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NEAR-INFRARED SPECTROSCOPY TO DETERMINE RESIDUAL MOISTURE IN FREEZE-DRIED PRODUCTS: MODEL GENERATION BY STATISTICAL DESIGN OF EXPERIMENTS

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Abstract. Moisture content (MC) is a critical quality attribute of lyophilized biopharmaceuticals and can be determined by near-infrared (NIR) spectroscopy as nondestructive alternative to Karl-Fischer titration. In this study, we create NIR models to determine MC in mAb lyophilisates by use of statistical design of experiments (DoE) and multivariate data analysis. We varied the composition of the formulation as well as lyophilization parameters covering a large range of representative conditions, which is commonly referred to as "robustness testing" according to quality-by-design concepts. We applied principles of chemometrics with partial least squares and principal component analysis. The NIR model excluded samples with complete collapse and MC > 6%. The 2 main components in the principal component analysis were MC (91%) and protein:sugar ratio (6%). The third component amounted to only 3% and remained unspecified but may include variations in process parameters and cake structure. In contrast to traditional approaches for NIR model creation, the DoE-based model can be used to monitor MC during drug product development work including scale-up, and transfer without the need to update the NIR model if protein:sugar ratio and MC stays within the tested limits and cake structure remains macroscopically intact. The use of the DoE approach and multivariate data analysis ensures product consistency and improves understanding of the manufacturing process.

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Abbreviations used: ADC, antibody drug conjugate; DoE, design of experiments; DP, drug product; EMA, European medicines agency; NIR, near-infrared; mAb, monoclonal antibody; MC, moisture content; MVDA, multivariate data analysis; NIPALS, nonlinear iterative partial least squares; PAT, process analytical technology; PCA, principal component analysis; PLS, partial least squares; QbD, quality by design; SEC/P, standard error of C ¼ calibration, P ¼ prediction; RMSEC/P/CV, root mean square error of C ¼ calibration, P ¼ prediction, CV ¼ cross validation; RPD, ratio of performance deviation; RER, range error ratio; SEM, scanning electron microscopy; SNV, standard normal variate.

Conflicts of interest: None.

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Introduction

Freeze-drying, also called lyophilization, is a well-established manufacturing process for parenteral drug product (DP) formulations. Lyophilization is used for many biological entities or thermo-sensitive

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molecules as a last step of DP manufacturing process to gently remove water from the product. This stabilizes the molecule chemically and physically while maintaining bioactivity. Because freeze-drying is relatively complex, time-consuming, and expensive, it is often used as a last resort if the active substance is unstable.[1-3] Residual moisture content (MC)constitutes an essential quality attribute as lyophilisates can be highly hygroscopic.[4,5] In general, MC should be as low as possible to avoid chemical and physical degradation of the product. However, below a certain MC threshold, degradation increases again and aggregation of the protein will start.[6-11]

MC can be determined by Karl-Fischer titration, which is a destructive, low-throughput method. Alternatively, MC can be determined by involving vibrational spectroscopy. Raman and near-infrared (NIR) spectroscopy have repeatedly proved suitable.[12-16] Especially, NIR spectroscopy offers a fast, reliable, and nondestructive technique[17,18] if adequate models have been established.[19-25] Because NIR spectroscopy can be used as a high-throughput method, it is in particular used as process analytical technology to assess and monitor moisture distribution of freeze dried materials and especially applied for in-line determination of MC during manufacture.[26-29] As adequate models are a prerequisite for the application of NIR, chemometrics principles as statistical tools are an essential part. Consequently, multivariate modeling for model generation using partial least squares (PLS),[30,31] main and interactions of individual principal components regression,[32] or spectral peak area analysis[33] are broadly used for MC determination by NIR spectroscopy. In this regard, the European Medicines Agency (EMA) established a guideline for the pharmaceutical industry for the use of NIR spectroscopy. The guideline provides data requirements and regulatory guidance for marketing authorization including development, calibration, and validation when used with chemometrics statistics for qualitative and quantitative analysis and in process analytical technology applications.[34]

Traditionally, NIR models during development of freeze-dried products are amended and updated by including increasing numbers of samples and samples with variable composition whose MC is verified by a reference method such as Karl-Fischer titration. This normally involves the testing of formulation variation at stable target process conditions or variation in process parameters at stable target formulation composition. In contrast to the traditional approach, we opted for a statistical design of experiments (DoE) approach to establish an NIR model based on inclusion of a large range of representative formulation variables and process parameters. Using this procedure, we pursued a robust NIR model, which does not need to be updated in response to each process variation during development. However, the prerequisite is to study the interplay between formulation and process parameters and resulting effects on MC because NIR spectra of samples with different MC are confounded by the composition of the formulation. While keeping freeze-drying process parameters constant, Lin and Hsu[19] for example studied the effect of buffer, surfactant, protein, and disaccharide concentration for different proteins highlighting the alteration of the NIR spectra. Grohganz and colleagues[35] reported the influence of various ratios of mannitol and sucrose on the NIR spectra as well as of other additives such as sodium chloride, calcium chloride, and histidine.

We aimed to assess the robustness of an NIR model to determine residual MC of mAb formulations. The initial NIR model was calibrated and tested for target conditions at various levels of residual MC obtained at different time points of a target freeze-drying cycle. We amended the model by including

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samples whose formulation covered a large range of representative compositions and by applying a large range of lyophilization process parameters, using a full-factorial design of experiments (DoE). Samples with protein concentrations between 20 and 30 mg/mL and sucrose concentration between 120 and 240 mM were prepared. The process parameters of the freeze-drying cycle that were varied comprised pressure (67-200 µbar), temperature (-20°C to +30°C), and cycle time. We estimated the reliability of the NIR model to predict residual MC by using PLS regression. Principal component analysis (PCA) was used to identify main variables of the NIR model and to visualize samples included in the NIR model.

Materials and Methods

FORMULATIONS

The test protein, mAb1 (IgG1, Mw=~148kDa), was provided by F. Hoffmann-La Roche (Basel, Switzerland) and was formulated in 20 mM histidine-histidine hydrochloride buffer pH 6.0 (Ajinomoto, Louvain-la-Neuve, Belgium) with the addition of 0.02% polysorbate 20 (Croda, Snaith, UK). A sample set was designed according to DoE principles covering a large range of representative formulation compositions: formulations containing protein concentrations of 20,25(target), and30 mg/m Landsucrose (FerroPfanstiehl,IL)levels of 120, 200, 220 (target), and 240 mM were prepared. Aliquots (10.6 mL)of the formulations were filled into 20mL transparent glass vials (Schott AG, Müllheim, Germany) and stoppered with teflonized D777-1 lyophilization stoppers (Daikyo, Tokyo, Japan).

LYOPHILIZATION

Samples were lyophilized on a Lyostar™ II (FTS Systems SP Scientific, Stone Ridge, NY). The freeze-drying process was monitored by Pirani and MKS pressure gauges, and product temperature was monitored by thermocouples.

For creation and testing of the NIR model, 80 vials of the intended formulation, which is referred to as "target formulation" in the following manuscript (i.e., 25 mg/mL protein and 220 mM sucrose), were lyophilized on the middle shelf of the lyophilizer at target freeze-drying conditions (Table 1). Primary and secondary drying temperatures were set to +5°C and 25°C, respectively, and vials were distributed across the shelf to cover middle, edge, and corner positions of the metal tray, and were randomized afterward for analysis. The remaining space on the tray was filled with placebo vials. We conducted 4 freeze-drying runs at target conditions but varying cycle times to achieve a broad range of MC, that is, stoppering of the vials after (a) when Pirani signal equaled MKS signal during primary drying, (b) at the end of primary drying, (c) when Pirani signal equaled MKS signal during secondary drying, and (d) at the end of secondary drying (Fig. 1).

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Table 1
Process Parameters of Target Freeze-Drying Process

Step	Temperature [°C]	Pressure [µbar]	Time [h]
Equilibration	+5	atm.	2
Freezing ramp	+5 to −40 with 1K/min	atm.	0.75
Holding time	−40	atm.	3
Vacuum	−40	133	–
Ramp to primary drying	−40 to +5 with 0.5K/min	133	1.5
Primary drying	+5	133	48
Ramp to secondary drying	+5 to +25 with 0.2 K/min	133	1.7
Secondary drying	+25	133	8
Stoppering	+25	760	–
Unloading	+25	atm.	–

atm., atmospheric pressure.

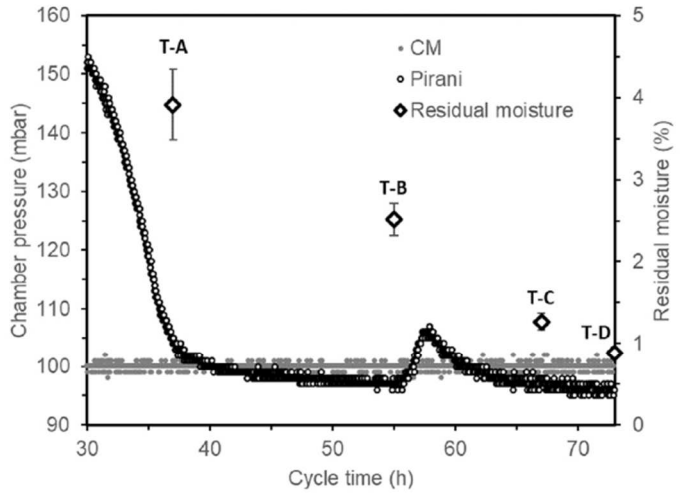

Figure 1. Pressure monitoring by MKS and Pirani pressure gauges and corresponding residual moisture levels measured by Karl-Fischer titration for target formulation and target lyophilization conditions at different lengths of the freeze-drying cycle. Stoppering for residual moisture determination was performed after (T-A) Pirani signals equaling MKS signal during primary drying, (T-B) end of primary drying, (T-C) Pirani signal equaling MKS signal during secondary drying, and (T-D) at the end of secondary drying.

In accordance with DoE principles, we conducted further lyophilization runs with the samples of different formulation composition (protein and sucrose) using freeze-drying parameters covering a large range of representative lyophilization process parameter settings (Table 2 and Table S-1 in the Supporting Information). A subset of samples was retained for stability testing at 5, 25, and 40°C for up to 13 months.

Table 2
Parameter Settings of Full-Factorial Design of Experiments

Parameter	Quantitative	Qualitative	Target	Minimum	Maximum	Additional
Protein conc. (mg/mL)	x	–	25	20	30	–
Sucrose conc. (mM)	x	–	220	200	240	120
Freeze-drying parameter[a]	–	x	5°C/25°C/133 µbar	−20°C/25°C/67 µbar	30°C/40°C/200 µbar	–
Primary and secondary drying times	–	x	48 h/8 h	Pirani = MKS signal/0 h	Pirani = MKS signal/8 h	–

[a] Primary drying temperature (°C)/secondary drying temperature (°C)/pressure (µbar).

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VISUAL INSPECTION

All freeze-dried samples were examined visually for cake defects such as shrinkage, cracks, meltback, or collapse.

SCANNING ELECTRON MICROSCOPY

We applied scanning electron microscopy (SEM; FEI, Grafel€ fing, Germany) to study the morphological properties of the lyophilized cake. The secondary electron detector, Everhart-Thornley detector, under high vacuum was used for image acquisition with an acceleration tension of 2 or 3 kV. Gold sputtering (for 120 s at an intensity of 50 mA) of the samples using a Leica EM SCD050 device (Leica, Heerbrugg, Switzerland) ensured good conductivity.

KARL-FISCHER TITRATION

Residual moisture was determined by coulometric Karl-Fischer titration using C30 Titrator from Mettler Toledo (Greifensee, Switzerland). Before titration, the complete vial content was subjected to extraction with anhydrous methanol (99.8%, Sigma Aldrich, Steinheim, Germany), and samples were vortexed and placed on a shaker for up to 3 h to ensure complete release of the entrapped water. Residual MC was expressed as the percentage of total solid content.

NEAR-IN FRARED SPECTROSCOPY

Freeze-dried samples were subjected to NIR spectroscopy using an Antaris Fourier Transform spectrometer (Thermo Fisher Scientific®, Madison, WI). The instrument was equipped with an InGaAs detector, Michelson interferometer, halogen NIR source, and an integrating sphere module. NIR spectra were acquired in diffuse reflection with 32 scans and a resolution of 8 cm^{1}, a gain of 1, and absence of an attenuator. Spectral range was 4000 to 10,000 cm^{1}, and a background spectrum was acquired every hour. Each sample was measured once through the bottom of the glass vial.

CHEMOMETRICS AND DATA ANALYSIS

DATA PREPROCESSING

NIR spectra were mathematically preprocessed by using standard normal variate (SNV) transformation[36] coupled with a first-derivative Norris Gap size of 7[37] before computation of the classification and regression models. This step corrects the effects unrelated to residual MC. During chemometric analysis, data were mean-centered to ensure interpretable results in terms of variance around the mean. Subsequently, nonlinear iterative partial least squares algorithm was applied.

PRINCIPAL COMPONENT ANALYSIS

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PCA is a multivariate data analysis tool widely used in the field of NIR spectroscopy for data visualization and unsupervised evaluation of experimental data.[38] PCA was applied to NIR preprocessed spectra and was further used to visualize the samples included in the NIR model based on the tested formulations and process parameters.

QUANTITATIVE ANALYSIS BY PARTIAL LEAST SQUARES

PLS analysis is a common regression approach for the creation of quantitative NIR models.[25] PLS was applied by using full cross-validation by nonlinear iterative partial least squares algorithm after preprocessing of the NIR spectra. We selected 4 PLS factors. The selection of the optimal number of components has been conducted for each model independently and based on the evolution of the root mean square error of cross-validation for each model.

MODEL CREATION

Three models were initially created based on a calibration set and a test set, which is referred to as "internal" validation according to EMA guidance[34] to check the performance of the model. Model no. 1 was based on 4 target runs using the target formulation and target process parameters but variable cycle lengths (Table S-2). We selected 28 vials for the calibration set and 12 vials for the test set. For the run that yielded the highest values of MC, we used 29 vials in the calibration set and 11 vials in the test set. The vial that contained the highest MC was included in the calibration set. "External validation" of the model according to EMA guidance[34] formally applying for market authorization only, which requires independent data sets, was not in scope of the present study. We further assessed whether our model still applied if process parameters and the composition of samples were modified. Model no. 2 was based on runs conducted at target conditions and all DoE runs except those without secondary drying and conservative cycle parameters (runs no. 1, 5, 9, and 13). The DoE runs comprised samples of different formulation composition (protein and sucrose) freeze-dried by covering a large range of representative lyophilization process parameter settings (Table 2 and Table S-1 in the Supporting Information). Lyophilization runs without secondary drying resulted in samples with high and variable MC and were thus excluded from the model. Consequently, calibration model no. 1 evolved into model no. 2. Three spectra of each run were used for the calibration set. The remaining 2 spectra of each run were added to the test set.

Model no. 3 was created by including all potential variabilities. For this, calibration model no. 2 was extended by 3 spectra of runs no. 1, 5, 9, and 13, which were the conservative lyophilization runs without secondary drying. The remaining 2 spectra of each run (no. 1, 5, 9, and 13) were added to the test set.

COMPARISON OF MODELS

Besides models no 1-3, we compared statistical means, for example, root mean square error (RMSE) of total 9 NIR models as summarized in the Supporting Information (Table S-2): we created 6 models in addition to models no. 1, 2, and 3 by including batch variability into the calibration and test sets (models no. 4-9). This means that calibration and test sets were completely independent as

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summarized in Table S-2 in the Supporting Information. The statistical results of these models highlight the importance of run selection when they are not embedded in the calibration set. For the 6 models, runs no. 1, 5, 9, and 13 (without secondary drying; conservative drying) were excluded from calibration and test sets.

STATISTICAL EVALUATION OF THE MODELS

In line with Clavaud et al.,[24] we determined the following parameters for each PLS model: slope, bias, intercept, R-square, standard error of calibration (SEC), standard error of prediction (SEP), root mean square error of calibration (RMSEC), root mean square error of prediction (RMSEP), ratio of performance deviation (RPD), and range error ratio (RER). Slope and intercept should be close to 1 and 0, respectively. A reliable model should yield an R-square close to 1 and RPD and RER values higher than 8 and 10, respectively. Ideally, RMSEC/SEC and RMSEP/SEP should be low, and bias values should be close to 0. In addition, the difference between RMSEC/SEC and RMSEP/SEP ratios should be small to avoid overfitting effects.

SOFTWARE

The "Result NIR Suite 3" package (Thermo Fisher Scientific®) with Result Integration software was used for NIR acquisition. Preprocessing and chemometrics were computed with Unscrambler 10.5 (CAMOs Software AS, Oslo, Norway). Statistical graphics were generated with RStudio version 3.2.3 and OriginLab 7.5 (OriginLab Corporation, Northampton, MA).

Results

NIR SPECTRA AND VISUAL INSPECTION

We evaluated the SNV-transformed NIR spectra of the 20 DoE runs and the 4 target runs. Figure 2a shows example spectra of samples with low (~1%) and high (~6%) values of MC as well as spectra of samples with the lowest sucrose concentration (120 mM). The detailed view (wavelength of approx. 4700 cm^{1}) corresponds to the OH band for sucrose[39] thus being characteristic for the sucrose concentrations of the various formulations. At a wave number of approx. 5150 cm^{1}, the combination band of OH stretching and HOH bending was observed. The first overtone of OH stretch was detected at a wave number of approx. 6900 cm^{1}. Both the combination band and the first overtone (see boxes 2 and 3 in Fig. 2a) are specific to the moisture bands. Because the aim of the study was to evaluate the impact of formulation modifications and alterations of process parameters on model robustness, the full-spectrum approach over the range of 4000-10,000 cm^{1} including regions sensitive to excipients was used for subsequent chemometric analysis using maximum spectral information. Further preprocessing of the SNV-NIR spectra by using the first derivative confirmed the sensitivity to excipient concentration in these regions.

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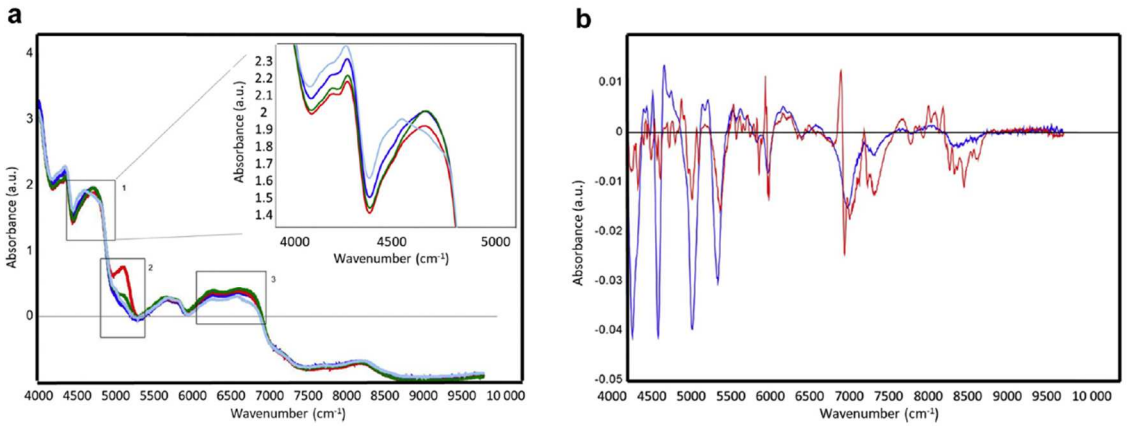

Figure 2. (a) Example of NIR spectra after SNV preprocessing for low-moisture samples (1%, dark blue line) and high-moisture samples (6%, red line) highlighting moisture-sensitive band around 5150 cm^{-1} (box no. 2) and 6900 cm^{-1} (box no. 3). The spectra also show samples with lower sucrose content (120 mM, pale-blue line) and samples with higher sucrose content (240 mM, other colors) showing adsorption bands around 4700 cm^{-1} sensitive to sucrose concentration (box no. 1). The red spectrum corresponds to the target run completed after primary drying (T-C), the dark blue spectrum to the completed target run (T-D), and the light-blue spectrum to the experimental DoE run no. 20. The green line corresponds to samples from DoE run no. 2. (b) Example of first derivative of SNV preprocessed NIR spectra of a collapsed sample (red line) in comparison to an intact sample (blue line). The red spectrum corresponds to a stability sample from DoE run no. 1 stored at 40°C for 13 weeks, and the blue spectrum corresponds to an intact sample of the target run (T-D).

In general, lyophilized samples appeared uniform and "elegant" without any macroscopic defects. So-called "dents" were irregularly observed in the bottom region of the vials that are inherent to sucrose formulations. Dents are characterized by slightly shrunken regions where cake has pulled away from the inner vial surface. However, for these samples, dents were classified as minor and solely observed at the edges of the vial. Only major dents interfering with the measuring position would be critical for subsequent data interpretation and NIR model robustness. NIR spectra of samples analyzed immediately after lyophilization (within 24 h) did not differ from those obtained 1 week after lyophilization. Some samples exhibited severe collapse of the lyophilized cake after storage at 40°C already after 1 month. These samples were produced by the conservative method without secondary drying and thus exhibited high MC. As water acts as plasticizer here, the samples were most likely stored above their glass transition temperature. Figure S-1 (Supporting Information) provides photographs of cake forms, that is, an elegant cake and a severely collapsed cake after storage at 40°C for 4 weeks. Cake collapse was readily identified on visual inspection and was confirmed by NIR spectra. Lyophilized cake structures that are impaired, for example, broken or melted with the product pulled away from the vial inner surface, directly and negatively impact data robustness. However, these samples can be directly identified by visual inspection beforehand. Thus, the mentioned samples were not used for model creation but were retained for moisture prediction in a second step (negative example). Figure 2b shows the first derivative of an NIR spectrum after SNV preprocessing of a collapsed cake, clearly indicating the loss of detail in the spectrum compared to the spectrum that results from a uniform and elegant cake.

PRINCIPAL COMPONENT ANALYSIS

The predictive power of an NIR model is defined by the selection of samples included in the calibration model. We developed a model that encompassed data points from a large range of representative formulation as well as lyophilization process parameters. Using PCA as a multivariate

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data analysis tool, the various parameters affecting MC as well as the samples included in the model can be visualized, and unsupervised data can be evaluated. Figure 3a shows the PCA plot including the initial target runs (i.e., runs T-A to T-D) and the DoE runs (i.e., runs no. 1 to 20). The plot shows that 97% of the variability in the data set is explained by the 2 first principle components (PCs). The first PC was residual MC associated with a variability of 91%, whereas the second PC (6%) represented the variability in sucrose concentration, more precisely protein:sugar ratio. Figure S-2 in the Supporting Information further highlights this dependency with increasing protein:sugar ratio for PC2 from left to the right. Figure 3b shows the PCA plot of the second and third PCs, clearly differentiating between formulation variables. The remaining variability of 3% (PC3) and of a fourth PC (1%, data not shown) was not specified but was thought to include the influence of aggressive and conservative cycle parameters (i.e., drying pressure and cycle time). These parameters could potentially influence cake structure of the samples. However, SEM pictures obtained for selected samples to study pore structure of the samples as a result of changes in cycle parameters (data not shown) could not be clearly linked to chemometrics results.

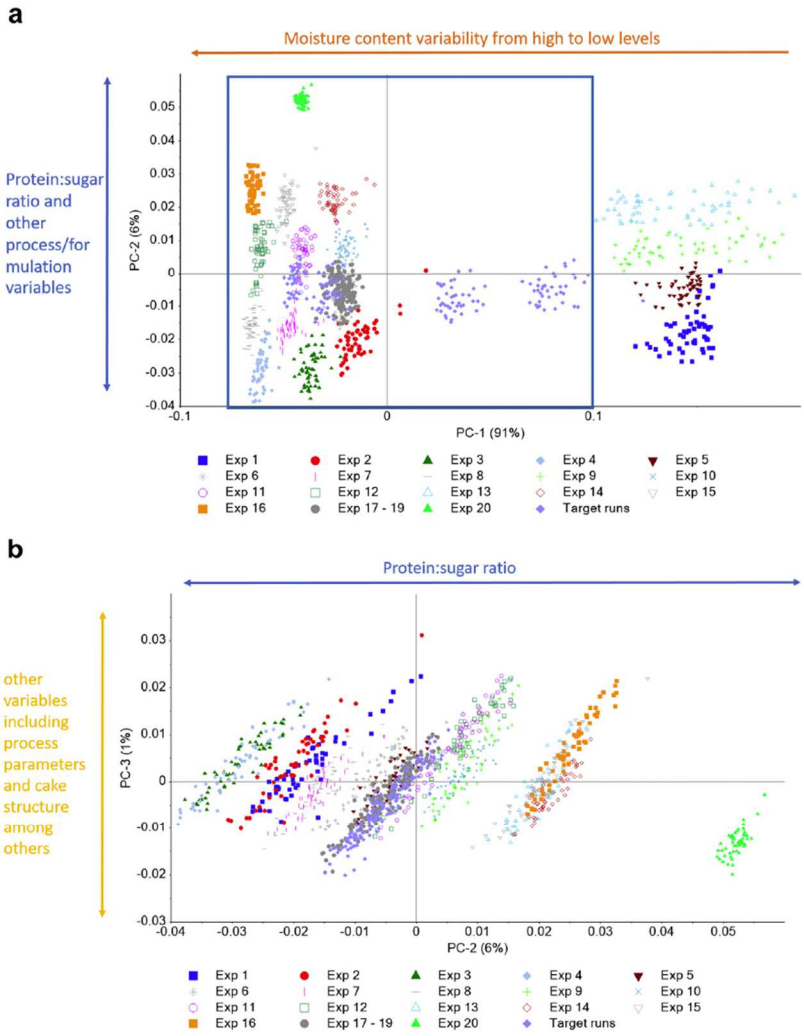

Figure 3. PCA plot visualizing (a) the effect of PC 1, representing residual moisture, compared to PC 2, (b) PC 2, representing the effect of protein:sugar ratio, compared to PC 3. The blue square in Figure 3a represents samples selected for the NIR regression model no. 2, which excludes samples with a moisture content of >6.35%.

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Homogeneity distribution of the experiments on the PCA plot is of interest. For all experiments with low residual MC (i.e., left side of the PCA, Fig. 3a), the data were grouped in homogenous clusters, whereas forexperimentswith high residual MC,sample distribution was heterogenous. In particular, broad clusters were observed for runs no. 9 and 13, suggesting largest MC variability. This finding is in line with the experimental conditions of runs no. 9 and 13 involving conservative drying at -20°C not followed by secondary drying.

Interestingly, we observed a relationship between run no. 6 and runs no. 17 to 19 (i.e., center points). The 4 runs presented similar profiles because they were located in the same area on the PCA plot (Fig. 3a). At 20% less protein, 10% less sucrose, 50% lower drying pressure, and a reduced manufacturing time, run no. 6 yielded MC similar to that achieved at target conditions. This finding may indicate based on similar moisture distribution that sublimation and desorption, which are linked to product resistance, may be similar, which may serve as a basis to conclude on how to adapt process parameter and formulation composition for future products. The blue square in Figure 3a represents samples that were finally selected for our NIR regression model, which excludes samples with a moisture content of >6.35% as elaborated in the following. Most importantly, the PCA plot allows us to identify future formulation/process conditions of interest before performing additional experiments or analytics.

NIR MODEL DEVELOPMENT

The process of lyophilization involves freezing, sublimation, and desorption with decreasing residual MC during each step. To obtain a broad range of moisture levels for NIR model calibration, we first varied the cycle length of samples manufactured at target lyophilization conditions. These samples were used for generation of the first model no. 1. Figure 1 indicates the loss of MC dependent on cycle time and highlights the relationship between pressure monitored by Pirani and MKS pressure sensors to confirm a good parameterization of the freeze-drying processes. We subsequently established 2 additional NIR models as also described in the Methods section: Whereas model no. 1 consisted of samples from the target runs at target composition but varied MC (equivalent to center points), model no. 3 included the target samples plus the DoE samples whose formulation and process parameters represent a large range of conditions (i.e., protein concentrations between 20 and 30 mg/mL, sucrose concentration between 120 and 240 mM, freeze-drying cycle pressure of 67-200 µbar, primary drying temperature of -20 to +30°C, and different cycle time; Tables 2 and S-1). However, model no. 2 proved most appropriate because it included a large range of composition and process modifications but specifically excluded the samples with highest MC (>6.35%) that were generated by the conservative lyophilization cycles excluding secondary drying.

NIR models were generated based on the first derivative of SNV preprocessed spectra. Cross-validation was used to determine the optimal number of components for the PLS analysis: Figure S-3 shows the residual variance (a) and the RMSE values (b) of cross-validated versus calibrated values indicating a plateauing value of the first one for more than 4 factors while the latter decreases. This implies that adding more factors to the model will not further decrease the residual variance or the RMSE to improve the model (overfitting) and justifies the use of 4 factors. PLS loading plots obtained

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for 4 factors are provided in Figures S-4a-d exemplarily for model no. 2. Plot (a) shows the loading plot for PC-1 that is linked to moisture indicated by water peaks at wave number at approx. 5150 cm^{1} and approx. 6900 cm^{1}. PC-2 (b) is mainly linked to protein:sugar ratio (and water) indicated by the sucrose peak around 4700 cm^{1}. A third and fourth component as justified by model generation and the loading plots (Figures S-4c and d) could not be linked to a single product attribute. However, we hypothesize that PC-3 is attributed to solid-state properties of the lyophilisate due to differences in the lyophilization process even if PC-3 includes information of moisture, sucrose, and other ones. Especially for the loading plot of 4 factors (D), noise effect starts in the region 7500-10,000 cm^{1}. Spectral information is clearly analyzable in the region 4000-7500 cm^{1} with limited noise effect and justifies the use of 4 PCs. Adding a fifth factor would include higher noise in loading plots (data not provided) implying that 4 factors are the best compromise.

Figure 4 compares the MC values obtained by Karl-Fischer titration with those predicted by NIR spectroscopy for the calibration and test sets of the 3 models and Table 3 summarizes the corresponding statistical results.

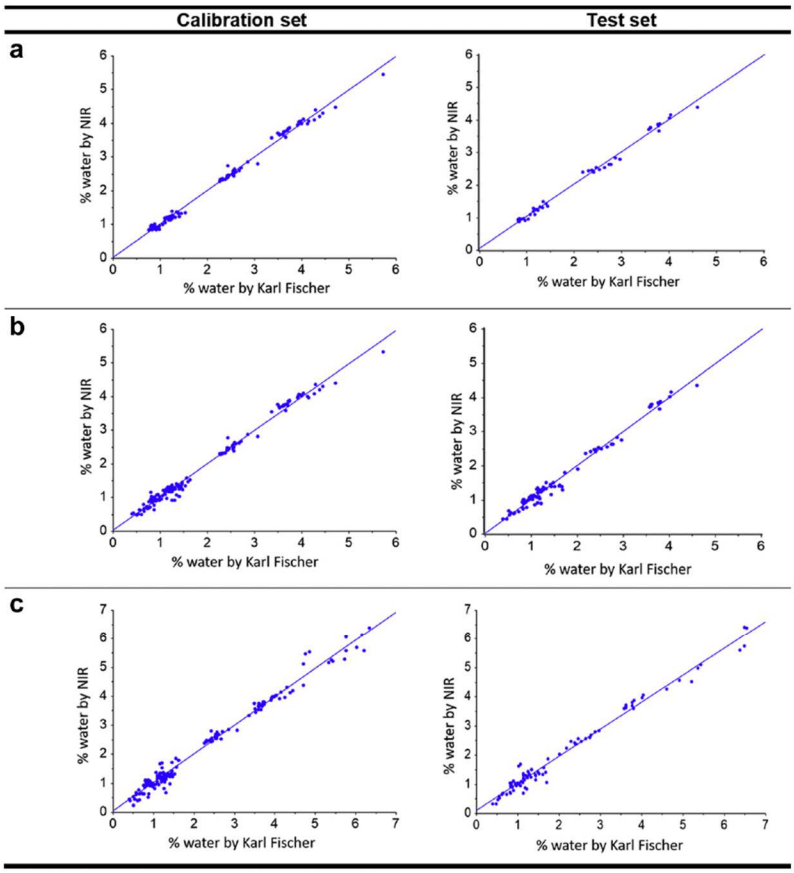

Figure 4. Correlation between residual MC measured by KF and predicted by NIR for calibration and test sets for (a) model no. 1 obtained with the 4 target cycles with different cycle lengths, (b) model no. 2 obtained with the target cycles and the 16 DoE runs (excluding conservative runs without secondary drying), (c) model no. 3 obtained with target cycles and the 20 DoE runs. Statistical parameters are summarized in Table 3.

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Model performances were first evaluated on the basis of RPD and RER values. An RPD higher than 8 was determined for models no.1 and 2 indicating that both models are suitable for quantitative studies.[40] The RER values obtained confirmed that both models exhibited high predictive performances. RPD and RER values for model no. 3 were lower than for the first 2 models, indicating that precision of this model was not sufficient. The additional statistical variables computed, such as slope, intercept, R-square, SEC, SEP, and residues values, predicted good performance for the 3 models (Table 3). Model no. 1 achieved SEC and SEP values of 0.10 and R-square values of 0.99, showing good correlation between NIR and KF values for MC. Intercept values close to 0 and a slope value close to 1 confirmed good correlation of the calibration and test sets, respectively. No bias was observed, thus confirming that there was no systematic error in the model. Similar data were obtained for models no. 2 and 3 but standard errors were higher (i.e., higher SEC and SEP values). To challenge model performance, we assessed the influence of altered composition and process parameters during DoE experimentation on the precision of MC determination. Because RMSE values between calibration and test set for the single models no. 1, 2, and 3 were equivalent (Table 3), variability in the calibration and test sets were concluded to be similar. Nevertheless, RMSE values are different between the models highlighting that samples of different variability originating from differences in formulation and process parameters were included in the 3 models. While model no. 1 was based on target process conditions and model no. 3 used target conditions and included all 20 DoE runs, model no. 2 was the same as model no. 3 with the exception of conservative cycle runs not including secondary drying. Thus, classifying the models according to their range in variability yielded the following ranking: model no. 3 > model no. 2 > model no. 1. Theoretically, larger sample variability results in higher RMSE values. However, model no. 1 that was associated with lowest variability yielded highest RMSEP value of 0.54% (Table 3). This high RMSEP value was a sign of overfitting and indicated that the setup of the calibration set had to be improved.

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Table 3
Statistical Results of Calibration (CAL) and Test (TEST) Sets of the Different NIR Models No. 1-3 Obtained With PLS Algorithm Using 4 Factors Including Results From Prediction of Residual Moisture Content for DoE Runs and Stress Stability Sets

Sample Set	Model No. 1						Model No. 2						Model No. 3					
	CAL	Test	DoE Runs	Stressed Set			CAL	Test	DoE Runs	Stressed Set			CAL	Test	DoE Runs	Stressed Set		
				a	b	c				a	b	c				a	b	c
Number of spectra	113	47	100	238	203	192	**161**	**79**	**100**	**238**	**203**	**192**	173	87	100	238	203	192
Slope (b)	0.99	0.99	0.99	0.86	0.86	0.95	**0.99**	**0.99**	**0.97**	**0.83**	**0.82**	**0.95**	0.98	0.93	0.97	0.89	0.87	1.18
Intercept (a)	0.01	0.05	−0.10	0.30	0.16	0.05	**0.02**	**0.01**	**0.02**	**0.37**	**0.26**	**0.12**	0.03	0.09	0.00	0.45	0.32	−0.04
R^2	0.99	0.99	0.93	0.82	0.94	0.78	**0.99**	**0.98**	**0.94**	**0.85**	**0.95**	**0.88**	0.99	0.98	0.98	0.72	0.86	0.26
RMSEC(P) (RMSECV)	0.10 *(0.11)*	0.10	0.54	0.71	0.24	0.19	**0.12** ***(0.14)***	**0.13**	**0.51**	**0.63**	**0.23**	**0.14**	0.19 *(0.24)*	0.23	0.30	0.88	0.37	0.35
SEC(P)	0.10	0.10	0.53	0.71	0.24	0.19	**0.12**	**0.13**	**0.50**	**0.63**	**0.23**	**0.13**	0.19	0.23	0.29	0.85	0.34	0.30
Ratio SEP/SEC	n.a.	1.00	5.3	7.1	2.4	1.9	**n.a.**	**1.08**	**4.17**	**5.25**	**1.92**	**1.08**	n.a.	1.21	1.53	4.47	1.79	1.58
Bias	0.00	0.03	−0.10	0.03	−0.04	−0.01	**0.00**	**0.00**	**−0.05**	**0.04**	**0.01**	**0.05**	0.00	−0.06	−0.08	0.24	0.14	0.18
RPD	n.a.	12.3	2.32	1.73	5.12	6.47	**n.a.**	**9**	**2.34**	**1.86**	**5.09**	**9.00**	n.a.	6.48	5.14	1.75	4.38	4.97
RER	n.a.	49.9	9.72	7.03	20.79	26.26	**n.a.**	**41**	**10.66**	**8.46**	**23.17**	**41.00**	n.a.	25.83	20.48	6.99	17.47	19.80

n.a., not applicable.
Spectra were preprocessed by SNV and first derivative.
"Model 2" represents in bold.
Italicized values are the RMSECV values.
[a] Including collapse samples observed for runs no. 1, 5, and 9.
[b] Excluding runs no. 1, 5, and 9.
[c] Excluding runs no. 1, 5, 9, and 13.

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Model 2 embeds more variability than model 1, namely all DoE runs (expected DoE runs 1, 5, 9, 13). Compared to model 1, the statistical differences between both models are very close but from a robustnesspointofview,onecanexpectthattherobustnessofmodel 2 in a potential commercial setting will be better than the robustness of model 1. Figure S-5 shows that model 1 and 2 performances are similar. However, the accuracy of the model 2 is superior regarding the stability samples. Only a complete validation will demonstrate that model 2 is suitable for a potential commercial setting, especially when completed with a total error and risk-based approach to assess the validity of the NIR method based on the predictive quality of future results that will be obtained in its routine use.

To mention, we generated additional models (model no. 4-9, Table S-2) besides model number 1-3 that are characterized by the calibration set being completely independent from the test set to highlight the importance of run selection, which need to be included in both the calibration as well as the test set. The idea was to elucidate whether the variability embedded in the calibrations model 4 to 9 could be sufficient to predict the excluded DoE runs with accuracy. Figure S-5 exemplarily compares the RMSE of the 9 calibration models. Highest RMSEP values were, for example, also obtained when predicting the stability samples for models no. 5 and 9, which was a sign of overfitting and indicated that the setup of the calibration set had to be improved. By contrast, for models no. 6-8, the RMSEP value was ± 2 times the RMSEC, but a factor of 2 is usually accepted. The poor predictive ability of models 4 to 9 is attributed to the inclusion of only a subset of the DoE into the calibrations set. These findings additionally confirm and suggest that the type of variability (i.e., type of run) had an important effect on the accuracy of the prediction despite similar RMSEC and root mean square error of cross validation values.

We conclude that model performance can be improved solely by including appropriate samples in the calibration model. This means that in sum, model no 2. proved best compromise in model performance from the 3 models generated based on the different points outlined previously.

NIR spectra collected during the DoE runs for which KF measurements were not performed were interpreted in a subsequent step using model no. 2. Here, we included samples subjected to 1-month stability testing at 5°C, 25°C, and 40°C. In these samples, physical and chemical properties like aggregation (initial: 1.8% ± 0.14, 13 M: 1.9% ± 0.14) or other quality attributes like subvisible particles or charge variants (data not shown) as well as solid-state properties were not significantly different. The predicted results were compared with the average KF values obtained for the same samples (Fig. 5). Most predicted values compared well with the reference values. For runs no. 3, 7, 10, and 11, however, the MC data obtained by KF titration (red dots) were outside the boxplots of the NIR data. The same difference was apparent for run no. 17 (target run). Although samples were randomized, the discrepancy between KF and NIR data might be explained as samples exhibit moisture distribution across the shelf during lyophilization due to radiation effects from the chamber walls with highest moisture values in the middle of the shelf and lowest values at the edges of the shelves. Thus, these results were considered sufficiently accurate and our data suggest that model no. 2 provides good prediction of MC in DoE samples for which KF titration data were unavailable.

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To note, we initially expected poor prediction of MC in samples with low sucrose concentration (run no. 20), but this was not confirmed. Low sucrose concentration had no impact on residual MC prediction by NIR spectroscopy especially if the samples were initially included in the calibration and test data sets.

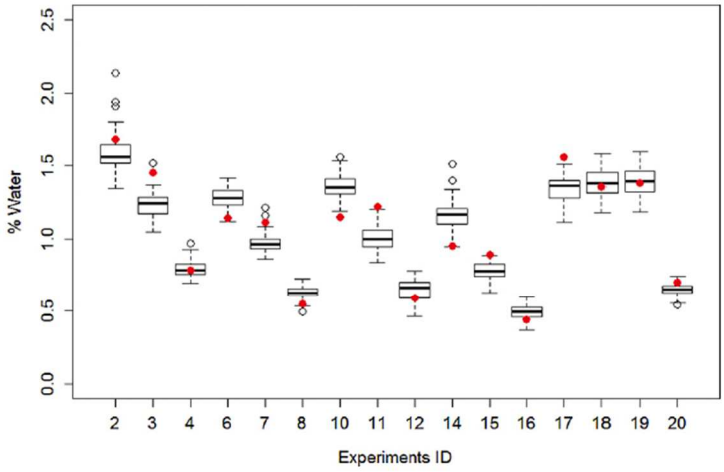

Figure 5. Boxplot of moisture content predicted by NIR spectra using model no. 2. Samples from the DoE runs which were not included in the calibration or test set of model no. 2 compared to average moisture content data from the DoE runs obtained from KF titration (red bullets).

In conclusion, models no. 2 provided the best prediction of MC because they contained the full range of modifications of both composition and process parameters. Nevertheless, collapsed cake structure seen visibly and confirmed by NIR spectra decreased the prediction by up to 0.88% (see model 3; Table 3 and Figure S-5), necessitating the elimination of the samples from analysis.

Discussion

DOE APPROACH AND PCA

In many cases, residual MC is a critical quality attribute of lyophilized DPs as it relates directly to their stability. Thus, MC must be monitored during all DP development stages. Traditional approaches when using NIR for MC determination require a frequent update of the NIR model including only those samples, which have been actually tested. These formulations have a given variability in composition and process parameter and comprise in most cases formulation variation at target freeze-drying conditions or process variability at target formulation. This limits the prediction performance of the NIR model although providing excellent process understanding. The DoE approach, in contrast, offers a resource conscious and fast procedure, aiming to eliminate the need to update the NIR model as development proceeds by covering a broad range of formulation and process conditions. In particular, changes to lyophilization process parameters and their optimization are common during process/product development and may also be required during scale-up and production. Moreover,

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use of another lyophilizer may necessitate adjustment of process parameters because product attributes are affected by the equipment used: lyophilizers tend to differ in size but also in radiation effects from the chamber wall. Thus, lyophilizer-specific properties are likely to influence drying behavior and require adaptation of the freeze-drying cycle and subsequent testing of product attributes. At the same time, these parameters depend on formulation composition, which requires a sophisticated approach when determining MC by NIR spectroscopy.

PCA allowed us to rapidly visualize the experimental values from the designed experiments and highlight those that were included in the NIR model. Consequently, the PCA allows us to identify formulation/process conditions of interest before performing additional analyses such as further DoE experiments or analytics. In our final model (model no. 2), samples from the 4 target cycles and 12 DoEs runs were selected excluding those with MC above 6.35% as visualized in the PCA plot. The PCs identified by PCA were moisture (PC1, 91%) and protein:sugar ratio (6%, PC2). Moisture variability not included in the calibration model interfered with the accuracy. Thus, stability samples that exhibited severe collapse, which also had moisture levels above the included ones into the NIR model, were outside the ranges of the NIR model. Severe collapsed cake structures were also clearly visible in the divergent NIR spectra (see Fig. 2b). While in PCA, the 2 main PCs (moisture content and protein:sugar ratio making up 97% of the variability) were clearly identified, the third PC remained unspecified. This applies also for the fourth PC. However, the remaining contributor to the overall variability was small (3% for PC3 and 1% for PC4) and did not appear to be solely related to the process parameters itself, nor solely related to cake appearance, pore structure (SEM), or amorphous/crystalline structure (data not shown). We hypothesize that neighboring clusters in the PCA plots exhibit similar drying behavior as elaborated in the Results section and may thus serve as a basis for adapting process and composition variables for future products. Ideally, however, a quantitative measure for the relationship between adjacent clusters should be established in further studies.

MANUFACTURE AND SELECTION OF SAMPLES FOR NIR MODELS

Appropriate manufacture of samples is a prerequisite for the establishment of a reliable NIR model. In particular, the relevant range of MC must be covered because the level of residual moisture critically affects the stability of the lyophilized DP. The conventional technique to produce samples with varying moisture levels is posthumidification performed in a laboratory.[23] Posthumidification of the samples can be done in 2 ways, that is, exposure of the samples to a controlled humid environment for a defined time,[36] or by spiking the lyophilized cake with small volumes of water that are absorbed by the lyophilisate overnight.[22] However, such humidification methods have certain limitations. Because the vials have to be opened when water is added, laboratory staff are at a risk of contamination, especially if highly potent products are involved. Moreover, the samples may be cross-contaminated with other substances if the laboratory conditions are inadequate. Thus, such procedures must be well established and validated to ensure reliable levels of residual MC.

Another method to adjust MC is to use varying cycle lengths (secondary drying), which is the method we chose in the present study. As an alternative, vials can be stoppered during the cycle to retain

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moisture. The later approach is based on the assumption that samples exhibit a similar physical structure but variable MC. The cake structure is mainly influenced by the drying temperature and pressure during primary drying and ideally remains almost unchanged during gentle secondary drying.

In this study, we evaluated different NIR models, all of which proved suitable for MC determination in freeze-dried products. However, prediction errors increased for 2 reasons for models covering highest moisture values, like for model no. 9 with 0.41%: First, coulometric Karl-Fischer titration applied to verify MC values is associated with an accurate and precise range up to ~5%-6% MC only. Method variability increases for higher moisture above this limit. Second, highest MC values (as included in model no. 9) were obtained by terminating the lyophilization cycle after primary drying. At this stage of the lyophilization cycle, distribution of MC among the samples is high and only decreases toward the end of secondary drying. In addition, MC depends markedly on the position of the vials on the shelf due to inhomogeneous drying behavior across the shelf especially during this stage of the lyophilization cycle. Thus, it was not useful to integrate all DoE runs in the final regression model.

Besides covering the appropriate moisture range in the NIR model, it is crucial to sensibly vary formulation and process parameters to obtain sufficient variability to be included in the model. In our study, we combined a large, representative range of process parameters with formulation parameters in a full-factorial DoE to obtain the largest variability in terms of sample composition and lyophilization. A common approach is to test the process parameters at a worst case away from intended (so-called "target") conditions, which is commonly referred to as "robustness testing" according to QbD principles. For these experiments, lower chamber pressure (lower heat transfer leading to longer cycles) and lower shelf temperatures compared to target conditions are used at the one end, which are commonly also referred to as "low/ low cycles." These processes eventually challenge sufficiently low residual moisture levels, which are dependent on cycle length in terms of completion of primary and secondary drying steps. Using high shelf temperature and high chamber pressure ("high/high" cycles) on the other end simulates worst-case conditions for cake collapse or cake meltback due to increased heat transfer. For robustness testing of formulation components, the concentration may generally vary within their specification of ± 10%, for example. In particular, the concentration of protein and bulking agent (e.g., sucrose used as lyoprotectant/cryoprotectant) significantly influences product resistance during the freeze-drying process. However, the product resistance also depends on the tested process parameters and linked to the overall cake structure and, finally, to residual MC. Thus, we propose that in addition to incorporating excipient variability into the NIR model, the interplay of product composition and freeze-drying parameters should be studied. Sucrose was used as cryoprotectant/lyoprotectant in this study as it has several advantages over other sugars like trehalose, and crystalline bulking agents like sugar alcohols (e.g., mannitol) when used in protein formulations. While crystalline excipients would be only favored for low protein formulations to improve cake appearance, protein stability would be inferior at protein concentrations typically needed for monoclonal antibodies (mg/mL range) requiring amorphous state of the excipient for optimal stabilization.[41] Trehalose on the other hand would be superior to sucrose because of its higher glass transition temperature; however, dimer formation in frozen drug substance (e.g., at -20°C) poses a severe challenge in drug product development as recently reported.[42] The use of other bulking agents

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than sucrose in general would need to be included in the model. Lin and Hsu reported that a change in buffer composition or surfactant concentration did not have any influence on the reliability of their NIR model.[22] Thus, we did not include these components in our NIR models.

We recommend the described approach for NIR model generation to permit the use of the NIR model to determine MC during development of a new DP: this means that the model can be used for high-throughput analysis with minimal resource consumption during cycle optimization, scale-up, and transfer without the need to update the NIR model as long as staying in the limit visualized in the PCA plot. Only 3%/1% of variability in the PCA plot (PC3/PC4) remains unspecified, which is considered negligible, and cannot be linked to defined product attributes. Thus, this approach is superior to traditional methodologies because the model can be used even if process parameters exceed those that were tested. This applies as long as staying with the tested limits of PC1 and PC2 (97% in total). In such cases, PCA analysis would readily identify any outliers. In addition, PCA enables to compare different lyophilization runs and thus helps to understand the changes in moisture distribution. Such knowledge facilitates troubleshooting as well as optimization or confirmation of cycle parameters. In addition, such information is essential if any equipment or the site of production of the DP are changed and enables us to rapidly conclude on product comparability with regard to moisture distribution. We would like to highlight that for market authorization, external validation of the model with independent data sets is required according to EMA guidance,[34] which was out of scope for the present study. If the model is intended to be used in a commercial setting, a complete validation will be required.

To summarize, the DoE and multivariate data analysis approach used in our study makes updating of the NIR model during DP development unnecessary, provided the conditions tested are within the limitations of the model as visualized in the PCA plot, which were identified as the tested range of protein:sugar ratio for the sugar included in the model and MC<~6%. Moreover, PCA shows the similarity of MC or its difference between 2 processes. Thus, if the variable conditions were taken into account in the calibration of the NIR model, predicted MC values are reliable. We hypothesize that this holds true even if another product is tested: Clavaud and colleagues[23] showed that the type, for example, mAbs or ADCs, and content of proteins in similar formulations may be merged into one regression model without affecting the prediction errors. In conclusion, the same model could be used for various conditions, as long as the protein:sugar ratio stays within the tested ranges of the model, and the specific disaccharide used in the formulation (e.g., sucrose) was included in the model. Consequently, a high-throughput global model based on the lyoprotectant/cryoprotectant most commonly used for lyophilized biopharmaceuticals could be applied, making application of drug-specific models redundant.

Conclusion

Moisture content is routinely measured as a critical quality attribute of freeze-dried formulations during DP development including scale-up and transfer activities using the time-consuming Karl-

Fischer titration with limited sample throughput. NIR spectroscopy is a nondestructive, high-throughput alternative if adequate models are available. Because the NIR model for MC determination is confounded by formulation composition, it is crucial to "train" (calibrate) and update the NIR model by including samples of a large representative range of formulation composition in relation to variable freeze-drying process parameters. We propose a DoE approach, which can be generated at any stage of drug development based on given target parameters. We propose to visualize samples included in the NIR model using PCA, which enables to modify process and formulation parameters affecting MC without the need to update the NIR model. This even applies to changes in process parameters outside the tested ranges due to the complex interplay between formulation and process parameters at different stages of the freeze-drying cycle, as long as the protein:sugar ratio and MC stays within the tested limits and the lyophilized cake remains macroscopically intact. The main factors affecting NIR spectra were identified as residual moisture (91%) and protein:sugar ratio (6%), with the remaining 3% in the PCA plot remaining unspecified. The PCA plot provides information on MC distribution in response to altered process parameters that could also include changes in lyophilization equipment. Thus, this approach constitutes a valuable tool to ensure product consistency during process optimization, scale-up, and transfer, as well as troubleshooting activities.

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Andrea Allmendinger and Eric Ziemons contributed equally to this study. Andrea Allmendinger was responsible for freeze-drying experiments, and Eric Ziemons was in charge of vibrational spectroscopic and chemometric activities.

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