. 2), this moisture did seem not have any impact on the potency at 20 °C. However, at 45 °C it appears that the combination of high stress temperature and moisture led to vials with unprocessed stoppers having the largest drop in potency. Both ampoules and vials with vacuum-oven dried stoppers, which had consistently low moisture (less than <1% w/w) throughout the course of the 12 months, were both able to retain slightly higher potency at this temperature. This suggests that a combination of both high temperature and moisture was required to have the greatest impact on the freeze-dried product potency. Ford and Dawson also saw similar results in which they found that high moisture alone was not enough to reduce enzymatic activity of freeze-dried alkaline phosphatase. It was only when combined together with high temperature storage that the higher moisture contributed to a reduction in activity [13].

Of course, this further leads to the question of how much moisture is acceptable in the cake for long term stability. Standard convention still maintains that the drier the cake then the better the stability. From our results ampoules and vials with vacuum-oven dried stoppers had the least amount of moisture and also the better potency at 45 °C compared to vials with unprocessed stoppers. However, for vials with unprocessed stopper at 20 °C, the relatively high moisture content of between 1.7 and 2.6 % w/w, did not have any impact on the potency compared with ampoules and vials with vacuum-oven dried stoppers. Stability here was highly dependent on the storage temperature. If a product is stored above the glass transition temperature (T_g) then there is a transition from a glassy brittle state to a more rubbery state with increased molecular flexibility and greater chance of instability. A study by Breen et al, investigating the stability of lyophilized humanized monoclonal antibody, showed that physical stability was not compromised by increasing moisture content as long as the storage temperature was below the T_g [23]. As moisture content increases then a subsequent reduction in the T_g is often observed. As shown at Table 2, the moisture content inside the vials with unprocessed stoppers will have reduced the T_g value. Since the vials at 45 °C storage temperature were closer to the reduced T_g value than the vials at 20 °C ($T_g - T$), it could possibly explain why there was a difference in potency (Table.2). If the storage temperature is closer to and approaches the T_g value then you would have increasing molecular flexibility and hence more chance of physical or chemical instability occurring. Hancock et al, even suggest that storage temperature must be at least 50 K below the T_g value of product in order to have negligible molecular motions [24].

Past studies have indicated that there may also be a "goldilocks region" of optimum moisture content [3]. This optimum region may be distributed in the form of a bell shaped curve which might also shift depending on the temperature of storage, so in fact whilst one moisture content might be acceptable for one storage temperature it might not be for another. However, if too much moisture is present or uptake occurs past a certain threshold, cake shrinkage or collapse can result, especially at higher temperatures [25]. Future studies could investigate the maximum moisture content permitted for freeze-dried B/Phuket antigen which might be acceptable up to room temperature storage without sacrificing potency, correlating it to the T_g. This of course would not be a general rule for all and will be dependent on a case by case basis for each freeze-dried antigen material. Additional further studies could also look at the

Table 2
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)	Tg (°C)	Tg − 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

influence of different size, formulation and treatment of rubber stoppers mapping their corresponding effects on long term moisture uptake and stability.

4. Conclusion

This study has shown that ampoules are the best long term storage format for a FD influenza antigen in maintaining both low moisture content and high potency at a range of temperatures. However, vials with vacuum-oven dried stoppers can be used as suitable alternative to provide comparable moisture levels and potency between storage at −20 °C to 20 °C over a 1 year period. Performance of vacuum-oven dried stoppers was slightly inferior to glass ampoules at 45 °C. Vials with unprocessed rubber stoppers also showed that at −20 °C they performed just as well as ampoules/vacuum stopper vials for both moisture integrity and potency, but at higher stress temperatures they displayed large increases in moisture and a drop in potency. At 20 °C, the increased moisture content in vials with unprocessed stoppers had no effect on potency, whilst at elevated temperature of 45 °C, higher moisture content was attributed to the larger potency losses. This suggests that antigen potency was highly dependent on storage temperature relative to moisture content and how close it was to the reduced glass transition temperature. Overall, the long term moisture uptake and stability of freeze-dried international reference material is of great importance and is highly dependent on the closure format, with vacuum-oven dried stoppers offering a potentially attractive option to glass ampoules.

Declaration of Competing Interest

None.

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