Development and validation of a portable AOTF-NIR measurement method for the determination of Baicalin in Yinhuang oral solution

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Abstract— **The present study was to develop and validate a hand-held AOTF-NIR measurement method enable to accurately determine low dose of Chinese Herbal Medicines (CHM). Yinhuang oral solutions were taken as an example, and an experimental protocol was then followed, involving two operators and independent production lots for data acquisition. On the basis of this protocol, partial least squares regression (PLS) was then carried out. This result in a calibration with standard errors of calibration (SEC) and determination coefficient (R^2) was equal to 146.6 μg/mL and 0.9945. In prediction step, prediction set gave standard errors of prediction (SEP) and R^2 of 87.7 μg/mL and 0.9923, respectively. Furthermore, a novel approach based on accuracy profile was used to validate the PLS model. The resulting accuracy profile clearly showed that PLS model was able to determine baicalin content, which the LLOQ was 2020 μg/mL. It was concluded that the hand-held AOTF-NIR measurement method could be used for complicated low content CHM, paving the way for portable environment.**

Keywords- Near-infrared spectroscopy; Accuracy profile; Yinhuang oral solution; Hand-held

I. INTRODUCTION

Near-infrared (NIR) region is situated between the red band of the visible light and the mid infrared. In the area of NIR (780–2526 nm) mainly vibrations of –CH, –OH, –SH and –NH bonds are observed [1-5]. All the absorption bands are the results of overtones or combinations of the fundamental mid-infrared bands. In recent year, NIR is generally chosen for its speed, low cost and non-destructive characteristic towards industrial analytical application [6-11]. On one hand, the interest in NIR has increased thanks to the instrument improvement and the development of hand-held that allow the portable measurements.

However, the sensitivity and precision of a hand-held NIR instrument is significantly lower than laboratory instrument's. In this paper, a hand-held AOTF-NIR for the analysis of Chinese herbal medicine (CHM) was described. It is fact that active pharmaceutical ingredients (APIs) in CHM are below 1%. Thus, hand-held AOTF-NIR instrument capable of determining the APIs with low content will be beneficial for portable application in CHM.

In this study, a CHM product, Yinhuang oral solution was used as an example and the use of partial least squares regression (PLS) was required to construct the math model. Goodness of fit of PLS model was evaluated according to global statistic quality parameters: low standard errors in cross-validation (SECV), low standard errors of prediction (SEP), high determination coefficient (R^2), and low bias [5, 12, 13].

After the development of PLS model, method validation scrutinizes the accuracy of results by considering both systematic and random errors. A novel approach named accuracy profile was introduced to obtain lower limit of quantification (LLOQ) measurement of the analytical assay from validation data [14-20]. Based on β-expectation tolerance intervals, the accuracy profile demonstrated the ability of PLS model to assess the analytical properties in term of accuracy, trueness, precision, LLOQ, risk and linearity. The aim was first to develop a reliable hand-held AOTF-NIR measurement method to determine baicalin content of Yinhuang oral solution. The second aim was to fully validate the method.

II. BASIC THEORY

The basic idea is the acceptability limit criterion, noted λ. It is assumed that end-users actually expect from an analytical procedure to return a result $\hat{Z}$ which differs of the unknown target value Z of less than λ. This requirement can be express by Eq. (1):

$$\left|Z-\hat{Z}\right|<\lambda \tag{1}$$

A procedure can be validated if it is very likely that the requirement given by Eq. (1) is fulfilled, i.e.:

$$P\left(\left|Z-\hat{Z}\right|<\lambda\right)\geq\beta \tag{2}$$

β being the probability that a future determination falls inside the acceptability limits. It is possible to compute the so-called "β -expectation tolerance interval" (β ETI). The βETI is given by

$$\delta\pm Q_t k_s S_R \tag{3}$$

Q_t is the β quantile of the Student's t-distribution, δ is the bias and k_s is the expansion factor:

$$k_S=\sqrt{1+\frac{1}{pnB^2}} \tag{4}$$

With

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$$B = \sqrt{\frac{R+1}{nR+1}}; \qquad R = \frac{S_B^2}{S_r^2} \tag{5}$$

S^2_B and S^2_r are the estimates of the between conditions variance and the within conditions variance (repeatability). The reproducibility variance, S^2_R is obtained as:

$$S_R^2 = S_r^2 + S_B^2 \tag{6}$$

It can be easily demonstrated that

$$k_s^2 S_R^2 = S_R^2 + \frac{nS_B^2 + S_r^2}{np} \tag{7}$$

The right term of the second member of Eq. (7) is the estimation of the variance of the overall mean that can be assimilated to the bias uncertainty for a nested design with p conditions for experiments and n replications within each condition. Thus

$$k_s^2 S_R^2 = S_R^2 + S_\delta^2 = u^2(Z) \tag{8}$$

And βETI can be given now by

$$\delta \pm Q_t u(Z) \tag{9}$$

Therefore, based on β-expectation tolerance intervals, the accuracy profile made possible a visual and reliable representation of the actual and future performance of the PLS model.

III. MATERIALS AND METHODS

A. Materials

Yinhuang oral solutions were purchased from Jvrong Pharmaceutical Group Co., Ltd. (Jiangsu, China) and deposited in the Key Laboratory of TCM-information Engineering of State Administration of Traditional Chinese Medicine (No.110201). Baicalin reference standards (lot number: 110777–201005) was supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade methanol was purchased from Tedia (USA). Deionized water was purified by Milli-Q water system (Millipore Corp., Bedford, MA, USA).

B. NIR equipment and software

The NIR spectra were collected in the transmission mode using Luminar 5030 Miniature "Hand-held" NIR Analyzer with SNAP software (Brimrose Corporation of America, Baltimore, MD), equipped with a transmission probe (Figure 1). Each spectrum was the average of 500 scans with a wavelength increment of 2 nm. The range of spectra was from 1100 nm to 2300 nm. Each sample was collected three times and the mean of three spectra was used in the following analysis procedure.

C. Reference method

The reference method used for the baicalin determination was the HPLC assay recommended by the Chinese Pharmacopoeia (ChP, 2010 Edition) for Yinhuang oral solution. An Agilent 1100 series HPLC apparatus, equipped with a quaternary solvent delivery system, an auto-sampler and a DAD detector, was used. The concentration of baicalin was analyzed by reverse-phase chromatography on an ODS column (150 mm × 4.6 mm, 5 μm, Waters) with isocratic elution of the mobile phase consisted of methanol, water and phosphoric acid (50:50:0.2,v/v) at a flow rate of 1.0 mL/min. A column temperature of ambient temperature, and detection wavelength at 274 nm was set.

Figure 1. the data collection.

D. Calibration and validation protocol

An experimental protocol was created for the calibration and validation steps in order to obtain a robust model. 9 samples of 3 lots (3 samples per lot) were measured by HPLC method and accurate content of baicalin was obtained in each sample. Then, 9 samples were diluted with water to create a series of baicalin concentration. Thus, 45 samples (belong to 3 lots) were obtained in the calibration set. The validation set was established in the same method as the calibration set (TABLE I).

TABLE I. VARIABILITY SOURCES INCLUDED IN THE CALIBRATION AND VALIDATION SETS

	Calibration set	Validation set
Variability sources	Amount of variability	
Solute	Baicalin	
Solvents	water	
Concentration levels(mg/mL)	5 (1, 2.5, 4, 5, 6.5)	4 (2, 3.5, 4.5, 6)
Operators	2	2
Days	2	2
NIR acquisition	3	3

E. Data pretreatmeats and software

To build the PLS calibration model, different spectra pre-process methods were compared based on the model's performance. Data analysis was performed by the Unscrambler 7.8 (CAMO Software Inc., Norway). The calculation of the accuracy profile based on the external validation set results were given by e. noval V3.0 (Arlenda, Liège, Belgium).

IV. RESULTS AND DISSCUSSION

A. Quantitative analysis of baicalin by HPLC method

HPLC method was recommended by the Chinese Pharmacopoeia for baicalin in Yinhuang oral solution. Figure 2 shows the Chromatograms of baicalin reference standard and Yinhuang oral solution. The retention time of the baicalin in the Yinhuang oral solution was the same with the reference standard. The calibration curve of the HPLC method was investigated before the real samples analysis. The results of the calibration curve exhibited good linearity (R^2 = 0.9990) within the baicalin range from 0.051 to 0.450 μg. It was concluded that the HPLC method satisfied the demand of quantitative analysis. Therefore, the reference values obtained using this method is accurate and can be used in NIR calibration.

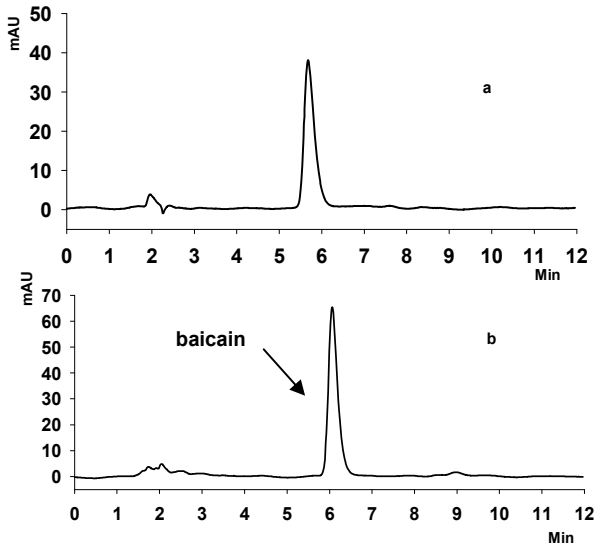

Figure 2. Chromatograms of baicalin reference standard (a) and Yinhuang oral solution (b).

B. Calibration of model

The raw spectra of Yinhuang oral solution show in Figure 3. As seen in the Figure 3, there were some fluctuations appear in the whole spectral region, which registered as a sharp peak in the region of 1369-1625 nm. Furthermore, it is generally known that the spectral pre-processing treatments and the number of latent factors are critical to obtain a quantitative model. To predict the baicalin concentration profile, the calibration model denoted as PLS was built based on different spectral preprocessing treatments, such as standard normal variate (snv) + Savitzky–Golay filter (s.g.), second derivative (2nd), baseline correction (bc) + s.g. The internal cross validation was used with a segment size of four. Raw spectra were obviously superior to others spectral preprocessing method for PLS model (Figure 4).

The number of factors relative to PLS required four. This treatment resulted in a calibration with SEC and R^2 equal to 146.6 μg/mL and 0.9945. In prediction step, prediction set gave

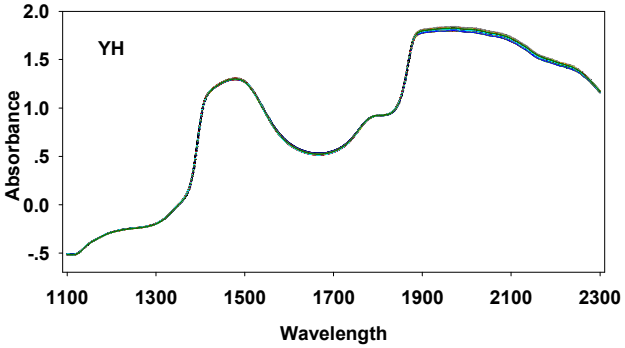

Figure 3. Raw NIR spectra of researched samples

SEP and determination coefficient of 87.7 μg/mL and 0.9923, respectively. The results showed a good agreement between the NIR predictions and the HPLC reference results for both the calibration and validation sets (Figure 5).

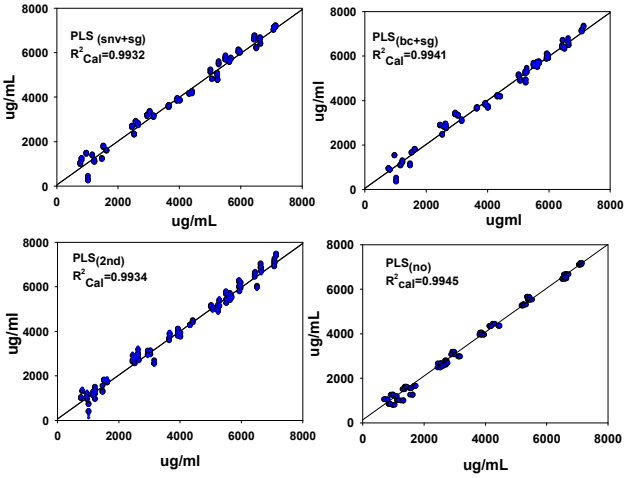

Figure 4. Correlation diagram of baicalin calibration models by different pre-processed methods.

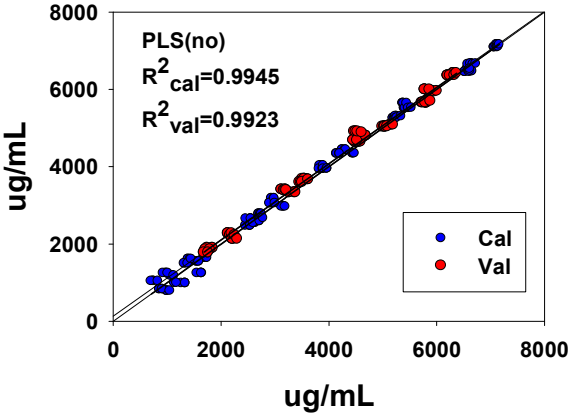

Figure 5. Baicalin NIR predictions versus the reference method results with raw spectra. X axis represents reference method results; Y axis represents NIR prediction value.

C. *Validation the models*

In order to obtain the accuracy and precision, the model predictive performance was evaluated with accuracy profile. This innovative approach used tolerance intervals as statistical methodology that the acceptance limits were set at ±15% while the maximum risk to obtain results outside these acceptance limits was set at 10%. Figure 6 displayed the accuracy profile computed with the external validation set results. As seen in Figure 6, the validation results were different from the ones displayed in Figure 5. It could be seen that model was relative stable and points in the line of acceptance limits from a concentration content ranging from 2 mg/mL to 6 mg/mL.

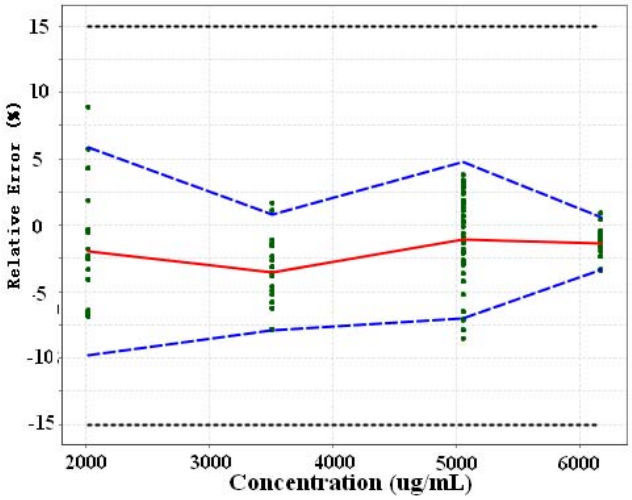

Figure 6. Accuracy profiles of PLS model. The plain line is the relative bias, the dashed lines are the β-expectations tolerance limits (β= 90%) and the dotted lines represent the acceptance limits (±15%).

TABLE II. ICH Q2(R1) VALIDATION CRITERIA OF PLS CALIBRATION MODEL

Trueness	Level (μg/mL)	Mean introduced concentration (μg/mL)	Relative bias (%)
	1979	2020	-2.009
	3384	3507	-3.513
	4997	5053	-1.113
	6081	6167	-1.379
Precision	Level (μg/mL)	Repeatability (RSD %)	Intermediate precision (RSD %)
	1979	4.273	4.425
	3384	2.484	2.484
	4997	3.465	3.465
	6081	1.063	1.115
Accuracy	Level (μg/mL)	Relative β-expectation tolerance limits (%)	Risk (%)
	1979	[-9.827 , 5.808]	0.5254
	3384	[-7.860 , 0.834]	0.008
	4997	[-6.989 , 4.762]	0.014
	6081	[-3.356 , 0.598]	0
LOQ	Lower LOQ (μg/mL)		Upper LOQ (mg/mL)
	2020		6167

In addition, Table II showed the ICH Q2(R1) validation criteria of the PLS method. The bias did not exceed ± 4%. The precision of the method was estimated by measuring repeatability and intermediate precision at the four concentration levels investigated. The repeatability and intermediate precision improved with growing baicalin content from the 1979 μg/mL content level. The risk was 0.5 for the 1979 μg/mL content level whereas it was 0 for the upper level.

The linear profile of the PLS model as a function of the introduced concentrations was shown in Figure 7. The regression equation was expressed as y = -59.06 + 0.9970 x with R^2=0.9920. The linearity of the results demonstrated that the β-expectation tolerance limits were included in the absolute acceptance limits for hand-held AOTF-NIR measurement method. From this point of view, the prediction result of PLS model in hand-held AOTF-NIR instrument showed good feasibility in application of Yinhuang oral solution.

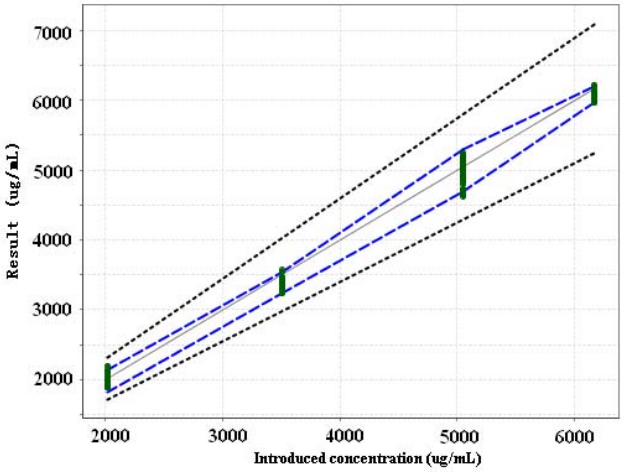

Figure 7. Linear profile of the PLS model. The dashed limits on this graph correspond to the accuracy profile, i.e. the β-expectation tolerance limits expressed in absolute values. The dotted curves represent the acceptance limits at ±15% expressed in the concentration unit. The continuous line is the identity line y = x.

D. *Uncertainty assessment*

Table III presented several uncertainty results of PLS model. The uncertainty is a parameter associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurement. The uncertainty of bias of the method at each concentration level of the validation standard, the uncertainty which combines the uncertainty of the bias with the uncertainty of the method obtained during the validation step, i.e. the intermediate precision standard deviation, and the expanded uncertainty which equals to the uncertainty multiplied by a coverage factor k = 2 representing an interval around the results where the unknown true value can be observed with a confidence level of 90%.

In addition, the relative expanded uncertainties (%) with the corresponding introduced concentrations were not higher than 10%, which means that with a confidence level of 90%, the unknown true value is located at a maximum of ± 10% around the measured result. For the upper level, the relative expended uncertainty was above 2.3%.

TABLE III. IESTIMATES OF MEASUREMENTS UNCERTAINTIES RELATED TO THE BAICALIN CONCENTRATION IN THE YINHUANG SOLUTION AT EACH CONCENTRATION LEVEL INVESTIGATED.

MIC (μg/ml)	UB (μg/ml)	U (μg/ml)	EU (μg/ml)	REU (%)
2020	21.11	91.83	183.7	9.094
3507	17.78	88.91	177.8	5.070
5053	25.27	176.9	353.8	7.002
6167	16.91	70.81	141.6	2.297

*Mean introduced concentration (MIC); Uncertainty of the bias (UB); Uncertainty(U); Expanded uncertainty(EU); Relative expanded uncertainty (REU).

V. CONCLUSIONS

Our study confirmed that hand-held NIR instrument could be used attributed to high accuracy and precision. Different pretreatment methods were used to construct the PLS model, then the model enable to quantify baicalin were developed. The PLS model was successfully validated baicalin ranged from 2020 μg/mL to 6167 μg/mL. The accuracy profile on the validation results demonstrated the accuracy of the NIR method, which LLOQ was 2020 μg/mL. Based on the present feasibility study, Hand-held AOTF-NIR measurement method has allowed the low concentration being determined by high accuracy and precision. The present quantitative method illuminated the hand-held NIR instrument could be used in complicated low content CHM system, paving the way for portable environment.

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