

Geographic origin classification and simultaneous determination of methylxanthines in vietnamese tea using chemometrics based on the near infrared reflectance spectroscopy

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Abstract

This paper reported the results of classification of geographic origin and simultaneous analysis of three methylxanthines (caffeine, theobromine, theophylline) in Vietnamese tea samples by the infrared reflectance spectrophotometry coupled with chemometrics. The spectral range was 10,000-4,000cm⁻¹ and each spectrum was measured at 2 cm⁻¹ intervals. For the purpose of geographic origin classification, this study used FT-NIR spectroscopy combined with Partial Least Squares Discriminant Analysis (PLS-DA), and Principal Component Analysis-Discriminant Analysis (PCA-DA). The ability to determine the origin of tea samples in the prediction set of PLS-DA model is 100%. Using the same IR spectral database combined with the partial least squares (PLS), three methylxanthines in tea samples are also quickly quantified. The PLS model based on the spectra of 24 tea samples in which the contents of 3 analytes were determined by high performance liquid chromatography- HPLC) were applied for simultaneous determination of caffeine, theobromine and theophylline in samples. The determination of methylxanthines in 7 tea samples in test set gave the good accuracy of the PLS model. The correlation coefficients (R²) in the prediction set were of 0.9582, 0.8894 and 0.9303 for theobromine, theophylline, and caffeine, respectively. This work demonstrated that infrared reflectance spectrophotometry combined with chemometrics could be applied to rapidly classify the geographic origin and simultaneous determination of main contents in green tea.

Nhận 20.05.2019

Được duyệt 14.06.2019

Công bố 26.06.2019

Keywords

caffeine, theobromine, theophylline, multivariable regression, tea, infrared reflectance spectrophotometry

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1 Introduction

Tea (*Camellia Sinensis L*) was discovered very early about 2700 BC. Tea becomes a cultural popular drink in almost every social activities and penetrates into daily life in Vietnam. Nowadays, tea have been varieties in the market not only from botanical standpoints but also in terms of quality attributes. Catechins, together with phenolic acids, are a group of polyphenols that are important factors in the taste of tea. Caffeine, theophylline, and theobromine are the main methylxanthines constituting the tea alkaloids, being important factors in the quality of teas. Many factors can contribute to the chemical composition and taste of tea, such as species, season, age of the leaves, climate and horticultural conditions. Thus, green teas cultivated in different geographical areas will present significant differences in their chemical compositions[1].

Traditionally, sensory evaluation is used to discriminate the geographic origin of tea. However, using sensory evaluation to identify tea is imprecise, as it can be easily influenced by other factors, including the environment and the mood of the evaluator[2,3]. So far, there have been many analytical methods have proved to be effective for quality control of tea. Several authors propose capillary electrophoresis as the technique to be used[4,5]. Many works have been reported including high-performance liquid chromatography (HPLC) determinations of these tea polyphenols with isocratic[6] and gradient elution[7-10]. However, the above chemical analysis methods are complex, time-consuming, labor-intensive, costly and require large amounts of organic solvents. Therefore, a rapid and accurate analytical method is required to discriminate the geographical indicator of tea origin. Fourier Transform Infrared (FT-IR) spectroscopy is a

powerful analytical tool because it is fast and non-destructive. Recently, IR spectroscopy has been applied for the simultaneous analysis of free amino acids, caffeine, total polyphenols and amylose in green tea[11-15].

Vietnam has 35 tea producing provinces with a total area of 125,000 hectares, most of them in the Northern Midlands, North Central and Central Highlands provinces. Every year, Vietnam's tea exports reach over US \$ 100 million. Vietnam has exported tea to 107 countries, ranking 7th in export volume (987.3 thousand tons in 2018), ranking 6th in export value. However, in our country the classification of different types of tea is still based on the sense[19].

In this study, we developed a method using IR spectroscopy to simultaneously analyze three methylxanthines and discriminate the geographic origin of Vietnamese tea. Statistical algorithm used in this paper was PLS. Pattern recognition techniques, such as PLS-DA and PCA-DA, were applied for classification purposes.

2 Material and methods

2.1 Instruments

A HPLC system (Shimadzu LC- 20A system) equipped with a dual wavelength absorbance detector and LiChrospher C-18 reverse phase (5 μ m x 250mm x 4.6mm) column was used. The mobile phase containing 85% buffer (potassium phosphate, pH 3.0) and 15% acetonitrile with 1.2ml min⁻¹ flow rate was used and the detector was set at 271nm.

The infrared reflectance analysis using Thermo scientific series Nicolet iS50 NIR was used. Each spectrum consists of 3000 values of intensities at 2cm⁻¹ intervals in the wavenumber range 10,000-4,000cm⁻¹. An Eureka HD-40 30L dehumidifier was used for removing water of samples before NIR analysis.

2.2 Sampling and sample preparation for analysis

A total of 57 green tea samples which have a identified geographical origin, directly taken in the process of harvesting and processing in the provinces of Thai Nguyen (23 samples), Lam Dong (14 samples), other provinces such as Ha Giang, Yen Bai, Tuyen Quang, Hoa Binh (20 samples) was collected. The original and botanical information of the samples were recognized by onsite collection (for setting up the model) or based on the package (for comparison of the geographical origin between predicted and trade result). About 100g of air-dried tea-leaves were kept at least 2 days in a dehumidifier at the 30% moisture before analyzing.

All the NIR analysis were carried out in a separated chamber with 30% moisture of air. The dried tea samples were ground in a laboratory grinder to obtain tea powder through to 240 (63 μ m) mesh BS sieves. Dry tea powder (about 5g) was put in to a sample cup in the standard procedure. Each tea sample was measured five times and then average of the five spectra

collected from the same tea sample was used for further analysis.

In order to obtain known and reference concentrations for setting up the multivariate models, methylxanthines contents in real samples were measured by reverse phase- high performance liquid chromatography (RP-HPLC). Because caffeine is very soluble in boiling water (66 g/100 mL), the methylxanthines were extracted out of tea samples by using boiling water. Approximately 2.0 g tea powders, accurately weighed, were extracted twice with 50mL double-boiling distilled water 95-100^oC [6], and let to stand for 5 minutes. The infusions were filtered with filter paper, and diluted to 100mL with double-distilled water. The tea brews were filtered through a 0.45 μ m membrane filter and analyzed immediately.

2.3 Spectral pre-treatment

In this study, the spectral pre-treatment was done using three algorithms: mean centering (MC), multiplicative scatter correction (MSC) and standard normal transformation (SNV). The MC is used for calculating the average spectrum of the data set. The MSC is the extraction algorithm and multiplied by the linear individual spectra with a mean score. SNV is a mathematical transformation method of the log (1/Intensity) spectra, used for removal of slope variations and to correct scatter effects[11]. After spectral pre-treatment, the PLS algorithm was applied for calculating the content of three methylxanthines in the tea samples.

2.4 Statistical analysis

Pattern recognition techniques, such as Partial Least Squares Discriminant Analysis (PLS-DA), and Principal Component Analysis-Discriminant Analysis (PCA-DA) were applied for classification purposes. Multivariate calibration of partial least square (PLS) was performed using *Matlab 2016a*. The values of coefficient of determination (R²) and root mean square error of calibration (RMSEC) were used as performance criteria for calibration model [16].

$$RMSEC = \sqrt{\frac{\sum_{i=1}^n (actual - calculated)^2}{N - f - 1}}$$

The smaller RMSEC value, the less uncertainty of calibration is [17]. Also, R² values and root mean square error of prediction (RMSEP) together can show how well the developed model for quantitative analysis of new samples; the lower the RMSEP value, the better the prediction performance of the model.

$$RMSEP = \sqrt{\frac{\sum_{i=1}^n (actual - calculated)^2}{M - 1}}$$

The term “actual” means the concentrations (determined by HPLC) of selected samples; and the term “calculated” refers to the concentrations calculated by the model using spectral data; N and M are the number of samples used in the calibration and validation sets, respectively; f is the number of factors used in the calibration model by PCA or PLS.



3 Results and discussion

3.1 Simultaneous analysis of three methylxanthines in tea samples

3.1.1 Analysis of methylxanthines by RP-HPLC

Prior to quantitative analysis by IR spectroscopy, the HPLC reference method has to be established. The contents of 3

methylxanthines in 32 tea samples (16 samples from Thai Nguyen, 6 samples from Lam Dong and 10 samples from other provinces) were quantified. The remaining amounts of samples were kept for IR analysis. Figure 1 shows the typical chromatograms of a standard solution and a tea sample. The results obtained after analyzing the tea samples, expressed in mg/g, on dry basis, are depicted in Table 1.

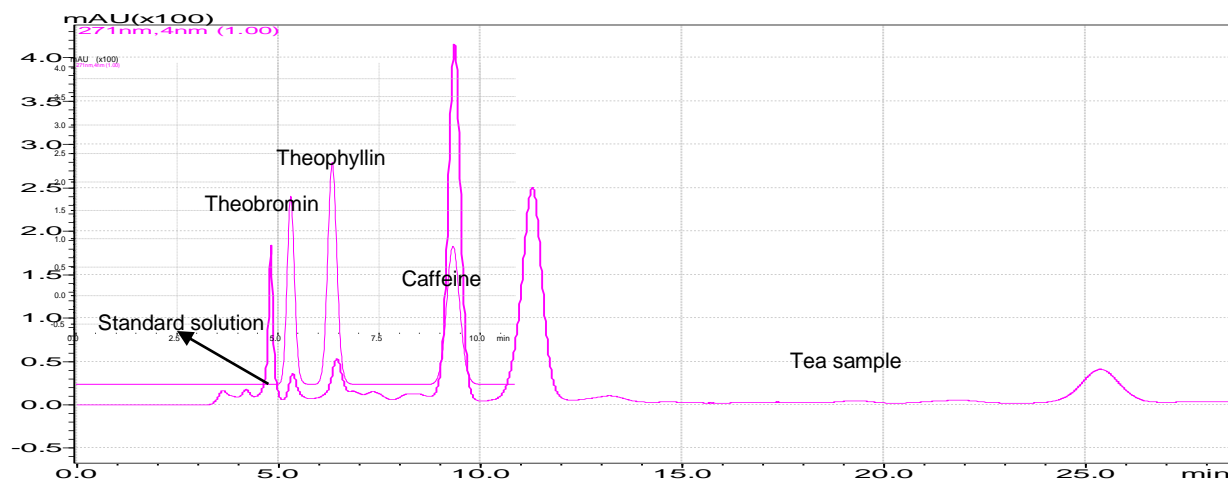


Fig. 1 Typical Chromatograms of a standard solution and a tea sample

Table 1 The contents of caffeine (CAF), theophylline (TP), theobromine (TB) in the analyzed tea samples (studied provinces: TN- Thai Nguyen; LD- Lam Dong; YB- Yen Bai; TQ- Tuyen Quang- HB- Hoa Binh)

No.	Region	Sample	Contents (mg/g)			No.	Region	Sample	Contents (mg/g)		
			TB	TP	CAF				TB	TP	CAF
1	Tan Cuong-TN	TN1	4.46	4.34	49.35	19	Hoa Ninh – LD	LD3	5.07	3.05	23.59
2	Tan Cuong-TN	TN2	6.01	4.32	44.02	20	Tam Chau – LD	LD4	1.96	3.39	17.93
3	Tan Cuong-TN	TN3	3.59	4.64	47.37	21	Tam Chau – LD	LD5	4.06	3.79	35.23
4	Tan Cuong-TN	TN4	2.94	4.44	45.47	22	Tam Chau – LD	LD6	2.49	3.25	23.93
5	Tan Cuong-TN	TN5	4.16	4.89	43.99	23	Mu Cang Chai – YB	CTK1	4.20	5.48	63.51
6	Tan Cuong-TN	TN6	3.34	4.43	56.26	24	Hong Ca – YB	CTK2	1.58	4.09	33.48
7	Tan Cuong-TN	TN7	4.53	5.03	70.16	25	Tran Yen – YB	CTK3	3.35	3.56	26.98
8	Tan Cuong-TN	TN8	4.52	4.95	68.00	26	Ham Yen- TQ	CTK4	2.93	5.27	61.77
9	Tan Cuong-TN	TN9	4.92	5.55	54.95	27	Ha Giang	CTK5	5.49	2.70	58.06
10	Tan Cuong-TN	TN10	5.12	6.79	77.72	28	Ha Giang	CTK6	3.31	2.38	79.39
11	Tan Cuong-TN	TN11	4.19	4.95	62.52	29	Lac Son –HB	CTK7	2.85	3.70	50.56
12	Tan Cuong-TN	TN12	3.36	6.92	77.01	30	Lac Thuy -HB	CTK8	3.20	4.35	50.73

13	Tan Cuong-TN	TN13	2.27	4.52	51.48	31	Lac Thuy -HB	CTK9	2.82	3.89	47.08
14	Dai Tu-TN	TN14	4.00	5.17	53.00	32	Lac Thuy -HB	CTK10	4.62	4.76	56.47
15	Dai Tu-TN	TN15	4.18	4.70	45.33						
16	Dai Tu-TN	TN16	3.42	5.14	64.46						
17	Di Linh -LD	LD1	3.61	1.34	21.78						
18	Bao Lam-LD	LD2	3.66	2.66	24.62						

Results in table 1 revealed the significant differences in methylxanthine's contents in samples collected in the three regions studied. Lam Dong tea tend to be distinguished by lower contents of methylxanthines compared to those from Northern Midlands. The methylxanthine contents of the studied tea samples may be influenced by the difference of climate, horticultural conditions.

3.1.2 Spectral pre-treatment

Figure 2-(a) shows FT-NIR spectra of 57 tea samples in infrared reflectance region ($10,000\text{ cm}^{-1}$ - $4,000\text{ cm}^{-1}$). The spectral region from $9,000\text{ cm}^{-1}$ to $4,500\text{ cm}^{-1}$ is known as the

functional group signal (such as C-H, O-H and N-H) with the intensive peaks that are caused by the stretch or deformation vibration. Therefore, the spectral regions from 9000 cm^{-1} to 4500 cm^{-1} were chosen for further making calibration models. Due to the changes of experimental conditions in IR measurements, algorithms of pre-treatment spectra are necessary to be applied.

The pre-treatment spectra obtained by three algorithms are shown in Fig. 2- (b,c,d). The MC pre-treatment spectra gave the better results in classification to SNV and MSC and therefore can be used for making calibration models.

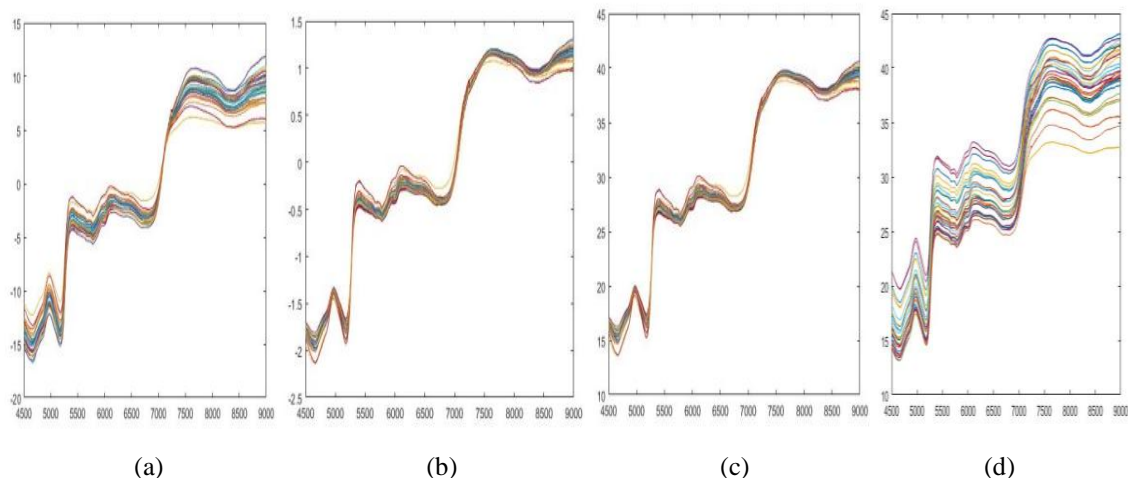


Fig. 2 IR spectra (Intensity versus wavenumbers) of green tea samples obtained from: (a) raw spectra, (b) MC pre-treatment spectra, (c) SNV pre-treatment spectra, (d) MSC pre-treatment spectra

3.1.3 PLS model for simultaneous quantitative analysis

The NIR spectra region contains bands that often overlap making it difficult to extract spectral signal of individual bands. Chemometrics has provided a way of overcoming these problems through empirical models that relates the multiple spectral intensities from many calibration samples to known analytes in these sample. Despite the lack of distinct peaks, it has been shown the PLS can extract relevant information for quantitative determination [5].

For the purpose of quantitative analysis, total 32 standard samples were randomly divided into two subsets. The first subset called calibration set (25 standard samples) was used for building model, while the other called prediction set (7 known samples) was used for testing the accuracy of model.

•Optimization of spectral Data

The PLS multivariate regression for simultaneous determination of CF, TB, TP in tea samples was based on the content matrix of 3 analytes in 25 standard samples determined by HPLC. The spectral signal of 25 samples at

2334 wavenumbers were the IR intensity in the spectral region of 9,000 -4,500cm⁻¹.

The accumulated percent variance explained by components in PLS is performed in Fig. 3. It is clear that first seven

components already explained for more than 95% of the total variance. Hence the calculation will be started from 7 components only.

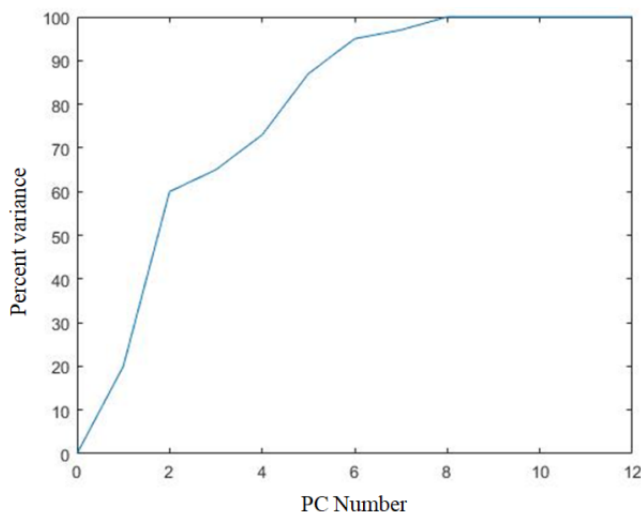


Fig. 3 Accumulated Percent variance explained by components for PLS calibration models as shown in Table 2, the maximum value of R² and minimum RMSEC, RMSEP values calculated with first 7 PLS components were better compared to 8 principal components (PC). Hence the further PLS calibrations would conduct with first seven components.

Table 2 RMSEC, RMSEP and R² values corresponding to 7 or 8 PLS components

No. of PC	R ²			RMSEC			RMSEP		
	TB	TP	CF	TB	TP	CF	TB	TP	CF
7	0.88	0.95	0.93	0.42	0.54	4.71	0.59	0.64	0.45
8	0.82	0.95	0.88	0.56	1.25	5.67	0.64	0.88	0.83

• *Validation of the quantitative model*

The calibration models were further validated using 7 tea samples having known concentrations by HPLC. The good models also were evaluated through the highest R² and lowest RMSEP. Figure 4 shows that there is a good match between three methylxanthine contents found in tea samples by HPLC (measured contents) with predicted content found

using multivariate models (correlation coefficients were 0.8893 to 0.9582 and intercepts were approximately to zero showed no system error happened). Therefore, it is possible to apply the PLS method to simultaneously quantify 3 methylxanthines in a tea sample without digestion and separation before analysis.

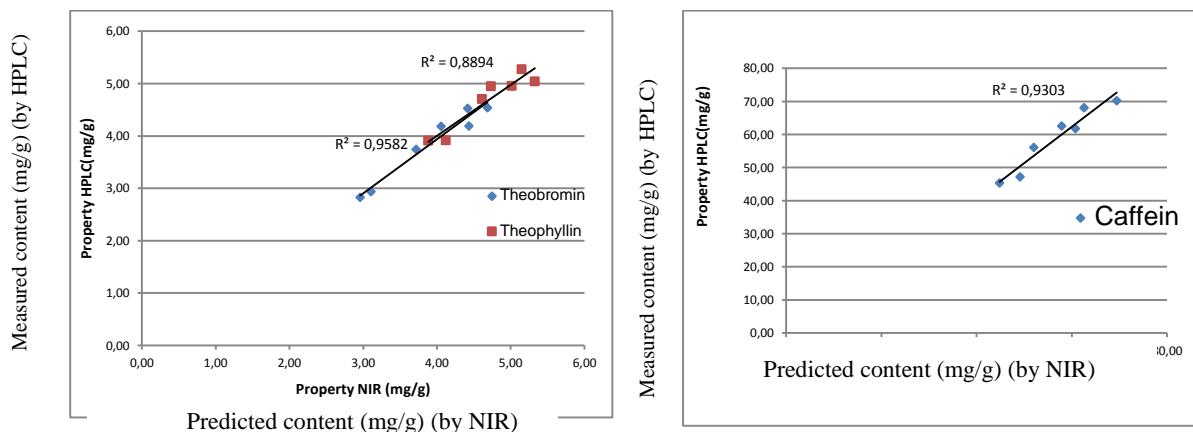


Fig. 4 Linear regression plot of measured versus predicted content of methylxanthines

3.2 Geographical Classification of Tea samples

In Northern Midlands (Thai Nguyen, Ha Giang, Yen Bai, Tuyen Quang, Hoa Binh), tea is grown on limestone Ferral soil, with tropical monsoon climate. In a climate with long lasting cold (5-6 months/year), tea grows relatively slowly, contributes to the slow accumulation of nutrients, making the tea in these provinces always have a strong taste. Compared to tea in the Northern Midlands Lam Dong tea is grown on fertile bazon soil so Lam Dong tea grows faster than Northern tea.

For chemometric calculations, the tea samples were divided into three groups: the green tea from Thai Nguyen (23 samples), Lam Dong (14 samples) and other provinces green (20 samples). Pattern recognition procedures were applied to these data sets, trying to classify the tea samples according to their geographical origin.

In this study, the supervised classification algorithms: Principal Component Analysis coupled with discriminate analysis-(PCA-DA) and PLS-DA were applied based on FT-IR spectra of 57 tea samples. The construction of the multivariate classification models was performed using a training set (51 samples). Each model was validated using the leave-one-out cross-validation technique. A test set (6 samples) was then used for final data evaluation and comparison to the classification models. The performance of the models was evaluated by accuracy, which is defined as the ratio of samples in the test set correctly assigned into their respective classes.

3.2.1 Selection of principal components

PCA is a statistical method to transform multiple indicators into several representative aggregative indicators. Redundancy information is reduced from a high-dimensional space to a low dimensional space by using PCA. The vectors obtained from each principal component are orthogonal. As shown in Figure 5, the first principal component (PC1) accounts for 99.91% of the variance. It is explained that the first component represented 99.91% of the information of the green tea samples and only the first PC was used to setup the classification model.

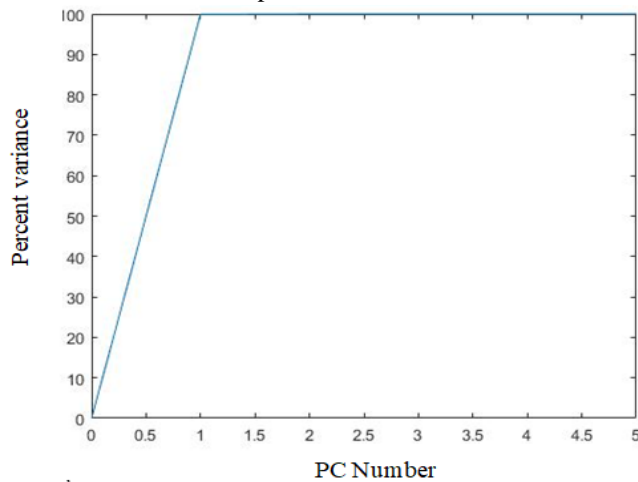


Fig. 5 Accumulated Percent variance explained by components for PCA classification model

3.2.2 Selection of multivariate model

To highlight the good performance of the algorithm, two supervised recognition algorithms, PCA-DA and PLS-DA were performed with only first PC. Figure 6 represents the recognition results obtained by the PCA-DA and PLS-DA approaches in training and prediction sets. The prediction set consists of six samples denoted by Thai Nguyen samples (TN1, TN2), Lam Dong samples (LD1, LD2), other province samples (CTK1, CTK2). PLS-DA typically outperforms Soft Independent Modeling of Class Analogy SIMCA in classification rates, provided that within-class variability is low, as class-separation is maximized. Compared with PCA-DA classification, the PLS-DA model was better able to deal with imbalance training samples and the prediction set. The ability to determine the origin of a tea sample in the prediction set shows PLS-DA can recognize tea's origin of sample with 100% while PCA-DA performed only 83.33%. Therefore, PLS-DA is the suitable method to determine the origin of a tea sample.

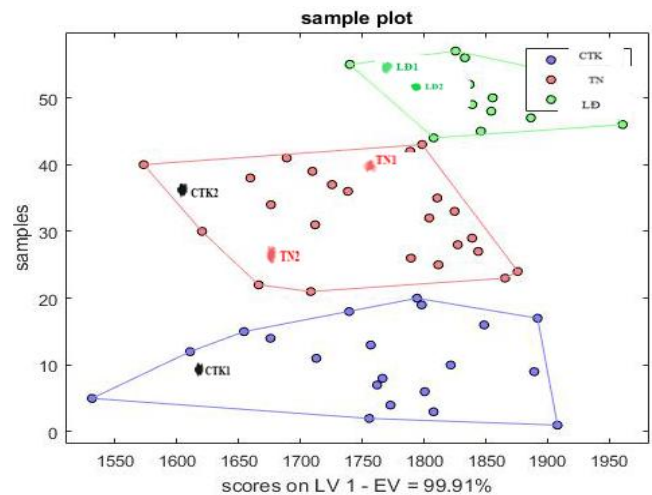


Fig. 6 PCA-DA sample plot for classification of green tea

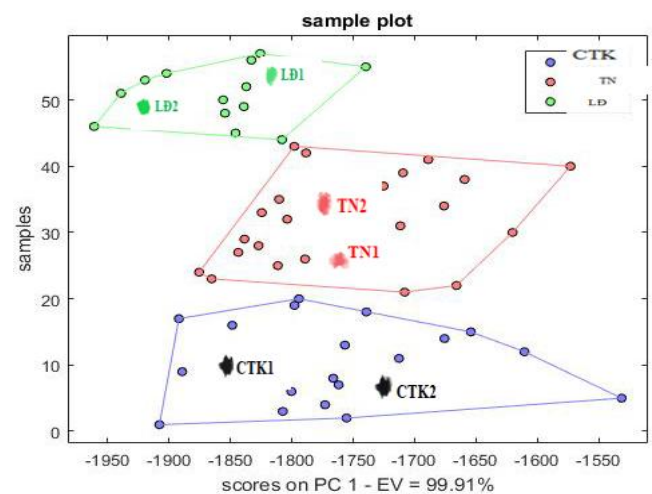


Fig. 7. PLS-DA sample plot for classification of green tea

4 Conclusions

The reflectance IR nondestructive spectroscopy technique coupled with the multivariate regression has a high potential to quantitative analysis of three methylxanthines as well as identify geographical origin of Vietnamese tea with the same spectra profile. For the purpose of quantitative analysis, the NIR spectral data are processed using a partial least squares calibration designed with a series of tea samples in which

methylxanthine concentrations were determined by a HPLC method. The statistical indicators for the prediction in validation sets of samples were good. This study used PLS-DA as a pattern recognition tool to develop an identification model. The PLS-DA algorithm outperforms the PCA-DA approaches in identifying the geographical origin of the tea samples. Therefore, NIR spectra analysis coupled with the multivariate regression can be used as an alternative approach to traditional methods for tea quality evaluation.

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Phân loại nguồn gốc địa lí và phân tích đồng thời các methylxanthine trong chè Việt Nam sử dụng phổ hồng ngoại phản xạ kết hợp với thống kê đa biến

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Tóm tắt Nghiên cứu này trình bày các kết quả phân loại nguồn gốc địa lí và xác định đồng thời ba methylxanthine (gồm caffeine, theobromine, theophylline) trong các mẫu chè Việt Nam dựa trên dữ liệu phổ hồng ngoại phản xạ kết hợp với thống kê đa biến. Phổ hồng ngoại được đo trong vùng 10000-4000cm⁻¹ với khoảng cách đo 2cm⁻¹. Thuật toán đa biến phân tích biệt thức kết hợp với bình phương tối thiểu từng phần (*Partial Least Squares Discriminant Analysis (PLS-DA)*) và phân tích cấu tử chính (*Principal Component Analysis-Discriminant Analysis (PCA-DA)*) được sử dụng để phân loại nguồn gốc địa lí. Các mô hình cho phép dự đoán đúng tuyệt đối nguồn gốc của mẫu chè chưa biết thuộc vùng địa lí nào đã khai báo trong mô hình. Cũng dựa trên cùng dữ liệu phổ IR của mẫu, bằng thuật toán PLS có thể định lượng nhanh ba methylxanthine trong mẫu chè. Mô hình PLS được xây dựng dựa trên dữ liệu phổ của 24 mẫu chuẩn (là các mẫu chè có kết quả hàm lượng 3 methylxanthine được phân tích bằng HPLC). Phép xác định các methylxanthine trong 7 mẫu chè (dùng làm mẫu kiểm tra) cho thấy mô hình có độ chính xác cao. Hệ số tương quan (R²) của các kết quả hàm lượng theobromine, theophylline và caffeine tính được theo mô hình và hàm lượng xác định theo phương pháp HPLC lần lượt là 0,9582, 0,8894 and 0,9303. Nghiên cứu này chỉ ra rằng với một lần đo phổ hồng ngoại phản xạ, khi kết hợp với thống kê đa biến có thể xác định nhanh các thành phần chính trong chè, cũng như có thể phân loại nguồn gốc địa lí các mẫu.

Keywords caffeine, theobromine, theophylline, hồi qui đa biến, chè, phổ hồng ngoại phản xạ