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Impact of the freeze-drying on the VHP content in the production of vaccines

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INTRODUCTION

The term “lyophilization” describes a process to produce a product that “loves the dry state”. Freeze-drying, also known as lyophilization, is a drying process, it has applications in food processing, agriculture, and pharmaceuticals. It is one among several dehydration processes, such as: air drying, chemical drying, and solvent drying. All of them effectively remove water from hydrated systems, but freeze-drying is the most reliable method nowadays for thermolabile materials.

“Freeze-drying is widely used for pharmaceuticals because enzyme/protein- or peptide-based drugs are more stable in solid form. In the freeze-dried solid state, physical or chemical degradation reactions are inhibited or sufficiently decelerated, this results in an improved long-term stability” [4].

Freeze-dried formulations not only have the advantage of better stability, they also provide easy handling during shipping and storage.

Formulation and filling of pharmaceutical drugs intended to be freeze-dried takes place in isolators. In order to avoid any undesired contamination during the filling operations, the inner surfaces of such isolators are regularly sterilized by using vaporized hydrogen peroxide (VHP). After the sterilization, VHP is removed from the isolator by aeration. Despite the purging with clean air, residuals of the VHP will remain in the containment atmosphere and they will potentially enter the drug product during the fill and finish process. This can trigger instability of the active pharmaceutical ingredients, excipients or entire drug product.

Understanding the impact and the consumption of hydrogen peroxide at each step of the freeze-drying process plays an important role in developing protein formulation, defining storage condition, and optimizing fill-finish process.

The purpose of this study was to investigate the hydrogen peroxide consumption in the vaccine during lyophilization. The present study is a spiking study in which the protein formulation was spiked with a specified amount of hydrogen peroxide and freeze-dried. The consumption of hydrogen peroxide, during the freeze-drying cycle, was monitored as a function of time and freezing temperature. The concentration of remaining hydrogen peroxide in the samples was evaluated by using Amplex UltraRed Method, this method allows determination of hydrogen peroxide down to 5 ng/mL.

CONTENT

SOMMARIO

Impatto della liofilizzazione sul contenuto residuo di VHP in una formulazione contenente un vaccino

La liofilizzazione o crioessiccamento è un processo tecnologico che permette l'eliminazione dell'acqua, o di un generico solvente, da una sostanza organica. Tra le diverse tecnologie di essiccazione, la liofilizzazione è sicuramente quella più adatta a sostanze termolabili. Viene principalmente utilizzata nell'industria farmaceutica in quanto i farmaci contenenti proteine sono più stabili in forma solida. Allo stato liofilizzato, le reazioni chimiche o fisiche di degradazione sono inibite o sufficientemente rallentate. In questo modo aumenta la stabilità a lungo termine del prodotto. La liofilizzazione oltre a una migliore stabilità dei prodotti, ne semplifica anche la gestione durante la loro distribuzione e stoccaggio.
Le fasi di formulazione e di riempimento dei preparati farmaceutici iniettabili, che sono liofilizzati, sono condotte in ambienti sterili che spesso ricorrono all'uso di isolatori. Al fine di evitare qualsiasi tipo di contaminazione, le superfici interne degli isolatori sono sterilizzate per esposizione a perossido di idrogeno vaporizzato (VHP). Quest'ultimo viene poi eliminato attraverso una fase di aerazione condotta utilizzando una corrente di aria sterile. A valle di questo trattamento, si è osservata la presenza di residui di VHP che permangono all'interno della camera di isolamento e possono contaminare i prodotti durante le fasi di formulazione e/o di riempimento e, di conseguenza, possono innescare fenomeni di instabilità o degradazione del prodotto stesso.

Liofilizzazione

La liofilizzazione è un processo tramite il quale il solvente viene rimosso per sublimazione e desorbimento, generalmente sotto vuoto. Il processo si divide in tre fasi: (i) congelamento; (ii) essiccamento primario; (iii) essiccamento secondario.
Durante la fase di congelamento, la formulazione liquida viene raffreddata fino a farla solidificare; in queste condizioni gran parte del solvente, solitamente acqua, si separa dal soluto sotto forma di giacchio. Durante l'essiccamento primario, il ghiaccio viene rimosso per sublimazione. La pressione all'interno del liofilizzatore è inferiore alla tensione di vapore del ghiaccio, mentre la temperatura aumenta per compensare il calore rimosso dalla sublimazione. Un certo quantitativo di acqua resta, dopo l'essiccamento primario, intrappolata nella matrice essiccata. Quest'acqua residua viene parzialmente asportata per desorbimento durante l'essiccamento secondario innalzando la temperatura.

Sterilizzazione via Perossido di Idrogeno

Il perossido di idrogeno è un agente sterilizzante di uso comune, molto efficiente nella rimozione dei microrganismi contaminanti, ma è anche caratterizzato da forti proprietà ossidanti. Gli isolatori, all'interno dei quali avviene la formulazione dei vaccini e il riempimento dei flaconi, vengono sterilizzati con perossido di idrogeno. Il perossido viene vaporizzato all'interno della camera, dopo un certo tempo di permanenza viene rimosso

tramite aerazione con aria sterile. Tracce di perossido possono restare all'interno della camera e diffondere nel vaccino durante la formulazione e il riempimento. In alcuni casi, residui di perossido presenti nell'isolatore possono adsorbirsi sul packaging primario (flaconi) e successivamente diffondere nel vaccino durante la fase di riempimento quando vengono a diretto contatto. Il perossido di idrogeno che va a contaminare il vaccino può indurre degradazione per ossidazione dei principi attivi, degli eccipienti o dell'intero vaccino. Può inoltre indurre: dimerizzazione, aggregazione, ossidazione, variazioni della struttura e cross-linking di grandi molecole proteiche ed eccipienti. Impattando così la stabilità, la sicurezza e l'efficacia del prodotto. La maggior parte del perossido di idrogeno che contamina i vaccini, viene rimosso con l'acqua durante il processo di liofilizzazione. La parte che rimane può però reagire con le proteine allo stato liofilizzato.
Inoltre, la liofilizzazione può contribuire ad aumentare l'ossidazione delle proteine in modo proporzionale alla concentrazione di perossido di idrogeno presente nel vaccino.

Obiettivi dello studio

Lo scopo di questa ricerca è stato quello di analizzare la variazione della concentrazione residua di HP nel vaccino durante il ciclo di liofilizzazione e stabilire quali sono i principali fattori che ne influenzano il consumo.

Metodi

Al fine di simulare la contaminazione che avviene negli isolatori, prima della liofilizzazione, quantità specifiche di perossido di idrogeno sono state aggiunte sia al vaccino che al suo placebo. Sono stati poi lasciati per due ore a temperatura ambiente ed esposti alla luce naturale in modo tale da simulare il processo produttivo. Trascorse le 2 ore, le formulazioni sono state distribuite in flaconi da 3 mL a 0.5 mL/flacone, parzialmente tappati (configurazione liofilizzazione) e poi liofilizzati.

Impatto del tempo di contatto tra il perossido e il vaccino/placebo

La concentrazione di perossido all'interno delle formulazioni liquide è stata misurata diverse volte dopo l'aggiunta del perossido, nelle due ore che precedono la liofilizzazione, per osservare la sua stabilità.

Impatto della concentrazione iniziale di perossido nel vaccino

È stato inoltre analizzato l'impatto della concentrazione iniziale di perossido sul suo consumo dopo liofilizzazione. Diverse soluzioni liquide di vaccino sono state addizionate con perossido fino ad ottenere concentrazioni finali di: 100, 200, 300, 400, 500 e 600 ng/mL. Il consumo di perossido di idrogeno è stato valutato alla fine del ciclo di liofilizzazione.

Consumo del perossido di idrogeno durante la liofilizzazione

Il consumo di perossido di idrogeno durante il processo di liofilizzazione è stato valutato in funzione di: (I) temperatura di congelamento; (II) presenza dell'antigene.

Per valutare l'impatto della temperatura di congelamento, lo stesso ciclo di liofilizzazione è stato ripetuto utilizzando due diverse temperature di congelamento: -45°C e -55°C.

Una soluzione acquosa di perossido di idrogeno, a bassa temperatura, forma un unico composto: il diidrato $H_2O_2 \cdot 2H_2O$ che congela a -52°C. Le due temperature sono state scelte perché:

- a -45°C il perossido è ancora attivo (non congelato);
- a -55°C il perossido è congelato come diidrato.

Per valutare l'impatto dell'antigene sul consumo di perossido durante la liofilizzazione, il vaccino e il suo placebo sono stato addizionati con una certa quantità di perossido di idrogeno e poi liofilizzati insieme nelle stesse condizioni. Durante ogni ciclo di liofilizzazione, metà dei flaconi conteneva il vaccino e l'altra metà il placebo.

Per ottenere l'andamento del consumo di perossido di idrogeno in funzione del tempo durante la liofilizzazione, il ciclo è stato interrotto in diversi punti come mostrato nella *Tabella 3.1,* ed è stata poi misurata la concentrazione residua di perossido nei vari campioni. Al termine di ogni ciclo, i flaconi liofilizzati sono stati conservati a -20°C fino alla valutazione della quantità di perossido residuo.

Consumo del perossido nel vaccino durante l'essiccamento secondario

Il consumo di perossido durante l'essiccamento secondario è stato anche valutato utilizzando il liofilizzatore Millrock. Quest'ultimo è equipaggiato con un braccio meccanico che permette, in qualsiasi momento, di chiudere alcuni flaconi mentre il processo va avanti. In questo modo, si arresta l'essiccamento per questi flaconi. Le temperature di congelamento testate sono: -45°C, -52°C e -57°C. È stata anche testata una temperatura più alta per quanto riguarda l'essiccamento secondario. In *Tabella 3.2* sono indicati i punti, durante l'essiccamento secondario, in cui l'essiccamento è stato interrotto per alcuni flaconi. È stata poi misurata la concentrazione residua di perossido nei vari campioni. Alla fine del processo, i flaconi liofilizzati sono stati conservati a -20°C fino alla valutazione della quantità di perossido residuo.

Metodo Amplex Ultra Red

La concentrazione di perossido di idrogeno all'interno dei campioni è stata determinata utilizzando il metodo Amplex UltraRed. L'analisi è stata effettuata aggiungendo il reagente di lavoro che contiene: il buffer (fosfato), il catalizzatore (HRP) e il reagente Amplex UltraRed. Il perossido, grazie alla presenza del catalizzatore, ossida l'Amplex UltraRed che passa da una condizione di non fluorescenza ad una di fluorescenza. La fluorescenza può essere misurata con un flurimetro adatto. Si ottiene una curva di taratura che correla la fluorescenza con la concentrazione di perossido. In questo modo è possibile misurare la concentrazione residua di perossido nei vari campioni liofilizzati. Questo metodo consente di calcolare la concentrazione di perossido all'interno di soluzioni acquose. Per questo motivo, la cake che si è formata a seguito della liofilizzazione, è stata ricostituita con 0.5 mL di acqua. Dopodiché, 2 flaconi sono stati mescolati insieme per ottenere il volume di liquido richiesto per effettuare la misura.

Risultati

Impatto del tempo di contatto tra il perossido e il vaccino/placebo

La concentrazione di perossido di idrogeno, nel placebo e nel vaccino, nelle 2 ore che precedono la liofilizzazione è mostrata in *Figura 4.1*.
Sia nel placebo che nel vaccino, la concentrazione di perossido è rimasta pressocché costante fino al termine delle due ore. Questo risultato indica che il tempo di contatto, tra il perossido e le due formulazioni liquide, non ha un impatto significativo sulla sua concentrazione durante il periodo di mantenimento prima della liofilizzazione.

Impatto della concentrazione iniziale di perossido nel vaccino

La *Figura 4.2* mostra il consumo di perossido (%) nei vaccini con diversa concentrazione iniziale dopo la liofilizzazione. Tutti i vaccini, dopo la liofilizzazione, hanno mostrato approssimativamente lo stesso consumo che è in media del 70.5% (SD = 2.5%). La velocità di consumo del perossido di idrogeno, ovvero la derivata nel tempo della concentrazione residua, non dipende dalla sua concentrazione iniziale.

Consumo del perossido di idrogeno durante la liofilizzazione

Vaccino - congelamento a -45°C *vs*. congelamento a -55°C

Il confronto in termini di concentrazione residua e di consumo di perossido di idrogeno nel vaccino durante la liofilizzazione, con le due diverse temperature di congelamento, è mostrato in *Figura 4.6*.
Le due curve che rappresentano il consumo di perossido hanno approssimativamente lo stesso andamento. Il consumo è più elevato nel caso di congelamento a -45°C rispetto a quello a -55°C.
Per entrambe le temperature di congelamento, durante l'essiccamento primario il perossido viene consumato maggiormente durante le prime 8 ore.
Nel caso in cui la temperature di congelamento è -45°C, 5 ore dopo l'inizio dell'essiccamento secondario, il consumo sembrerebbe raggiungere uno stazionario. Tutto ciò indica che è stato raggiunto il limite inferiore della concentrazione di perossido, quindi non è più possibile continuare a rimuovere il perossido attraverso l'essiccazione e/o l'ossidazione delle proteine. Mentre nel caso in cui la temperature di congelamento è -55°C, si ha un minor consumo durante tutto il processo, per questo motivo il consumo aumenta linearmente fino alla fine del processo. Con questa temperatura di congelamento non viene raggiunto il limite inferiore della concentrazione di perossido.

Placebo - congelamento a -45°C *vs*. congelamento a -55°C

Il confronto in termini di concentrazione residua e di consumo di perossido di idrogeno nel placebo durante la liofilizzazione, con le due diverse temperature di congelamento, è mostrato in *Figura 4.9*.
Anche con il placebo, il consumo è più elevato nel caso di congelamento a -45°C rispetto a quello a -55°C. Le due curve che rappresentano il consumo del perossido durante la liofilizzazione hanno lo stesso andamento: il consumo raggiunge uno stazionario durante l'essiccamento primario che viene mantenuto fino alla fine dello step. Quando comincia

l'essiccamento secondario, il consumo riprende ad aumentare per poi raggiungere un nuovo stazionario alla fine dell'essiccamento secondario.
Nel caso in cui la temperature di congelamento è -45°C, il consumo raggiunge più velocemente lo stazionario durante l'essiccamento primario. Infatti, con -45°C lo stazionario viene raggiunto dopo 8.5 ore. Mentre nel caso in cui la temperature di congelamento è -55°C, lo stazionario viene raggiunto dopo 15.25 ore. Nonostante questa differenza durante l'essiccamento primario, durante l'essiccamento secondario, per entrambe le temperature il consumo di perossido ha raggiunto lo stazionario nello stesso momento (dopo 5 ore dall'inizio dell'essiccamento secondario) .

Placebo *vs*. vaccino – temperatura di congelamento -55°C

La *Figura 4.10* mostra il consumo di perossido di idrogeno nel vaccino e nel placebo durante il processo di essiccazione. La temperatura di congelamento è -55°C.

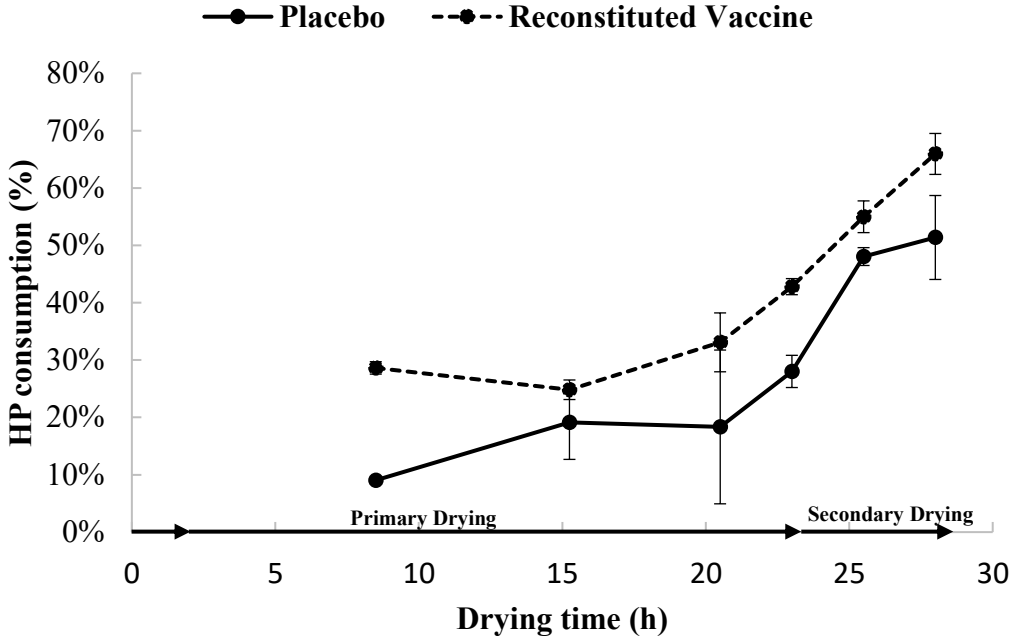

Figura 4.10 Consumo percentuale di perossido di idrogeno (%) durante la liofilizzazione. Temperatura di congelamento -55°C.

Nel vaccino il consumo di perossido di idrogeno è più elevato rispetto a quello nel placebo. Questo risultato indica che la presenza dell'antigene ha un impatto sul consumo di perossido. È possibile che all'interno del vaccino, il perossido venga parzialmente consumato dalle reazioni con le proteine. Per questo motivo il consumo è del 14.6% più elevato rispetto a quello nel placebo, questa differenza in termini di consumo è dovuta molto probabilmente alle reazioni di ossidazione.
Inoltre, nel placebo il perossido viene rimosso solamente attraverso il processo di essiccazione. Quando la concentrazione di perossido raggiunge il valore minimo, si ha lo stazionario. Nel caso del vaccino, invece, anche se viene raggiunto il valore minimo di concentrazione per quanto riguarda il processo di essiccazione, il consumo di perossido continua a causa delle reazioni di ossidazione.

Placebo *vs*. vaccino – temperatura di congelamento -45°C

La *Figura 4.11* mostra il consumo di perossido di idrogeno nel vaccino e nel placebo durante il processo di essiccazione. La temperatura di congelamento è -55°C.

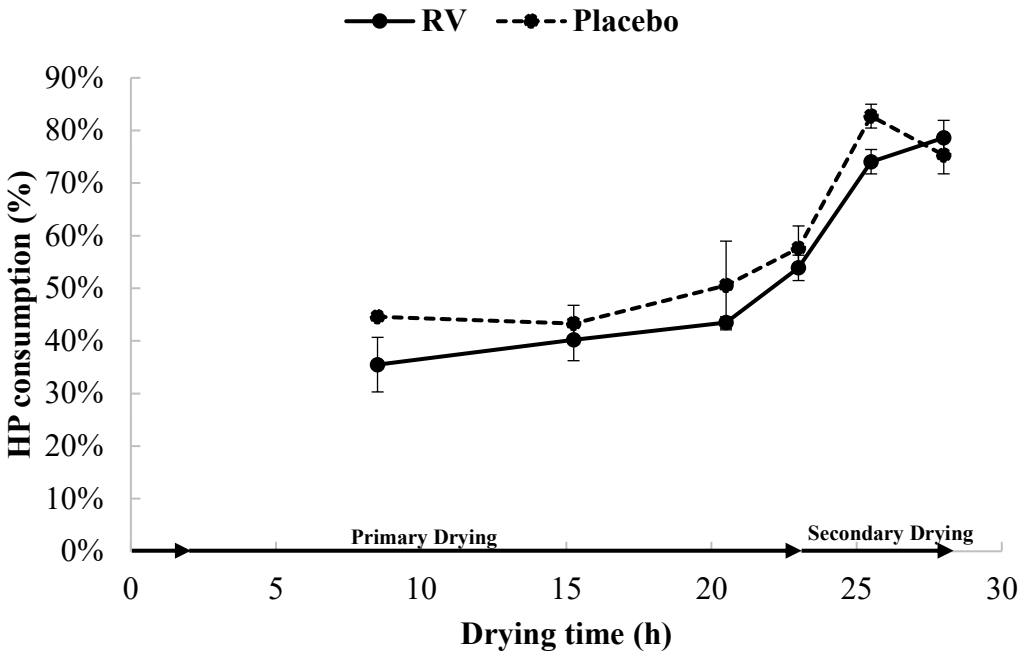

Figura 4.11 Consumo percentuale di perossido di idrogeno (%) durante la liofilizzazione. Temperatura di congelamento -55°C.

Lo stesso consumo è stato registrato sia nel vaccino che nel placebo. Con questa temperatura di congelamento, l'antigene non ha nessun impatto sul consumo di perossido. Inoltre, questo risultato indica che nel vaccino il perossido viene rimosso prevalentemente dal processo di essiccazione, come accade nel placebo, e non dalle reazioni di ossidazione.

Consumo del perossido nel vaccino durante l'essiccamento secondario

Temperatura di congelamento -52°C

La *Figura 4.12* mostra il consumo di perossido di idrogeno nel vaccino durante l'essiccamento secondario. In questo caso la temperatura di congelamento è -52°C, questa è la temperatura di congelamento che si ha nel ciclo standard di questo vaccino.

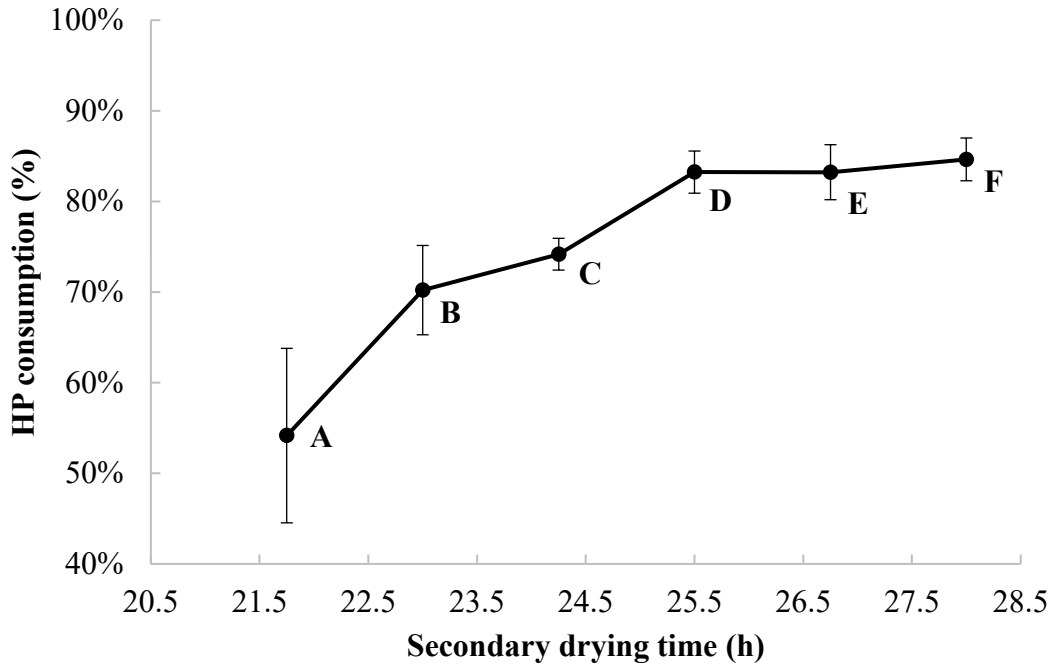

Figura 4.12 Consumo del perossido di idrogeno (%) nel vaccino durante l'essiccamento secondario.

Il consumo aumenta linearmente nel tempo finché non viene raggiunto un stazionario. Tale stazionario viene raggiunto 5 ore dopo l'inizio di questo step.

Durante l'essiccamento secondario, il perossido viene rimosso con l'acqua per evaporazione. Il processo segue i seguenti step: (I) il perossido diffonde attraverso il prodotto; (II) evaporazione e (III) condensazione (nel condensatore). La presenza del plateau indica che lo step limitante è la diffusione del perossido attraverso il campione e non la sua evaporazione dalla superficie. Lo stazionario è stato raggiunto quando il consumo è del 83.5%. Inoltre, il fatto che la curva di consumo di perossido *vs*. tempo (vedi *Figura 4.12*, senza considerare lo stazionario) sia lineare conferma che la velocità di consumo non dipende dalla sua concentrazione.

Lo stesso ciclo di liofilizzazione, con la stessa temperatura di congelamento (-52°C), è stato ripetuto con una temperatura più alta per quanto riguarda l'ultimo step di essiccamento secondario. La temperatura dell'ultimo step di essiccamento secondario del ciclo standard è 40°C. La temperatura più alta testata è 45°C. In *Figura 4.13* viene mostrato il confronto in termini di consumo di perossido tra le due temperature di essiccamento secondario.

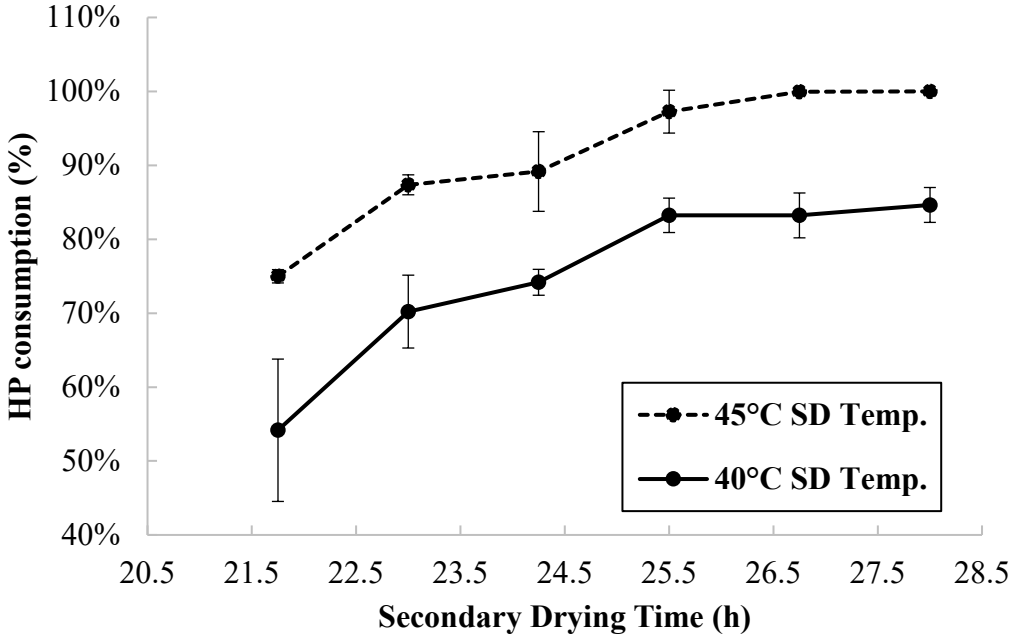

Figure 4.13 Consumo di perossido di idrogeno (%) durante l'essiccamento secondario, temperatura di congelamento -52°C. Confronto tra le due temperature dell'ultimo step di essiccamento secondario: 40°C e 45°C.

Sebbene le misure della concentrazione residua di perossido nei campioni siano corrette, sorgono dubbi circa l'accuratezza dei risultati (curva 45°C SD Temp.) quando vengono comparate le due curve. Queste ultime dovrebbero essere sovrapposte fino a 23 ore (h) (tempo di essiccamento secondario), perché fino a questo istante di tempo i due cicli sono esattamente identici, e iniziare a divergere soltanto dopo. Infatti a 23 ore (h), una ciclo rimane a 40°C mentre l'altro aumenta fino a 45°C. Nonostante questa considerazione, alcune conclusioni possono comunque essere tratte considerando la concentrazione residua di perossido nel vaccino dopo la liofilizzazione.

Nel caso in cui la temperatura di essiccamento secondario è di 45°C, tutto il perossido presente è stato rimosso. Perciò, aumentando la temperatura di essiccamento secondario, aumenta la velocità di rimozione del perossido.

Confronto tra diverse temperature di congelamento

Il consumo di perossido durante l'essiccazione secondaria è stata anche valutata con altre temperature di congelamento: -45°C e -57°C, come mostrato in *Figura 4.14:*

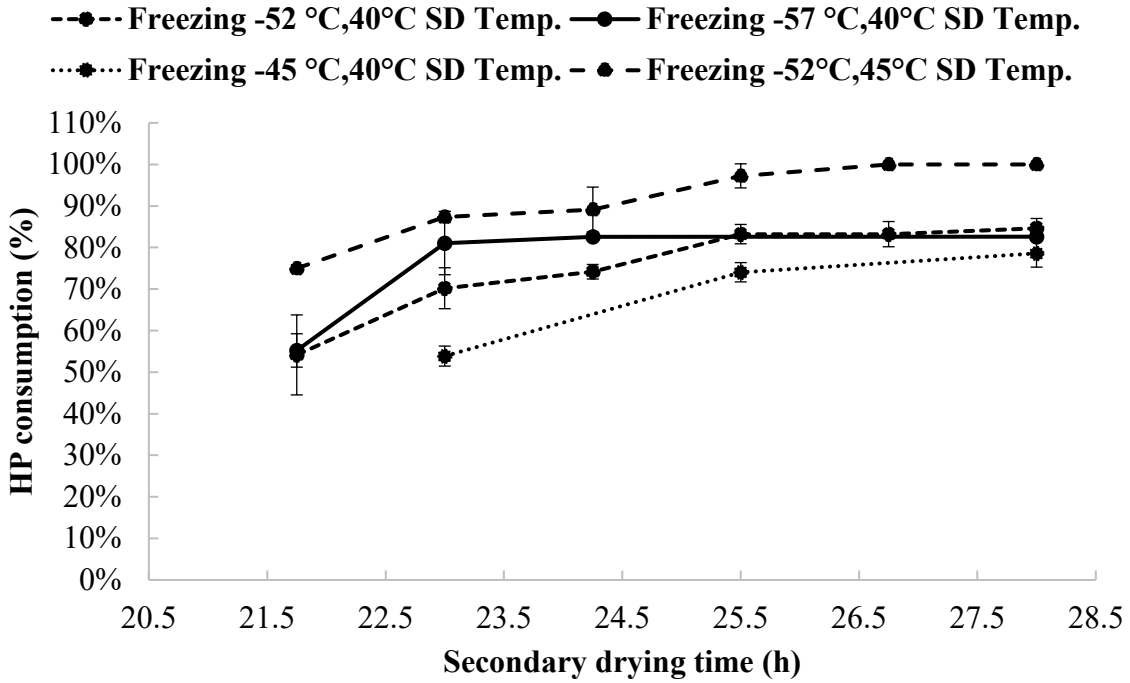

Figura 4.14 Confronto in termini di consumo di perossido tra diversi cicli di liofilizzazione con diverse temperature di congelamento.

Nel caso in cui la temperatura di essiccamento secondario sia 40°C, indipendentemente dalla temperatura di congelamento, il perossido non viene completamente eliminato dal vaccino. Infatti il consumo aumenta linearmente per poi raggiungere uno stazionario e mantenerlo fino alla fine del ciclo. Con una temperatura di congelamento di -57°C e -52°C, si ha lo stesso consumo che è intorno all'83%. Mentre con una temperatura di congelamento di -45°C il 78.6% del perossido viene consumato.

Nel caso in cui la temperatura di essiccamento secondario sia 45°C, tutto il perossido contenuto nel campione viene rimosso.

Questi risultati indicano che il consumo di perossido dipende principalmente dalla temperatura di essiccamento secondario piuttosto che da quella di congelamento.

Conclusioni

Durante le due ore che precedono la liofilizzazione (dopo l'aggiunta del perossido), il tempo di contatto del perossido con le formulazioni liquide (FB e P) non ha mostrato un impatto significativo sulla sua concentrazione. Tuttavia, in letteratura scientifica è stato riportato che il perossido di idrogeno, nel placebo, può degradare in presenza di eccipienti comunemente utilizzati come mannitolo, saccarosio e sorbitolo. Nonostante l'esatto meccanismo non sia ancora chiaro, è possibile che tracce di ioni metallici presenti in soluzione , come zinco o ferro, che nel vaccino sono legati alle proteine, nel placebo sono disponibili per catalizzare la degradazione del perossido. Nel vaccino invece, il consumo di perossido è probabilmente dovuto soltanto alle reazioni di ossidazione con le proteine [14].

È stato osservato che la maggior parte (50-80%) del perossido presente nella formulazione viene rimosso durante la liofilizzazione. Inoltre, la velocità di consumo del perossido non dipende dalla sua concentrazione iniziale.

Nel caso in cui la temperatura di congelamento sia -55°C, la presenza dell'antigene ha un impatto sul consumo di perossido. Infatti, il consumo nel placebo è minore rispetto a quello nel vaccino. Il consumo del perossido non è dovuto solo al processo di liofilizzazione, ma è anche dovuto ad altri meccanismi come l'ossidazione delle proteine. Mentre con una temperatura di congelamento di -45°C, si ha lo stesso consumo sia nel placebo che nel vaccino. Perciò, con questa temperatura di congelamento, l'antigene non ha nessun impatto sul consumo.

Nel caso in cui la temperatura di essiccamento secondario sia 40°C, indipendentemente dalla temperatura di congelamento, il consumo di perossido aumenta con il tempo per poi raggiungere uno stazionario mantenuto fino alla fine del processo. L'aumento di temperatura a 45°C, ha consentito di eliminare tutto il perossido presente nel vaccino. Il consumo di perossido durante la liofilizzazione dipende maggiormente dalla temperatura di essiccamento secondario piuttosto che da quella di congelamento. Aumentando la temperatura di essiccamento secondario, aumenta il consumo di perossido.
Per questa configurazione con specifiche condizioni di processo, una specifica composizione del vaccino e una concentrazione del perossido al suo interno di 150 ng/mL. Aumentando la temperatura di essiccamento secondario di soli 5°C, è possibile eliminare tutto il perossido che contamina il vaccino, e in questo modo, si evitano possibili reazioni tra il perossido e le proteine allo stato liofilizzato durante lo stoccaggio.

Chapter 1

Fundamentals of freeze-drying

1.1. Basic principles

Freeze-drying/Lyophilization is a process by which a solvent is removed by sublimation and desorption, generally under reduced pressure. A typical freeze-drying process consists of three stages: freezing (solidification of the solvent), primary drying (ice sublimation), and secondary drying (desorption of unfrozen water). The main steps of the freeze-drying process are shown in *Figure 1.1* [1].

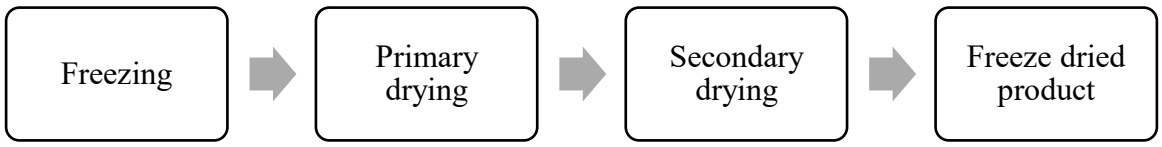

Figure 1.1 Schematic of the main steps of the freeze-drying process.

During the freezing the liquid formulation is cooled below the solidification temperature and most of the solvent, typically water, is separated from the solutes to form ice. By the end of this step, the freeze-concentrate solution usually contains only about 20% of water (w/w), or less than 1% of total water in the solution before ice formation [2]. During primary drying the ice is removed by sublimation, the chamber pressure is well below the vapor pressure of the ice, whereas the chamber temperature is raised to supply the heat removed by ice sublimation. The primary drying stage is the longest stage of the entire process.

After the formulation has been freeze-concentrated to the limit and the ice has been sublimed, any residual unfrozen water (bound water) can be removed by desorption. The bound water is removed during the secondary drying by heating the product under vacuum.

1.2. Freezing

Freezing is the first step of freeze-drying. The liquid formulation is cooled and ice crystals of pure water form. During the freezing, phase separation into ice and freeze-concentrated solution occur. As the freezing step continues, the concentration and the viscosity of the remaining liquid increase, inducing inhibition of further crystallization [3]. To ensure the total solidification, the drug phase should be below Tg' (glass transition temperature associated with maximum freeze concentration) if amorphous, or below Teu (eutectic temperature) if it is in the crystalline state [2]. At the end of the freezing the formulation solidifies, yielding an amorphous, crystalline, or combined amorphous-crystalline phase.

Cooling involves formation of ice nuclei then those nuclei begin to grow.
"The number of ice nuclei formed, the rate of ice growth and the ice crystals' size depend on the degree of supercooling. The higher the degree of supercooling, the higher the rate of nucleation and the faster the effective rate of freezing. A high degree of supercooling leads to high number of small ice crystals and larger ice specific surface area, in contrast, a lower degree of supercooling leads to a lower number of large ice crystals" [4].

As the ice crystals grow, the concentration of the solute constituents of the formulation is increased in the interstitial region between the growing ice crystals, this is the "cryoconcentration".

In crystalline material the system can be completely solidified only below Teu and the interstitial region between the ice crystals matrices consists of an intimate mixture of small crystals of ice and solute. In case of amorphous material, the water continues to freeze beyond the eutectic melting temperature and the system becomes increasingly viscous until no further freezing occurs. This is called vitrification and occurs at Tg'. The interstitial region in amorphous solids consists of solid solution and unfrozen, amorphous water [4]. Eutectics are mixtures of substances that freeze at lower temperature (eutectic temperature) than the surrounding water. Eutectic point is the point where all the three phases (solid, liquid and gaseous phases) coexist. The other type of frozen product instead of forming eutectics, the suspension becomes increasingly viscous as the temperature is lowered and the products freeze at the glass transition point forming a vitreous solid. Freezing is a critical step, since the microstructure of the dried product usually depends on the microstructure established by the freezing process [5].

Freezing perturbates the hydration shell around the protein molecules. This hydration shell is composed of water molecules directly bound to the protein, and such shell is essential for maintaining the three-dimensional fold of protein molecules. As the freezing process continues, water molecules are removed out of the protein surface leading to desiccation of protein molecule [10]. The compounds that protect proteins, such as glucose and amino acid salts, seem to act mechanically, strengthening the hydration layer and the native conformation of the proteins; this reinforcement seems to counteract long-range disturbances and perhaps also the diffusion of oxygen molecules responsible of the protein oxidation during the freeze-drying [13].

1.2.1. Shelf-ramped freezing

In shelf-ramped freezing the filled vials are either directly loaded on the cooled shelves or loaded at ambient temperatures, and then the temperature is decreased linearly with time to the hold temperature. By using this freezing method, the samples show a high degree of supercooling, this condition leads to an extremely fast ice crystal growth when the nucleation temperature is reached and thereby many small ice crystals form [4].

1.2.2. Pre-cooled shelf method

In pre-cooled shelf method, the filled vials are placed on the shelf of the freeze-dryer that has already cooled to the desired final shelf temperature resulting in higher nucleation temperatures compared to the shelf-ramped freezing. Furthermore, when applying the pre-cooled shelf method, the freezing rate after ice nucleation is slower compared to shelf-ramped freezing due to the lowered degree of supercooling [4].

1.3. Primary drying

This is the stage at which ice separated from the solute phase is removed by sublimation. The pressure chamber of the freeze-dryer is reduced well below the vapor pressure of ice and heat must be applied to the product to encourage the removal of water in the form of vapor.

In this process:

1. heat is transferred from the shelf to the frozen solution.
2. The ice sublimes and the water vapor formed passes through the dried portion of the product to the surface of the sample.
3. The water vapor is transferred from the surface of the product through the chamber to the condenser.
4. The water vapor condenses on the condenser.

At the end of sublimation step a porous plug is formed, its pores correspond to the spaces that were occupied by ice crystals [4]. The temperature of the sample is a critical parameter, it is characterized by a collapse temperature Tc. The macroscopic collapse temperature of the formulation (Tc) is the temperature above which the freeze-dried product loses macroscopic structure and collapses during freeze drying. Tc is usually about 2°C higher than Tg' or equals the eutectic temperature (Teu) if solutes are crystallized in the frozen solution [2]. In order to produce an acceptable freeze-dried product, it is always required to process the primary drying at a temperature lower than Tc.

The sublimation rate is the mass of ice sublimed (g) per unit time (hour), which can be represented by *Equation (1.1)*.

$$\frac{dm}{dt} = \frac{P_{ice} - P_c}{R_p + R_s} \qquad (1.1)$$

dm/dt is the mass transfer rate for the water vapor, Pice (Torr) is the equilibrium vapor pressure over ice at the product temperature, Pc is the chamber pressure, Rp (Torr·h/g) is the dry product layer resistance to vapor transfer, while Rs is the resistance of the stopper. The pressure difference between the vapor pressure of ice and the partial pressure of water in the chamber (Pi) is the driving force for ice sublimation. Pi corresponds to the chamber pressure during primary drying [8]. Besides the chamber pressure affects the rate of sublimation, but not the driving force [7].

The optimized conditions regarding ice sublimation stage depend on a balancing of coupled heat and mass transfer within the product, which can be expressed by *Eq. (1.2)*. Heat supplied to the product = heat required for sublimation.

$$K_V(T_s - T_i) = \Delta H(dm/dt) \qquad (1.2)$$

where Kv is the heat transfer coefficient, Ts and Ti are the temperatures of the shelf and the product, respectively, (*dm/dt*) is the rate of mass transfer, ΔH is the latent heat of sublimation of ice per unit mass at the appropriate temperature Ti.

The energy absorbed during the sublimation process must be compensated by a supply of energy from the heated shelf to the product. If the energy supplied is not enough, the product temperature will decrease and sublimation will slow down. If, on the other hand, the shelf temperature is raised excessively, causing the product temperature to rise to above Tc, then ice will melt back into the freeze concentrate, causing structural collapse and possible chemical deterioration [6].

1.3.1. Heat transfer mechanisms

The thermal energy is supplied to the shelves in order to balance the latent heat of sublimation absorbed by the ice. Heat enters the product by one of several mechanisms:

i. Radiation from ‘‘heated’’ surfaces.
ii. Conduction within and between solid phases.
iii. Convection.

The magnitudes of the various mechanisms to the total heat transfer depend on the quality of the contact between solid phases, the chamber pressure and the temperature. The major contribution to heat transfer is due to collisions between gas molecules (convection), and this effect becomes more pronounced with increasing chamber pressure [7]. Thereby an increase in pressure is expected to accelerate sublimation.

1.3.2. Factors influencing ice sublimation

Ice sublimation is also influenced by additional factors [7]:

- ice crystal size distribution;
- solid content (solution concentration);
- formation of an amorphous, glassy product “skin”;
- fill depth.

The effects of cooling rate on ice crystal size distribution are shown in *Table 1.1*:

Table 1.1 Effect of cooling rate on ice crystal dimensions and primary drying duration [7].

Cooling rate	**Nucleation rate**	**Number of ice crystals**	**Size of ice crystals**	**Ice sublimation time**
Low	Low	Small	Large	Shorter
High	High	Large	Small	Longer

Rapid cooling leads to small ice crystals and a uniform texture in the dried product, such a product is more difficult to freeze-dry. Coarse structures are usually preferred as they favor ice sublimation due to the less restrictive channel in the matrix. High solid content causes the decreasing of the porosity of the dried portion of the plug and hence raises the resistance to further sublimation. Furthermore, high solid contents, in extreme cases, would produce an almost impermeable glassy product skin at the surface of the frozen solution. This film coating would reduce the ice sublimation rate. The effect of fill depth can be demonstrated by considering the resistance of the dried cake to further sublimation. An increasing in the cake thickness (fill volume) give rise to increasing of the resistance to sublimation.

1.4. Secondary drying

The last stage of freeze drying is termed “secondary drying”, it involves the removal of any residual unfrozen water from the product which did not sublimate off as ice during the freezing. The ‘bound water’ (to protein molecules) may be adsorbed on the surface of the crystalline product or is in the solute phase either as hydrate water in a crystalline hydrate

or dissolved in an amorphous solid to form a solid solution [3]. For amorphous systems, the amount of unfrozen water remaining after the primary drying may be typically 20–30% w/w or even > 50% w/w. For crystalline systems, where water only exists in the form of a thin adsorbed layer on the crystal surfaces, much lower amounts of water remain [7]. Secondary drying aims at reducing the residual moisture content to a level optimal for stability, which is usually less than 1%. The products are kept at "high" temperature for a certain period in order to allow the removal of residual water by diffusion from the product filaments and its subsequent desorption and condensation (in the condenser). Desorption drying is realized by raising shelf temperature and reducing chamber pressure to a minimum. The temperature must be chosen while keeping in view the thermal stability of proteins, since protein polymerization or biodegradation may result from using high processing temperature during secondary drying.

The non-linearity of the drying isotherms (*Figure 1.2*) indicates that the rate-limiting step is the diffusion within the sample and not the evaporation from the surface. At a given temperature, the water content decreases with time and then tends to a plateau. Raising the temperature will increase the rate of water removal until a new rate-limiting step is reached. The presence of the plateau demonstrates that products cannot be adequately dried to low water contents, unless very extended and uneconomical drying periods are employed.

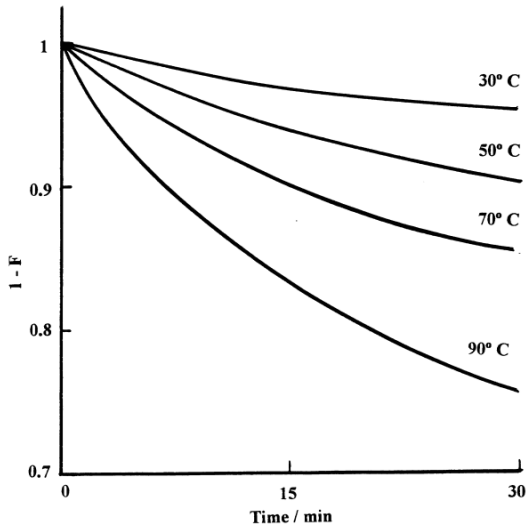

Figure 1.2 Isothermal drying profiles of Ficoll glass film of 0.08-mm thickness, where F is defined as the fractional extent of drying, the mass of water removed at time t, divided by the initial water content. Thus F = 1 corresponds to the initial water content, 8.87% in this case [7].

In contrast to primary drying, the chamber pressure does not appear to affect the secondary drying rate.

1.5. Formulation of freeze-drying

The freeze-drying process involves destabilization forces that may induce spontaneous unfolding of the protein and denaturation e.g., cold shock, ice-water interfaces, pH changes during freezing, dehydration stress, etc. Therefore, a liquid solution that is to be freeze-dried to yield an acceptable solid dosage form contains several components. In order to maximize

stability, biological activity and safety of a freeze-dried product, the identification of the right formulation conditions, the right excipients in optimal quantities, and the right dosage is essential. Excipients are substances that are combined with a drug to render it suitable for administration, they should have no pharmacological actions themselves [7].

In pharmaceutical freeze-drying operations various types of excipients are added prior to freeze-drying for specific purposes; *Table 1.2* presents some examples of the excipients commonly used [3].

Table 1.2 Examples of commonly used excipients in freeze-drying of pharmaceutical products.

Type	**Substance**
Bulking agents	Hydroxyethyl starch, trehalose, mannitol, lactose, and glycine
Buffers	Phosphate, tris HCl, citrate, and histidine
Stabilizers	Sucrose, lactose, glucose, trehalose, glycerol, mannitol, sorbitol, glycine, alanine, lysine, polyethylene glycol, dextran, and PVP
Tonicity adjusters	Mannitol, sucrose, glycine, glycerol, and sodium chloride

Bulking agents

These agents are used to provide bulk to the formulation in order to achieve a mechanically more robust plug. This is important in cases in which very low concentrations of the active ingredient are used. Bulking agents used must be able to crystallize during the freeze-drying process as only in this state they have a neutral effect on product stability.

Buffers

Buffers are required in pharmaceutical formulations to adjust pH changes during freezing.

Stabilizers

Stabilizers that protect proteins against freezing stress are called cryoprotectants, whereas those giving protection against drying stress are called lyoprotectants. Disaccharides are used as bulking agent but also as cryoprotectant agents as they vitrify at a specific temperature. The drug and water molecules are immobilized in the viscous glassy matrix, this immobilization prevents the protein aggregation and protects them against the mechanical stress of ice crystals. Furthermore, another cryoprotectant stabilization mechanism is the particle isolation hypothesis, in which sugars isolate individual particles in the unfrozen fraction, thereby preventing aggregation during

freezing above Tg′. In this case, the vitrification is not required. As regards lyoprotectants, the stabilization mechanism is the water replacement hypothesis, in which the formation of hydrogen bonds between a lyoprotectant and the product takes place. These lyoprotectants preserve the native structures of nanoparticles by serving as water substitutes [3,5,9].

Tonicity adjusters

Excipients such as mannitol, sucrose, glycine, glycerol, and sodium chloride are good tonicity adjusters. These substances are used to yield an isotonic solution, when required, and control osmotic pressure.

1.6. Equipment

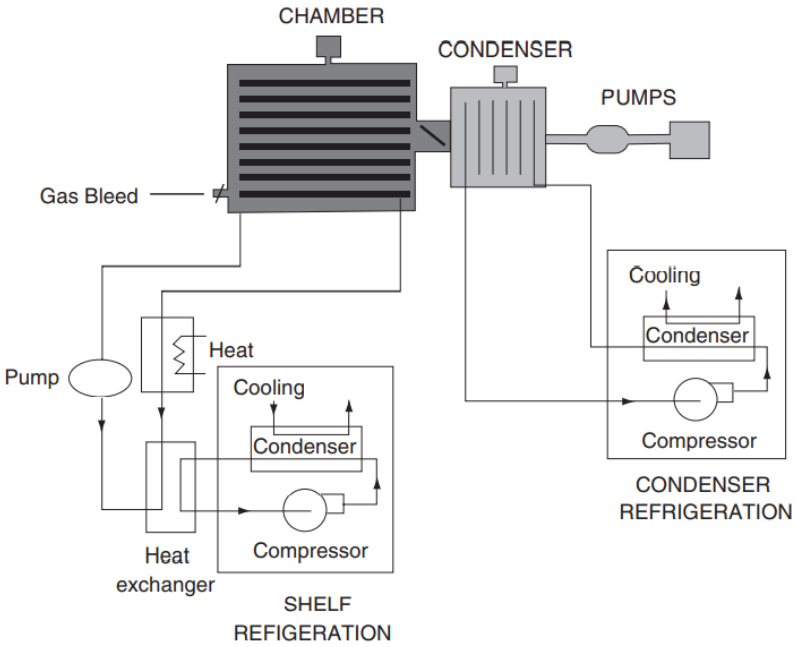

Figure 1.3 Schematic of a typical industrial freeze-drying plant [7].

A typical configuration of a pharmaceutical freeze-dryer is shown in *Figure 1.3*. It consists of a vacuum chamber that contains shelf or shelves for processing product acting as a heat exchangers. The shelf temperature is controlled with a heat exchange fluid circulating through the shelf that removes energy from the product during freezing, and supplies energy to the product during the primary and secondary drying. The formulation to be treated is contained in vials or syringes which are placed on the shelf. A condenser designed to trap the solvent, typically water vapor, during the drying steps. It may be in a vessel separate to the chamber or within the same chamber as the shelves. Also, the condenser is equipped with its own compressor and heat exchange system. A refrigeration system to cool down the formulation during the freezing. A vacuum system able to maintain the chamber pressure well below the saturation vapor pressure of ice during the primary drying and to reduce the chamber pressure to a minimum (when required) during the secondary drying. The freeze-dryer is equipped with a control system acting on - temperature, pressure and time, since the entire process cycle can essentially be controlled by only these three parameters [5].

Chapter 2

VHP

Hydrogen peroxide is a chemical compound (H_2O_2). It is the simplest peroxide (a compound with an oxygen-oxygen single bond) and in its pure form is a colorless liquid, slightly more viscous than water. Hydrogen peroxide is a strong oxidizer and it is used as a bleaching agent and disinfectant. Also, it might act as a reducing agent, for example it reduces manganese from the MnO_4^- state to Mn^{++}.

2.1. Physical properties [15]

The boiling point of pure hydrogen peroxide at atmospheric pressure is 150.2 °C as shown in *Table 2.1* and *Figure 2.1*

Table 2.1 Water-Hydrogen peroxide boiling point at 1 atm.

H_2O_2 concentration		**T (°C)**
molar fraction	**% in weight**	
0	0	100.0
0.1	17.34	103.0
0.2	32.07	106.9
0.3	44.73	111.5
0.4	55.73	116.7
0.5	65.37	122.3
0.6	73.90	128.2
0.7	81.50	134.0
0.8	88.31	139.7
0.9	94.44	145.1
1.0	100.00	150.2

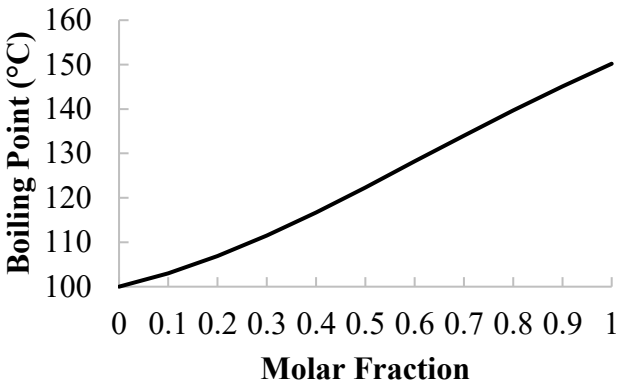

Figure 2.1 Water-Hydrogen peroxide boiling point at 1 atm.

Pure water has a melting point of 0 °C and pure hydrogen peroxide of -0.41 °C (272.75 K). The solid-liquid equilibrium diagram of water-hydrogen peroxide is relatively simple, as shown in *Figure 2.2*. The two components form a single addition compound: the dihydrate $H_2O_2 \cdot 2H_2O$ that melts at -52 °C.

Table 2.2 Recommended liquid coordinates values for water-hydrogen peroxide solution.

% H_2O_2	**T (°C)**	**% H_2O_2**	**T (°C)**
0	0	50	-52.2
5	-2.9	55	-53.3
10	-6.4	60	-55.5
15	-10.3	61.2	-56.1 (eutectic)
20	-14.6	65	-49.0
25	-19.5	70	-40.3
30	-25.7	75	-32.3
35	-33.0	80	-24.8
40	-41.4	85	-17.9
45	-51.7	90	-11.5
45.2	-52.2 (eutectic)	95	-5.6
48.6	-52 ($H_2O_2 \cdot 2H_2O$)	100	-0.41

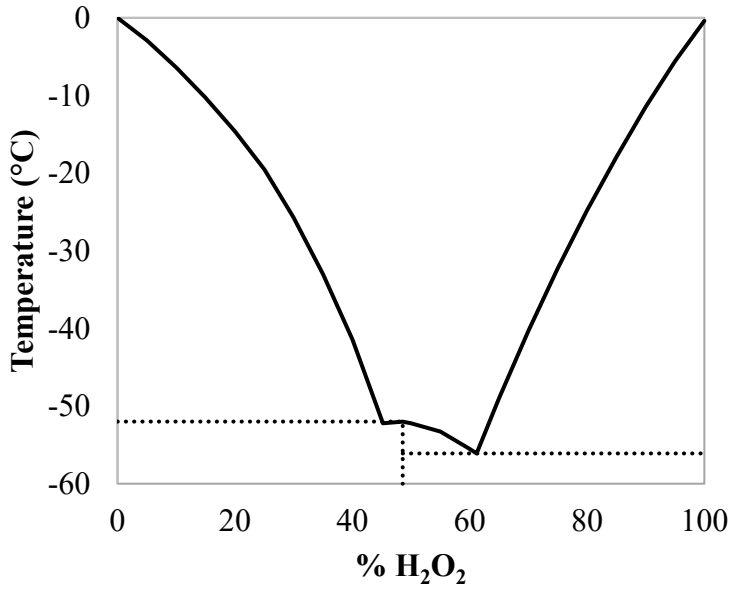

Figure 2.2 Equilibrium diagram of water-hydrogen peroxide system.

Table 2.3 and *Figure 2.3* show the density of hydrogen peroxide at atmospheric pressure and at 0 °C and 25 °C.

Table 2.3 Recommended density values for water-hydrogen peroxide solution.

% H_2O_2	Density (Mg/m^3)	
	0 °C	**25 °C**
0	0.9998	0.9971
5	1.0193	1.0145
10	1.0393	1.0324
15	1.0598	1.0507
20	1.0804	1.0694
25	1.1014	1.0885
30	1.1226	1.1081
35	1.1441	1.1282
40	1.1661	1.1487
45	1.1883	1.1698
50	1.2110	1.1914
55	1.2342	1.2137
60	1.2579	1.2364
65	1.2822	1.2598
70	1.3071	1.2839
75	1.3326	1.3086
80	1.3589	1.3339
85	1.3855	1.3600
90	1.4136	1.3867
95	1.4421	1.4142
100	1.4709	1.4425

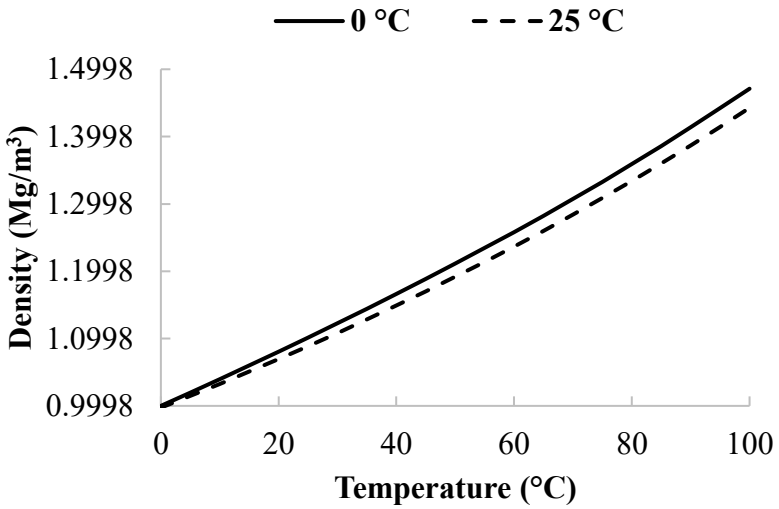

Figure 2.3 Density diagram of water-hydrogen peroxide system at 0°C and 25°C.

2.2. Line sterilization considerations

Formulation and filling of biological products occur in isolators in order to avoid contamination and thereby to ensure the maximum level of sterility. In this context, vaporized hydrogen peroxide (VHP) is a commonly used agent for the decontamination of the internal surfaces of the aseptic fill–finish installations. VHP has several advantages compared to other sterilizing agents; it is easier to use, less corrosive, less toxic to humans, easier to remove from the equipment and less pollutant to the environment [10]. VHP is a very efficient agent in killing viable microorganisms but it is also characterized by strong oxidizing property.

The VHP sterilization process consists of:

- conditioning, vaporization or nebulization of hydrogen peroxide in the closed isolator used for aseptic filling and primary packaging (vials and stoppers). The VHP is introduced under defined conditions (concentration, temperature, relative humidity, exposure time) in order to achieve a high sterility level;
- decontamination (working time of the VHP);
- aeration, to reduce the VHP level to very low concentrations before starting formulation and filling. The maximum permissible level of VHP during aseptic filling of pharmaceutical products, as defined by the U.S. Occupational Safety and Health Administration is 1 ppm v/v for the duration of 8 hours [11].

Residuals of the VHP, if present in the containment atmosphere or desorbing from the inner surface of the isolator or from sterilized primary packaging, will potentially enter the drug product during the fill and finish process. This can trigger instability of the active pharmaceutical ingredients, excipients or entire drug product. Hydrogen peroxide may induce dimerization, aggregation, oxidation, conformational changes and chemical cross-linking of large molecule drugs and excipients, thereby impacting the long-term product stability, safety and efficacy.

Many factors such as vial size, vial opening diameter, fill volume, time between liquid fill and stopper closing, application of polymer and silicon layering on the primary packaging, residual VHP level, etc., affect the hydrogen peroxide adsorption and the dissolved amount in the product [10,11].

2.3. Adsorption of VHP on the primary packaging

Hydrogen peroxide (HP) traces from the isolator could adsorb onto the primary packaging during the filling process and subsequently it can be released into the filled product. Desorption of adsorbed hydrogen peroxide from the vials and stoppers takes place after filling through the direct contact. The HP is adsorbed on the siliconized layer covering the glass vials as bulk glass does not have a significant adsorption capacity [12]. Also, the closures/stoppers are made of rubber, both sorption of HP into and desorption out of the stopper depend upon the solubility and diffusion coefficient of HP into rubber.
Adsorption of HP on the primary packaging depends on several factors - physical dimensions; concentration of VHP in the isolator atmosphere; relative humidity (RH) in the isolator chamber; exposure time to the VHP contaminated atmosphere [11].

The VHP level has the biggest impact, followed by RH. An increase in RH leads to a decrease of VHP adsorption, this implies the existence of a competitive adsorption between HP and H_2O molecules in vapor phase on the glass vials and closures. At ambient temperature, H_2O molecules temporarily occupy free sites of the siliconized layer of the glass vials, diffuse and penetrate inside the layer. This leaves fewer unoccupied sites for HP, resulting in a low HP adsorption [11]. The exposure time does not have a relevant impact on the HP desorption.

Additionally, the HP adsorption and its subsequent desorption into the filled drug product might also depend upon the filled level and the formulation properties.

2.4. VHP during lyophilization

Typically, the majority (50–80%) of the hydrogen peroxide contaminating the formulation is removed during the lyophilization process. The remaining HP can react with proteins in the lyophilized state. Besides, while proteins are still in liquid state (prior to lyophilization) the reaction rate can be even faster than in the solid state [10].

2.4.1. Protein oxidation induced by VHP

It is well known that HP can oxidize proteins and protein oxidation often leads to structural change, aggregation, loss of activity. Lyophilization can significantly increase protein oxidation in an HP concentration-dependent manner, W. Cheng et al. [14] fund a linear relationship ($R^2 > 0.99$) between the spiked HP concentration and oxidation level before and after lyophilization. After lyophilization the HP level significantly decrease, the loss is due to the lyophilization process itself and the oxidation of proteins. The oxidation of the protein due to the HP take place mainly during the freeze-drying process.

Chapter 3

MATERIALS AND METHODS

3.1. Materials

The Placebo is a sucrose-based formulation (5% sucrose), and the drug substance (DS) contains the active protein.
Primary packaging: 3mL glass vials (Müller + Müller-Holzminden, Germany), 13-mm Helvoet elastomeric stoppers (bromobutyl Stoppers) and Tear-Off Aluminum Seal. Vials and stoppers were siliconized for processing reasons. The vial dimensions were as follows: height 31 mm, opening diameter 9.75 mm, and vial diameter 16.25 mm.
Hydrogen peroxide stabilized solution (35% weight/weight) was purchased from Solvay Chemicals through SA International.

3.2. Sample preparation and freeze drying

The Final Bulk (FB) was freshly prepared by mixing Placebo and DS.
The hydrogen peroxide solution was two times diluted with water for injection (WFI) to reduce its concentration to 153 ng/mL. The HP diluted solution was spiked to the FB and Placebo in order to have 150 ng/mL as final HP concentration. The spiked formulation was gently mixed and then left for 2 hours on the bench, at room temperature and exposed to the natural light, to mimic the industrial process. After 2 h, the spiked formulation was dispensed into 3 mL siliconized glass vials at 0.5 mL/vial (2.4 mm fill depth) and partially stoppered (freeze-dryer stopper configuration).
The remaining part of the spiked formulation, which has been used to produce the freeze-dried samples, was placed in 1.5 mL Eppendorf tubes and stored at -20 °C to be used as control in order to evaluate the magnitude of the HP consumption during the freeze-drying.
The freeze-dryer is a lab-scale freeze-dryer, it consists of a vacuum chamber containing 3 shelves and, on the bottom, the condenser to trap the solvent. The freeze-drying cycle has an overall duration of 28 hours. The filled vials were loaded onto a freeze-dryer shelf that was precooled to the freezing temperature. The vials were frozen for 1 before the temperature was ramped to -12 °C, then to -24 °C and held for 5 hours. Then during the secondary drying the temperature was ramped to 40 °C and held until the end of the cycle. The chamber pressure was 1 bar during the freezing and then reduced to 70 µbar up to the end of the cycle.

3.3. Experimental plan (I) – HP consumption during the freeze-frying cycle

In order to evaluate the HP consumption with time throughout the freeze-drying cycle, it was interrupted in 6 points, as it is shown in *Table 3.1*.

Table 3.1 Freeze-drying cycle interruption points.

Point	Duration	Description
1	8H30	freezing + 40% of primary drying
2	15H15	freezing + 75% of primary drying
3	20H30	freezing + total primary drying
4	23H	freezing + primary drying + 33% of secondary drying
5	25H30	freezing + primary drying + 60% of secondary drying
6	28H	total cycle

The vials of each group were automatically fully stoppered, manually crimped with aluminum seals and stored at -20 °C until the HP residual concentration was evaluated.

3.3.1. Impact of the freezing temperature

To investigate the impact of the freezing temperature on the HP consumption during the freeze-drying, the same cycle was carried out with two different freezing temperature: -45 °C and -55 °C.

An HP-H_2O solution, at low temperature, forms a single solid addition compound: the dihydrate $H_2O_2 \cdot 2H_2O$ that freezes at -52 °C (see *Figure 2.2*). These two temperatures have been chosen because:

- at -45°C the HP it is still active (not frozen) as the dihydrate $H_2O_2 \cdot 2H_2O$ freezes at -52 °C;
- at -55 °C the HP is frozen as dihydrate $H_2O_2 \cdot 2H_2O$.

3.3.2. Impact of the antigen

To investigate the impact of the presence of the antigen on the HP consumption during the freeze-drying, two liquid formulations were freeze-dried together:

- Placebo;
- Final Bulk = Placebo + Drug Substance (containing the antigen).

During each freeze-drying cycle, half of the vials contained Placebo, and the other half contained the FB.

3.4. Impact of the HP initial concentration

The impact of the initial HP concentration on its consumption during the process, more specifically on the consumption-rate, was also investigated. Freshly prepared FB solutions were spiked with HP to a final concentration of 100, 200, 300, 400, 500 and 600 ng/mL. The sample preparation and the freeze-drying cycle are the same described above, the only

difference is the HP concentration. The freeze-drying cycle has -45 °C as freezing temperature and the HP consumption (with these concentrations) was evaluated at the end of the freeze-drying cycle (see point 6, *Table 3.1*.).

3.5. Impact of contact time HP-FB/Placebo

Placebo and FB were spiked with HP and then, after 2 hours, freeze-dried. A study was carried out to investigate the stability of HP within 2 hours after the spiking, when it is at room temperature and exposed to the natural light. The formulation was spiked in order to have a concentration of HP of 150 ng/mL. The HP concentration was measured different times in the 2 hours after the spiking.

3.6. Experimental plan (II) – HP consumption during the Secondary Drying

The HP consumption during the secondary drying was also evaluated by using the Millrock freeze-dryer. The latter is a lab-scale freeze-dryer, it is equipped with a mechanical arm that allows, at any time, to close vials while the freeze-drying process is going on. In this way, the drying is interrupted for those vials. The freeze-drying cycle is the same described above.

The tested freezing temperatures are: -45 °C, -52 °C (standard freezing cycle for this vaccine formulation) and -57 °C. It was also investigated the HP consumption with a higher secondary drying temperature. In *Table 3.2* the points, during the secondary drying, where the drying was interrupted for some vials:

Table 3.2 Interruption points during the secondary drying.

Point	% of Secondary Drying	% of the total cycle
A	16.7%	77.7%
B	33.33%	82.1%
C	50%	86.6%
D	66.7%	91.1%
E	83.33%	95.5%
F	100%	100 %

At the end of the drying process, the vials were automatically fully stoppered, manually crimped with aluminum seals and stored at -20 °C until the HP residual concentration was evaluated.

3.7. Hydrogen Peroxide Assay

The HP concentration in the samples was determined on microplates with 96 wells, using the Amplex UltraRed Method. The analysis was carried out by adding the working reagent which contains phosphate buffer, Horse Radish Peroxidase (HRP) and Amplex UltraRed reagent which has been solved in Dimethyl Sulfoxide (DMSO). HP together with the catalyst HRP, oxidizes the Amplex UltraRed from non-fluorescing to a fluorescing substance. This fluorescence can be measured with a suitable fluorimeter. An internal calibration curve for each plate is obtained and the respective HP concentration can be calculated out.

This method allows the evaluation of the HP concentration in an aqueous solution, for this reason the cake was reconstituted with 0.5 mL of WFI. Then 2 vials (of the same group) were mixed in order to get one sample with a suitable volume of liquid to realize the HP evaluation. With this method, 6 samples per plate can be analyzed, on each plate the first two samples in all the evaluations were: I) FB/Placebo non spiked as blank and II) FB/Placebo spiked (not freeze-dried) as control. Each sample was analyzed in triplicate, and the analysis of the data was performed on the averaged value.

Chapter 4

RESULTS

4.1. Impact of the contact time in the Pre-Lyo holding period

HP concentration in the FB and Placebo during the Pre-Lyophilization holding period (2 hours) was analyzed several times in order to check the HP stability, as it is shown in *Figure 4.1*.

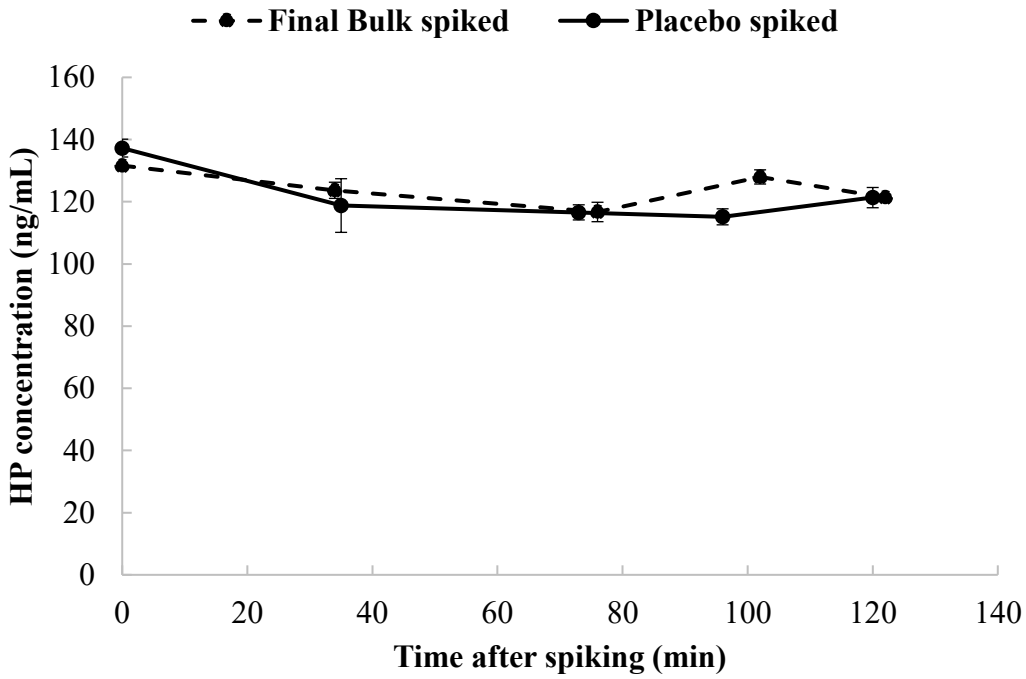

Figure 4.1 HP level in FB and Placebo during the Pre-Lyophilization holding period (2 hours).

In both Placebo and FB the HP concentration remained quite constant during the Pre-Lyo holding period.

This result suggests that the contact time of HP with the liquid formulations (FB and P) does not show a significant impact on the HP concentration within the 2 hours after the spiking.

4.2. Impact of the initial concentration of HP

Figure 4.2 shows the HP consumption (%) in the protein formulation spiked with different HP concentration and then freeze-dried.

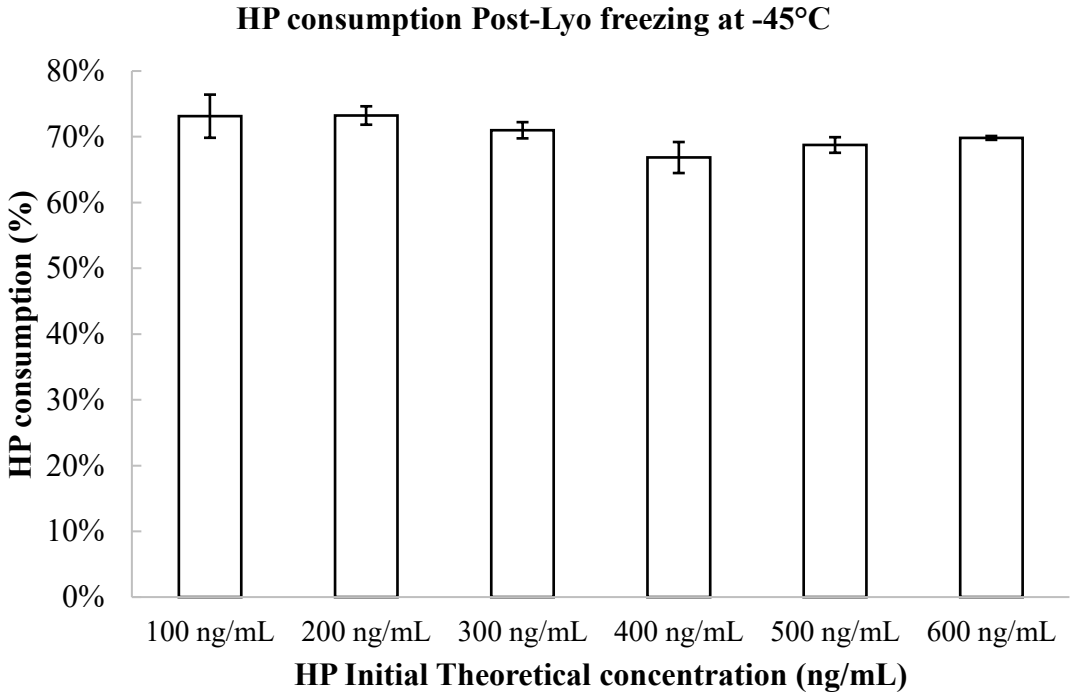

Figure 4.2 HP consumption (%) in the protein formulations after the freeze-drying. Final Bulks spiked with 100-600 ng/mL.

All the spiked formulation – after the freeze-drying process – showed approximately the same HP consumption that is on average 70.5% (SD = 2.5%). The HP consumption-rate does not depend on its initial concentration.

4.3. HP consumption during the freeze-frying cycle

4.3.1. Impact of the freezing temperature

All the vials contained elegant cakes, except for the samples subjected to 8H30 of freeze-drying (see point 1, *Table 3.1).* Regarding those samples, only the vials placed at the edges of the tray (tray containing the filled vials and then placed on the freeze-dryer shelf) contained elegant cakes.

4.3.1.1. Protein formulation - Freezing at -55 °C

The relative residual HP concentration in the protein formulation and the percentage of consumption of HP during the freeze-drying process (with -55 °C as freezing temperature) are shown in *Figure 4.3*.

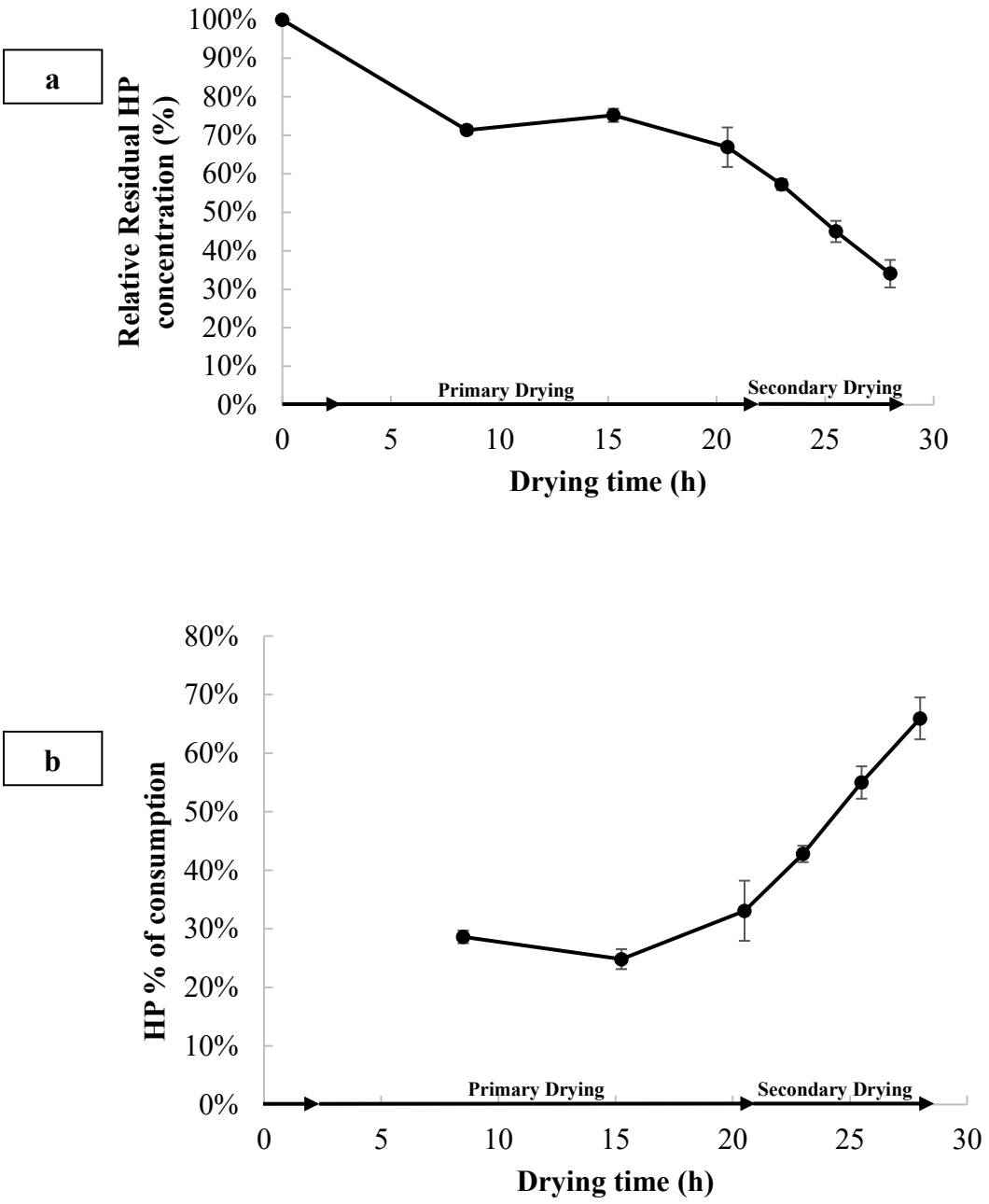

Figure 4.3 Relative HP concentration and percentage of consumption in the spiked protein formulation, (a) shows the average HP concentration change during the drying process. (b) shows the HP percentage of consumption change during the drying process.

After the freeze-drying the HP concentration in the protein formulation decreased. The residual HP concentration in the samples decreased to 30%-36.6% (34.1% on average, SD = 3.6 %) of the initial value at the end of the drying process.
The HP percentage of consumption increased with time, at the end of the process the 66% of the HP is removed, *Table 4.1* shows the HP consumption during the drying steps:

Table 4.1 HP percentage of consumption (%) in the protein formulation during the drying steps.

	HP % consumption
During Primary Drying	33.1%
During Secondary Drying	32.9%
Overall consumption	66%

After 8.5 hours of freeze-drying, the HP consumption increased to 28.6% on average (SD = 1.1%) while at the end of the primary drying (20.5 h) to 33.1% on average (SD = 5.1%). This indicates that during the primary drying the most HP removal takes place within 8 hours from the beginning of the step, after that only a small increment of consumption may occur.
During the secondary drying the HP consumption increased linearly with time, with this drying step the 32.9% of HP is removed by desorption.

For a given configuration with predefined process conditions, a predefined composition of the liquid formulation and a predefined HP concentration in it, HP consumption during the drying process can be estimated. The proposed model was obtained by fitting the data with a polynomial of order 2 (R = 0.9966, by using the software Excel). The model fit is shown in *Figure 4.4*:

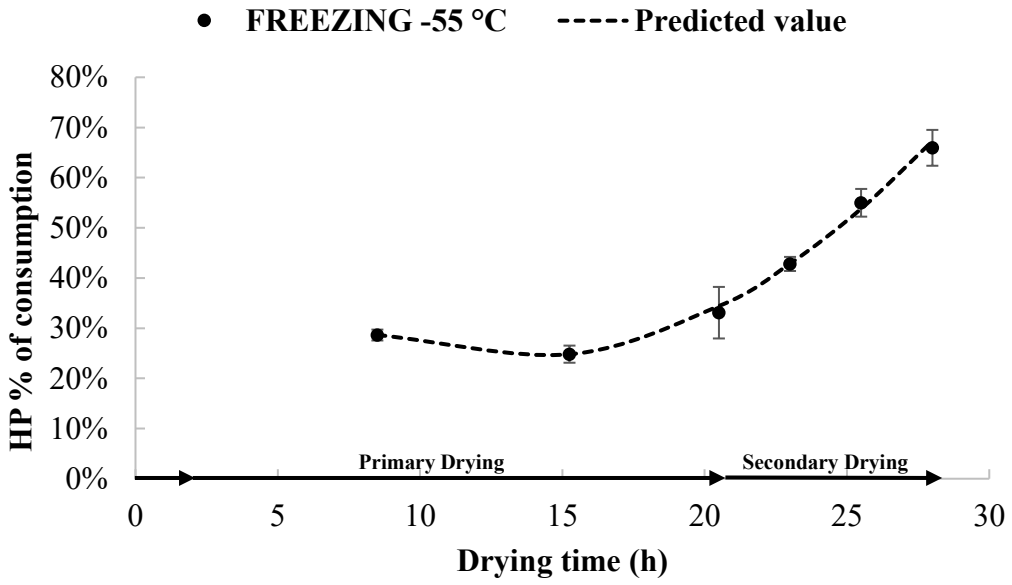

Figure 4.4 Fitting (R^2=0.9966) on HP consumption data as a function of time (h). The dashed bar represents the predicted value by the model, the dots represent the experimental values obtained.

4.3.1.2. Protein formulation - Freezing at -45 °C

The relative residual HP concentration in the protein formulation and the percentage of consumption of HP during the freeze-drying process (with -45 °C as freezing temperature) are shown in *Figure 4.5*.

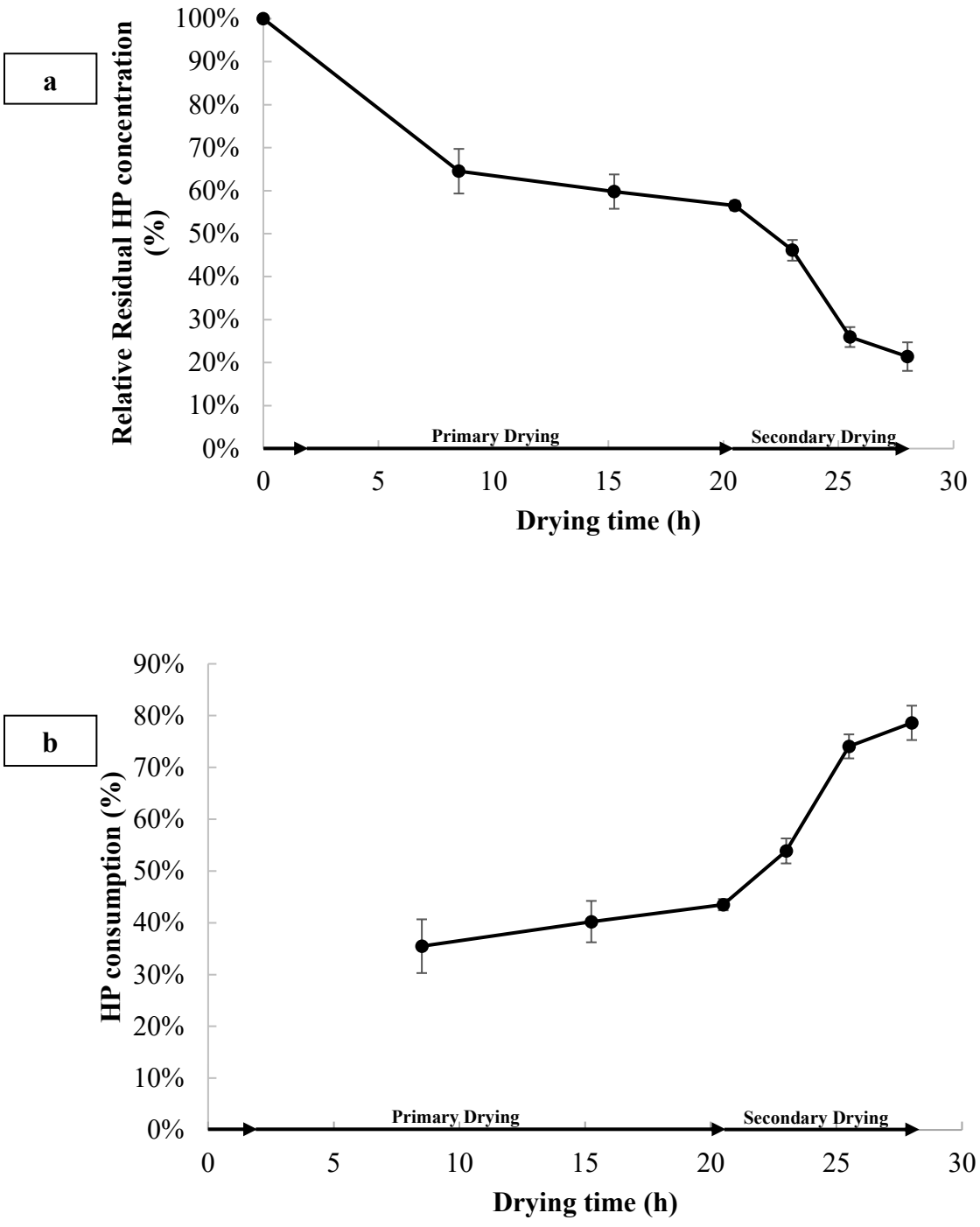

Figure 4.5 Relative HP concentration and percentage of consumption in the spiked protein formulation, (a) shows the average HP concentration change during the drying process. (b) shows the HP percentage of consumption change during the drying process.

At the end of the freeze-drying the HP concentration in the protein formulation significantly decreased to 17.6%-23.5% (21.4% on average, SD = 3.3%) of the initial value. *Table 4.2* shows the HP consumption during the drying steps:

Table 4.2 HP percentage of consumption (%) in the protein formulation during the drying steps.

	HP % consumption
During Primary Drying	43.5%
During Secondary Drying	35.1%
Overall consumption	78.6%

During the primary drying the 43.5% of the initial HP concentration was removed. Also, with this freezing temperature, during the primary drying the most HP removal took place within 8 hours from the beginning of the step. During the secondary drying the HP consumption increased approximately linearly with time, after 5 hours (the secondary drying started) it seemed to turn to a plateau.

4.3.1.3. Protein formulation - Freezing at -45 °C vs. freezing at -55 °C

The comparison in terms of relative residual HP concentration and percentage of consumption of HP in the protein formulation during the two freeze-drying processes are shown in *Figure 4.6.* The only difference between them is the freezing temperature: one is -45°C, and the other one is -55°C.

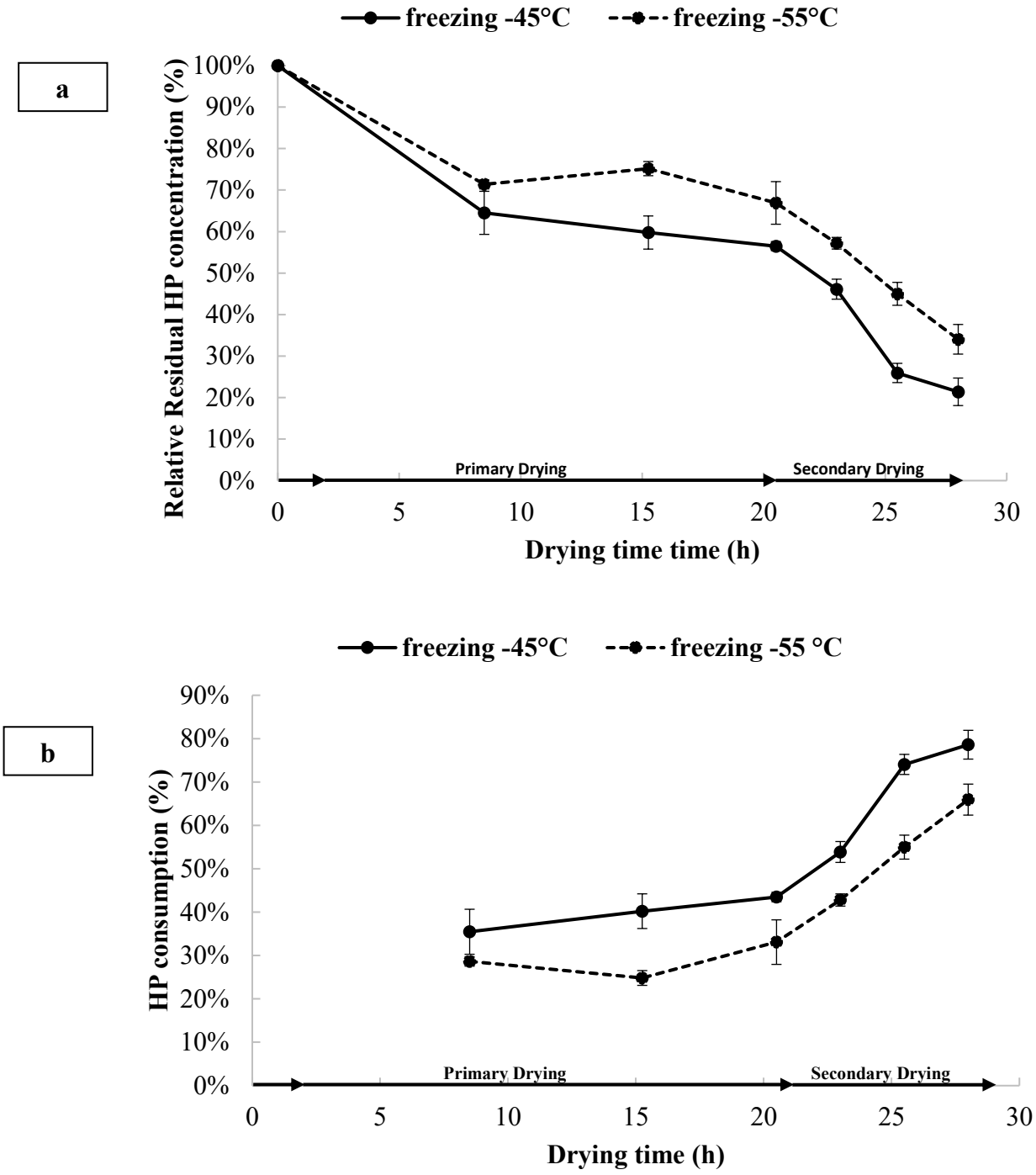

Figure 4.6 Relative HP concentration and percentage of consumption in the spiked protein formulation with two freezing temperatures (-45°C and -55°C). (a) shows the average HP concentration change during the drying process. (b) shows the HP percentage of consumption change during the drying process.

The HP consumption for both freezing temperature (-45°C and -55°C) has approximately the same trend. Throughout the entire drying process, the HP consumption with freezing at -45 °C was higher than with freezing at -55 °C. *Table 4.3* shows the HP consumption during the drying steps for the two freezing temperatures.

Table 4.3 HP percentage of consumption (%), with freezing at -45 °C and -55 °C, during the drying steps.

	HP % consumption	
	Freezing at -45 °C	Freezing at -55 °C
During Primary Drying	43.5%	33.1%
During Secondary Drying	35.1%	32.9%
Overall consumption	78.6%	66%

During the primary drying the most HP removal took place within 8 hours from the beginning of the step for both freezing temperatures.
In case of freezing at -45 °C – 5 hours after the secondary drying started – the HP consumption seemed to turn to a plateau. This means that the lower limit of HP concentration was reached, hence no further HP might be lost by the drying process and/or by the oxidation reactions. While with freezing at -55 °C, a lower HP loss took place during the drying, therefore the HP consumption increased linearly with time until the end of the process. The lower limit of HP concentration was not reached with this freezing temperature (-55%).
The reason for the difference, in terms of magnitude of HP consumption is that a HP-H_2O solution at low temperature forms a single solid addition compound: the dihydrate $H_2O_2 \cdot 2H_2O$ which freezes at -52 °C. Therefore, with -45 °C as freezing temperature, the HP is not frozen hence still and/or more active.

4.3.1.4. Placebo - Freezing at -55 °C

The relative residual HP concentration in the Placebo and the percentage of consumption of HP during the freeze-drying process (with -55 °C as freezing temperature) are shown in *Figure 4.7.*

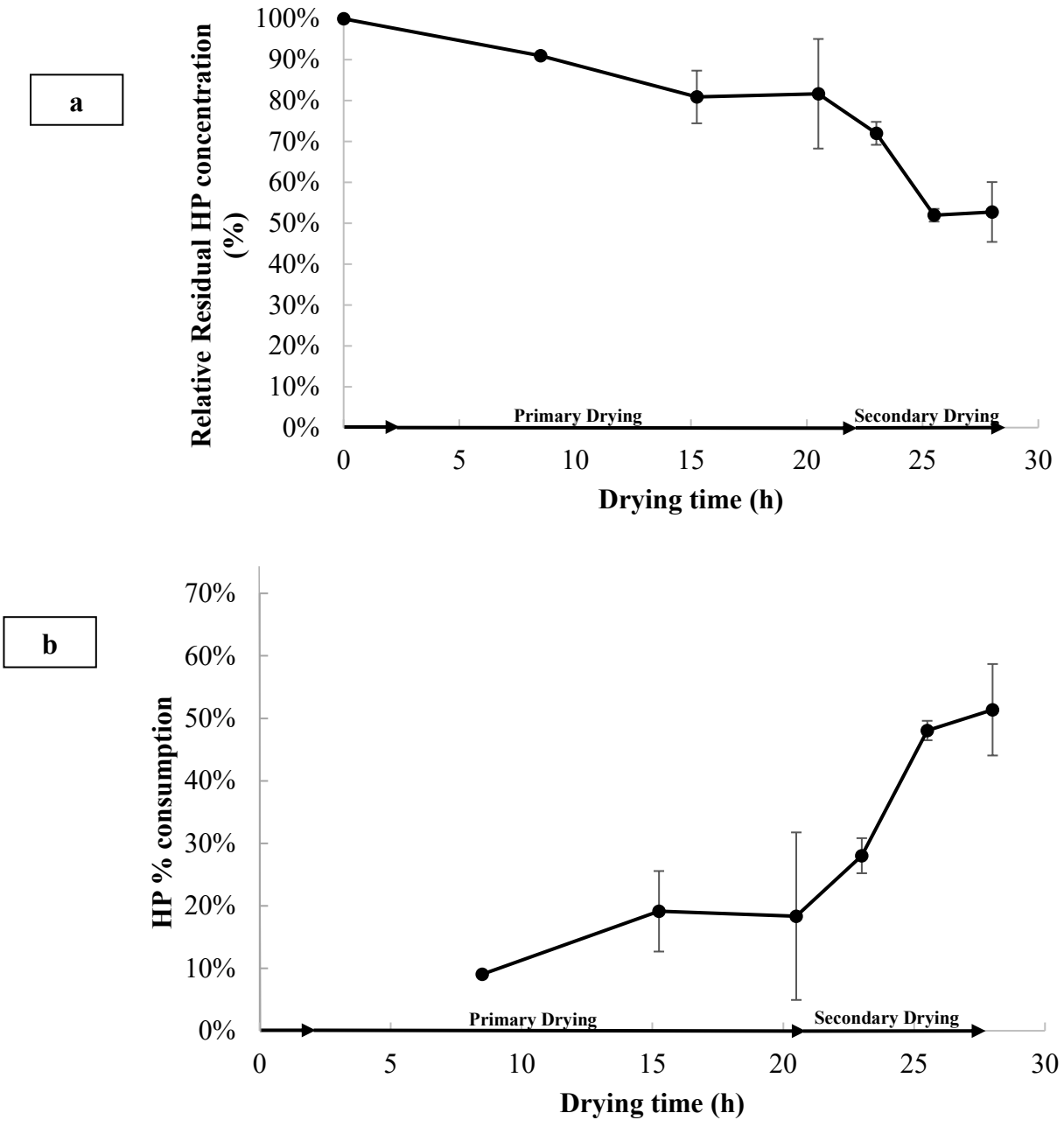

Figure 4.7 Relative HP concentration and percentage of consumption in the spiked Placebo, (a) shows the average HP concentration change during the drying process. (b) shows the HP percentage of consumption change during the drying process.

After the freeze-drying, the HP concentration in the Placebo decreased to 61%-47% (52.7% on average, SD = 7.3%) of the initial value.
The HP percentage of consumption increased with time, at the end of the process the consumption was 51.4%, *Table 4.4* shows the HP consumption during the drying steps:

Table 4.4 HP percentage of consumption (%) in the Placebo during the drying steps.

	HP % consumption
During Primary Drying	18.3%
During Secondary Drying	33.1%
Overall consumption	51.4%

The HP consumption after 15.25 hours of drying turned to a plateau until the end of the primary drying (20.5 h of drying). The plateau was reached when the HP consumption was 19.1% on average (SD = 6.4%). This indicates that there is a lover limit of HP concentration, when this limit is reached, no further HP may be removed during this step. When the secondary drying started, the HP consumption started again increasing, after 5 hours (of secondary drying, 25.5 h overall drying process) it seemed to turn to another plateau.

4.3.1.5. Placebo - Freezing at -45 °C

The relative residual HP concentration in the Placebo and the percentage of consumption of HP during the freeze-drying process (with -45 °C as freezing temperature) are shown in *Figure 4.8.*

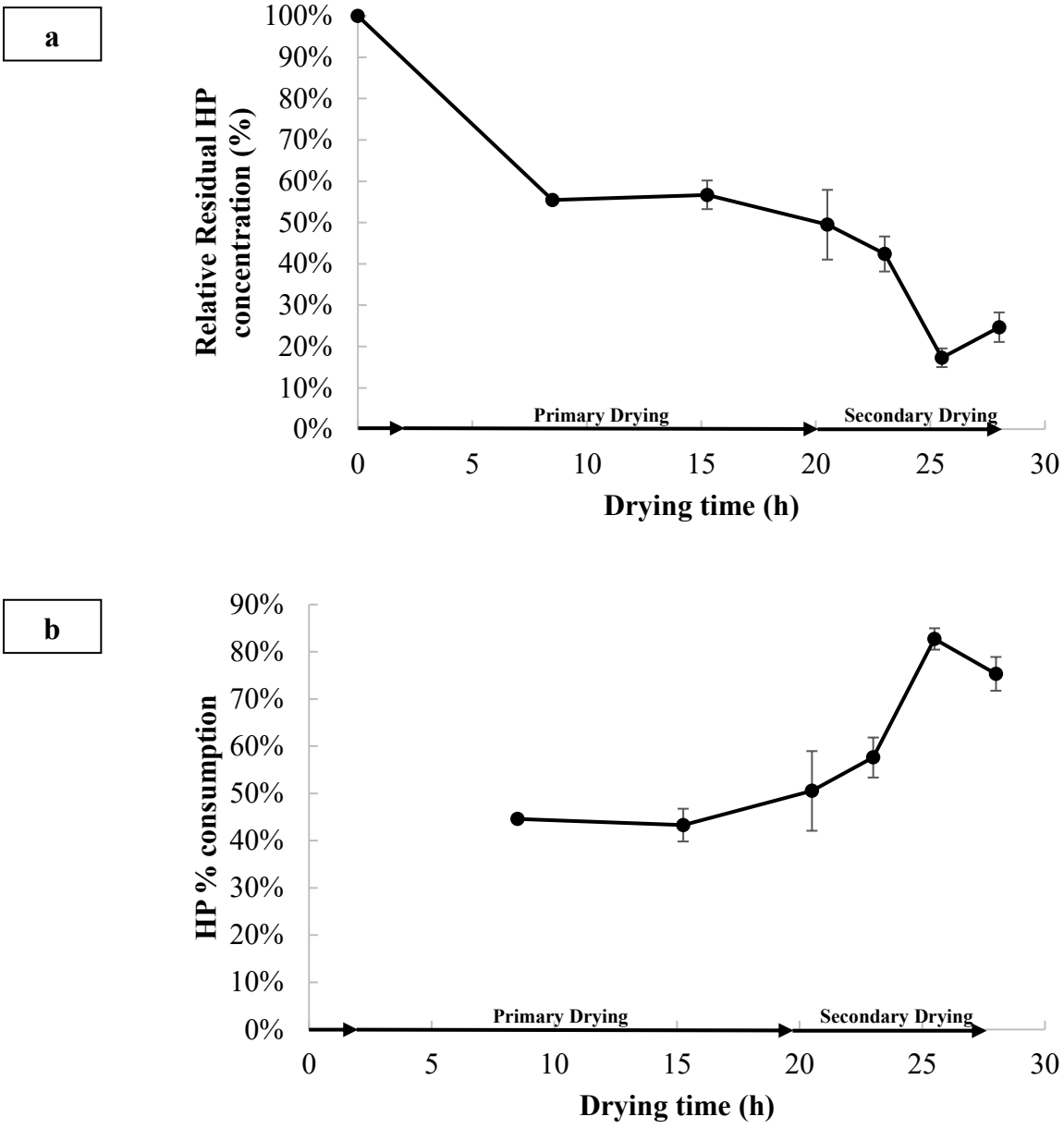

Figure 4.8 Relative HP concentration and percentage of consumption in the spiked Placebo, (a) shows the average HP concentration change during the drying process. (b) shows the HP percentage of consumption change during the drying process.

At the end of the freeze-drying process the HP concentration in the Placebo decreased to 28.8%- 22.2% (24.7% on average, SD = 3,6%) of the initial value. *Table 4.5* shows the HP consumption during the drying steps:

Table 4.5 HP percentage of consumption (%) in the Placebo during the drying steps.

	HP % consumption
During Primary Drying	50.5%
During Secondary Drying	24.8%
Overall consumption	75.3%

The HP consumption, after 8.5 hours of drying turned to a plateau that seemed to last until the end of primary drying (20.5 h of drying). Such a plateau was reached when the HP consumption was circa 50% of the initial value. When the secondary drying started, the HP consumption started again increasing, after 5 hours (of secondary drying, 25.5 h overall drying process) it seemed to turn to another plateau.
With -45 °C as freezing temperature the most HP consumption took place during the primary drying.

4.3.1.6. Placebo - Freezing at -45 °C vs. freezing at -55 °C

The comparison in terms of relative residual HP concentration and percentage of consumption of HP in the Placebo during the two freeze-drying processes are shown in *Figure 4.9.* The only difference between them was the freezing temperature: -45°C and -55°C.

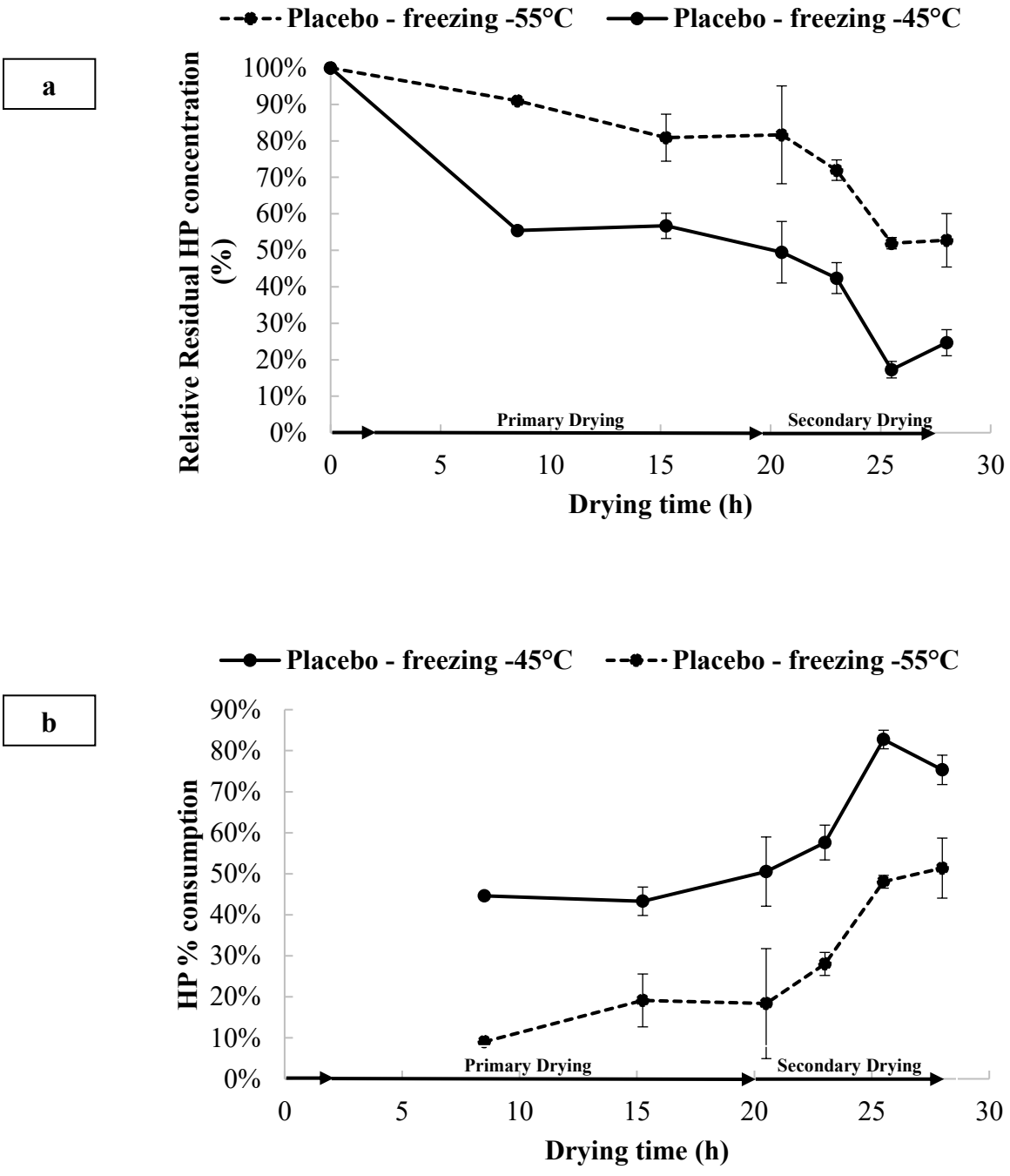

Figure 4.9 Relative HP concentration and percentage of consumption in the spiked Placebo with two freezing temperatures (-45°C and -55°C). (a) shows the average HP concentration change during the drying process. (b) shows the HP percentage of consumption change during the drying process.

During the freeze-drying time the HP consumption for both freezing temperature (-45°C and -55°C) has approximately the same trend. Throughout the entire drying process, the HP consumption with freezing at -45 °C was higher than with freezing at -55 °C. *Table 4.6* shows the HP consumption during the drying steps for the two freezing temperatures.

Table 4.6 HP percentage of consumption (%), with freezing at -45 °C and -55 °C, during the drying steps.

	HP % consumption	
	Freezing -45 °C	Freezing at -55 °C
During Primary Drying	50.5%	18.3%
During Secondary Drying	24.8%	33.1%
Overall consumption	75.3%	51.4%

In case of freezing at -45 °C, the HP consumption appeared to reach faster the plateau during the primary drying. Indeed, with freezing at -45 °C the plateau was already reached after 8.5 hours of drying, on the other hand, with freezing at -55 °C, the plateau was reached after 15.25 hours. Even though this difference during the primary drying, during the secondary drying the HP consumption reached the plateau at the same time (5 h after the secondary drying started) for both freezing temperatures.

The difference in terms of magnitude of HP consumption is due to the same reason explained above with protein formulation.

With -45 °C as freezing temperature, the most HP consumption occurred during the primary drying; while, with -55 °C as freezing temperature it occurred during the secondary drying. Regarding the overall HP consumption, with -45 °C as freezing temperature an additional 23.9% of HP was consumed.

4.3.2. Impact of the antigen

4.3.2.1. Placebo vs. RV - Freezing -55 °C

Figure 4.10 shows the average relative residual HP level and the consumption of HP in the protein formulation (Reconstituted Vaccine = RV) and Placebo over drying time, freezing temperature = -55 °C).

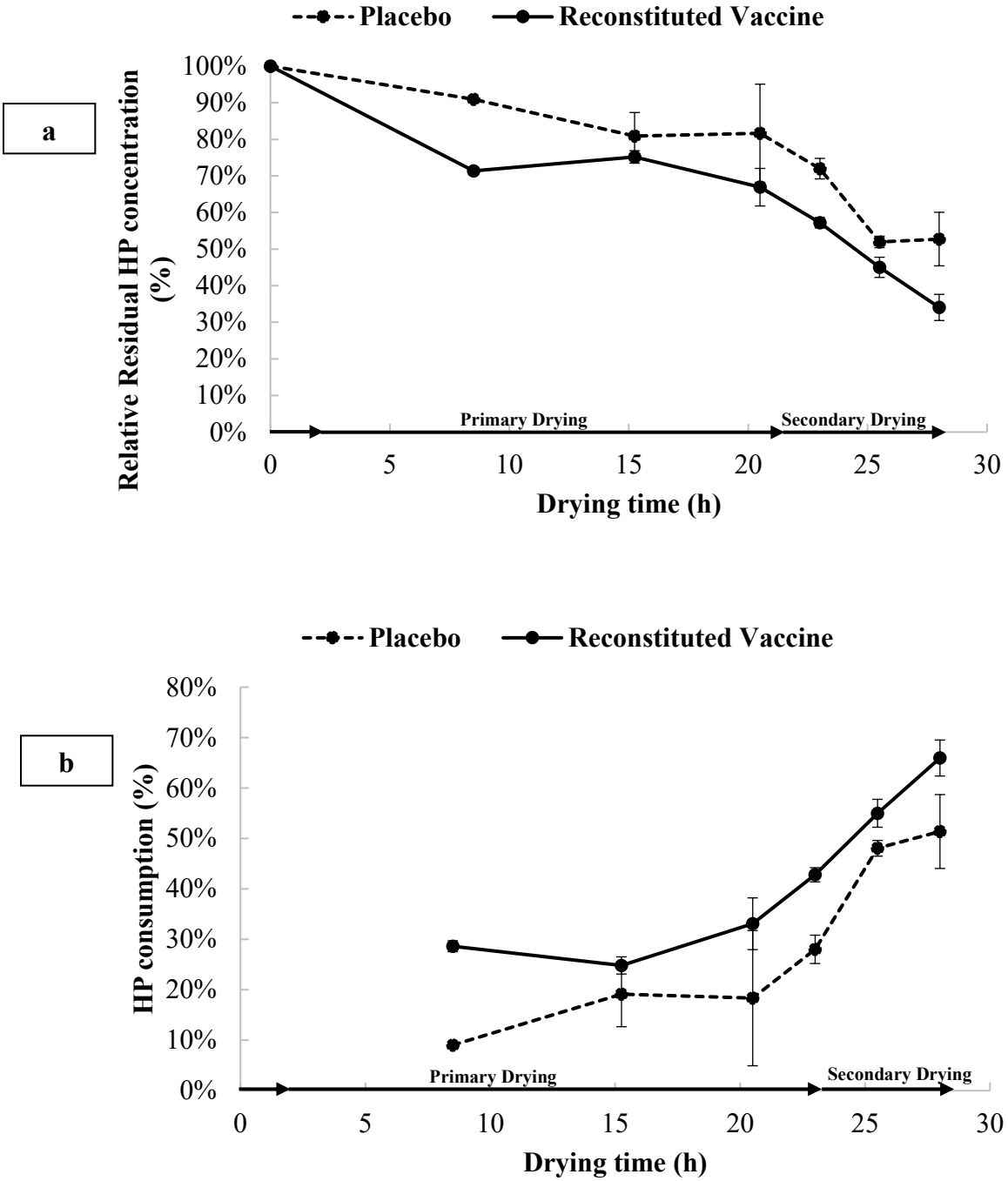

Figure 4.10 Relative HP concentration and percentage of consumption in Placebo and RV (freezing temperature = -55 °C), (a) shows the average HP concentration change during the drying process. (b) shows the HP percentage of consumption change during the drying process.

During the freeze-drying time the HP consumption in both formulations has approximately the same trend. The plateau during the primary drying is more visible in the Placebo than in the protein formulation, while during the secondary drying, only the Placebo shows a plateau. *Table 4.7* shows the HP consumption during the drying steps in the Placebo and RV.

Table 4.7 HP percentage of consumption (%) in the Placebo and RV during the drying steps.

	HP % consumption	
	Placebo	**RV**
During Primary Drying	18.3%	33.1%
During Secondary Drying	33.1%	32.9%
Overall consumption	51.4%	66%

Throughout the entire drying process, the HP consumption in the protein formulation was higher than in Placebo. However, during the secondary drying (see *Table 4.4*), in both formulations the same HP consumption occurred.

These results suggest that the presence of the antigen impacts the drying process in terms of HP consumption. It is possible that in the protein formulation the HP loss was partially due to the reactions between HP and proteins. Therefore, an additional 14.6% of HP in RV was consumed and this was likely due to those oxidation reactions.

Furthermore, in the Placebo the HP was only removed by the drying process, hence when the HP concentration reached the lower limit, the plateau occurred. On the other hand, in the protein formulation, even if the lower limit of concentration for the drying process was reached, the HP consumption continued because of the oxidation reactions.

4.3.2.2. Placebo vs RV - Freezing -45 °C

Figure 4.11 shows the average relative residual HP level and the consumption of HP in the protein formulation (Reconstituted Vaccine = RV) and Placebo over drying time, freezing temperature = -45 °C).

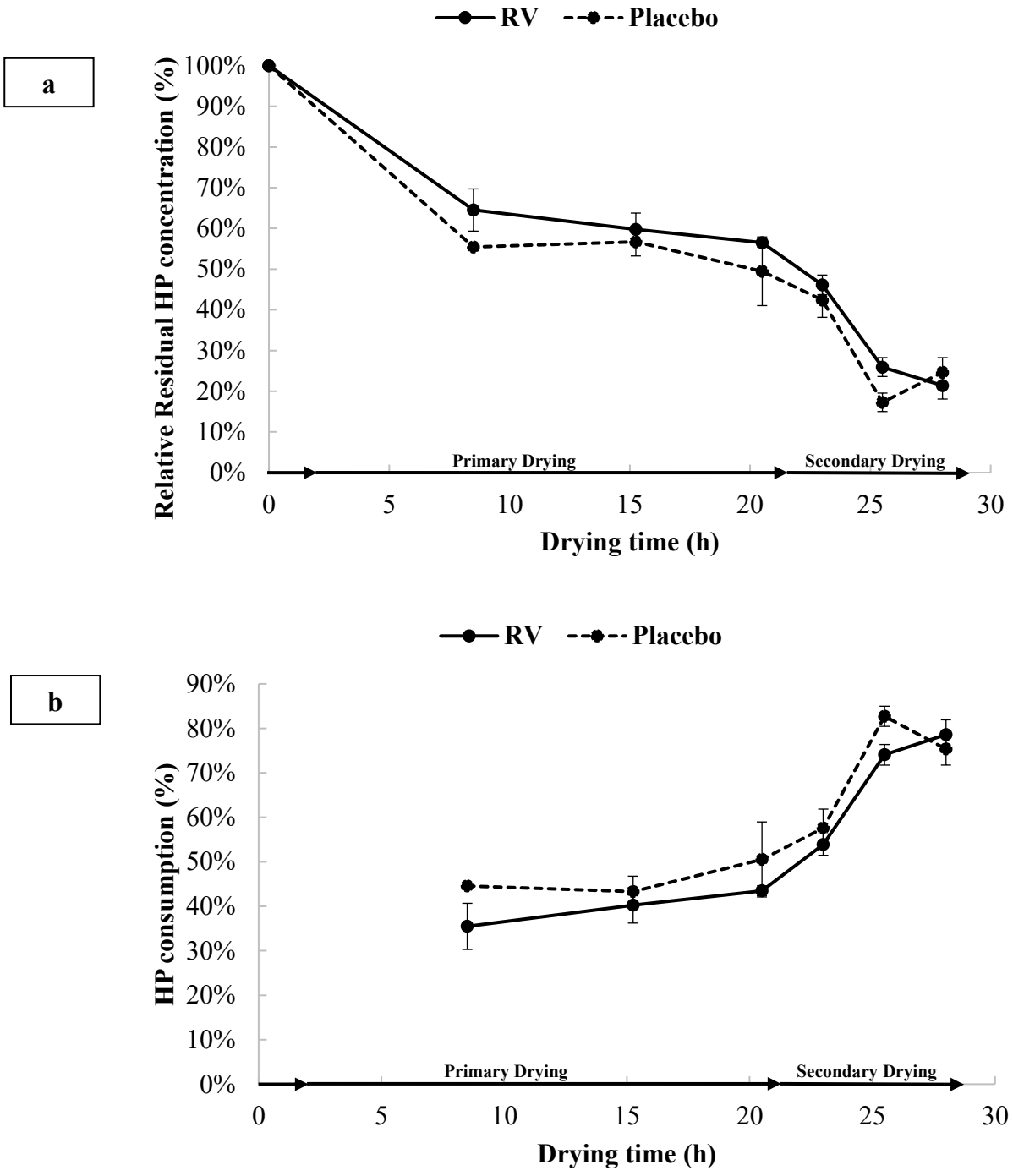

Figure 4.11 Relative HP concentration and percentage of consumption in Placebo and RV (freezing temperature = -45 °C), (a) shows the average HP concentration change during the drying process. (b) shows the HP percentage of consumption change during the drying process.

During the freeze-drying time the HP consumption in both formulations has approximately the same trend. The HP consumption in the Placebo showed a plateau during the primary drying, while in the protein formulation the consumption during the same step increased linearly. At the end of the secondary drying, in both formulations the HP consumption seems to turn to a plateau.

Table 4.8 HP percentage of consumption (%) in the Placebo and RV during the drying steps.

	HP % consumption	
	Placebo	**RV**
During Primary Drying	50.5%	43.5%
During Secondary Drying	24.8%	35.1%
Overall consumption	75.3%	78.6%

Almost the same consumption occurred in Placebo and in RV, this indicates that in RV the HP was mostly removed by the drying process like in the Placebo, and not by the oxidation reactions. Only 3.3% more of HP was consumed in the protein formulation, most probably by the oxidation reactions. With this freezing temperature, the presence of the antigen does not impact the drying process in terms of HP consumption.

4.4. HP consumption in RV during the secondary drying

4.4.1. Freezing at -52 °C

All the vials contained elegant cakes, except for the samples subjected to 21H45 of freeze-drying (see point A, *Table 3.2*).

Figure 4.12 shows the average HP consumption in the protein formulation (RV) over secondary drying time:

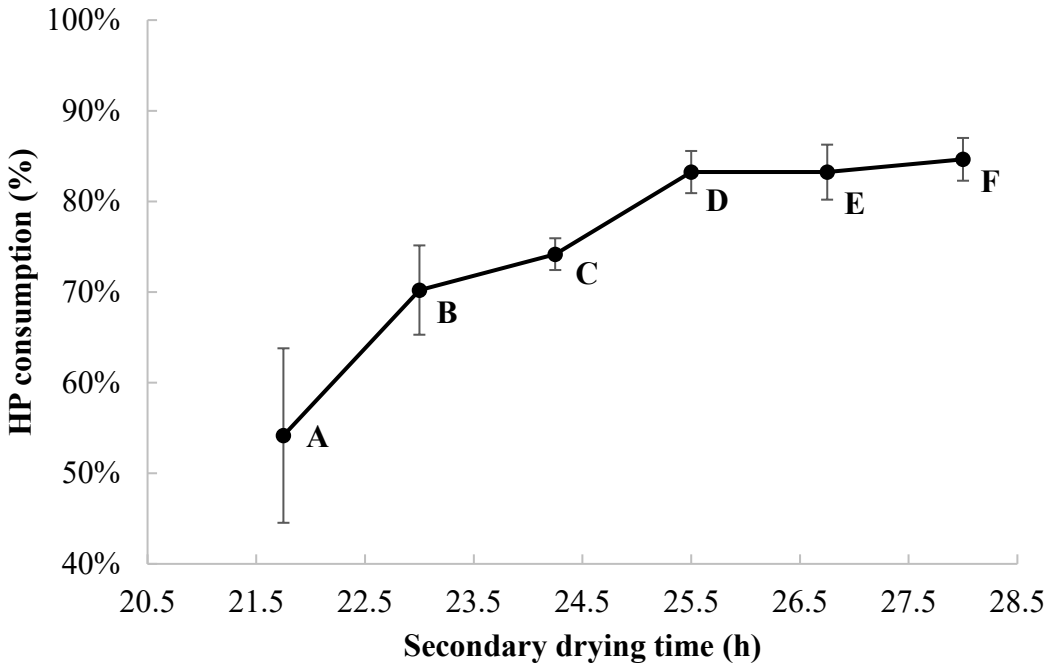

Figure 4.12 HP percentage of consumption change during the secondary drying time.

The HP concentration decreased linearly with time until it reached a plateau. Such a plateau was reached 5 hours after the secondary drying started. There is a lower limit of the HP concentration. When this limit is reached, no further HP can be removed during the drying process.

During the secondary drying, the HP is removed together with water by evaporation. The steps are the following: (I) HP diffusion through the product filaments; (II) evaporation and (III) condensation (in the condenser). The presence of the plateau indicates that the rate-limiting step is the HP diffusion within the sample and not the evaporation from the surface. For the same reason, the percentage of consumption of HP increased linearly with time until it turned to a plateau. The plateau was reached with a consumption of circa 83.5% of the initial HP value.

Furthermore, the fact that the curve HP concentration *vs.* time (see *Figure 4.9*, without considering the plateau) is linear confirmed that the HP consumption rate does not depend on the HP concentration.

The freeze-drying cycle, with the same freezing temperature (-52°C), was also carried out with a higher temperature of the last step of the secondary drying. The temperature of the last step of secondary drying of the standard freeze-drying process is 40°C, the higher temperature tested was 45°C. In *Figure 4.13* the comparison between the two secondary drying temperatures.

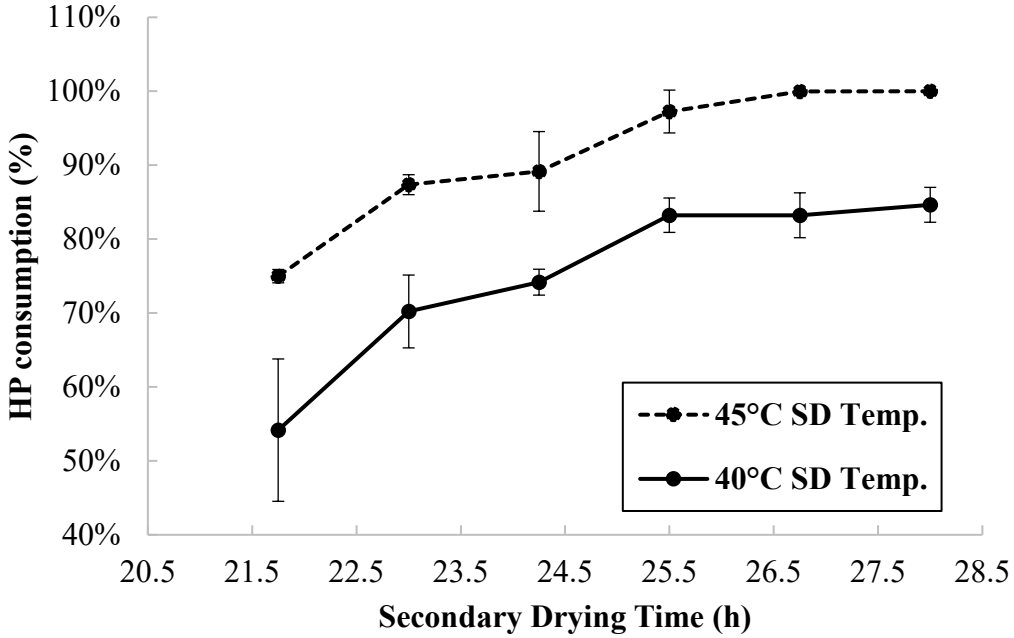

Figure 4.13 HP consumption (%) during the secondary drying, freezing temperature -52°C. Comparison between two temperatures of the final step of the secondary drying: 40°C and 45°C..

Even though the evaluations of the HP residual concentration in the samples are correct, a doubt can arise about the accuracy of these results (45°C SD Temp.) when the two curves are compared. Both of them should overlap each other until 23 h (secondary drying time) as they refer to the same conditions. They should start to diverge only from this point on, because at 23 h, one cycle remains at 40°C and the other increases to 45°C. Despite of this consideration, some conclusions can still be made considering the HP residual concentration of the vaccine at the end of the freeze-drying cycle.

In case of 45 °C as secondary drying temperature, all the HP present in the formulation was removed. Therefore, raising the temperature increased the rate of HP removal.

4.4.2. Comparison between different freezing temperatures

All the vials contained elegant cakes, except for the samples subjected to 21H45 of freeze-drying (see point A, *Table 3.2*).

The HP consumption during the secondary drying was also evaluated with different freezing temperature: -45°C and -57°C, as it is shown in *Figure 4.14*.

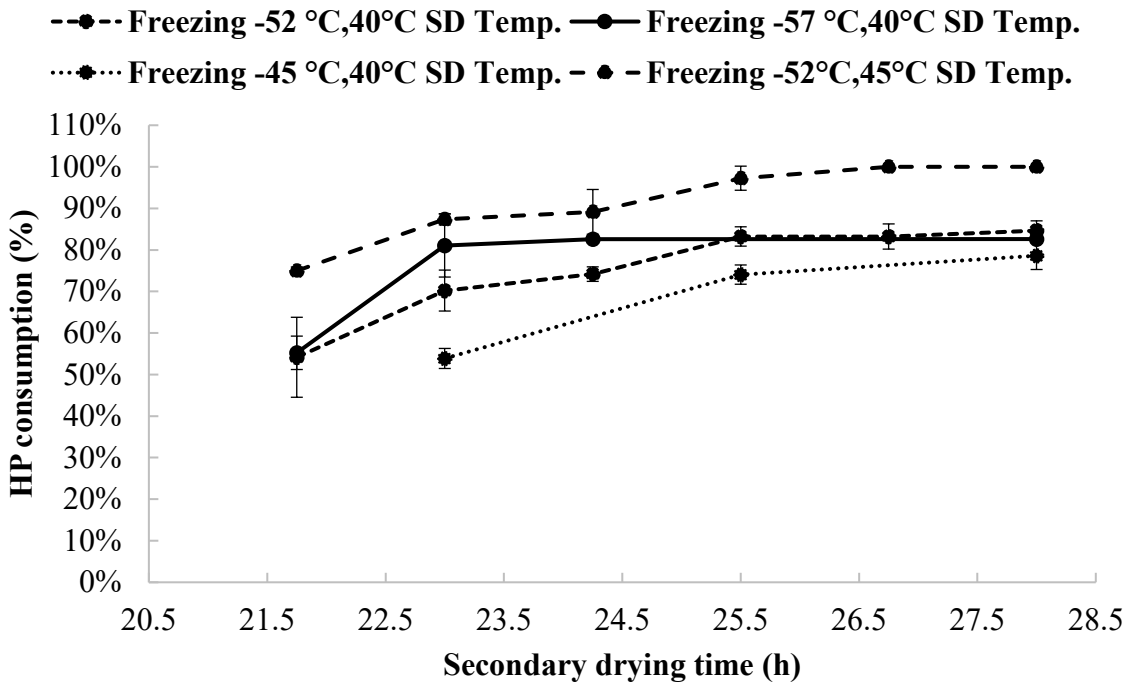

Figure 4.14 Comparison in terms of HP consumption between different freeze-drying cycle.

In case of 40 °C as the temperature of the last step of the secondary drying, regardless the freezing temperature, the HP consumption increased with time and then, 5 hours after the secondary drying started, it remained constant.
With freezing at -57°C and -52 °C, the same HP consumption occurred, that is around 83%. While with freezing at -45 °C, the 78.6% of HP is consumed.

In case of 45°C as the temperature of the last step of the secondary drying, all the HP was consumed.

These results suggest that the HP consumption depends more on the secondary drying temperature than on the freezing temperature

Chapter 5

Conclusions

This study was conducted to systematically investigate the HP consumption during the freeze-drying and establish a thorough understanding of the factors influencing it.
During the Pre-Lyophilization holding period (2 hours), the contact time of HP with the liquid formulations (FB and P) does not show a significant impact on the HP concentration. However it has been mentioned in the literature [14] that the HP in Placebo in the presence of commonly used excipients, such as mannitol, sucrose, and sorbitol may degrade. Although the exact mechanism remains unclear, it is possible that a trace amount of metal ions remaining in the solution, such as zinc, or iron, may be bound to the protein in the vaccine but are freely available in the Placebo formulation to catalyze the HP degradation [14]. Instead in the protein formulation, the HP consumption is likely due to the oxidation reactions with proteins.

It was observed that the majority (50–80%) of the HP present in the formulation is removed during the freeze-drying process. Also, the consumption-rate of the HP does not depend on its concentration since 6 different formulations – spiked with different HP concentration (from 100 to 600 ng/mL) – showed the same HP consumption after the drying.

In case of freezing at -55°C, the presence of the antigen impacts the freeze-drying in terms of HP consumption. Indeed, the HP consumption in the Placebo is lower than that in the protein formulation. The consumption of HP in the protein formulation is not only due to the drying process, but it is also due to other unclear mechanisms such as oxidation reaction with proteins. While with freezing at -45°C, the same HP consumption occurred in the Placebo and in the protein formulation. Therefore, the antigen does not impact the HP consumption.

In of 40 °C as the temperature of the last step of the secondary drying, regardless the freezing temperature, the HP consumption increased and then – 5 hours after the secondary drying started – it turned to a plateau. Raising the temperature to 45°C, all the HP present in the protein formulation was consumed. The HP consumption depends more on the temperature of the final step of the secondary drying than on the freezing temperature. Raising the secondary drying temperature, increases the HP consumption.
For this configuration with predefined process conditions, predefined composition of the liquid formulation and predefined HP concentration in it. By raising the secondary drying temperature with only 5°C, it is possible to remove all the HP contaminating the Final Bulk, and in this way, avoid any possible reaction between the HP and the proteins in the lyophilized state during the storage.

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