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Validation of a NIR quantification method for the determination of chlorogenic acid in *Lonicera japonica* solution in ethanol precipitation process

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ABSTRACT

The feasibility of near-infrared spectroscopy (NIRS) for chlorogenic acid content analysis in ethanol precipitation process of water extract of *Lonicera japonica* was verified in this work. A calibration and validation set was designed for the conception and evaluation of the method adequacy. An experimental protocol was then followed, involving two different NIR instruments for data acquisition. On the basis of this protocol, the model was developed based on partial least squares regression (PLS) and the determination coefficient (R^2_{cal} and R^2_{val}), standard error of calibration and prediction (SEC and SEP) were 0.9962, 0.9955, 111.1 μg/mL and 107.1 μg/mL for Holographic Grating NIR instrument, and 0.9984, 0.9971, 53.6 μg/mL and 83.3 μg/mL for Fourier Transform NIR instrument. However, such above criteria did not clearly demonstrate the model's prediction error over each analyzed content range. Consequently, a novel approach based on accuracy profile which allowed the acquisition of the lower limit of quantification (LLOQ) was used to validate the robustness and accuracy of PLS model. The resulting accuracy profile showed that PLS model was able to determine chlorogenic acid content by two NIR systems, whose LLOQ was about 1550 μg/mL. It was concluded that the two NIR systems were suitable for use as Process Analytical Technology (PAT) to understand ethanol precipitation process of water extract of *Lonicera japonica*.

1. Introduction

With the issuance of Process Analytical Technology (PAT) guidance for industry in September 2004, the FDA is encouraging pharmaceutical manufacturers to adopt new technologies for timely measurement of critical product and process attributes in the manufacturing environment. Near-infrared spectroscopy (NIRS) has been demonstrated as one of many efficient tools used in a PAT capacity for real time monitoring of quality parameters, providing valuable information and ultimately to ensure better in-process quality control in synthetic medicine field [1].

However, it is well known that NIRS has high detection limit and low sensitivity [2,3], and thus a NIR model being able to quantify active pharmaceutical ingredients (API) in a pharmaceutical formulation should be developed and validated. Accuracy profile is proposed as an efficient and reliable validation method to evaluate the quantitative performance of the model by considering systematic and random errors. Based on β-expectation tolerance intervals, accuracy profile gives a lower limit of quantification (LLOQ) measurement of the validated assay [4]. In previous studies, a NIR model was validated for the quantitative determination of API or moisture in synthetic medicine field by accuracy profile (Table shows in supplementary material) [5–9]. The validation result demonstrated that in synthetic medicine field the NIR model had robust and accurate performance due to relative high-dose API content.

Chinese Herbal Medicine (CHM) has its own characteristic compared with synthetic medicine. One of the most important differences is the lower-dose API content in CHM, which is below 1%. In recent years, a number of studies have reported the determination of API in CHM by NIR [10–16]. Conventionally, a robust NIR model can be evaluated according to the following criteria: low standard error of calibration (SEC), low standard error of prediction (SEP), high determination coefficient (R^2), and low bias. Nevertheless, those criteria do not allow the assessment of the model ability to quantify accurately over the entire content range especially for CHM. Thus, an analysis method capable of validating the prediction accuracy and obtaining the LLOQ of NIR model in low-dose API will be beneficial for NIR application in CHM.

Ethanol precipitation is well-known as an important process in CHM product, which is used to extract API from concentrated liquid and precipitate most polysaccharides, proteins, lipids, etc. The efficiency of this process can be affected by a lot of factors, like the

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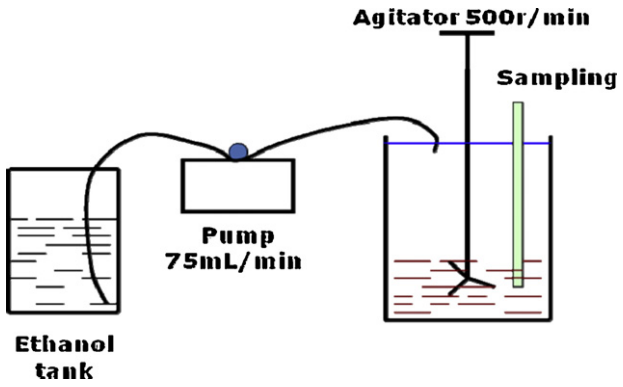

Fig. 1. A schematic of ethanol precipitation process.

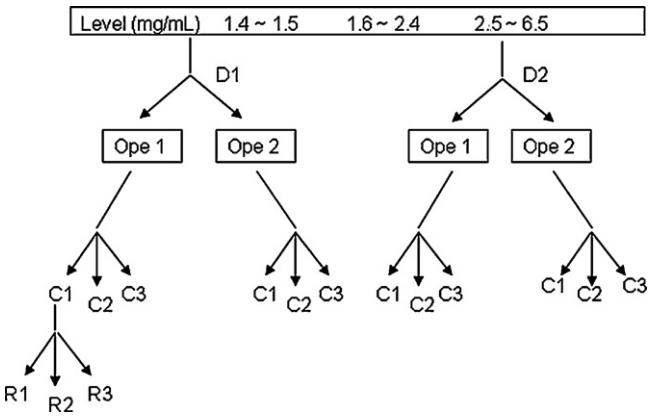

Fig. 2. Schematic illustration of the calibration/validation protocol. D means day; Ope represents operator; C represents independent series; R represents different NIR measurements of the same sample (repetitions).

final ethanol concentration, the speed of stirring, the initial density of the extract, the temperature and the pH of liquid, etc. Therefore, an efficient monitoring of ethanol precipitation process can ensure product quality and guide further optimization of this process. Traditionally, ethanol precipitation process has been monitored using manual procedures, and the result of precipitation process is characterized in terms of the transfer rate of API, the yield and the purity of extract in the supernatant. These techniques are time-consuming and occasionally call for experienced analysts and/or technicians [17].

To the best of our knowledge, few studies have been reported on the determination of API in ethanol precipitation process in CHM system by NIRS. Additionally, the validation of NIR method in CHM by accuracy profile has not been reported before. In the present work, the chlorogenic acid content of *Lonicera japonica* in ethanol precipitation process was monitored by two types of NIR instruments combined with partial least squares regression (PLS) model. The prediction accuracy of PLS model was necessary to be validated because chlorogenic acid content in ethanol precipitation process of water extract of *L. japonica* was very low. The validated result illuminated the feasibility of the NIR method, and provided a guideline of NIR accurate measurement in ethanol precipitation process in CHM system.

2. Materials and methods

2.1. Materials

L. japonica was purchased from Ben Cao Fang Yuan Medicine Co., Ltd. (Beijing, China), and deposited in the Key Laboratory of TCM-information Engineering of State Administration of Traditional Chinese Medicine (No. 110402). Chlorogenic acid reference standard (lot number: 110753–200413) 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).

2.2. Ethanol precipitation process

L. japonica was extracted with 10-fold water decocting twice, once per hour, and condensed into 0.5 g of crude drug per milliliter. The condensed liquid was gradually precipitated by adding ethanol for 20 min, and the final ethanol/water ratio was constant, which was 75% (Fig. 1). Samples collected at different time were centrifuged at 5976 g. The supernatant liquid was analyzed by NIR and HPLC. The concentration ranged from 1400 μg/mL to 6500 μg/mL.

2.3. NIR equipment and software

The NIR spectra were collected in the transmission mode using the XDS rapid liquid analyzer with VISION software (Foss Holographic Grating (HG) NIR System, Silver Spring, MD, USA). Each spectrum was the average of 32 scans with a wavelength increment of 0.5 nm. The range of spectra was from 400 nm to 2500 nm. Another NIR system, Fourier Transform (FT) NIR was used with Thermonic resulting software (Thermo Nicolet Corporation) over a wavenumber range 4000–10,000 cm^{-1}, using 32 scans, 16 cm^{-1} resolution per spectrum and recorded as absorbance with air as the reference standard. The sample was hold in a circular sample cuvette with solid cap (8 mm in diameter). Each sample was analyzed three times and the mean of three spectra was used in the following analysis.

2.4. Reference method

The reference method used for the chlorogenic acid determination was HPLC assay recommended by the Chinese Pharmacopoeia (ChP, 2010 Edition) for *L. japonica*. 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 chlorogenic acid was analyzed by reverse-phase chromatography on an ODS column (250 mm × 4.6 mm, 5 μm, Agilent) with isocratic elution of the mobile phase consisted of methanol, water with 0.1% formic acid (20: 80, v/v) at a flow rate of 1.0 mL/min. A column temperature of ambient temperature and detection wavelength at 327 nm were set.

2.5. Calibration and validation protocol

An experimental protocol was created for the calibration and validation sets in order to obtain a robust model (Fig. 2). To obtain robust NIR model, we repeated the experiment six times using different batches of *L. japonica* solution containing 120 samples. 60 samples from 3 series of ethanol precipitation process were included in the calibration set. Then the other 3 new series were used as the validation set. The validation set was built with the same method as the calibration set.

2.6. Calibration of models

To build PLS calibration model, the optimum preprocessing method was selected based upon the lowest SEC, prediction residual error-sum squares (PRESS) and standard error of cross

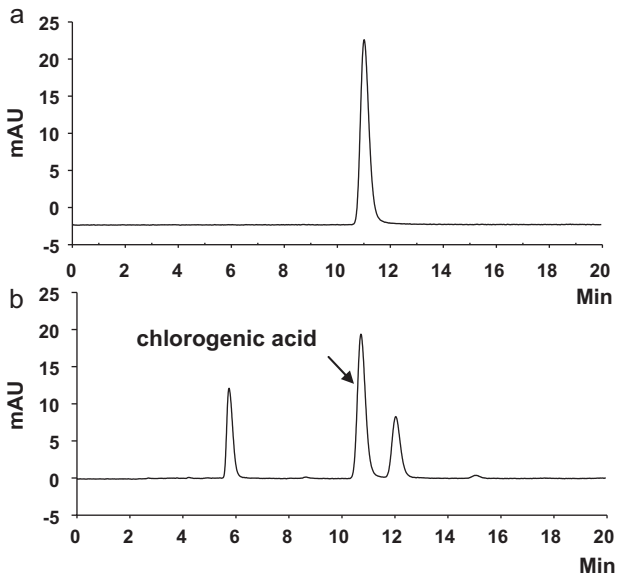

Fig. 3. The chromatograms of chlorogenic acid reference standard (a) and a sample solution in ethanol precipitation process (b).

validation (SECV). Cross validation was executed with a segment size of four, and a PRESS plot was produced. Usually, the first minimum value on the PRESS plot is used to determine the optimum number of factors with the best prediction for the cross validation samples. Data analysis was performed by TQ analyst software package (Version 8.0, Thermoscientific, Madison, USA) and VISION software (Version 3.5, Foss, Silver Spring, MD, USA). The calculation of accuracy profile based on the external validation set results was given by e. noval V3.0 (Arlenda, Liège, Belgium).

3. Results and discussion

3.1. Quantitative analysis of chlorogenic acid by HPLC method

HPLC method was carried out under the regulation of Pharmacopoeia of the People's Republic of China, vol. 1(2010 Edition). It was concluded that the method satisfied the demand of quantitative analysis. Therefore, the reference values obtained using this method were accurate and could be used in NIR calibration model. Fig. 3 shows typical HPLC chromatograms of chlorogenic acid and that of a sample during ethanol precipitation process. The retention time of the chlorogenic acid in sample solution of ethanol precipitation process was the same with the reference solution. The calibration curve of the HPLC method was investigated before the

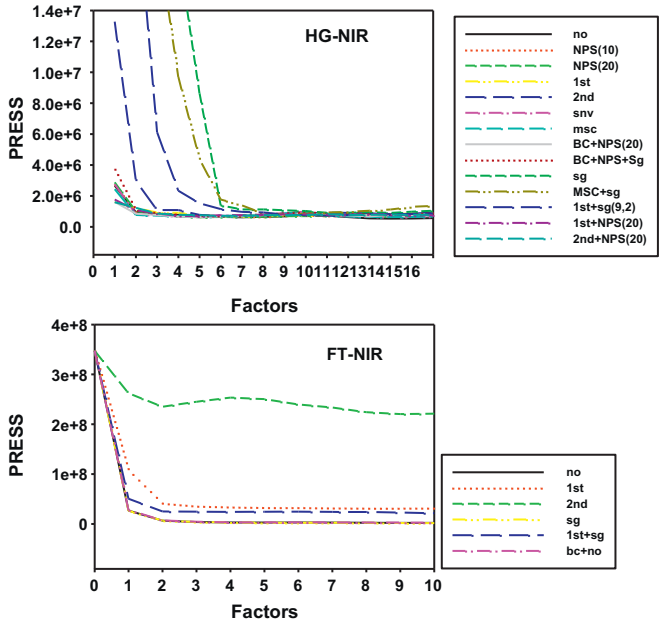

Fig. 5. Effect of number of PLS latent factors on PRESS values for two NIR instruments.

real sample analysis. The calibration curve exhibited good linearity (R^2 = 0.9990) within the line range from 0.0074 μg to 0.026 μg (Data show in supplementary material).

3.2. Comparison of different spectral pretreatment methods

PLS is a full-spectral calibration method and has a built-in capacity to deal with over-determined problem of full-spectrum calibration. The raw NIR spectra of sample solutions are shown in Fig. 4. As seen in the raw spectra, there were large fluctuations in the region of combinations of fundamental vibrations for HG-NIR spectra. Therefore, the spectra region of 1100–1900 nm was selected for HG-NIR and full-spectra (4000–10,000 cm^{-1}) for FT-NIR (It should be noted that the unit conventionally used in HG-NIR and FT-NIR are different). Furthermore, in PLS model, it is generally known that the spectral preprocessing treatments and the number of latent factors are critical parameters. The optimum number of latent factors is determined by the lowest PRESS value. Fig. 5 shows on the PRESS as a function of latent factors for determining chlorogenic acid contents vs different spectral preprocessing methods. Baseline correction (BC, 1800 nm), N-point smooth (NPS, segment: 20 nm) for HG-NIR system and no spectral preprocessing treatment for FT-NIR system

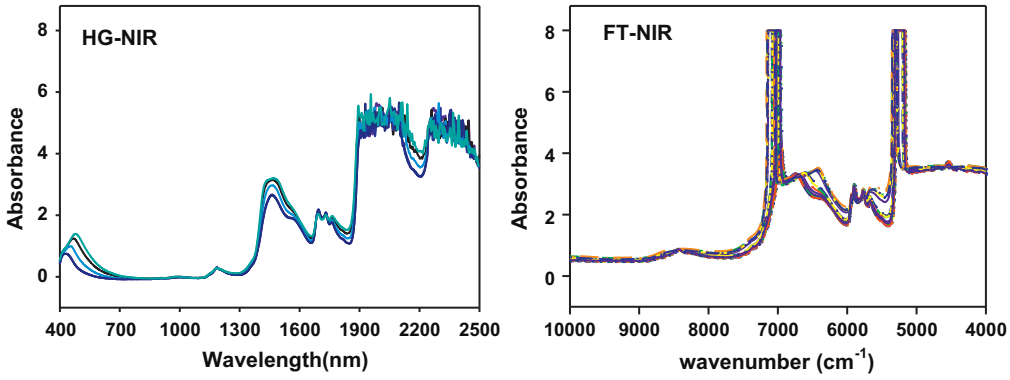

Fig. 4. Raw spectra of *Lonicera japonica* solution in ethanol precipitation process for two NIR instruments.

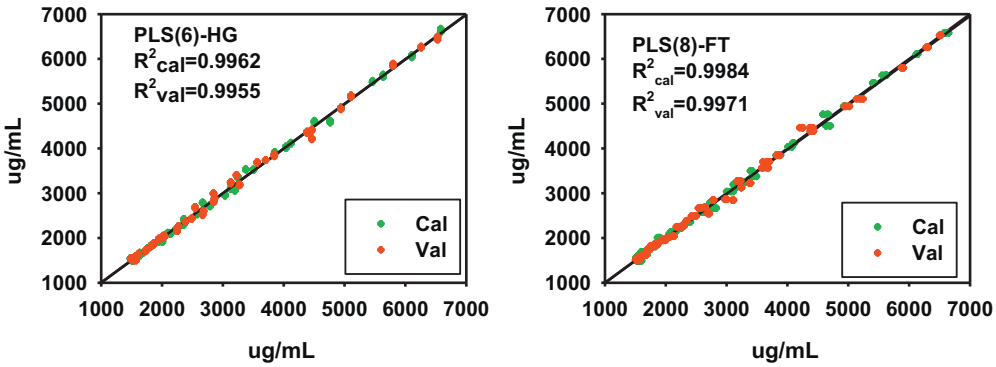

Fig. 6. Chlorogenic acid NIR predictions versus the reference method results for two NIR instruments. *X*-axis represents reference method result; *Y*-axis represents NIR prediction value.

were superior to other spectral preprocessing method for PLS model.

3.3. Prediction results of the NIR method

In order to build robust PLS model, the calibration set had to contain variability that the model might meet the future routine analysis of *L. japonica* solution in ethanol precipitation process. Therefore, 3 sources of variability containing ethanol concentration, stirring speed and stirring time were integrated in the calibration set. To predict chlorogenic acid concentration profile, the optimum calibration denoted as PLS was built based on HG-NIR system and FT-NIR system.

Firstly, for HG-NIR system, the number of factors required for PLS was six. The SEC, SECV, and R^2 were 111.1 μg/mL, 152.1 μg/mL, and 0.9962, respectively. The calibration subjected to prediction gave SEP and R^2 value of 107.1 μg/mL and 0.9955 (Fig. 6, HG-NIR). Secondly, for FT-NIR system, the PLS calibration was developed using eight latent factors. The SEC, SECV, and R^2 were 53.6 μg/mL, 70.3 μg/mL, and 0.9984, respectively. In prediction process, the SEP and R^2 were 83.3 μg/mL and 0.9971 (Fig. 6, FT-NIR). The results of the two instrument systems showed a good agreement between the NIR predictions and HPLC results for both the calibration and validation sets.

3.4. Validation of the models

Based on above criteria, it was not enough to assess the PLS model to quantify API over the whole chlorogenic acid content range. Thus, accuracy profile was used to finally evaluate the quantitative performance of the two PLS models in order to obtain the analytical properties such as accuracy, trueness, precision, LLOQ, range and linearity. Fig. 7 depicts accuracy profile result obtained with two NIR systems. The acceptance limits were set at ±10% while the maximum risk to obtain results outside these acceptance limits was set at 5%. As seen from Fig. 8, these β-expectation tolerance limits were fully included within the ±10% acceptance limits. Therefore, each future result had at least 95% probability to fall within the ±10% acceptance limits. Furthermore, PLS model (FT-NIR) was relative stable, while the model (HG-NIR) showed that the prediction results of some samples had large bias, whose relative errors were above 10%. Nevertheless, the relative errors still satisfied the analysis needs.

Table 1 shows the ICH Q2(R1) validation criteria of PLS model for HG-NIR. As seen from table, the repeatability in R.S.D.% presented similar change trend with the intermediate precision in R.S.D.%. The repeatability and intermediate precision of the lowest concentration level were 4.49% and 4.60%, relatively large but still satisfactory. Furthermore, the relative bias was less than ±1.25%, low and stable. The same perfect result was also obtained by FT-NIR system in term of accuracy, trueness, precision, LLOQ, range and linearity etc (Table 2).

The linear profile of PLS model is shown in Fig. 8. A linear regression model was fitted on the results as a function of the introduced concentrations in order to obtain the following equations: for HG-NIR, the regression equation was expressed as $y = 5.892 + 0.9976x$ with $R^2 = 0.9906$; for FT-NIR, the regression equation was $y = -13.66 + 1.010x$ with $R^2 = 0.9959$. The linearity of

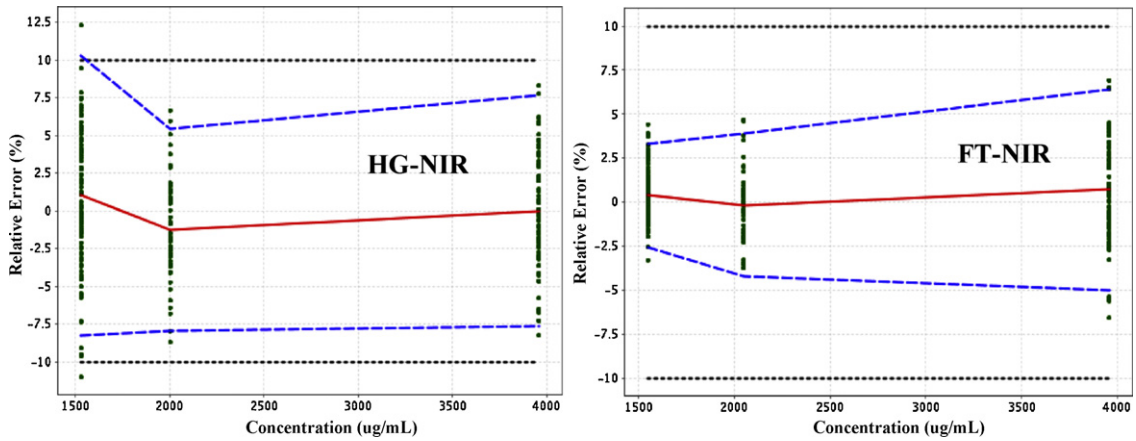

Fig. 7. Accuracy profile of PLS model. The plain line is the relative bias, the dashed lines are the β-expectations tolerance limits ($\beta = 95\%$) and the dotted lines represent the acceptance limits (±10%).

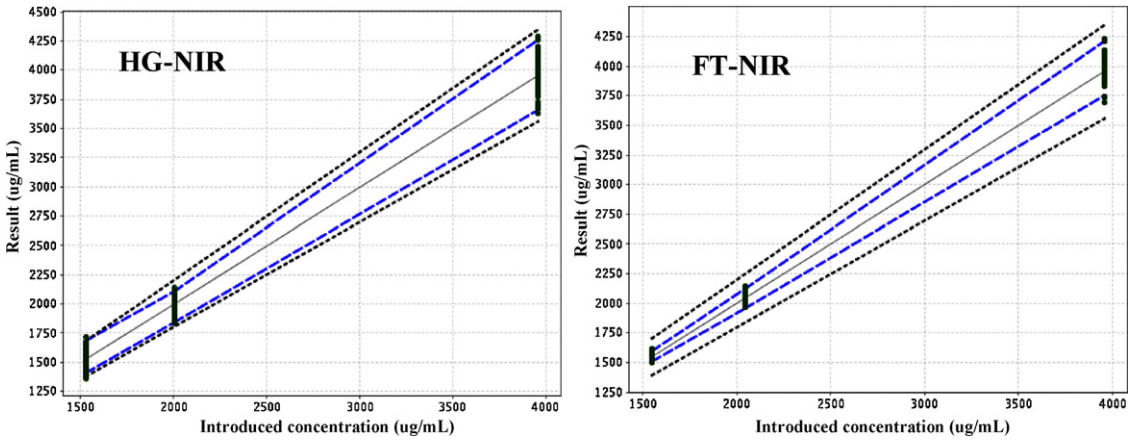

Fig. 8. Linear profile of the PLS model. The dashed limits on this graph correspond to the accuracy profile, i.e. the β-expectation tolerance limits expressed in absolute values (β = 95%). The dotted curves represent the acceptance limits at ±10% expressed in the concentration unit. The continuous line is the identity line $y = x$.

the results demonstrated that the β-expectation tolerance limits were included in the absolute acceptance limits for two NIR instruments. Furthermore, it is well known that the slope and intercept are close to 1 and 0 respectively confirming the absence of proportional and constant systematic error of the model. From this point of view, the prediction result of PLS model in HG-NIR instrument showed larger systematic error than the result in FT-NIR instrument.

3.5. Uncertainty assessment

Table 3 presents several uncertainty results of PLS model for two NIR instruments: the uncertainty of bias of the PLS model at each concentration level of the validation standard, the uncertainty which combined the uncertainty of the bias with the uncertainty of the PLS model obtained during the validation step, i.e. the intermediate precision standard deviation, and the expanded uncertainty which equaled to the uncertainty multiplied by a coverage factor k(k = 2)representing an interval around the results where the unknown true value of chlorogenic acid in *L. japonica* solution in ethanol precipitation process could be observed with a confidence level of 95%.

Table 1
ICH Q2(R1) validation criteria of PLS calibration model for HG-NIR.

Trueness

Region (μg/mL)	Mean introduced concentration (μg/mL)	Relative bias (%)
1.40–1.59	1531	1.01
1.60–2.49	2003	−1.25
2.50–6.50	3956	0.01

Precision

Region (μg/mL)	Repeatability (RSD %)	Intermediate precision (RSD %)
1.40–1.59	4.49	4.60
1.60–2.49	3.19	3.28
2.50–6.50	3.82	3.82

Accuracy

Region (μg/mL)	β-Expectation tolerance limits (μg/mL)	Risk (%)
1.40–1.59	[1405,1688]	3.83
1.60–2.49	[1844,2111]	0.63
2.50–6.50	[3654,4259]	1.10

LOQ

Lower LOQ (μg/mL)	Upper LOQ (μg/mL)
1556	3956

Table 2
ICH Q2(R1) validation criteria of PLS model for FT-NIR.

Trueness

Range (μg/mL)	Mean introduced concentration (μg/mL)	Relative bias (%)
1.40–1.59	1531	0.49
1.60–2.49	2003	−0.23
2.50–6.50	3956	0.71

Precision

Range (μg/mL)	Repeatability (RSD %)	Intermediate precision (RSD %)
1.40–1.59	1.38	1.38
1.60–2.49	1.94	1.96
2.50–6.50	2.86	2.86

Accuracy

Range (μg/mL)	β-Expectation tolerance limits (μg/mL)	Risk (%)
1.40–1.59	[1496,1581]	0.00
1.60–2.49	[1919,2078]	0.00
2.50–6.50	[3757,4211]	0.11

LOQ

Lower LOQ (μg/mL)	Upper LOQ (mg/mL)
1531	3956

Table 3
Estimates of measurements uncertainties related to the chlorogenic acid concentration by two NIR technologies at each concentration level investigated. Mean introduced concentration (MIC); uncertainty of the bias (UB); uncertainty (U); expanded uncertainty (EU); relative expanded uncertainty (REU).

MIC (μg/mL)	UB (μg/mL)	U (μg/mL)	EU (μg/mL)	REU (%)
1531[a]	10.47[a]	71.18[a]	142.4[a]	9.30[a]
2003[a]	11.38[a]	66.70[a]	133.4[a]	6.66[a]
3956[a]	16.89[a]	152.0[a]	304.0[a]	7.68[a]
1531[b]	2.38 [b]	21.30[b]	42.60[b]	2.78[b]
2003 [b]	5.723 [b]	39.68[b]	79.36[b]	3.96[b]
3956 [b]	12.66 [b]	113.9[b]	227.9[b]	5.76[b]

[a] Represents HG-NIR result.
[b] Represents FT-NIR result.

In addition, the relative expanded uncertainties (%) with the corresponding introduced concentrations were not higher than 10% for HG-NIR instrument and FT-NIR instrument, which meant that with a confidence level of 95%, the unknown true value of chlorogenic acid was located at a maximum of ± 10% around the measured result. It illustrated that the PLS model presented low relative expended uncertainty for the two instruments.

4. Conclusions

The PLS model based on FT-NIR and HG-NIR was built in order to determine the chlorogenic acid content in *L. japonica* solution in ethanol precipitation process. The results including both the calibration and validation sets showed a good agreement between the NIR predictions and HPLC results for two types of NIR system. The PLS model was validated using a new approach based on the accuracy profile. Indeed, according to the calculated tolerance interval, each future result of PLS model would be included within the ±10% acceptance limits with a probability of at least 95%. Finally, the combination of PLS model and accuracy profile method was successfully used for accurate determination of chlorogenic acid content in *L. japonica* solution in ethanol precipitation process.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2011.12.005.

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