Rapid detection of adulteration in *Anoectochilus roxburghii* by near-infrared spectroscopy coupled with chemometric methods

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Abstract To determine the authenticity of *Anoectochilus roxburghii*, this study presents an application of near-infrared spectroscopy and chemometric methods for evaluating adulteration of *A. roxburghii* with two cheaper adulterants, i.e. *C. Goodyera schlechtendaliana* and *Ludisia discolor*. Partial least squares discriminant analysis models were built for the accurate classification of authentic *A. roxburghii* and *A. roxburghii* adulterated at 5–100% (w/w) levels. Partial least squares regression models were used to predict the level of adulteration in the *A. roxburghii*. After by compared different spectral pre-treatment methods, and using interval PLS and synergy interval PLS for variable selection, optimum models were developed. These results show that the NIR spectroscopy combined with chemometric methods offers a simple, fast, and reliable method for classifying and quantifying the adulteration of *A. roxburghii*.

Keywords *Anoectochilus roxburghii* · Near-infrared spectroscopy · Chemometric methods · Partial least squares discriminant analysis · Synergy interval partial least squares regression

Introduction

Anoectochilus roxburghii, a perennial herb of the *Orchidaceae* *Anoectochilus*, is mainly distributed in China, Japan, Sri Lanka, India, and Nepal. In China mainly produced in Fujian, Jiangxi, Guizhou, Zhejiang, Guangdong, and Guangxi provinces (Ye et al. 2017). It is rich in polysaccharides, microelements, amino acids, alkaloids, flavonoid compounds, and other chemical compounds. *Anoectochilus roxburghii* is not only used in drinks and health foods but also widely used in Chinese medicine for clearing away heat and cooling blood, dehumidification and detoxification, regulating the body's immune system and other effects (Li et al. 2012; Liu et al. 2014b; Zeng et al. 2016). It can also be used to treat acute and chronic hepatitis, diabetes, tumors and other disorders (Shao et al. 2013; Zhang et al. 2015). In recent years, the growing demand for *A. roxburghii* on the domestic and international markets, its restricted growing conditions, slow rate of growth, low rate of seed germination, its long-term predatory exploitation, and the serious destruction of its habitat, have led to a shortage in supply and consequently a very high selling price (Hsieh et al. 2016). These are the reasons why *A. roxburghii* products are being adulterated with two other herbs, *G. schlechtendaliana* and *L. discolor* (Lv et al. 2015), which greatly reduce its pharmacological activity. Distinguishing between authentic and adulterated *A. roxburghii* by visual observation is difficult because of their similar appearance. Adulterated *A. roxburghii* could lead to consumers suffering an economic loss and even harm to their health. Thus, a rapid and accurate method for authenticating *A. roxburghii* and its quality is a major issue in the food and medicinal herb trade (Ma et al. 2017).

The methods most commonly used to identify adulteration include nuclear magnetic resonance (NMR) (Siddiqui

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et al. 2017), high-performance liquid chromatography (HPLC) (Hurst and Pfeifer 2014; Liu et al. 2014a), microscopy (Dattatreya et al. 2011), fingerprint identification (Donno et al. 2016) and DNA detection (Lv et al. 2015). However, these methods generally have some shortcomings: complex sample preparation, use of toxic chemicals, damage samples during analysis, and have long analysis times (Xu et al. 2013). Therefore, these methods are not suitable for use in markets that require rapid detection of *A. roxburghii*. But, near-infrared spectroscopy is a fast, simple, non-destructive and effective analytical tool (Li-Guo et al. 2014; Popa 2016), it has been widely used for qualitative and quantitative analyses of food and pharmaceutical commodities (Huang et al. 2016; Marquez et al. 2016), such as milk powder mixed with melamine (Li et al. 2016), Lotus root starch adulterated with cheaper starch (Xu et al. 2013), honey mixed with syrups (Bázár et al. 2016; Yuan et al. 2015), and camellia oil adulterated with soybean and corn oils (Shao et al. 2014). However, there have been no studies about the use of NIR spectroscopy for measuring and quantifying the adulteration of *A. roxburghii*. Therefore, the present study investigated whether NIR spectroscopy can be used for rapidly identifying the adulteration of *A. roxburghii*.

Materials and methods

Authentic and adulterated *A. roxburghii* samples

A total of 69 authentic *A. roxburghii* samples were collected from the six main producing areas of China: the provinces of Fujian (37 samples), Jiangxi (8 samples), Guizhou (8 samples), Zhejiang (6 samples), Guangdong (6 samples), and Guangxi (4 samples). Samples of *G. schlechtendaliana* and *L. discolor* were obtained from a local market in Hangzhou. The voucher numbers of the plants here studied are kept at the herbarium of traditional Chinese Medicine the Zhejiang A&F University. All samples were dried in an oven at 60 °C overnight. After drying, the *G. schlechtendaliana* and *L. discolor* were mixed with authentic *A. roxburghii* (from Fujian province) at levels of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% (wt%, based on the total weight of *G. schlechtendaliana*, *L. discolor* and *A. roxburghii*). Each level was repeated three times. All samples were homogenized to a powder using a mill then sieved through a No. 80 mesh. A 2-g sample was prepared for the NIR spectroscopy study.

Collection of NIR spectra

A multipurpose analyzer MATRIX-F FT-NIR spectrometer (Bruker Optik GmbH, Ettlingen, Germany) was used to

collect the spectral data of all samples. The data were processed with OPUS 7.0, Smica-P 2013 and Matlab 2013 software package. The sample was placed in a circular sample cup (40 mm diameter, 25 mm height) and the sample spectrum collected in the diffuse reflection mode. All spectra were measured using the air background spectrum as a blank control. The samples were scanned in the spectral region from 12,000 to 4000 cm^{-1} with 32 scans at a nominal resolution of 16 cm^{-1} . The spectrum of each sample was measured three times and the mean of three measurements used for the multivariate analysis to minimize any effects of inhomogeneity.

Qualitative analysis

First, principal component analysis is used for preliminary exploratory analysis to data reduction, outlier detection, and pattern recognition, as well as for evaluation of the variable influence (Petrakis and Polissiou 2017; Marquez et al. 2016). Then, the NIR spectra were analysed using PLS-DA. The results were used to distinguish of adulterated and identified adulterants. At the same time, by compared different pretreatment methods (first derivative, second derivative, SNV, and second derivative + SNV), the optimal classification model was selected (Popa and Petrus 2017). The classification performance parameters was estimated by calculated predictability, sensitivity, specificity, and stability (Ma et al. 2017; Popa 2015).

Quantification analysis

PLS is a multivariate statistical data analysis method commonly used for NIR spectral analysis (Jong 1993). Quantitative analysis of *G. schlechtendaliana* and *L. discolor* in the adulterated *A. roxburghii* was carried out using PLS model. To establish a reliable, accurate, and stable model (Popa et al. 2015), compared with different data preprocessing methods, included smoothing, first derivative, second derivative and SNV, and different wavenumber ranges were evaluated for model. The interval PLS (iPLS) and synergy interval PLS (siPLS) were applied to remove irrelevant spectral variables. The use of iPLS is intended to 10, 20 and 30 intervals and calculate a local PLS model for one of the intervals. The siPLS was allowed to develop PLS models based on combination of 2, 3, or 4 intervals.

The PLS, iPLS, and siPLS calibration models were evaluated using the coefficient of determination (R^2) and the root mean square error of cross validation (RMSECV). The predictive ability of the calibration model was tested by computing the root mean standard error of prediction (RMSEP) (Zhong and Qin 2016; Petrakis and Polissiou 2017).

Results and discussion

Sample analysis

The dried *A. roxburghii*, *G. schlechtendaliana* and *L. discolor* samples (Fig. 1) were very similar in appearance, making it difficult to distinguish the authentic from the adulterated *A. roxburghii* using only visual observation.

Spectral analysis

The raw NIR spectra of *A. roxburghii*, and *A. roxburghii* adulterated with *G. schlechtendaliana* and *L. discolor* are shown in Fig. 2a, b. This shows that the NIR spectral curves of the authentic and adulterated *A. roxburghii* samples exhibited similar trends with absorbance bands in the range 12,000–4000 cm^{-1} . The spectra were shown the first overtone of N–H stretching or O–H stretching at 7100–6000 cm^{-1} . Other peaks were assigned as follows: at around 4320 cm^{-1} , the combination absorbance of C–C and C–H stretching; at around 4750 cm^{-1} , the combination of C–O stretching and O–H deformation; at around 5180 cm^{-1} , the combination of the baseband of O–H stretching and the first overtone of C–O deformation; and at 6000–5380 cm^{-1} , the first overtones of C–H stretching in various groups (Xu et al. 2013). Overall, the absorbance intensity of authentic *A. roxburghii* was higher than that of the adulterated *A. roxburghii* which exhibited relatively large fluctuations. However, the spectral differences between the authentic and adulterated *A. roxburghii* were subtle and could not be distinguished just using the naked eye. Therefore, the spectral data were used for qualitative and quantitative analysis analysis with SIMCA-P 2013 and MATLAB 2013.

Qualitative analysis

The NIR spectra were analyzed using PCA, it was not possible to distinguish between the authentic and the adulterated *A. roxburghii*. Because the cumulative contribution rate of the first two principal components was low, it indicates that the classification does not represent all the sample information. Thus, to improve the differentiation of samples, the PLS-DA supervised classification method was used to distinguish between the authentic and adulterated *A. roxburghii*. PLS-DA score plots were obtained of the first derivative, second derivative, SNV, and second derivative + SNV pretreatment (Fig. 3a–d). As shown in Fig. 3a, b were signs of separation as compared to Fig. 3c, but Fig. 3a had a partial overlap between the samples with lower adulterated content. And Fig. 3b showed a clear separation between the authentic and adulterated *A. roxburghii*, but the adulterated *G. schlechtendaliana* and *L. discolor* samples were overlapping with each other. In general, the first derivative, second derivative, and SNV pretreatments could not distinguish between authentic *A. roxburghii*, and *A. roxburghii* adulterated with *G. schlechtendaliana* and *L. discolor* samples. However, it could be seen from Fig. 3d that the second derivative + SNV appeared to be the best pre-treatment method for distinguishing among samples. As Fig. 3d displayed three sample groups were obviously separate: the authentic *A. roxburghii* clustered to the right of the central axis, whereas the *A. roxburghii* adulterated with *G. schlechtendaliana* grouped in the top left, and the *A. roxburghii* adulterated with *L. discolor* clustered in the bottom left. The t_1 played an important role in distinguishing between authentic and adulterated *A. roxburghii* samples, while t_2 had a greater effect on the separation between *A.*

Fig. 1 Samples of *A. roxburghii* (a), *G. schlechtendaliana* (b), and *L. discolor* (c) after drying. Scale bar = 1 cm

Fig. 2 NIR spectra of *A. roxburghii* samples: authentic (a) and adulterated with *G. schlechtendalana* and *L. discolor* (b)

Fig. 3 Score plot from PLS-DA using first derivative (a), second derivative (b), SNV (c), and second derivative + SNV (d) preprocessing

roxburghii samples adulterated with *G. schlechtendalana* and *L. discolor*. Moreover, the goodness of fit (R^2Y) and the predictive ability (Q^2) of the PLS-DA model were 0.941 and 0.851, respectively (Ma et al. 2017), this showed that the stability and predictability of the model were better. Thus, it is illustrated that all authentic and adulterated *A. roxburghii* samples can be correctly classified by using the PLS-DA model, and selection of an appropriate pre-treatment method could improve the classification performance.

Quantitative analysis

After successfully distinguishing authentic *A. roxburghii* from all the *A. roxburghii* samples, the PLS model using the raw spectra, smoothing, first derivative, second derivative and SNV pretreatments, was used for further statistical analysis to determine the proportion of *G. schlechtendalana* and *L. discolor* in adulterated *A. roxburghii*. The spectral data of *A. roxburghii* adulterated with different proportions of *G. schlechtendalana* and *L.*

Table 1 The results of the PLS models with different spectral pretreatments

Model	Adulterated content	Spectra pretreatments	LV	Rc ²	RMSECV (%)	Rv ²	RMSEP (%)
PLS	<i>G. schlechtendaliana</i> adulteration of <i>A. roxburghii</i>	Raw spectra	4	0.9947	3.2656	0.9867	5.1942
		Smoothing	4	0.9946	3.2881	0.9868	5.1819
		First derivative	4	0.9955	2.9873	0.9919	4.0131
		Second derivative	4	0.9986	1.6828	0.9833	7.3317
		SNV	4	0.9991	1.3626	0.9922	4.4575
	<i>L. discolor</i> adulterated in <i>A. roxburghii</i>	Raw spectra	3	0.9834	5.7367	0.9901	5.8667
		Smoothing	3	0.9832	5.7720	0.9898	5.8994
		First derivative	3	0.9900	4.4672	0.9851	5.7889
		Second derivative	3	0.9896	4.5561	0.9754	7.1977
		SNV	3	0.9996	1.3626	0.9982	2.4364

Rc², coefficient of correlation in calibration set; Rv², coefficient of correlation in validation set; RMSECV, root mean square error of cross validation; RMSEP, root-mean-square error of prediction; LV, latent variables

Table 2 Performance results of PLS models using different wavenumber ranges

Adulterated content	Model	Intervals	LV	Rc ²	RMSECV (%)	Rv ²	RMSEP (%)
<i>G. schlechtendaliana</i> adulteration of <i>A. roxburghii</i>	iPLS10	6	5	0.9865	5.2088	0.9933	3.7832
	iPLS20	12	5	0.9808	6.1749	0.9935	3.6112
	iPLS30	16	5	0.9813	6.1340	0.9938	4.4837
	siPLS10	7, 9	6	0.9974	2.3020	0.9972	2.8976
		4, 5, 9	5	0.9973	2.3159	0.9940	3.8713
		4, 5, 7, 9	5	0.9969	2.4798	0.9929	4.2468
	siPLS20	10, 18	4	0.9983	1.8536	0.9960	3.2684
		11, 18, 19	4	0.9985	1.7361	0.9969	2.8938
		7, 10, 18, 19	5	0.9987	1.6168	0.9967	2.9115
	siPLS30	10, 26	5	0.9982	1.8895	0.9947	3.5435
		14, 26, 27	3	0.9981	1.9440	0.9941	3.8697
		5, 25, 26, 29	8	0.9988	1.6107	0.9895	4.6867
<i>L. discolor</i> adulterated in <i>A. roxburghii</i>	iPLS10	5	5	0.9481	10.2849	0.9785	6.8705
	iPLS20	13	5	0.9977	2.1537	0.9992	1.3429
	iPLS30	19	3	0.9971	2.4311	0.9952	3.2355
	siPLS10	6, 7	5	0.9473	10.2561	0.9742	8.9136
		6, 7, 8	6	0.9991	1.3217	0.9987	1.7697
		5, 6, 7, 9	6	0.9988	1.5459	0.9995	1.3693
	siPLS20	13, 19	5	0.9991	1.3370	0.9996	1.0748
		11, 13, 17	6	0.9994	1.1327	0.9992	1.4049
		11, 13, 15, 16	6	0.9992	1.2420	0.9986	1.8316
	siPLS30	19, 26	9	0.9992	1.2743	0.9981	2.3271
		21, 26, 29	5	0.9993	1.1738	0.9994	1.7745
		13, 17, 19, 26	8	0.9995	1.0204	0.9989	1.4990

Rc², coefficient of correlation in calibration set; Rv², coefficient of correlation in validation set; RMSECV, root mean square error of cross validation; RMSEP, root-mean-square error of prediction; LV, latent variables

discolor (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100%, wt%) were analyzed by PLS. The spectra ranging between the wave numbers of 12,000–4000 cm⁻¹ were used for the

quantitative analysis. The PLS analysis results are shown in Table 1. According to the criteria of the R² and RMSE values for the prediction set and training sets in the PLS

Fig. 4 Predicted levels (%) of *G. schlechtendalians* (a, b) and *L. discolor* (c, d) in adulterated samples *A. roxburghii* using NIR with siPLS for the calibration and validation sets

model, the latent variables was chosen as 4. The R^2 values (R^2 for the calibration set) of the first derivative, second derivative, and SNV spectra were greater than those for the raw spectra, with RMSECV and RMSEP values being smaller than those for the raw spectra. We also found that the R^2 value was greatest and closest to 1 for the SNV pretreatment with the RMSECV and RMSEP values being the smallest at 1.3626 and 4.4575%, respectively. The PLS analysis results for *A. roxburghii* samples adulterated with *L. discolor* were similar to samples adulterated with *G. schlechtendalians*. When the LV was 3, we found that the R^2 value for SNV was greatest with RMSECV and RMSEP values being smallest with values of 1.3626 and 2.4364%, respectively. This indicated that pretreatment can improve the performance of the PLS model, and that using the SNV treatment for quantitative analysis was best for the calibration model and most satisfactory for the prediction model.

Improve the performance of the selected PLS model by using the iPLS and siPLS variable selection methods. Table 2 showed the statistical indicator for better iPLS and siPLS calibration models of *A. roxburghii* adulterated with *G. schlechtendalians* and *L. discolor*, respectively. The

RMSEP values obtained for the models developed a different interval (10, 20, 30) model after the iPLS algorithm were high and low compared to the values for the full spectrum model. This result showed that choosing the appropriate interval to avoid spectral noise information, but only select a range, will lose some useful information. So the use of siPLS variable selection method can better improve the performance of the model. As shown in Table 2, the models that presented better performance by compared RMSECV values and RMSEP values were based on siPLS in all cases. The optimal siPLS model of *G. schlechtendalians* adulteration of *A. roxburghii* was 20 intervals and 11, 18 and 19 intervals were combined. At this point, RMSEP value was at least 2.8938, RMSECV value was 1.7361. However, the optimal siPLS model of *L. discolor* adulteration of *A. roxburghii* was 20 intervals and 13 and 19 intervals were combined. RMSEP value was at least 1.0748, RMSECV value was 1.3370. Figure 4 illustrates the linear relationship between the predicted values from the optimal siPLS model and the actual adulteration level of *G. schlechtendalians* and *L. discolor* in *A. roxburghii*. As shown in calibration R^2 and validation R^2 value of 0.9985 and 0.9969 for *G. schlechtendalians* and 0.9991

and 0.9996 for *L. discolor*, respectively. The results showed that spectral preprocessing and variable selection have improved the performance of PLS model.

Conclusion

In this study, a qualitative analysis method to discriminate between authentic *A. roxburghii* and *A. roxburghii* adulterated with *G. schlechtendaliana* and *L. discolor* and to quantify the level of adulteration using NIR combined with chemometric methods. The classification model using PLS-DA combined with second derivative + SNV method exhibited excellent performance to classify three categories of sample correctly. After identification, NIR coupled with a PLS calibration model was established to quantify the level of adulteration by *G. schlechtendaliana* and *L. discolor* in *A. roxburghii* samples. And the effects of different pretreatment methods and iPLS and siPLS variable selection methods on model performance are compared. These results suggested that the appropriate pretreatment method and variable selection can effectively improve the performance of the model. The combination of NIR spectroscopy and chemometric methods offers a fast, simple, and reliable method for classifying and quantifying adulteration in *A. roxburghii* for use in a quality control system.

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