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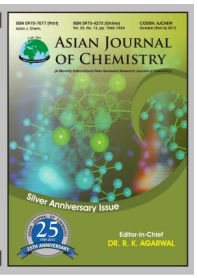

Study on Discriminating Flue-Cured Tobacco by Volatile Compounds Related to Geographical Origin and Cultivar

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To distinguish the geographical origins and cultivars of flue-cured tobacco, 35 samples cultivated in three districts (Liangshan, Yibin and Gunagyuan) of Sichuan Province (southwest of China) were collected. Volatile compounds were analyzed with gas chromatography/mass spectrometry (GC/MS) and 56 different volatile compounds were tentatively identified and semi-quantified. The data was subjected to SIMCA-P+13.0 Software. Principal component analysis (PCA) and partial least squares discrimination analysis (PLS-DA) were used to predict the tobacco's geographical origin or cultivar using soft independent modeling of class analogy (SIMCA) statistical techniques. Both PCA and PLS-DA indicate that all samples could be classified into three clusters coincident to the three geographical origins. The PLS-DA have an average recognition ability of 91.4 %. However, analysis on discriminating tobacco cultivars by volatiles revealed that it is difficult to find any volatile as a distinctive mark to identify these six tobacco cultivars cultured in Sichuan Province.

Key Words: Classification, Tobacco, Cultivar, Volatile, Geographical origin.

INTRODUCTION

Characteristics of agro-product quality have long been linked with their geographical origin. Identification of the geographical origin of agro-products is effective in protecting the quality and safety of agro-product[1]. Increased demand for high-quality and authentic agro-products has given rise to the need to identify and trace the geographical origin of agro-products. Chemical composition and content of agro-products are dominant factors for product quality and can serve as geographical indications of origin. Many modern analytical instruments used in analyzing mineral content[2-4], isotope content and ratio[5,6], DNA analysis[7,8]and volatile compounds[9,10], the collection of numerous data about the chemical characteristics of agro-products is possible. Furthermore, when combined with chemometrics, the geographical origin of agro-products can be discerned[11].

It is generally assumed that plant volatiles could attract pollinators[12], increase tolerance[13], serve as plant defenses[14,15] and also act in plant-plant communication. However, characterizing the composition and content of volatiles in the same kind of agro-products could be very diverse, due to the differences of many influencing factors such as variety, processing technology, environmental factors, *etc*. Examination of the volatiles might be considered as a strategy enabling products' authentication since composition is known to vary widely with multiple factors involved in agro-production. For example, depending on the difference in volatiles, milk samples were classified correctly into groups which were consistent with the type of forage eaten by the cattle[16]. Characterization of volatiles was also highly suitable for varietal discrimination of hops (Humulus lupulus L.)[17] or wines according to varieties[18]. Environmental factors such as light, temperature and moisture status can greatly affect the emission of volatiles, including the yield and composition of essential oils[19,20]. Studies also show that geographical origins of coffee beans[21], honeys[22,23], olive oils[24], green teas[25],cheese[26] and many other agro-products could be discriminated based on the diversity of volatiles.

As far as flavour and taste are concerned, volatiles are one of the most important influencing factors, especially for tobacco. Many volatile compounds in tobacco can be transferred from cut tobacco leaf to smoke by volatilization without any structural change.

Flue-cured tobacco (*Nicotiana tabacum* L.) is one of the most important commercial crops. The major volatile compounds reported in flue-cured tobacco are neophytadiene, aromatic ketones, aromatic alcohols, aliphatic acids and aromatic aldehydes. These compounds usually comprise more than 90 % of the total volatile compounds and contribute to the aroma of flue-cured tobacco[27]. Volatile compounds in

tobacco are affected widely and deeply by many factors related to growing area[28-30]. It is interesting to clarify whether the geographical origin of tobacco could be discriminated or traced based on the difference of volatiles and whether geographical indications of volatiles could be taken as a quality-mark of flue-cured tobacco.

However, few efforts have been made to identify tobacco's geographical origin and cultivar by analyzing volatile compounds. Furthermore, no information exists in the literature on classification of flue-cured tobacco planted in Sichuan Province. In this study we are trying to discriminate the geographical origin and cultivars of flue-cured tobacco planted in Sichuan Province according to volatile compounds.

EXPERIMENTAL

Tobacco samples: Tobacco leaves were harvested by hand and cured in bulk curing barns with the standard three-phase curing process (including yellowing phase, leaf drying phase and stem drying phase). A total of 35 flue-cured tobacco samples (ranked as C3F) were collected after the curing period from three districts, including Liangshan, Guangyuan and Yibin. They are located in the south-west, north and south area of Sichuan Province, respectively, which have different climatic conditions. Sampling was carried out during October and November in 2011. The sample group was composed of six tobacco cultivars, including Yunyan 85, Yunyan 87, Yunyan 97, Honghuadajinyuan, Zhongyan 103 and K326. All information about samples is displayed in Table-1.

Sample preparation: Tobacco leaves were dried at 40 ºC for 6 h and ground into powder. Volatile compounds were extracted with simultaneous distillation-solvent extraction (SDE) apparatus. For each extraction, 20 g of tobacco sample, 10 g sodium sulphate and 300 mL ultra-pure water were placed in a 1 L flask and heated with a boiling water bath, 50 mL dichloromethane was added to a 100 mL flask heated with 40 ºC water bath and the temperature of the circulating cooling water system was operated at 8 ºC. Steam distillation was stopped after 2 h, while solvent extraction was continued for further 15 min. The extract was concentrated to 1 mL at 10 ºC using nitrogen-purge apparatus. The concentrated solution was dehydrated with anhydrous sodium sulphate for at least 12 h, of which 2 µL was injected into the GC-MS system for analysis.

GC-MS conditions: The auto system Shimaszu QP 2010 GC-MS was employed for the analysis of volatile components. Low-bleed GC-MS column Rtx-5Ms, (5 % diphenyl/95 % dimethyl polysiloxane,30 m × 0.25 mm ID × 0.25 µm as used to resolve the volatiles. In terms of GC temperature programming, the oven temperature was set at 50 ºC and kept there for 2 min, then raised to 110 ºC at a ramp of 8 ºC/min and kept there for 2 min, then raised to 150 ºC at of 3 ºC/min and kept there for 2 min, then raised to 200 ºC at of 5 ºC/min and kept there for 5 min and finally raised to 240 ºC at 10 ºC/min and kept there for 2 min. The carrier gas was helium. Mass spectrometry was operated at 230 ºC in electron impact mode (70 eV), scanning from m/z 40-600 in 0.3 s with an 0.2 s scanning interval time, the temperature of the GC-MS interface was 250 ºC and the voltage of the photoelectric multiplier tube (PMT) was 200 V.

Validation parameters for the GC-MS method: The linearity and sensitivity of the method were investigated using available reference standard phenylethyl acetate, with concentrations ranging from 5 to 200 µg mL/L. Each point on the calibration curve, expressed as peak area, was obtained from a minimum of three replicates of measurements (RSD < 0.02). The relationship between the peak area and standard concentration was determined by linear regression with $R^2 > 0.99$.

Semi-quantitative and qualitative of volatile: Data analysis was performed using GCMS solution (Shimadzu, JAP). After peak smoothing and aligning of GC spectra, the quantification of the internal standard was done manually for each sample, then the peak area was corrected with reference to the internal standard. Semi-quantitative analysis was based on the peak area percentage method using GC-MS total ion chromatograms. Peak area percentage was calculated by comparing each peak area to the total peak area in the same sample, without considering calibration factors. GC peaks with relative peak area exceeding 0.1 % were extracted. Identification of selected peaks was based on the NIST05.L MS library (National Institute of Standards and Technology, Gaithersburg, USA).

Statistical analysis: Comparison of the means was achieved using a one-way analysis of variance (ANOVA) using the SPSS 17.0 statistics software (SPSS Inc.). Data was centered and pareto scaled after importing to SIMCA software. Principal component analysis (PCA) and partial least

TABLE-1
TOBACCO SAMPLES FROM SICHUAN PROVINCE

No.	Geographical origin	Cultivar	No.	Geographical origin	Cultivar	No.	Geographical origin	Cultivar
1	LS	Y87	13	YB	Y97	25	GY	Y85
2	LS	Y85	14	YB	K326	26	GY	Y85
3	LS	Y85	15	YB	HD	27	GY	Y85
4	LS	Y85	16	YB	K326	28	GY	Y85
5	LS	Y87	17	YB	HD	29	GY	Y85
6	LS	Y85	18	YB	HD	30	GY	Y85
7	LS	Y87	19	YB	Z103	31	GY	Y87
8	LS	Y87	20	YB	Z103	32	GY	Y87
9	LS	Y85	21	YB	Y97	33	GY	Y87
10	LS	Y87	22	YB	K326	34	GY	Y87
11	LS	Y85	23	YB	Z103	35	GY	Y87
12	LS	Y87	24	YB	K326	–	–	–

Origin: LS-Liangshan district, YB-Yibin district, GY- Guangyuan district Tobacco Cultivar: Y85-Yunyan85, Y87-Yunyan87, Y97-Yunyan97, HD-Honghuadajinyuan, Z103-Zhongyan103, K326.

squares discrimination analysis (PLS-DA) were performed with SIMCA-P version 13. PCA was used to overview data clustering trends and to identify outliers with Ellipse, Hotelling's T2 (95 %). PLS-DA was carried out to reveal the relationship between samples and variables (tobacco volatiles). The leave out cross validation method was used to test the prediction classification ability.

RESULTS AND DISCUSSION

A total of 56 volatile compounds were detected with GC-MS and identified (Table-2) by matching with NIST05.L MS library (similarity ratio > 80 %). The volatile compounds covered on average 95.46 % of the total peak area recovered from the GC spectra. Volatile compounds were semi-quantified by means of peak area percentage (not shown).

Geographical discrimination of flue-cured tobacco using volatile compound analysis: Principal component analysis (PCA) was used to provide an overview of the capacity of the variables (volatile compounds) to discriminate tobacco samples from different regions. All data derived from 56 volatile compounds of 35 samples were subjected to a PCA-class model with geographical origins taken as class ID. In referencing three independent PCA score plots (not shown) for each sample group from different regions, no samples were identified as possible outliers. After applying PCA to the raw data set of 35 samples, four PCs were extracted according to the NIPALS algorithm (R2X = 0.942, Q2 = 0.547). The percentage of variance explained by each PC was 46.5, 29.5, 11.5 and 6 %, respectively. The PCA-X score scatter plot (not shown) reveals that they could be classified into three clusters coincident to the three sampled geographical origins.

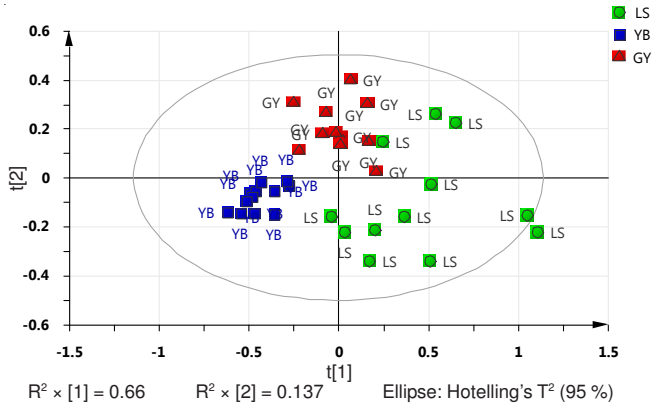
Supervised PLS-DA was carried out to reveal the relationship between volatile compounds and the three clusters. The initial PLS-DA model was calculated using all 56 volatile compounds. The R2X and Q2 X values of the PLS-DA models described the model quality, R2X indicates how well the model fits and Q2X indicates the model predictability. The first PLS-DA model was established using two components and revealed RX2 (cum), RY2 (cum) and Q2 (cum) values of 0.80, 0.62 and 0.54, respectively. The score plots of PLS-DA show correlation of samples and the weights scatter plot of PLS-DA reveal the relationship between variables and their loading. The PLS-DA score plot (Fig. 1a) also displays a clear separating trend in the three classes, according to the sample origins as well as the PCA-X score scatter plot. However, the PLS-DA loading plot (Fig. 1b) failed to show the correlation between variables and sample groups clearly because of the serious overlap of most variables. To decrease overlap and to improve the interoperating ability of the PLS-DA loading plot, unimportant variables for the PLS-DA were reduced. Variable importance for the projection (VIP) plot of the PLS-DA (Fig. 2) displayed the contribution value of each variable and using the VIP > 1, nine variables were judged as being important.

PLS-DA was performed again with the nine variables (far left in Fig. 2) with VIP > 1. In the PLS-DA loading plot, X-variables situated in the vicinity of the dummy Y-variables have the highest discriminatory power between the classes. Based on the loading (Fig. 1d) and score plots (Fig. 1c) of the

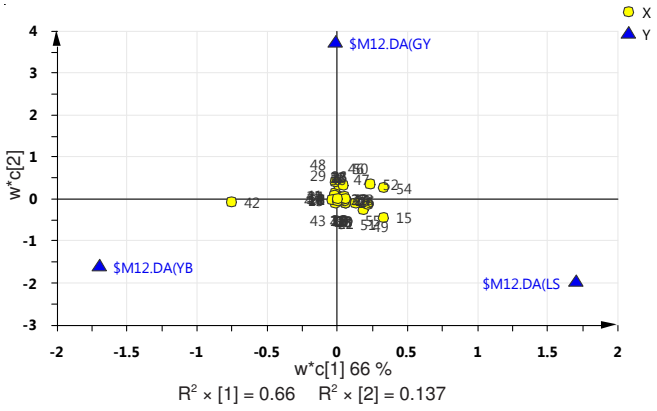
TABLE-2
TENTATIVELY IDENTIFIED VOLATILE COMPOUNDS IN FLUE-CURED TOBACCO FROM SICHUAN

No.	Compound	RT	No.	Compound	RT
1	2-Butylfuran	6.446	29	Cedrol	26.880
2	2,3-Dihydro-benzofuran	7.251	30	Megastigmatrienone C	27.084
3	6-Methyl-2-heptanone	8.326	31	Megastigmatrienone D	27.437
4	Benzyl alcohol	10.471	32	4-(3-Hydroxy-1-butenyl)-3,5,5-trimethyl-2-cyclohexen-1-one	27.958
5	Benzeneacetaldehyde	10.776	33	Heneicosane	28.877
6	Acetophenone	11.410	34	Heptadecane	29.272
7	Nonanal	12.342	35	Norphytane	29.432
8	Phenylethyl alcohol	12.645	36	Palmitaldehyde	29.765
9	2,6,6-Trimethyl-2-cyclohexene-1,4-dione	13.481	37	Hexa-hydrofarnesol	30.155
10	Nonanoic acid	16.434	38	Tetradecanoic acid	30.990
11	Indole	17.360	39	Isopulegyl acetate	31.230
12	4-Ethenyl-2-methoxy phenol	17.850	40	Anthracene	31.930
13	Farnesol	17.992	41	Phytane	32.336
14	Hexahydrofarnesol	18.783	42	Neophytadiene	33.305
15	Nicotine	18.865	43	Hexahydrofarnesyl acetone	33.431
16	Solanone	19.289	44	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	33.860
17	Hexahydropseudoionone	20.412	45	3,7,11-Trimethyl-2,10-dodecadien-1-ol	34.179
18	Myosmine	21.276	46	Farnesyl acetone	35.518
19	Geranyl acetone	21.950	47	Palmitic acid, methyl ester	35.632
20	Nicotine-N'-oxide	22.859	48	3,7,11,15-Tetramethyl-2-hexadecene	35.856
21	2,4-*bis*(1,1-Dimethylethyl) phenol	23.776	49	Hexadecanoic acid	36.718
22	6-Methoxy-3-methylbenzofuran	24.171	50	3-(4,8,12-Trimethyltridecyl)furan	37.037
23	Dihydroactinidiolide	24.679	51	17-(Acetyloxy)-(4β)-kauran-18-al	41.430
24	Megastigmatrienone A	25.501	52	β-4,8,13-Duvatriene-1,3-diol	42.095
25	Megastigmatrienone B	26.018	53	Phytol	43.090
26	Hexadecane	26.322	54	α-4,8,13-Duvatriene-1,3-diol	43.465
27	Diethyl Phthalate	26.416	55	Linolenic acid, methyl ester	44.503
28	Myristaldehyde	26.789	56	Phthalic acid, dioctyl ester	64.193

RT: Retention time.



(a)



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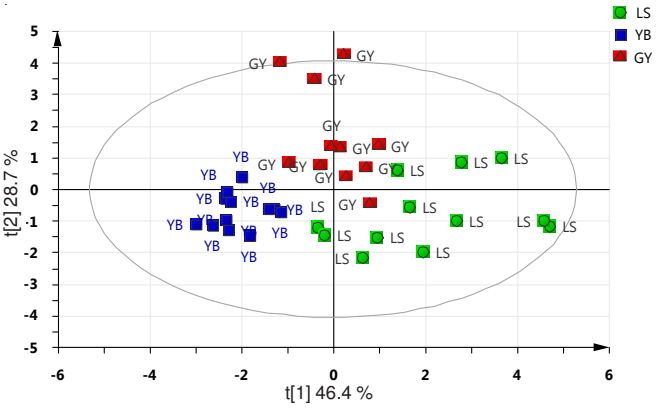

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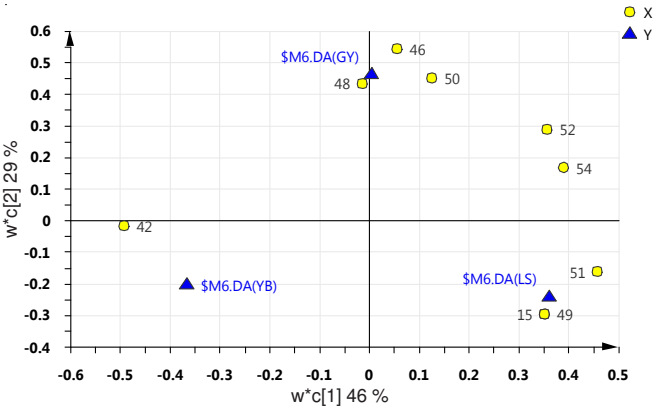

(d)

Fig. 1. PLS-DA score plot and loading plot

second PLS-DA, it is easy to find that Var 42 is the most important variable to discriminate YB samples; Var 48, Var 46 and Var 50 are the most important three variables to discriminate GY samples; Var 15, Var 49, Var 51, Var 52 and Var 54 are the most important variables for discriminating LS samples.

Contribution scores and the analysis of variance (ANOVA) of the variables of the second PLS-DA are shown in Table-3, displayed on the left and right sides, respectively. The contribution scores of PLS-DA show the weighted difference between certain groups and the average of three groups. It also indicates which variables in a group deviate most from the group average. The signs of contribution scores (positive = higher and negative=lower content than average) indicate in which direction the variables deviate. ANOVA highlighted statistically significant differences ($p < 0.05$) of the nine variables among the sample groups produced in three geographical areas. This can explain why they are so important in discriminating geographical origin.

Validity and reliability of the second PLS-DA models were tested by permutation tests and analysis of variance testing of cross-validated predictive residuals (CV-ANOVA). Permutation tests were performed with 20 random reclassifications. The permutation plot displays the correlation coefficient between the original y-variable and the permuted y-variable on the x-axis *versus* the cumulative R2 and Q2 on the Y-axis and draws the regression line. The criteria for validity of PLS-DA models

TABLE-3
CONTRIBUTION SCORES AND ANOVA OF VARIABLES IN THE SECOND PLS-DA

Var ID	Contribution			ANOVA					
				LS		YB		GY	
	LS	YB	GY	Mean	SD	Mean	SD	Mean	SD
15	0.9601*	-0.5342	-0.4077	3.70a	2.92	0.29b	0.29	0.43b	0.64
42	-1.2915*	1.4103*	-0.0036	79.41c	5.17	91.82a	1.37	85.07b	1.96
46	-0.2268	-0.3437	1.4474*	0.23a	0.04	0.14b	0.04	0.22a	0.06
48	-0.2120	-0.1503	0.9157*	0.43b	0.20	0.48b	0.33	1.20a	1.09
49	0.9658*	-0.5373	-0.4114	1.16a	0.97	0.05b	0.05	0.09b	0.07
50	-0.0619	-0.3796	1.0058*	0.61b	0.19	0.391b	0.16	0.97a	0.61
51	1.3004*	-1.0635*	-0.1128	2.21a	0.53	1.01c	0.26	1.39b	0.30
52	0.5222	-0.9935*	0.4226	2.69a	1.15	0.96b	0.48	2.67a	0.87
54	0.7085	-1.0212*	0.1484	4.53a	2.04	1.49b	0.65	3.82a	1.10

In the left side of Table-3, variables in one column (LS, YB or GY) marked with "*" are discriminating variables to identify samples' geographical origin. In the right part of Table-3, variables in one row with different letters are statistically different ($p < 0.05$), a > b > c (= significantly different contents). standard deviations (SD).

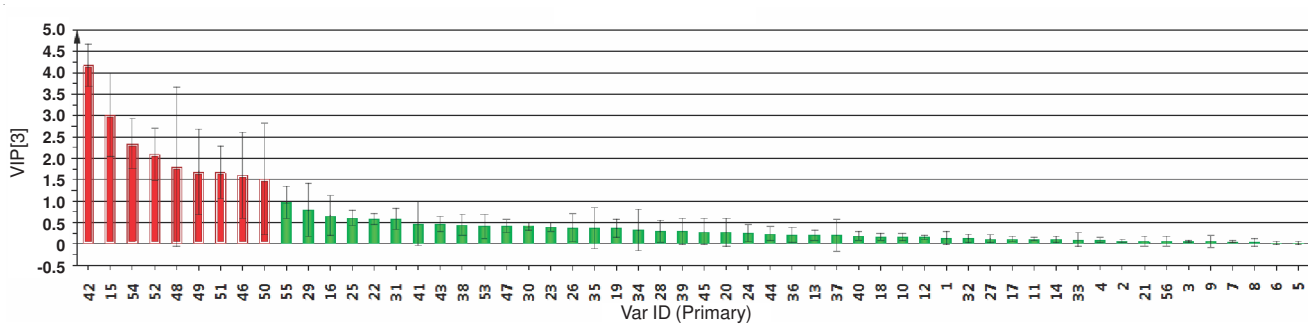

Fig. 2. VIP analysis on 56 variables of the first PLS-DA. Note: column marked in red represents variables with variable influence of projection (VIP) values exceeding 1

are all blue, Q2-values to the left are lower than the original points to the right or the blue regression line of the Q2-points intersect the vertical axis (on the left) below zero. Fig. 3 indicates that all of the second PLS-DA models with nine variables have good fitness and predictability. Analysis of variance of seven-fold cross-validation predictive residual (CV-ANOVA) show the P = 1.84612e-006 < 0.05. The misclassification test (Table-4) indicates that the second PLS-DA can discriminate tobacco samples from the three districts with a correct classification rate of 91.43 %. This supports the view that environmental conditions (climate, rainfall, soil quality) and culture technology are closely related to geographical origin, which directly affect the character of volatiles[31].

TABLE-4
MISCLASSIFICATION TEST OF THE SECOND PLS-DA

Origin	Sample members	Result			
		Correct (%)	LS	YB	GY
LS	12	83.33	10	2	0
YB	12	100	0	12	0
GY	11	90.91	1	0	10
Total	35	91.43	11	14	10

Note: Fishers prob. = 3.1e-012.

Variety discrimination of flue-cured tobacco using volatile compound analysis: In this study, all samples belong to six varieties, respectively, with the sample numbers ranging from 2 to 11. The score plot of PCA to determine primary observation of the six varieties failed to reveal separation between varieties (Fig. 4). Moreover, the PLS-DA model could not be established with SIMCA due to the fact that no significant principle component could be extracted. Considering that samples from Liangshan and Guangyuan are very suitable for analyzing the difference in volatiles between Y85 and Y87, further attempts to detect the difference between Y85 and Y87 samples on volatiles by OPLS-DA was carried out, because both of them have six samples of Y85 and five or six samples of Y87. However, no predictive components could be extracted from the Liangshan and Guangyuan samples. Therefore, no volatile could be identified as a useful characteristic to discriminate tobacco varieties in this study.

Conclusion

Volatile profiling based on GCMS spectra was used to analyze the differences among tobacco samples extracted from three districts with the aim of finding markers useful for identifying geographical origin. The results demonstrate that a

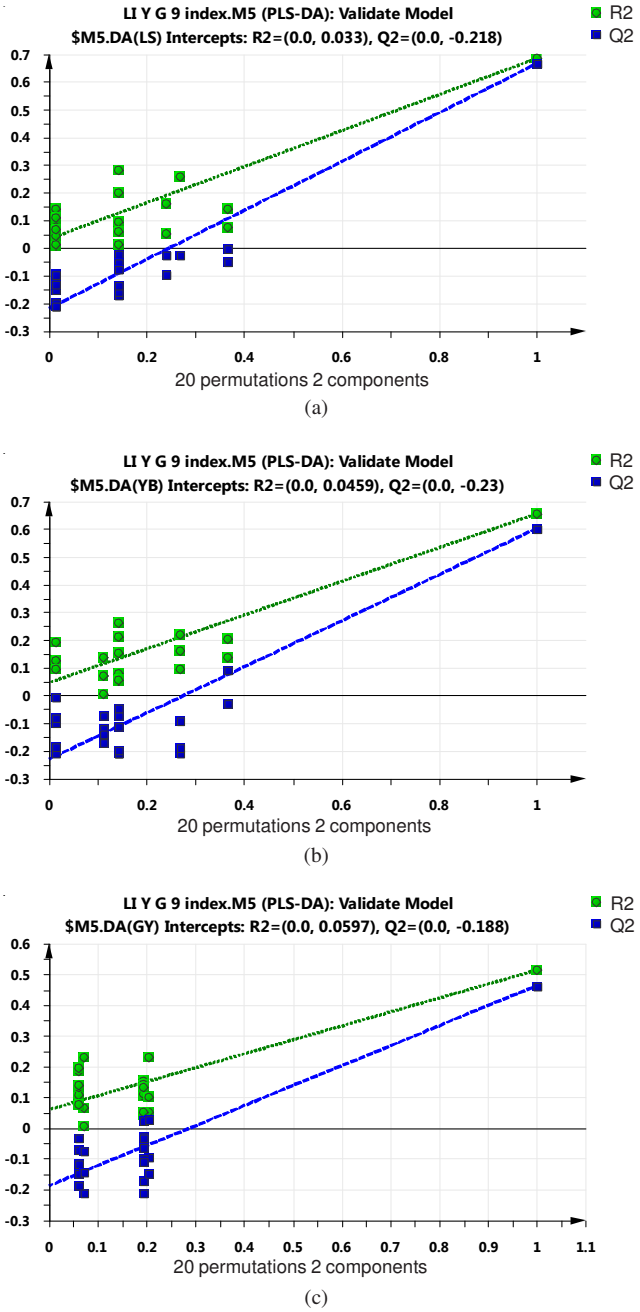

Fig. 3. Results from response permutation test of PLS-DA models. The vertical axis gives the R2 (green) and Q2 (blue) values of the original model (far right) and the Y-permuted models further to the left. The horizontal axis shows the correlation between the permuted y-vectors and the original y-vector for the selected. (a): LS, (b): YB, (c): GY

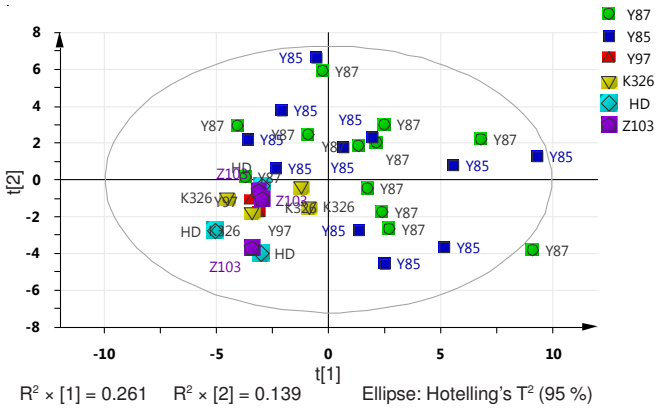

Fig. 4. PCA-X score plot of six tobacco varieties

combination of GCMS and PLS-DA multivariate analyses allows comparisons of overall volatile fingerprints and that this technique can be applied to identify differences between tobacco samples.

Distinct separations between tobacco samples from three districts were observed in chemometric analyses using PCA and PLS-DA. Nine volatile components were selected as candidate biomarkers that could be used to quickly and easily differentiate tobacco samples. However, no volatile component was selected as a biomarker to identify tobacco varieties in this study. In summary, this study demonstrates that GCMS-based volatile fingerprinting is a useful tool for distinguishing origins of tobacco samples, coupled with multivariate statistical analysis. The reasons for the differences in volatile profiles in tobacco leaves sampled from different geographical origins are not fully understood, and further investigations are needed.

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