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Application of near-infrared spectroscopy for the rapid analysis of *Lonicerae Japonicae Flos* solution extracted by water

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A rapid quantitative analytical method for three components of *Lonicerae Japonicae Flos* solution (*Lonicera Japonica* Thumb.) extracted by water was developed using near-infrared (NIR) spectroscopy and the partial least-squares (PLS) method. The NIR spectra of 81 samples collected from a production line were obtained. The concentrations of secologanic acid, chlorogenic acid and galuteolin were determined by using high-performance liquid chromatography-diode array detection as the reference method. Several pretreatment methods for the NIR spectra were used during PLS calibration. The most appropriate latent variable number of the PLS factor was selected based on the standard error of cross-validation (SECV). The performance of the final PLS models was evaluated according to SECV, standard error of prediction (SEP) and determination coefficient (R^2). The compounds secologanic acid, chlorogenic acid and galuteolin had SEP values of 0.030, 0.061 and 1.668 μg/mL, respectively and R^2 values over 0.85. This work shows that NIR spectroscopy is a rapid and convenient method for the analysis of *Lonicerae Japonicae Flos* solution extracted by water. The proposed method can help in the application of process analytical technology in the pharmaceutical industry, particularly in traditional Chinese medicine injections.

Keywords: *Lonicerae Japonicae Flos*; Qingkailing injection; near-infrared; partial least-squares; rapid analysis.

1. Introduction

Qingkailing (QKL) injection is widely used in traditional Chinese medicine to treat upper respiratory tract infections and serious influenza.[1,2] The drug has shown satisfactory efficacy as a defense against serious acute respiration symptom in 2003 and A/H1N1 flu since 2009. However, recent reports on the adverse drug reactions of QKL injection have necessitated improvements in its quality. Several articles have reported various analytical methods for the raw medicinal materials and end products in QKL injection.[3–8] However, analytical studies on the extraction process of QKL injection, especially specific for *Isatidis Radix, Lonicerae Japonicae Flos* or Gardeniae Fructus, are lacking. Thus, the development of a rapid and accurate method for analyzing such components is necessary.[9,10]

Process analytical technology (PAT) for the pharmaceutical industry was put forward by the US FDA to obtain quality information on products in a timely manner or as close to real time as possible. Many PAT methods have been introduced to guarantee the quality and consistency of products.[11,12] As regards PAT research on traditional Chinese medicine injections (TCMIs), two articles have reported the use of ultraviolet (UV) spectroscopy for the rapid analysis of QKL injection intermediates.[13,14]

Near-infrared (NIR) spectroscopy, especially online NIR spectroscopy, is an ideal alternative method for such purpose. NIR spectroscopy has been widely used in many analytical areas, such as food and pharmaceuticals.[15,16] This method is more useful than other analytical techniques because of its rapid speed, accurate prediction, eco-friendliness and low cost.[17–21]

This work aims to establish an NIR method for the rapid determination of the concentrations of three active ingredients, chlorogenic acid, secologanic acid and galuteolin, of *Lonicerae Japonicae Flos* solution extracted by water using high-performance liquid chromatography-diode array detection (HPLC-DAD) as the reference method.

2. Experimental Methods

2.1. *Sample preparation*

Samples were obtained from a TCM pharmaceutical factory (Shineway Pharmaceutical Co., Ltd., Shijiazhuang, China). Approximately 10 mL of the solution extracted by water was used as the test sample in the liquid preparation of the QKL injection. The test samples mentioned above were obtained from a production line in five batches. A total of 81 samples were collected.

2.2. *HPLC analysis*

A gradient elution HPLC method was established to determine the concentrations of the three active compounds. An Agilent 1100 HPLC system (Agilent Technologies, USA) comprising a vacuum degasser, a quaternary pump, an autosampler, a thermostatic column compartment and a DAD was used. Separation was performed on a Phenomenex Luna C_{18} column (4.6 mm × 250 mm with 5 μm particle size) at 30°C. The mobile phase consisted of (A) 0.1% HCOOH–H_2O and (B) acetonitrile (v/v). The gradient program is shown in Table 1. The flow rate of the mobile phase was 0.5 mL/min. The detection wavelengths for secologanic acid, chlorogenic and galuteolin were set to 240, 325 and 350 nm, respectively, because these components possess satisfactory UV absorption. The chemical structures of the three analytes are shown in Fig. 1. The samples in this research were diluted at a ratio of 1:5 (v/v) prior to centrifugation and 10 μL of the supernatant fluid was passed through a Millipore filter (0.45 μm) and then injected into the HPLC system for analysis.

2.3. *NIR apparatus and software*

The NIR spectra of the samples were obtained at 1-nm intervals over the spectral region from 1000 to 2500 nm using a SupNIR-4510 instrument (Focused Photonics, Inc., Hangzhou, China) equipped with an optical fiber transflectance adapter and a sample pretreatment system. The samples were scanned using a 2-mm path length and equilibrated at 75°C before scanning to ensure that the samples were

Table 1. Gradient elution program.

Time/min	B (%)	A (%)
0–3	5 → 5	95 → 95
3–13	5 → 15	95 → 85
13–15	15 → 15	85 → 85
15–40	15 → 30	85 → 70
40–50	50 → 5	70 → 95

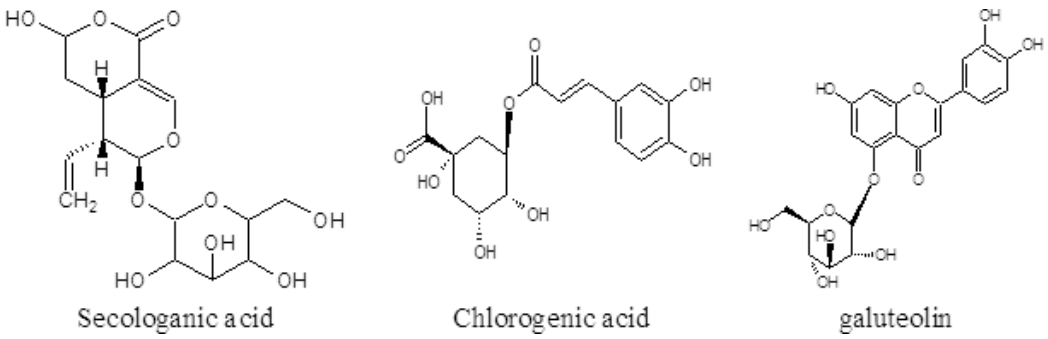


Fig. 1. Chemical structures of the investigated analytes.

analyzed under the same conditions. Each spectrum was obtained by averaging 16 scans and all spectra were recorded in SCV format. The NIR instrument was controlled by a compatible PC and a THUNIR online workstation was used for data acquisition.

All considerations, including selection of the spectral wavebands, mathematical pretreatments and partial least-squares (PLS) regression, were performed using THUNIR software (Version 3.0, Tsinghua University, Beijing, China) and Unscrambler V9.7 (Demo, CAMO Inc., Trondheim, Norway).

2.4. *Spectral data pretreatment*

Spectral pretreatment is essential to obtain reliable, stable and accurate models because NIR data may contain noise, background information and other interferences. The spectral bands were selected initially from 1000 to 1800 nm. In this research, several data preprocessing methods, including derivation, multiplicative scatter correction (MSC), standard normal variate (SNV) transformation, Savitsky–Golay (SG) smoothing and normalization, were applied to minimize interference effects. The outcomes of the different pretreatment methods were compared and the optimized combination was selected independently.

2.5. *Calibration of models*

PLS regression, the most common algorithm for calibrating models, has been well documented in the literature.[22,23] The performance of the final PLS model was evaluated in terms of standard error of calibration (SEC), standard error of cross-validation (SECV) and determination coefficient $[R^2(M)]$.[24,25] Selection of the number of latent variables (LVs) was based on the minimization of SECV, which corresponds to the predictive error obtained at the cross-prediction stage. A total of 10 spectra were selected for the predicted set and a PLS regression model was built with the remaining spectra of the calibration set. The SECV value decreased with increasing LV number. When the SECV approached a constant value, the optimum LV numbers were obtained.

3. Results and Discussion

3.1. *Determination of the active ingredients using HPLC-DAD and division of calibration and prediction sets*

A total of 81 samples were analyzed using the methods described in Sec. 2.2. All three active compounds can be analyzed accurately because of the distinct separation of the peaks (see the chromatogram in Fig. 2). Standard curves were established and methodology parameters were investigated prior to online sample analyses (see Table 2).

All samples were separated into a calibration set and a prediction set. For each model, the calibration and prediction sets consisted of 71 and 10 samples, respectively. Statistics of the active ingredients in

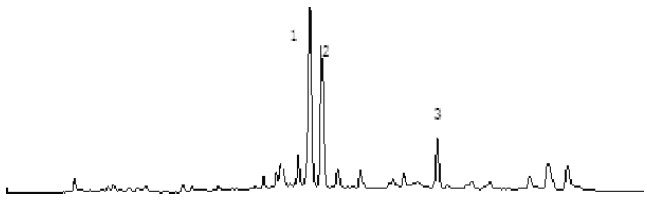


Fig. 2. HPLC chromatogram of Lonicerae Japonicae Flos solution extracted by water under optimized conditions. The peaks marked 1, 2 and 3 are secologanic acid, chlorogenic acid and galuteolin, respectively.

Table 2. Calibration curves and methodology parameters of the reference method.

Compound	Calibration curve	$R^2(L)$	Linearity ranges (mg/mL)	Recovery (%, $n = 3$)
Secologanic acid	$Y = 1925.69x + 0.24$	0.998	0.0817–1.634	99.5
Chlorogenic acid	$Y = 2884.19x + 6.82$	0.999	0.0976–2.928	102.3
Galuteolin	$Y = 2476.78x - 0.06$	0.999	0.0010–0.05045	98.7

Table 3. Statistics of active ingredient content in calibration and prediction sets.

Active ingredient	Average value	Calibration set	Prediction set
Secologanic acid	0.3794 mg/mL	0.091–0.580 mg/mL	0.097–0.571 mg/mL
Chlorogenic acid	0.5340 mg/mL	0.134–0.868 mg/mL	0.165–0.862 mg/mL
Galuteolin	10.69 μg/mL	1.662–21.47 μg/mL	4.167–20.77 μg/mL

the calibration and prediction sets are listed in Table 3. The calibration set ranges covered the larger scale and the concentrations were evenly distributed in both data sets.

3.2. *NIR spectral features*

The raw and preprocessed spectra of the *Lonicerae Japonicae Flos* solution extracted by water are shown in Fig. 3. As shown in the derivative

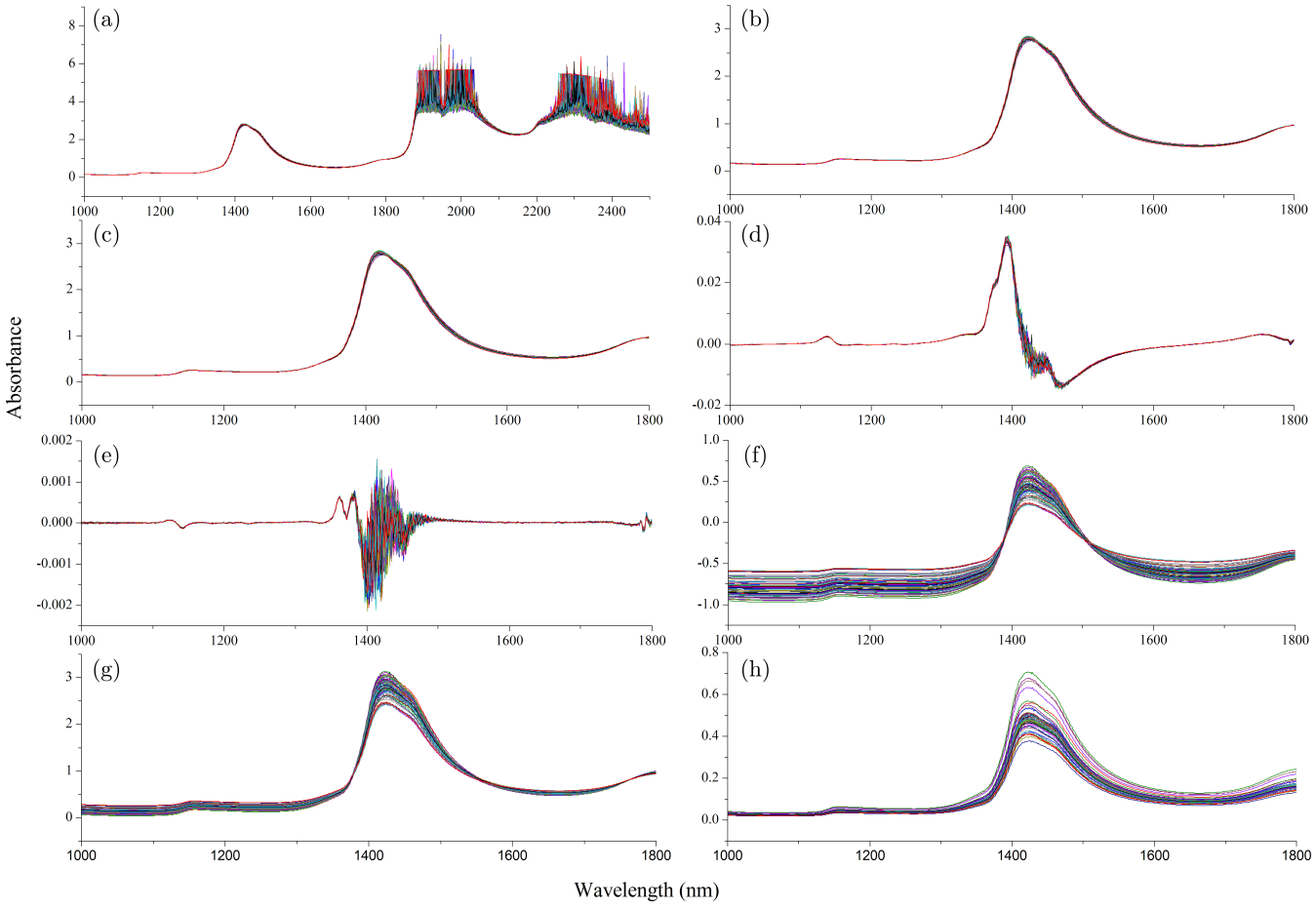


Fig. 3. Raw NIR spectra of full wavelength (a), raw NIR spectra (b) and spectra preprocessed using SG smoothing (c), one-dimensional convolution (d), two-dimensional convolution (d), SNV (e), MSC (f) and normalization of selected wavelength (g) of test samples.

absorption spectra, three sharp peaks appeared in the regions 1100 to 1170 nm, 1300 to 1420 nm and 1450 to 1600 nm, which corresponded to the second overtone of C–H in $–CH_2$, the second overtone of the carbonyl group, and the stretching and deformation vibrations of the O–H bond,[26] respectively. The three wavebands appeared far and wide in the three active substances and the NIR spectra reflected concentration information. These results can be used as basis for the quantitative NIR analysis of the *Lonicerae Japonicae Flos* solution extracted by water.

3.3. *Principal component analysis (PCA)*

PCA is a suitable method to classify different samples based on the score of the spectra and can be used to verify the consistency of the spectra.[27] Data were processed using Unscrambler V9.7. The score plot of the spectra (see Fig. 4) showed no obvious outliers and no groupings appeared in the sample set. The results showed that the five batches of *Lonicerae Japonicae Flos* solution extracted by water were under the same conditions.

3.4. *Calibration of models*

3.4.1. *Regression method*

PLS was selected for the quantitative analysis of the corresponding models. Using secologanic acid as an example, the performance parameters of the optimized calibration models are listed in Table 4. Three models were developed using the PLS method. During PLS calibration, the most influential factors included waveband selection, LV number and spectrum pretreatment method. These factors are discussed below.

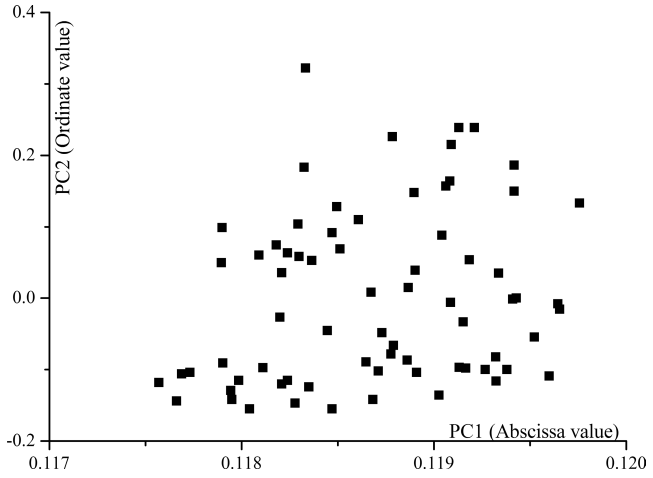


Fig. 4. PCA score plot of raw NIR spectra of *Lonicerae Japonicae Flos*.

Table 4. Comparison of different calibration models of secologanic acid concentration developed with different pretreatment spectra.

Pretreatment methods	LVs[a]	Calibration		Cross-validation	
		R^2(C)	SEC[b]	R^2(CV)	SECV[c]
Raw spectra	4	97.695	0.032	96.902	0.037
SG Smoothing	4	97.695	0.032	96.902	0.035
One DC[d]	6	99.103	0.056	96.381	0.145
Two DC	1	36.391	0.196	27.993	0.224
SNV[e]	5	98.593	0.067	96.739	0.089
MSC[f]	5	98.593	0.051	96.739	0.074
Normalization	5	98.593	0.049	96.739	0.070

[a]LV: latent variable number.
[b]SEC: Standard error of calibration.
[c]SECV: Standard error of cross validation.
[d]DC: dimension convolution.
[e]SNV: standard normal variate.
[f]MSC: multiple scatter correction.

3.4.2. *NIR waveband selection*

The useful bands of NIR ranged from 1000 to 2500 nm, which corresponded to the first or second overtone. The intensity of NIR absorption decreased as the overtone increased. To obtain more information, the useable band was selected to include the whole region rather than the short-wave NIR (SW-NIR) or common NIR region.[28] As shown in Fig. 3, most of the spectral regions were smooth, but noise was introduced at the latter part by the spectral differentiation process due to optical fiber absorption. The selected waveband for this study ranged from 1000 to 1800 nm, which covered both SW-NIR and common NIR regions. The correlation coefficients of the spectra were considered for band selection. Secologanic acid was used as an example (see Fig. 5). The variables with high coefficients (> 0.4) were parts of the regions selected. Similar conditions can be used for the two other compounds.

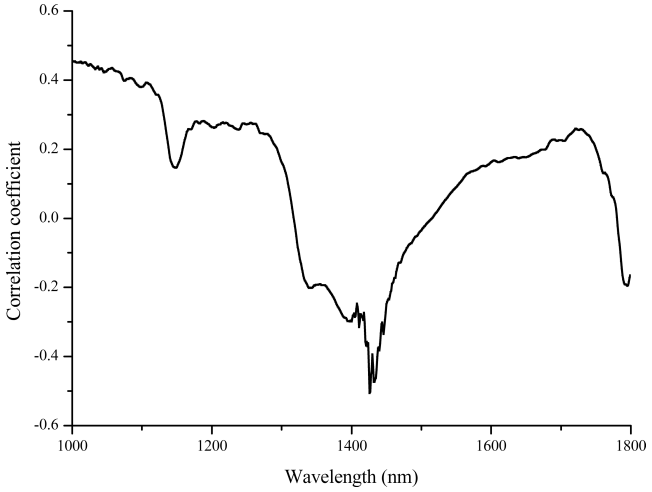


Fig. 5. Correlogram of the NIR spectra and secologanic acid concentration.

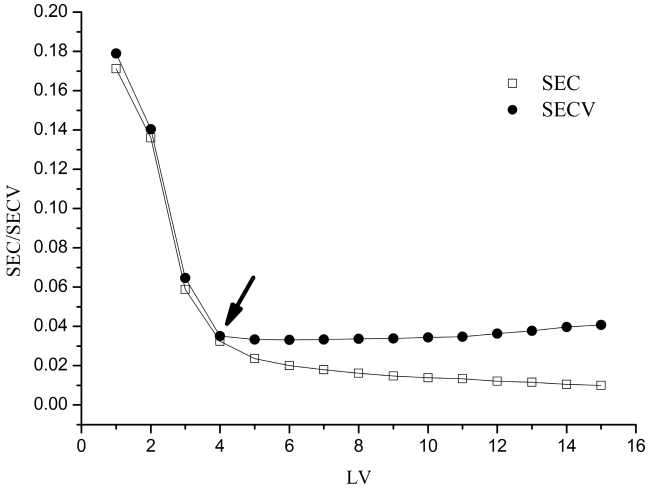


Fig. 6. Relational graph of LV numbers and SECV of secologanic acid.

3.4.3. *Comparison of different pretreatment methods for the spectra*

Data pretreatment is important to determine appropriate quantitative models. Derivation, MSC, SNV, SG smoothing and normalization are methods used to correct variations in light scattering due to differences in particle size distribution.[29] The selection standard was the representation of the models, which was determined by SECV. Using the calibration model of secologanic acid as an example, the calibration results using different pretreatment methods are shown in Table 4 and SG smoothing was selected. Spectral pretreatment methods for the other calibration models were also analyzed. All images that were conveniently and intuitively pretreated are shown in Fig. 3.

3.4.4. *Determination of the optimum LV numbers*

An optimum LV number is critical in building an appropriate model to avoid "under-fitting" and "over-fitting."[30] In this research, the optimum number of factors was determined using SECV, which was calculated using the leave-one out method. Using the secologanic acid calibration model as an example, the determination method for the optimum LV numbers and the correlation diagram of the SECV with the optimum LVs are shown in Fig. 6.

3.4.5. *Establishment and prediction of the calibration models*

All spectra were pretreated with the corresponding method and three calibration models were subsequently established. The parameters SEC, SECV and R^2 were used to evaluate the model. A good model should have low SEC and SECV, as well as high R^2, because high SEC and SECV and low R^2 indicate that too many LVs have been introduced to the system, which may lead to the unnecessary inclusion of noise in the model. The performance parameters of all three models are shown in Table 5. The R^2 values of the calibration were higher than 0.85, indicating that the models had good predictive and inferential abilities.

3.5. *Application of the models for analysis of unknown*

The PLS models built according to the previous procedures were used to simultaneously detect the remaining NIR spectra and obtain the outcomes of the three active compounds. The concentrations of secologanic acid, chlorogenic acid and galuteolin, the most important quality indicators of QKL injection, are shown in Table 6 and Fig. 7. The higher the content, the better the predictive capacity of the method.

As shown in Table 6 and Fig. 7, NIR spectroscopy may be used for routine analysis in the

Table 5. Parameters of the final calibration models.

Model	LV[a]	SPNIRS[b]	Bands/nm	SEC[c]	SECV[d]	CD[e]
Secologanic acid	4	SG smoothing	1000 to 1595	0.032	0.035	97.699
Chlorogenic acid	3	SG smoothing	1298 to 1595	0.061	0.065	95.687
Galuteolin	6	SG smoothing	1000 to 1297	2.471	3.870	85.291

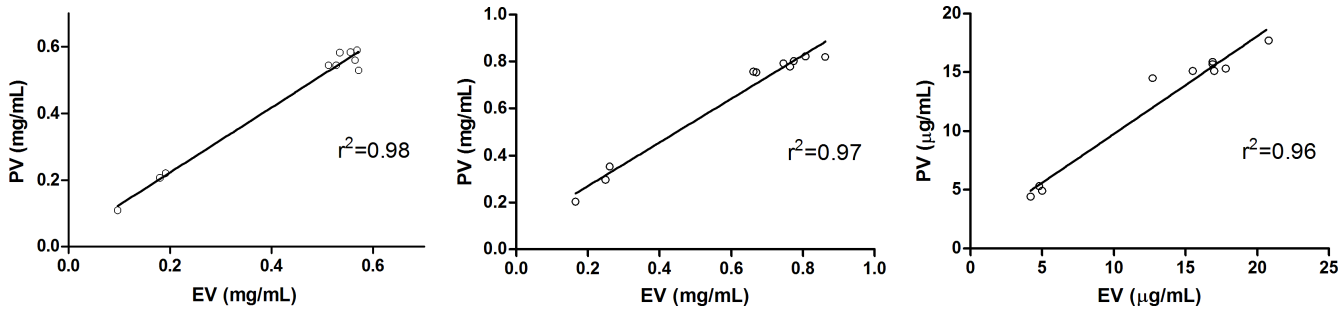
[a]LV: latent variable number.
[b]SPNIRS: spectra pretreatment in NIR spectroscopy.
[c]SEC: standard error of calibration.
[d]SECV: standard error of cross validation.
[e]CD: coefficient of determination.

Table 6. Experimental vs. predictive values of the three active compounds.

	Secologanic acid/mg/mL			Chlorogenic acid/mg/mL			Galuteolin/μg/mL		
Samples	EV[a]	PV[b]	Ea[c]	EV	PV	Ea	EV	PV	Ea
Spec_21313	0.564	0.559	−0.005	0.862	0.820	−0.042	12.7	14.5	1.8
Spec_21355	0.097	0.109	0.012	0.165	0.203	0.038	4.2	4.4	0.2
Spec_21431	0.571	0.529	−0.042	0.764	0.778	0.014	17.8	15.3	−2.5
Spec_21457	0.180	0.206	0.026	0.249	0.296	0.047	4.8	5.3	0.5
Spec_21479	0.191	0.220	0.029	0.261	0.353	0.092	5.0	4.9	−0.1
Spec_21711	0.534	0.582	0.048	0.746	0.792	0.046	16.9	15.7	−1.2
Spec_21737	0.555	0.583	0.028	0.775	0.802	0.027	16.9	15.9	−1.0
Spec_22448	0.568	0.589	0.021	0.807	0.822	0.015	20.8	17.7	−3.1
Spec_22657	0.512	0.544	0.032	0.662	0.757	0.095	15.5	15.1	−0.4
Spec_22669	0.527	0.544	0.017	0.670	0.754	0.084	17.0	15.1	−1.9
SEP[d]		0.030			0.061			1.668	
SEC/SEP		1.080			1.013			1.482	

[a]EV: experiment values.
[b]PV: predicted values.
[c]Ea: absolute error.
[d]SEP: standard error of prediction.

manufacturing industry because of the stable, feasible and ascendant models established. The concentration of the three active compounds was rapidly determined and overmastered real time during QKL manufacturing. The results can be used to examine in advance whether or not a product meets specification criteria and judge the quality of a final product. Limitations on the application described above may



(a) (b) (c)

Fig. 7. Experimental values vs. predictive values: secologanic acid (a), chlorogenic acid (b) and galuteolin (c).

be considered as a PAT application and regarded as a first step in bringing about a modern, automated, digital and integrated system.

4. Conclusion

In this research, an NIR-based method was established to quickly and accurately determine the concentrations of secologanic acid, chlorogenic acid and galuteolin. These three active ingredients in *Lonicerae Japonicae Flos* solution were extracted by water for QKL injection and their concentrations were determined using HPLC-DAD as the reference method. The model established above can be used successfully in the production line of QKL injection to improve the efficiency of quality control. NIR spectroscopy may be an alternative method to the wet analysis method, which is widely used at present. The method illustrated in this study proposes a feasible and rapid means for analyzing water-extracted solutions of QKL injection that may be applied in similar contexts. NIR spectroscopy may be an applicable tool for the fulfillment of PAT in TCMI and other TCM manufacturing industries. All research efforts can be helpful in the modernization and globalization of TCM.

Acknowledgments

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