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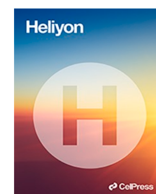

Research article

Study of metabolite differences of flue-cured tobacco from Canada (CT157) and Yunnan (Yunyan 87)

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A B S T R A C T

In order to comprehend the dissimilarities in tobacco quality between Canada and Yunnan, a comparison of the aroma components was conducted using GC-MS and HPLC analysis, coupled with orthogonal partial least squares discriminant analysis (OPLS-DA). The study revealed the detection of a total of 81 aroma components and 22 non-volatile components in both varieties of tobacco leaves. Specifically, there were 102 components of Canada tobacco leaves and 103 components of Yunnan tobacco leaves. Subsequently, a screening was performed on these two types of tobacco leaves, identifying 51 differential components, which accounted for approximately 49.5 % of the overall components detected. Among these, Canada tobacco exhibited a higher concentration of 22 components, comprising roughly 36.4 % of the total, which were primarily composed of semi-volatile organic acids and sesquiterpenes. On the other hand, Yunnan tobacco was characterized by a comparatively higher content of 43 components, constituting approximately 63.6 %, including fatty acid esters, phenols, diterpenes, sugars, and amino acids. Comparatively, Canada tobacco demonstrated elevated levels of fatty acids and sesquiterpenes, while the content of fatty acid esters and diterpenes was relatively lower. These distinctions in aroma components potentially contribute to the varied sensory aroma profiles exhibited by the two types of tobacco.

1. Introduction

Tobacco is a widely cultivated economic crop with a long history in the Americas, Asia, and Africa [1]. In both developing and developed nations, tobacco cultivation serves as a livelihood for numerous individuals, while the tobacco industry itself provides economic support for certain countries. Although predominantly used as a raw material in the cigarette industry, it is important to acknowledge that tobacco boasts a diverse chemical composition and tremendous potential for application in realms such as medicine and spices [2–4]. Consequently, exploring the industrial applications of tobacco should extend beyond its exclusive use in cigarette production, instead focusing on the advancement of alternative industrial uses. Upon maturation, tobacco lacks aroma; however, through a process of flue-curing, it undergoes a series of intricate chemical reactions in which certain precursors undergo transformation, leading to the generation of a substantial amount of aromatic substances [5]. This phenomenon leads to the exquisite and distinctive fragrance of tobacco. Considering the remarkable yield of tobacco, it can be argued that tobacco serves as a valuable source for obtaining distinct flavors in daily chemical essence. However, a practical matter arises due to the diverse range of tobacco varieties as a crop, and the composition of tobacco leaves varies across different regions [6–8]. In China, the Yunnan region holds great significance in tobacco production due to its moderate rainfall, temperature, and ample sunlight. Yunnan's tobacco output accounts for approximately 40 % of the nation's total production, leading to substantial income for numerous farmers. It is widely believed that the

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tobacco aroma in Yunnan production areas is more abundant and refined [9,10], thereby enticing more tobacco merchants to utilize Yunnan tobacco leaves. Simultaneously, tobacco leaves obtained from regions in Native America, including Ontario in southern Canada, are acknowledged to possess a favorable scent [11]. Consequently, a comprehensive examination and comparison of the metabolic elements present in tobacco cultivated in Yunnan Province, China and Canada would foster an enhanced comprehension of their prospective practicality.

Metabolomics is a novel tool that is designed to identify and quantify small molecule metabolites. It employs modern detection techniques to comprehensively analyze and detect as many metabolites as possible in food, plant, and biological samples [12–16]. These techniques, such as principal component analysis (PCA), orthogonal partial least squares discriminant analysis (OPLS-DA), and other methods, have been widely utilized in various fields, including species identification [17–19], changes in ingredients before and after food processing [20,21], traceability of herb medicine origin [22,23], and differentiation of tobacco aroma types [24,25]. Hence, this paper intends to utilize GC-MS, HPLC detection methods, and multivariate statistical methods to examine the distinctions in primary and secondary metabolic components between the two tobaccos, and investigate the underlying causes of their style characteristics.

2. Materials and methods

2.1. Materials

Sixteen flue-cured tobacco leave samples of the middle section were gathered from Canada (variety CT157, eight samples, produced in 2021, Ontario) and Yunnan province (variety Yunyan 87, four samples were produced in 2021 from Kunming city, others were produced in 2022 from Qujing city) in China. Tobacco leaves were ground into powder using a grinder, and then passed through an 80-mesh sieve. Prior to extraction, all samples were stored at −20 °C.

2.2. Reagents

HPLC-grade acetonitrile, methanol and dichloromethane were purchased from J T Baker. 2,6-Dichlorotoluene, *trans*-2-hexenoic acid, nitrobenzene and adipic acid as internal standard, sugars and organic acids as standard were purchased from J&K Scientific, with purity are above 99 %. AccQ Fluor reagent and amino acid standards were purchased from Waters. Distilled water was purified using the Millipore Milli-Q system.

2.3. Determination of volatile components

Volatile components were prepared using a simultaneous distillation extraction (SDE) method described previously, with a minor modification [26]. Briefly, 30.0 g of tobacco powder were placed in a 1000 mL round bottom flask mixed with 400 mL of deionized water, and flask was mounted on the sample port of SDE. A 250 mL flask with 100 mL dichloromethane was linked to solvent port of the SDE. The sample was distilled and extracted for 2.5 h to obtain the dichloromethane extract containing aroma components. Further, the mixed aroma components were separated into neutral, alkaline, and acidic aroma components [27]. The neutral, alkaline, and acidic aroma components extracts were added 10.0 g of anhydrous Na_2SO_4, refrigerated overnight, filtered and concentrated on a atmospheric distillation unit to 1 mL for GC-MS analysis, with 2,6-dichlorotoluene, nitrobenzene and *trans*-2-hexenoic acid as internal standard, respectively. Repeated three times for each sample and taken the average quantitative result.

The GC-MS determination was performed on an Agilent 8860-5977B. Chromatographic separation was conducted using a DB-wax capillary column (60 m × 0.25 mm, 0.25 μm) from Agilent. The column flow of carrier gas (helium, 99.999 %) was 1.2 mL/min 1 μL of sample was injected into column with a 10:1 split ratio at 280 °C. The oven procedure was 50 °C for 2 min and then raised at 3 °C/min to 280 °C and kept for 5 min. The transfer line and the ion source temperature were 280 °C and 240 °C, respectively. The ionization was performed in electron ionization mode at 70 eV. The mass scan range was 33–500 *m/z* with a 5 scans/s scan speed under full-scan mode.

2.4. Determination of monosaccharide and disaccharide

The methods used for monosaccharide and disaccharide analysis were similar to those described previously, with a minor modification [28]. Briefly, 1 g of tobacco sample and 30 mL of 50 % acetonitrile aqueous solution were placed in a 100 mL flask. After shake at 180 r/min in a shaker for 40 min, 1.0 mL of the extract was passed through a 0.2 μm PTFE syringe filter for HPLC analysis.

The HPLC analysis was performed on a waters 2695 pump system coupled with an waters 2874 evaporative light-scattering detector (ELSD) and a X-Bridge Amide separation column (4.6 mm × 150 mm, 3.5 μm). Column temperature was set on 45 °C. Mobile phase A was acetonitrile, and mobile phase B was water which containing 0.2 % triethylamine with a ratio of 75/25(v/v). The injection volume was 10 μL, and the flow rate was 1 mL/min. ELSD parameters: nitrogen flow rate was 2.2 L/min, drift tube temperature was 45 °C and gain was set at 10.

2.5. Determination of amino acids

AccQ•Tag derivatization method used here was reported previously [29,30]. Briefly, 1g of the sample was placed in a 50 mL

Table 1
Volatile components from the two tobacco leaves.

Entry	Retention time (min)	Component	Match	Relative content (μg/g)		*P* value
				Canada	Yunnan	
1	17.87	2-Hexenal	95.2	0.329	0.369	0.196
2	19.60	Coffee furanone	95.2	0.931	1.01	0.482
3	22.06	Methyl heptenone	95.8	0.374	0.434	0.002
4	23.55	*cis*-3-Hexenol	95.7	0.346	0.260	0.030
5	27.14	3-Furaldehyde	97.3	6.44	6.23	0.399
6	28.32	(E,E)-2,4-Heptadienal	88.6	0.268	0.310	0.192
7	28.92	2-Acetylfuran	96.2	0.763	0.760	0.937
8	29.91	Benzaldehyde	98.0	1.11	0.834	<0.001
9	30.15	(E)-2-Nonenal	90.7	0.257	0.446	<0.001
10	30.34	Linalool	98.0	1.34	0.990	<0.001
11	32.30	5-Methylfurfural	95.5	0.789	1.07	<0.001
12	32.78	(E,Z)-2,6-Nonadienal	89.1	0.387	0.351	0.021
13	34.77	Cyclocitra	89.1	0.904	1.02	0.135
14	36.30	Benzeneacetaldehyde	97.6	4.13	4.40	0.238
15	36.97	3-Furanmethanol	94.6	1.53	1.442	0.257
16	39.08	L-Terpineol	88.6	0.602	0.592	0.449
17	39.15	4-Oxoisophorone	92.4	0.515	0.583	0.103
18	40.57	Vlencene	88.9	0.137	0.097	0.010
19	41.53	Solanone	92.8	20.1	19.3	0.729
20	47.33	β-Damascone	98.3	1.46	1.28	0.045
21	47.83	β-Damascenone	94.7	7.68	9.90	0.017
22	50.29	Geranyl acetone	96.5	3.52	3.68	0.633
23	52.27	Benzyl alcohol	96.2	10.1	9.06	0.220
24	54.90	Phenylethyl alcohol	97.1	5.33	4.63	0.076
25	57.39	Neophytadiene	96.0	160	263	<0.001
26	59.94	2-Acetyl pyrrole	96.6	3.40	3.15	0.069
27	62.77	Methyl tetradecanoate	94.5	1.52	2.25	0.004
28	71.42	Methyl Pentadecanoate	94.9	0.835	1.76	<0.001
29	72.39	Phytone	95.4	2.35	2.93	0.008
30	74.52	Megastigmatrienone A	94.0	5.08	5.23	0.772
31	75.97	Geranyllinalool	92.9	1.79	3.60	<0.001
32	78.57	Megastigmatrienone B	96.7	12.4	18.4	0.002
33	81.51	Methyl palmitate	96.5	21.6	59.4	<0.001
34	84.69	Nootkatone	86.7	7.71	5.68	0.006
35	85.78	Megastigmatrienone C	93.3	2.27	3.03	<0.011
36	89.65	Megastigmatrienone D	81.1	17.2	20.9	0.042
37	93.79	Dihydroactinidiolide	96.5	3.83	3.61	0.518
38	99.84	Farnesyl acetone	93.3	4.47	4.40	0.870
39	104.91	Methyl stearate	95.6	2.73	5.81	<0.001
40	106.60	Methyl oleate	99.1	4.41	11.5	<0.001
41	109.70	Solavetivone	92.5	5.40	2.02	<0.001
42	110.01	Methyl linoleate	94.3	7.07	17.9	<0.001
43	114.39	Methyl linolenate	97.8	8.38	24.8	<0.001
44	29.63	Isobutyric acid	89.8	0.113	0.139	0.015
45	32.69	2-Methylbutyric acid	93.3	1.84	2.79	<0.001
46	34.65	Pentanoic acid	94.6	1.44	0.973	<0.001
47	37.37	MCP	91.8	0.130	0.083	<0.001
48	37.59	Hexanoic acid	86.8	2.54	1.66	<0.001
49	37.73	Tiglic acid	93.4	0.602	0.670	0.010
50	38.28	Guaiacol	84.7	0.926	0.909	0.812
51	40.37	Heptanoic acid	83.4	0.500	0.454	0.171
52	41.86	2-MethylPhenol	96.6	0.053	0.588	<0.001
53	41.98	Phenol	94.8	0.097	1.001	<0.001
54	43.04	Octanoic acid	95.4	1.82	1.53	0.006
55	43.68	2,4-Dimethylphenol	94.1	0	0.104	<0.001
56	44.04	4-Cresol	97.4	0.404	0.704	0.006
57	45.58	Nonanoic acid	91.6	1.01	0.842	<0.010
58	46.72	4-Vinyl guaiacol	93.4	5.97	3.43	0.001
59	48.35	Decanoic acid	85.0	0.765	0.649	0.008
60	51.74	Undecanoic acid	90.5	0.987	0.878	0.433
61	56.01	Lauric acid	88.4	1.29	0.54	<0.001
62	69.00	Tetradecanoic acid	94.8	18.4	7.94	<0.001
63	78.27	Pentadecanoic acid	93.5	8.65	4.63	<0.001
64	85.16	Palmitic acid	97.2	130	96.4	0.003
65	89.74	Heptadecanoic acid	90.6	2.76	1.78	0.001
66	92.28	Linolenic acid	86.5	4.99	6.38	0.118

(*continued on next page*)

Table 1 (*continued*)

Entry	Retention time (min)	Component	Match	Relative content (μg/g)		*P* value
				Canada	Yunnan	
67	94.84	Octadecanoic acid	95.2	9.15	6.68	0.001
68	96.54	Oleic Acid	95.9	18.2	15.4	0.041
69	16.54	Pyridine	98.2	1.05	1.58	<0.001
70	20.59	2-Propylpyridine	91.1	0.031	0.021	0.093
71	22.16	2-Acetyl-1-pyrroline	82.2	0.093	0.109	0.445
72	24.44	Trimethylpyrazine	88.4	0.051	0.128	<0.001
73	25.93	4-Isopropylpyridine	70.4	0.052	0.049	0.763
74	27.56	4-Ethenylpyridine	81.5	0.041	0.057	<0.001
65	29.46	2,3-Cyclopentenopyridine	93.7	0.121	0.110	0.271
76	33.85	2-Acetylpyridine	94.7	0.063	0.066	0.123
77	40.08	3-Pyridinecarboxaldehyde	94.5	0.188	0.162	0.028
78	48.42	3-Acetylpyridine	90.2	0.372	0.185	<0.001
79	56.46	Quinoline	79.9	0.106	0.110	0.778
80	56.65	4-Methylquinoline	95.2	0.038	0.041	0.534
81	70.12	2,3′-Bipyridine	84.9	0.838	0.351	<0.001

volumetric flask, added 30 mL of 0.01 mol/L hydrochloric acid solution, sonicated for 30 min, diluted to 50 mL with water, centrifugation for 5 min on a centrifuge with a speed of 5000 rpm to obtained the supernatant for later use. 10 μL of the supernatant was mixed with 70 μL of AccQ Flour borate buffer in the derivative tube. Immediately after this, 20 μL of AccQ reagent (10 mM in acetonitrile) were added. The mixture was vortex whirled for 10 s and heated at 55 °C for 10 min. Finally, derivatives were analyzed by HPLC. The derivation of amino acid standards was using the same method above.

The analytical column was a AccQ·Tag amino acid analysis column (150 mm × 3.9 mm). Mobile phase A was 0.1 mol/L sodium acetate buffer solution (pH 6.5), mobile phase B was acetonitrile, and mobile phase C was water. The gradient elution, with flow rate of 1.0 mL/min, was started at 100 % A, 0–0.5 min, mobile phase B increased from 0 to 2 %, then increased to 5 % B 0.5–13 min, to 9 % B from 13 to 19 min, to 17 % B from 19 to 29.5 min, to 60 % B and 40 % C from 29.5 to 36 min, after 36 min A was 100 % and kept for 9 min. Fluorescence excitation wavelength was 250 nm, emission wavelength was 395 nm.

2.6. Determination of nonvolatile organic acids

Nonvolatile organic acids were analyzed according to the literature [31]. Briefly, 1g of the sample was placed in a 150 mL round bottom flask mixed 40 mL of 5 % sulfuric acid methanol solution and 200 μL adipic acid (20 mg/mL) internal standard solution. The mixture was refluxed at 60 °C for 2 h, and then cooled to room temperature. 10 mL of the supernatant was added 20 mL of saturated NaCl aqueous solution, extracted with dichloromethane (3 × 10 mL), and the combined organic phase was dried over about 4.0 g of anhydrous Na_2SO_4. 1.0 mL of the extract was passed through a 0.2 μm PTFE syringe filter for gas chromatography (GC) analysis. The derivation of organic acid standards was using the same method above.

GC analysis was carried out on an Agilent 6890 GC system equipped with a flame ionization detector (FID). A HP-INNOWax column (30 m × 250 μm, 0.25 μm) was used as separation column. Carrier gas was helium with a flow rate of 1.0 mL/min 1 μL of sample was injected into column with a 5:1 split ratio at 260 °C. The oven procedure was 50 °C for 2 min and then raised at 10 °C/min to 220 °C and kept for 10 min. FID temperature was 280 °C, hydrogen and air flow rate was 40 mL/min and 400 mL/min, respectively.

2.7. Sensory evaluation

Sensory evaluation of the tobacco samples was following reference [32]. Tobacco leaf samples were rolled into cigarette sticks using an automatic cigarette-rolling machine. Cigarette samples were equilibrated under a condition with the temperature of 22 ± 1 °C and the humidity of 60 ± 3 % for 48 h before the sensory evaluation. The sensory quality indicators mainly included flavor characteristics, aroma, miscellaneous gas, aftertaste, smoky concentration, diffusiveness, irritation and softness, which were evaluated by seven assessors with more than 5 years of sensory evaluation experience. All the participants written an informed consent form. Furthermore, the sensory evaluation was approved by the ethics committee of Zhengzhou University of Light Industry (202305). The quality and style characteristics were evaluated on a 5-point scale, and with high score indicated strong characteristics.

2.8. Statistical analysis

Data processing was performed using Excel, SPSS 19, and multivariate statistical software SIMCA14.1. PCA was performed on two sets of samples to predict the stability and reliability of the model using the OPLS-DA method. Differential metabolites were screened using multidimensional statistical variable importance in project (VIP) values, independent sample T-test P values, and difference multiple FC values.

Table 2
Nonvolatile components from the two tobaccos.

Entry	Retention time (min)	Component	Content (mg/g)		*P* value
			Canada	Yunnan	
1	3.99	Fructose	88.7	104	<0.001
2	4.23	Glucose	75.3	95.9	<0.001
3	6.32	Sucrose	3.50	12.8	<0.001
4	7.51	Maltose	1.10	1.90	0.179
5	6.07	Asp	0.295	0.337	<0.001
6	7.80	Glu	0.183	0.161	<0.001
7	12.01	Ser	0.085	0.099	0.003
8	14.88	Arg	0.023	0.039	<0.001
9	15.17	Gly	0.086	0.097	<0.001
10	15.55	Thr	0.007	0.008	0.031
11	17.06	Pro	6.75	9.41	<0.001
12	17.81	Ala	0.870	0.853	0.379
13	22.86	Val	0.051	0.065	<0.001
14	28.07	Ile	0.008	0.013	<0.001
15	28.76	Leu	0.015	0.029	0.335
16	31.72	Phe	0.167	0.214	<0.001
17	32.26	His	0.130	0.128	0.801
18	35.38	Lys	0.076	0.150	0.364
19	36.26	Tyr	3.71	3.60	0.095
20	2.31	Oxalic acid	12.0	12.1	0.833
21	8.76	Malic acid	50.7	49.2	0.605
22	17.47	Citric acid	10.1	4.80	<0.001

3. Results and discussions

3.1. Analysis of volatile components

Initially, we conducted an analysis of the volatile aroma components present in eight tobacco leaves from Canada and eight tobacco leaves from Yunnan, utilizing GC-MS. If the aroma components in tobacco are classified according to their acid-base properties, they can be divided into the neutral components, acidic components, and alkaline components. Neutral components are represented by a series of degradation products of cembrane and carotenoids such as solanone, damascone, and megastigmatrienone, while acidic components are mainly phenolic substances and fatty acids, while alkaline components are mainly pyrazine and pyridine. Due to the relatively low levels of alkaline and acidic components in tobacco leaves, they may be overlooked if tobacco extracts are directly examined. Therefore, that is necessary to conduct preliminary separation of the three types of components prior to analysis. The analysis results are shown in Table 1. By utilizing the NIST 20 mass spectrometry database and incorporating a retention index, a total of 81 identifiable aroma components were detected for qualitative analysis of the separated peaks, because of in the metabolic profiling analysis, the absolute amounts of metabolites are not required. Among them, 43 neutral aroma components were identified. Notably, the Canada tobacco exhibited higher content of nootkatone and solavetivone, both of which belong to the sesquiterpenoid ketones. On the other hand, the Yunnan tobacco displayed higher levels of neophytadiene, geranyllinalool, megastigmatrienone, and several long-chain fatty acid esters such as methyl palmitate and methyl stearate. Out of the total acidic aroma components, 24 were found in the Canada tobacco and 25 were found in the Yunnan tobacco, comprising volatile acids, semi-volatile acids, and phenols. The presence of acids, particularly long-chain fatty acids, was significantly greater in Canada tobacco compared to Yunnan tobacco. In terms of alkaline components, 13 compounds were discovered in both types of tobacco, predominantly pyridine and quinoline compounds.

3.2. Analysis of nonvolatile components

The quantification of sugars, amino acids, and nonvolatile organic acids in two types of tobacco was conducted using high-performance liquid chromatography and gas chromatography. These essential components are undergo intricate Maillard reactions and caramelization reactions during the preparation, processing, and combustion of tobacco leaf, which have a notable influence on the flavor of tobacco. As shown in Table 2, the sugar concentration in Canada tobacco is considerably lower compared to that of Yunnan tobacco. Yunnan tobacco exhibit a total sugar content of 215.02 mg/g. The amino acid content in Yunnan tobacco is generally higher or comparable to that found in Canada tobacco, with the exception of asparagine. Moreover, the presence of nonvolatile organic acids in Canada tobacco is similar to that of Yunnan tobacco, but the oxalic acid content is significantly elevated.

3.3. Analysis of differences in composition between two types of tobacco leaves

Firstly, PCA was performed on two tobacco samples from Canada and Yunnan, in order to obtain a preliminary understanding of the overall compositional differences between each group of samples and the level of variation within each group. The score plot is shown

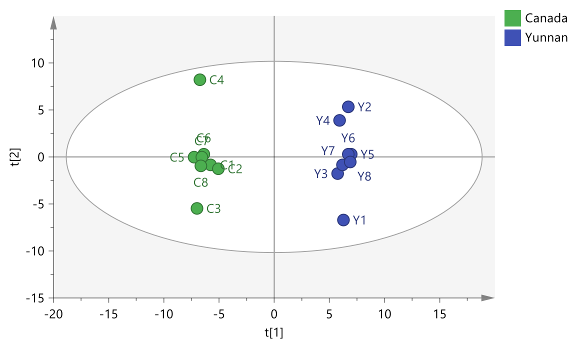

Fig. 1. PCA score plot for the two tobacco.

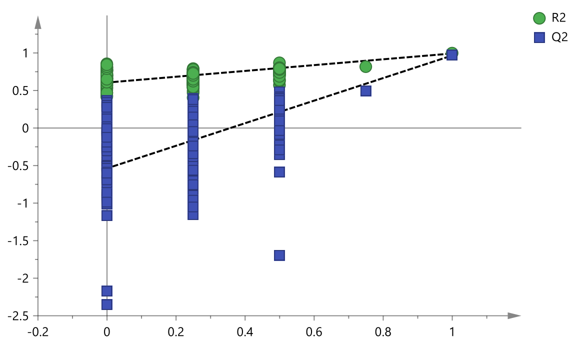

Fig. 2. Cross-validation plot of OPLS-DA with 200 permutation test for two tobacco leaves.

in Fig. 1, with each point representing one independent sample. It can be seen that the cumulative contribution rate of the two principal components reaches 90.1 %, and the separation trend between the two groups of samples is obvious, indicating a significant difference between Canada tobacco and Yunnan tobacco. Furthermore, the supervised pattern recognition method OPLS-DA was used to further process the data, extract the main information of variables with low correlation, and remove irrelevant differences to screen for differential variables. The model exhibited high interpretability variables, with R2 values of 0.556 and 0.993 for the X and Y dimensions respectively. The predictability Q2 value was 0.966, indicating the model's strong stability and predictive capabilities. Thus, it effectively reflects the differences in aroma components between the tobacco samples from Canada and Yunnan.

To verify whether there is over fitting, the model was sorted 200 times in response, and the results are shown in Fig. 2. The intercepts of R2 and Q2 are 0.606 and −0.537, respectively, indicating that the model is effective and there is no over fitting phenomenon.

Based on the results of OPLS-DA, the VIP values were utilized to represent the strength of the impact of inter group differences of corresponding aroma components on the classification discrimination of samples in each group of the model. It is widely accepted that substances with VIP values of one or higher exhibit significant differences. Screening was conducted on Yunnan and Canada tobacco leaves, and 51 differential compounds were obtained. The results are shown in Table 3. Overall, the differential components between Yunnan tobacco and Canada tobacco accounted for 49.5 % of the total components (103 types), indicating a significant difference between the two types of tobacco. The types of compounds with most differences are fatty acids and amino acids, accounting for 19.6 % and 17.64 % respectively, followed by fatty acids ester (17.73 %) and alkaloids (9.8 %). For volatile components, the fatty acid content of Canada tobacco leaves is significantly higher than that of Yunnan tobacco leaves. Tetradecanoic acid, lauric acid, and pentadecanoic acid have higher VIP and FC values. The fatty acid ester content of Canada tobacco leaves is significantly lower than that of Yunnan tobacco leaves, but the FC values are relatively small. It is worth noting that solavetivone has a relatively high content in Canada tobacco, with both VIP and FC values, indicating the possible presence of more vetispirane-type compounds in Canada tobacco. For non-volatile components, the differential compounds include Glu, Pro, Arg, Ile, Phe, Val, fructose, sucrosse, glucose, and citric acid.

In order to compare the quantitative information of components, the differences were processed (log2FC), and the top 30 differentially expressed metabolic components were shown in Fig. 3. Seven fatty acids (lauric acid, tetradecanoic acid, pentadecanoic acid, octadecanoic acid, heptadecanoic acid, hexanoic acid, pentanoic acid), two phenols (MCP, 4-vinyl guaiacol), three terpenes (solavetivone, valencene, linalool), two pyridine alkaloids (2,3′-bipyridine, 3-acetylpyridine) and citric acid were significantly higher in Canada tobacco than in Yunnan tobacco. Three phenols (2,4-dimethylphenol, 2-methylphenol and phenol), five fatty acid esters (methy linolenate, methyl palmitate, methyl linoleate, methyl pentadecanoate and methyl stearate), two diterpenes (geranyllinalool, neophytadiene) were significantly higher in Yunnan tobacco than that of Canada tobacco.

Table 3
Differential components between two tobacco($P < 0.01$).

Entry	Differential components	VIP	kind	FC
1	Tetradecanoic acid	1.42	+	2.32
2	Glu	1.40	+	1.14
3	Phenol	1.39	–	0.09
4	Solavetivone	1.39	+	2.72
5	Pro	1.37	–	0.72
6	2,3′-Bipyridine	1.37	+	2.39
7	Lauric acid	1.37	+	2.41
8	Arg	1.36	–	0.59
9	Ile	1.35	–	0.62
10	2-MethylPhenol	1.35	–	0.09
11	Citric acid	1.34	+	2.11
12	4-Ethenylpyridine	1.33	–	0.72
13	Methyl palmitate	1.33	–	0.36
14	3-Acetylpyridine	1.32	+	2.01
15	Fructose	1.32	–	0.85
16	Trimethylpyrazine	1.31	–	0.40
17	Methyl Pentadecanoate	1.31	–	0.47
18	Methyl Linolenate	1.31	–	0.34
19	Phe	1.28	–	0.78
20	Val	1.26	–	0.78
21	Pyridine	1.26	–	0.67
22	Methyl linoleate	1.25	–	0.39
23	2,4-Dimethylphenol	1.25	–	0.01
24	Pentadecanoic acid	1.25	+	1.87
25	Sucrose	1.25	–	0.27
26	2-Methylbutyric acid	1.25	–	0.66
27	5-Methylfurfural	1.24	–	0.74
28	Asp	1.21	–	0.88
29	Hexanoic acid	1.21	+	1.54
30	MCP	1.20	+	1.57
31	Methyl oleate	1.20	–	0.40
32	Pentanoic acid	1.20	+	1.48
33	(E)-2-Nonenal	1.19	–	0.58
34	Geranyllinalool	1.18	–	0.50
35	Methyl stearate	1.16	–	0.47
36	Linalool	1.16	+	1.35
37	valencene	1.16	+	1.41
38	Benzaldehyde	1.16	+	1.33
39	Gly	1.15	–	0.89
40	Glucose	1.12	–	0.79
41	4-Vinyl guaiacol	1.118	+	1.74
42	Methyl tetradecanoate	1.114	–	0.67
43	Heptadecanoic acid	1.11	+	1.55
44	Megastigmatrienone B	1.10	–	0.67
45	Neophytadiene	1.09	–	0.61
46	Octadecanoic acid	1.09	+	1.74
47	Ser	1.08	–	0.86
48	palmitic acid	1.06	+	1.35
49	Phytone	1.03	–	0.80
50	Octanoic acid	1.02	+	1.37
51	Nootkatone	1.00	+	1.36

3.4. The influence of differential components on the aroma style of the two tobacco

Among the tobacco leaves produced in China, Yunyan 87 is the closest in sensory quality to Canada tobacco, but there are still some differences (Fig. 4). Yunnan tobacco is a typical fresh flavor style, with good aroma quality and quantity, outstanding sweetness and green, but a slightly more irritation. On the other hand, the tobacco leaves from Canada exhibit a delicacy, diffusive and softness characteristic, satisfactory aroma, accompanied by a moderate smoke concentration.

The difference in sensory quality and aroma style between the two varieties of tobacco leaves is probably associated with the types and concentrations of their aroma components. There is minimal variation in alkaline components, but a noteworthy distinction in neutral and acidic aroma components between Canada and Yunnan tobacco. The degradation products of carotenoids, such as megastigmatrienone, β-damascone, β-damascenone, along with the degradation products of cembrane, such as solanone, are considered important influencing factors in the formation of tobacco aroma style. However, there is no significant difference in the content of these compounds between the two tobaccos. Canada tobacco exhibits similarities to Yunnan tobacco in terms of aroma quality and quantity, while also possessing a fresh flavor profile. Moreover, the concentration of semi-volatile fatty acids in Canada tobacco leaves is significantly higher than that of Yunnan tobacco leaves. Previous research [33] indicated that these semi-volatile

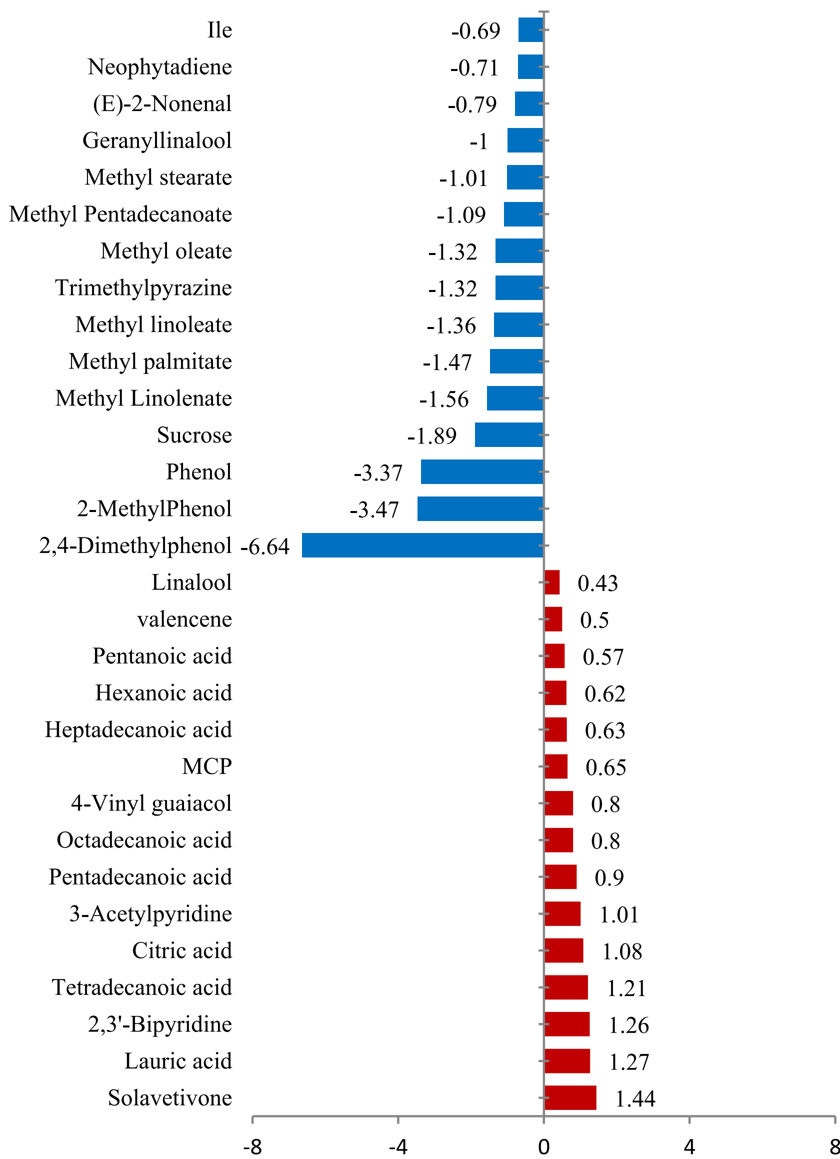

Fig. 3. Thirty most significantly differential components between the two tobacco.

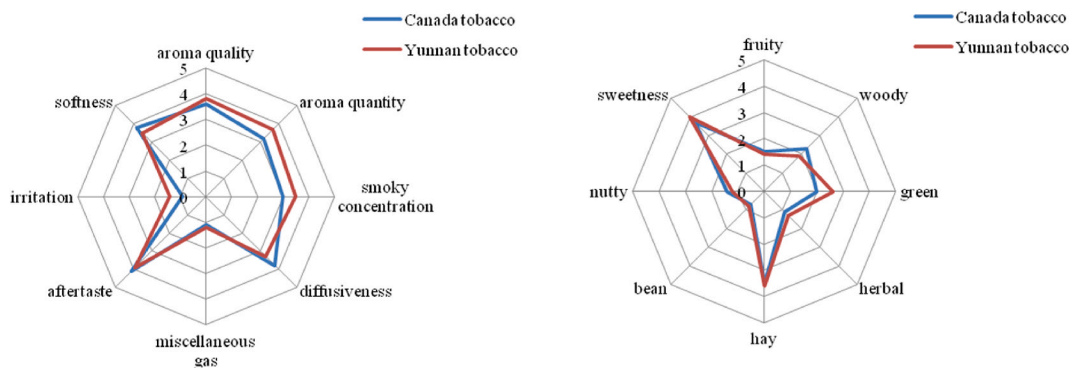

Fig. 4. Sensory quality profile of the two tobacco.

fatty acids contribute to smoke softening, enhancing its smoothness and transparency, and are vital factors for the development of a robust flavor in tobacco. Yunnan tobacco had a sweeter flavor, which was probably owing to the presence of higher concentrations of fatty acid esters (fresh sweet) than those in Canada tobacco [34]. Additionally, Canada tobacco contains higher levels of solavetivone nootkatone and valencene, which are structurally similar and therefore can be considered to have the same biosynthetic pathway. Valencene, being a precursor, can be biotransformed into nootkatone, both of which have a fresh citrus aroma [35]. Solavetivone gives Canada tobacco more woody characteristics [36]. Consequently, Canada tobacco acquires a distinctive aroma style that combines robust and fresh flavor.

In addition to aroma components, nonvolatile components also make significant contributions to the tobacco aroma style and smoke characteristics. Nonvolatile components, including sugars, amino acids, and organic acids, are fundamental substances of tobacco leaves. These substances have no aroma, but can release a series of aroma components such as furanone, maltol, pyrazine via pyrolysis, Maillard reaction, caramelization. Compared to Canada tobacco, a typical characteristic of Yunnan tobacco is its high sugar and amino acid content, which can be used as important index to evaluate the sensor quality [37]. Sugar gives Yunnan tobacco smoking better sweetness, while amino acids give Yunnan tobacco smoking more aroma characteristics. As for organic acids, the citric acid content in Canada tobacco is higher than that in Yunnan tobacco. Although citric acid is considered an adverse substance to tobacco smoke, it can reduce the impact of nicotine, which may be related to the softness and delicacy of Canada tobacco. In this sense, non-volatile compounds can also reflect the differences in aroma styles between Yunnan and Canada tobacco.

4. Conclusions

The volatile aroma components and nonvolatile components in tobacco leaves can be analyzed effectively by employing the GC-MS and HPLC detection methods. A total of 81 volatile metabolites and 22 non-volatile metabolites were identified in tobacco leaves from Canada and Yunnan. These metabolites exhibited distinguishable differences, which were accurately determined through OPLS-DA analysis. Additionally, VIP analysis resulted in the identification of 51 differential compounds. The content of semi-volatile acidic components and specific sesquiterpenes in Canada tobacco leaves were found to be significantly higher compared to Yunnan tobacco leaves. Conversely, Yunnan tobacco leaves exhibited significantly higher levels of fatty acid esters, phenols, and diterpenes. These differences in components between the two tobacco were closely associated with their respective aroma styles.

Data availability statement

Data is contained within the article.

CRediT authorship contribution statement

Jinxin Tie: Writing – original draft, Methodology, Investigation, Formal analysis. **Shitou Li:** Funding acquisition, Data curation. **Wenmiao He:** Visualization. **Yongsheng Li:** Investigation. **Fu Liao:** Supervision. **Jingjing Xue:** Data curation. **Bing Bai:** Writing – review & editing, Methodology. **Jing Yang:** Project administration. **Jizhong Wu:** Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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