



A Design of Experiment approach to predict product and process parameters for a spray dried influenza vaccine



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ABSTRACT

Spray dried vaccine formulations might be an alternative to traditional lyophilized vaccines. Compared to lyophilization, spray drying is a fast and cheap process extensively used for drying biologicals. The current study provides an approach that utilizes Design of Experiments for spray drying process to stabilize whole inactivated influenza virus (WIV) vaccine. The approach included systematically screening and optimizing the spray drying process variables, determining the desired process parameters and predicting product quality parameters. The process parameters inlet air temperature, nozzle gas flow rate and feed flow rate and their effect on WIV vaccine powder characteristics such as particle size, residual moisture content (RMC) and powder yield were investigated. Vaccine powders with a broad range of physical characteristics (RMC 1.2–4.9%, particle size 2.4–8.5 μm and powder yield 42–82%) were obtained. WIV showed no significant loss in antigenicity as revealed by hemagglutination test. Furthermore, descriptive models generated by DoE software could be used to determine and select (set) spray drying process parameter. This was used to generate a dried WIV powder with predefined (predicted) characteristics. Moreover, the spray dried vaccine powders retained their antigenic stability even after storage for 3 months at 60 °C. The approach used here enabled the generation of a thermostable, antigenic WIV vaccine powder with desired physical characteristics that could be potentially used for pulmonary administration.

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1. Introduction

Many existing vaccines are currently distributed and administered in a liquid form. Liquid vaccines need to be stored at 2–8 °C to remain stable. This dependency on a steady cold chain makes vaccine distribution complex and expensive, especially in developing countries (Wang, 1999). Dried vaccines can overcome this requirement for a cold chain, as they possess a longer shelf life at elevated temperatures (Geeraedts et al., 2010; Smith et al., 2015).

Abbreviations: QbD, quality by design; DoE, Design of Experiments; QTTP, quality target product profile; WIV, whole inactivated influenza virus; XRD, X-ray diffractometry; DSC, differential scanning calorimetry; RMC, residual moisture content.

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Moreover, dry powder vaccine formulations have the potential to be used for alternative vaccine delivery routes, such as the intranasal, pulmonary or oral routes (Dicko et al., 2000; Giudice and Campbell, 2006; Tonnis et al., 2012; Amorij et al., 2010).

An established method to produce dried biologics is spray drying. Spray drying has the advantage over traditional drying techniques (such as freeze-drying) that it is relatively fast and has lower operating costs. Moreover, it results in a dispersible fine powder compared to a dry cake as obtained by freeze-drying, which may enable further powder handling and usage for alternative delivery routes.

Powders with different physicochemical and morphological properties can be obtained by spray drying. The powder properties depend on the applied process parameters and composition of the liquid feed (Crowe et al., 1994; Jain and Roy, 2008). The spray drying process consists of nebulization of a liquid product, generating aerosols, into a heated gaseous drying medium, resulting in a dry powder (Fig. 1). The large surface area of the aerosols results in a relative rapid drying process. Depending on

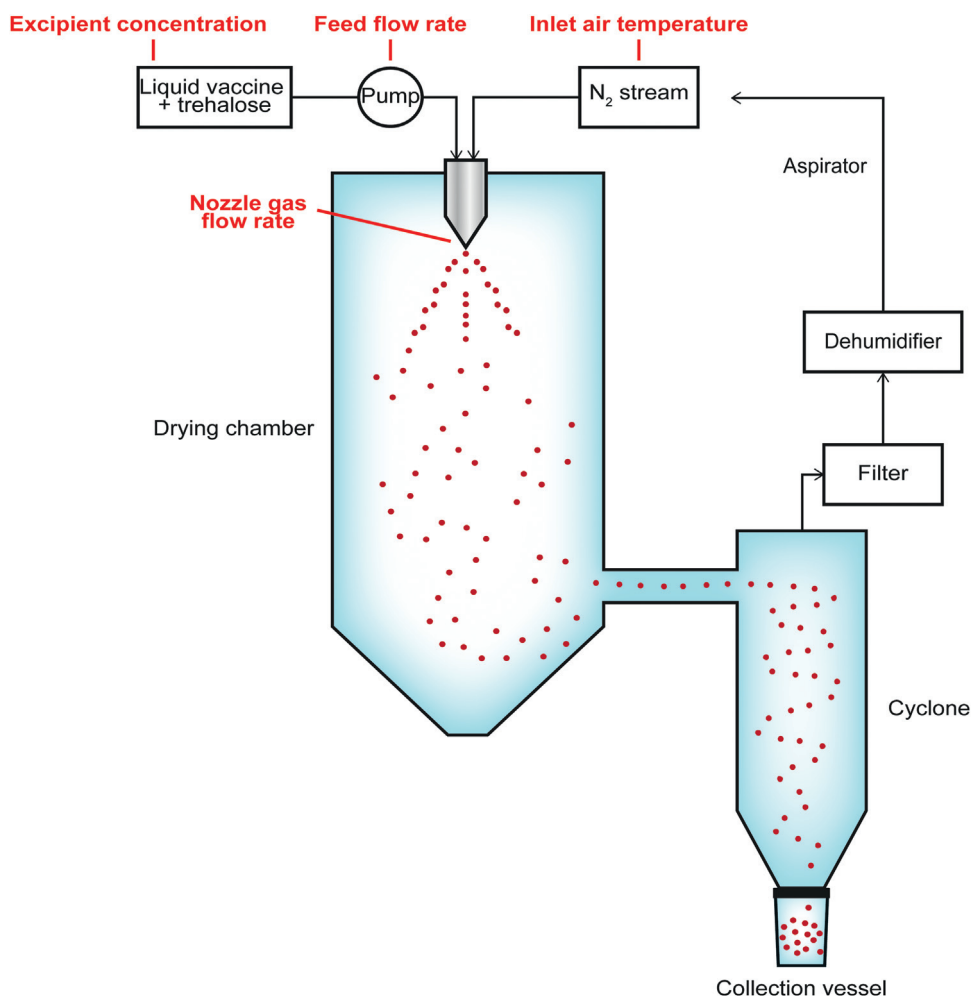


Fig. 1. Spray drying process: A liquid influenza vaccine (whole inactivated influenza virus vaccine) was spray dried with trehalose as an excipient to produce a powder vaccine. Investigated process parameters are indicated in red. Aspirator capacity was kept fixed at $22 \text{ m}^3/\text{h}$ (highest aspirator setting possible).

the size of the spray drier and airflow rate, the drying may take between 0.2–30 s (Anon., 1999). During drying, the protein in evaporating droplets may experience reversible or irreversible denaturation. This could be due to the loss or weakening of hydrogen bonds and simultaneous increase in hydrophobic interactions during evaporation of water. However, the self-cooling effect of droplets due to water evaporation prevents the temperature increase of the droplet surface above the wet bulb temperature (temperature of drying aerosols achieved through evaporation cooling) (Katja, 2011). Thus, spray drying may be an appropriate procedure for drying thermolabile vaccines and has been used to produce experimental dry powder vaccines against measles (Lin et al., 2011; Ohtake et al., 2010), influenza (Lovalenti et al., 2016; Saluja et al., 2010; Scherliess et al., 2014; Sou et al., 2015), tuberculosis (Wong et al., 2007) and hepatitis B (Chen et al., 2010). Moreover, dry powdered measles vaccine has showed promising results in phase 1 clinical trials (MVDP author group et al., 2014). Therefore, spray drying might be a suitable alternative method to obtain dry powders of a variety of vaccines (Amorij et al., 2008).

The spray-drying process used to produce these powders with desired product characteristics is usually optimized by a one-factor-at-a-time (OFAT) approach, where the effect of process parameters on the product are assessed in a linear fashion, one-at-a-time. This consumes a lot of time and resources. Moreover, it

requires a large number of experiments and interactions between parameters are frequently missed. A Design of Experiments (DoE) approach can be used instead in order to systematically screen and optimize processes. DoE is a structured approach that can be used to identify critical and non-critical parameters, and their respective interactions, of a production process. Moreover, it can be used to quantify the impact of raw materials and process parameters on the product characteristics and quality (Cook et al., 2013). Several studies have employed a DoE approach to investigate and optimize the spray drying process of proteins (Prinn et al., 2002; Maltesen et al., 2008) and liposomal adjuvants (Ingvarsson et al., 2013). However, the potential of utilizing DoE for producing spray-dried powder vaccines has not been explored so far.

To maintain the structural integrity during and after the drying process, biological products such as proteins or vaccines often require excipients that act as stabilizers in their formulation. The sugar trehalose is an excipient commonly used for stabilizing vaccines, due to its good protein-stabilizing characteristics (Geeraedts et al., 2010; Ogain et al., 2011). Including trehalose in the vaccine formulations for spray drying might therefore be essential to obtain a stable vaccine product after spray drying. Previously, spray drying of influenza vaccines has been described using various sugars, Maa et al. (Maa et al., 2004) were first to describe the use of trehalose, Sou et al. (2015) combined trehalose with leucine, whereas Scherliess et al. (2014) showed the

superiority of trehalose over mannitol. Audouy et al. (2011) and Saluja et al. (2010) described the use of the spray drying process with inulin as stabilizer.

The goal of this study was to investigate the use of a DoE approach to systematically screen and optimize the spray drying process parameters (inlet air temperature, nozzle gas flow rate, and feed flow rate) and predicting the process settings needed to achieve the targeted product quality parameters like outlet temperature, particle size, powder yield and residual moisture content. As a model antigen, whole-inactivated influenza virus (WIV) vaccine was used, with trehalose as a stabilizing excipient. Using DoE software, an experimental design was generated to assess the impact of various process parameters (inlet air temperature, nozzle gas flow rate, and feed flow rate) and trehalose concentrations on the final product characteristics (particle size, powder yield and residual moisture content). Regression models were fitted on the measured output parameters, and the prediction power of the model was assessed by selecting three untested combinations of process parameters within the investigated design space. Finally, the antigenic recovery and thermostability of the obtained spray-dried vaccines was assessed.

2. Materials and methods

2.1. Influenza vaccine

Influenza A/PR8/34 WIV was obtained by inactivating egg-propagated influenza virus with β -propiolactone as described previously (Hendriks et al., 2011). The bulk vaccine was concentrated with Centriprep centrifugal filters (Millipore) with a molecular weight cut-off (MWCO) of 10 kDa, formulated in HBS (20 mM HEPES, 125 mM NaCl, 9 mM CaCl₂, 5 mM MgCl₂). The final WIV stock contained 800 μ g/mL HA that was determined by surface plasmon resonance as described previously (Hendriks et al., 2011). The vaccine solution to be spray dried was prepared by mixing the WIV stock with D-(+)-trehalose dihydrate (Sigma-Aldrich) solution in PBS (resulting in weight ratio hemagglutinin protein (HA)/trehalose: 1/400). The ratio of protein to sugar was chosen based on previous research experience with spray-freeze drying of influenza vaccines (Geeraedts et al., 2010). The trehalose solution was filtered using a 0.45 μ m Millex-HV filter (Millipore) prior to mixing with WIV.

2.2. Spray drying of influenza vaccine formulation

WIV powders were produced using a Büchi mini spray-drier B-290 in conjunction with a high performance cyclone and a B-296 dehumidifier (both from Büchi Labortechnik AG). All the experiments were performed in a closed loop configuration using nitrogen as drying medium.

Nitrogen being inert in nature was preferred to avert any unwanted reaction that might occur in the presence of air as drying medium. A two-way nozzle with orifice diameter of 0.7 mm was used in a co-current mode with nitrogen as atomizing gas. The nitrogen pressure was set constant at 5 bar. The spray drying parameters were varied in accordance with the experimental design matrix (Table 2). The feed flow rate is displayed in percentage (%) on the equipment and feed flow rate of 5, 10 or 15% corresponding to experimentally determined flow rates ranged from 1.0, 3.4 and 4.5 mL/min, respectively. An atomizing airflow of 7.3 – 17.5 L/min corresponds to a setting from 30 to 50 mm (normal liter [L_n] is the volume at 0 °C and 1 atm). The aspirator rates were set at 22 m³/h in all experiments; this corresponded to instrument setting of 100%.

After spray drying the spray dried product was collected and, aliquoted (100 mg) in vials (3 mL vial, Nuova Ompi) in a glove box under a relative humidity of <3% (Terra Universal Inc, Series 100) and sealed. The yield was defined as the ratio between the amount of powder obtained and the amount of substance introduced in the liquid feed (Eq. (1)) (Maltesen et al., 2008). The weight of buffer salts was not included in yield calculation as they were constant for all formulations.

$$\text{Powder Yield}[\%] = \frac{\text{Collected powder weight}[\text{g}]}{\text{Trehalose in the feed}[\text{g}]} * 100 \quad (1)$$

2.3. Target product profile

The quality target product profile (QTPP) was defined, and process parameters that may have an impact on residual moisture content (RMC), particle size, powder recovery and outlet temperature were determined from previous studies on spray drying (Prinn et al., 2002; Ingvarsson et al., 2013; Ogain et al., 2011). The QTPP describes the characteristics of the final product (Table 1). The parameters investigated were inlet temperature, atomization airflow rate (nozzle pressure), feed flow rate and feed excipient concentration. Additional factors such as raw materials and operator were kept constant. The investigated space would result in a design space where one or more of the targeted product profile could be achieved.

2.4. Design of Experiments (DoE)

The DoE model was prepared and evaluated using MODDE 10.0 (Umetrics AB). Models were fitted with multiple linear regression (MLR) and adjusted by removing non-significant model terms. Prior to our study, screening experiments were performed using a full factorial design to determine the most relevant input process parameters that affected the output process and product parameters. Since the weight ratio of HA/trehalose was 1/400, it was decided to find the appropriate process input parameter ranges without using the antigen. Thereby, assuming that such low

Table 1

Quality targeted product profile. Describes the desired quality target profile for a dry powder Whole inactivated influenza virus vaccine.

Desired response	Target	Rationale
Particle size	1–5 μ m	Particle size that is suitable for inhalational and parenteral administration after reconstitution.
Residual moisture content	3% or less	Within the prescribed limits for dried biologicals (May et al., 1992; The Food and Drug Administration, 1990)
Powder Yield	70% or more	Powder yield should be sufficient to make the process economically feasible.
Stability	Less than 10% loss in HA titers during storage for 12 months at 2–8 °C	Powder vaccine should be at least as stable as the described stability for whole inactivated liquid influenza vaccines (Farnsworth et al., 2011; Kumru et al., 2014). The human dose 15 μ g of HA can be calculated theoretically based on powder yield of the vaccine.

Table 2

Experimental design matrix. The design matrix shows the input parameters set for spray drying (inlet temperature, nozzle gas flow rate, feed flow rate, trehalose concentration) and the output parameters that were experimentally determined [Particle size (X_{50} median particle size) and span, outlet temperature, RMC and powder yield]. Aspirator was set at maximum instrument setting.

Exp No.	Inlet temp (°C)	Nozzle gas flow rate (L/min)	Feed flow rate (mL/min)	Trehalose conc (mg/mL)	Particle size [X_{50}] (μm)	Size (span) ($X_{90} - X_{10}$)/ X_{50}	Outlet temp (°C)	Moisture content (%)	Powder Yield (%)
1	110	7.3	1.0	100	6.1	1.7	67	4.2	63
2	160	7.3	1.0	100	6.9	2.0	91	1.4	72
3	110	17.5	1.0	100	2.4	2.1	58	2.4	82
4	160	7.3	4.5	100	7.3	2.0	78	3.8	53
5	110	17.5	4.5	100	5.7	2.0	48	7.7	64
6	160	17.5	4.5	100	3.2	1.5	63	3.1	72
7	110	7.3	1.0	150	6.8	1.3	63	4.1	61
8	110	17.5	1.0	150	2.9	1.6	61	2.6	75
9	160	17.5	1.0	150	2.9	1.2	83	1.9	69
10	110	7.3	4.5	150	8.5	1.8	53	2.9	42
11	160	7.3	4.5	150	7.9	1.8	76	3.6	52
12	160	17.5	4.5	150	3.1	1.5	73	2.3	69
13	110	12.4	3.4	125	4.5	1.8	55	3.7	69
14	160	12.4	3.4	125	4.3	2.0	78	2.4	70
15	135	7.3	3.4	125	7.5	1.9	68	4.9	57
16	135	17.5	3.4	125	3.3	2.1	65	3.1	76
17	135	12.4	1.0	125	3.7	1.8	74	2.4	77
18	135	12.4	4.5	125	4.2	1.6	61	3.3	69
19	135	12.4	3.4	100	3.8	2.1	67	2.9	78
20	135	12.4	3.4	150	4.2	2.1	62	2.8	62
21	135	12.4	3.4	125	4.1	1.6	63	3.1	67
22	135	12.4	3.4	125	4.5	2.0	66	3.2	72
23	135	12.4	3.4	125	4.4	2.1	66	3.5	73

antigen weight ratio would not significantly influence the output parameters. The model was further optimized using a Central Composite Family (CCF) design, including WIV as the central antigen. A reduced CCF design was used for optimization, consisting of in total 23 experimental runs. The choice of a reduced CCF design was made due to fewer experimental runs giving the same amount of information as the CCF. To reduce systematic errors, all the experiments were completely randomized.

2.5. Antigen characterization

2.5.1. WIV hemagglutination titer

To determine the hemagglutination titer of the WIV in dried vaccine powder, a hemagglutination assay was performed as described previously by Soema et al. (2014). Spray-dried vaccines were reconstituted accordingly in purified water (MilliQ) corresponding to a HA concentration of 0.1 $\mu\text{g}/\mu\text{L}$. Finally, PBS was added to the reconstituted vaccine to obtain a 1:10 dilution. Diluted vaccine solution were transferred to a 96-wells V-bottom plate (Greiner) and serially diluted two-fold with PBS. All the wells consisted of 50 μL after dilution. Next, an equal volume of a 1% suspension of turkey erythrocytes (Harlan laboratories) was added to the wells and the plates were incubated for 1 h at room temperature. The titer was determined by visual observation of agglutination of RBC in the wells and subsequently expressed as the reciprocal of the highest dilution that yielded complete hemagglutination. The titers were determined on reconstituted powders just after spray drying ($t=0$) and were repeated on stored vaccine powder over a period of 3 months at intervals of one month. HA titers (activity) of the samples at each condition were calculated relative to the starting liquid mixture of WIV with excipient at 4 °C. The Log₂ HA titers were expressed in percentage for comparison between different samples.

2.5.2. Dynamic light scattering (DLS)

The size of WIV after reconstitution was measured using a Zetasizer Nano-ZS system (Malvern Instruments). DLS

measurements were done in triplicate with 0.2 mL of the reconstituted WIV samples. Samples were prepared with vaccine powder reconstituted by gentle shaking in purified water (MilliQ) (HA 0.1 $\mu\text{g}/\mu\text{L}$) at an operating temperature of 25 °C. Homogeneity of the size distribution was reflected in the polydispersity index (PdI).

2.6. Residual moisture content (RMC)

The RMC of spray dried influenza vaccine samples was determined using a C30 Compact Karl Fischer Coulometer (Mettler-Toledo). Samples of approximately 100 mg of dried powder vaccine in vials were reconstituted in 1 mL HYDRANAL Coulomat A (Sigma-Aldrich) and subsequently injected into the titration vessel. Each sample was measured in triplicate. The relative and absolute moisture content were calculated from the standard plot, based on the weight of the dried product in the vial, volume of the reconstituted reagent, volume of extracted sample injected into the titration vessel and the blank titration.

2.7. Physical characterization of the vaccine powders

2.7.1. Geometric particle size

The geometric particle size (X_{50} defined as the median particle size) of spray dried powder product was analyzed by laser diffraction with a Helos system (Sympatec GmbH). The powder was dispersed into the Helos system using an aspirator dispersing system operated at a dispersing pressure of 1.0 bar. The vaccine powder was measured with lens having a measuring range of 0.1/0.18–35 μm . Furthermore, to check for any aggregates the dried vaccine powder was measured again with a lens having a measuring range of 4.5–875 μm . Moreover, increasing the dispersion pressure to 5 bar did not result in change of the measured particle size distribution, which indicates that the size distribution of the primary particles was obtained at 1 bar. Results are the mean of three measurements. The span of the produced powders was calculated using the equation: $\text{span} = (X_{90} - X_{10})/X_{50}$. The span is a

measure for the homogeneity/polydispersity of the particle size distribution.

2.7.2. Differential scanning calorimetry

Modulated differential scanning calorimetry (mDSC) was conducted using a TA DSC Q100 (TA instruments). Samples weighing between 10.0–15.0 mg were crimped in hermetically sealed pans for measurement. The glass transition temperature (T_g) was determined by modulated DSC (MDSC); the samples were cooled to 10 °C and then heated to 180 °C at a rate of 2.0 °C/min. The modulation amplitude was set at ± 0.318 °C every 60 s. The midpoint in deflection in the reverse heat flow was taken as the T_g .

2.7.3. Scanning electron microscopy (SEM)

The morphology of the particles was visualized using a JSM 6301F scanning electron microscope (JEOL). Samples (Experiment 1, 7, 8, 11 and 12) were prepared by placing the powders on double-sided sticky carbon tape on a metal disk. Subsequently, the particles were coated with a gold layer of approximately 10 nm using a Balzers 120 B sputtering device (Balzer UNION). The voltage used for analysis was 10 kV with a spot size of 7–8. Images were taken at a magnification of 1000 \times and 5000 \times .

2.7.4. Powder X-ray diffractometry (XRD)

Vaccine powder samples (Experiment 1, 7, 8, 11 and 12) were analyzed by an D2 Phaser desktop X-ray diffractometer equipped

with a LynxEye Si strip one-dimensional detector (both from Bruker AXS). The samples were exposed to Cu K α (X-rays) at an angular ranging was from 5° to 60° 2 θ ; with a step size of 0.01° and a dwell time of 0.5 s. The crystalline status of the powder was assessed qualitatively by examination of the resulting diffraction patterns. The upper limit of detection of crystallinity is 2% of the total sample volume.

3. Results

A DoE approach was used to systematically investigate the effects of feed flow rate, inlet temperature, nozzle gas flow rate (nozzle pressure) and trehalose concentration (which were established to be the important input process parameters in prior screening studies) on the output parameters (outlet temperature) and vaccine powder characteristics. To gain more insight in the relation between input parameters on the responses, a reduced CCF design was adopted for further optimization of process parameters (Table 2). After spray drying and analyzing the formulations of the CCF design, MLR models were fitted for each output parameter. Valid models were obtained for outlet temperature, particle size, residual moisture content and powder yield, described in model fit (R^2), prediction power (Q^2), reproducibility and a valid model.

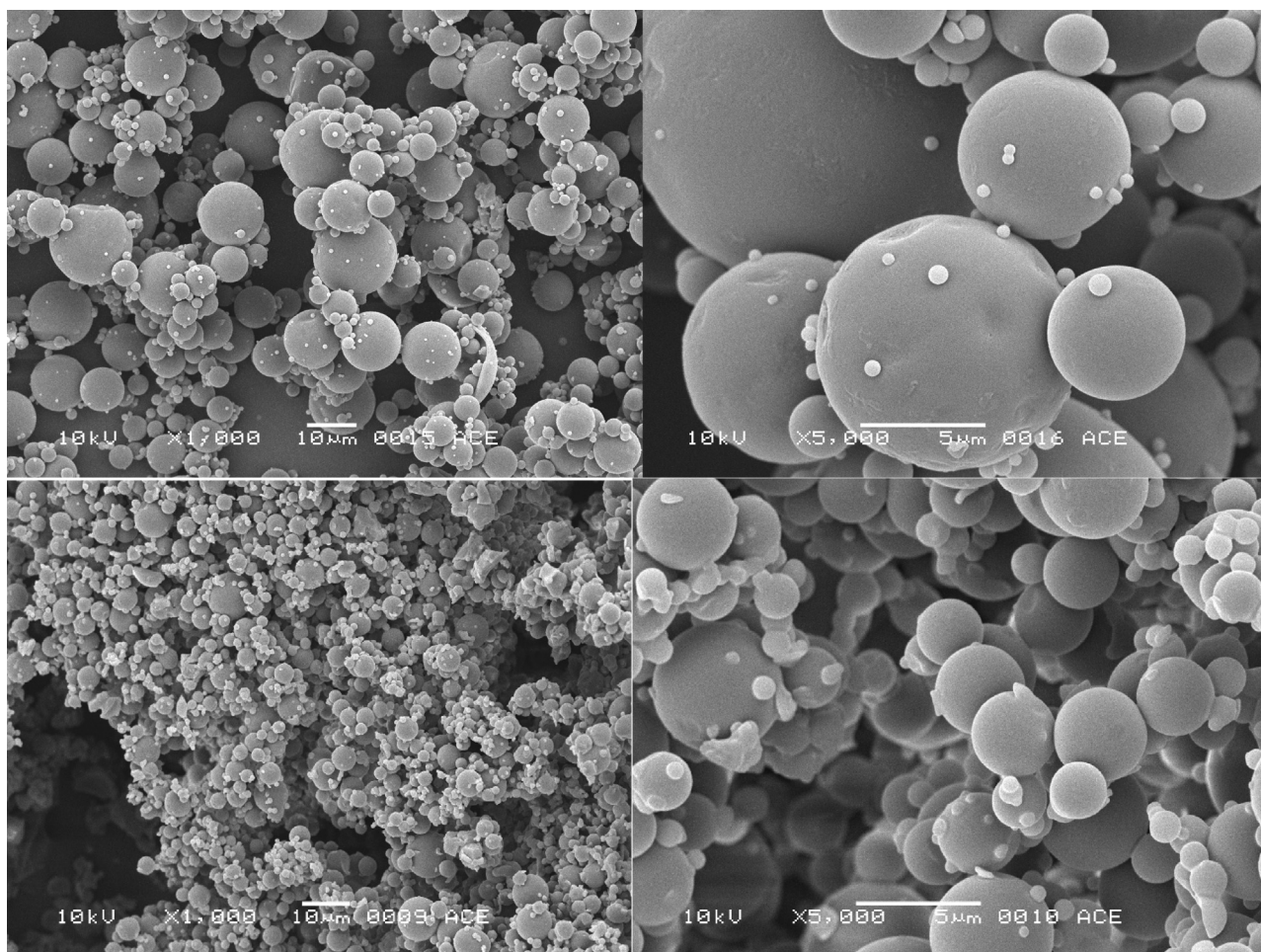


Fig. 2. Powder morphology of spray dried WIV powders. Scanning electron micrographs of representative spray dried trehalose-stabilized WIV vaccines [Top: Run 12 (X_{50} : 3.1 μ m), Bottom: Run 8 (X_{50} : 2.9 μ m)]. Magnification images left (1000 \times) and right (5000 \times).

3.1. Physical characteristics of obtained vaccine powders

3.1.1. Particle shape and size

All the experiments in the current design yielded white to off-white powders after spray drying. No visible aggregates could be observed in the powders by eye. SEM was performed on the powders to provide particle form and surface morphology. The powders showed a relatively homogenous surface morphology. Typically, they were spherical with a smooth surface (Fig. 2). In addition, the particle size of the acquired powder vaccines was measured by laser diffraction. The powders had a median volume diameter (X_{50}) between 2.4 μm and 8.5 μm , with narrow size distributions (Table 2). To investigate the relationship between the input parameters and the particle size, a MLR model was fitted on the data (Fig. 3A). The model fit and prediction power ($R^2 = 0.965$, $Q^2 = 0.917$) were good, and model reproducibility and validity were sufficient. The regression coefficient plot (Fig. 3B) shows that the nozzle gas flow rate (nozzle pressure), inlet air temperature and feed flow rate were relevant to particle size. The effect of the two most influential factors, feed flow rate and nozzle pressure, on the particle size is shown in Fig. 3C. An increase in feed flow rate

resulted in bigger particles, whereas an increase in nozzle pressure resulted in a decreased in particle size (summarized in Table 6).

3.1.2. Powder yield

The influence of spray drying process parameters on the yield of the vaccine powder was assessed. The powder yields ranged from 42% to 82%, and approximately half of the runs in the design had a yield of >70%, which was the targeted yield in the QTPP (Table 2). Applying a MLR model resulted in a good model fit, prediction power, reproducibility and validity (Fig. 4A). Three input parameters (nozzle pressure, feed flow rate and trehalose concentration) were found to affect the yield (Fig. 4B). An increase in nozzle pressure increased the powder yield, whereas increasing the feed flow rate and trehalose concentration decreased the powder yield. The effect of two most influential factors nozzle pressure and feed flow rate on predicted powder yield is shown in contour plot, Fig. 4C. In general, high nozzle pressure and lower feed flow rate with other parameters fixed favors higher powder yield.

3.1.3. Residual moisture content

The moisture content of the produced powders was between 1.2% and 4.9% (Table 2). One outlier was detected (experiment No

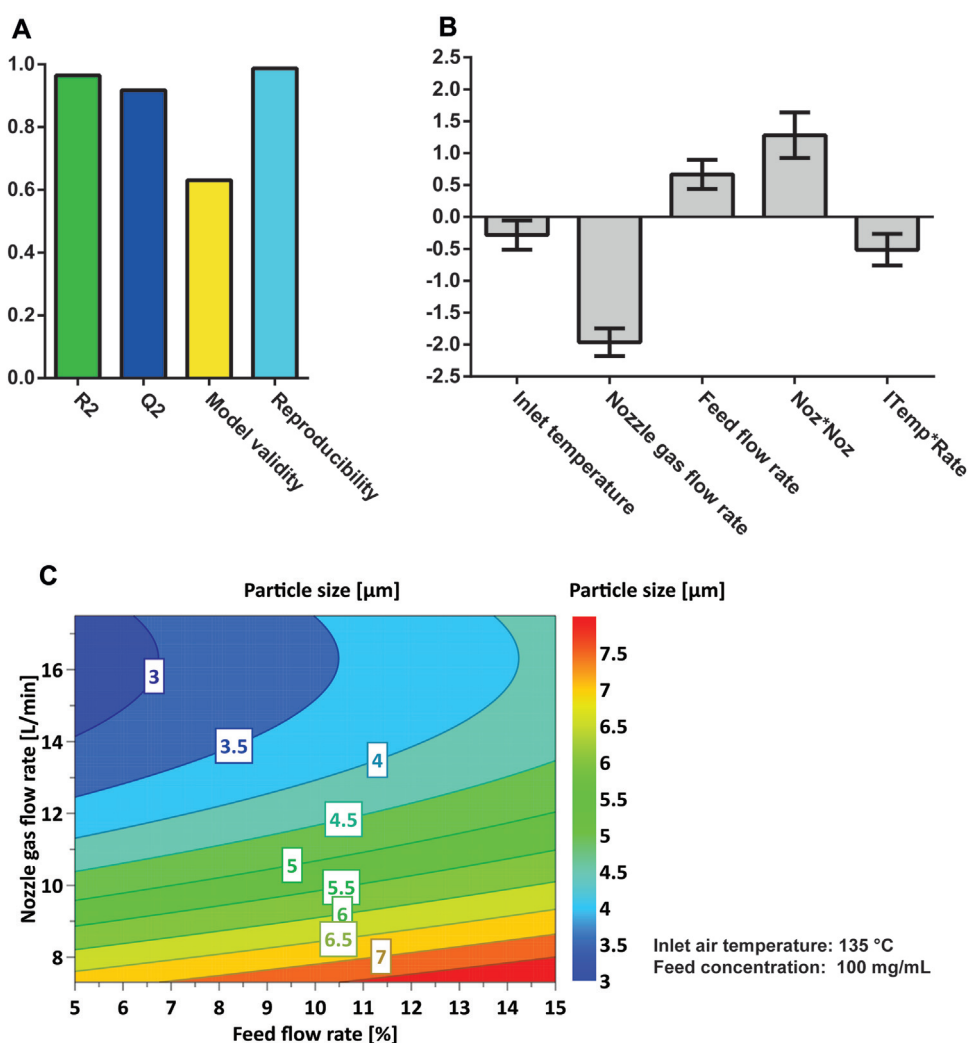


Fig. 3. Regression model for particle size. A: Summary of fit plot for particle size. R^2 (Goodness of fit, 1 = perfect model) ($R^2 = 0.964$). Q^2 (Goodness of prediction, values greater than 0.5 is a good fit) ($Q^2 = 0.917$). Model validity (value greater than 0.25 indicates good fit). Reproducibility (greater than 0.5 indicates a small experimental error) B: Regression coefficients for particle size. C: Response contour plots for particle size. The effect of the two most influential factors (as observed from Fig B) was taken into account and other factors were kept constant. The color regions represent the predicted response (particle size) for defined parameter settings. Aspirator was set at maximum instrument setting.

Table 3

Thermostability of spray dried formulations. Spray dried formulations were stored up to 3 months at 60 °C, and their antigenicity was subsequently determined by hemagglutination (HA). Liquid WIV was also stored at 60 °C as a control. The HA titers were measured in triplicate; since no difference in the triplicate of the titers was observed no standard deviations are shown. Number 1–23 represent the different formulations spray dried according to the design described in Table 2.

Type	Day 0 HA titers (%)						Day 30 HA titers (%)	Day 60 HA titers (%)	Day 90 HA titers (%)
	D0	D1	D2	D3	D4	D5			
Liquid WIV stored at 60 °C (negative control)	100	72	56	45	34	0			
Experiment 1	100						100	100	96
Experiment 2	100						104	106	96
Experiment 3	100						104	100	96
Experiment 4	94						104	104	96
Experiment 5	90						101	100	92
Experiment 6	86						105	104	102
Experiment 7	87						98	98	96
Experiment 8	100						108	105	96
Spray dried 9	93						105	112	102
Experiment 10	96						105	100	98
Experiment 11	93						108	115	105
Experiment 12	100						101	100	92
Experiment 13	100						105	105	102
Experiment 14	96						105	105	98
Experiment 15	96						107	100	98
Experiment 16	100						104	98	96
Experiment 17	100						104	104	96
Experiment 18	100						112	105	102
Experiment 19	100						108	98	96
Experiment 20	94						104	98	96
Experiment 21	87						95	98	96
Experiment 22	96						94	100	98
Experiment 23	100						102	105	102

Table 4

Prediction power of DoE model. Three experimental input parameters were chosen from the predicted design space (Fig. 9). Point A was selected for proof-of-principle purposes for a formulation that would result in targeted product specifications (within optimum area of predicted design space). Point B and C do not meet all criteria for targeted product (outside optimum area of design space). The rationale for choosing B and C was to confirm the prediction abilities of the model within the design space but outside the optimum area. Aspirator was set at maximum instrument setting for the three experimental runs.

Exp No.	Inlet temp (°C)	Nozzle gas flow rate (L/min)	Feed flow rate (mL/min)	Trehalose conc (mg/mL)
Within optimum area(A)	120	12.4	1.0	100
Outside optimum area (B)	135	10.5	1.0	125
Outside optimum area spot (C)	132	9.0	1.0	125

5), which was subsequently excluded from the regression model. Fitting a MLR regression model on the RMC data resulted in a good model fit, prediction power, model reproducibility and validity (Fig. 5A). The resulting regression coefficient plot shows that many

process parameters influence the RMC of the spray-dried vaccine powders (Fig. 5B). The effect of two most influential factors nozzle pressure and inlet temperature on predicted RMC is shown in contour plot, Fig. 5C. An increase in inlet temperature or nozzle

Table 5

Assessment/qualification of the prediction models. The acquired model from experimental design described in Table 2 predicted the particle size, residual moisture content, outlet temperature and powder yield. Predictions are expressed as mean \pm 95% confidence intervals. The upper and lower ranges were taken from the model. Data for particle size and RMC represent mean \pm SD of one sample measured in triplicate N = 3 (observed).

Output parameters	Predicted	Predicted lower range	Predicted upper range	Observed
Particle size(μ m)				
A	3.38	3.00	3.77	3.08 \pm 0.1
B	4.23	3.88	4.57	4.07 \pm 0.1
C	5.88	5.55	6.21	5.78 \pm 0.1
Residual moisture content (%)				
A	2.1	1.8	2.5	2.4 \pm 0.03
B	2.7	2.4	3.0	2.7 \pm 0.02
C	3.6	3.3	4.0	2.8 \pm 0.02
Outlet temperature (°C)				
A	67	65	69	67
B	74	73	76	76
C	75	73	77	75
Powder yield (%)				
A	80	76	84	77
B	73	70	77	73
C	66	63	69	64

Table 6

Summary of main findings. Description of the relationships between process input parameters and product output parameters in this study for the spray drying process (Büchi, B-290 and 0.7 mm orifice nozzle) of a whole inactivated influenza vaccine. Aspirator capacity was kept fixed at 22 m³/h [highest aspirator setting possible (of used equipment)].

Parameter/Dependence	Nozzle pressure ↑	Inlet air temperature ↑	Feed flow rate ↑	Trehalose concentration ↑
Particle Size	↓↓↓ Greater energy for dispersion, smaller particles after atomization	↓ More evaporation after shell formation	↑ More fluid needs to be dispersed	No effect in the investigated range
Powder Yield	↑ Smaller particles, improved drying, lesser deposition in drying chamber	↑↑ Eventually dryer product but chances of sticking at high temperatures	↓ Higher water content to evaporate, more sticking but dependent on other variables	↓ Bigger droplets for drying, more deposition in drying chamber
Residual moisture content	↓ Smaller droplets thus better drying	↓↓ Lower relative humidity in air	↑↑ More water for evaporation leads to higher partial pressure	↓ Less water evaporated, lower partial pressure
Outlet Temperature	↓ More nitrogen inflow in system for heating.	↑↑↑ Directly proportional as available energy for drying increases proportionally with rise in inlet temperature	↓↓ More water needs to be evaporated	↑ Less water needs to be evaporated as more excipient in the droplet
Antigenicity	No effect in the investigated range	No effect in the investigated range	No effect in the investigated range	No effect in the investigated range

pressure resulted in a decrease of RMC, while an increase in feed flow rate increased the RMC of the powders.

3.1.4. Amorphous nature of dried powder vaccine

We had observed some samples with high RMC and since water is a potent plasticizer for sugar glasses. We wanted to confirm the amorphousness of the samples. The amorphous nature of the spray dried vaccine powders was determined by assessing the glass transition temperatures (T_g) with DSC and crystallinity by X-ray diffraction (XRD). T_g analysis of the vaccine powders gave values between 45 °C and 83 °C for the highest and lowest RMC-containing powders, respectively (Supplementary Table 2). The X-ray diffraction (XRD) profiles of the excipient (trehalose dihydrate) and spray dried influenza vaccines are shown in Fig. 6. The profiles indicate that solid trehalose dihydrate was crystalline prior to spray drying, as peaks were observed. On the other hand, no peaks were detected in the spray dried vaccine powders, indicating that the powders were amorphous.

3.2. Outlet temperature

The outlet air temperature was determined during the spray drying process of each experiment in the design. During spray drying of the influenza vaccine formulations the outlet temperature was found to be between 48 °C and 91 °C (Table 2). A MLR regression model was fitted to the outlet temperature data, which resulted in a good model fit (Fig. 7A). The obtained regression coefficient plot showed that the outlet temperature was most affected by the inlet temperature (Fig. 7B). In addition, the relationship between inlet air temperature and feed flow rate is depicted in response contour plot for outlet temperature (Fig. 7C). It was observed that on increasing the feed flow rate and decreasing inlet air temperature (keeping other factors constant) resulted in decrease of the outlet temperature.

3.3. Antigen recovery and stability after spray drying

Next to the physical powder characteristics, the antigen characteristics were determined after spray drying. First, the particle size of WIV was determined for each spray dried formulation. Dynamic light scattering was performed on reconstituted vaccine powders. All samples dissolved readily and gave clear opalescent liquid, which did not contain any visible aggregates. The particle size of WIV remained unchanged for all formulations, with an average size of 175 nm and polydispersity

indexes below 0.08 (Supplementary Table 1), indicating that the WIV particles were intact and not aggregated during or after spray drying or reconstitution.

To assess the antigenicity of the WIV antigens after spray drying, a hemagglutination assay was performed on the reconstituted vaccine powders directly after spray drying. Overall, loss of antigenicity was minimal after spray drying, with antigenic recoveries ranging from 86% to 100% (after ²log transformation) (Table 3). Due to these minimal changes in antigenicity, no MLR model could be fitted on the antigenic recovery data.

In addition to the recovery of the antigen immediately after spray drying, an accelerated stability study was performed on the vaccine powders. Liquid WIV vaccine stored under refrigerated conditions was taken as a control group. The powder vaccine samples from the experimental design were sealed under a nitrogen environment (100 mg per vial) and stored at 60 °C for 3 months. The change in hemagglutinin titers upon storage was assessed by hemagglutination assay after reconstitution of the powder vaccines. Minimal or no loss in antigenicity was observed after storage for 3 months at 60 °C for all spray dried samples (Table 3). In contrast, liquid WIV vaccine lost all its hemagglutinin binding capacity after 5 days of storage at 60 °C. This indicates that spray dried WIV powder vaccines are much more resistant to heat stress than conventional liquid vaccines.

3.4. Prediction power of the DoE model

An advantage of the generated MLR models is that they can be used to predict process or product parameters (in this case outlet temperature, powder particle size, powder yield, and RMC) for combinations of the different input process parameters that have not been tested yet. As previously described, the study obtained valid models for outlet temperature, particle size, powder yield and moisture content. The structured approach helped in identifying a design space where the established QTPP can be achieved (Fig. 9). The prediction ability of the model was tested by selecting input parameters that would result in predefined product specifications for three untested formulations. The calculation for prediction are based on statistical values from the model calculated by MODDE software (Lundstedt et al., 1998). Experiment A was selected in the sweet spot region, which would result in a product according to the defined QTPP. Experiments B and C were selected outside the sweet spot, which would yield powder vaccines that would not meet all the QTPP criteria (Table 4). Using the selected target product characteristics as input, the acquired

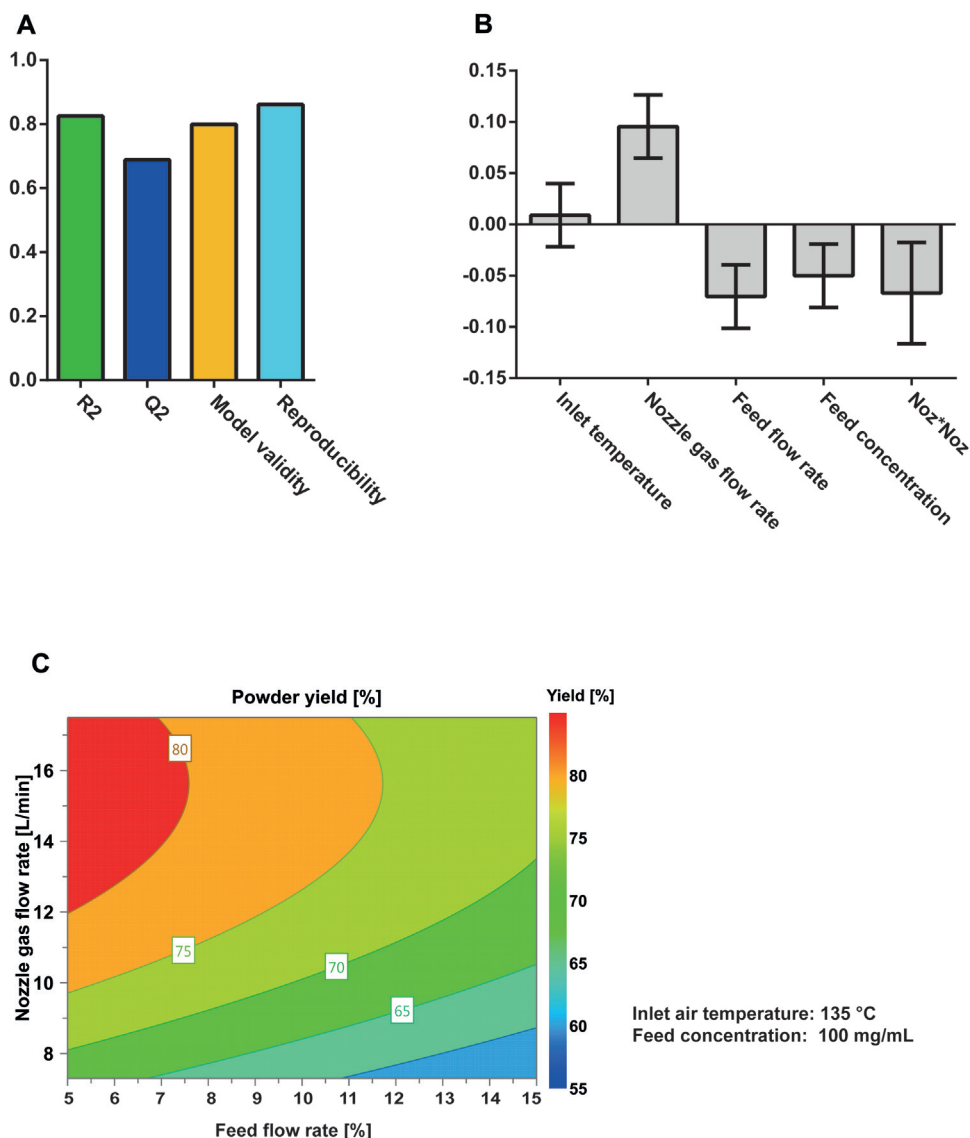


Fig. 4. Regression model for yield. A: Summary of fit plot for powder yield. R² (Goodness of fit = 0.825). Q² (Goodness of prediction = 0.688). B: Regression coefficients of powder yield after model refinement. Aspirator was set at maximum capacity. C: Response contour plots for powder yield. The effect of the two most influential factors (as observed from B) was taken into account and other factors were kept constant. The color regions represent the predicted response (powder yield) for defined parameter settings. Aspirator was set at maximum instrument setting.

DoE model gave spray-drying parameters that would yield the desired product (Table 4). For experiment A, the experimentally acquired data indeed correlated in range with the predicted values for particle size (predicted 3.38 μm , observed 3.08 μm), RMC (predicted 2.1%, observed 2.4%), powder yield (predicted 80.2%, observed 77%) and outlet temperature (predicted 67.1 °C, observed 67 °C) (Table 5). The predicted parameters thus yielded a powder vaccine that met all the criteria of the QTP. The output parameters (particle size, powder yield and outlet temperature) of experiments B and C also concurred with the responses predicted by the model, with the exception of the RMC for experiment C, which was lower (2.8%) than the predicted (3.6%) RMC. These data indicate that the predictions made by the model were accurate.

4. Discussion

The present study demonstrates the usefulness of a DoE approach to understand the effects of spray drying process parameters and excipient concentration on the production of

stable spray dried influenza vaccines. The main findings are summarized in Table 6.

4.1. Effect of nozzle pressure (atomization gas flow rate)

In general, the nozzle pressure (7.3–17.5 L/min) was the most influential process parameters for the studied characteristics of the obtained powder vaccine. An increase in gas (nitrogen) pressure on the nozzle resulted in decreased particle size, which can be related to the increased energy available for breaking up the liquid jet by the nozzle, thus forming smaller particles during atomization. This was in line with previous studies from Maltesen et al. (Maltesen et al., 2008), who reported a decrease in particle size of spray dried product from 26 μm to 9.8 μm when increasing the nozzle pressure in the range from 7.3 to 17.5 L/min. In addition, the interaction factor (Noz*Noz) (Fig. 3B) showed an opposite effect compared to the main factor nozzle pressure, indicating that the relationship between nozzle pressure and particle size could be non-linear outside the studied range.

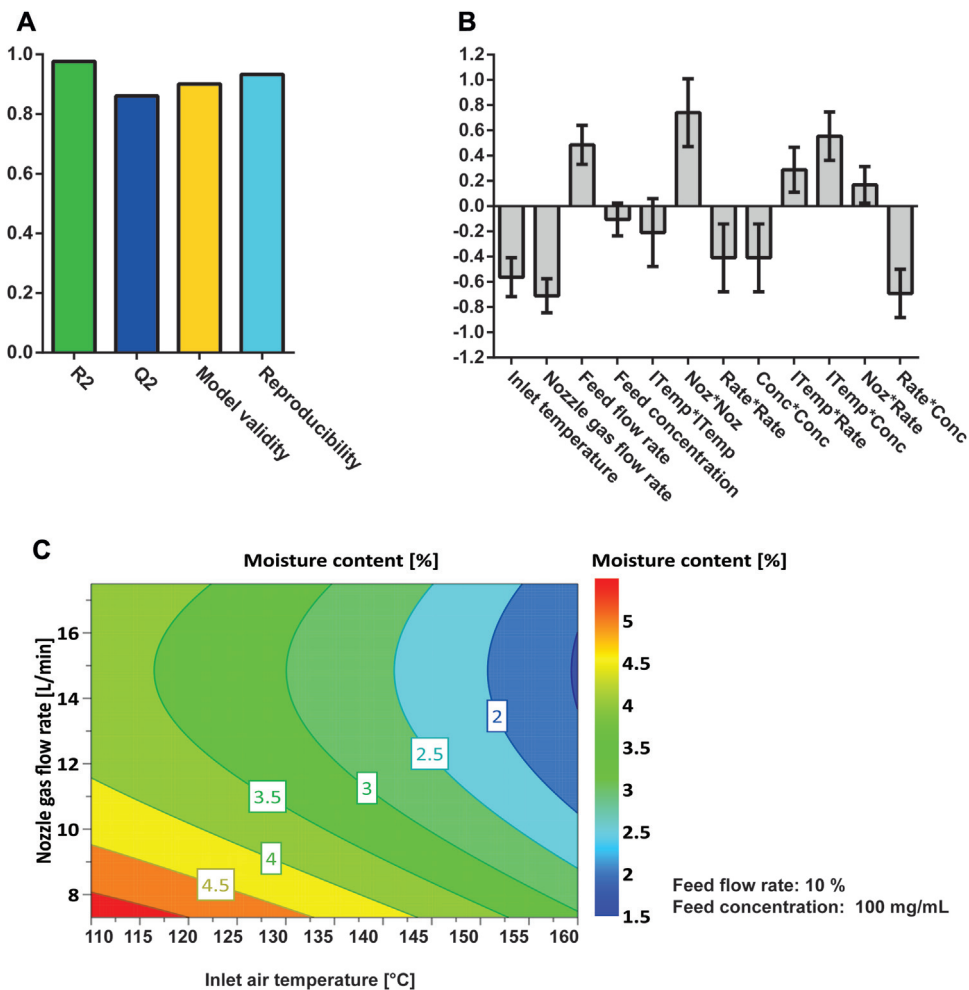


Fig. 5. Regression model for residual moisture content. A: Summary of fit plot for residual moisture content. R² (Goodness of fit = 0.976). Q² (Goodness of prediction = 0.860). B: Regression coefficients of residual moisture content after model refinement. Aspirator was set at maximum capacity. C: Response contour plots for residual moisture content. The effect of the two most influential factors (as observed from Fig B) were taken into account and other factors were kept constant. The color regions represent the predicted response (RMC) for defined parameter settings. Aspirator was set at maximum instrument setting.

The role of nozzle gas pressure on powder yield was evident. A decrease in nozzle pressure decreases the imparted kinetic energy of the gas on the dispersed liquid, resulting in bigger droplets with smaller surface area for drying, that increases deposition in the drying chamber and hence decreases the yield. This deposit was visually observed on the inside wall of the drying chamber which increased with decreasing nozzle pressure. These results are consistent with research by Maury et al. (2005), which observed a trend of decreasing powder yield (from 60% to 40%) with decreasing the nozzle pressure from 13.4 L/min to 8.3 L/min at a fixed inlet temperature of 170 °C. Furthermore, the model for residual moisture content shows an increase in RMC of the powder with decreasing nozzle pressure. This result is likely to be related to the formation of larger particles at decreasing nozzle pressure, which leads to a reduced specific surface area for evaporation.

Although not being the most influencing parameter (in contrast to the inlet air temperature), the nozzle pressure appears to reduce the outlet temperature. This relation is consistent with the data obtained by Maa et al. (1997), who observed a similar relation with nozzle pressure, while spray drying a IgE class monoclonal antibody with trehalose.

4.2. Effect of inlet air temperature

The inlet air temperature for the drying was varied between 110 °C and 160 °C and had a significant effect on vaccine powder characteristics. It was observed that the outlet air temperature increases linearly with the increase in inlet air temperature. Moreover, this correlation is consistent with observations by Maltesen et al. (2008) who reported linear increase in outlet temperatures while varying the inlet temperature between 75 °C and 220 °C. In the current study, the maximum outlet temperature observed was 91 °C. This is below the theoretical glass transition temperature of trehalose (117 °C) (Hinrichs et al., 2001). Furthermore, water is known to be a potent plasticizer for sugar glasses (Hinrichs et al., 2001), which reduces the T_g values and could lead to crystallization of sugar during storage. However, the amorphous nature of the powder vaccine was confirmed by X-ray diffraction (discussed later).

Besides that, an increase of inlet temperature decreases the RMC of the powder. This observation could be attributed to the increased energy available for water evaporation during the drying process. As expected, the model shows that a higher inlet temperature would lead to better drying.

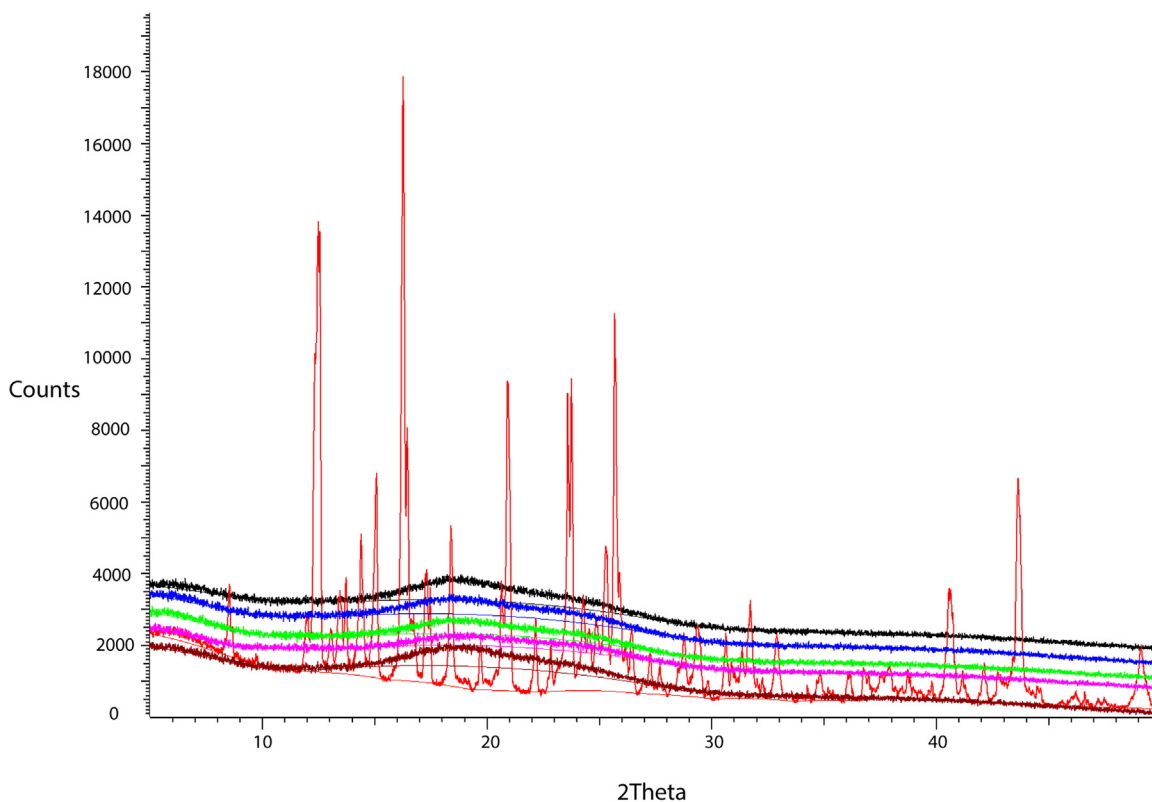


Fig. 6. X-ray diffraction (XRD). The patterns of spray dried vaccine samples in comparison with trehalose dihydrate (red). Vaccine product: black (run1), green (run5), brown (run 10), blue (run11), pink (run22). Trehalose dihydrate, the starting material is crystalline as depicted by the sharp peaks in the figure (red). In contrary, the spray-dried vaccine powder was amorphous with no sharp peaks. The formulations for XRD evaluation were selected based on their residual moisture content and anticipated potential of trehalose to crystallize in the product (predicted Tg vs T outlet). Run 1 and run 5 had RMC in higher range (4–7%) and run10, 11 and 22 had RMC in lower ranges (2–3%).

4.3. Effect of liquid feed flow rate

Increasing the feed flow rate avails more fluid for dispersion per unit of energy available for atomization. This results in insufficient water evaporation and thus higher moisture content. This was observed in our study (experiment 5). Furthermore, increased water available for evaporation leads to a decrease in outlet temperature (Table 6).

In addition, increasing the feed flow rate decreases the powder yield in this model to 42% at highest feed flow rate. However, a decrease in yield with high feed flow rate can be compensated with increasing nozzle pressure as observed in experiment 6 (powder yield: 72%). Thus, it could be inferred that nozzle pressure in combination with feed flow rate is significantly influencing the powder yield.

4.4. Effect of excipient concentration

In the current investigated parameter combinations, the excipient concentration alone did not play a significant role on the product characteristic. However, the excipient concentration significantly influenced the product characteristics when also other process parameter were changed. Trehalose was used in the concentration range of 50 mg/mL–150 mg/mL. In general, increasing the trehalose concentration increases density and decreases porosity of the particles. Furthermore, increasing trehalose concentration may lead to more deposition in the drying chamber and thereby a reduced powder yield (Adler and Lee, 1999). In addition, the concentration and solubility of excipients affects the particle morphology. The precipitation kinetics and possible

crystallization (although not the case in our study) could play an important role and are affected by the rate of evaporation of water during drying (Vehring, 2008; Vehring, 2007).

4.5. Amorphousness of the powder

Spray drying of trehalose resulted in an amorphous powder as observed in several previous studies (Baldinger et al., 2012; Zhu et al., 2014). For several sugars the amorphous glassy state may reduce the molecular mobility to the included compounds such as vaccine antigens (Tonnis et al., 2015). The limited mobility reduces protein aggregation/unfolding and chemical degradation reactions.

Although the reported glass transition temperature of fully amorphous trehalose is 117°C, we observed Tg values as low as 43°C for the formulation with highest moisture content. This reduced Tg is explained by the fact that water is a potent plasticizer for sugar glasses (Hinrichs et al., 2001). However, with the current understanding of the drying process, the RMC can be decreased to an acceptable level (<3%). Moreover, the spray dried vaccine formulations with high RMC stayed amorphous as confirmed by powder XRD. However, it cannot be excluded that during storage for longer time, trehalose might crystallize to some extent.

4.6. Antigenicity and antigen stability

The antigen recovery of influenza vaccine after spray drying was above 86% for all experiments in the investigated design space. Due to limited variation in antigenicity between the different formulations during drying no regression model could be fitted

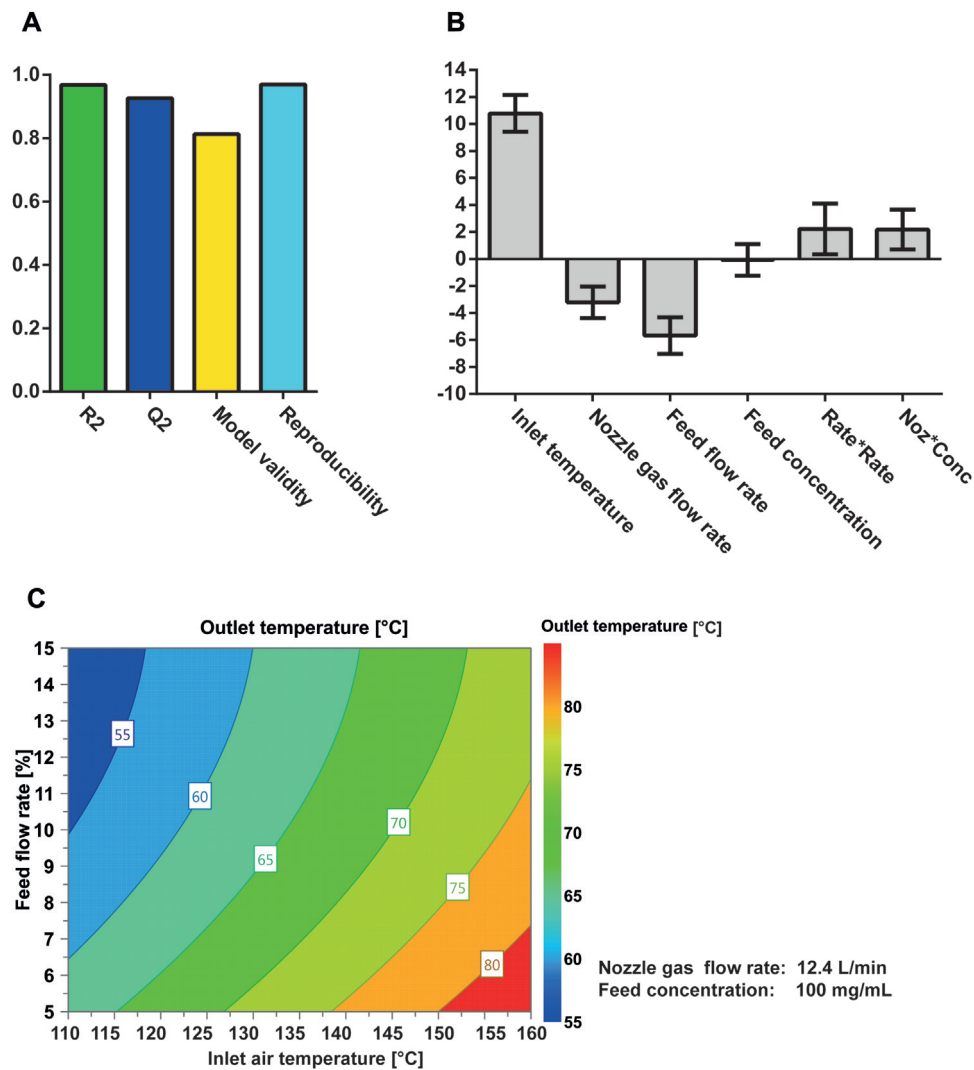


Fig. 7. Regression model of outlet temperature. A: Summary of fit plot for residual outlet temperatures. R^2 (Goodness of fit = 0.968). Q^2 (Goodness of prediction = 0.926). B: Regression coefficients of outlet temperatures after model refinement. C: Response contour plots for outlet temperature. The effect of the two most influential factors (as observed from Fig B) was taken into account and other factors were kept constant. The color regions represent the predicted response (outlet temperature) for defined parameter settings. Aspirator was set at maximum instrument setting.

on the data. Thus, the influence of the investigated process parameters on antigenic recovery could not be assessed. Trehalose was a suitable excipient to stabilize influenza vaccine during and after the spray drying process. The powder formulations remained stable for 3 months of storage at 60 °C, on the other hand liquid WIV stored at 60 °C completely lost its activity within 5 days of storage. While this is the first report on the stability of spray dried WIV vaccine, other studies have shown that spray dried influenza subunit antigens remain stable at elevated temperatures when trehalose was used as the main excipient. [Zhu et al. \(2014\)](#) observed trehalose containing influenza vaccine formulation protected the antigen during storage at 50 °C for 2 months. Furthermore, [Sou et al. \(2015\)](#) showed that trehalose and leucine containing influenza vaccine formulations had stable HA titers after being stored at 40 °C for 2 months. Other drying methods such as freeze-drying ([Soema et al., 2014](#)), spray freeze drying ([Lundstedt et al., 1998](#)) and foam drying ([Maury et al., 2005](#)) have also yielded stable dried influenza vaccines. Besides this, the potential of spray dried influenza vaccines for pulmonary administration has been demonstrated by several previous preclinical studies ([Saluja et al., 2010](#); [Scherliess et al., 2014](#); [Sou et al., 2015](#)).

4.7. Prediction of spray-drying process parameter combinations for a specific Quality Target Product Profile

The acquired model predicted particle size, outlet temperature, moisture content and powder yield accurately for the dried vaccine within the obtained design space. Formulation A, chosen from the sweet spot (Inlet temperature 120 °C, Nozzle gas flow rate 12.4 L/min, feed flow rate of 1.0 mL/min and trehalose concentration of 100 mg/mL) resulted in a product with the desired quality product profile (QTPP). Even with varying the settings of nozzle gas flow rate (experiment B and C to 10.5 and 9.0 L/min respectively) outside the sweet spot region but within the predicted design space resulted in a product within the defined target product profile. The only exception observed was for experiment C, where the RMC varied from the predicted value. It could be the case, that handling of powder during collection and analysis contributed to this variation. Although, the residual moisture content of all the produced powders was less than 3%, which is the upper-limit for dried biologicals in European Pharmacopoeia ([May et al., 1992](#)). This indicates towards a well-defined design space and good prediction abilities of the obtained model. One can presume that any other experiments performed within the experimental

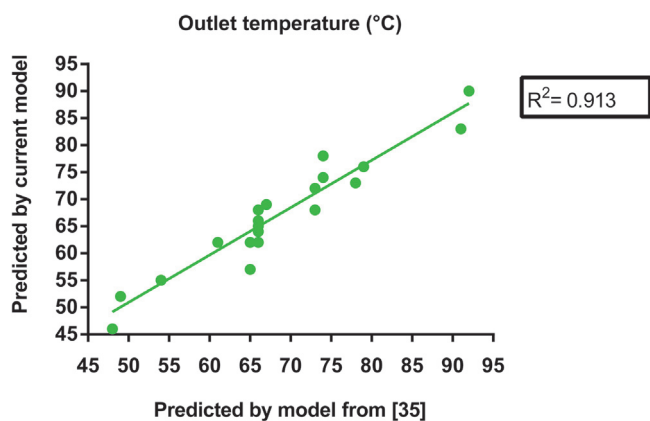


Fig. 8. Predicted values for outlet temperatures by two different models. The values predicted from our model in comparison with values predicted from the model by Grasmeyer et al. (Grasmeyer, 2013).

boundaries of the design space would accurately predict the product characteristics. Furthermore, the WIV vaccine antigenicity remained unaffected during drying and subsequent reconstitution within the current design. The stability of WIV outside the current spray drying design space cannot be predicted and might be different outside the design space. At this stage, we cannot model the antigenicity of the vaccine because of limited variation in antigenicity between the different formulations; a further expansion of the design space to more extreme spray drying conditions may induce differences in antigenicity. However, it is debatable if such extreme conditions would ever be used in an actual production setting. Furthermore, a good correlation was found between the outlet temperature predicted by a mathematical model from Grasmeyer et al. and the outlet temperature predicted by model described in the current study (Fig. 8, $R^2=0.91$) (Grasmeyer, 2013). This combination of findings provides some conceptual premise that could be applied for regulating the physical powder characteristics during spray drying for other vaccine candidates. The biological/antigenic stability these

candidate vaccines might vary depending on their ability to withstand extremes of spray drying conditions. One needs to accurately assess this by performing spray drying runs including these individual vaccine candidates.

In conclusion, the current study successfully demonstrates the application of QbD principles and the DoE approach in the development of a dry powder influenza vaccine formulation. This approach provided an overview of the impact of process parameters that affect the spray dried vaccine product characteristics as summarized in Table 6. The approach gave a descriptive model of a design space in which trehalose based WIV vaccine powder with a product profile of particle size 1–5 μm , powder yield above 70% and residual moisture content below 3% were produced using Büchi mini spray-drier B-290. The produced spray dried influenza vaccine contained WIV that retained its antigenicity, and was thermostable for several months upon storage at 60 °C. Finally, the descriptive model was suitable to define and subsequently select process settings to produce a vaccine powder with predefined characteristics, as confirmed by experiments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijpharm.2016.08.022>.

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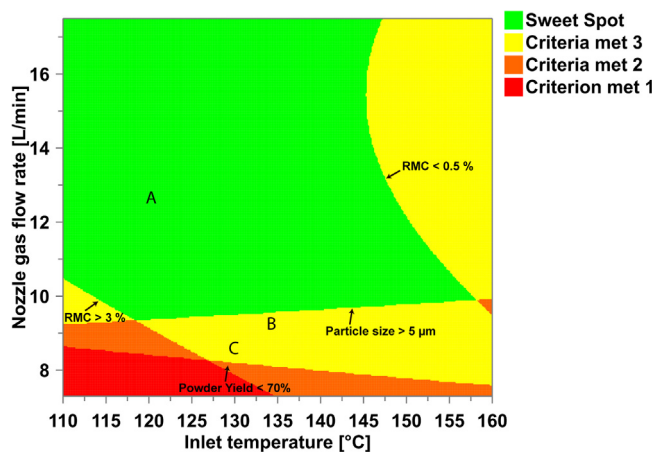


Fig. 9. The predicted design space. The parameters feed flow rate at 1.0 mL/min and feed excipient concentration at 100 mg/mL were fixed for the predicted design space. The criteria were set for particle size range of 1–5 μm , the outlet temperature below 100 °C, residual moisture content between 1 and 3% and the process yield above 70%. The criteria for powder vaccine were set in accordance with QTTP. Color code: Red (one criteria met), orange (two criteria met), yellow (three criteria met), green (all criteria met/sweet spot). The feed concentration and feed flow rate for the current design were predicted as 100 mg/mL and 1.0 mL/min respectively. Points A, B and C represent the spray drying runs that were performed to confirm the prediction abilities of the model. In all experiments aspirator was set at maximum equipment setting.

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