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# The use of near-infrared spectroscopy in the efficient prediction of a specification for the residual moisture content of a freeze-dried product

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**Abstract**

The purpose of this study is to improve the efficiency in the search for a suitable specification for the residual moisture content in a freeze-dried product. A near-infrared spectroscopic (NIRS) method was developed for the measurement of the residual moisture content. Samples with a wide range of residual moisture contents were stored for two months at 8, 50, and 60°C. Because of the non-destructive character of the NIRS method initially the residual moisture content and, subsequently, the content of the active ingredient could be measured in the same sample vials after storage. Plots of the residual moisture content against the content of the active ingredient were made for storage at 50 and 60°C. For this only 69 samples were needed in the stability study for the assay determination while traditionally a five-fold of samples is needed because of the high intra-batch variability of the residual moisture content. The plots at 50 and 60°C were combined with the Arrhenius relationship between degradation rate constant and temperature. The maximal allowable residual moisture content was calculated for product shelf-lives of 2 and 3 years and storage temperatures of 20, 25, and 30°C, respectively. 

*Keywords:* Near-infrared spectroscopy; Freeze-dried product; Residual moisture; Stability testing

## 1. Introduction

The shelf life or expiration date of a drug product is defined as the time period in which the drug content remains above a specified fraction of the labelled drug content and in which the content of decomposition products remain below a specified level. This shelf life may be predicted by directly monitoring the drug content in the drug product as a function of time at several specified storage temperatures. Regression analysis and subsequently use of the Arrhenius equation may then be used to estimate the rate constant at the specified storage temperatures and to predict drug stability [1,2].

For most freeze-dried products, the decomposition proceeds through hydrolysis reactions. Since

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moisture is involved in such reactions, the rate constant is often strongly dependent on the residual moisture content of the drug product and specifications should be given. If one has quantitative insight in the way the residual moisture has an effect on the stability of the product, one may predict the rate constant for any combination of storage temperature and residual moisture content of the drug product.

One approach would be to collect sets of samples at various intervals in the freeze-drying process. Mean moisture contents can be assigned to every set by Karl Fischer titration of several samples from every set. Since Karl Fischer titration is a destructive technique, the stability can only be correlated to the moisture content by measuring the stability on other samples from these sets. Due to the high variability of the moisture content within every set of samples, the predictive value of such studies is rather limited. A more advanced approach would be the use of a technique that is non-destructive either in the determination of the residual moisture content or in the determination of the stability, i.e. the concentration of the active drug, of the freeze-dried product. One such technique is near-infrared spectroscopy (NIRS).

NIRS has been used extensively in the agricultural and food industries for the past thirty years [3–5]. Although slowly accepted, recent advances in instrumentation and multivariate data analysis have made NIRS an attractive analytical method for use in the pharmaceutical industry, both as a qualitative and a quantitative method [6–9]. Multivariate data analysis is especially necessary for near-infrared spectra because highly overlapping absorption bands, descending from weak overtone and combination bands, hamper the assignment of a signal to certain functional groups. The weakness of the absorption bands in the near-infrared region provides useful sampling advantages with respect to the fundamental bands in the mid-infrared region. Even more, near-infrared spectra of samples can be collected through glass which opens the way for fast and non-invasive analysis of e.g. freeze-dried formulations in glass vials [10–12]. Because NIRS is non-destructive, additional testing such as stability tests can be performed in the same sample.

In this report, the development and application of the NIRS method for the determination of residual moisture content is described. This NIRS method was used for an accurate and fast estimation of the moisture specification for a freeze-dried product.

## 2. Materials and methods

### 2.1. Samples

The injectable is a freeze-dried product which contains, beside the active compound, citric acid monohydrate, disodium phosphate dihydrate and mannitol. Aliquots of 1.5 ml of a 2% w/w solution of the drug substance were filled into 6R DIN glass vials, semi stoppered with PH 4104 stoppers, and subsequently freeze-dried. The weight of the freeze-dried cake is approx. 296 mg. Samples with a wide range of residual moisture content were obtained by opening the freeze-drier at four different times in the secondary drying phase. After recording the near-infrared spectra of 152 samples a subset of 25 samples was selected and analysed by Karl Fischer titration immediately. The remaining 127 samples were stored for 56 days at 8, 50, and 60°C in the dark and then measured by NIRS and, subsequently, analysed by either Karl Fischer titration (58 samples) or HPLC (69 samples). See Fig. 1 for schematic illustration of experimental setup.

At an earlier stage of the product development, the Arrhenius relation between decomposition rate and temperature had been established for samples with a specific moisture content. The drug substance is a compound with two ester groups. It was found that the hydrolysis of one of the esters is the primary decomposition reaction. Furthermore, it was estimated that the decomposition of the active substance was a first-order reaction. The equation for the first-order reaction is:

$$k = \frac{\Delta \ln[\text{drug substance}]}{\Delta t}$$

wherein $k$ is the decomposition rate at a specific temperature and $t$ is the time period.

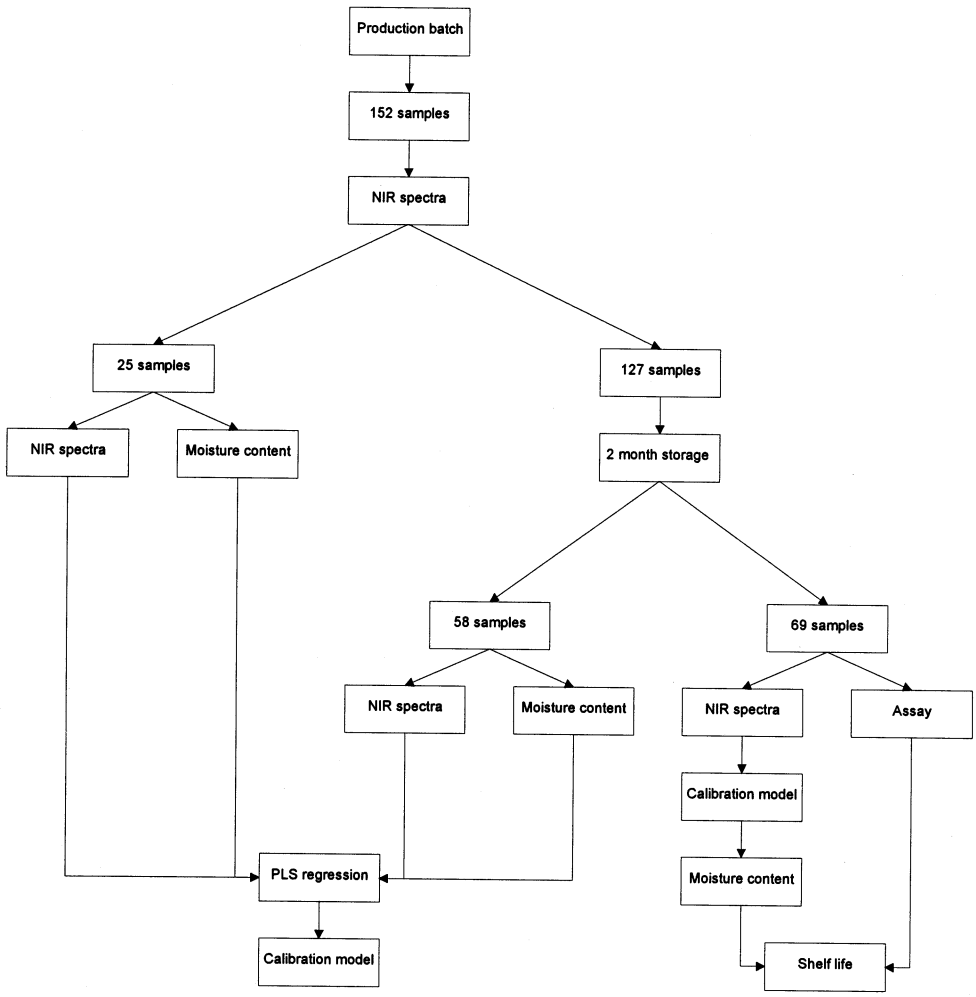


Fig. 1. Schematic illustration of experimental setup.

### 2.2. Near-infrared reflectance spectroscopy

An NIRSystem Model 6500 spectrophotometer (Perstorp Analytical, Silverstone, USA) configured with a reflection module, was used to collect the near-infrared reflectance spectra. The software packages NSAS 3.30 and $IQ^2$ 1.11 (Perstorp Analytical, Silverstone, USA) were used to collect and analyse the data. The spectrophotometer was placed in a horizontal position with the optic window facing upwards and fitted with the horizontal set-up module. An iris assembly was used to reproducibly centre the unopened vials on the spectrophotometer window. The spectra were compensated for background absorption by subtraction of a reference spectrum of a 6R DIN glass vial filled with dried magnesium oxide powder. The spectra were measured between 1100 and 2500 nm, at 2 nm increments, using a resolution of about 10 nm bandwidth. After averaging over 32 scans, the spectra were transformed to second derivative spectra in order to remove absorbance offsets (segment size 20, gap size 0).

All sample vials were individually marked and scanned through the bases of the glass vial prior to analysis either by Karl Fischer titration or by HPLC.

### 2.3. Karl Fischer titration

The residual moisture content of the samples was determined by means of a coulometric Karl Fischer titration method. The samples were dissolved in 1.5 ml anodic reagent, suspended for approx. 15 min, and then 0.2 ml of this solution was injected into the titration vessel.

### 2.4. HPLC

The content of the drug substance in the samples was determined by means of a reversed-phase HPLC method and UV detection (Table 1). After the samples were reconstituted in 10 ml of water for injection, 0.2 ml of the reconstituted sample was diluted with 1.8 ml of acetonitrile. In order to precipitate the mannitol, the solution was stored for about 15 min in a freezer at $-18$°C. Since phase separation may have occurred the samples were mixed. The mannitol was removed by filtration using syringes and Millex-$HV_4$, 4 mm luer filter units.

### 2.5. Data analysis

Partial least squares or PLS regression [13] was used to model the relation between the residual moisture content and the near-infrared reflectance spectra as obtained for the 83 samples that were used for Karl Fischer titration. In general, the model is of the form

$$y_i = b_0 + b_1 \cdot x_{i1} + b_2 \cdot x_{i2} + \ldots + b_m \cdot x_{im}$$

Table 1
HPLC conditions for the determination of the contents of the drug substance and decomposition products

| | |
|---|---|
| Column | Hypersil silica, 250 × 4.6 mm |
| Mobile phase | Acetonitrile-tetramethylammonium hydroxide (pH 7.4; 0.024 M) (87.5:12.5 v/v) |
| Flow rate | 2.0 ml $min^{-1}$ |
| Column temperature | 40°C |
| Detection wavelength | 210 nm |
| Injection volume | 10 μl |

where $y_i$ is the moisture content in the $i$th sample, $x_{im}$ is the spectral measurement at the $m$th wavelength in the $i$th sample, and $b_m$ is the regression coefficient for the $m$th wavelength. In PLS regression the regression coefficients are obtained in such a manner that the resulting estimates are stable. An optimal calibration model, i.e. an optimal number of PLS factors, was compiled by means of cross-validation testing. Therefore, a subset of calibration samples was omitted during calibration. After calibration, the residual moisture content of the omitted calibration samples was estimated. This procedure was repeated until all calibration samples were omitted once. Based on a minimum mean standard error, i.e. the mean difference between the Karl Fischer results and the estimated results, an optimal number of PLS factors was selected.

The performance of the optimal PLS model was evaluated by means of trueness, precision and linearity. Trueness, expressed as bias, is the mean difference between the Karl Fischer results ($y$) and the estimated results as obtained by the optimal PLS model ($\hat{y}$) for $N$ calibration samples:

$$\text{bias} = \frac{\sum_{i=1}^{N} (\hat{y}_i - y_i)}{N}$$

Precision, expressed as standard error of estimation (SEE), is the standard deviation of the mean difference between the Karl Fischer results and the estimated results as obtained by the optimal PLS model for $N$ calibration samples:

$$\text{SEE} = \sqrt{\frac{\sum_{i=1}^{N} (\hat{y}_i - y_i)^2}{N-1}}$$

The standard error of estimation is a combination of the errors of the Karl Fischer titration, the errors of the NIRS method, and the sampling errors. Because no known validation samples were available, the more realistic standard error of prediction (SEP) could not be calculated. Furthermore, it should be emphasised that from a theoretical point of view the standard error has to be corrected for the bias. Linearity, expressed as correlation ($r^2$), is the correlation between the Karl Fischer results and the estimated results as

obtained by the optimal PLS model for $N$ calibration samples.

Finally, the calibration model was used to predict the residual moisture content in the 69 samples that were used for HPLC.

## 3. Results and discussion

From one batch of the freeze-dried product sufficient samples were taken at different intervals of the freeze-drying process in order to obtain samples with a wide range of residual moisture contents. After recording the near-infrared spectra of all samples a subset of samples was selected and analysed by Karl Fischer titration in order to calibrate the NIRS method. The remaining samples were stored for 2 months at different temperature conditions. After this period the samples were measured by NIRS and subsequently analysed by either Karl Fischer titration for calibration purposes or HPLC to assay the remaining concentration of the active ingredient. On basis of the obtained results an NIRS method for the determination of the residual moisture content was developed. See Fig. 1 for schematic illustration of experimental setup.

### 3.1. Near-infrared spectra

Fig. 2a shows representative near-infrared reflectance spectra of freeze-dried samples with a varying residual moisture content. The observed offsets in the reflectance spectra are caused by variations in the particle size, variations in the compaction of the freeze-dried solid and from slight optical aberrations in the glass vials. The dip at about 1360 nm is probably caused by Wood's anomalies. Wood's anomalies are spurious absorbances that are associated with any grating monochromator operating in the single-beam mode. In order to reduce these effects, the second derivative spectrum as shown in Fig. 2b is used for further analysis. The band at about 1920 nm, which mainly reflects the residual moisture content, is a combination of the fundamental OH stretch with OH deformation. The band at about 1410 nm is the first overtone of OH stretch. The

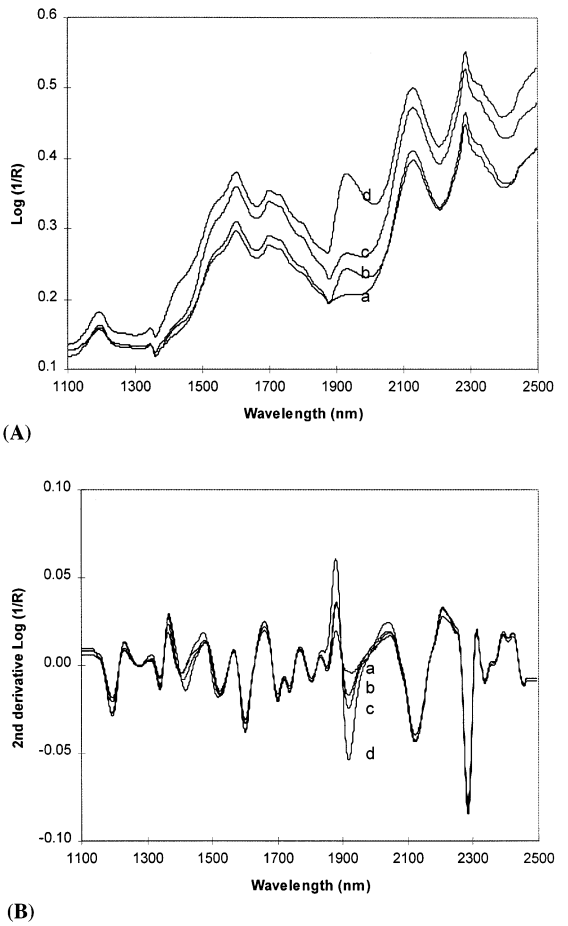


Fig. 2. Representative near-infrared reflectance spectra (A) and second derivative spectra (B) of freeze-dried samples with a residual moisture content of 0.5 (a), 2.5(b), 5.0 (c), and 10.6 mg vial$^{-1}$ (d).

position of these bands is strongly dependent on the hydrogen bonding environment. It is observed for freeze-dried samples that these bands shift to a higher wavelength at increasing moisture content. At increasing moisture content there is more free water present in the samples which vibrates more easily than bonded water, i.e. band shift to a higher wavelength. Therefore, it is concluded that in this case a multivariate calibration method such as PLS regression is preferred over single or dual wavelength calibration methods.

### 3.2. Partial least squares regression for moisture determination

Based on the 83 samples that were used for Karl Fischer titration an optimal PLS model of four factors was compiled by means of cross-validation testing. Deducing the true number of PLS factors is a difficult problem because of experimental errors in both the Karl Fischer titration and the NIRS method. In general, the more PLS factors included in the calibration model the less rugged the model will be for small spectral differences. In this case, both the calibration and test spectra are obtained from samples taken from one batch and therefore no significant spectral differences are expected. If the model should be used for future samples, e.g. samples taken from additional production batches, less PLS factors are preferred.

From Fig. 3 it can be seen that the estimated regression coefficients, as obtained by PLS regression, contain structural information. In this case, the spectral ranges around 1410 and 1920 nm, i.e. the first overtone of OH stretch and a combination of the fundamental OH stretch with OH deformation, seems to be important to model the relation between the residual moisture content and the near-infrared reflectance spectra. Furthermore, the remaining spectral ranges seems to be necessary to model small spectral differences that could not be eliminated by using the second derivative spectra.

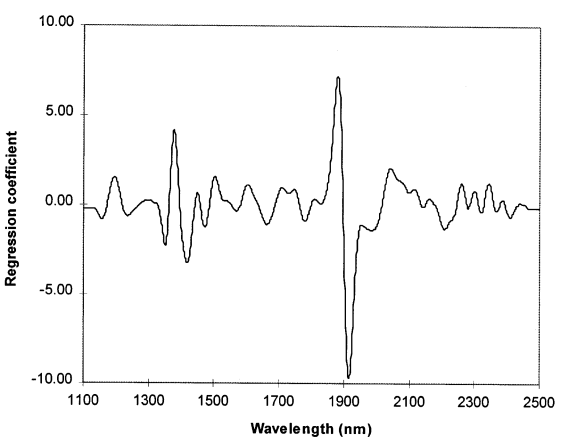


Fig. 3. Regression coefficients for the optimal PLS model.

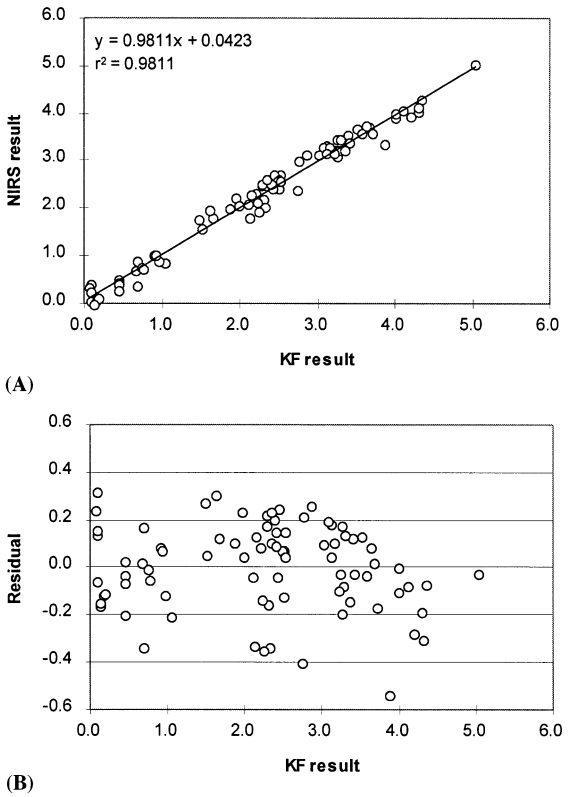


Fig. 4. The moisture content (A) and the residual moisture content (B) as estimated by near-infrared reflectance spectroscopy versus the content as determined by coulometric Karl Fischer titration for 83 samples (expressed as mg moisture per vial).

The overall range of residual moisture content as determined by Karl Fischer titration is 0.1–5.0 mg vial$^{-1}$ moisture (Fig. 4A). With respect to the individual samples the differences between the results, as obtained by Karl Fischer titration and the NIRS method, ranges from about $-0.6$ to 0.4 mg vial$^{-1}$ moisture (Fig. 4B). No significant mean difference was calculated, i.e. the bias is less than 0.01 mg vial$^{-1}$ moisture. The standard error of estimation is 0.02 mg vial$^{-1}$ moisture. At a level of 1.0 mg vial$^{-1}$ moisture this correspond with a relative standard deviation of 2%. The correlation between the results of both methods is 0.98 (Fig. 4A). Based on these figures, it was concluded that the NIRS method could be used for the prediction of the residual moisture content in the samples used for stability testing.

### 3.3. Stability versus moisture content

Finally, the NIRS method was used to predict the residual moisture content in the 69 samples that were submitted for stability testing, i.e. HPLC analysis. From the resulting data set, a relation between the residual moisture content and decomposition rate at 50 and 60°C could be determined (Fig. 5).

From an accelerated stability study of samples with a certain moisture content that covered more temperature conditions (8, 20, 30, 40, 50 and 60°C) and a period of 12 months, the Arrhenius relation between decomposition rate and temperature had been established earlier. The results of that study indicated a shelf-life, based on a maximum decrease of the active drug substance with 5%, of 351 days at 30°C. This means that when a shelf-life of 3 years (1095 days) is desired, that the reaction rate constant should decrease 3.12 fold.

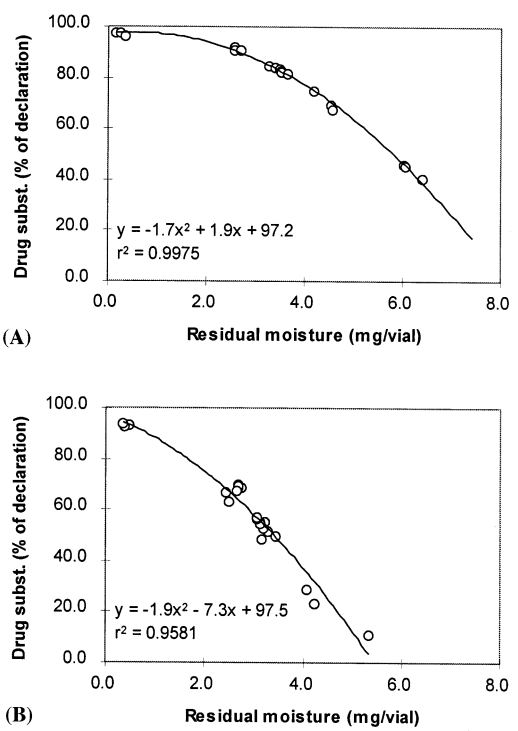


Fig. 5. Content of the drug substance as function of actual residual moisture content after 56 days at 50°C (A) and 60°C (B), respectively.

Table 2
Maximal allowable residual moisture contents (after 2 months) that result in shelf lives of 2 and 3 years at room temperatures calculated by using the 50°C (A) and the 60°C data (B).

| Temperature (°C) | Maximal residual moisture content | |
|---|---|---|
| | 2 years shelf life (mg vial$^{-1}$ moisture) | 3 years shelf life (mg vial$^{-1}$ moisture) |
| (A) | | |
| 20 | 3.27 | 2.70 |
| 25 | 2.36 | 1.88 |
| 30 | 1.60 | 1.10 |
| (B) | | |
| 20 | 2.86 | 2.22 |
| 25 | 1.83 | 1.32 |
| 30 | 1.06 | 0.69 |

In the same accelerated stability study a reaction rate constant of $1.6 \cdot 10^{-3}$ days$^{-1}$ was found at 50°C. It was found that the decomposition of the drug substance was a first order reaction and, therefore, the reaction rate constant found at 50°C should also be decreased 3.12 fold in order to obtain a shelf-life of 3 years at 30°C. In this way it can be calculated that the content of the drug substance may not become lower than 97.18% after 56 days at 50°C. This value is fitted in the equation given in Fig. 5A and gives a specification for the moisture content of 1.10 mg vial$^{-1}$. In other words a residual moisture content of 1.10 mg vial$^{-1}$ would render a product shelf life of 3 years at a storage temperature of 30°C. The same procedure was repeated for storage temperatures of 20 and 25°C and for an aimed shelf life of 2 years. The maximal allowable moisture contents for those situations are listed in Table 2A. The 60°C data set was also used to determine maximal allowable moisture contents. Results are listed in Table 2B.

Based on the statistical figures of the relation between the residual moisture content and decomposition rate at 50 and 60°C it was concluded that the relation at 50°C is the most reliable. The decomposition at 60°C is so dramatic, viz. the amount of the drug substance decreased with about 80% for samples with a residual moisture

content of 4 mg vial$^{-1}$, that the extrapolation of the results to storage conditions of 20, 25, and 30°C is quite speculative. Furthermore, it should be emphasised that the freeze-dried cake of some samples stored at 60°C was shrunken which made the NIRS results less reliable.

## 4. Conclusions

It was shown that NIRS offered good possibilities for the determination of the residual moisture content in freeze-dried samples. The developed NIRS method, applicable for the studied freeze-dried product with a residual moisture content between approx. 0.1 and 5.0 mg vial$^{-1}$, shows a standard error of estimation of approx. 0.02 mg vial$^{-1}$ and a correlation with the Karl Fischer titration of approx. 0.98. Based on these figures, it was concluded that the NIRS method could be used as a quantitative method for the estimation of the residual moisture content in freeze-dried samples that were submitted for stability testing. Whereas the samples used for calibration of the NIRS method and the samples used for stability testing were descended from the same production batch, it was not necessary to validate the NIRS method extensively including batch to batch variation.

Because the residual moisture content and the content of the active ingredient could be measured in the same samples, only a limited number of samples were needed in the stability study. It should be realised that when the moisture content and the assay cannot be determined in the same vial, about a five-fold of samples is needed because of the high intra-batch variability of the moisture content. Based on the relation between the residual moisture content and the content of the active ingredient at different storage temperatures combined with the earlier established Arrhenius relationship between degradation rate constant and temperature, it was possible to predict the residual moisture content specification for product shelf lives of 2 and 3 years and storage temperatures of 20, 25, and 30°C based on two months stability data.

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