

Application of near infrared spectroscopy in monitoring the moisture content in freeze-drying process of human coagulation factor VIII

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As an important process analysis tool, near infrared spectroscopy (NIRS) has been widely used in process monitoring. In the present work, the feasibility of NIRS for monitoring the moisture content of human coagulation factor VIII (FVIII) in freeze-drying process was investigated. A partial least squares regression (PLS-R) model for moisture content determination was built with 88 samples. Different pre-processing methods were explored, and the best method found was standard normal variate (SNV) transformation combined with 1st derivation with Savitzky–Golay (SG) 15 point smoothing. Then, four different variable selection methods, including uninformative variable elimination (UVE), interval partial least squares regression (iPLS), competitive adaptive reweighted sampling (CARS) and manual method, were compared for eliminating irrelevant variables, and iPLS was chosen as the best variable selection method. The correlation coefficient (R), correlation coefficient of calibration set (R_{cal}), correlation coefficient of validation set (R_{val}), root mean square errors of cross-validation (RMSECV) and root mean square errors of prediction (RMSEP) of PLS model were 0.9284, 0.9463, 0.8890, 0.4986% and 0.4514%, respectively. The results showed that the model for moisture content determination has a wide range, good linearity, accuracy and precision. The developed approach was demonstrated to be a potential for monitoring the moisture content of FVIII in freeze-drying process.

Keywords: Near infrared spectroscopy; freeze-drying; moisture content determination.

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1. Introduction

Human coagulation factor VIII (FVIII), whose relative molecular mass (M_r) is about 300 kD, is synthesized by the liver cells as a plasma protein. FVIII, a large glycoprotein of 2332 amino acids is organized in 6 structural domains: A1-A2-B-A3-C1-C2, is easily cleaved by protease at the B-A3 junction or within the B domain, forming a light chain (A3-C1-C2, M_r about 73 kD) and a heavy chain (A1-A2 and indefinite B domain, M_r about 90–220 kD). Two chains form a metal ion-dependent heterodimer by noncovalent binding.^{1–3} As an important coagulation factor in intrinsic coagulation pathway, FVIII is defective and deficient in hemophilia A patients. Nowadays, quite a lot of hemophilia A patients are treated with a periodical injection of FVIII, which has become a short but essential clinical drug.⁴

Currently, the production of FVIII is acquired mainly through two approaches: extracting from fresh plasma⁵ and gene recombination technique. However, the first method, including the steps of separation, purification, viral inactivation/removal and freeze-drying, is the main procedure to get the FVIII products in China and many other countries. Freeze-drying,⁶ allowing water to sublimate from solid to vapor and excluding from the products, is one of the main downstream process in the manufacturing of FVIII. The moisture content of final product, which has a pivotal impact on the stability of FVIII products, is a major critical quality attribute (CQA) of freeze-drying products.^{7,8} According to the requirements of Chinese Pharmacopoeia (Ch.P. 2010 Edition, Volume III),⁹ the residual moisture must be below 3% in the industrial production of FVIII. Besides, it has been reported that one of the most important parameters affecting the freeze-drying products was moisture content. High-level and zero-level residual moisture can compromise the stability of product and lead to a loss of biological activity.^{10,11} Currently, due to the impossibility of sampling and moisture content determination during manufacturing process, the condition and time of freeze-drying is determined by experience to get qualified products whose moisture content meets the quality requirement. In addition, the method for determining moisture content, Karl Fischer (KF) titration analysis,¹² is laborious, destructive and time consuming. The analysis results are only obtained after complete freeze-drying process, which is lagging behind the manufacturing

process. Moreover, only a few samples are selected to measure the moisture content, and the analysis results might not represent the quality of the entire batch. In order to dynamically monitor the end-point of freeze-drying and control the moisture content in a suitable range, it is momentous to develop an effective and fast quantitative technique for measuring moisture content in manufacturing process. Near infrared spectroscopy (NIRS) might be an ideal choice for this aim.

Based on molecular vibration (combinations and overtone) of hydrogen bound like C–H, N–H and O–H, NIRS, due to its simple, rapid, low-cost and nondestructive nature, has been widely applied in many fields, such as agriculture,¹³ food¹⁴ and petrochemical industry.¹⁵ In pharmaceutical industry, as a powerful process analytical technology (PAT) tool,¹⁶ NIRS has been successfully applied to process monitoring, including blending,¹⁷ granulation,¹⁸ drying,¹⁹ coating²⁰ and so on.^{21,22} Recently, the study on monitoring moisture in freeze-drying by NIRS has attracted considerable attention and many scholars have confirmed the feasibility for monitoring the process of freeze-drying by NIRS.^{7,8,23}

In this paper, the feasibility for moisture content determination of FVIII by NIRS was attempted. First, after different times of freeze-drying, near infrared (NIR) spectra were directly collected without destroying the samples, and the moisture content was measured by KF method. Then, partial least squares regression (PLS-R) was used for establishment of NIR model. To optimize the PLS-R model, several pre-processing techniques were compared and four different variable selection methods, including uninformative variable elimination (UVE),²⁴ interval partial least squares regression (iPLS),²⁵ competitive adaptive reweighted sampling (CARS)²⁶ and manual method, were investigated to seek the informative variables related to moisture content. Additionally, several validation characteristics of NIR model, such as range, linearity, accuracy and precision were also evaluated.

2. Materials and Methods

2.1. Materials

Lyophilized FVIII product (including FVIII, mannitol and many other auxiliary materials) was provided by Shandong Taibang Biological Products Co., Ltd. (Taian, China). Methanol was purchased from

Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). KF reagent was purchased from Tianjin Kermel Chemical Reagent Co., Ltd. (Tianjin, China). The deionized water was obtained from a Millipore Milli-Elix/RiOs ultra-pure water system (Bedford, MA, USA). All reagents were analytical grade.

2.2. Freeze-drying process

Several bottles of lyophilized FVIII were taken in each experiment, which were loaded into a number of 50 mL glass penicillin bottles, then 10 mL deionized water was added into each bottle to dissolve it completely. The potency of FVIII product was about 20 IU/mL. Then, samples were pre-frozen for over 2 h in the -80°C ultra-low temperature freezer. The primary freeze-drying process was carried out in an Alpha 1-2 LDplus freeze dryer (Martin Christ, GER) and the drying time ranged from 12 h to 48 h under about -20°C . Fractional samples with drying time over 24 h were selected to second freeze-drying process (parsing drying) in a VD-53 vacuum drying oven (Binder, GER). The parsing drying was performed at 32°C from 5 h to 24 h. Finally, 88 samples with different moisture contents, which cover all the possible moisture contents in predicting unknown sample, were obtained.

2.3. NIR measurement

Diffuse reflectance spectra were collected by an Antaris II Fourier-transform near infrared spectrometer (Thermo Fisher Scientific, USA) with InGaAs detector. All samples were loaded in penicillin bottles, and the spectra were directly collected through the bottle material after freeze-drying without the transfer of sample. Each sample spectrum was the average of 32 scans with a resolution 8 cm^{-1} and a range from 10,000 to 4000 cm^{-1} , and the diameter of window width of light was 1 cm. The spectra were obtained by integrating sphere module at ambient temperature with air as the reference. Three spots from bottom of the bottle were used to collect the spectra, and every sample spectrum was the average of three spectra.

2.4. Moisture content determination

The residual moisture was measured by KF titration analysis with KF-4 automatic moisture

analyzer (Nanjing Kehuan analytical instrument Co., Ltd, China). The instrument should be checked to make sure it is in normal status before it is operated. Then, methanol was added into the reaction cup with a suitable volume submerged electrode sheet and the stir was turned on. The moisture in methanol was removed before calibration of the instrument. The steps of calibration were as follows: (1) precise amount of $10\ \mu\text{L}$ deionized water was added into the reaction cup; (2) the titration started once the switch was turned on, and its end point would reach automatically; (3) the consumption volume of KF reagent was automatically recorded by instrument; (4) the titer (F) was calculated by using the following Eq. (1).

$$F = W/A, \quad (1)$$

where W was the weight of water (the unit was mg); A was consumption volume of KF reagent (the unit was mL).

After an accurate calibration of the instrument, the residual moisture content in the samples was measured as follows: (1) about 0.2 g sample was precisely weighted and directly added into reaction cup as a solid; (2) titration was begun and the consumption volume of the KF reagent was recorded; (3) moisture content was calculated by using the following Eq. (2).

$$(\text{Moisture content})\% = A \times F/W_s \times 100\%, \quad (2)$$

where A was the same meaning in Eq. (1) (the unit was mL); F was the titer; W_s was the weight of the sample (the unit was mg).

2.5. Data analysis

The quantitative analysis models were built using PLS-R. A total of 59 samples of calibration set and 29 samples of validation set were acquired by SPXY algorithm²⁷ written by Roberto Kawakami Harrop Galvao. Spectra were pre-processed with several different pre-processing techniques, including Savitzky-Golay (SG) smoothing, SG-derivative, standard normal variate (SNV) transformation and multiplicative scatter correction (MSC), to eliminate background and irrelevant information interference. Then, in order to remove the interference of irrelevant variables and improve the prediction performance of the model, four different methods, including UVE, CARS, iPLS and manual method, were applied to select the variables for modeling.

The venetian blinds cross validation²⁸ was carried out to determine the optimal number of latent variables (LVs). The performance of model for quantitative analysis was evaluated by several model parameters, including correlation coefficient (R), root mean square errors of cross-validation (RMSECV) and root mean square errors of prediction (RMSEP).

All data analysis was performed in MATLAB 2010a (Mathworks Inc., USA) and PLS-Toolbox (Eigenvector Research, USA).

3. Results and Discussion

3.1. Measurement of moisture content

In this study, the range of moisture contents in 88 samples, which were measured by KF method, was between 5.3031% and 0.9996%. The specific distribution of moisture contents is shown in Table 1.

3.2. Spectral investigation

Figure 1 shows the raw spectra of 88 samples with different residual moisture contents. By observing

Table 1. Distribution of moisture contents.

Sample sets	Numbers			
	of sample	Range (%)	Mean (%)	SD ^a
Calibration	59	0.9996–5.3031	2.8325	1.0376
Validation	29	1.1101–5.2047	2.9005	0.9860

^aSD standard deviation.

these raw spectra, we could easily find that it is difficult to search the characteristic spectral bands. In addition, the spectra exhibit obvious baseline drift because of scattering of light and physical information of the samples. In view of these problems, the specific spectral transform methods, such as SG smoothing, SG-derivative, SNV and MSC, were all attempted to improve the spectral quality. The purpose of SG smoothing is to remove high frequency noise. Derivatives combined with SG smoothing are used to remove unimportant baseline signal and enhance the spectral resolution. Both SNV and MSC are common methods to eliminate the effect of surface scattering, optical path changes and solid particle size on diffuse reflection spectra. PLS-R models with different pre-processing methods were established to compare with the corresponding results. To find the best spectral pre-processing methods, the model parameters, including R , RMSECV, RMSEP, were compared. As shown in Table 2, the appropriate methods for pre-processing were SNV and 1st derivation with SG smoothing (windows size: 15, polynomial order: 2) owing to higher R , lower RMSECV and lower RMSEP. After the data pre-processing, the spectral baseline drift was effectively eliminated and the spectral resolution was obviously improved (see Fig. 2).

3.3. Establishment of PLS-R model

PLS-R models were built with the variables selected by four methods mentioned above for establishing a simple quantitative analysis model with good predictive ability and robustness. The number of LVs

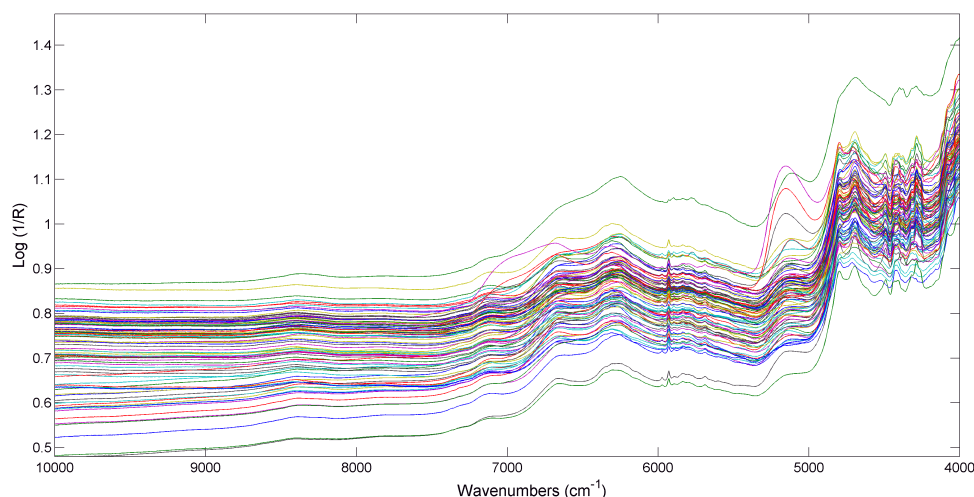


Fig. 1. Raw spectra of 88 samples.

Table 2. Model results with different pre-processing methods.

Pre-processing methods	R	RMSEC (%)	RMSECV (%)	RMSEP (%)	$nLVs^a$
None	0.8276	0.5885	0.7154	0.5339	5
SG 15 ^b	0.8246	0.5938	0.7227	0.5370	5
SNV	0.8608	0.5171	0.6156	0.5174	6
MSC	0.7906	0.6392	0.8256	0.5885	5
1st-derivative+SG	0.8295	0.5770	0.6689	0.5523	3
SNV+1st-derivative+SG	0.9033	0.4233	0.6138	0.4593	5
MSC+1st-derivative+SG	0.9022	0.4256	0.6149	0.4616	5

^a $nLVs$ - number of latent variables.

^bSG-Savitzky-Golay smoothing (windows size: 15, polynomial order: 2).

was determined based on RMSECV calculated by venetian blinds cross validation.

In manual method, characteristic absorption bands of water,²² including the absorption of the first overtone of O–H stretching vibration (6950 cm^{-1}) and the combination of stretching and bending vibrations (5150 cm^{-1}), were both selected for PLS-R model. Table 3 shows the results of PLS-R models built with different regions by manual selection. Model based on the region of $5292\text{--}4933\text{ cm}^{-1}$ has higher R and lower RMSECV than that of full spectrum. However, as compared with the model results of full spectrum, lower R and higher RMSECV are acquired with models based on other regions selected by manual method, and the predictive ability of models cannot be improved. The possible reason for this result is the effect of mannitol, which was added as a protective agent in

freezing process. The regions of characteristic absorbance of O–H were selected as modeling bands by manual selection. However, O–H is rich in mannitol, which affects the predictive ability of the model. So the optimal model cannot be obtained by using characteristic absorbance of O–H.

Table 4 shows the results of PLS-R model established by different variable selection methods. Four different methods could apparently reduce the variables for modeling and obtain higher R and lower RMSECV in comparison with that of full spectrum. However, only iPLS method has obtained lower RMSEP, indicating that this method could effectively improve the predictive ability and the robustness of an optimized model. The gray shaded areas in Fig. 2 are the variable regions selected by iPLS method. The selected spectral regions, including $7471\text{--}7660\text{ cm}^{-1}$, $6121\text{--}6310\text{ cm}^{-1}$, $4964\text{--}5153\text{ cm}^{-1}$

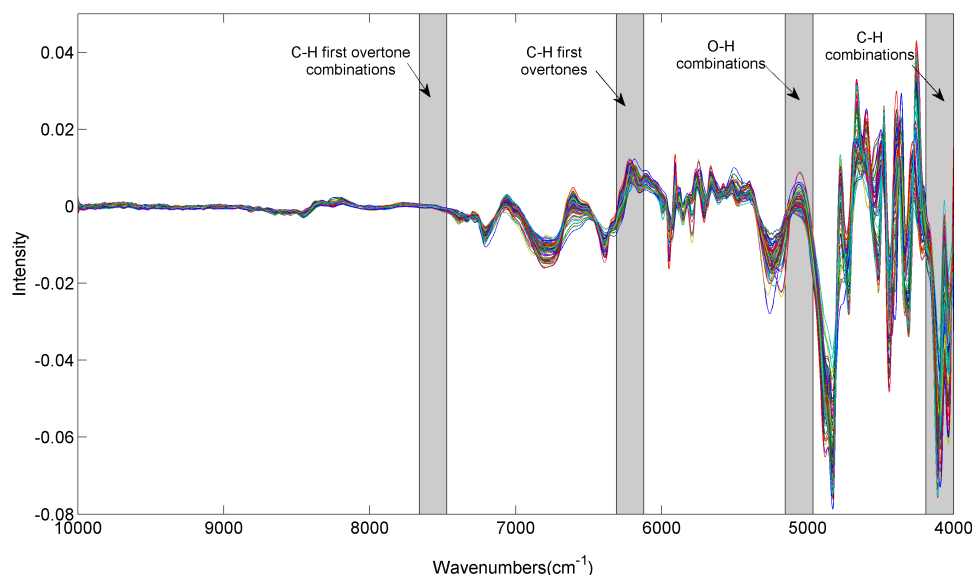


Fig. 2. Pre-processed spectra with SG1st derivative and SNV and variable selection by iPLS (gray shaded area).

Table 3. Model results with different regions by manual selection.

Region	R	RMSEC (%)	RMSECV (%)	RMSEP (%)	$nLVs$
10,000–4000 cm^{-1}	0.9033	0.4233	0.6138	0.4593	5
5292–4933 cm^{-1}	0.9061	0.4023	0.4755	0.4772	8
7239–6692 cm^{-1}	0.7829	0.6143	0.7669	0.6599	6
5292–4933 cm^{-1} and 7239–6692 cm^{-1}	0.8724	0.4925	0.6388	0.5027	6

Table 4. Model results with different variable selection methods.

Variable selection methods	R	RMSEC (%)	RMSECV (%)	RMSEP (%)	$nLVs$	nVs^a
None	0.9033	0.4233	0.6138	0.4593	5	1557
UVE	0.9203	0.3570	0.4771	0.4626	7	270
iPLS	0.9284	0.3327	0.4986	0.4514	7	200
CARS	0.9230	0.3034	0.4020	0.5249	4	46
Manual	0.9061	0.4023	0.4755	0.4772	8	94

^a nVs number of variables for modeling.

and 4000–4189 cm^{-1} , correspond to the absorption of C–H first overtone combinations, C–H first overtones, O–H combinations and C–H combinations, respectively. The regions for modeling cover both characteristic absorption of O–H and C–H. The reason for this was that H_2O was attached to the solid matters, and the change of solid matters absorption arose with different moisture contents. In this study, solid matters contain carbohydrate and protein, which are rich in C–H groups. Figure 3 shows the optimized model results with pre-processed spectra and iPLS variable selection. The red line in Fig. 3 shows the fitted curve between moisture value measured by KF and moisture value

predicted by NIR model, and the green line is the standard curve, whose correlation coefficient is 1. The corresponding R , correlation coefficient of calibration set (R_{cal}), correlation coefficient of validation set (R_{val}), RMSECV and RMSEP were 0.9284, 0.9463, 0.8890, 0.4986% and 0.4514%, respectively.

3.4. Evaluation of the model

3.4.1. Range

Ensuring a relatively wide range to cover the scope of moisture content in the process of freeze-drying is

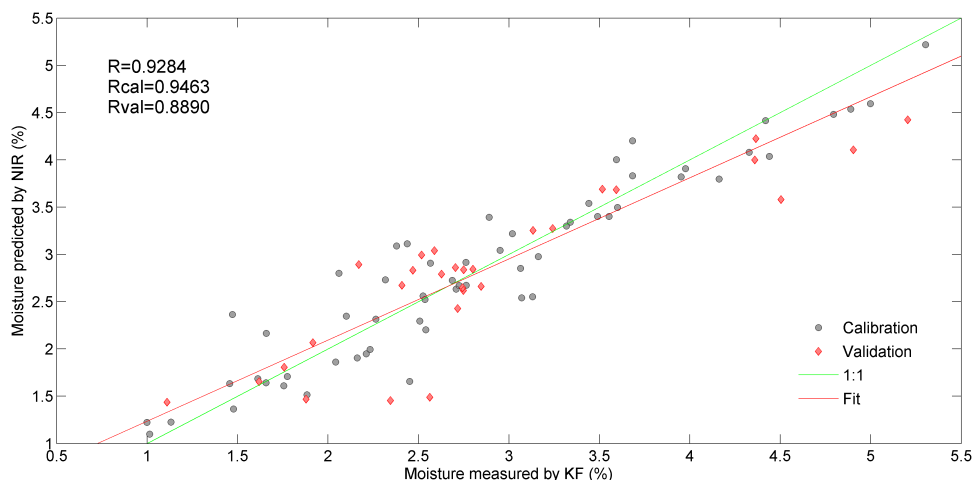


Fig. 3. Relationship between the reference and predicted value.

Table 5. Statistical results of paired t -test.

Sample set	Method	n^a	Mean (%)	SD (%)	t -test (0.05)	
					t	P
Calibration set	NIR	59	2.833	0.982	0.000	1.000
	KF	59	2.833	1.038		
Validation set	NIR	29	2.819	0.861	0.974	0.339
	KF	29	2.900	0.986		

^a n number of samples.

Table 6. Statistical results of precision.

Assay	Predicted value of the established PLS model (%)		
	Sample 1 (3.5164%)	Sample 2 (2.7171%)	Sample 3 (1.6195%)
1	3.6745	2.5829	1.6100
2	3.7173	2.5019	1.5706
3	3.6786	2.4300	1.6324
4	3.5907	2.6511	1.6326
5	3.6933	2.3963	1.6306
6	3.5407	2.5604	1.7387
mean	3.6492	2.5204	1.6358
SD	0.06824	0.09644	0.05577
RSD (%)	1.87	3.83	3.41
χ^2 (0.05, 2)	1.75 (critical value 5.99)		

very critical. In this study, the range of moisture contents that could be measured by NIR model was determined by maximum and minimum value of samples in the calibration set, which was 5.3031% and 0.9996%, respectively. The NIR model could completely meet the requirement of the range of moisture content determination during manufacturing process.

3.4.2. Linearity

The linearity of NIR model was evaluated by the correlation between moisture content measured by KF method and those predicted by NIR model. The correlation coefficient of the optimized model was 0.9284 (see Fig. 3), showing better linearity, which demonstrated that the two methods for determination of moisture content had a good correlation.

3.4.3. Accuracy

To investigate the accuracy of the NIR model, a paired t -test between predicted values (NIR model)

and reference values (KF method) was performed.^{19,29} The analysis was carried out in the calibration set and the validation set, respectively. The statistical result of paired t -test was listed in Table 5. For a significance level of 0.05, t value was also obviously lower than t critical value for both sets. Consequently, it could be concluded that two analytical methods for determination of moisture content had no significant difference, demonstrating good accuracy of NIR model.

3.4.4. Precision

The precision of the model was evaluated by repeatability tests,²⁹ which were performed by repeated measurement of spectra in 10 min. Three samples were selected from the validation set to represent relatively high (3.5164%), medium (2.7171%) and low (1.6195%) moisture contents. Six spectra of each sample mentioned above were collected and the moisture content was calculated by PLS model built above. A chi-square (χ^2) test was performed to evaluate whether the repeatability described by standard deviation belonged to the same population. Under the confidence level of 95%, the χ^2 (0.05, 2) of NIR model was lower than the critical value (see Table 6), which suggested that the standard deviation of repeatability belonged to the same population. Relative standard deviation (RSD) of 3 samples was lower than 5%, showing a good repeatability and validating the precision of model (see Table 6).

4. Conclusions

This paper presented a new method to monitor the moisture content of FVIII using NIRS. After pre-processing of SNV and 1st derivative with SG smoothing as well as the variable selection using iPLS method, the optimized PLS model was finally established for the residual moisture content determination. The results of this paper demonstrated the feasibility of rapid moisture content determination of FVIII. Furthermore, the samples were packed in penicillin bottles, and the spectra were directly collected without the pretreatment of the samples after freeze-drying. Therefore, the study provides a useful reference for the moisture content determination of FVIII, and it may be valuable for the establishment of in-line monitoring system of

FVIII freeze-drying process. Once the in-line monitoring system was built, the time of freeze-drying would be greatly optimized, which will lead to the significantly reduction of the activity loss and the production costs of FVIII.

However, due to the inhomogeneity of the bottom of penicillin bottles, the quality of spectra might be affected and the model will not be optimal. In addition, considering the acquisition of spectra is offline, further work needs to be done for in-line monitoring of freeze-drying process by NIRS in practice.

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