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Moisture assay of an antifungal by near-infrared diffuse reflectance spectroscopy

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Abstract

Near-infrared (NIR) diffuse reflectance spectroscopy was employed in the method development and validation of a moisture assay for the novel antifungal caspofungin acetate. Spectra were obtained over the entire spectral region available (950–1650 nm) using an InGaAs photodiode array detector equipped with a diffuse reflectance probe. No sample pre-treatment was required and the analysis time was less than 1 min. Primary reference data were obtained using a Karl Fischer (KF) titration (coulometric, volumetric or both). The investigated range of water content was 2.6–9.9% (w/w) with a standard error of prediction (SEP) of 0.2%. The predictive capabilities of the partial least-squares (PLS) regression calibration model used in the moisture assay were verified using independent test sets. The NIR predicted values of the developed method were equivalent to the reference method sets and the prediction error was equivalent to the reference method error. These results reveal that the predictive model constructed by means of a PLS regression is valid, rugged and could be used to determine moisture levels on-line in caspofungin acetate drug substance.

Keywords: Near-infrared spectroscopy; Diffuse reflectance; Moisture content; Partial least-squares regression

1. Introduction

The determination of water content in pharmaceuticals is an important part of the manufacturing and storage processes, as some compounds may degrade rapidly under high or low moisture conditions. In recent studies, it was found that moisture can diminish the efficacy of drug compounds and in some cases is responsible for the formation of degradation products [1]. Recent advances in the study of near-infrared (NIR) spectrometry have led to quantitative determination of not only water, but also other constituents (i.e. organic solvents). These advances are based largely on the availability of sophisticated statistical and chemometric methods, and powerful computers, and software that can account for all the

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natural vibrational combinations and overtones present in the NIR region (780–2500 nm) [2]. These absorptions are broader, less intense and more difficult to assign to functional groups compared with bands in the mid-IR region (2500–25 000 nm; 4000–400 cm^{-1}). On the other hand, the NIR absorptions are linear over a wide dynamic range and samples can be measured in reflectance or transmission mode without any sample pre-treatment.

All the basic vibrations of the water molecule are mid-IR and NIR active with absorbance maxima at 2700, 1900, and around 1400 nm [3]. Due to hydrogen bonding, these bands are broad and may experience wavelength shifts due to matrix interactions. The significant absorptivity of water in the NIR region of the spectrum (compared with other substances) makes the technique ideal for the determination of moisture content [4,5]. Also it has been shown that water absorbs at all NIR wavelengths [1]. This effect makes partial least-squares (PLS) regression a particularly good chemometric method for the quantitative analysis of water, because, it uses the entire NIR spectrum in order to determine its concentration.

In this work, we report the development and validation of a NIR method that could provide an accurate and fast moisture determination for this novel antifungal drug substance without any sample pre-treatment. The challenge was to develop a non-invasive method for moisture determination in caspofungin acetate, which has a property, heretofore unseen in water analyses, of quickly changing its water content based on the atmosphere's percent relative humidity. NIR testing during manufacturing only becomes practical when a large number of batches of material are to be tested. The time and effort involved in developing the method would not be justified if only a few batches were in need of testing. A non-invasive spectroscopic method would alleviate the need for sample preparation and manual handling, as is necessary in the currently used Karl Fischer (KF) analysis. In general, no matter how simple the preparation, each step requires an analyst's time and provides an opportunity for analyst error.

2. Experimental

2.1. Materials

Dry KBr (99 + % pure FT-IR grade from Spectra-Tech, Inc., Shelton, CT) was used as a reference material for diffuse reflectance measurements. The caspofungin acetate drug substance was synthesized by Merck Research Laboratories, Merck & Co., Inc., Rahway, NJ. The water titrant in the volumetric KF determination was Hydranal®-Composite 5 (Riedel-de Haën AG, Seelze, Germany).

2.2. Samples

A total of 49 samples of caspofungin acetate from 13 different batches were used in this study. The samples were stored in glass vials at −70 or −20 °C when not in use. At ambient temperature, the gain or loss of water in caspofungin depends on the percent relative humidity (%RH) of the environment in which the sample is stored or handled. Measures were taken to minimize the time exposed to the atmosphere, and careful measurements of the humidity levels were taken in order to evaluate any errors resulting from the hygroscopicity of caspofungin. The moisture level was deliberately increased or decreased by exposing the samples to environments of varying relative humidities. The moisture content of the resulting samples was found to vary between 2.5 and 15.0% (w/w).

2.3. Instrumentation

NIR spectra (950–1650 nm) were recorded using an optical solutions PS-2 portable diode array spectrometer that included a thermoelectrically cooled 256-element InGaAs photodiode array detector and an optically stabilized tungsten–halogen light source (Optical Solutions, Folsom, CA). An FDR-320 series diffuse reflectance probe (Axiom Analytical Inc., Irvine, CA) was connected to the spectrometer via a bifurcated fiber-optic bundle (2 m in length) equipped with SMA fiber optic connectors. The spectral range of the FDR-320 probe was 0.9–2.5 μm, and the length and diame-

ter were 33 and 25 mm, respectively. The active diameter of the fiber optic was 2.4 mm at the probe and 1.7 mm at the source and detector terminals. The light source drift was 0.0005 AU over a period of 6 h. The spectrograph resolution, spectral bandwidth and the wavelength stability were 3 and 7 and ± 0.05 nm, respectively. The probe is capable of obtaining spectra up to a maximum distance of approximately 6 mm from a sample. In this work the probe was operated in a contact mode with the sample or the sample container (glass or ACLAR® 22C liner). The reference KF titrations were done using a Metrohm 701 KF Titrino and 703 Ti Stand (Brinkman Instruments, Inc., Westbury, NY).

2.4. Procedure

The NIR probe was mounted on a ring stand with a clamp in a near vertical position. Each spectrum was obtained by averaging 20 full spectral (950–1650 nm) scans. During the data collection the amount of time spent on each element of the array was 0.1 s (integration time). A background scan and a reference spectrum were collected every time the spectrometer was turned-on. A background scan is a record of incident light that may enter the fiber optic bundle, probe and detector. The background spectrum represented 0% transmittance and was collected by blocking the source lamp. The reference spectrum represented 100% transmittance and was collected by presenting the probe to a dry KBr powder placed on the top of an upside-down glass beaker. The sample spectra were collected in the same way, replacing the KBr powder with caspofungin acetate. The glass beaker, spatula and probe were washed after each sample presentation with dry methanol and dried under nitrogen. Spectra were recorded by the instrument data system.

Coulometric and/or volumetric KF titrations were used as reference methods for all the moisture determinations. In the coulometric KF titration, HYDRANAL®-Coulomat A (anolyte) and HYDRANAL®-Coulomat (catholyte) were used in the coulometric titration cell. Following a 'blank titration' (titration of the cell to dryness) the sample was added and titrated to dryness. In the volumetric method, analytical grade anhydrous methanol was used as the working medium in the titration vessel. A one-component KF reagent, HYDRANAL®-Composite 5, used and consumed the equivalent of 5 mg of water per ml titrant.

2.5. Software

In all cases NIR data was processed using the GRAMS/32 V.4.14 (Galactic Industries Corporation, Salem, NH) program. For the calibration and prediction of water in caspofungin, PLS regression and multiple linear regression (MLR) methods were applied [6]. The chemometric software used to build the calibration model and predict the unknown moisture level was PLSPLUS/IQ V.3.03 (Galactic Industries Corporation), which is based on the published works of Kowalski and Seasholtz [7] and Haaland and Thomas [8]. The calibration equations for all models were stored electronically and validated by generating results on known, independent lots of caspofungin.

3. Results and discussion

3.1. Near-IR calibration using partial-least squares regression

When infrared light is projected onto a sample of caspofungin acetate that contains water, some of it is selectively absorbed while the remainder is scattered. Vibrational excitation of the –OH groups in the water molecule and –CH and –OH groups in the caspofungin molecular structure results from this absorption. The main features of the caspofungin acetate spectrum in the region from 950 to 1650 nm are attributed to the second O–H overtone bands from the hydroxyl groups in caspofungin, the second O–H overtone of the O–H stretching in water, and the second C–H overtone of the methyl groups in caspofungin. With greater levels of moisture in caspofungin, more absorption occurs with a corresponding reduction in the amount of diffusely reflected light.

The spectra were evaluated using PLS regression and MLR methods [7,8]. These two chemometric methods are the most widely used multivariate methods in building calibration models. The MLR method assumes that concentration is a function of absorbance and the analysis is performed using selected wavelengths having the highest correlation. This number is relatively small, because, it cannot exceed the number of calibration mixtures used in the analysis. Determining which and how many wavelengths to include in the model is very important and must be done with great care in order to develop a robust method. In addition, co-linearity problems could affect the accuracy of the model when too many wavelengths are included in the analysis.

PLS is a full-spectrum method that extracts principal components from whole wavelength regions and correlates the spectral data in these regions with the concentration of the constituent of interest. One of the main advantages of PLS is that the resulting spectral vectors are directly related to the water level, because, the spectral decomposition and the regression against the concentration of water occur simultaneously and not as two separate steps. In contrast, in MLR the vectors merely represent the most common spectral variations of the data ignoring the relation to the water concentration until the final regression. Another advantage of PLS is that, because it is a full-spectrum method, efficient outlier detection methods are available from spectral residuals. Finally, the use of a photodiode array detector rather than a conventional scanning spectrophotometer eliminated the wavelength shift problems and resulted in more rugged PLS calibration models. The most accurate model using the PLS regression chemometric method was constructed by using, (a) the entire spectral region (950–1650 nm) and (b) a process that determined what type of data treatment produced a model with the best possible predictive capabilities.

3.2. Calibration model using cross-validation

Samples from 13 different lots of caspofungin, synthesized over a 3-year period, were used in this study. From these, 33 samples were used to build the calibration model and 16 samples were used to validate it. The final calibration model was constructed using the spectra and KF values from all the 33 samples that where included in the initial calibration set. The first step in these multivariate analyses involves mean centering of the entire spectrum in order to normalize all the spectra. Mean centering means that the average spectrum is calculated from all the calibration spectra and then subtracted from every calibration spectrum. As a result, small shifts in the instrument's performance will not affect the calibration. It also serves the purpose of removing redundant information, enhancing the sample-to-sample differences and improving the overall ruggedness of the model.

All the spectra were processed using a Savitzky–Golay second derivative function utilizing 11 data-points. The purpose of generating the second derivative of each spectrum is to minimize any effects of baseline shifting due to particle size differences or other solid-state characteristics [9]. The advantage of looking at the derivative curve is that, it highlights much of the hidden information within the spectrum. A simple comparison of two spectra with their derivatives can illustrate this point clearly. The spectra of two samples with water levels of approximately 6 and 9%, respectively, are shown in Fig. 1A. The relevant information to be extracted from these spectra is not immediately apparent. However, the second derivatives of these two spectra (Fig. 1B) reveal many of the hidden spectral features. The value of the derivative function at a particular wavelength is the absolute intensity due solely to the molecular absorption, since the background absorption is subtracted out. The number of data points used in the derivation minimizes the influence of noise in the calibration model and increases the robustness of the model. The use of less than 11 data-points contributed to noise peaks in the second derivative spectrum, while more than 11 data-points smoothed out the important peak features containing the spectral influence of water.

Cross-validation is a calibration technique that removes one sample and predicts its value on the basis of a model of all the other samples. This technique allows us to calculate the number of factors to be used in the treatment of each spec-

trum. Factors are the number of loading vectors used by the model to evaluate and map an unknown spectrum against the vector map of all the known spectra already in the calibration set. The number of factors employed in the treatment of each spectrum has a similar effect as the number of data-points described previously. A model that includes more or less factors than are actually necessary to predict the constituent concentrations is called 'over-fit' or 'under-fit', respectively.

In this study the optimum number of factors was calculated by using the predicted residual error sum of squares (PRESS) value for every possible factor. The PRESS value was the sum of the squared difference between the predicted and the known concentrations. It was calculated by building calibration models with different number of factors and then predicting some samples of known concentration against the model. The number of factors where the PRESS plot reached

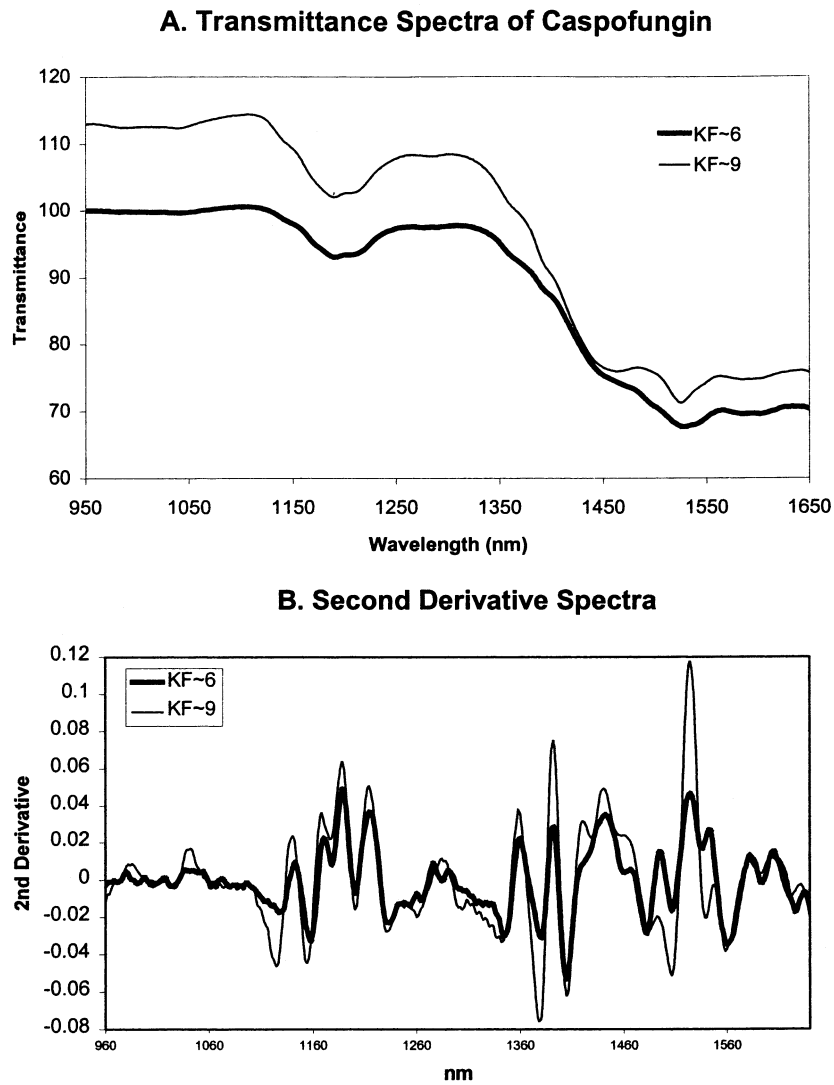

Fig. 1. (A) Example of spectral baseline shifting. (B) Example of the corrective capabilities of second derivative data treatment of two samples with water levels of approximately 6 and 9% (w/w).

Table 1
Calibration model information for moisture assay in caspofungin

Number of samples (spectra)	33
Number of factors	3
Correlation coefficient (R)	0.9003
Standard error of cross-validation	0.54%
Range of calibration	2.63–9.89%

a minimum was three and was chosen as the optimum number of factors for the calibration model described in this study. Building models with more or less than three factors and applying these models to predict moisture levels further supported this result.

Other parameters such as spectral region, removal of outliers, variance scaling, and baseline correction were examined with respect to their effects on moisture prediction. Any attempt to use only a particular region instead of the full spectrum decreased the predictive capabilities of the model. The PLS regression analysis of the caspofungin acetate spectra favors the water-influenced wavelengths around 1475 nm, as indicated by the factor weights in this area. MLR analysis using multiple wavelengths around this region did not predict the moisture levels as accurately as the PLS regression analysis. This is a result of the water influence over the entire NIR spectrum of caspofungin. Also, attempts to remove outliers from the calibration model resulted in worse than expected predictions. None of the other parameters had a positive influence on the predictions, so they were not applied to the calibration model. These results emphasized the need to incorporate into the model all possible moisture concentrations and manufacturing process variables in order to achieve a robust calibration. Overall, PLS models gave more accurate predictions due to their ability to treat interfering and overlapping peaks of complex matrices better than MLR [10].

The details of the most successful calibration model using PLS regression analysis are shown in Table 1. This calibration was represented graphically by plotting the KF values of the samples whose spectra were used to build the calibration model versus the predicted values by the model based on those same spectra (Fig. 2).

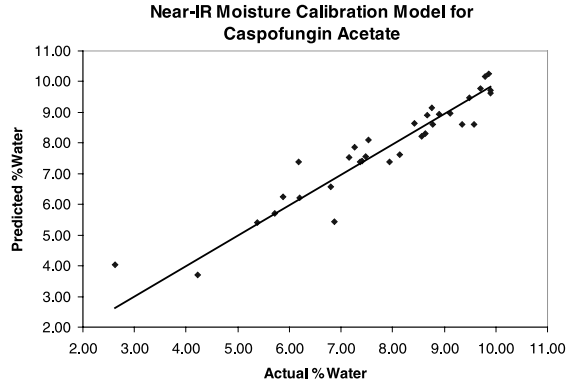

Fig. 2. Full cross-validation model plotting the KF values vs. the predicted values using PLS regression.

3.3. Validation of the near-IR calibration model

3.3.1. Measurement precision

The measurement precision of the instrument and the probe was determined by recording ten spectra from the same sample presentation. The results are shown in Table 2. The difference between the predicted (NIR) and actual (KF) water level for a sample is defined as the residual. As the predictive capability of the model is increased the residuals approach zero. The standard deviation (S.D.) of the associated residuals (NIR–KF) shown in Table 2 was 0.08.

Table 2
Measurement precision of the NIR calibration model

KF (% water)	NIR	Residuals
8.16	8.06	−0.10
8.16	8.10	−0.06
8.16	8.16	0.00
8.16	8.10	−0.06
8.16	7.96	−0.20
8.16	7.98	−0.18
8.16	7.99	−0.17
8.16	7.93	−0.23
8.16	7.96	−0.20
8.16	7.93	−0.23
	Bias	−0.14
	S.D.	0.08
	R.S.D. (%)	1.00

Table 3
Method precision of the NIR calibration model

KF (% water)	NIR	Residuals
8.11	8.01	−0.10
8.11	8.15	0.04
8.11	8.33	0.22
8.11	8.31	0.20
8.11	8.45	0.34
8.11	8.32	0.21
8.11	8.34	0.23
8.11	8.44	0.33
8.11	8.31	0.20
8.11	8.18	0.07
	Bias:	0.17
	S.D.	0.13
	R.S.D. (%)	1.57

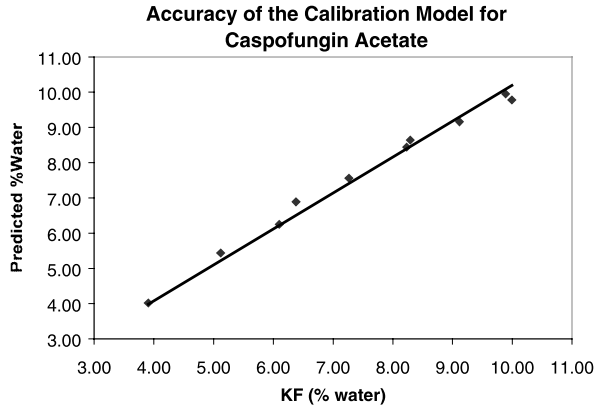

Fig. 3. Accuracy of the NIR calibration model using ten samples from lots A–D used in the calibration study.

3.3.2. Method precision

The method precision was demonstrated by taking ten independent measurements of the same lot of caspofungin. In each single measurement the sample was introduced into the probe and the spectrum was recorded. After each spectrum collection the probe was cleaned and a new aliquot of the same sample was introduced into the probe for the next measurement. The residuals (NIR–KF) ranged from −0.10 to 0.34 as shown in Table 3. The error associated with this technique of recording NIR spectra was quantified with a S.D. of 0.13.

3.3.3. Accuracy

The accuracy of the calibration model was demonstrated using ten samples from four lots (A–D) of caspofungin that were previously used to build the calibration model. Their KF values were determined again during the recording of their NIR spectra. The calibration model was then used to predict the moisture levels from these new spectra. The plot of actual (KF) versus predicted (NIR) values is shown in Fig. 3 ($R^2 = 0.992$). The residuals (NIR–KF) ranged from −0.21 to 0.51 with a S.D. of 0.20 as shown in Table 4.

3.3.4. Thickness of sample

The thickness of the sample that the light penetrates is an important variable and easily controlled. The effect of the sample-thickness variability in the prediction was demonstrated by increasing the amount of powder presented to the probe. The thickness of eight aliquots from the same lot that were presented to the probe was increased incrementally from 0.5 to 5.0 mm. Spectra were recorded until no more visible light from the probe was observed to penetrate through the sample at 5.0 mm thickness. The residuals (NIR–KF) ranged from −0.97 to 0.14 and are shown in Table 5. The decrease in error of prediction due to the powder's thickness is an obvious and straightforward trend.

Table 4
Accuracy of NIR calibration model

Lot	KF (% water)	NIR	Residuals
A	3.90	4.02	0.12
B	5.12	5.44	0.32
C	6.10	6.25	0.15
A	6.38	6.89	0.51
A	7.26	7.56	0.30
C	8.23	8.44	0.21
B	8.29	8.64	0.35
D	9.12	9.16	0.04
C	9.89	9.95	0.06
B	9.78	9.78	−0.21
		Bias	0.19
		S.D.	0.20

Table 5
Sample-thickness effects on the prediction

Sample thickness (mm)	KF (% water)	NIR	Residual
0.5	9.29	8.32	−0.97
1.0	9.29	8.34	−0.95
1.5	9.29	8.57	−0.72
2.0	9.29	8.91	−0.38
2.5	9.29	9.20	−0.09
3.0	9.29	9.23	−0.06
4.0	9.29	9.43	−0.14
5.0	9.29	9.27	−0.02

3.3.5. Verification (prediction) of the near-IR calibration model

The final test on the validity and accuracy of the calibration model required the moisture prediction of samples not included in the calibration set. These samples represented by caspofungin lots synthesized by different chemical processes and they were not included in the calibration set. In this study six samples from five different lots (E–I) that were not included in any of the previous studies were used as the verification set. The residuals (NIR–KF) of this verification set ranged from −0.27 to 0.11 with a S.D. of 0.10 as shown in Table 6. The plot of actual (KF) versus predicted (NIR) values is shown in Fig. 4.

3.3.6. Prediction through the caspofungin container system

The feasibility of taking measurements through glass or ACLAR® 22C liner was investigated. It was found that NIR spectra can readily be recorded through the walls of glass vials or ACLAR® 22C liner with minimal interference or spectral scatter from the glass or the liner. The average prediction results using the 'through-liner' and 'through-glass' techniques were 8.32 and 8.34, respectively (Table 7). These results are in agreement with the KF result of 8.31, and the prediction result of 8.30 obtained using the directly in contact with the sample technique as described previously. The residuals (NIR–KF) ranged from −0.05 to 0.10 with a standard deviation of 0.08. Even though the calibration model did not include any spectra taken through the liner, it was possible to predict the water level accurately by recording a reference spectrum through the liner and collecting spectra in that manner. The agree-

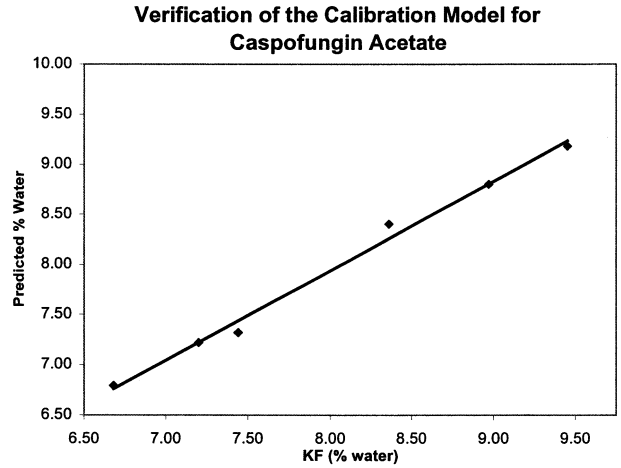

Fig. 4. Verification of the predictive capabilities of the NIR calibration model using six samples from lots E–I that were not used in the calibration study.

Table 6
Prediction of the NIR calibration model

Lot	KF (% water)	NIR	Residuals
E	6.68	6.79	0.11
F	7.20	7.22	0.02
F	7.44	7.32	−0.12
G	8.36	8.40	0.04
H	8.97	8.80	−0.17
I	9.45	9.18	−0.27
		Bias	−0.02
		S.D.	0.10

Table 7
Spectral prediction through ACLAR® 22C liner and glass

KF (% water)	Spectral acquisition		
	In-contact	Through-liner	Through-glass
8.31	8.34	8.32	8.26
8.31	8.29	8.38	8.41
8.31	8.26	8.26	8.36
	Average 8.30	Average 8.32	Average 8.34

ment of these results was another demonstration of the predictive capabilities of the calibration model.

3.3.7. Ruggedness of the calibration model

The age of the caspofungin samples ranged from 2 months to 3 years. Thus, variability due to compound age, storage conditions and the synthetic scheme were built into the calibration model. This is certainly a positive characteristic of the prediction model if one is going to use this model to determine water in older materials or materials made by different chemistry routes employed throughout the development and manufacturing.

During the recording of spectra for the calibration model, the amount of powder that was presented to the probe was varied depending on the available amount (mg to g) of that particular lot of caspofungin. This variable is easily controlled during construction of the calibration model. Samples of greater thickness produce superior diffuse reflectance spectra and thus minimize predictive error.

The plot of the calibration model that was created does not fit a perfect line (Fig. 2), but is a scattering of data very unlike the traditional 'calibration-curve' spectroscopists are more familiar with. The important aspect of the calibration model is not how well it fits a line, but how well it can predict the concentration of the constituent(s) of interest. This model has been able to demonstrate a predictive capability with residuals from the reference method having a comparable standard deviation to that of the reference KF titrimetric method.

Since, the calibration model accounted for variables such as age of sample, sample-thickness and ambient percent relative humidity, the standard error of calibration (SEC) of 0.5% is realistic. The positive effect of these variables can be seen in the standard error of prediction (SEP) which was calculated to be 0.2%. The prediction (verification) samples were predominantly recent lots and the amount of material was sufficient to present an adequate amount to the probe during the spectra collection. This is why the predictions are more accurate than the SEC would seem to let them be and are better represented by a SEP which combines the two verification data sets (Tables 4 and 6) to give a SEP of 0.2%. Having a SEC greater than the SEP is an indication that the model does not require the inclusion of any additional datapoints.

4. Conclusions

The NIR spectroscopic analysis of water by diffuse reflectance using an InGaAs photodiode array detector can generate accurate and precise data for moisture sensitive compounds. It is an efficient, non-invasive and non-destructive technique. It is imperative to establish an accurate, valid, and robust calibration model incorporating all possible variations in the samples and collection of spectra. Calibrations built in this manner will predict reasonably well in the presence of future variations not represented in the calibration. This is the most valuable property of a robust NIR calibration model.

The method that is reported in this work determines water content (weight percent) using NIR diffuse reflectance spectroscopy in the entire NIR region. It was demonstrated to be an equivalent method to the reference KF titrimetric method currently used for moisture analysis. The robustness and accuracy of the model was demonstrated by its ability to predict the moisture level for future on-line or at-line monitoring during the manufacturing of caspofungin acetate drug substance.

This method shows great potential for future on-line or at-line water determination measurements during the manufacturing of caspofungin. The powder may be in contact with the probe or spectra may be collected through glass or some other type of packaging material. A similar approach could be employed for other hygroscopic drug substances. With the trend toward real-time monitoring and control of the processes, it appears to be advantageous to employ NIR methodologies in moisture and organic solvent determinations.

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