NIR rapid assessments of Chinese material medica: Simultaneous determination of three major active components of licorice

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Abstract

Objective: A rapid and nondestructive method was used to quantitatively predict the content of three main active components (glycyrrhizin, liquiritin and isoliquiritin) in licorice by near infrared spectroscopy (NIRS). **Methods**: Diffuse reflectance spectra of licorice powder were obtained, the contents of glycyrrhizin, liquiritin and isoliquiritin were analyzed simultaneously by high-performance liquid chromatography (HPLC). The partial least squares (PLS) regression algorithm was used to establish the quantitative models. Several pretreatments such as multiplicative scatter correction (MSC), first derivative, second derivative and Savitzky-Golay (SG) smoothing were utilized to correct the scattering effect and eliminate the baseline shift in all spectra. The calibration equations produced the highest determination of coefficient values (R^2), the lowest root mean square error of calibration (RMSEC) and the lowest root mean square error of prediction (RMSEP) were used for the determination of glycyrrhizin, liquiritin and isoliquiritin. **Results:** The R^2 of glycyrrhizin, liquiritin and isoliquiritin were 0.999, 0.996 and 0.999, respectively. The RMSEC of glycyrrhizin, liquiritin and isoliquiritin were 1.14 mg/g, 0.77 mg/g and 0.068 mg/g respectively. The RMSEP of glycyrrhizin, liquiritin and isoliquiritin were 4.92 mg/g, 2.06 mg/g and 0.35 mg/g respectively. **Conclusions:** The results indicated that the NIRS method could be used for the rapid assessment of licorice.

Keywords: Near infrared, Process analytical technology, Glycyrrhizin, Liquiritin, Isoliquiritin, Licorice, Partial least squares, high-performance liquid chromatography

1. Introduction

Traditional Chinese Medicine (TCM) plays an important role in clinical therapy in China for thousands of years and has been utilized widely as health products around the world. Owing to the complicated chemical components of TCM, traditional analytical methods such as chromatography and electrophoresis are not only time-consuming, but also destructive methods, for they need complex pretreatment and long analysis time. However, near infrared spectroscopy (NIRS) which is mainly based on the overtone and combination bands arising from fundamental vibrations in the mid-infrared region, provides a fast, nondestructive method, requiring no sample or minimal preparation and producing no waste in contrast with traditional analytical methods. In recent years, NIRS combined with chemometrics technique have attracted considerable attention for the purpose of quantification and qualification analysis in herbal medicine [1-6].

Glycyrrhzza Radix et Rhizoma, also known as licorice, is a Chinese herb medicine, which has been widely used in the treatment of spleen and stomach weak, lassitude, feeble, severe palpitation and etc. in China for over 2000 years [7]. Chemistry and pharmacology studies show that the main active constituents of licorice are triterpenoid saponin and flavonoid. Glycyrrhizin, the main triterpenoid saponin active ingredient has wide pharmacological activities such as liver protection, anti-anaphylaxis, anti-inflammation and etc. The main active flavonoid ingredients known as liquiritin,

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isoliquiritin, liquiritigenin also have wide pharmacological activities as anti-ulcerative, bacteriostasis, antiviral, antioxidation and etc. [8-10].

In China, licorice has a wide range of sources, including different areas, different production modes (wild or cultivate) and different cultivation conditions. Previous studies showed that licorice from different sources had significant differences in the active constituents [11, 12]. Therefore, it is necessary to determine the main active contents in raw material to ensure the safe and efficacy of herbal medicinal products.

Several methods have been applied to determine the active constituents in licorice, such as thin-layer chromatography (TLC) [5], high-performance liquid chromatography (HPLC) [5, 13], and capillary zone electrophoresis (CZE) [14]. However, all the methods involved sample extractions and it took a long time to complete the whole analysis. Therefore, there is an increasing demand for a reliable and fast analytical method to evaluate the quality of licorice. In regard to analysis time, cost and environmental issues, the potential of using NIRS to speed up the quality control of TCM was explored.

Licorice was used as an example to demonstrate the feasibility of NIRS for multi-components analysis. As far as we know, NIRS has been demonstrated that it can be used in licorice qualitative and quantitative analysis [15], but the existing report was based on a limited number of samples and it only used in the prediction of glycyrrhizin content. In view of these shortages, we did a further study here. Comparing to the existing report, we have obtained more samples and they are more representative for they are acquired from nearly all the licorice main production areas in China.

Supported by Engineering Research Center of Good Agricultural Practice (GAP) for Chinese Crude Drugs of Ministry of Education, 7 provinces and 21 counties were investigated. NIRS has used in the prediction of glycyrrhizin, liquiritin and isoliquiritin contents here while the existing report only used in the prediction of glycyrrhizin content. In this paper, a NIRS method of rapid quantitative determination of glycyrrhizin, liquiritin and isoliquiritin in licorice samples was established through multivariate calibration regression.

2 Materials and Methods

2.1 Materials

110 wild licorice samples were collected from 21 counties of 7 provinces in China in 2008 and 2010 respectively. All of the samples were identified as the root and rhizome from Glycyrrhizia uralensis Fisch. by Dr. WANG Wenquan (Institute of Medicinal Plant Development, Chinese Academy of Medical Science & Peking Union Medical College). The samples' details were shown in Table 1. After being cleaned by brushing off soil dust from the surface, the licorice samples were crushed into pieces, and then milled into powder with a grinder. The final powder samples were prepared by passing through 60-mesh sieve. To ensure that moisture was not an interfering factor, all powder samples were dried for 24 hours at an oven of 50 °C. Standard of glycyrrhizin was purchased from the National Institute for the Control of Pharmaceutical and Biological products (Beijing, China).

Standards of liquiritin and isoliquiritin were purchased from the Shanghai Ronghe Medical Technology Co., Ltd (Shanghai, China). HPLC-grade acetonitrile was purchased from Merck (Germany). Deionized water was purified by Milli-Q water system (Millipore Corp., Bedford, MA, USA). Other reagents were analytical grade.

Table 1: The samples' details of licorice

Province	County	Number	Province	County	Number
Ningxia	Gaoshawo	3	Shanxi	Fanzhi	6
Ningxia	Hongsipu	9	Shaanxi	Suide	3
Gansu	Longxi	4	Shaanxi	Zizhou	6
Inner Mongolia	Ejinaqi	2	Xinjiang	Gongliu	3
Inner Mongolia	Chifeng	3	Xinjiang	Hejing	4
Inner Mongolia	Etuokeqian	10	Xinjiang	Jimunai	2
Inner Mongolia	Guyang	6	Heilongjiang	Anda	3
Inner Mongolia	Hangjiqi	11	Heilongjiang	Zhaozhou	3
Inner Mongolia	Tuyouqi	6	Jilin	Tongyu	7
Inner Mongolia	Tongliao	3	Jilin	Zhenlai	3
Inner Mongolia	Wuchuan	3			

2.2 NIR equipment and software

A FT-NIR spectrometer (Nexus FT-NIR spectrometer, Thermo Nicolet Co., USA) equipped with an integral sphere diffuse accessory, Results signal acquisition software and TQ Analyst software was used to acquire the diffuse

reflectance spectra of licorice powder. Following was the scanning parameters: the resolution was set as 4 cm^{-1}, scanning 32 times, the scanning range was 4,000-10,000 cm^{-1}. Sample was measured four times, and then we calculated the average spectrum to establish models. Diffuse reflectance spectra of licorice samples were shown in Fig. 1.

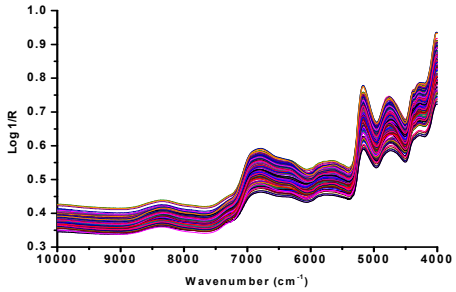

Figure 1: NIR spectra of licorice samples

2.3 HPLC analysis

2.3.1 Extraction

Licorice powder (0.1 g) was extracted by ultrasonic method (30 min, 250 W, 40 °C) with 50 ml 70% ethanol. After being extracted, the mixture was filtered through filter-paper, and then the filtrate was filtrated through a 0.45 μm filter. Finally, the solution was transferred into an HPLC vial prior to HPLC analysis[16].

2.3.2 HPLC equipment and chromatography conditions

The HPLC analysis was performed on a Waters HPLC system, equipped with a degasser, a quaternary pump (Model 1525) and a UV detector (Model 2489). A Breeze 2 ChemStation for LC System was used for data acquisition and integration. Separation was achieved on a Dikma Diamond C18 column (250 mm × 4.6 mm, 5 μm). The gradient elution system consisting of solvent A (acetonitrile) and solvent B (0.05% phosphoric acid water solution) was carried out at the flow rate of 1.0 mL/min and the injection volume was 10 μL. The mobile phase elution was programmed according to the gradient elution program shown in Table 2. The absorbance was measured at wavelength of 237 nm (detection of glycyrrhizin and liquiritin) and 365 nm (detection of isoliquiritin). The chromatographic peaks were identified by comparing the retention time with known standards. The HPLC chromatograms of licorice standards and sample were shown as Fig. 2.

Table 2: Gradient elution program for HPLC

Time（min）	Solvent A（%）	Solvent B（%）
0~5	20	80
5~30	20→50	80→50
30~33	50→100	50→0
33~36	100→80	0→20
36~40	80→20	20→80

2.4 Data preprocessing

The prediction of glycyrrhizin, liquiritin and isoliquiritin contents were established based on HPLC data set as reference in the wavenumber range of 4,000-10,000 cm^{-1} using the PLS algorithm. HPLC data was divided into a calibration set and a validation set. The optimum number of factors used for calibration model was determined by the root mean square error of cross-validation (RMSECV). Several pretreatment methods including multiplicative signal correlation (MSC), normalization (DN) and derivative method were investigated for the optimization of calibration model. The relative performance of the constructed model was assessed by the determination of coefficient (R^2) and the root mean square error of calibration (RMSEC). The predictive ability was evaluated using root mean square error of prediction (RMSEP)[17-19].

3 Results and Discussion

3.1 Quantitative analysis of licorice by HPLC method

All of the 110 licorice samples were used to establish the NIR models. The quantitative results of all the licorice samples were shown in Table 3. From the table, we can see that the concentration ranges of active constituents in

licorice were wide. Therefore, there was no need for other exerted labor to expand the range of active constituents. The samples were divided into a calibration and a validation set, including 66 and 44 samples respectively.

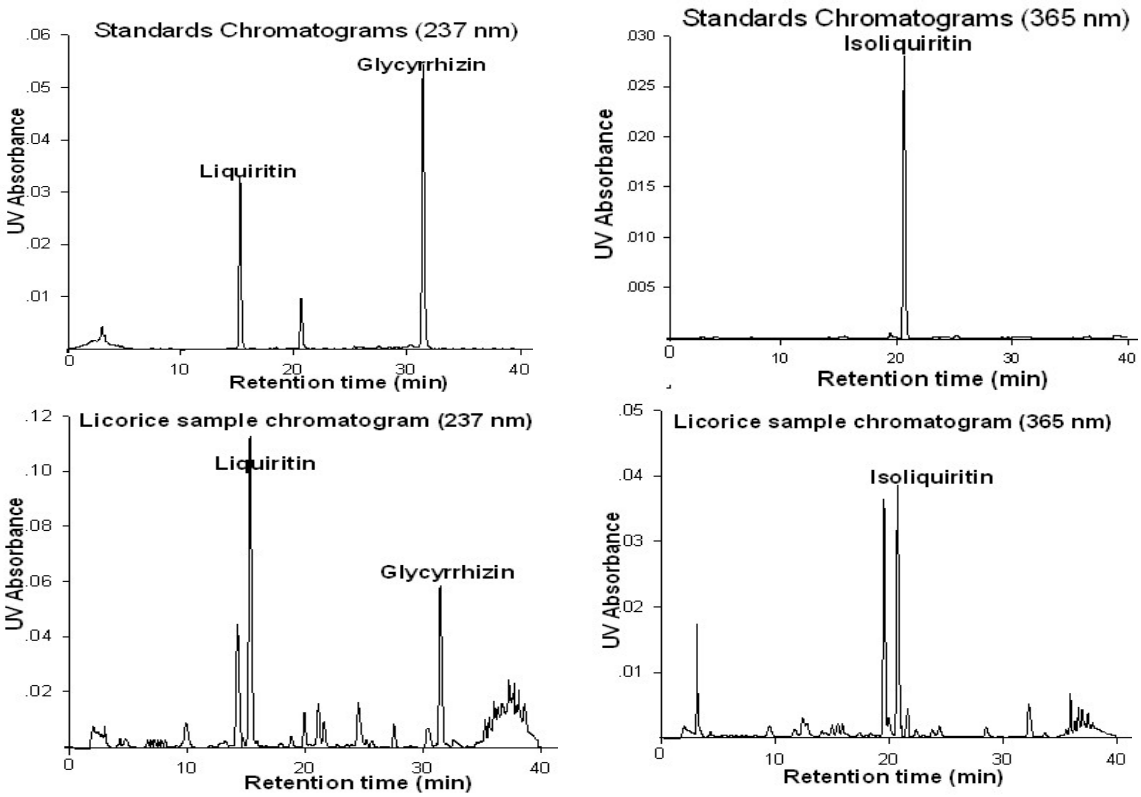

Figure 2: HPLC chromatograms of licorice standards and sample.

As seen from Table 4, the concentration ranges of glycyrrhizin, liquiritin and isoliquiritin contents were spanned over the range in both sets, therefore the distribution of the samples was appropriate in the calibration and validation sets. Quantification of the glycyrrhizin, liquiritin and isoliquiritin contents in licorice samples were performed in the NIR region of 4,000–10,000 cm^{-1}. The best calibration equation for each analysis was selected in terms of R^2, RMSECV and RMSEP.

Table 3: HPLC results of 110 licorice samples

components	samples	content(mg/g)	Mean	Standard deviation
glycyrrhizin	110	5.23-105.42	38.63	22.86
liquiritin	110	7.22-36.88	13.23	8.60
isoliquiritin	110	0.24-7.67	2.48	1.57

Table 4: Statistic results of calibration and validation sets

components	usage	Samples（n）	Content (mg/g)	Mean (mg/g)	Standard deviation(mg/g)
glycyrrhizin	Calibration set	66	5.23- 105.42	40.12	24.53
	Validation set	44	10.14- 95.73	36.40	20.47
liquiritin	Calibration set	66	7.22- 36.88	13.00	8.92
	Validation set	44	8.00- 34.72	13.58	8.19
isoliquiritin	Calibration set	66	0.25-7.67	2.59	1.63
	Validation set	44	0.24-6.38	2.31	1.47

3.2 Optimization of spectral pretreatments

Several data pretreatment methods and their combination were investigated here for the optimization of calibration model. As seen from the Table 5, for glycyrrhizin quantification analysis, the result showed that PLS models with MSC and first derivative pretreatments provided the most acceptable results. For liquiritin quantification analysis, PLS models with first derivative pretreatment provided the most acceptable results. For isoliquiritin quantification analysis, PLS models with second derivative and SG smoothing pretreatments provided the most acceptable results.

Table 5: The effects of different pretreatments on the calibration performance

components	Pretreatment	R^2	RMSEC(mg/g)	RMSEP(mg/g)	Factor numbers
glycyrrhizin	Raw spectra	0.925	9.27	9.25	7
	First derivative	0.998	1.38	5.77	10
	First derivative + SG smoothing	0.984	4.41	5.14	10
	MSC+ First derivative	0.999	1.14	4.92	10
	Second derivative	0.998	1.52	10.2	5
	Second derivative + SG smoothing	0.992	3.06	6.8	7
	MSC+ Second derivative	0.999	0.692	9.56	6
liquiritin	Raw spectra	0.899	3.88	3.13	7
	First derivative	0.996	0.77	2.06	9
	First derivative + SG smoothing	0.971	2.1	2.35	9
	MSC+ First derivative	0.996	0.789	2.18	9
	Second derivative	0.987	1.41	5.66	4
	Second derivative + SG smoothing	0.981	1.71	2.68	7
	MSC+ Second derivative	0.991	1.22	5.66	4
isoliquiritin	Raw spectra	0.929	0.598	0.73	10
	First derivative	0.992	0.204	0.46	8
	First derivative + SG smoothing	0.935	0.576	0.539	6
	MSC+ First derivative	0.967	0.413	0.537	6
	Second derivative	0.999	0.0698	1.09	6
	Second derivative + SG smoothing	0.999	0.0685	0.35	10
	MSC+ Second derivative	0.988	0.248	1.04	4

3.3 Effect of factor numbers

In PLS algorithm, it is generally known that the number of PLS factors is critical parameter. Including more PLS factors in the model will fit the training set better, but the prediction for other samples may become worse. This phenomenon is called over-fitting. Here, we have discussed the effect of different main factor numbers (1-20). The result was shown as Fig. 3. We can see that the optimum number of factors for glycyrrhizin, liquiritin and isoliquiritin calibration models were 10, 9 and 10 respectively.

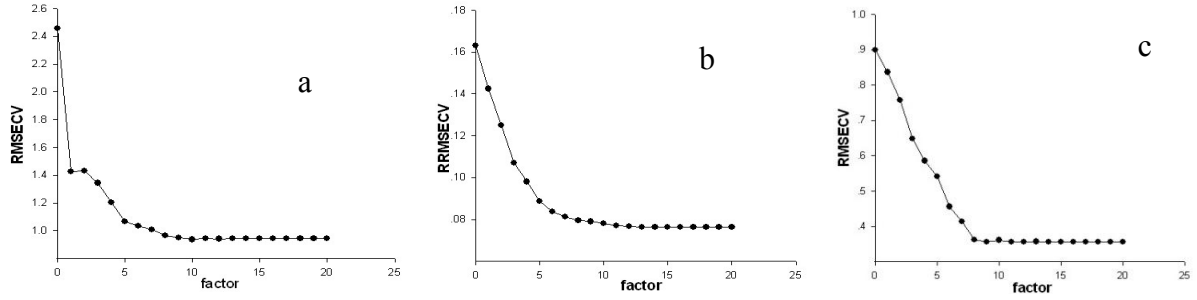

Figure 3: The effects of different factor numbers on the calibration performance, (a) glycyrrhizin, (b) liquiritin, (c) isoliquiritin.

3.4 Calibration model and assessment

For the quantification analysis of glycyrrhizin, the results showed that PLS models with MSC and first derivative pretreatments and 10 main factors provided the most acceptable results, the RMSEC approximately equaled to 1.14 mg/g, the RMSEP approximately equaled to 4.92 mg/g and the R^2 equaled to 0.996. For the quantification analysis of liquiritin, the results showed that PLS models with first derivative pretreatment and 9 factors provided the most acceptable results, the RMSEC approximately equaled to 0.77 mg/g, the RMSEP approximately equaled to 2.06 mg/g and the R^2 equaled to 0.996. For the quantification analysis of isoliquiritin, the results showed that PLS models with second derivative and SG smoothing pretreatments and 10 factors provided the most acceptable results, the RMSEC approximately equaled to 0.068 mg/g, the RMSEP approximately equaled to 0.35 mg/g and the R^2 equaled to 0.999.

The regression plots between reference HPLC measurement and NIRS prediction valued for glycyrrhizin, liquiritin and isoliquiritin were depicted in Fig. 4. The results suggested that the quantitative NIR models developed can be implement in the quality control of Chinese herbal medicine, especially in handling with a large amount of herbal samples in a short period of time, as the only sample treatment is grinding and drying the samples.

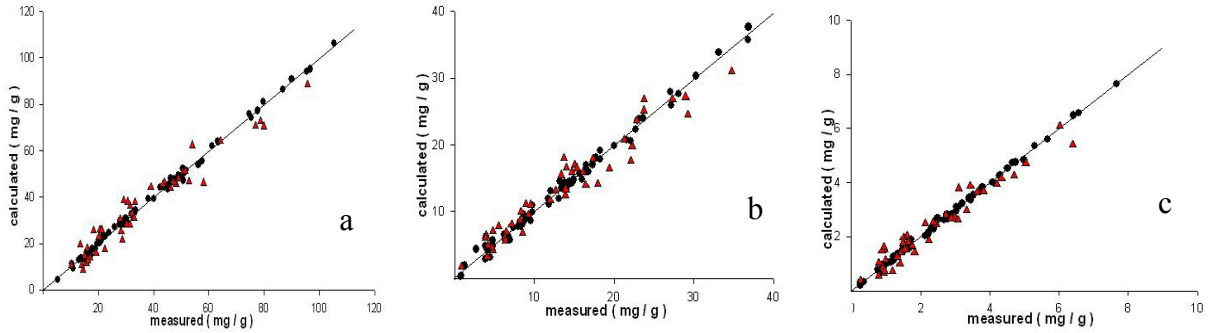

Figure 4: Regression plots for analyses of glycyrrhizin(a), liquiritin(b) and isoliquiritin(c), (●—calibration; ▲ —validation).

4 Conclusions

The first application of NIRS to simultaneously, accurately determination the glycyrrhizin, liquiritin and isoliquiritin in licorice powder were reported. The overall results showed the feasibility of NIRS to quantification of multi-components. Compare to traditional methods such as HPLC, TLC, CEZ, NIRS methods are non-invasive, more rapid, more environmentally friendly and less time-consuming, cost-consuming. Therefore based on this study, NIRS could be possibly applied in the quality control of licorice.

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