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Effect of Fermentation Duration on the Chemical Compounds of *Coffea arabica* from Ultra Performance Liquid Chromatography–Triple Quadrupole Mass Spectrometry and Gas Chromatography–Mass Spectrometry Analysis During the Washed Processing

Xiaojing Shen [1], Qi Wang [2], Tingting Zheng [3], Biao Yuan [1], Zhiheng Yin [1], Kunyi Liu [2,*] and Wenjuan Yuan [1,*]

[1] College of Science, Yunnan Agricultural University, Kunming 650201, China; 2013017@ynau.edu.cn (X.S.)
[2] School of Wuliangye Technology and Food Engineering, Yibin Vocational and Technical College, Yibin 644100, China; mumu202107@163.com
[3] Pu'er Institute of Pu-erh Tea, Pu'er 665000, China
* Correspondence: ben.91@163.com (K.L.); 2018020@ynau.edu.cn (W.Y.)

Abstract: The washed process is one of the traditional post-harvest processes of coffee beans, which include selective harvesting, flotation, pulping, submerged fermentation underwater, washing, and drying operations. During the washed processing, fermentation underwater can remove coffee mucilage and change metabolites by microorganisms. Therefore, coffee fermentation is a key factor influencing coffee's flavor. To compare the influence of fermentation duration in an open environment of *Coffea arabica* in 48 h during the washed processing on the coffee's flavor, the sensory characteristics of the coffee at different fermentation durations were evaluated using the Specialty Coffee Association of America (SCAA) cupping protocol. Moreover, ultra performance liquid chromatography–triple quadrupole mass spectrometry (UHPLC–MS/MS) and gas chromatography–mass spectrometry (GC–MS) were combined to analyze and compare the chemical compounds of coffee samples from fermentation durations of 24 h (W24) and 36 h (W36) during the washed processing method. The results showed that W36 had the highest total cupping score with 77.25 in all different fermentation duration coffee samples, and 2567 non-volatile compounds (nVCs) and 176 volatile compounds (VCs) were detected in W36 and W24 during the washed processing method. Furthermore, 43 differentially changed non-volatile compounds (DCnVCs) and 22 differentially changed volatile compounds (DCVCs) were detected in W36 vs. W24. Therefore, suitable fermentation duration in an open environment is beneficial to coffee flavor, judging by chemical compound changes. For the washed primary processing of *C. arabica* from Yunnan, China, 36 h fermentation was the suitable fermentation duration in an open environment, which presented potential value as the reference for washed coffee processing in the food industry.

Keywords: *Coffea arabica*; washed processing method; fermentation duration; coffee flavor

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1. Introduction

Coffee, tea, and cocoa are the three top beverages in the world. Consumers are very interested in the special coffee flavor. However, a cup of a high-quality coffee beverage is comprehensively affected by many factors, such as genetic attributes, growing conditions, harvesting, post-harvest coffee processing, storage, roasting, and the brewing steps of coffee beverages [1–4]. Coffee beans are surrounded by skin, pulp, mucilage, parchment, and silver skin. Therefore, post-harvesting primary processing (wet, dry, or semi-dry processing) is the first step of coffee processing used to obtain green coffee beans [5] which can significantly influence coffee's flavor [6,7]. Microbial communities and chemical compounds showed significant differences compared to primary processing [8,9]. For

example, levels of fructose, glucose, arabinose, and other free low-molecular-weight sugars in green coffee beans undergoing wet processing were lower than in those undergoing dry processing [10]. However, levels of glutamic acid and aspartic acid in green coffee beans undergoing wet processing were higher than in those undergoing dry processing [11]. Moreover, microbial species were different compared across different primary processes. For bacteria, *Leuconostoc*, *Lactiplantibacillus*, *Klebsiella*, and *Weissela* were the main bacteria in wet processing, while *Enterobacter*, *Bacillus*, *Tatumella*, and *Pseudomonas* were the main bacteria in dry processing [12].

Coffea arabica and *Coffea robusta* are two popular marked coffee species worldwide [13]. Dry processing is often used for *C. robusta*, while wet processing is often used for *C. arabica* [5]. Washed or wet processing, a traditional post-harvest coffee processing technique, includes de-pulping, fermentation, washing, drying, and other main operations [2]. Fermentation is a critical operation, as it can remove the pulp and mucilage by enzymes from the coffee fruit and microflora from the environment, and since mucilage is composed of protein, this reduces sugar, pectates, and ash [14,15]. However, traditional coffee fermentation is carried out in a developed environment; microbial communities often are influenced by environmental factors, such as the coffee region, temperature, altitude, pH, and so on. Therefore, the control of fermentation conditions is a prerequisite for improving coffee quality, such as suitable fermentation duration, processing type, application of soaking, etc. [2,16]. For example, a long fermentation duration would produce positive fruity and acid notes along with negative cereal and floral notes in the coffee flavor [2]. Fermentation leads to the change in volatile compounds related to coffee flavor [15]. Previous studies have showed that 143 non-volatile compounds were differentially changed in a 36 h fermentation compared to non-fermentation [6].

To provide a reference for further understanding the influence of fermentation on coffee's flavor during the washed processing method, the Specialty Coffee Association of America (SCAA) cupping protocol was used to evaluate coffee flavor and characteristics by ten attributes (fragrance/aroma, flavor, aftertaste, acidity, body, balance, overall impression, uniformity, sweetness, and clean cup). Furthermore, ultra performance liquid chromatography–triple quadrupole mass spectrometry (UHPLC–MS/MS) and gas chromatography–mass spectrometry (GC–MS) were combined to analyze and compare the differences of non-volatile compounds and volatile compounds at different fermentation durations of coffee beans in this paper. Then, the different changed compounds after 24 h vs. 36 h durations in *C. arabica* from Yunnan province were analyzed.

2. Materials and Methods

2.1. Materials and Chemical Standards

The raw materials were mature coffee cherries from *C. arabica*, which were harvested and collected from Pu-er City, Yunnan Province, China. According to the washed processing method, the mature coffee cherries were processed, followed by sorting in water, de-pulping of the exocarp, and fermentation under water. A total of 1 kg of de-pulping coffee was spontaneously fermented under 1 L of sterile water (the pH was 6.8, the content of dissolved oxygen was 0.17 mg/L) at room temperature ranging from 18 to 25 °C in an open environment. During fermentation under the water, four coffee samples from different fermentation durations were collected at fermentation times of 12 h, 24 h, 36 h, and 48 h, respectively. An unfermented control (UC) sample was taken at the start of the fermentation. Then, these five coffee samples were washed and dried under the sun to obtain green coffee. Finally, these green coffee beans were roasted using a IKWA Pro V3 coffee bean roaster (IKAWA Ltd., London, UK) under medium roasting conditions (10–15 min roasting time, 210–220 °C roasting temperature) to obtain roasted coffee beans by certified professionals from Anke Coffee Limited Company (Kunming, China). The roasted coffee samples were stored at −20 °C for the subsequent analysis. To clearly distinguish the different coffee samples, they were marked as UC, W12 (fermentation duration of 12 h), W24 (fermentation duration of 24 h), W36 (fermentation duration of 36 h), and W48 (fermentation duration

of 48 h), respectively. The chemical reagents including methyl alcohol, acetonitrile, and propyl alcohol were high-performance liquid-chromatography-grade and purchased from Fisher Co., Ltd. (Shanghai, China).

2.2. Cupping Analysis

The cupping analysis of five coffee samples was conducted based on the Specialty Coffee Association of America (SCAA) cupping protocol. Ten attributes (fragrance, flavor, aftertaste, acidity, body, balance, uniformity, sweetness, cleanliness, and score) were categorized and scored based on quality by nine certified professionals (five males and four females, aged 18 to 35 years) with expertise in cupping analysis from Anke Coffee Limited Company (Kunming, China) [6]. Each coffee sample was independently given a triplicate score and its coffee characteristics described by a professional under blind conditions. In brief, uniformity, sweetness, and clean cup were used to reflect the absence of defects with 10 points. Other attributes were scored based on their quality on a scale of 6 to 10 points at intervals of 0.25 points. Simultaneously, the characteristics of fragrance, aroma, aftertaste, and body were assessed in the detailed description.

2.3. Analysis of Non-Volatile Compounds by UHPLC–MS/MS

According to the result of the cupping analysis, non-volatile compounds (nVCs) from W24 and W36 were analyzed using UHPLC–MS/MS. In order to extract non-volatile compounds to 50 mg of roasted coffee powder sample, 0.4 mL of 80% methanol was added. Then the mixture was centrifugated at 13,000× *g* for 15 min at 4 °C to obtain the supernatant. A total of 2 μL of supernatant was injected into a UHPLC-Q Exactive system (Thermo Fisher Scientific, Bremen, Germany) with HSS T3 C18 column (2.1 × 100 mm, 1.8 μm; Waters Corporation, Milford, MA, USA) at a 0.4 mL/min flow rate [17,18]. A mixture of (A) 0.1% formic acid in water: acetonitrile (95: 5, *v*/*v*) and (B) 0.1% formic acid in acetonitrile: isopropanol: water (47.5: 47.5: 5, *v*/*v*) was the mobile phase under the gradient elution: 0–5% B for 0–0.1 min, 5–25% B for 0.1–2 min, 25–100% B for 2–9 min, and 100% B for 9–13 min, and 100–0% B for 13–13.1 min, then 0% B for 13.1–16 min. Mass spectra were carried out in an electrospray ionization (ESI) source at –2800 V in negative mode and 3500 V in positive mode and scanned in the range of 70–1050 *m/z.* Data filtering, peak detection, alignment, and calculations were performed using Analyst 1.6.3 software (AB Sciex, Framingham, MA, USA). To facilitate the identification/annotation of metabolites, accurate *m/z* ratios were obtained for each precursor ion. Total ion chromatograms and extracted ion chromatograms of QC samples were exported to give an overview of the metabolite profiles of all samples. Metabolites were characterized by searching internal and public databases (MassBank, KNApSAcK, HMDB, MoTo DB, and METLIN) and comparing their *m/z* values, retention times, and fragmentation patterns with those of the standards and comparing their *m/z* values, retention times and fragmentation patterns with those of the standards. The chromatographic peak area of each was calculated. Positive and negative data were combined to obtain a combined data set.

2.4. Analysis of Volatile Compounds by GC–MS

According to the result of the cupping analysis, volatile compounds (VCs) of W24 and W36 were evaluated using GC–MS. Firstly, the extract volatile compounds to 50 mg of roasted coffee powder sample, 0.5 mL of 80% methanol was added. The mixture was ground at –20 °C and added 0.2 mL trichloromethane to ultrasonic extraction for 30 min. Then the extract was quiescence at –20 °C and centrifugated at 13,000× *g* for 15 min at 4 °C to obtain the supernatant. 80 μL 15 mg/mL methamphetamine hydrochloride pyridine solution was added to the supernation for reaction for 90 min at 37 °C. 80 μL Bis (trimethylsilyl) trifluoroacetamide (1% chlorotrimethylsilane) was added and reacted for 60 min at 70 °C following a shocking for 2 min. The GC–MS analysis was carried out referencing Shen et al. [6], using a model 8890B GC instrument with a DB–5MS (40 m × 0.25 mm × 0.25 μm) capillary column and 5977B mass spectrometer (Agilent, Santa Clara, CA, USA). In brief, the carrier

gas was 99.999% helium at a 1.0 mL/min column flow. The column temperature program was set to 60 °C held for 0.5 min and then raised to 310 °C at an 8 °C/min rate. Mass spectra were recorded in electron impact (EI) ionization mode at 70 eV and scanned in the range *m/z* 50–500. Chroma TOF 4.3X software (LECO Corporation, St. Joseph, MI, USA) and the LECO-Fiehn Rtx5 database were utilized for raw peak extraction, baseline filtering and calibration, peak alignment, deconvolution analysis, peak identification, and peak area integration. Metabolite identification considered both mass spectrum and retention index matches. Peaks detected in fewer than 50% of QC samples or with an relative standard deviation greater than 30% in QC samples were removed. Following normalization of the original peak area data to the total peak area, further analysis was conducted.

2.5. Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics v28.0.1.1 (SPSS Inc., Chicago, IL, USA). The data were expressed as mean ± standard deviation. LSD analysis of variance was used for statistical analysis, and $p < 0.05$ was considered significant. The analysis method for differentially changed compounds was as follows. Firstly, the response intensity of the sample mass spectrum peaks was normalized using the sum normalization method, and variables with a relative standard deviation (RSD) > 30% of QC samples (equal volumes of all samples) were removed, followed by log10 calculations. Then, variable importance in projection (VIP) analysis ranked the overall contribution of each variable to the orthogonal partial least square discriminant analysis (OPLS-DA) model, and the variables with VIP > 1.0, $p < 0.05$, fold change (FC) > 1.5 or FC < 0.67 were classified as differentially changed non-volatile compounds (DCnVCs), and VIP > 1.0, $p < 0.05$, FC > 1.1 or FC < 0.9 were classified as differentially changed volatile compounds (DCVCs) [19].

3. Results

3.1. The Results of Cupping Analysis and Sensory Characteristics of Different Fermentation Durations

The cupping test is a very important evaluation method for coffee flavor and characteristics. Each coffee sample was evaluated by three certified professionals with expertise in cupping analysis based on the SCAA cupping protocol. If the total score of the cupping analysis is in the range of 70.00–79.00, it is considered premium [20]. *C. arabica* is the main cultivated species of coffee in Yunnan province, China, which is a well-known cultivation base of *C. arabica* in the world [21]. All coffee samples from different fermentation durations were premium with total scores of 75.25 ± 0.14 (UC), 75.75 ± 0.25 (W12), 76.50 ± 0.14 (W24), 77.25 ± 0.25 (W36), and 76.00 ± 0.14 (W48), respectively. The scores of detailed attributes were evaluated as shown in Figure 1. According to the score, the score of fragrance/aroma in different coffee samples was 6.75, which was not influenced by the fermentation duration. The score of 10 for uniformity, sweetness, and clean cup means these coffee samples had an absence of defect. The scores of UC and W12 were significantly lower than others ($p < 0.05$) and had a weak roast nut aroma, short aftertaste, and low tea body. This indicates that a short fermentation duration was not beneficial for the forming of coffee flavor. Body, sweetness, and balance were promoted by lengthening fermentation duration. Meanwhile, the scores of W24 and W36 were significantly higher than other fermentation duration samples ($p < 0.05$), and W36 had the highest sensory score and had obvious roast cereal and nut aromas, orange acid, medium tea body, and low coffee sweet. Roasted nut flavors often relate to pyrazine, such as 2,5-dimethylpyrazine, 2,6-dimethylpyrazine,2,3-diethyl-5-methylpyrazine, 2-ethyl-3,5-dimethylpyrazine, and 67-dihydro-5-methyl-5H-cyclopentapyrazine, while carbohydrates are related to the sweetness of coffee, such as sucrose, glucose, fructose [22].

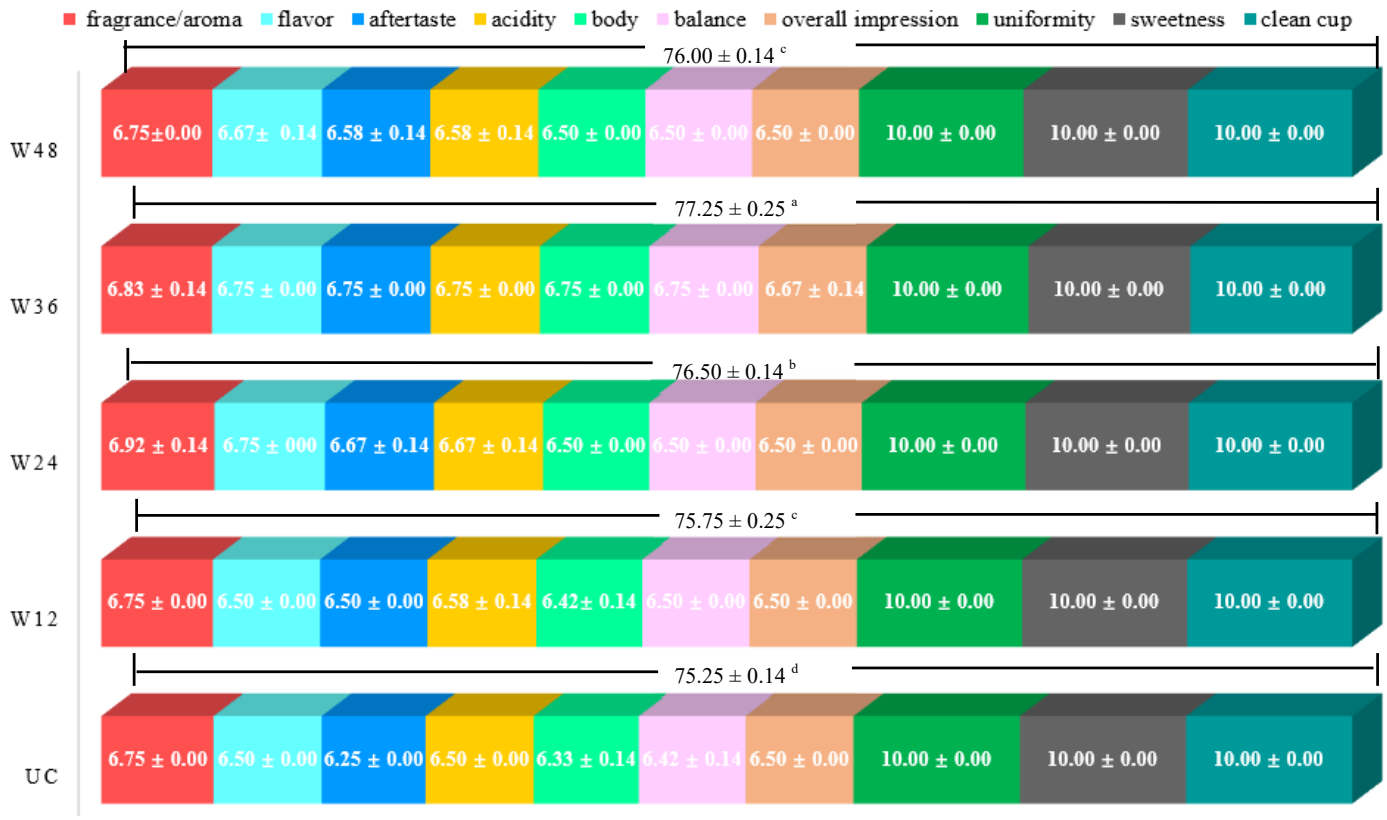

Figure 1. The scores of the coffee cupping test from fermentation duration of *C. arabica* in Yunnan Province. Different lowercase superscripts indicate significant differences between comparisons ($p < 0.05$).

3.2. The Result of Non-Volatile Compound Analysis by UHPLC–MS/MS

Non-volatile compounds in roasted coffee beans are very important to coffee flavor. The non-volatile compounds in roasted coffee beans, such as trigonelline, chlorogenic acids, carboxylic acids, carbohydrates, polymeric polysaccharides, lipids, protein, melanoidins, and minerals, can contribute to the overall aroma, astringency, bitterness, sweetness, and other sensory properties of coffee beverages [22]. Based on the result of cupping test, W36 had the highest score following with W24. W36 showed significant differences from other fermentation duration samples with a value of p low than 0.05. Among them, the value of p between W36 with W24 was 0.023, which was the highest in all groups. A total of 2567 non-volatile compounds (nVCs) were detected in W24 and W36 using UHPLC–MS/MS (Table S1), which were classified as 17 super-classes, shown in Figure 2. They included 502 lipids and lipid-like molecules (comprising 19.56% of the total number of nVCs), 487 organoheterocyclic compounds (18.97%), 461 organic acids and derivatives (17.96%), 353 organic oxygen compounds (13.75%), 301 phenylpropanoids and polyketides (11.73%), 255 benzenoids (9.93%), 72 nucleosides, nucleotides, and analogues (2.80%), 36 alkaloids and derivatives (1.40%), 31 organic nitrogen compounds (1.21%), nine hydrocarbons (0.35%), four lignans, neolignans, and related compounds (0.16%), four hydrocarbon derivatives (0.16%), two homogeneous non-metal compounds (0.08%), two organic 1,3-dipolar compounds (0.08%), one organohalogen compound (0.04%), one acetylide (0.04%), and 46 others (1.79%).

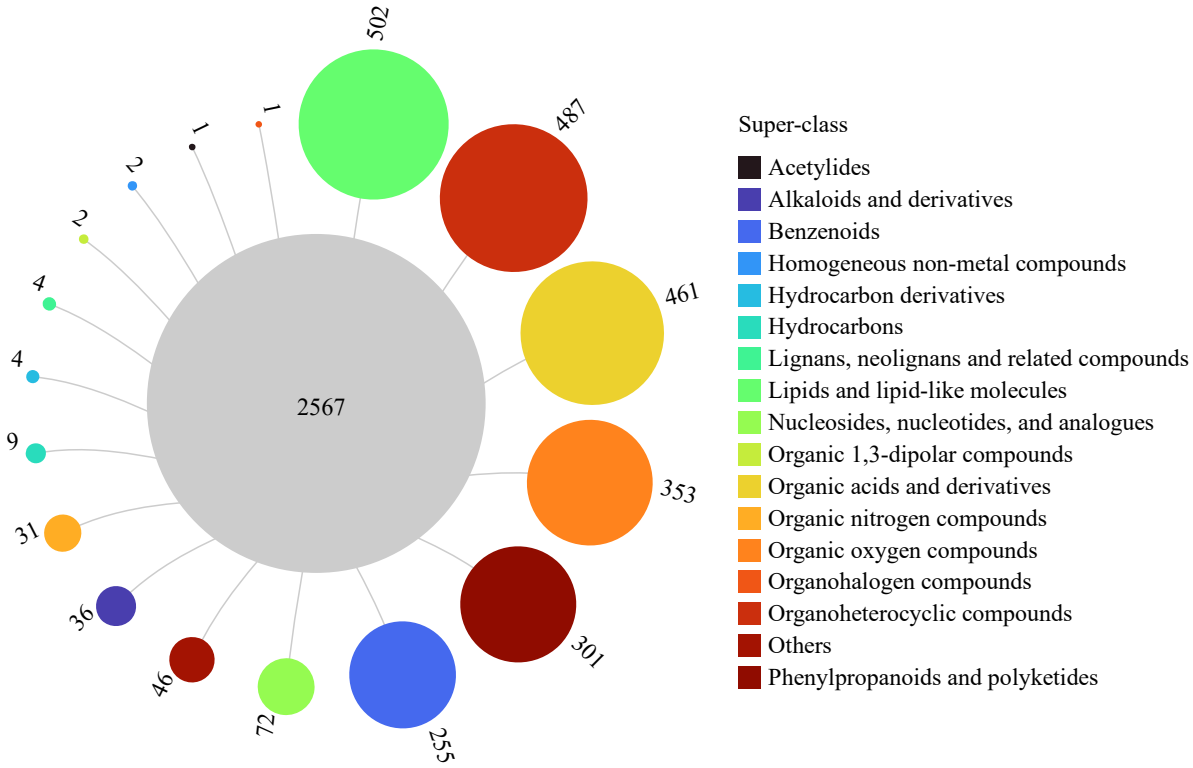

Figure 2. Super-classes of non-volatile compounds (nVCs) from the fermentation duration of *C. arabica* in Yunnan province. The circles represent super-classes of nVC, different colors represent different super-classes, and different sizes represent the different numbers of non-volatile compounds.

These nVCs were further grouped into 162 classes. These classes mainly included 386 carboxylic acids and derivatives (comprising 15.04%), 352 organooxygen compounds (13.71%), 198 fatty acyls (7.71%), 148 prenol lipids (5.77%), 136 benzenes and substituted derivatives (5.30%), 89 flavonoids (3.47%), 79 steroids and steroid derivatives (3.08%), 64 coumarins and derivatives (2.49%), 64 glycerophospholipids (2.49%), 62 phenols (2.42%), 60 indoles and derivatives (2.34%), 52 pyridines and derivatives (2.03%), 51 cinnamic acids and derivatives (1.99%), 38 benzopyrans (1.48%), 31 organonitrogen compounds (1.21%), 28 keto acids and derivatives (1.09%), 28 imidazopyrimidines (1.09%), 26 quinolines and derivatives (1.01%), 23 lactones (0.90%), 23 isoflavonoids (0.90%), 22 purine nucleosides (0.86%), 22 naphthalenes (0.86%), 21 hydroxy acids and derivatives (0.82%), 21 pyrimidine nucleoside (0.82%), 19 phenylpropanoic acids (0.74%), 18 diazines (0.70%), 18 dihydrofurans (0.70%), 17 pyrans (0.66%), 13 peptidomimetics (0.51%), 13 piperidines (0.51%), 12 azoles (0.47%), 12 heteroaromatic compounds (0.47%), 11 pyrrolidines (0.43%), 11 phenol ethers (0.43%), 11 macrolides and analogues (0.43%), 10 pteridines and derivatives (0.39%), and 10 benzodioxoles (0.39%). Quinic acids, mono-caffeoylquinic acids, di-caffeoylquinic acids, tri-caffeoylquinic acids, and feruloylquinic acids, such as caffeoylquinic acid, isoquinoline, 4-*O*-p-coumarylquinic acid, 1-*O*-caffeoylquinic acid, 5-caffeoylquinic acid, 3-*O*-feruloyquinic acid, 4-*O*-caffeoyl-3-feruloylquinic acid, 1,3-dicaffeoylquinic acid, 1,5-dicaffeoylquinic acid, and 3-caffeoyl-4-feruloylquinic acid, were identified as common compounds in coffee at different fermentation durations.

To compare the change in nVCs, the differentially changed non-volatile compounds (DCnVCs) (VIP > 1.0, $p < 0.05$, FC > 1.5 or FC < 0.67) between W36 and W24 were analyzed. The result was shown in Figure 3, in which significantly increased DCnVCs were represented by red circles and significantly decreased DCnVCs were represented by blue circles. A total of 43 DCnVCs were detected in W36 vs. W24. They were related to 16 lipids and lipid-like molecules, six organic oxygen compounds, three organoheterocyclic compounds, three benzenoids, three organic acids and derivatives, two nucleosides, nucleotides, and analogues,

two phenylpropanoids and polyketides, one organic nitrogen compound, one hydrocarbon, and six others. Among them, the regulative levels of 12 DCnVCs were decreased significantly (VIP > 1.0, $p < 0.05$, and FC < 0.67), including lipids and lipid-like molecules (six DCnVCs, PA(18:1(9Z)/18:2(9Z,12Z)), PA(18:1(9Z)/16:0), PA(16:0/18:2(9Z,12Z)), 20-hydroxy-leukotriene E4, deoxynivalenol, and DG(18:1(11Z)/18:3(9Z,12Z,15Z)/0:0)), organic acids and derivatives (two DCnVCs, imazamox and Thr-lue), organic oxygen compounds (one DCnVC, 4-O-beta-D-glucosyl-4-coumaric acid), organoheterocyclic compounds (one DCnVC, citrusinine I), and phenylpropanoids and polyketides (two DCnVCs, dihydromethysticin and 3-demethylsimmondsin 2″-(Z)-ferulate). Among these related decreased DCnVCs, four DCnVCs, PA(18:1(9Z)/18:2(9Z,12Z)), PA(18:1(9Z)/16:0), imazamox, and PA(16:0(9Z)/18:2(9Z,12Z)), were significantly decreased with a value of FC less than 0.2.

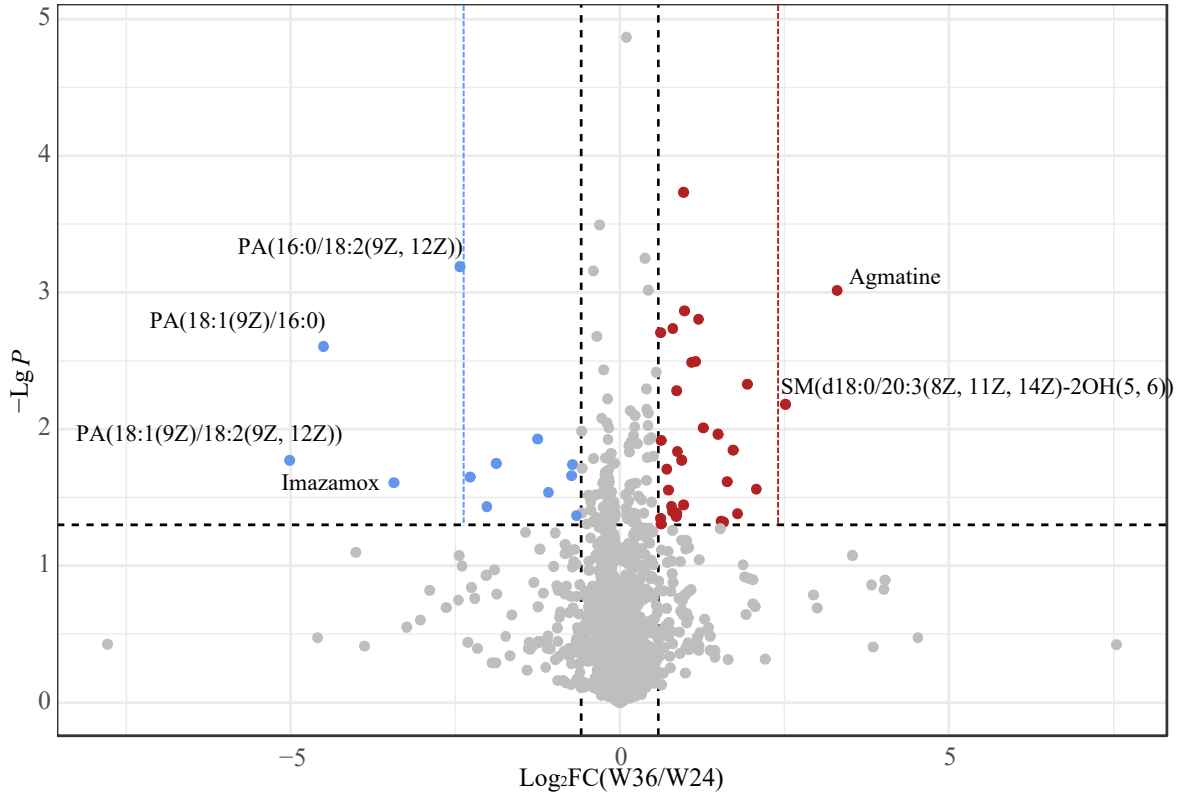

Figure 3. The differentially changed non-volatile compounds (DCnVCs) between W36 and W24. Red circles represent significantly increased differentially changed non-volatile compounds, blue circles represent significantly decreased differentially changed non-volatile changed compounds, gray circles represent non-significantly changed non-volatile compounds.

Meanwhile, 31 DCnVCs were relatively increased significantly (VIP > 1.0, $p < 0.05$, and FC > 1.5), including lipids and lipid-like molecules (10 DCnVCs, neoconvallatoxoloside, PC(20:1(11Z)/14:0), PS(15:0/24:1(15Z)), tetranor 12-HETE, ganosporeric acid A, porrigenin A, PI(16:0/18:2(9Z,12Z)), sebacic acids, azelaic acid, and apo-12′-zeaxanthinal), organic oxygen compounds (five DCnVCs, gomphrenin II, CMP-2-aminoethylphosphonate, 3-methylthiopropyl-desulfoglucosinolate, glucomannan, and 2,3-butanediol glucoside), benzenoids (three DCnVCs, tiapamil, neopine, and salutaridinol), nucleosides, nucleotides, and analogues (two DCnVCs, 5′-methylthioadenosine, and cytidine 5′-monophosphate-N-acetylneuraminic acid), organoheterocyclic compounds (two DCnVCs, fenoldopam and merbarone), organic acids and derivatives (one DCnVC, tyrosyl-tyrosine), hydrocarbons (one DCnVC, vinylcyclohexene), organic nitrogen compounds (one DCnVC, agmatine), and others (six DCnVCs, SM(d18:0/20:3(8Z,11Z,14Z)-2OH(5,6)), PG(6 keto-PGF1 alpha/i-17:0), PG(22:6(5Z,7Z,10Z,13Z,16Z,19Z)-OH(4)/20:1(11Z), PI(18:1(9Z)/18:1(12Z)-

O(9S,10R)), 16alpha-hydroxy DHEA 3-sulfate, and PI(18:1(9Z)-O(12,13)/18:1(11Z))). Among these related increased DCnVCs, two DCnVCs, SM(d18:0/20:3(8Z,11Z,14Z)-2OH(5,6)) and agmatine, were significantly increased with a value of FC over five. Non-volatile compounds in coffee play a key function in coffee flavor. For example, lipid compounds can contribute to the perceived texture and mouthfeel of coffee beverages. Carbohydrates can impact the sweetness. Trigonelline, caffeine, and chlorogenic acids can impact the bitterness [22]. Although trigonelline, caffeine, and chlorogenic acids did not show significant changes in these results, the function of these DCnVCs will be worthy of attention.

3.3. The Result of Volatile Compound Analysis by GC–MS

Aroma-volatile chemicals in roasted coffee are the most important quality determinant. Although more than 1000 volatile compounds including hydrocarbons, alcohols, aldehydes, ketones, carboxyclic acids, esters, pyrazines, pyrroles, and pyridines, have been identified in coffee, only a small number of them contribute to the coffee flavor and aroma [22]. In total, 176 volatile compounds (VCs) from 19 classes were confirmed in W36 vs. W24 (Table S2). The percentage of these 19 classes of VC were shown in Figure 4. The dominating classes were acids (48 VCs, comprising 39.28 in W24 and 40.61% in W36, respectively), alcohols (28 VCs, comprising 21.46% and 19.91%, respectively), hydrocarbons (22 VCs, comprising 12.89% and 12.11%, respectively), aldehydes (six VCs, comprising 6.22% and 614%, respectively), lactones (four VCs, 5.08% and 5.02%), ketones (20 VCs, comprising 2.89% and 2.57%, respectively), and ethers (10 VCs, comprising 0.64% and 0.59%, respectively). Acids, alcohols, and hydrocarbons were the dominating non-volatile compounds.

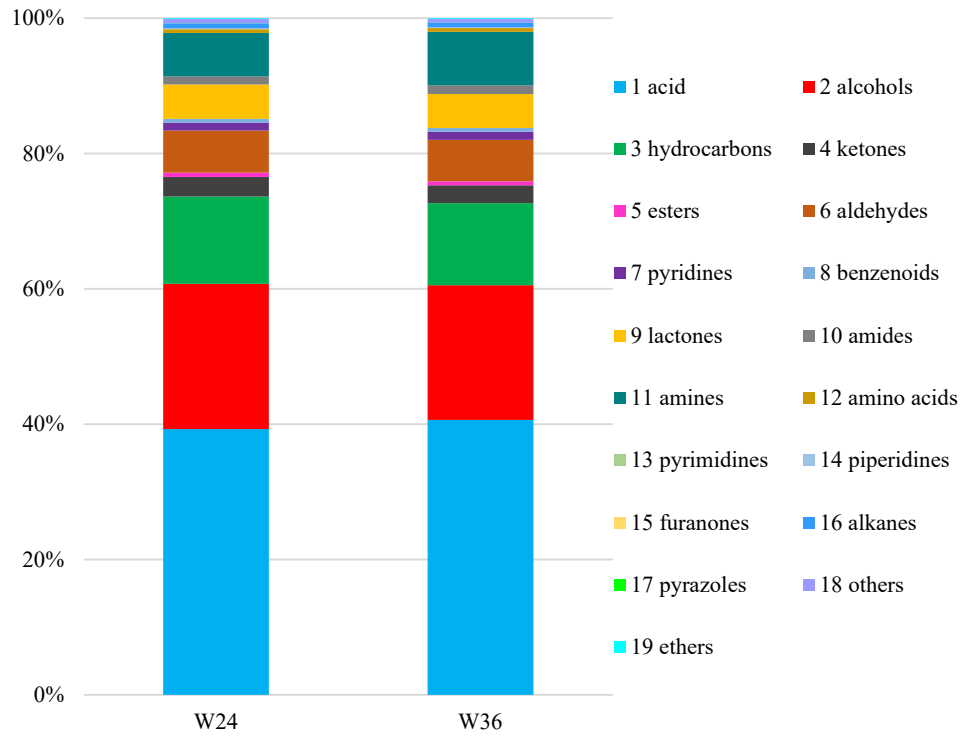

Figure 4. Percentage of volatile compounds from fermentation duration of *C. arabica* in Yunnan province.

In W24, the percentage of quininic acid was a maximum of 6.15% ± 1.13%, followed by 3-[(tetrahydro-2H-pyran-2-yl)oxy-]-benzenamine and 2-ethylhexanal ethylene glycol acetal with 6.06% ± 3.43% and 5.53% ± 0.67%, respectively, while 3-[(tetrahydro-2H-pyran-2-yl)oxy-]-benzenamine was 7.54% ± 0.53% in W36, followed by quininic acid (7.00% ± 0.21%) and 2-ethylhexanal ethylene glycol acetal (5.46% ± 0.26%). Nine VCs, isochlorogenic acid, glycolic acid, 3-pyridinol, lactic acids, myo-inositol, galactinol, sucrose,

D-(+)-trehalose, hexonic acid, and 3-deoxy-gamma-lactone were also significant VCs with a percentage value over 3.00%.

Moreover, 22 significantly differentially changed volatile compounds (DCVCs) were detected, as shown in Figure 5. These DCVCs came from seven alcohols (2,3-butanediol, phloroglucinol, hexitol, butan-1-ol, pyrogallol, D-mannitol, and D-erythro-sphingosine), five acids (pipecolic acid, 3-hydroxypropionic acid, nicotinic acid, 2-oxovaleric acid, and lactic acid), three hydrocarbons (trehalose, ethylalpha-D-glucopyranoside, and 1,6-anhydro-glucose), three ketones (1-(5-ethyl-2-hydroxy-4-methoxyphenyl)-2-(3,4-methylenedioxy-phenyl)-ethanone, methylhydroquinone, and 1bata,12,12-trimethyl-7,11-dioxapentacylo [15.3.0.0(4,16).0(5,10)]eicos-13-en-20-ol-8-one), two amides (2,3,6,7,8,8a-hexahydro-1,4-dioxo-pyrrolo [1,2-s]prrazine-3-propanamide and benzalaniline), one pyrimidine (4-(2-hydroxy-5-nitrophenyl)pyrimidine), and one aldehyde (2-fluoro-3-hydroxy-4-methoxy-benzaldehyde). Based on DCVCs between W36 and W24, only three DCVCs including benzalaniline, ethylalpha-D-glucopyranoside, and 2,3-butanediol were significantly reduced with prolonged fermentation duration; other DCVCs were significantly accumulated in roasted coffee. Although the contribution of these DCVCs to coffee flavor is not confirmed in the current research report, their function is worthy of further study to find directly odor-volatile compounds in coffee beverages.

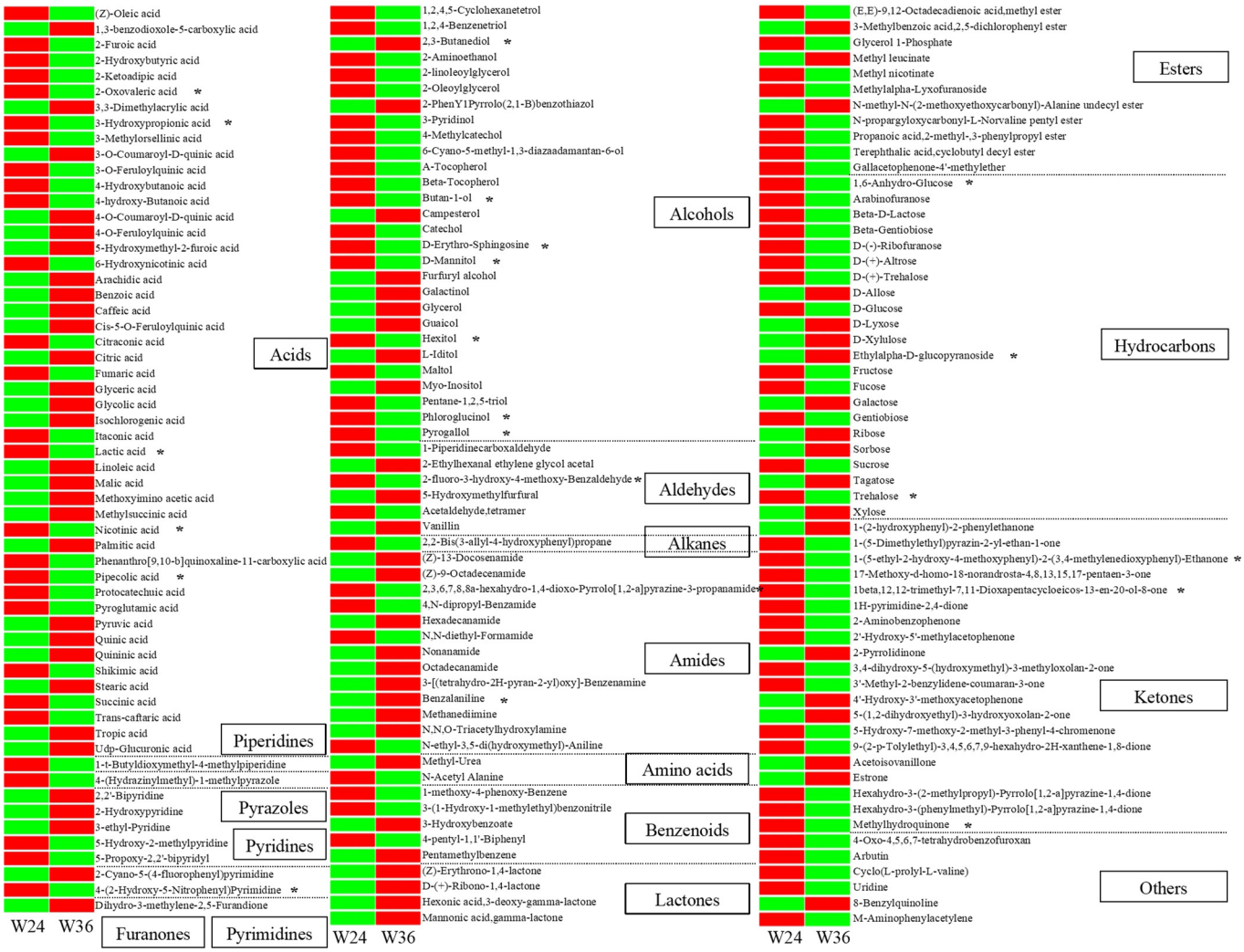

Figure 5. The volatile compounds (VCs) and their differently changed volatile compounds (DCVCs) between W36 and W24. "*" represents significantly differentially changed volatile compounds. Red represents that the content of VCs was higher, green represents that the content of VCs was lower.

4. Discussion

Coffee flavor is easily influenced by several factors, such as post-harvest primary processing [5,9]. From the surface to the interior, coffee beans are surrounded by skin, pulp, mucilage, parchment, and silver skin. To obtain green coffee beans, these five layers surrounding coffee beans must be peeled by mechanical or other methods. The first step to obtaining green coffee beans is post-harvest treatment, which is a crucial step for the chemical composition of coffee beans and the sensory quality of the coffee beverage [5]. According to previous studies, the post-harvest primary processing method can significantly affect the chemical compounds of roast and green coffee beans [23,24]. In roasted coffee beans, 1-*O*-caffeoylquinic acid, 5-hydroxymethylfurfural, and sugar alcohol can serve as discriminant marker compounds for distinguishing different primary processing methods for *C. arabica* in Yunnan [6].

In the wet processing, coffee cherries with the skin removed were submerged in fermentation for 12–36 h to remove the pulp [5]. Fermentation is a multifactorial and important process in coffee processing which has often been influenced by environmental conditions [16]. Microorganisms (yeasts, bacteria, and filamentous fungi) are present in coffee fermentation, which come from coffee cherries, soil, air, water, etc. [12]. During the washed processing of *C. arabica* from Yunnan province, the chemical compounds of coffee in fermentation were changed with the change in the structure of the microorganisms, and some chemical compounds showed a strong positive or strong negative correlation with the microorganisms [18]. For example, L-quinate showed a strong positive correlation with *Leuconostoc*, *Metschnikowia*, and *Apiotrichum*. 3-deazaadenosine showed a strong negative correlation with *Tatumella* and a positive correlation with *Burkholderia*, *Dyella*, and *Candida*. In addition, microorganisms also showed interaction with other microorganisms. For example, *Metschnikowia* and *Apiotrichum* fungi genera were extremely strongly positively correlated with *Leuconostoc* [18]. Furthermore, these microorganisms can produce important enzymes for degrading pectin [15]. Furthermore, coffee fermentation can produce a positive or negative impact on coffee flavor and aroma [25]. Coffee beans themselves contain all kinds of chemical compounds [26]; in some of these compounds are coffee flavor precursors such as sugar, proteins, amino acids, and phenolic compounds [4,25]. These coffee flavor precursors can form coffee aroma by Maillard reaction, Strecker degradation, caramelization reaction, fragmentation reaction, etc. [4]. During coffee fermentation, microbial activity, the extent of fermentation, and microorganisms' diverse metabolites can influence flavor precursors, such as the concentrations of free sugars and free amino acids [27]. Therefore, the selection of appropriate specific microorganisms for coffee fermentation has become a popular method to improve the sensory profile of coffee beverages by producing extracellular enzymes, volatile and non-volatile metabolites, and pH changes [23,28]. However, controlling the coffee fermentation process is a major key factor and challenge for improving coffee flavor by microorganisms [3], because underfermentation, overfermentation, or failing fermentation would develop spoilage microorganisms, producing adverse effects on the coffee's aroma and flavor. Moreover, fermentation time, temperature, pH conditions, moisture content, and other fermentation conditions can influence coffee quality [29,30]. For example, overfermentation would produce undesirable chemical compounds, such as notably propionic and butyric acids [3]. These compounds would feature an onion taste [3]. Therefore, fermentation duration is one of the main useful fermentation conditions that is important for coffee flavor. Coffee fermentation duration is often uncertain; a complete coffee fermentation needs 12–36 h [5]. In addition, the study on the fermentation method has become an effective way to improve coffee quality [31,32] and special microbial fermentation also been used as a starter to improve coffee flavor [20,33].

Based on the study of the fermentation duration of *C. arabica* from Yunnan province, China, the roasted coffee beans with a 36 h fermentation showed the highest cupping score and the best sensory character, which was significantly different from other fermentation coffee samples with a value of p lower than 0.05. Meanwhile, the results of differentially changed non-volatile and volatile compound analysis found 43 DCnVCs and 22 DCVCs

between fermentation at W36 and W24. Although all these compounds did not contribute to the coffee flavor, the chemical compounds' constitutions showed differences.

5. Conclusions

The nVCs, VCs, and coffee flavor of roasted *C. arabica* beans from Yunnan province at different fermentation durations during the washed processing methods were compared in this analysis. The fermentation duration of 36 h showed the highest total cupping score. Furthermore, a total of 2567 nVCs from 17 super-classes and 176 VCs from 19 classes were identified in W24 and W36, respectively. Among these lipids and lipid-like molecules, organoheterocyclic compounds, organic acids and derivatives, organic oxygen compounds, phenylpropanoids and polyketides, and benzenoids dominated the nVCs, comprising 91.90%. Acids, alcohols, hydrocarbons, aldehydes, and lactones dominated the VCs. Moreover, 43 nVCs were significantly differentially changed non-volatile compounds, and 22 were significantly differentially changed volatile compounds between W36 and W24. Coffee fermentation is one of the key steps in coffee processing. These results imply that fermentation duration can influence coffee flavor by the change in chemical compounds. For the washed primary processing of *C. arabica* from Yunnan province, a fermentation duration of 36 h may be the suitable fermentation duration, which presents potential value as a reference for the washed processing of coffee in the food industry. This study will provide the possibility for further study on coffee fermentation to improve coffee flavor and coffee quality by changing coffee chemicals.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/fermentation10110560/s1, Table S1: The peak area of non-volatile compounds in W24 and W36. Table S2: The peak area of volatile compounds in W24 and W36.

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