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Investigation of the Chemical Compositions in Tobacco of Different Origins and Maturities at Harvest by GC−MS and HPLC−PDA-QTOF-MS

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Supporting Information

ABSTRACT: Tobacco samples of a same cultivar grown in different plantations in China were evaluated for their chemical compositions at different maturities for the first time. This was accomplished by a comprehensive and reliable method using gas chromatography−mass spectrometry (GC−MS) and high performance liquid chromatography−photodiode array-quadrupole time-of-flight mass spectrometry (HPLC-PDA-QTOF-MS) to analyze the fat-soluble and polar components in 12 batches of tobacco samples of three origins and four maturities. The GC−MS analyses showed that tobacco samples harvested at 40 days after transplantation exhibited more fat-soluble components, while those harvested at 100 days after transplantation exhibited the least fat-soluble components. Tentatively, identification of the main components as well as quantitative analyses of eight reference compounds, including five alkaloids, two polyphenols, and a coumarin, was performed by the developed HPLC−QTOF-PDA method. Results showed significant differences among origins and maturities in the contents of these compounds. The nicotine contents showed great variety among the 12 tobacco samples. The highest nicotine content were found in a sample from Zhengzhou harvested at 40 days after transplantation (ZZ-T with 25399.39 ± 308.95 μg/g), and the lowest nicotine level was detected in a sample from Zunyi harvested at 60 days after transplantation (ZY-X with 1654.49 ± 34.52 μg/g). The highest level of rutin was found in a Jiangchuan sample harvested at 60 days after transplantation (JC-X with 725.93 ± 40.70 μg/g), and the lowest rutin content was detected in a Zunyi tobacco sample harvested at 60 days after transplantation (ZY-X with 87.42 ± 2.78 μg/g). The developed method provided a convenient approach which might be applied for rapid maturity evaluation and tobacco flavor identification and also holds the potential for analysis of compounds present in other plants.

KEYWORDS: *tobacco, chemical compositions, maturities, planting areas, HPLC−QTOF-PDA-MS*

INTRODUCTION

Nicotiana (Solanaceae) species contain a diverse array of chemical composition. The most important groups of which are alkaloids, phenolic compounds, and terpenoids.[1−5] Alkaloids in tobacco have been widely recognized for their contributions to tobacco quality and usability.[6] Among more than 20 different alkaloids found in tobacco,[7] nicotine is the most abundant and accounts for widespread human use of tobacco products throughout the world. The minor and less potent alkaloids, including nornicotine, anabasine, and anatabine, are also pharmacologically active.[8] The nature and underlying neurobiology associated with nicotine, the principal tobacco pyridine alkaloid, and its role in pleasurable sensations and continuation of tobacco use have been reviewed by Ashton et al.[9] The quantitative analysis of tobacco alkaloids has always been of great interest to tobacco scientists. The most frequently used technique for the quantification of tobacco alkaloids has been gas chromatography. The first study had been developed with packed columns in 1962, when it was proposed asa method for the determination of alkaloids in tobacco.[10] In 1965, a study comparing packed and capillary column results for the analysis of various alkaloids was presented.[11] Cai et al.[12] developed a novel fast megabore capillary gas chromatographic (MCGC) method for analysis of seven nicotine related alkaloids in tobacco and cigarette smoke. As a powerful analytical tool, high pressure liquid chromatography (HPLC) has also been used for the determination of alkaloids in recent years.[13−15] Ciolino et al.[16−18] developed a reversed phase ion-pair liquid chromatographic method for the determination of nicotine in commercial tobacco products.

In addition to alkaloids, polyphenols are also important components in tobacco, which greatly affect the odor and taste of smoking.[19] The content of polyphenols is significant to cigarette blend and quality control in tobacco processing. Therefore, the separation and quantification of polyphenols in tobacco have caused extensive concern in the past years. Court first developed a HPLC method for the determination of seven

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polyphenols in tobacco leaves in 1977.[20] Snook et al.[21,22] made a good idea for improving above HPLC method and quantified seven polyphenols. In 1993, Achilli et al.[23] for the first time realized the separation of chlorogenic acid and rutin. The efforts of the separation of alkaloids and polyphenols are summarized in Table 1.

Table 1. Summarization of the Efforts on the Detection of Alkaloids and Polyphenols

year	author	target compound	separation mean
1962	Quin et al.[10]	alkaloids	GC with packed columns
1965	Massingill et al.[11]	alkaloids	GC with packed and capillary columns
2003	Cai et al.[12]	alkaloids	GC with megabore capillary column
1984	Sudan et al.[15]	alkaloids	reversed-phase HPLC
1985	Mousa et al.[13]	alkaloids	HPLC with electrochemical detection
1998	Sellergren et al.[14]	alkaloids	liquid−liquid extraction followed by reversed-phase HPLC using gradient elution
1999	Ciolino et al.[16−18]	alkaloids	reversed-phase ion-pair HPLC
1977	Court[20]	polyphenols	reversed-phase HPLC
1982	Snook et al.[21]	polyphenols	reversed-phase HPLC
1993	Achilli et al.[23]	polyphenols	reversed-phase HPLC

It is well recognized that chemical composition of tobacco is highly associated with the flavor of tobacco products and the risk of smoking.[24] Many methods have been developed to determine tobacco flavoring or harmful constitutions.[25−29] Currently, the chemical compositions of tobacco leaves have attracted a considerable amount of attention throughout the world.[30−33] In view of the fact that the unique combination of tobacco maturation stages and cultivation conditions result in unique secondary metabolites, comprehensive studies on the chemical constituents of tobacco sample of different origins harvested at different maturation stages might provide valuable information to identify superior cultivation condition and the appropriate time to harvest tobacco. However, to our knowledge, no such study was yet reported. In this study, a comprehensive high pressure liquid chromatography−photo-diode array-quadrupole time-of-flight mass spectrometry (HPLC−PDA-QTOF-MS) method was developed for the quantitative determination of the main polyphenols and alkaloids as well as qualitative profiling of other secondary metabolites in 12 batches of tobacco samples of three origins and four maturation stages. The reliability and adaptability of the method were verified by determination of linear range, recovery, and reproducibility with tobacco samples. We expect our work would provide a scientific basis and guidance for artificial cultivation and harvest of tobacco and, thereby, improve the quality of tobacco.

EXPERIMENTAL SECTION

Chemicals and Reagents. HPLC grade acetonitrile and methanol were purchased from Fisher Co. (Fisher Scientific, USA). Deionized water obtained from a Milli-Q water system (Milli-Pore Corp., Bedford, MA, USA) was used for sample preparation procedures and HPLC analyses. Other chemicals were of analytical grade. Eight reference compounds, namely nornicotine (**1**), neonicotine (**2**), anatabine (**3**), nicotine (**4**), myosmine (**5**), scopoletin (**6**), rutin (**7**), and quercetin (**8**) (Figure 1), were isolated and purified in our laboratory and were identified based on UV, IR, MS, ^{1}H NMR, and

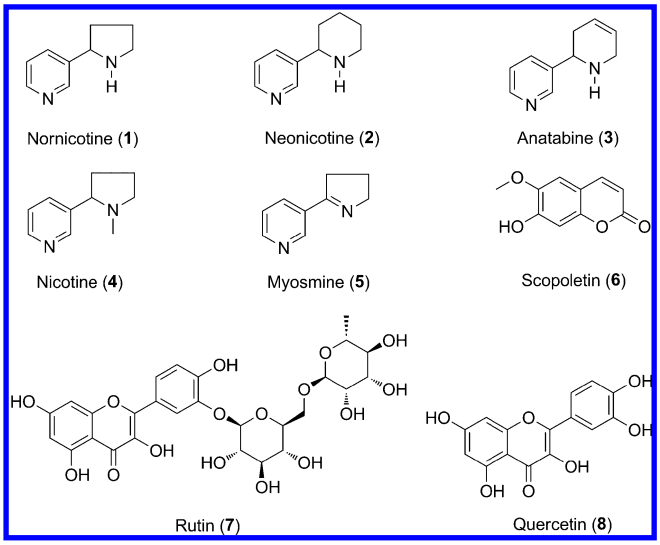

Figure 1. Chemical structures of reference compounds **1**−**8**.

^{13}C NMR analyses. The purity of these compounds was determined to be more than 98% by HPLC analysis. Among them, compounds **1**, **2**, **3**, **4**, and **5** are alkaloids, compounds **7** and **8** are polyphenols, and compound **6** is a coumarin.

Plant Material and Plantation Conditions. Seeds of a tobacco cultivar (*Nicotiana tabacum* "k326") were obtained from the Seed Center of Yunnan Academy of Tobacco (Yunnan, China). Seeds were planted and raised within a germination cabinet in 25 ± 1 °C. Thirty days after germination, the seedlings were transplanted into the pots (50 cm diameter × 50 cm deep). Soil for the potted plants was composed of the same red soil and humus (3:1). These plants were grown at three sites in China: Zhengzhou of Henan province (34°72′ N, 113°65′ E; elevation 110 m), Zunyi of Guizhou province (27°07′ N,106°88′ E; elevation 1000 m), and Jiangchuan of Yunnan province (24°17′ N,102°45′ E; elevation 1730 m). Each site had different temperature, humidity, and rainfall characteristics (Figure S-1 of the Supporting Information). Seedlings were transplanted between 23 April and 6 May of 2012. During the experiment, none of the plants experienced any water or nutrient stresses.

Tobacco leaf samples were collected from the three cultivation places at four maturities. The tobacco samples ZZ-T, ZZ-X, ZZ-C, and ZZ-C2 were collected from Zhengzhou, the samples ZY-T, ZY-X, ZY-C, and ZY-C2 were collected from Zunyi, and the JC-T, JC-X, JC-C, and JC-C2 samples were collected from Jiangchuan. Those sample names marked with -T, -X, -C, and -C2 suffixes represent they were harvested at 40 days (rosette stage), 60 days (squaring stage), 85 days (physiological maturation stage), and 100 days (harvest maturation stage) after transplantation, respectively.

Sample Preparation. The tobacco samples were sun dried on site right after collection and then fine-ground for further extraction. For HPLC analysis, an accurately weighted 1.0 g of the ground powder of each sample and 10 mL of methanol was added to a flask in an ultrasonic bath for 30 min. The extraction process was repeated three times. The extracted solutions were combined, filtered, and evaporated under vacuum. The residue was diluted to volume with methanol in a 25 mL volumetric flask. Then the solutions were filtered through a 0.22 μm PTFE syringe filter into HPLC vials for HPLC−PDA-MS analysis.

Trichloromethane was used to extract the fat-soluble components in the tobacco leaves. Then 1.0 g tobacco powder was introduced into a 25 mL flask, followed by the addition of 10 mL of trichloromethane. The extraction was performed under ultrasonication for 30 min. The mixture was filtered through a filter paper, and the solution was transferred to a 50 mL round-bottomed flask and evaporated to

Table 2. Fat-Soluble Compositions of Tobacco Samples from Three Different Origins Harvested at Four Maturation Stages by GC−MS

no.	t_R (min)	CAS	name	ZZ-T	ZZ-X	ZZ-C	ZZ-C2	ZY-T	ZY-X	ZY-C	ZY-C2	JC-T	JC-X	JC-C	JC-C2
1	3.31	000108-87-2	methylcyclohexane	13.94	23.43	15.74	20.19	26.56	15.30	22.05	14.19	16.70	15.74	16.51	14.93
2	3.45	001640-89-7	ethylcyclopentane	1.01	1.71	1.14	1.55	1.93	1.12	1.61	0.80	1.26	1.14	1.21	1.13
3	4.04	000108-88-3	toluene	0.82	1.04	0.91	1.07	1.58	0.91	1.33	0.80	0.66	0.96	0.84	0.77
4	6.42	000108-93-0	cyclohexanol	4.36	7.16	5.04	6.70	8.14	4.55	6.54	6.70	5.53	4.98	5.14	5.66
5	11.85	000054-11-5	nicotine	2.24	0.00	0.40	0.84	0.44	1.52	6.57	0.84	1.52	0.12	0.05	0.14
6	13.97	003076-04-8	2-propenoic acid, tridecyl ester	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.17	0.00	0.11	0.00
7	14.07	000139-08-2	tetradecyldimethylbenzylammonium chloride	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.18	0.00	0.14	0.00
8	14.42	000486-56-6	cotinine	0.07	0.00	0.08	0.00	0.00	0.28	0.11	0.00	1.53	0.00	0.10	0.00
9	15.27	000473-55-2	dihydropinene	3.40	5.03	4.56	2.36	2.64	3.11	0.00	0.18	5.78	2.52	2.90	3.96
10	15.79	056554-74-6	1,4-eicosadiene	0.00	1.33	0.41	0.66	0.00	0.00	0.00	2.36	1.63	0.32	0.41	1.10
11	16.87	000057-10-3	*n*-hexadecanoic acid	2.45	2.19	2.05	2.83	3.45	2.82	0.00	0.66	10.33	3.54	3.92	2.65
12	17.03	000473-55-2	dihydropinene	0.11	0.00	0.19	2.80	0.00	0.00	0.00	1.83	0.00	0.07	0.00	0.00
13	19.45	005208-51-5	[1*S*,3*S*,(+)]-1-methyl-3-isopropenyl-4-cyclohexene	1.35	0.00	0.61	0.09	0.00	0.80	0.70	2.80	1.54	0.25	0.55	0.16
14	19.98	000506-44-5	linolenic alcohol	2.42	1.23	1.44	0.73	2.26	2.75	1.77	0.09	4.63	1.31	2.51	1.25
15	20.32	000057-11-4	octadecanoic acid	1.22	0.10	0.69	0.75	0.43	1.15	0.91	0.73	1.12	0.52	0.73	0.49
16	20.68	000638-58-4	tetradecanamide	2.36	0.84	1.04	0.29	0.82	1.95	0.00	0.75	4.13	0.59	1.87	0.47
17	22.51	000077-94-2	butyl citrate	5.51	3.44	9.79	1.04	2.40	12.59	8.00	0.29	1.65	0.24	1.65	0.18
18	24.98	000301-02-0	oleamide	3.99	3.83	1.37	8.09	2.59	2.15	2.71	1.04	15.26	0.68	0.22	1.63
19	26.69	000119-47-1	2,2′-methylenebis(6-*tert*-butyl-4-methylphenol)	0.56	0.00	0.00	1.34	0.63	0.38	0.43	8.09	1.07	0.24	0.18	0.17
20	30.34	024468-13-1	2-ethylhexyl chloroformate	0.18	0.00	0.21	1.06	0.00	1.01	0.00	1.34	1.30	0.17	0.28	0.32
21	34.27	000593-49-7	heptacosane	1.83	1.71	2.32	2.32	1.46	1.31	1.95	1.06	1.49	2.52	2.16	2.10
22	36.94	000112-84-5	erucylamide	0.77	0.92	0.58	0.94	0.45	0.50	1.27	2.32	1.80	0.26	0.34	0.83
23	39.38	000593-45-3	octadecane	0.90	0.68	0.35	0.92	3.11	1.46	1.05	0.94	0.48	2.16	1.50	1.17
24	40.55	000630-03-5	nonacosane	2.58	2.37	3.14	3.76	3.22	2.04	3.04	0.92	0.49	4.17	3.69	4.23
25	42.24	000593-49-7	heptacosane	3.53	3.14	2.01	2.20	0.32	3.67	0.60	3.76	1.56	6.24	4.33	3.81
26	43.14	000593-45-3	triacontane	0.75	0.74	1.63	2.74	1.13	0.62	1.94	2.20	1.35	1.51	1.53	2.19
27	44.52	055470-98-9	10-heptyl-10-octylicosane	6.26	6.21	6.29	5.31	12.66	8.86	0.00	2.74	0.23	10.09	7.98	9.17
28	45.27	000646-31-1	tetracosane	10.43	9.35	12.69	11.25	10.61	8.33	17.89	5.31	0.36	15.13	15.70	18.40
29	46.06	101191-41-1	vitamin E	0.58	0.07	1.40	0.78	0.35	0.67	2.11	11.25	0.00	0.14	1.13	0.24
30	46.63	055470-98-9	1-triacontanol	7.55	8.51	5.87	4.26	0.00	5.95	0.00	0.78	0.30	8.34	6.37	7.37
31	47.06	055320-06-4	11-decylheneicosane	1.68	1.87	3.14	2.06	0.00	1.53	3.78	4.26	0.07	2.27	2.47	3.56
32	48.38	000297-35-8	cyclotriacontane	3.59	4.00	4.14	4.12	5.72	4.41	0.00	2.06	1.30	4.21	2.95	3.61
33	48.90	000630-03-5	nonacosane	6.75	6.52	6.73	6.09	1.43	5.80	8.31	4.12	0.52	7.96	6.38	7.43
34	49.87	000084-80-0	phytonadione	1.00	1.07	0.84	0.29	5.20	0.78	2.22	6.09	1.23	0.41	2.15	0.31

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dryness at 45 °C under reduced pressure. The residue in the round-bottomed flask was dissolved in 2 mL of cyclohexane with ultrasonication. The solution was then filtered through a 0.22 μm PTFE syringe filter before GC-MS analysis.

GC−MS Analysis. The fat-soluble components were analyzed on an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA) coupled to an Agilent 5975C mass spectrometer. Helium was used as the carrier gas at a flow rate of 1 mL/min. The GC analyses were performed on a capillary column (30 m × 0.22 mm inner diameter × 1.00 μm d_f). The split ratio was 1:10. The oven temperature was held at 50 °C for 4 min, followed by a ramp of 15 °C/min to 140 °C, then increased to 200 °C at 30 °C/min, followed by a ramp of 2 °C/min to 250 °C and held for 2 min, and then increased to the final temperature of 290 °C at 5 °C/min for 10 min and the inlet was set to a temperature of 240 °C. Temperature of the interface, ion source and quadrapole of the mass spectrometer were set at 280, 230, and 150 °C, respectively. The mass range of m/z 50−700 was set for all GC-MS analyses. Compound identification was based on comparisons to mass spectra of authentic reference compounds by reference to the National Institute of Standards and Technology (NIST) library and published data.

HPLC−PDA-QTOF-MS Analysis. The HPLC analysis was performed on a Waters ACQUITY UPLC I Class system (Waters Corp., MA, USA), equipped with a binary solvent delivery system, a 48-vial autosampler, and a photodiode array detector (PDA). The separation of the main components was achieved on a TSK gel ODS-100Z column (150 mm × 4.6 mm, 3 μm). The mobile phases consisted of solvent A (deionized water with 10 mM ammonium acetate) and solvent B (acetonitrile). The gradient elution was applied at a flow rate of 1.2 mL/min as follows: 3% B (0−4 min), 3−10% B (4−8 min), 10% B (8−12 min), 10−15% B (12−16 min), 15% B (16−18 min), 15%−60% B (18−34 min), 60%−99% B (34−35 min), and 99% (35−37 min). The column and autosampler temperature were maintained at 60 and 15 °C, respectively. The injection volume was 5 μL, and the UV wavelength was set at 254 nm for the detection of **1**−**5**, and **8**. While for the detection of **6**, the UV wavelength of 340 nm was used.

Stock solutions containing eight reference compounds were prepared and diluted to appropriate concentrations for construction of calibration curves. Resulting solutions were filtered through 0.22 μm nylon syringe filters, and aliquots of 5 μL were injected in the chromatographic system for analyses. The standard solutions were analyzed in triplicate, and peak areas were used as an analytical signal. The calibration curves were constructed by plotting the peak areas versus the concentrations of standards.

The method was validated for linearity, sensitivity, repeatability, and accuracy. The limits of detection (LOD) and limits of quantification (LOQ) under the present conditions were determined at an S/N (signal-to-noise) of about 3 and 10, respectively. Intra- and interday variations were chosen to determine the precision of the developed assay. For intraday variability test, three different amounts (high, middle, and low level) of reference compounds were analyzed for six replicates within 1 day, while for interday variability tests, the same mixed standards were examined in duplicates in consecutive 3 days. Variations were expressed by the relative standard deviation (RSD) of the data. Recovery was used to evaluate the accuracy of the method. Three different amounts of the standard solutions were added to sample ZY-C2, and the recovery was measured in triplicate. For comparison, unspiked ZY-C2 sample was concurrently prepared and analyzed. The recovery was calculated as follows: recovery (%) = (amount found − amount original)/amount spiked × 100%. For the stability test, the sample solution was analyzed using the established method at 0, 2, 4, 6, 8, 10, and 12 h, respectively, the peak areas of eight analytes were recorded, and the RSD of peak areas at different times were calculated.

Mass spectrometric analysis was performed on a Synapt G2 QTOF-MS (HDMS, Waters, USA) operating in electrospray ionization (ESI) positive mode. The HPLC elution was split 3:1 using a flow splitting tee, resulting in flow rate of 0.3 mL/min to MS analysis. The mass spectrometer was operated with the following parameters: capillary voltage of 2.5 kV, cone voltage of 40 V, source temperature of 110 °C, desolvation temperature of 300 °C, and desolvation gas (N_2) flow of 700 L/h. The mass range was set at m/z 50−1000 with a scan time of 0.25 s and an interscan time of 0.02 s. LC−MS/MS analysis was performed by a collision energy ramp from 15 to 35 eV in the mass range of m/z 50−1000 in MS^E mode. To ensure the accuracy of the measured mass, leucine−enkephalin (with a reference mass at m/z 556.2771 in positive mode) was used as the lock-mass compound at a concentration of 500 pg/μL and a flow rate of 5 μL/min. The UPLC and Synapt G2 system operations were carried out using MassLynx 4.1 software (Waters, Manchester).

Statistical Analysis. Cluster analysis is a common technique for statistical data analysis which provides means for classifying a given set of objects into groups (clusters) in such a way that the similarities between objects could easily be determined. In this study, hierarchical cluster analysis (HCA) was performed to reveal the similarities between tobacco samples of different origins and maturities according to their fat-soluble constituents. The fat-soluble constituents of the 12 tobacco samples were subject to hierarchical cluster analysis based on the 34 most abundant fat-soluble constituents in the samples obtained by GC-MS. The percentage peak areas (PPAs) of these peaks in 12 batches of tobacco samples were used as variables for the HCA calculation. HCA was performed using Euclidean distance and average linkage without standardizing the variables. Statistical analysis was performed by Minitab for Windows, release 12 (Minitab Inc., State College, PA, USA).

RESULTS AND DISCUSSION

Influence of Origin and Maturity on the Fat-Soluble Composition. The fat-soluble composition is one of the important parameters to understand the variation among tobacco samples of different origins and maturities. More than 100 compounds were detected in 12 batches of tobacco samples through GC−MS analyses, and the percentage compositions of 34 most abundant compounds were tentatively identified and summarized in Table 2. Obvious variation can be found in fat-soluble composition among all samples. For instance, higher contents of cyclohexanol, butyl citrate, vitamin E, and cyclotriacontane were found in the Zhengzhou and Zunyi tobacco samples in comparison to those from Jiangchuan. However, Zunyi and Jiangchuan samples showed higher contents of phytonadione, octadecane, and linolenic alcohol, while the Zhengzhou and Jiangchuan tobacco samples had higher contents of heptacosane, 1-triacontanol, 11-decylheneicosane, tetracosane, dihydropinene, 10-heptyl-10-octylicosane, tetradecanamide, and nonacosane. During maturity, the 10-heptyl-10-octylicosane contents in Zhengzhou tobacco samples were almost constant (6.26, 6.21, 6.29, and 5.31% for ZZ-T, ZZ-X, ZZ-C, and ZZ-C2, respectively), while that of Zunyi tobacco samples showed significant reduction (12.66, 8.86, 0, and 2.74% for ZY-T, ZY-X, ZY-C, and ZY-C2, respectively). However, the Jiangchuan tobacco sample showed an approximate increased 10-heptyl-10-octylicosane content (0.23, 10.09, 7.98, and 9.17 for JC-T, JC-X, JC-C, and JC-C2, respectively) during maturity. In contrast to 10-heptyl-10-octylicosane content, the Zunyi tobacco samples showed the highest methylcyclohexane content (ZY-T with 26.56%) among all the tobacco samples, while the Zhengzhou tobacco sample showed the lowest methylcyclohexane content (ZZ-T with 13.94%). These results clearly demonstrated the influences of different origins from the same region and different regions on fat-soluble compositions during maturity. The GC-MS profile clearly shows that tobacco samples of all three origins harvested at rosette stage exhibited more fat-soluble components, while those harvested at harvest maturation stage exhibited the least

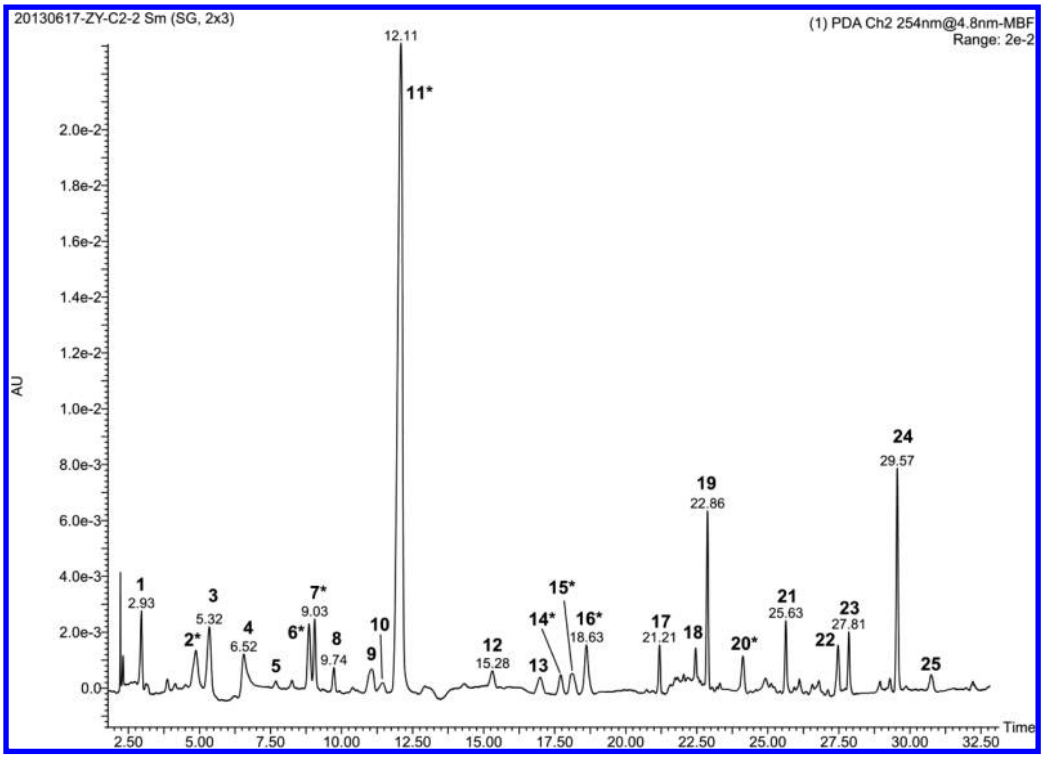

Figure 2. Chromatogram of a representative chromatogram of the tobacco samples (ZY-C2; detected at 254 nm). Peaks 2, 6, 7, 11, 14, 15, 16, and 20 were identified as reference standards **1**−**8**, and the tentative identification of the rest peaks can be found in Table 4.

Table 3. Retention Time, Regression Equation, Correlation Coefficient, Linearity Range, LOD, LOQ, Precision, Repeatability, and Recovery by HPLC−QTOF-MS for the Eight Analytes

							precision RSD (%)			
analyte	t_R (min)	calibration curve	r^2	linear range (μg/mL)	LOD (ng)	LOQ (ng)	intraday (n = 6)	interday (n = 6)	stability (RSD %, n = 6)	recovery (%)
nornicotine (**1**)	4.99	$y = 24.45x + 29.22$	0.9997	0.2−200	0.2	0.67	0.78	1.28	2.01	99.61
neonicotine (**2**)	8.27	$y = 21.54x - 37.84$	0.9999	0.2−200	0.2	0.67	0.52	0.70	0.94	98.90
anatabine (**3**)	9.09	$y = 20.11x - 75.47$	0.9995	0.2−200	1.0	3.3	0.94	1.35	0.40	100.71
nicotine (**4**)	12.02	$y = 14.70x - 35.84$	0.9996	0.5−200	1.4	4.5	1.34	3.01	1.75	99.51
myosmine (**5**)	18.35	$y = 34.83x - 24.06$	0.9997	0.5−200	1.0	3.2	0.41	0.75	3.67	99.31
scopoletin (**6**)	17.94	$y = 65.44x - 23.50$	0.9995	0.5−200	0.2	0.71	0.67	1.03	3.21	99.36
rutin (**7**)	18.82	$y = 44.89x - 88.48$	0.9995	0.5−200	1.5	4.9	2.62	3.66	0.39	98.40
quercetin (**8**)	24.87	$y = 81.45x - 368.88$	0.9990	0.5−200	0.5	1.6	3.04	3.46	1.01	101.22

fat-soluble components. The GC−MS profile of the fat-soluble components of the 12 batches of tobacco samples of three origins and four maturities is presented in Figures S-2−S-4 of the Supporting Information.

Validation of HPLC Method. All 12 batches of the tobacco sample were analyzed with the developed chromatographic condition, and the chromatogram of the eight standards and a representative chromatogram of the tobacco samples are illustrated in Figure 2. The chromatograms of the all the tobacco samples can be found in Figures S-4−S-6 of the Supporting Information.

The regression equation, correlation coefficient, linear range, LOD, LOQ, precision, repeatability, and recovery for the eight standards are summarized in Table 3. The correlation coefficient values (r^2 > 0.999) indicated appropriate correlations between the investigated compound concentrations and their peak area within the test ranges. The relative standard deviation (RSD) of peak area of each standard was within the range of 0.76−3.0% and 0.70−3.7% for intra- and interday repeatability and within 0.39−3.67% for stability. All the results demonstrated that the developed method is reproducible, precise, and stable. The spike recoveries were within the range of 98.8−102.7%, demonstrating superior performance of the method in both recovery and accuracy.

Variation in the Content of Alkaloids and Polyphenols between Samples of Different Origins during Maturity. Variation of the contents of nicotine and rutin in all tobacco samples is shown in Table 5. Of the five alkaloids and two polyphenols, nicotine and rutin showed the highest content in all tobacco samples, and thus nicotine and rutin were chosen as representative alkaloid and polyphenol compounds in the following discussion.

Affected by many factors such as growth conditions and leaf stalk position, the nicotine contents in tobacco varies from 1% to 3% of the total weight of tobacco.[34,35] Our results clearly showed that the nicotine content varied nearly 40-fold, ranging from 0.66 to 25.40 mg/g, in the tobacco samples of different origins and maturities (Table 5). As illustrated in Table 5,

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Table 4. Identification of Main Chemical Constituents in ZY-C2 by HPLC-QTOF-MS (in ESI+ Mode; * Represents Reference Standards)

no.	t_R (min)	measured mass (m/z)	main fragment ions (relative intensity)	formula	exact mass	Δ (ppm)	tentative identification
1	2.93	136.0617	136.0617 (100), 119.0351 (51)	$C_5H_5N_5$	135.0545	−0.51	adenine
2*	5.22	149.1071	149.1071 (100), 132.0811 (37), 130.0649 (55), 117.0570 (23)	$C_9H_{12}N_2$	148.1000	−1.19	nornicotine (**1**)
3	5.32	179.1178	179.1178 (100), 132.0810 (65), 130.0649 (62), 117.0570 (43)	$C_{10}H_{14}N_2O$	178.1106	−0.43	nicotine-1′-*N*-oxide
4	6.52	251.1386	251.1386 (100), 234.1126 (55), 163.0391 (39),	$C_{13}H_{18}N_2O_3$	250.1317	−1.51	caffeoylputrescine (isomer 1)
5	7.63	325.1758	325.1758 (14), 163.1230 (100), 130.0653(15), 106.0650 (21)	$C_{17}H_{20}N_6O$	324.1699	−4.25	*N*-{2-[4-(2-pyrimidinyl)-1-piperazinyl]ethyl}-1,3-benzoxazol-2-amine
6*	8.52	163.1229	163.1229 (100), 146.0961 (30), 130.0645 (32), 118.0643 (61), 117.0572 (31)	$C_{10}H_{14}N_2$	162.1157	−0.47	neonicotine (**2**)
7*	9.03	161.1069	161.1069 (100), 144.0806 (42), 117.0571 (35)	$C_{10}H_{12}N_2$	160.1000	−2.35	anatabine (**3**)
8	9.74	251.1383	251.1383 (100), 234.1123 (46), 163.0388 (45)	$C_{13}H_{18}N_2O_3$	250.1317	−2.70	caffeoylputrescine (isomer 2)
9	11.01	177.1019	177.1019 (100), 159.0886 (8), 146.0598 (14), 120.0803 (10), 118.0643 (12),	$C_{10}H_{12}N_2O$	176.0950	−2.14	cotinine
10	11.44	193.0493	193.0498 (100), 178.0261 (82), 150.0310 (24), 135.0437 (80), 133.0284 (67), 122.0360 (18), 107.0489 (40)	$C_{10}H_8O_4$	192.0423	−1.44	herniarin
11*	12.11	163.1228	163.1228 (20), 132.0805 (52), 130.0648 (100), 120.0805 (10), 117.0571 (45), 106.0649 (15)	$C_{10}H_{14}N_2$	162.1157	−1.09	nicotine (**4**)
12	15.28	293.1493	293.1493 (100), 275.1373 (23), 227.1163 (11), 161.1069 (25), 132.0802 (21), 130.0651 (18),	$C_{15}H_{20}N_2O_4$	292.1423	−0.95	*rac-trans*-3′-hydroxymethylnicotine hemisuccinate
13	16.84	411.1993	411.1993 (100), 249.1581 (43)	$C_{22}H_{26}N_4O_4$	410.1954	−8.23	unknown
14*	17.63	193.0491	193.0491 (100), 178.0258 (82), 165.0549 (8), 150.0309 (21), 137.0595 (25), 133.0283 (65), 122.0362 (14), 105.0333 (10)	$C_{10}H_8O_4$	192.0423	−2.48	scopoletin (**5**)
15*	18.23	147.0914	147.0914 (100), 130.06567 (21), 117.0578 (35), 105.0453 (37), 104.0501 (15)	$C_9H_{10}N_2$	146.0844	−1.89	myosmine (**6**)
16*	18.63	611.1607	611.1607 (61), 465.1030 (44), 303.0501 (100)	$C_{27}H_{30}O_{16}$	610.1534	0.04	rutin (**7**)
17	21.21	314.1383	314.1383 (23), 238.1651 (11), 201.0461 (100), 135.0441 (27)	$C_{18}H_{19}NO_4$	313.1314	−1.20	feruloyltyramine
18	22.46	515.2611	515.2611 (13), 423.1979 (32), 303.0493 (43), 201.0461 (100)	$C_{29}H_{38}O_8$	514.2580	−8.12	unknown
19	22.86	191.1433	191.1433 (81), 175.1165 (36), 161.1071 (38), 135.0439 (100), 107.0487 (71)	$C_{13}H_{18}O$	190.1358	1.18	*β*-damascenone
20*	24.52	303.0497	303.0497 (63), 285.0391 (13), 257.0441 (31), 229.0491 (68), 201.0543 (35)	$C_{15}H_{10}O_7$	302.0426	−0.58	quercetin (**8**)
21	25.63	235.1441	235.1441 (13), 218.1174 (33), 161.1069 (100), 135.0442 (10)	$C_{13}H_{18}N_2O_2$	234.1368	0.10	*N*-Coumaroylputrescine
22	27.51	419.2039	419.2039 (100)	$C_{24}H_{26}N_4O_3$	418.2005	−9.27	unknown
23	27.81	497.3020	497.3020 (100), 466.2593 (10), 306.1596 (8), 161.1071 (35), 130.0651 (10)	$C_{29}H_{40}N_2O_5$	496.2937	2.06	1-(4-ethylphenyl)-*N*-{4-[(4-ethyl-1-piperazinyl)methyl]phenyl}-3,6-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine-4-carboxamide
24	29.57	276.1957	276.1957 (22), 234.1851 (100), 217.1581 (13), 177.1271 (9)	$C_{17}H_{25}NO_2$	275.1885	−0.28	1-(3-methoxybenzoyl)-2,2,6,6-tetramethylpiperidine
25	30.75	275.2115	275.2115 (100), 257.2007 (8), 149.1068 (63), 132.0803 (12)	$C_{17}H_{26}N_2O$	274.2045	−1.01	4-(1,2,4,4a,5,6-hexahydro-3*H*-pyrazino[1,2-*a*]quinolin-3-yl)-2-methyl-2-butanol

among tobacco samples, the content of nicotine showed certain trend along the maturation stages. The content of nicotine in tobacco samples of each origin harvested at rosette stage was in a medium level (except for ZZ-T, which showed the highest nicotine level among all the tobacco samples) and in a dramatic decrease to reach the lowest level at squaring stage, reached the highest level at physiological maturation stage, and then fell sharply at harvest maturation stage. For the Zhengzhou tobacco samples, the nicotine contents ranged from 5655.62 ± 230.88 to 25399.39 ± 308.95 μg/g. The highest nicotine content of 25399.39 ± 308.95 μg/g was found in the rosette stage sample (ZZ-T) and in a dramatic decrease to reach the lowest level (ZZ-X with 5655.62 ± 230.88 μg/g) at squaring stage. Subsequently, the nicotine level showed an upward trend and reached a higher level at the physiological maturation stage and then decreased at the harvest maturation stage. The lowest nicotine level of 660.16 ± 24.78 μg/g was detected in a Zunyi tobacco sample (ZY-X). For the Zunyi tobacco samples, the highest nicotine content of 12215.15 ± 122.95 μg/g was detected in the sample harvested at physiological maturation stage. Similar to the Zunyi tobacco samples, the highest nicotine level (21409.02 ± 176.10 μg/g) in the Jiangchuan tobacco sample was also observed in the sample collected at physiological maturation stage.

The rutin contents of the tobacco samples harvested at different maturation stages varied greatly. At rosette stage, the rutin content ranged from 103.86 ± 8.57 to 201.87 ± 9.16 μg/g, while those ranged from 87.42 ± 2.78 to 734.66 ± 27.42 μg/g at squaring stage. The rutin contents at physiological maturation stage ranged from 90.30 ± 4.80 to 725.93 ± 40.70 μg/g, while those ranged from 164.00 ± 5.08 to 243.44 ± 6.79 μg/g at harvest maturation stage. The highest level of rutin of 734.66 ± 27.42 μg/g was found in the Jiangchuan tobacco sample harvested at squaring stage (JC-X), which was more

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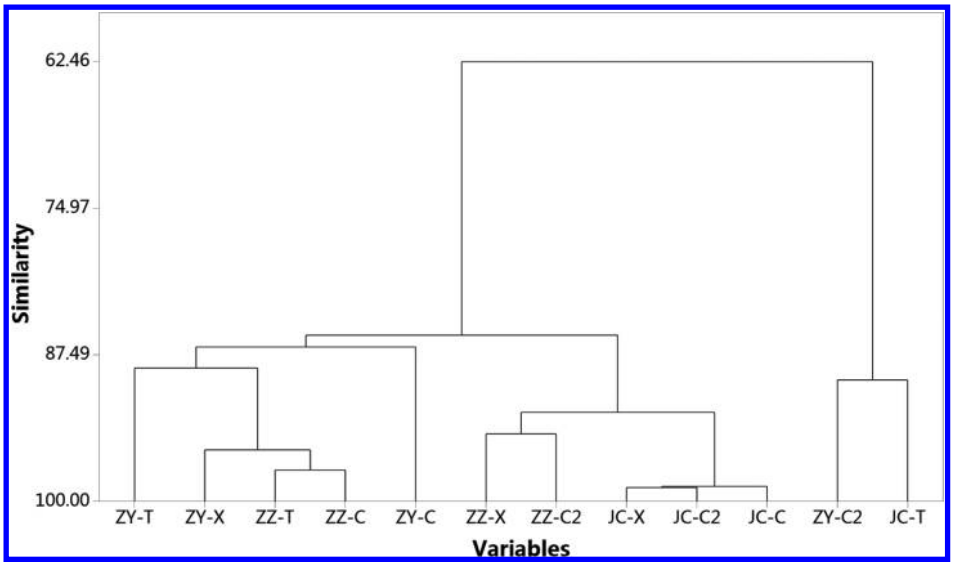

Figure 3. Dendrogram of hierarchical cluster analysis of 12 tobacco samples.

than 3-fold of that detected in the sample harvested at rosette stage (JC-T with 201.87 ± 9.16 μg/g). The lowest rutin content (ZY-X with 87.42 ± 2.78 μg/g) was detected in the Zunyi tobacco sample harvested at squaring stage, which was only approximately 70% amounts of that detected in the same origin harvest at harvest maturation stage (ZY-C2).

Among the Zhengzhou tobacco samples, the rutin content varied from 158.99 to 725.93 μg/g. The rutin content of the Zhengzhou tobacco samples harvested at physiological maturation stage was the highest (ZZ-C with 725.93 ± 40.70 μg/g) and was almost constant during rosette stage (ZZ-T with 186.28 ± 3.57 μg/g), squaring stage (ZZ-X with 158.99 ± 2.16 μg/g), and harvest maturation stage (ZZ-C2 with 164 ± 5.08 μg/g). For the Zunyi tobacco samples, the rutin contents varied from 87.42 ± 2.78 to 243.44 ± 6.79 μg/g. In the first three stages, the rutin contents did not vary with maturity (103.86 ± 8.57, 87.42 ± 2.78, 90.30 ± 4.80 μg/g for rosette stage, squaring stage, and physiological maturation stage, respectively). The maximum rutin content (ZY-C2 with 243.44 ± 6.79 μg/g) of the Zunyi tobacco samples was found at the harvest maturation stage. Compared to the tobacco samples from the other two origins, the Jiangchuan tobacco samples were found to have relatively high contents of rutin ranging from 201.87 ± 9.16 to 734.66 ± 27.42 μg/g. Generally, tobacco samples at the squaring stage had the highest level of rutin, which was about 3.5-fold over that measured in rosette stage.

Identification of the Main Constituents in Tobacco by HPLC-ESI-QTOF-MS. The difference of the chemical compositions is one of the important parameters to understand the variation among tobacco samples harvested from different origins and maturation stages. Unequivocal and tentative identification of the target compounds as well as the unknown constituents in the tobacco samples were performed by means of HPLC−ESI-QTOF-MS and MS/MS. Upon obtaining the data from MS and MS/MS analysis, a total of 25 compounds with relative high abundance were detected in 12 batches of the tobacco samples. Compounds at the retention times (t_R) of 5.0, 8.3, 9.1, 12.0, 17.9, 18.4, 18.8, and 24.9 min were unequivocally identified to be the reference compounds **1**−**8**, respectively. Other peaks were tentatively identified via searching for the precursor ion and fragmentation ions in MassBank Mass Spectral Database and literatures. We further performed empirical molecular formulas matching with those of the published compounds of tobacco in databases, such as PubMed of the U.S. National Library Medicine and the National Institutes of Health, SciFinder scholar of American Chemical Society, Chinese National Knowledge Infrastructure (CNKI) of Tsinghua University, and ChemSpider database when the precursor ion and fragmentation ions were not found in MassBank and literatures. When several empirical molecular formulas matched the same formula those isomeric compounds previously reported from tobacco would be preferentially selected as the putative ones. All the identified compounds in the tobacco samples were summarized in Table 4.

Statistical Analysis. To examine the change in similarity of fat-soluble compounds displayed in the 12 tobacco samples of different origins during maturity, a hierarchical cluster analysis based on the composition of major fat-soluble constituents was performed. The hierarchical cluster analysis is a multivariate procedure that allows the classification of variables into groups based on Euclidean distances between cases.[36] The dendogram of the 12 tobacco samples is shown in Figure 3. Two major clusters, viz., clusters 1 and 2, can be found in Figure 3. Cluster 1 was composed of 10 samples, while cluster 2 was composed of two samples. Cluster 1 formed two subclusters, viz., ZY-T, ZY-X, ZZ-T, and ZZ-C and ZZ-C2, JC-X, JC-C2, and JC-C. From the dendogram, it can be inferred that the tobacco samples of the same origin showed more similarity in the fat-soluble composition. The Jiangchuan tobacco sample showed more similarity during squaring stage, physiological maturation stage, and harvest maturation stage (i.e., JC-X, JC-C, and JC-C2). These clusters show that similarity mainly exists between samples of the same origin rather than different origins. In addition, it can be useful for farmers to choose the appropriate harvest stage of tobacco with desired content of components as well as to choose alternate origins with more similarity in the absence of a desired variety at the time of cultivation.

In conclusion, the presented study provided a comprehensive analysis in fat-soluble compositions as well as polar constituents in 12 batches of tobacco leaf samples of three origins at four maturations. The GC-MS analyses showed that tobacco samples harvested at rosette stage exhibited the most fat-

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Table 5. Contents of Eight Reference Compounds in Each Tobacco Sample (μg/g)

no.	sample code	nornicotine	neonicotine	anatabine	nicotine	scopoletin	myosmine	rutin	quercetin
1	ZZ-T	175.30 ± 9.00	NQ	570.78 ± 18.11	25399.39 ± 308.95	NQ	101.73 ± 7.25	186.28 ± 3.57	156.91 ± 9.29
2	ZZ-X	45.79 ± 4.33	NQ	536.80 ± 28.25	5655.62 ± 230.88	112.59 ± 5.78	97.18 ± 4.16	158.99 ± 2.16	151.69 ± 3.60
3	ZZ-C	87.37 ± 6.06	NQ	433.20 ± 24.83	11737.14 ± 270.77	122.01 ± 7.34	267.53 ± 10.03	725.93 ± 40.70	125.60 ± 2.16
4	ZZ-C2	64.63 ± 4.27	NQ	260.82 ± 15.19	8326.26 ± 155.95	228.79 ± 6.55	64.64 ± 3.86	164.00 ± 5.08	146.58 ± 3.42
5	ZY-T	NQ	NQ	167.62 ± 15.37	1654.49 ± 34.52	36.41 ± 2.74	NQ	103.86 ± 8.57	NQ
6	ZY-X	NQ	NQ	601.86 ± 14.57	660.16 ± 24.78	37.58 ± 3.12	NQ	87.42 ± 2.78	170.11 ± 5.75
7	ZY-C	97.27 ± 5.41	94.68 ± 8.10	420.36 ± 8.99	12215.15 ± 122.95	338.52 ± 12.85	72.89 ± 4.59	90.30 ± 4.80	143.71 ± 10.61
8	ZY-C2	123.84 ± 6.62	89.96 ± 8.71	354.89 ± 18.65	8835.33 ± 215.52	124.95 ± 5.11	117.04 ± 10.70	243.44 ± 6.79	126.93 ± 1.38
9	JC-T	NQ	93.96 ± 10.14	179.60 ± 2.15	5070.59 ± 211.84	14.37 ± 3.32	NQ	201.87 ± 9.16	169.70 ± 8.12
10	JC-X	221.66 ± 11.58	98.08 ± 9.47	384.31 ± 18.11	12653.36 ± 241.26	99.66 ± 8.64	NQ	734.66 ± 27.42	169.29 ± 8.45
11	JC-C	126.56 ± 5.02	116.26 ± 4.08	850.91 ± 20.54	21409.02 ± 176.10	165.84 ± 10.52	80.91 ± 8.86	542.70 ± 33.01	149.54 ± 4.91
12	JC-C2	64.87 ± 3.85	92.66 ± 7.45	507.79 ± 13.84	10868.21 ± 263.04	94.36 ± 8.01	55.79 ± 6.82	237.33 ± 11.23	132.66 ± 1.69

soluble components, while those harvested at harvest maturation stage exhibited the least fat-soluble components. In addition to GC-MS analysis, an HPLC-QTOF-MS method was developed for the quantitative analyses of eight main compounds in tobacco including five alkaloids and two polyphenols. The nicotine contents varied greatly in the 12 tobacco samples. The Zhengzhou tobacco sample harvested at rosette stage was found to have the highest nicotine content (ZZ-T), and the lowest nicotine level was detected in Zunyi tobacco samples harvested at squaring stage (ZY-X). The highest level of rutin was found in the Jiangchuan tobacco sample harvested at squaring stage (JC-X), and the lowest rutin content was detected in the Zunyi tobacco sample harvested at squaring stage (ZY-X). In addition to quantitative analyses, the main compounds in the tobacco samples were also unequivocally or tentatively identified. The developed method provided a convenient approach which might be applied for rapid maturity evaluation, tobacco flavor identification, and also holds the potential for analysis of compounds present in other plants.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The monthly average temperatures, total irradiances (average of 20 years), monthly average relative humidity, and monthly rainfall of May and June in 2012 in Zhengzhou, Zunyi, and Jiangchuan; GC-MS profile of fat-soluble compositions of tobacco samples planted in Zhengzhou, Zunyi, and Jiangchuan harvested at four maturities; HPLC-PDA (254 nm) profile of aroma composition of tobacco planted in Zhenzhou, Zunyi, and Jiangchuan harvested at four maturities. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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