

## Research Article

# Application of Fourier Transform Near-Infrared Spectroscopy Combined with High-Performance Liquid Chromatography in Rapid and Simultaneous Determination of Essential Components in Crude Radix Scrophulariae

Xiaomeng Li,<sup>1</sup> Dansi Fang,<sup>2</sup> Xiaodong Cong,<sup>1,5</sup> Gang Cao,<sup>1,4</sup> Hao Cai,<sup>3,4</sup> and Baochang Cai<sup>1,3,4</sup>

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**Abstract.** A method is described using rapid and sensitive Fourier transform near-infrared spectroscopy combined with high-performance liquid chromatography–diode array detection for the simultaneous identification and determination of four bioactive compounds in crude Radix Scrophulariae samples. Partial least squares regression is selected as the analysis type and multiplicative scatter correction, second derivative, and Savitzky–Golay filter were adopted for the spectral pretreatment. The correlation coefficients (*R*) of the calibration models were above 0.96 and the root mean square error of predictions were under 0.028. The developed models were applied to unknown samples with satisfactory results. The established method was validated and can be applied to the intrinsic quality control of crude Radix Scrophulariae.

**KEY WORDS:** crude Radix Scrophulariae; FT-NIRS; HPLC-DAD; quality control; quantification.

## INTRODUCTION

Radix Scrophulariae known as “Xuanshen” in Chinese, derived from the dried root of *Scrophularia ningpoensis* Hemsl, is one of the most widely and commonly used herbs within traditional Chinese medicine (TCM) (1,2). Pharmacological studies and clinical practice have demonstrated that Radix Scrophulariae possesses various bioactivities, including antichronic inflammatory, antihypertensive, abirritative, anti-spasmodic, antihepatitis B virus, and immunological enhancement. The efficacy of this herb results from the synergetic action of the various constituents covering iridoid glycosides, phenylpropanoid glycosides, as well as cinnamic acid (CA). Harpagoside (HG) is a characteristic and active constituent of Radix Scrophulariae and some researchers also showed that HG has some pharmacological effects which are closely related to the nourishment of yin for lowering fire (3–5).

Several analytical methods have been developed to investigate the contents of the main components in Radix Scrophu-

lariae, for instance, high-performance liquid chromatography (HPLC) coupled with different detectors, such as diode array detection (DAD), ion trap mass spectrometry, and electrospray ionization time-of-flight mass spectrometry (6–8).

Near-infrared spectroscopy (NIRS) is currently widely applied to quantify active pharmaceutical ingredients in many pharmaceutical formulations (9–12). It provides a rapid, non-destructive method without complicated sample preparation. Thus, it plays a significant role in the quality control of TCM. To date, for Radix Scrophulariae, only the quantification of HG and CA by NIRS has been reported (13) and those alone do not fully reflect the chemical characteristics of Radix Scrophulariae.

In the present study, a new application based on NIRS and HPLC-DAD, partial least squares (PLS) regression, and multiplicative signal correction (MSC) was developed for the simultaneous determination of four main components including HG, CA, acteoside (AT), and angraside C (ARC) for the first time. The precision and accuracy of the new NIR method were validated, comparing with the standard HPLC method. On the basis of this new NIRS quantitative model, 10 batches of Radix Scrophulariae samples were also analyzed. This technology shows satisfactory results and could be broadly used for the quality control of TCM.

## MATERIALS AND METHODS

### Samples and Reagents

A total of 51 crude Radix Scrophulariae from different areas in China were collected and analyzed (Table I). These samples were authenticated by Professor Yun Zhang

<sup>1</sup> Research Center of TCM Processing Technology, Zhejiang Chinese Medical University, Hangzhou, People’s Republic of China.

<sup>2</sup> Zhejiang Chinese Medical University, Hangzhou, People’s Republic of China.

<sup>3</sup> Engineering Center of State Ministry of Education for Standardization of Chinese Medicine Processing, Nanjing University of Chinese Medicine, Nanjing, People’s Republic of China.

<sup>4</sup> National First-Class Key Discipline for Traditional Chinese Medicine of Nanjing University of Chinese Medicine, Nanjing, People’s Republic of China.

<sup>5</sup> To whom correspondence should be addressed. (e-mail: congxiaodong199@yahoo.com.cn)

**Table I.** The Origin and Respective Voucher Number of Crude Radix Scrophulariae

Sample no.	Origin
1	Dongyang, Zhejiang A
2	Dongyang, Zhejiang B
3	Dongyang, Zhejiang C
4	Dongyang, Zhejiang D
5	Dongyang, Zhejiang E
6	Dongyang, Zhejiang F
7	Panan, Zhejiang A
8	Henan A
9	Dongyang, Zhejiang G
10	Hunan
11	Dongyang, Zhejiang H
12	Anhui
13	Panan, Zhejiang B
14	Dongyang, Zhejiang I
15	Panan, Zhejiang C
16	Dongyang, Zhejiang J
17	Xianju, Zhejiang A
18	Xianju, Zhejiang B
19	Chongqing
20	Shandong A
21	Dongyang, Zhejiang K
22	Jinyun, Zhejiang A
23	Jinyun, Zhejiang B
24	Jinyun, Zhejiang C
25	Jinyun, Zhejiang D
26	Jinyun, Zhejiang E
27	Jinyun, Zhejiang F
28	Panan, Zhejiang D
29	Panan, Zhejiang E
30	Panan, Zhejiang F
31	Panan, Zhejiang G
32	Fuyang, Zhejiang
33	Zhejiang I
34	Henan
35	Shandong B
36	Zhejiang II
37	Jiangxi
38	Hubei
39	Zhejiang III
40	Zhejiang IV
41	Zhejiang V
42	Shandong C
43	Shandong D
44	Zhejiang VI
45	Panan, Zhejiang D
46	Panan, Zhejiang E
47	Panan, Zhejiang F
48	Panan, Zhejiang G
49	Henan B
50	Henan C
51	Henan D

(Research Center of TCM Processing Technology, Zhejiang Chinese Medical University, Hangzhou, China). Voucher specimens were deposited at the Research Center of TCM Processing Technology. The reference standards of HG, CA, AT, and ARC were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purities of all four standards were  $\geq 98\%$  by HPLC analysis. The structures of these four

compounds were shown in Fig. 1. The HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany), phosphoric acid was purchased from Tedia (Fairfield, Ohio, USA), and ultrapure water was prepared by a Milli-Q50 SP Reagent Water System (Millipore, Billerica, Massachusetts, USA) for the preparation of samples and buffer solutions. Other reagents were of analytical grade.

### Standard Solutions and Sample Preparation

Primary stock standard solutions of the four compounds were prepared by dissolving them with methanol, respectively, to get a concentration of 0.0543 mg/mL (AT), 0.0284 mg/mL (ARC), 0.0204 mg/mL (HG), and 0.0275 mg/mL (CA). Working mixed standard solutions were prepared daily by mixing the stock solutions and diluted to a series of standard mixed solutions with methanol. The solutions were all stored at 4°C.

All crude Radix Scrophulariae samples were milled into powder with a grinder and then dried at 60°C for 2 h prior to analysis to avoid the effect of moisture. The powder of crude Radix Scrophulariae samples were precisely weighed (0.500 g) and transferred into dark brown volumetric flasks. They were extracted with 50 mL of 50% methanol in an ultrasonic bath for 45 min. Additional 50% methanol was added to make up the volume lost to evaporation. The extracts were filtered through a 0.45- $\mu\text{m}$  membrane prior to injection.

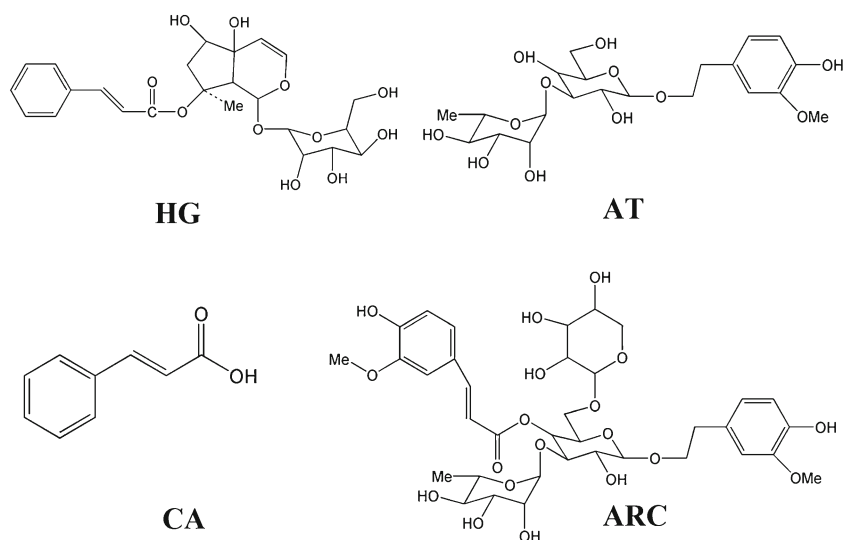
### HPLC Analysis

Chromatographic analyses were carried out on an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, California, USA) with diode array detector. An Agilent Zorbax Extend C<sub>18</sub> (250×4.6 mm, 5  $\mu\text{m}$ ) was used with a flow rate of 1.0 mL/min. Detection wavelength was set at 280 nm. The injection volume was 10  $\mu\text{L}$  and the column temperature was maintained at 30°C. The mobile phase was composed of (A) aqueous phosphoric acid (0.03%, *v/v*) and (B) acetonitrile using a gradient elution of 3–6% B at 0–8 min, 6–15% B at 8–18 min, 15–20% B at 18–25 min, 20–35% B at 25–35 min, 35–47% B at 35–38 min, 47% B at 38–40 min, 47–75% B at 40–45 min, and 75–80% B at 45–50 min.

### NIR Spectra Collection

The NIR spectra of Radix Scrophulariae powder samples were collected using a Thermo ANTARIS II FT-NIR spectrometer (Thermo Electron Co., Madison, Wisconsin, USA) equipped with an InGaAs detector, an integrating sphere, sample cup, and rotary tables. The system was operated by the RESULT operating system and the spectral data were analyzed by the TQ Analyst software package (version 8.0).

The spectra were obtained with an 8  $\text{cm}^{-1}$  resolution over a wavelength range of 10,000–4,000  $\text{cm}^{-1}$  with 64 scans per spectrum. Both sample and background scan times were 32 s, and air absorption is recorded as a reference standard. An average spectrum was obtained after each sample was measured three times. Powdered samples were weighed precisely each time and put in the sample cup. A slight tap was needed to make the powder more uniform before the measurements.



**Fig. 1.** The chemical structures of four active components in crude *Radix Scrophulariae*

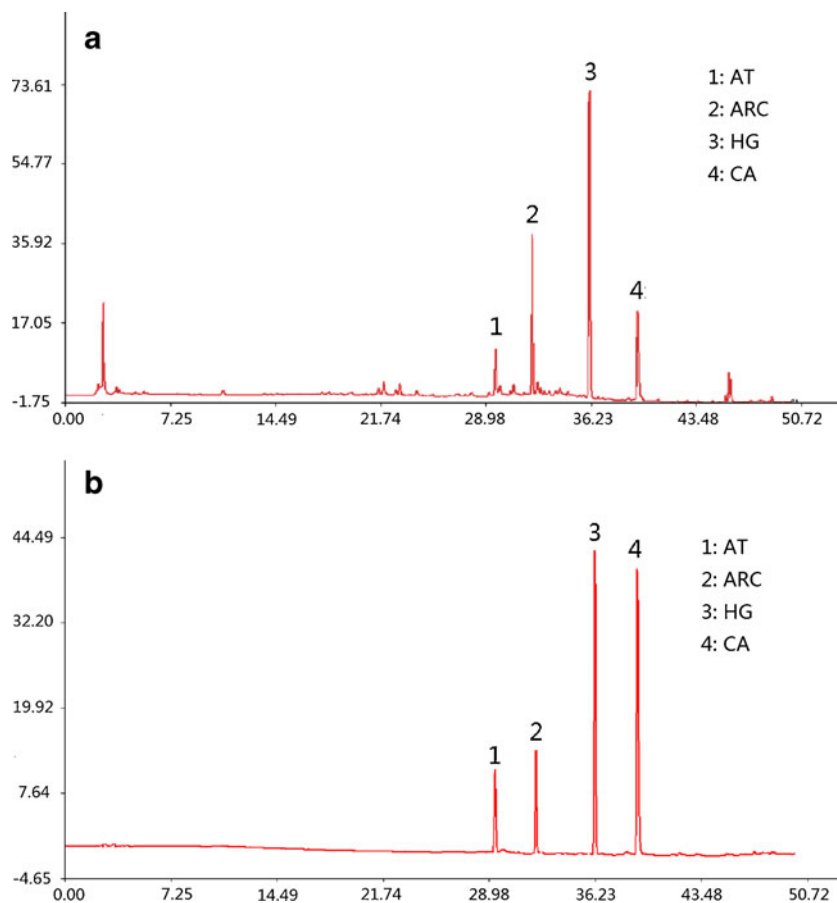
### Preprocessing Method

In this work, several data preprocessing methods, including derivation, MSC, Savitzky–Golay (SG), first derivative (1-Der), and second derivative (2-Der) were investigated for NIR spectra in order to minimize the interfering effect such as correction of scatter effect, elimination of baseline shift,

systematic noise, path length differences, and smoothing spectrum, etc.

### Parameters for Evaluating Models

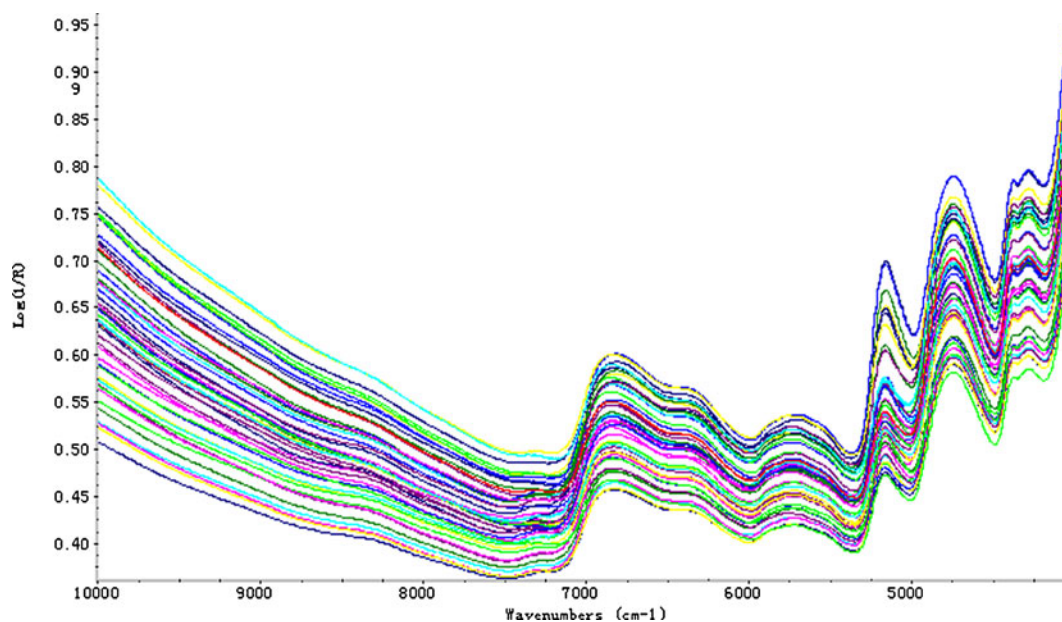
To build a robust PLS calibration model, the optimum preprocessing method was selected based upon root mean



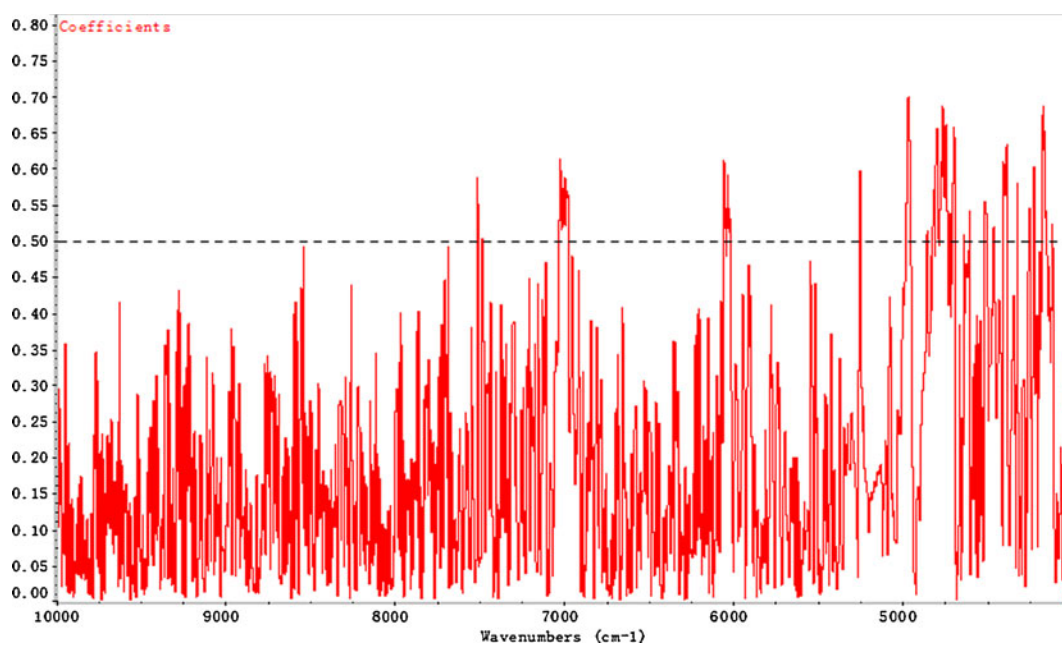
**Fig. 2.** Typical HPLC-DAD chromatogram of **a** crude *Radix Scrophulariae* and **b** mixed standard solution

**Table II.** The Methodology Parameters and the Calibration Plots of the Reference Method ( $n=6$ )

Compounds	$t_R$ (min)	Regression equation	$R^2$	Linearity range ( $\mu\text{g/mL}$ )	LOD ( $\mu\text{g/mL}$ )	Repeatability RSD%	Recovery %
HG	36.091	$y=28,831.93x - 0.071$	0.9999	2.55–30.60	0.05	1.13	99.39
CA	39.462	$y=17,970.24x - 1.2931$	0.9999	3.44–41.25	0.09	1.21	99.75
AT	29.681	$y=2,233.28x - 0.7159$	0.9999	6.78–81.45	0.16	0.92	99.75
ARC	32.196	$y=4,126.60x - 0.4145$	0.9998	3.55–42.60	0.07	1.00	99.85



**Fig. 3.** The original NIRS spectra of crude Radix Scrophulariae



**Fig. 4.** The correlogram of the NIR spectra and the AT concentrations

**Table III.** The Impact of Different Pretreatment Methods on the Model

Pretreatment methods	<i>R</i>	RMSEC	RMSEP	RMSECV
Original spectra	0.943	0.0544	0.0527	0.0720
1-Der+no smoothing	0.924	0.0629	0.0434	0.0823
1-Der+SG	0.919	0.0647	0.0428	0.0824
1-Der+Norris	0.867	0.0816	0.0520	0.0884
2-Der+no smoothing	0.999	0.0018	0.0784	0.0500
2-Der+SG	0.999	0.0089	0.0273	0.0236
2-Der+Norris	0.960	0.0457	0.0435	0.0361

square error of calibration (RMSEC), root mean square error of prediction (RMSEP), root mean square error of cross-validation (RMSECV), prediction residual error sum of squares (PRESS), and correlation coefficients (*R*) (14).

RMSEC and RMSEP indicate the capability of the developed NIRS model. Generally, the values of RMSEC and RMSEP are better approaching to zero. The *R* values are suitable for most application models with values of 0.92–0.96 including quality control and those above 0.98 for all applications. PRESS and RMSECV were used to determine the optimum number of principal components. Usually, PRESS is plotted as a function of the number of PLS factors. When the PRESS value reaches minimum, its corresponding PLS factors is selected as the optimum one for a PLS quantitative model.

## RESULTS AND DISCUSSION

### Quantitative Analysis of HG, CA, AT, and ARC by HPLC Method

Figure 2 shows a typical HPLC-DAD chromatogram of crude Radix Scrophulariae extract solution and four mixed standard solutions. The HPLC method was validated by defining the linearity, limits of quantification and detection, identification and

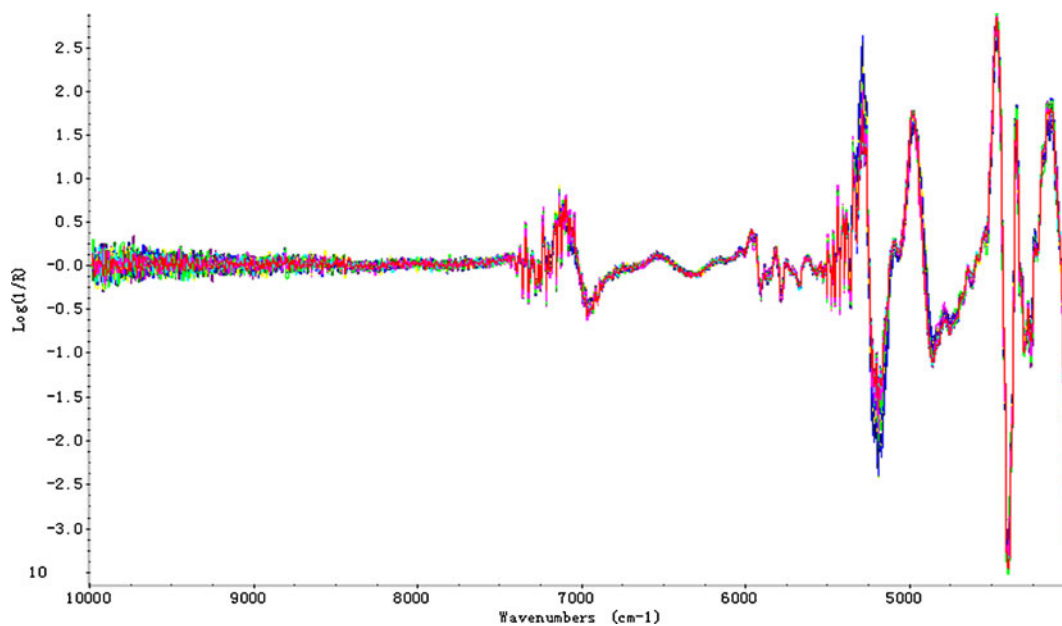
quantification of the analytes, repeatability, precision, stability, and recovery. Four calibration curves were constructed from peak areas of the reference standards *versus* their concentrations. All criteria for assessing the suitability of the system are listed in Table II. The calibration curves of four standards show good linearity ( $R^2 > 0.9998$ ), covering the range of quantification. These data demonstrated the suitability of this HPLC method for simultaneous quantification.

### Spectral Analysis

PLS is a full-spectrum calibration method and all four NIRS models were based on it. Figure 3 shows the raw NIR spectra of 51 batches of crude Radix Scrophulariae over the 10,000–4,000  $\text{cm}^{-1}$  range. Intensive spectral peaks of original spectra are mainly in the range of 7,400–4,000  $\text{cm}^{-1}$ . These are caused by the stretch and vibration of the C–H, N–H, and O–H groups that are the typical chemical groups of HG, CA, AT, and ARC. Therefore, the region of 8,000–4,000  $\text{cm}^{-1}$  was selected for the PLS calibration model. The correlation coefficients of the spectra also verified the accuracy of band selection. Taking AT as an example, the correlogram of *R* and wave numbers (WN) is shown in Fig. 4, in which it can be clearly seen that the WN with higher coefficients ( $>0.5$ ) are mainly distributed in the selected band region.

### Calibration of NIRS Models

The integrating sphere module was adopted as the sampling technique based on diffusion reflection. MSC was used to correct the light scattering effects due to particle size distribution by adjusting the spectra based on ranges of wavelengths carrying no specific chemical information (15,16). Considering some influence factors like baseline shift, systematic noise, intense band overlapping, and difference between various samples (17), 1-Der and 2-Der are quite needed. Derivative processing was used to correct the baseline shift and reinforce spectral resolution. Meanwhile, smooth process-

**Fig. 5.** NIRS spectra pretreated with 2-Der

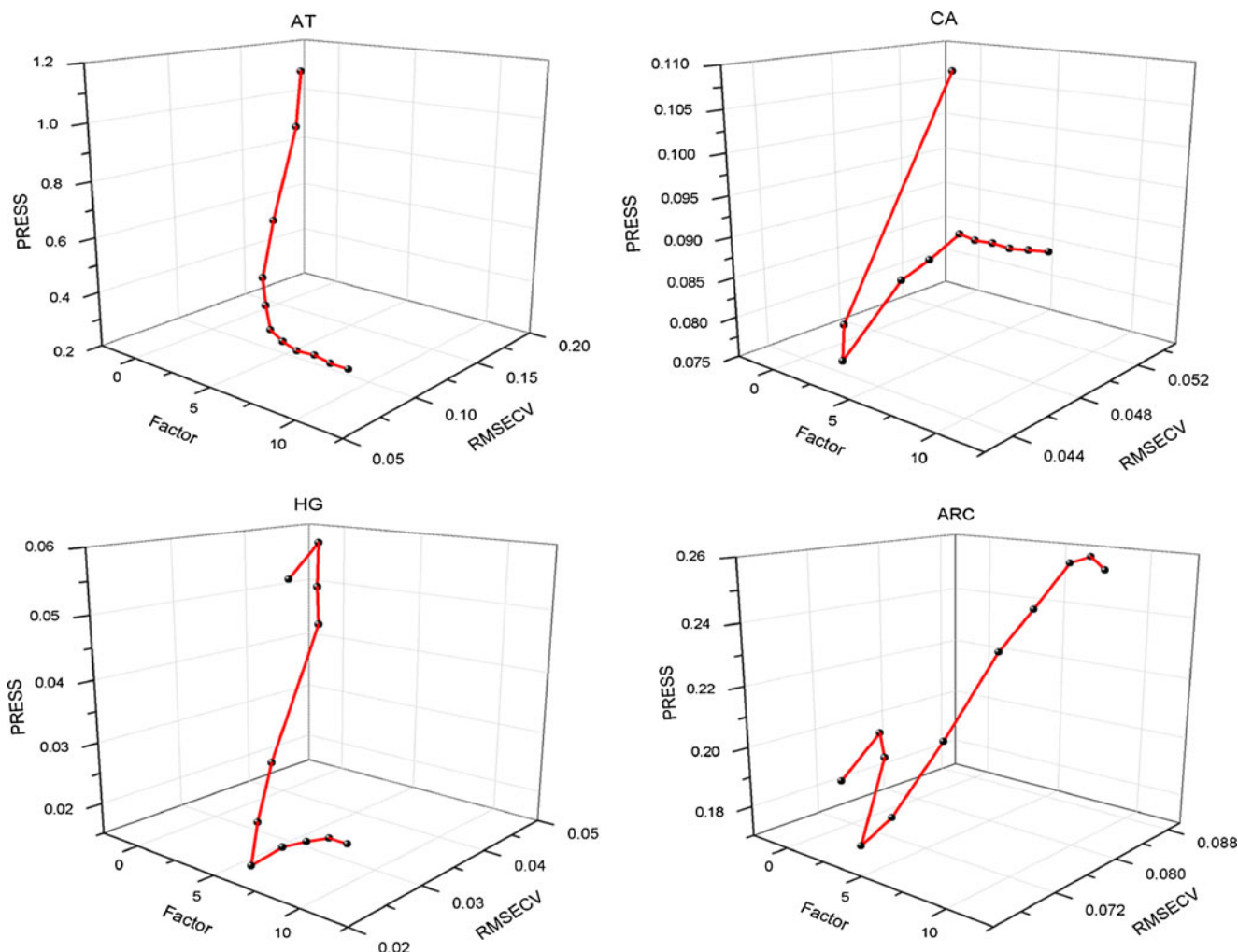


Fig. 6. Correlogram of number of PLS factors and PRESS and RMSECV values for calibration models

ing was also introduced to eliminate enhanced noise signals (18). Two classical smoothing techniques, SG filter and Norris derivative filter, were investigated and compared. Whether these processing methods qualified for the models or not are evaluated by  $R$ , RMSEC, RMSEP, and RMSECV. Taking AT as an example, the comparison of different methods was listed in Table III. After comparison of diverse spectra pretreatments, 2-Der with SG filter was demonstrated as an optimum pretreatment and its spectra were illustrated in Fig. 5. After pretreatment with 2-Der, there was no distinct change over the wavelength range of  $10,000\text{--}7,400\text{ cm}^{-1}$  which mostly contains baseline information.

The number of PLS factors would significantly affect the precision of a quantitative model. An optimum choice should fully reflect the spectral changes resulting from the changes in the concentration of the measured components and exclude the spectral noise. The three-dimensional correlograms for factors, PRESS, and RMSECV were illustrated in Fig. 6. The optimal number of factors were 6, 2, 7, and 3 for HG, CA, AT, and ARC, respectively.

### Results of Calibration Model

Generally, RMSEC, RMSEP, RMSECV, and correlation coefficients ( $R$ ) are used for evaluating an NIR model.

RESEC, RESEP, and RMSECV close to 0 and  $R$  approaching to 1 indicate a robust model. Table IV shows the results of these four evaluating parameters as well as the corresponding number of PLS factors for each model. It can be concluded that the coefficients of determination are all above 0.96. RMSEC, RMSEP, and RMSECV are below 0.045, indicating a satisfactory correlation between the HPLC-calculated value and the model-predicted value.

Figure 7 shows the correlogram between the actual reference values obtained by HPLC and NIRS-predicted concentration values. The results show a good linearity and suggest that the established models can be applied to the determination of main components in crude Radix Scrophulariae.

Table IV. The Results of RMSEC, RMSEP, RMSECV, and  $R$  of Four Quantitative Models

Compounds	$R$	RMSEC	RMSEP	RMSECV	Factor
HG	0.9670	0.0101	0.0225	0.0227	6
CA	0.9618	0.0141	0.0244	0.0442	2
AT	0.9985	0.0089	0.0273	0.0236	7
ARC	0.9613	0.0189	0.0219	0.0328	3

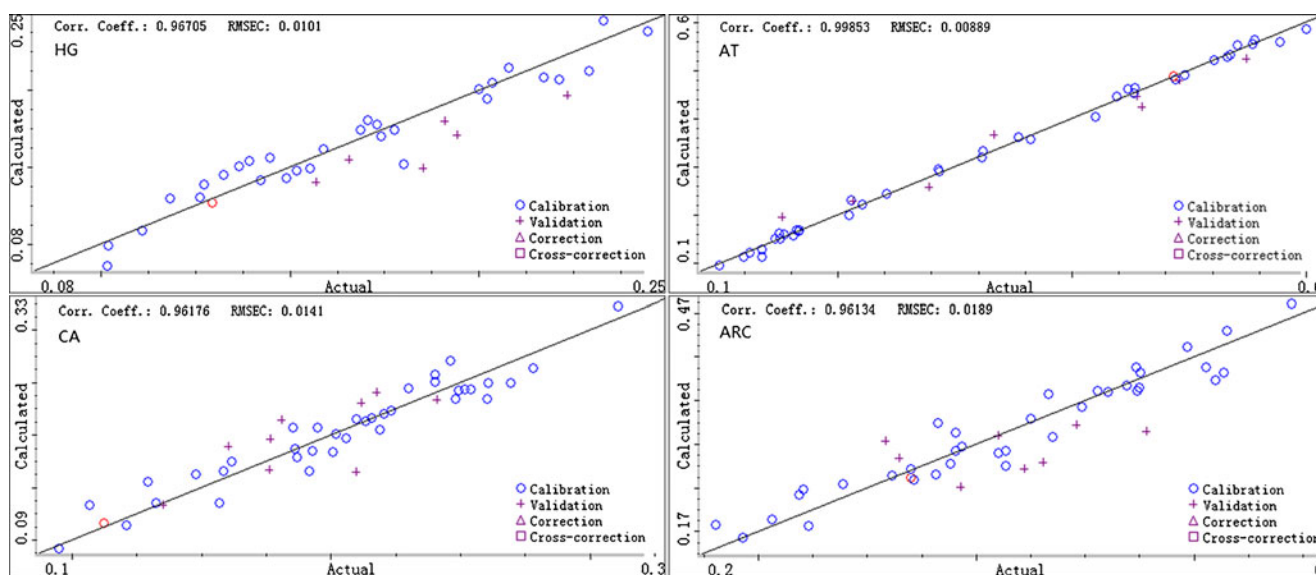


Fig. 7. Correlation diagrams between the NIR-predicted values and the reference values of HG, CA, AT, and ARC concentrations

### Validation of Developed NIRS Model

Four separate NIRS models for the quantification of HG, CA, AT, and ARC have been developed corresponding to their reference HPLC methods. To investigate their robustness, 10 batches of unknown crude *Radix Scrophulariae* have been picked up and analyzed by the models. The concentration results were compared with those analyzed by HPLC, showing good prediction accuracy with  $R$  exceeding 0.9. The correlations between measured values by HPLC and NIR prediction values for HG, CA, AT, and ARC were showed in Fig. 7. Tailed  $t$  test has also been applied to validate the results obtained by the HPLC and NIR methods. Taking HG as an example, the value of  $t$  is 0.927, less than  $t_{(0.05,9)}=2.262$  with the significance level of 0.05 which convincingly indicates the accuracy of the established NIR models.

### CONCLUSIONS

In this study, PLS-NIRS models have been developed for the simultaneous determination of the four main components, namely, HG, CA, ARC, and AT, in crude *Radix Scrophulariae* with HPLC-DAD analysis as reference method. The overall results proved the feasibility of quantification and quality control of TCM with shorter time and less pretreatment. Good and optimized models were established. This suggests that the major components in original TCM could be analyzed quickly and simultaneously without extraction. This technique could greatly accelerate the development of quantification methods and improve the efficacy of the chemical analysis of TCM especially in routine quality control of the herbs.

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