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Comprehensive Comparison of Two Color Varieties of Perillae Folium by GC-MS-Based Metabolomic Approach

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Abstract: Perillae Folium (PF), the leaf of *Perilla frutescens* (L.) Britt, is extensively used as culinary vegetable in many countries. It can be divided into two major varietal forms based on leaf color variation, including purple PF (*Perilla frutescens* var. *arguta*) and green PF (*P. frutescens* var. *frutescens*). The aroma of purple and green PF is discrepant. To figure out the divergence of chemical composition in purple and green PF, gas chromatography–tandem mass spectrometry (GC-MS) was applied to analyze compounds in purple and green PF. A total of 54 compounds were identified and relatively quantified. Multivariate statistical methods, including principal component analysis (PCA), orthogonal partial least-squares discrimination analysis (OPLS-DA) and clustering analysis (CA), were used to screen the chemical markers for discrimination of purple and green PF. Seven compounds that accumulated discrepantly in green and purple PF were characterized as chemical markers for the discrimination of the purple and green PF. Among these 7 marker compounds, limonene, shisool and perillaldehyde that from the same branch of the terpenoid biosynthetic pathway were with relatively higher contents in purple PF, while perilla ketone, isoegomaketone, tocopheryl and squalene on other branch pathways were higher in green PF. The results of the present study are expected to provide theoretical support for the development and utilization of PF resources.

Keywords: perilla leaf; chemical composition; GC-MS; multivariate statistical analysis; biosynthetic pathway

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1. Introduction

Perilla frutescens (L.) Britt. is an annual herbal plant that belongs to the family of Lamiaceae [1,2]. The leaf of *P. frutescens* (L.) Britt, also called Perillae Folium (PF), has been extensively used in many countries as a culinary vegetable. Based on plant leaf color variation, PF can be divided into two major varietal forms that are circulated in China, including purple PF (*P. frutescens* var. *arguta*) and green PF (*P. frutescens* var. *frutescens*) [3]. *P. frutescens* var. *arguta* and *P. frutescens* var. *frutescens* are considered the same species in plant taxonomy, but there are large differences in practical application. Purple PF is widely used as a natural food pigment and a genuine medicinal plant for the treatment of food poisoning, coughs and gastritis [4–6]. Purple PF is believed to have efficacy in exterior relief, dispersing cold, easing stomach pain, reducing phlegm and relieving coughs and asthma [7]. Traditionally, it has been used to alleviate a variety of symptoms, including coughs, colds, fever, allergies and some intestinal diseases [8,9]. Unlike purple PF, green PF is consumed only as a vegetable or industrial preservative and is not used as a traditional Chinese medicine in China [3].

Phytochemical studies indicate that PF is rich in volatile compounds [10–12], flavonoids [13,14], anthocyanins [15], fatty acids [16,17] and phenolic compounds [18,19]. Compounds and extractions of PF showed various biological activities, such as antioxidant, antimicrobial, antiallergic, antidepressant, anti-inflammatory and anticancer effects [20–24]. Metabolites in foods or natural herbs differ by varietal forms, which may produce effects on their quality and effectiveness. Therefore, it is necessary to clarify the chemical differences of different PF. Huang et al. [25] compared the content and composition of the volatiles of purple and green PF, obtained by SFE, HS-SPME and hydrodistillation. A total of 64 volatile compounds were identified in purple and green PF by GC-MS, with 29 components simultaneously found in both of them. Tabanca et al. [26] identified 27 volatile compounds in purple and green PF by GC-MS, with only 8 compounds present simultaneously in both of them. Fan et al. [27] reported that a total of 57 nonvolatile chemical components and 105 volatile chemical components were characterized in leaves, stems and seeds of different varieties of perilla by ultrahigh-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS/MS) and GC-MS. Furthermore, 27 nonvolatile constituents and 16 volatile constituents were identified as potential markers for discriminating perilla between different varieties. Deguchi et al. [28], using high-performance liquid chromatography (HPLC), reported that the main phenolic compound rosmarinic acid content was higher in green PF compared with purple PF. Zheng et al. [29] investigated the difference in the chemical compositions between green PF and purple PF by rapid resolution liquid chromatography coupled with quadruple time-of-flight mass spectrometry (RRLC-Q/TOF-MS), and revealed that flavonoids and anthocyanins in particular had higher contents in purple PF. Additionally, their results showed that the purple PF had more pronounced antioxidative activities than the green PF.

In the present study, purple PF and green PF were compared and distinguished from the aspect of chemical composition by the GC-MS-based metabolomic approach. In addition, multivariate statistical methods, including principal component analysis (PCA), orthogonal partial least-squares discrimination analysis (OPLS-DA) and clustering analysis (CA) were used to screen the chemical markers between purple and green PF.

2. Results and Discussion

2.1. Compounds Identification

In this study, the chemical profiling of n-hexane extract in 12 batches of purple PF and 10 batches of green PF (sample information see in Table 1) was achieved by GC-MS. The representative total ion chromatogram (TIC) of the two varietal forms of PF is shown in Figure 1. With reference to the NIST17 database, 54 compounds were identified by comparing their mass spectra. Most of the identified compounds belong to monoterpenes and sesquiterpenes. The retention time, retention index, molecular weight and molecular formula of the identified compounds are summarized in Table 2.

Table 1. The information of collected purple Perillae Folium (Z1–Z12) and green Perillae Folium (B1–B10).

No.	Source	Specimen No.	No.	Source	Specimen No.
Z1	Hebei Province	PF201908Z01	Z12	Imported from Japan	PF201908Z12
Z2	Hebei Province	PF201908Z02	B1	Gansu Province	PF201908B01
Z3	Hebei Province	PF201908Z03	B2	Gansu Province	PF201908B02
Z4	Guizhou Province	PF201908Z04	B3	Gansu Province	PF201908B03
Z5	Hebei Province	PF201908Z05	B4	Hebei Province	PF201908B04
Z6	Hebei Province	PF201908Z06	B5	Gansu Province	PF201908B05
Z7	Hebei Province	PF201908Z07	B6	Hebei Province	PF201908B06
Z8	Hebei Province	PF201908Z08	B7	Gansu Province	PF201908B07
Z9	Sichuan Province	PF201908Z09	B8	Gansu Province	PF201908B08
Z10	Shanxi Province	PF201908Z10	B9	Gansu Province	PF201908B09
Z11	Gansu Province	PF201908Z11	B10	Liaoning Province	PF201908B10

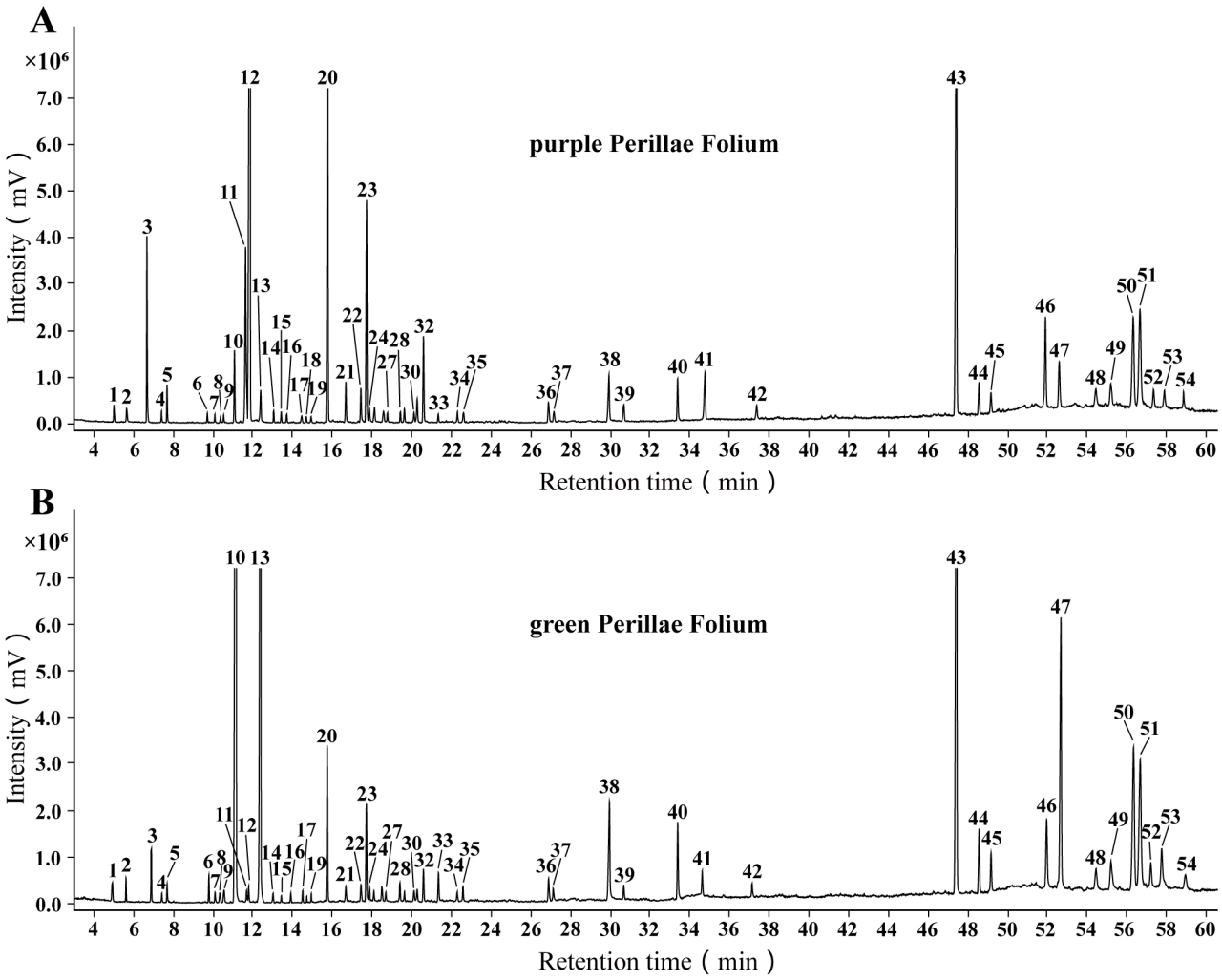

Figure 1. The typical total ion chromatograms of n-hexane extracts of (**A**) purple Perillae Folium and (**B**) green Perillae Folium by GC-MS. The number of peaks was consistent with those of compounds in Table 2.

Table 2. The information of the compounds in purple PF and green PF by GC-MS.

Peak No.	Retention Time (min)	Compounds	Molecular Weight	Molecular Formula	Retention Index	VIP	*p*-Value
1	5.01	α-Pinene	136	$C_{10}H_{16}$	918	0.11	***
2	5.66	Pseudolimonene	136	$C_{10}H_{16}$	964	0.12	***
3	6.45	D-limonene	136	$C_{10}H_{16}$	1018	1.02	***
4	7.52	α-Terpinene	136	$C_{10}H_{16}$	1083	0.09	***
5	7.69	Linalool	154	$C_{10}H_{18}O$	1093	0.13	-
6	9.71	α-Terpineol	154	$C_{10}H_{18}O$	1193	0.24	***
7	10.01	Perilla alcohol	152	$C_{10}H_{16}O$	1207	0.06	***
8	10.1	Egomaketone	166	$C_{10}H_{14}O_2$	1210	0.34	***
9	10.54	Nerol	154	$C_{10}H_{18}O$	1229	0.14	*
10	11.21	Perilla ketone	166	$C_{10}H_{14}O_2$	1257	5.78	***
11	11.71	Shisool	154	$C_{10}H_{18}O$	1277	1.06	***
12	11.87	Perillaldehyde	150	$C_{10}H_{14}O$	1284	2.56	***
13	12.45	Isoegomaketone	164	$C_{10}H_{12}O_2$	1307	2.03	***
14	13.28	Methyl perillate	180	$C_{11}H_{16}O_2$	1339	0.07	***
15	13.43	γ-Elemene	204	$C_{15}H_{24}$	1344	0.20	***

Table 2. *Cont.*

Peak No.	Retention Time (min)	Compounds	Molecular Weight	Molecular Formula	Retention Index	VIP	*p*-Value
16	13.94	Eugenol	164	$C_{10}H_{12}O_2$	1363	0.20	***
17	14.51	α-Copaene	204	$C_{15}H_{24}$	1385	0.06	-
18	14.77	β-Bourbonene	204	$C_{15}H_{24}$	1395	0.18	***
19	14.94	β-Elemene	204	$C_{15}H_{24}$	1401	0.05	*
20	15.77	β-Caryophyllene	204	$C_{15}H_{24}$	1431	0.45	***
21	16.66	Perillic acid	166	$C_{10}H_{14}O_2$	1464	0.22	***
22	17.44	β-Copaene	204	$C_{15}H_{24}$	1492	0.19	-
23	17.74	Cis-α-Bergamotene	204	$C_{15}H_{24}$	1503	0.63	-
24	17.88	Bicyclogermacrene	204	$C_{15}H_{24}$	1508	0.28	**
25	18.09	α-Farnesene	204	$C_{15}H_{24}$	1516	0.17	***
26	18.51	Myristicin	192	$C_{11}H_{12}O_3$	1531	0.03	*
27	18.59	δ-Cadinene	204	$C_{15}H_{24}$	1532	0.05	*
28	19.43	Elemicin	208	$C_{12}H_{16}O_3$	1565	0.05	-
29	19.64	Nerolidol	222	$C_{15}H_{26}O$	1572	0.15	-
30	20.12	Espatulenol	220	$C_{15}H_{24}O$	1590	0.10	***
31	20.27	β-Caryophyllene oxide	220	$C_{15}H_{24}O$	1595	0.11	-
32	20.59	α-Patchoulene	204	$C_{15}H_{24}$	1607	0.36	***
33	21.35	Apiol	222	$C_{12}H_{14}O_4$	1636	0.07	-
34	22.16	Isoelemicin	208	$C_{12}H_{16}O_3$	1666	0.03	-
35	22.62	Isoaromadendrene epoxide	220	$C_{15}H_{24}O$	1683	0.03	**
36	26.89	Phytyl acetate	338	$C_{22}H_{42}O_2$	1849	0.29	**
37	27.04	Pentadecanone	268	$C_{18}H_{36}O$	1855	0.05	***
38	29.91	Palmitic acid	256	$C_{16}H_{32}O_2$	1973	0.38	***
39	30.67	Ethyl palmitate	284	$C_{18}H_{36}O_2$	2005	0.03	*
40	33.39	Phytol	296	$C_{20}H_{40}O$	2119	0.24	*
41	34.89	α-Linolenic acid	278	$C_{18}H_{30}O_2$	2181	0.06	-
42	37.32	Glycidyl palmitate	312	$C_{19}H_{36}O_3$	2283	0.08	***
43	47.41	Squalene	410	$C_{30}H_{50}$	2705	1.60	***
44	48.56	Nonacosane	408	$C_{29}H_{60}$	2754	0.40	*
45	49.16	1-Heptatriacotanol	537	$C_{37}H_{76}O$	2779	0.38	***
46	51.91	Hentriacontane	436	C31H64	2894	0.98	***
47	52.61	Tocopheryl	430	$C_{29}H_{50}O_2$	2923	1.01	***
48	54.44	Campesterol	400	$C_{28}H_{48}O$	3000	0.20	***
49	55.2	β-Stigmasterol	412	$C_{29}H_{48}O$	3031	0.12	*
50	56.33	Dotriacontane	450	$C_{32}H_{66}$	3079	0.87	***
51	56.68	γ-Sitosterol	414	$C_{29}H_{50}O$	3093	0.39	***
52	57.42	β-Amyrin	426	$C_{30}H_{50}O$	3124	0.17	-
53	57.98	β-Amyrone	424	$C_{30}H_{48}O$	3148	0.04	-
54	58.7	α-Amyrin	426	$C_{30}H_{50}O$	3178	0.27	-

"-" represent no significant difference. VIP, variable importance in projection. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2.2. Chemical Comparison of Purple and Green PF

In this work, all 54 detected compounds were found in both purple and green PF, with their contents varying. To further specify the difference of the n-hexane extract profiles of purple and green PF, multivariate statistical methods, including PCA, OPLS-DA and CA, were used to analyze the data.

PCA is an unsupervised pattern recognition method to visualize grouping trends and outliers. PCA was performed with 54 compounds used as independent variables. As shown in the PCA scores plot (Figure 2A), all samples were clearly separated into two groups corresponding to purple PF and green PF. The first two components explained 68.5% of the total variance. PCA results indicated that the purple PF and green PF samples were indeed different in terms of the content of identified compounds.

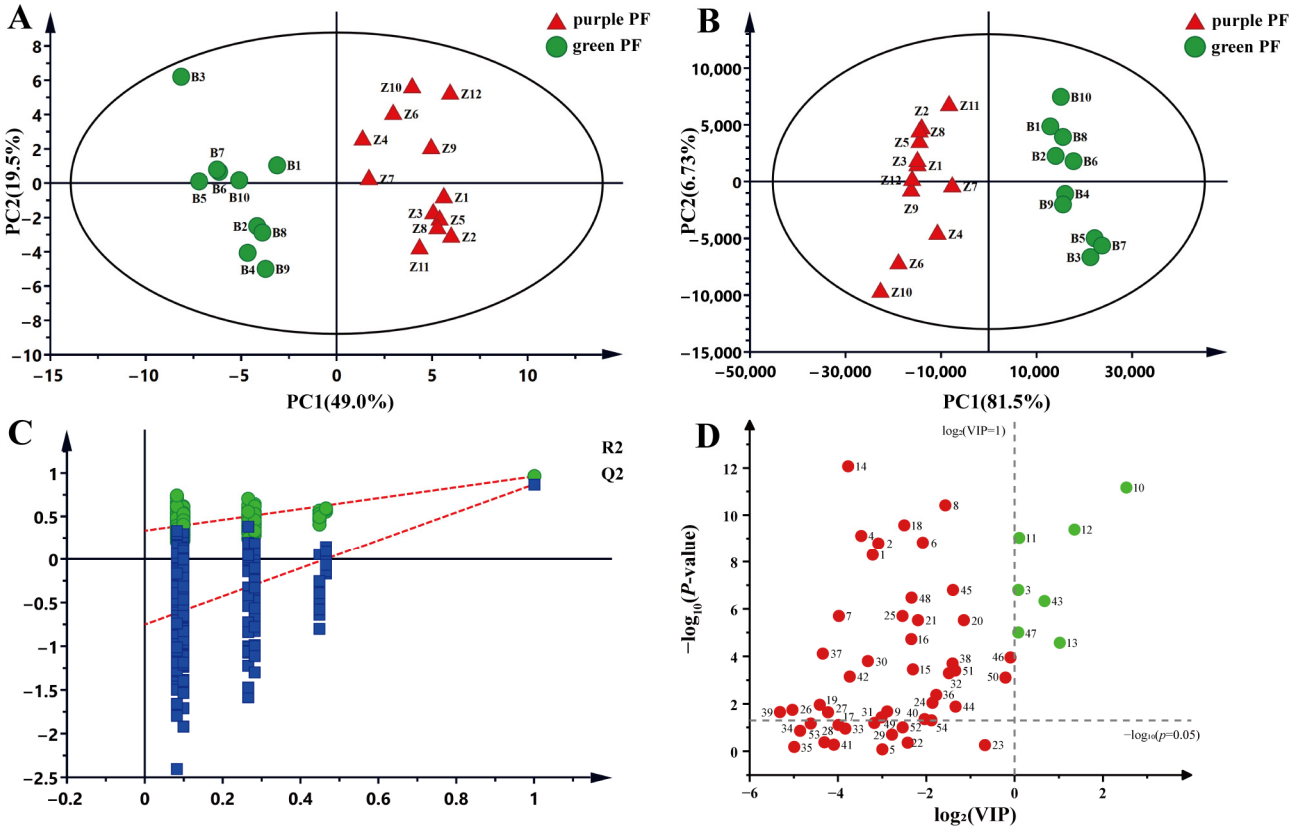

Figure 2. Determination of differential compounds from two PF varieties. (**A**) Unsupervised PCA score plot of purple and green PF samples. PC1 occupies 49.0% and PC2 19.5% of total variance. (**B**) Supervised OPLS-DA score plot of purple and green PF samples. PC1 occupies 81.5% and PC2 6.73% of total variance. (**C**) Permutation test at 200 times used for the discrimination between the two PF varieties. (**D**) Scatter plot of *p*-value and VIP value. The green points show differential compounds with VIP > 1, $p < 0.05$.

OPLS-DA is a supervised pattern recognition method that can be used to analyze, classify and reduce the dimensionality of complex datasets. To filter out the differential components of the two varietal forms of PF, the GC-MS data were analyzed by OPLS-DA. The OPLS-DA scores plot (Figure 2B) shows that purple PF and green PF can also be clearly classified into two groups. Further to validate the model of OPLS-DA, a permutation test ($n = 200$) was conducted. The results of R2Y (cum) = 0.962 and Q2 (cum) = 0.870 (Figure 2C), indicated good classification and predictability of the OPLS-DA model. By using the metabolite features with VIP > 1 and $p < 0.05$, 7 compounds, including D-limonene (3), perilla ketone (10), shisool (11), perillaldehyde (12), isoegomaketone (13), squalene (43) and tocopheryl (47), were screened out as potential chemical markers for distinguishing purple PF and green PF (Figure 2D). The relative peak areas (%) of potential chemical markers in purple and green PF were calculated (Table 3). The results indicated that perilla ketone (10) was the most abundant compound in green PF, with relative peak areas of 27.50 ± 3.01%, while perillaldehyde (12) was the most abundant compound in purple PF, with relative peak areas of 31.72 ± 3.12%.

CA is a multivariate statistical method to classify samples or indicators, and a heatmap was used to show the relative concentration trends of compounds across all samples. In order to visualize the differences in metabolic profiles between the two varieties of PF, the peak areas of 54 compounds were used to construct a heatmap. The heatmap (Figure 3) showed that the two PF varieties could be clearly distinguished on the basis of the clustering relationships of the identified compounds, consistent with the results of PCA and OPLS-DA. Among the 54 compounds, the content of perillaldehyde (12), shisool (11), D-limonene (3),

perillic acid (21), α-terpineol (6), perilla alcohol (7), terpinene (4) and α-pinene (1) in purple PF was significantly higher than that of green PF, while isoegomaketone (13), squalene (43), dotriacontane (50), tocopheryl (47), hentriacontane (46), perilla ketone (10) and perilla ketone (8) had higher content in green PF. Specifically, the main identified components in purple and green PF were perillaldehyde (12) and perilla ketone (10), respectively. According to the classification principles of volatile oil chemotypes of PF in previous studies [30,31], all purple PF samples of volatile oil chemotypes were PA (perillaldehyde), and all green PF samples were PK (perilla ketone).

Table 3. The relative peak areas (%) of the potential chemical markers in purple PF and green PF.

No.	Retention Time (min)	Retention Index	Compounds	Purple PF ($\bar{X} \pm$ SD, n = 12, %)	Green PF ($\bar{X} \pm$ SD, n = 10, %)
3	6.45	1018	D-limonene	5.12 ± 1.23	0.20 ± 0.04
10	11.21	1257	Perilla ketone	2.15 ± 0.97	27.50 ± 3.01
11	11.71	1277	Shisool	5.41 ± 0.86	0.05 ± 0.02
12	11.87	1284	Perillaldehyde	31.72 ± 3.12	0.60 ± 0.21
13	12.45	1307	Isoegomaketone	0.13 ± 0.07	5.71 ± 0.80
43	47.41	2705	Squalene	4.44 ± 0.88	7.32 ± 0.76
47	52.61	2923	Tocopheryl	4.81 ± 0.67	7.00 ± 0.68

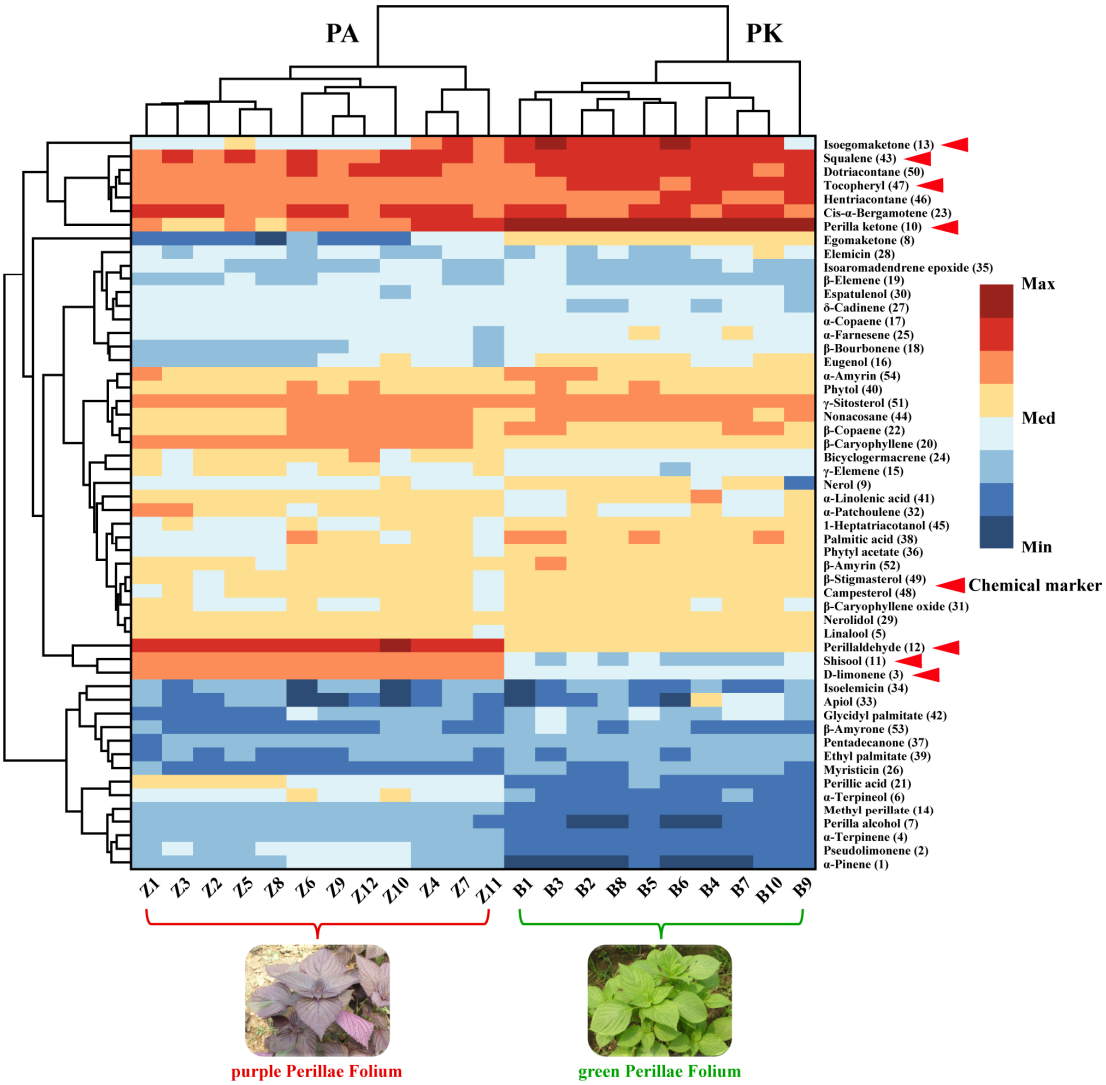

Figure 3. The relative concentration trends of identified compounds in purple PF and green PF.

Considering the biosynthetic information of potential chemical markers, perilla alcohol (7), shisool (11) and perillaldehyde (12) that metabolized from limonene (3) all had higher contents in purple PF samples, whereas egomaketone (8), perilla ketone (10) and isoegomaketone (13) that derived from geranial together with squalene (43) and tocopheryl (16) had higher contents in green PF (Figure 4).

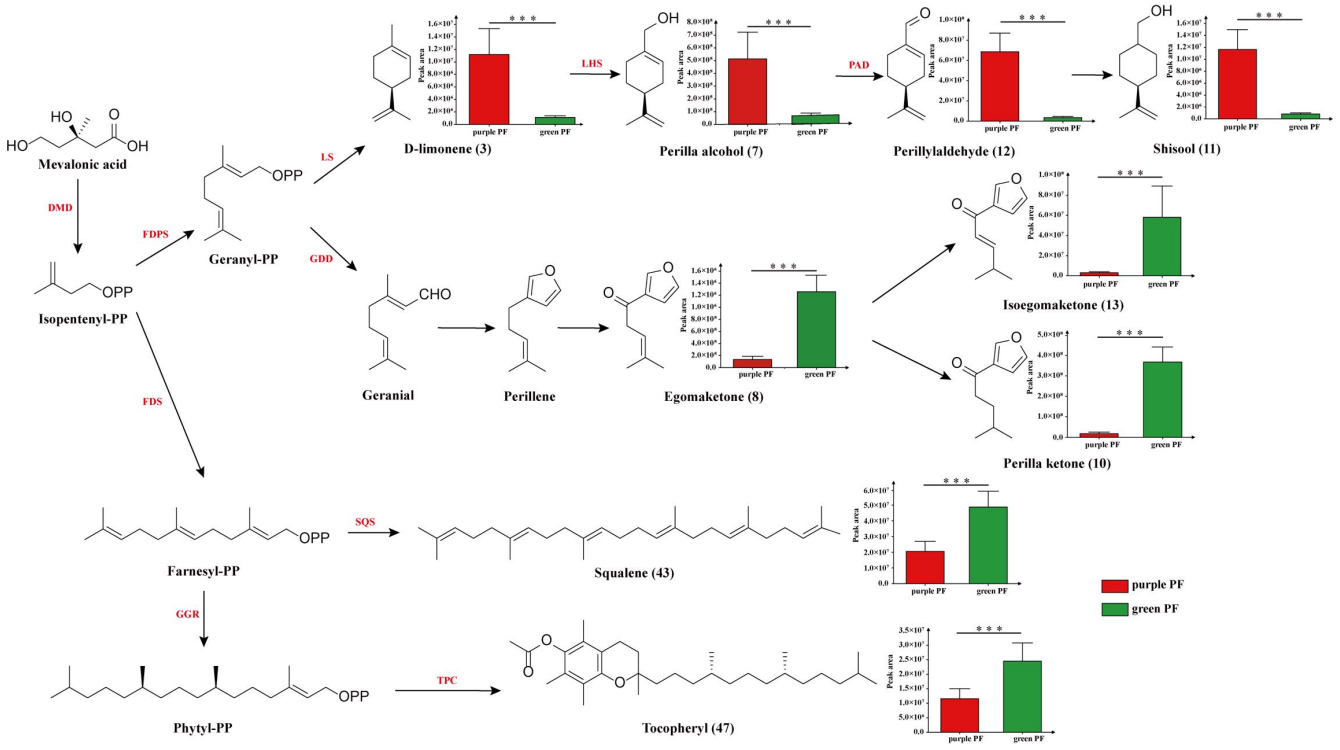

Figure 4. Putative biosynthetic pathways of the main terpenoids in perilla. Metabolites are written in black letters, whereas enzymes are written in red letters. DMD, diphosphomevalonate decarboxylase; FDPS, farnesyl diphosphate synthase; LS, limonene synthase; LHS, limonene hydroxylase; PAD, perillylalcohol dehydrogenase; GDD, geranyl diphosphate diphosphohydrolase; FDS, farnesyl diphosphate synthase; SQS, squalene synthase; GGR, geranylgeranyl reductase; TPC, tocopherol C-methyltransferase. *** $p < 0.001$.

Generally, the potential mechanisms of differences in chemical composition are related with genes encoding biosynthetic enzymes and regulatory proteins [32]. Zheng et al. [29] reported that the conserved gene sequences of ITS2 (internal transcribed spacer 2) are consistent in green and purple PF, which suggests that it is reasonable to classify them as the same species of *P. frutescens* (L.) Britt from the perspective of plant taxonomy. Therefore, the obvious differences in the chemical composition between the two varieties of PF may relate with nonconserved gene regions and downstream regulatory proteins. Previous research had found quite different levels of the PFLC1 gene encoding limonene synthase in different perilla chemotypes [33]. The content difference of identified terpenoids in purple and green PF might be related with the expression of key genes encoding limonene synthase (LS), geranyl diphosphate diphosphohydrolase (GDD) and farnesyl diphosphate synthase (FDS).

3. Materials and Methods

3.1. Plant Material

A total of 12 batches of purple PF (*P. frutescens* var. *arguta*) and 10 batches of green PF (*P. frutescens* var. *frutescens*), were collected from Hebei Academy of Agriculture and Forestry Sciences in Shijiazhuang (China 38°06′41.7″ N, 114°45′35.8″ E) on 30 August 2019 and identified by Yuguang Zheng, professor in the field of identification of Chinese

Medicine. The origins of the 22 samples are listed in Table 1. The harvested leaves were air-dried in the dark at room temperature for 2 weeks to acquire consistently low water content. All voucher specimens were deposited in dry, dark room of Traditional Chinese Medicine Processing Technology Innovation Center of Hebei Province, Hebei University of Chinese Medicine with their specimen number (see Table 1).

3.2. Metabolite Extraction

Plant materials of each batch were pulverized and screened through 60-mesh sieves. The powdered sample was extracted according to an ultrasonic extraction protocol [34] with some modification. A total of 0.1 g of the powdered sample was extracted with 1 mL of n-hexane by means of sonication (power, 300 W; frequency, 40 kHz) for 15 min at room temperature. The extract was then centrifuged at 13,000 rpm for 10 min at room temperature. A total of 1μL of supernatant was injected into the GC-MS for analysis.

3.3. GC-MS Analysis

The GC-MS analysis was performed with an Agilent 7890B GC coupled with 5977B MSD mass detector (Agilent Technologies, Santa Clara, CA, USA). The GC-MS instrument coupled with an Agilent HP-5MS 5% phenyl methyl siloxane capillary column (30 m × 0.25 mm, 0.25 μm film thickness, Agilent, Santa Clara, CA, USA). Helium (≥99.999%) was used as carrier gas at a constant flow rate of 1.0 mL·min^{-1}. A total of 1 μL of the prepared supernatant solution was injected in split mode with the split ratio set to 2:1 at a temperature of 250 °C. The oven temperature program was initially set at 45 °C, then raised to 100 °C at a rate of 10 °C·min^{-1} and subsequently raised to 280 °C at a rate of 4 °C·min^{-1}, then finally held for 10 min. The quadrupole mass detector was operated in electron impact (EI) mode at 70 eVwith a mass range of 50–500 m/z. A total of 22 batches of samples were randomly analyzed with three replicates to ensure system stability throughout the analysis. n-Alkane standard solution (C_8–C_{20}, 40 mg·L^{-1}, Sigma-Aldrich, Buchs, Switzerland) was analyzed under the same condition for retention index (RI) calculation.

3.4. Data Processing and Statistical Analysis

The identification of metabolites in purple and green PF were achieved by comparing the obtained mass spectra with reference mass spectra from the National Institute of Standards and Technology 17 (NIST17) library. The peaks in all the samples were aligned and matched by using Agilent MassHunter analysis program (Agilent, Santa Clara, CA, USA). The RI of all the identified compounds was calculated by comparing their corresponding peak retention time to that of n-alkanes (C8–C20) [35,36]. Finally, the resulting data matrix consisting of sample codes, variables and peak areas was extracted and used for statistical analysis.

The obtained data matrix was imported into SIMCA P13 software (Umetrics, Umea, Sweden) for principal component analysis (PCA) and orthogonal partial least-squares discrimination analysis (OPLS-DA). Cluster analysis (CA) was performed with Origin Pro 2020 (OriginLab Corporation, Northampton, MA, USA) software. *p*-value was calculated by independent-samples *t*-test with IBM SPSS Statistics 23.0 (IBM, Armonk, NY, USA) software.

4. Conclusions

In this study, a GC-MS-based metabolomics method for rapid discrimination of differential metabolites between purple and green PF was established. The chemical compositions of n-hexane extracts of purple and green PF were investigated and a total of 54 compounds were identified by comparison of their mass spectra with NIST17 library. Among them, 7 differential compounds between the two varieties of PF were screened and characterized using multivariate statistical methods and heatmap visualization analysis. The results indicated that purple PF and green PF samples could be distinguished from each other according to the relative content of these marker compounds. This study may offer data support for research and exploitation of purple and green PF, and provide a feasible method for the authentication of purple and green PF.

Author Contributions: J.C.: methodology, software, validation, formal analysis, writing—original draft preparation, writing—review and editing; D.Z.: investigation, supervision, writing—review and editing; Q.W.: writing—review and editing; A.Y.: validation, formal analysis; Y.Z.: conceptualization, writing—review and editing, funding acquisition; L.W.: conceptualization, supervision, writing—review and editing, funding acquisition; project administration. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds are available from the authors.

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