Profiling of volatile and non-volatile compounds in Dianhong by a combined approach of static headspace GC-MS and UPLC-MS

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ABSTRACT

The systematic identification of volatile organic compounds (VOCs) and non-VOCs will facilitate understanding the characteristic aroma and bioactive constituents of Dianhong. This article reported for the first time the volatile and non-volatile composition profiles of Dianhong from four main producing areas. A combined total of 42 VOCs were identified from four Dianhong samples by static headspace GC-MS. Thirteen VOCs were found to be important common components contributing to the characteristic aroma of Dianhong. The non-VOCs of Dianhong were evaluated by UPLC-ESI-MS, and Dianhong samples from four producing areas shared almost the same component profiles. Thirty-four components including derivatives of quinic acid, gallic acid, and catechin, flavone C-glycosides, flavonol O-glycosides, and alkaloids were characterized. Finally, catechin, gallic acid, 5-O-gallloylquinic acid, and theobromine were characterized as the main non-VOCs in Dianhong. Dianhong samples from four main producing areas showed similar VOCs and non-VOCs profiles under our optimized GC-MS and LC-MS conditions.

KEYWORDS

Black tea; static headspace; volatile organic compounds; GC-MS; LC-MS

Resumen

Considerando que la identificación sistemática de los compuestos orgánicos volátiles (VOC) y no volátiles facilitará la comprensión del aroma característico y los componentes bioactivos del Dianhong, este artículo da a conocer por primera vez los perfiles de composición volátil y no volátil del Dianhong proveniente de cuatro zonas productoras principales. Empleando GC-MS estática de espacio de cabeza en cuatro muestras de Dianhong, se identificó un total combinado de 42 VOC, comprobándose que 13 VOC son componentes comunes importantes que contribuyen al aroma característico del Dianhong. Los compuestos orgánicos no volátiles del Dianhong fueron evaluados mediante UPLC-ESI-MS, constatándose que las muestras provenientes de cuatro zonas productoras presentaron casi los mismos perfiles de componentes. Se caracterizaron 34 componentes, incluidos los derivados del ácido quínico, el ácido gálico y la catequina, los glucósidos C de las flavonas, los glucósidos O de los flavonoles y los alcaloides. Por último, se identificó que la catequina, el ácido gálico, el ácido 5-O-gallloylquinico y la teobromina son los principales compuestos orgánicos no volátiles del Dianhong. En nuestras condiciones optimizadas de GC-MS y LC-MS, las muestras de Dianhong de las cuatro principales zonas productoras exhibieron perfiles de compuestos orgánicos volátiles (VOC) y no volátiles similares.

PALABRAS CLAVE

Té negro; espacio de cabeza estático; compuestos orgánicos volátiles; GC-MS; LC-MS

1. Introduction

Dianhong (the black tea in Yunnan) is one of the famous black tea (fermented tea) in the world, which is produced from fresh leaves of large-leaf tea species [Camellia sinensis (Linn.) var. assamica (Masters) Kitamura] cultivated in Yunnan province of China. Dianhong is mainly produced in approximate 20 counties such as Lincang, Baoshan, Simao, Xishuangbanna, Dehong, and Honghe located in Yunnan province (Ren et al., 2012). It can be categorized into congou tea and broken-leaf tea, both of which are processed through manufacturing practices of withering, rolling, fermentation, and drying (Liu et al., 2017). With over 70 years’ development, a unique flavor of Dianhong is gradually formed with potential health benefits. Unless otherwise indicated, Dianhong is referred to the congou black tea in Yunnan in this study.

Tea aroma is one of the important indicators influencing tea quality, and is well known to be determined by the plant species and the production processes. To date, there have been a few reports on volatile organic compounds (VOCs) of Dianhong. For example, VOCs were investigated and compared between congou and broken-leaf of Dianhong (Lv et al., 2013; Ren et al., 2012), or between different sampling methods (such as steam distillation and headspace solid-phase micro-extraction, HS-SPME) (Ma et al., 2017; Yu et al.,...
2018). However, some easily volatile components might be seriously lost during extraction and concentration of VOCs using distillation methods (Qin et al., 2016). In addition, HS-SPME sampling method may result in a discriminatory effect on VOCs that only absorbed selectively by given sorbents packed in SPME cartridges. Therefore, the static HS method was used for direct sampling and analysis of VOCs present in Dianhong, though the sensitivity of this method is relatively low (Qin et al., 2016). The static HS method coupled to gas chromatography-mass spectrometry (GC-MS) can truly reflect the VOCs, especially for those high volatile compounds that are the major contributors to tea aroma.

Besides the VOCs, the non-volatile compounds of various tea have been extensively studied. Catechins, flavonoids, and phenolic acids are reported to be main constituents of tea, and to exert antioxidant and lipid-lowering activities (Koo & Noh, 2007). In the past decade, with the development of liquid chromatography-mass spectrometry (LC-MS) methods, chemical constituents of many types of black tea have been profiled and further identified. However, there is insufficient recognition of chemical constituents present in Dianhong. Early in 2004, Menet and co-workers studied only theaflavins and thearubigins in Dianhong using MALDI-TOF-MS (Menet et al., 2004). Thereafter, Chen et al. revealed the presence of theaflavin trigallate and tetragallate in Dianhong using LC-ESI-LTQ-MS (Chen et al., 2012). Until recently, a UHPLC-ESI-HRMS study on components profiling demonstrated that a total of 106 components including catechin, flavonoids, phenolic acids, and amino acids were detected in Dianhong collected from Fengqing county in Yunnan province (Chen et al., 2020). However, no component comparison has ever been performed for Dianhong from different producing areas.

It is therefore of interest to investigate systematically the VOCs and non-VOCs of Dianhong collected from different producing areas across Yunnan province. In this study, we have qualitatively evaluated of the static HS in VOCs profiling of Dianhong collected from four producing areas including Fengqing, Lincang, Dehong, and Baoshan in Yunnan province. Several sampling factors such as heating temperature, heating time, and sample amounts were optimized for the static HS sampling. The non-VOCs were also studied by an optimized ultra-performance liquid chromatography-ion trap-tandem mass spectrometry (UPLC-IT-MS$^3$) \((n = 3)\) method. To our knowledge, this is the first report on systematic profiling and comparison of VOCs and non-VOCs of Dianhong collected from four main producing areas by optimized static HS GC-MS and LC-MS methods.

2. Materials and methods

2.1. Materials and reagents

HPLC-grade methanol used for extraction and chromatographic separation was purchased from CINC High Purity Solvents Co., Ltd. (Shanghai, China). LC-MS-grade methanol used for mass spectrometry analysis was purchased from Merck (Darmstadt, Germany). HPLC-grade formic acid was obtained from Aladdin (Shanghai, China). Dianhong sample 1 (DH-1, the producing area: Fengqing County of Lincang City in Yunnan, 24°13’ to 25°02’ N latitude and 99°31’ to 100°13’ E longitude) was obtained from Yunnan Noupu Commercial & Trading Co, Ltd. ( Kunming, Yunnan, China). Dianhong sample 2 (DH-2, the producing area: Mang City of Dehong Prefecture in Yunnan, 24°05’ to 24°39’ N latitude and 98°05’ to 98°44’ E longitude) was obtained from Zhicheng Tea Co., Ltd. (Mang City, Yunnan, China). Dianhong sample 3 (DH-3, the producing area: Baoshan City in Yunnan, 24°08’ to 25°51’ N latitude and 98°25’ to 100°02’ E longitude) was obtained from Fengxi Tea Co., Ltd. (Baoshan City, Yunnan, China). Dianhong sample 4 (DH-4, the producing area: Menghai County of Xishuangbanna Dai Autonomous Prefecture in Yunnan, 21°28’ to 22°28’ N latitude and 99°56’ to 100°41’ E longitude) was obtained from Simao District Tea Factory (Pu’er City, Yunnan, China). All the four Dianhong samples were cooked tea and produced in April to Jun in 2020. Tea samples were ground to fine powders using a grinding mill prior to GC-MS and LC-MS analysis.

2.2. Static headspace (HS) extraction

The static HS extraction was conducted on a Shimadzu AOC-5000 Plus headspace autosampler, connected to a Shimadzu QP2010 Ultra GC-MS system (Kyoto, Japan). Each Dianhong sample (2.0 g) was powdered and transferred into 20-mL headspace vials. The vials were sealed with Teflon-coated rubber stoppers and aluminum crimp caps, and placed into the headspace autosampler. The sample vials were heated at 100 °C for 30 min in the vial heating block. Then, 1.0 mL of the headspace gas was automatically taken and injected into GC-MS. Duplicate analysis was conducted for each sample.

2.3. Sample preparation for LC-MS analysis

An accurately weighed powder (20 g) of each Dianhong sample was placed into 200 mL hot distilled water at 100°C, and allowed to stand for 10 min. The mixture was then centrifuged at 4000 rpm for 10 min, and the supernatant was collected and subjected to evaporation under vacuum in a rotary evaporator at 45°C. The obtained residue was re-dissolved in 3 mL of water-methanol (2:1). After filtration through 0.22 μm membranes, the filtrates were analyzed by LC-MS. The analysis was conducted in duplicate for each sample.

2.4. GC-MS analysis

GC-MS analysis was conducted on a Shimadzu GCMS-QP2010 Ultra system equipped a single quadrupole mass spectrometer (Kyoto, Japan). The separation was conducted on a DB-5 MS column (30 m × 0.25 μm × 0.25 mm, Agilent Technologies, Santa Clara, CA, USA). The column temperature was initially maintained at 32°C for 5 min, after which it was raised to 200°C at 10°C/min (held for 5 min), and then programmed to 260°C at 20°C/min (held for 2 min). The injector temperature was set at 230°C and desorption was carried out in a split mode as 10:1. Helium was used as the carrier gas with a constant flow rate of 1.02 mL/min, and nitrogen as the make-up gas for purging the sample loop. Mass spectrometry analysis was conducted in a full-scan mode with a scan range of 35–450 m/z. The electron impact ionization was used at 70 eV. The ion source temperature was 230°C, and the GC-MS interface temperature was 250°C. The VOCs were identified by computer matching of their MS fragments with those in the NIST-MS library. Each individual
VOC was relatively quantified by normalized against total peak areas of VOCs.

2.5. Ultra-performance liquid chromatography (UPLC)-MS$^a$ analysis

The UPLC analysis was performed using a Dionex Ultimate 3000 UPLC system (ThermoFisher Scientific, Waltham, MA, USA) equipped with an electrospray ionization (ESI) source and a linear ion trap as the mass analyzer. Chromatographic separation was performed with a Waters Acquity UPLC HSS T3 column (2.1 mm $\times$ 100 mm, 1.8 $\mu$m) maintained at 30°C. Gradient elution was conducted with two mobile phases consisting of 0.1% formic acid (A) and methanol (B) using the following gradient: 0–2 min, 0–2% B; 2–6 min, 2–11% B; 6–12 min, 11% B; 12–13 min, 11–16% B; 13–22 min, 16–17% B; 22–26 min, 17–80% B; and 26–40 min, 80–100% B. The flow rate was set at 300 $\mu$L/min throughout the gradient. The injection volume was 1.0 $\mu$L, and the effluents were monitored by a PDA detector, with two preferential channels as the detection wavelengths, 254 nm (channel A) and 270 nm (channel B). The ESI source and MS$^a$ parameters were automatically optimized. The negative and positive ion modes were used with the condition as follows: spray voltage, $\pm$3.5 kV; sheath gas (N$_2$) flow rate, 40 au; auxiliary gas (N$_2$) flow rate, 10 au; capillary temperature, 330°C. Helium was used as the collision gas, and the collision energy was set to 30 V. The MS detector was programmed to perform a full scan and a data-dependent scan. For the full scan MS analysis, the spectra were recorded in the range of $m/z$ 50 to 1000. The data-dependent MS$^a$ mode for MS data collection was carried out automatically on the most abundant fragment ion in MS$^a$($^1$).

3. Results and discussion

3.1. Optimization of static headspace GC-MS analysis of VOCs in Dianhong

3.1.1. Effect of sample amounts on VOC profile of Dianhong

Sample amount is a determinant factor influencing the detection sensitivity of static HS methods. Thus, the effect of sample amounts in headspace vials on the VOC profile of Dianhong was firstly investigated using the tea sample of DH-1. The sample amounts of 1.0, 2.0, and 3.0 g per vial were checked under a heating temperature of 100°C for 30 min. According to the graph (Figure S1), the total ion current (TIC) profiles obtained for the three sample amounts were very similar, differing only in the relative intensities of each individual VOCs. The sample amount effect can be more clearly observed by examining 15 main VOC peaks with different structure types (Figure S2). The peak intensities increased with sample amounts from 1 g/vial to 2 g/vial, but decreased at 3 g/vial. This was probably due to that the emission of VOCs was impacted by the increased highness of tea samples in vials. Thus, the sample amount of 2 g/vial was selected for subsequent experiments.

3.1.2. Effect of sample heating temperatures on VOC profile of Dianhong

Heating temperature is an important parameter contributing to sampling efficiency in static HS methods. In this study, the effect of heating temperatures (50, 80, and 100°C) was investigated with a fixed heating time of 30 min using the tea sample of DH-1. As shown in Figure S3, the peak intensities and numbers were significantly increased as the temperature was increased from 50 to 100°C. The Dianhong sample exposed to heating temperature of 50°C produced only very few VOCs with the low-boiling point to be sampled. After this temperature point, some less volatile components ($t_{R} = 10–15$ min) appeared at 80°C, though their peak intensities need to be enhanced. Consequently, 100°C enabled the VOCs ranging from the low to high-boiling points to be extracted with considerable intensities. To guarantee the detection sensitivity of trace VOCs, a temperature of 100°C was thus chosen as the optimized heating temperature in the further experiments. This temperature is consistent with the frequently used temperature that people make black tea habitually.

3.1.3. Effect of sample heating time on VOC profile of Dianhong

The effect of heating time (15, 30, 45, and 60 min) was investigated with an optimal heating temperature of 100°C using the tea sample of DH-1. As shown in Figures S4 and S5, different types of VOCs showed different trends over time. For components with low-boiling point, their peak intensities (such as peaks 1–5) were increased with increasing time. However, for some VOCs (such as peaks 6–15) with medium and high-boiling points, their relative intensities were not obviously increased from 30 to 60 min. Therefore, a heating time of 30 min was selected for subsequent analysis to reduce the analysis time.

3.2. Identification of VOCs in four Dianhong samples by static headspace GC-MS

Under the optimized static HS conditions described above, 2.0 g fine powders of each Dianhong sample were subjected to static HS coupled to GC-MS analysis. Typical chromatograms of VOCs of four Dianhong samples obtained by static HS were shown in Figure 1. VOC profiles of the four Dianhong samples (especially for the latter three samples) were found intuitively similar from their observed TIC chromatograms. A combined total of 42 VOCs were separated and identified from four Dianhong samples (DH-1–DH-4), which were quantified as normalized peak areas and summarized in Table 1. Among these VOCs, 13 common components were found in all four Dianhong samples including acetone, dimethyl sulfide, 2-methyl propanal, acetic acid, 3-methyl butanal, 2-methyl butanal, 1-penten-3-ol, hexanal, benzaldehyde, benzeneacetalddehyde, cis-linalool oxide, trans-linalool oxide, and linalool. These common VOCs accounted for 89.39%, 89.70%, 89.73%, and 94.39% of the total VOCs of DH-1–DH-4, respectively, confirming that these VOCs of the four Dianhong samples were quite similar. This further suggested that these 13 VOCs can be used as important common components contributing to the characteristic aroma of Dianhong. In addition, five of these 13 common VOCs including 2-methyl propanal (20.19–30.32%), 2-methyl butanal (16.38–25.06%), 3-methyl butanal (10.32–16.87%), acetone (3.09–7.82%), and acetic acid (2.81–16.14%) were recognized as their main VOCs that might dominate the aroma of all the four tea samples. It is worthy of note that all the non-common VOCs contributed less than 2% of the total VOCs in the corresponding Dianhong samples.
Additionally, the main VOCs contained in all the four tea samples have lower-boiling point and higher volatility (with $t_r$ less than 5.00 min). These highly volatile components of the four Dianhong samples accounted for 80.54%, 77.65%, 81.19%, and 85.98% of the total VOCs of DH-1–DH-4, respectively. This is the unique advantage of the static HS methodology for sampling these types of VOCs (Qin et al., 2016). However, these highly volatile components showed a relatively poor separation due to their huge abundance. Further trial-and-error optimization of the programmed temperature condition, capillary columns of different polarity (DB-Heavy WAX and DB-5 MS), and split ratio were attempted without an obvious improvement in peak resolution.

In 2017, a report on VOCs of Yunnan black tea formulated with rose fragrance showed that linalool, linalool oxide, memantine, β-ionone, pentadecane, and hexadecane were the main VOCs detected by headspace SPME-GC-MS method, and each of these individual VOCs accounted for over 5% of the total VOCs (Liu et al., 2017). In the current study, linalool and its oxide were also detected as the main volatile components of four Dianhong samples, and their relative contents were found to be 4.25%, 9.95%, 7.58%, and 6.44% in the total VOCs of DH-1–DH-4, respectively. The other main VOCs found in that study were not detected in our study, probably due to the lower volatility that cannot be captured by static HS method. In the same year, Ma et al reported that linalool (35.86–40.20%) was the most abundant volatile component of Dianhong, followed by trans-2-hexenal and hexanal, by two distillation methods of VOCs (Ma et al., 2017). The latter two VOCs were also found in all four Dianhong samples with relative contents of 0.21–1.86% and 0.52–7.74%, respectively. Similarly, linalool and its oxide were found as the predominant VOCs in Dianhong in another study (Ren et al., 2012) using water distillation, along with some main components such as 2-trans-hexenal, phenylacetaldehyde, n-hexanal, benzylalcohol, and geraniol. This is in agreement with ours which most of these VOCs, such as phenylacetaldehyde (0.53–1.83%), n-hexanal (0.52–7.74%), and benzylalcohol (0.63–0.67%), were detected in the four Dianhong samples. These three VOCs possess hyacinth-like, green grass-like, and rose scent, respectively, probably providing a strong sweet aroma to Dianhong. However, a recent study conducted by Yu et al. found that α-terpineol, β-pinene, dioctyl isophthalate, 3,7-dimethylocta-1,6-dien-3-yl-2-aminobenzoate, and methyl linolenate were the main contributors to Dianhong VOCs through simultaneous distillation extraction (Yu et al., 2018). These VOCs were totally different from those of literatures and our study. Based on the results described above, the VOCs reported in these literatures were obtained with the traditional water distillation, followed by CH$_2$Cl$_2$ or CHCl$_3$ extraction, and vacuum concentration. It was therefore concluded that relatively low-volatile components were mostly captured by these reported methods, due to the substantial losses of highly volatile components during sampling and preparation of VOCs. In addition, we have attempted to prepare the essential oil of Dianhong by traditional hydrodistillation extraction method using 100 g DH-1 sample, but unfortunately inadequate amount of oils was obtained for further GC-MS analysis.

Tea aroma is recognized as one of key factors influencing tea quality, which achieved over 40% contribution of overall quality of tea (Lu et al., 2015). The combined and interacted actions of all VOCs determine the aroma characteristic of tea samples. However, these VOCs exert different types of fragrance depending on their contents and odor threshold. As the common components with relative higher abundance in Dianhong, linalool and its derivatives exhibited strong floral fragrance (Mao et al., 2018), which was proposed to contribute to the background aroma of Dianhong (Takeo, 1983). And those high volatile and high abundant VOCs such as 3-methyl-butanal, 2-methyl-butanal, and hexanal have strong fruit fragrance, which might constitute the characteristic aroma of Dianhong. In addition, although some pungent components such as acetone and acetic acid had relative high contents in the current study, the overall aroma of Dianhong were not obviously affected, indicating their lower odor thresholds. It should be noted that the formation of abundant acetic acid is
Table 1. GC-MS data of volatile organic compounds (VOCs) of four Dianhong (DH) samples extracted by static headspace.

| tR (min) | Identified compounds | Formula | DH-1 (%) | DH-2 (%) | DH-3 (%) | DH-4 (%) |
|---------|----------------------|---------|----------|----------|----------|----------|
| 1.863   | Acetone              | C2H4O   | 6.43     | 4.08     | 3.09     | 7.82     |
| 1.980   | Dimethyl sulfide     | C4H10S  | 17.38    | 1.51     | 4.54     | 1.13     |
| 2.173   | 2-Methyl propanol    | C4H10O  | 20.19    | 21.21    | 24.99    | 30.32    |
| 2.363   | Acetic acid          | C2H4O    | 0.51     | 3.20     | 1.02     | 0.53     |
| 2.407   | 3-Methyl-2-butanone  | C6H14O   | 0.60     | ND       | ND       | ND       |
| 3.163   | Acetic acid          | C2H4O    | 2.81     | 16.14    | 7.57     | 3.80     |
| 3.237   | 3-Methyl-butanol     | C6H14O   | 11.37    | 10.32    | 16.87    | 15.33    |
| 3.390   | 2-Methyl-butanol     | C6H14O   | 16.38    | 19.40    | 20.11    | 25.06    |
| 3.757   | 1-Penten-3-ol        | C5H10O   | 1.49     | 1.02     | 1.42     | 0.87     |
| 4.060   | 1,4-Dimethyl-4-pentenyl acetate | C10H20O2 | 3.38 | 0.77 | ND | ND |
| 4.083   | 5-Methyl-1-hexyne    | C7H14O   | ND       | ND       | 1.58     | 1.10     |
| 7.280   | Hexanal              | C6H12O   | 7.74     | 0.52     | 1.47     | 1.37     |
| 7.983   | Methyl pyrazine      | C7H12N   | 0.40     | ND       | ND       | ND       |
| 8.157   | Furfural             | C4H6O    | 0.90     | ND       | ND       | ND       |
| 8.313   | 3-Methyl-butanoic acid | C7H14O   | ND       | 0.70     | ND       | ND       |
| 8.807   | 2-Methyl-butanoic acid | C7H14O   | ND       | 0.65     | ND       | ND       |
| 8.810   | (E)-2-Hexenal        | C6H12O   | 1.86     | ND       | 0.81     | 0.21     |
| 8.860   | 3-Hexen-1-ol         | C6H12O   | ND       | 0.63     | 1.41     | ND       |
| 9.253   | 1-Hexanol            | C6H12O   | ND       | 0.57     | ND       | 0.16     |
| 10.050  | Heptanal             | C7H14O   | 0.40     | ND       | ND       | ND       |
| 10.053  | 2-(2-Methoxyethyl)-1-hexanol | C8H16O2 | ND | ND | ND | 0.19 |
| 10.059  | 2-Heptanol           | C7H14O   | ND       | 0.32     | ND       | ND       |
| 10.067  | 3-Ethyl-2-pentanol   | C8H16O   | ND       | ND       | 0.31     | ND       |
| 11.433  | Benzaldehyde         | C6H8O    | 0.31     | 0.52     | 0.32     | 0.93     |
| 11.707  | Hexanoic acid        | C6H12O   | ND       | 0.41     | 0.47     | ND       |
| 12.027  | 2-Pentyl-furan       | C10H22O  | 1.15     | ND       | ND       | 0.42     |
| 12.040  | 1-(3-Cyclohexen-1-yl)-22- dimethyl-1-propanone | C15H26O | ND | 0.55 | ND |
| 12.397  | 2-Hexenoic acid      | C8H14O   | ND       | 0.55     | ND       | ND       |
| 12.867  | D-Limonene           | C10H16O  | ND       | 0.74     | 0.48     | ND       |
| 12.960  | Benzyl alcohol       | C9H10O   | ND       | 0.67     | 0.63     | ND       |
| 13.153  | Benzeneacetaldehyde  | C8H10O   | 0.53     | 1.83     | 0.75     | 0.77     |
| 13.217  | 1-Ethyl-1-pyrole-2-carboxaldehyde | C8H13NO | 0.37 | ND | 0.74 | ND |
| 13.460  | 1-(2-Pyrol-2-yl)-ethanone | C9H14NO | ND | 0.68 | ND | ND |
| 13.673  | cis-Linalool oxide   | C10H16O1 | 0.72 | 1.83 | 1.07 | 1.93 |
| 13.980  | trans-Linalool oxide | C10H16O1 | 1.51 | 2.73 | 2.78 | 3.45 |
| 14.173  | Linalool             | C10H18O  | 2.02     | 5.39     | 3.73     | 1.06     |
| 14.233  | Hotrienol            | C12H16O  | ND       | 0.41     | ND       | ND       |
| 14.241  | Dodecanol            | C12H26O  | 0.41     | ND       | ND       | ND       |
| 14.437  | Phenylethyl alcohol  | C8H10O   | ND       | 0.84     | 0.64     | 0.25     |
| 15.493  | trans-Linalool 3,7-oxide isomer | C10H16O2 | 0.19 | ND | ND | 0.98 |
| 15.520  | trans-Linalool 3,7-oxide | C10H16O2 | ND | 1.05 | 1.05 | 0.27 |
| 15.847  | Methyl salicylate    | C10H12O3 | 0.19 | 0.75 | ND | ND |
| 16.687  | Geraniol             | C10H16O  | ND       | 0.34     | 0.27     | ND       |
| Total   |                      |          | 99.24    | 98.67    | 99.08    | 97.97    |

\(t_R\) retention index; ND, not detected; The relative content (%) normalized to the sum of areas of all peaks in total ion current of GC-MS. 

\(t_R\) índice de retención; ND, no detectado; el contenido relativo (%) fue normalizado a la suma de áreas de todos los picos en la corriente iónica total de GC-MS.

probably due to the microbial metabolism during the fermentation of Dianhong, as reported that saccharomyces in black tea could transform glucose and fructose into acetic acid and other metabolites (Yuan et al., 2017).

In conclusion, the VOCs of Dianhong will be influenced by multiple factors, namely VOC sampling methods, tea processed manners, and tea formulation. We proposed that the numbers and types of higher volatile components sampled by static HS should be characterized as the basic aroma of Dianhong. This is the first report on VOCs profile of Dianhong using static HS coupled to GC-MS.

3.3. Identification of non-VOCs of Dianhong by UPLC-MS®

3.3.1. General considerations

The hot water extract of Dianhong was concentrated in vacuo to dryness and reconstructed in a mixture of water and methanol (2:1) for UPLC-MS analysis. As shown in Figure 2, multiple peaks of different intensity occurred scatteredly in the 40-min chromatography detected at 254 nm.

The water-methanol extracts of four Dianhong samples were analyzed by the UPLC-ESI-MS® method at 254 nm. As shown in Figure 2, the UPLC-PDA chromatograms of four Dianhong samples were quite similar, differing only in the peak intensity. Most of the main components were detected with the retention times of 0–20 min, and a large peak at \(t_R = 9.86\) min predominated in the chromatograms. The similar UPLC-MS TIC chromatograms in the negative and positive ionization modes were shown in Figures 3 and 56. Also noted from Figure 56 was that the components in Dianhong generated more abundant ions in the negative ion mode than in the positive ion mode, especially for those with \(t_R\) of 10–40 min, which is probably due to the presence of catechins, flavonoids, and phenolic acids in Dianhong. Therefore, the structural characterization of components by UPLC-MS® was mainly acquired in the negative ion mode, aided or confirmed by the positive ion mode. The 34 peaks were identified or tentatively identified on the basis of their
UV data, $M^+$ ($n = 2–3$) fragmentation data, and by comparison with reported MS data. These identified compounds can be classified into six classes including quinic acid derivatives (peaks 1–4, 7, 10, 13, 18, and 20), gallic acid derivatives (peaks 5, 6, 11, 14, 16, and 23), catechin derivatives (peaks 15, 17, and 27), flavonol $O$-glycosides (peaks 26, 28–33, and 35), flavone $C$-glycosides (peaks 19, 21, 22, 24, and 25), and other components (peaks 8, 9, and 12). The retention time, UV $\lambda_{\text{max}}$ (PDA), quasi-molecular ions, $M^+$ fragments, and identification of these peaks were given in Table 2.

### 3.3.2. Quinic acid (or shikimic acid) derivatives in Dianhong

A total of nine quinic acid derivatives including peaks 1–4, 7, 10, 13, 18, and 20 were detected in Dianhong. Peaks 1 and 3 showed the same deprotonated ion at $m/z$ 191 [M-H]$, and $M^+$ fragments at $m/z$ 173 [M-H$_2$O-H]$^+$, 155 [M-2 H$_2$O-H]$^+$, 127 [M-3 H$_2$O-H]$^+$, and 111 [M-H$_2$O-HCOOH-H]$^+$, but only differed in the relative intensities of $M^+$ fragments. All these fragments were diagnostic fragments of quinic acid (Guo et al., 2018). Thus, peaks 1 and 3 were identified as quinic acid and its isomer. Peak 2 exhibited a quasi-molecular ion at $m/z$ 173 [M-H]$, and its $M^+$ fragment at $m/z$ 155 [M-H$_2$O-H]$^+$. Taken together with its $M^+$ fragment at $m/z$ 127, it was indicative of a quinic acid derivative with the loss of a hydroxyl group. Thus, peak 2 was proposed as dehydroxyquinic acid, namely shikimic acid (Li et al., 2021). Peak 4 displayed a parent ion at $m/z$ 335 [M-H]. Its $M^+$ fragments at $m/z$ 245 [M-90-H]$^+$ and 173 [M-162-H]$^+$ were the result of the $\mathrm{O}_2X$ cross-ring cleavage and loss of a hexosyl group, respectively. The $M^+$ fragmentation pattern corresponding to the fragments at $m/z$ 173 and 155 were consistent with those of peak 2. Thus, peak 4 was identified as shikimic acid O-hexoside (Marzouk et al., 2019). The $M^+$ spectra and the proposed fragmentation pathway of peak 4 were shown in Figure S7. This is the first report on detection of shikimic acid and its O-hexoside in Dianhong. Peak 7 showed the deprotonated molecule [M-H]$^-$ at $m/z$ 343 as the base peak, and [2 M-H]$^-$ dimer at $m/z$ 687 in high abundance. Its $M^+$ product-ion mass spectrum of $m/z$ 343 displayed a fragment at $m/z$ 191 [M-152-H]$^-$ corresponding to the deprotonated quinic acid after the loss of a galloyl group. Its $M^+$ fragment of $m/z$ 191 confirmed the presence of quinic acid in peak 7. Thus, this major peak was assigned as galloylquinic acid, which might be 5-O-galloylquinic acid due to the same fragments reported in Keemun black tea (Kelebek, 2016). Peak 10 displayed a quasi-
molecular ion at m/z 353 and MS² fragments at m/z 191 [M-162-H]- and 179 [M-174-H]-. The loss of 162 Da was ascribed to a caffeoyl group instead of a hexose, because of the online PDA absorption (221 and 275 nm) and the MS² fragment at m/z 135 produced from the caffeoyl group. The MS³ experiment of m/z 191 confirmed the identification of quinic acid. Thus, peak 10 was tentatively identified as 5'-caffeoylquinic acid, the MS fragmentation pattern and data of which were highly consistent with the reported data (Gu et al., 2019; Kelebek, 2016). Peaks 13, 18, and 20 all showed deprotonated molecular ions at m/z 337 [M-H]- and a similar fragmentation behavior. They displayed abundant MS² fragment ions at m/z 163 (for coumaroyl group) and 173 (for dehydroxyquinic acid). The assignment of a coumaroyl group was confirmed by the presence of the MS³ fragment ion at m/z 119 arising from its parent ion at m/z 163 due to the loss of a carboxyl group in peak 13. The quinic acid moiety in the three peaks was also evidenced by their typical MS² or MS³ ions shown in Table 2. Thus, peaks 13, 18, and 20 were tentatively identified as coumaroyl quinic acid or its position isomers (Kelebek, 2016).

### 3.3.3. Gallic acid derivatives in Dianhong

The gallic acid derivatives including peaks 5, 6, 11, 14, 16, and 23 showed the similar type of fragmentation behavior. The mass spectrum of peak 5 showed a deprotonated ion
at m/z 169 fragmenting on MS² to yield an almost exclusive fragment ion at m/z 125 due to the loss of carboxyl group. Peak 5 was thus assigned as gallic acid (Fathoni et al., 2017; Kelebek, 2016). Peak 6 had a deprotonated ion at m/z 331 that dissociated to yield a MS³ fragment at m/z 169 [M-162-H]- as the most abundant ion, indicating the loss of a hexose unit. The presence of another intensive MS² fragment at m/z 271 [M-60-H]- corresponding to the 0.3X cross-ring cleavage of a hexose confirmed the identification of a hexosyl instead of a corumaryl group (also 162 Da). In addition, the MS³ experiment from the ion at m/z 169 showed the same fragments found in peak 5. Thus, peak 6 was identified as gallic acid O-hexoside (Fathoni et al., 2017; Kelebek, 2016; Rio et al., 2004). Peaks 11 and 14 were identified as digalloylated hexoside and its isomer, because their deprotonated molecule ions [M-H]- displayed at m/z 483. Their MS²-3 fragment ions differed only in ion intensities. In the case of peak 11, MS² fragments at m/z 331 [M-152-H]- and 483 [M-60-H]- corresponded to the loss of a galloyl unit and the 0.3X cross-ring cleavage of a hexose, respectively. Its MS³ experiment of m/z 483 yielded fragment ions at m/z 271 [M-2galloyl-H]- and 169 corresponding to the gallic acid unit, consistent with that reported previously in digalloylated glucoside (Scoparo et al., 2012). Peak 16 showed a deprotonated molecule at m/z 633 [M-H]- and weak MS² fragments at m/z 463 [M-170-H]- (loss of a gallic acid unit) and 301 [M-(170+162)-H]-, corresponding to ellagic acid after a further loss of a hexose from the fragment at m/z 463. The ion at m/z 301 was assigned as ellagic acid instead of quercetin based on the MS² experiment of the ion at m/z 301, showing typical fragments at m/z 257, 229, and 185 ascribed to ellagic acid (Fathoni et al., 2017). Thus, peak 16 was tentatively identified as ellagoyl-galloyl-hexoside, probably ellagoyl-galloyl-glucoside reported previously in black tea (Kuhent et al., 2010). The MS²-3 spectra and the proposed fragmentation pathway of peak 16 were given in Figure S8. Peak 23 had a deprotonated ion at m/z 683, which was 152 Da higher than that of peak 14. This suggested that peak 23 possessed one more galloyl group, which was confirmed by MS³ fragment at m/z 313 [M-2galloyl-H]- dissociated from m/z 465 [M-170-H]-. Thus, peak 23 was tentatively identified as trigalloylated glucoside, consistent with the similar MS fragmentation characteristic observed for the same compound in Keemun black tea (Guo et al., 2018).

3.3.4. Catechin derivatives in Dianhong

Three catechin derivatives including peaks 15, 17, and 27 were detected. Peak 15 showed the deprotonated ion at m/z 289 [M-H]- and its deprotonated dimer ion at m/z 579 [2 M-H]-. The MS² fragment ions at m/z 245 (base peak), 205, and 179, typical fragment masses of (+)-catechin or (-)-epicatechin (Verloop et al., 2016), indicated losses of (CH₃)OH, five hydroxyl groups, and C-ring unit, respectively. Thus, peak 15 was tentatively identified as catechin or its stereoisomer, epicatechin, which was found as the most abundant components in all the four Dianhong samples. Peak 17 gave the quasi-molecular ion at m/z 577 [M-H]- and distinctive MS² fragments at m/z 425 [M-152-H]- and 407 [M-170-H]- that are matched with a galloyl group. The MS³ base peak at m/z 273 [M-2×152-H]- was indicative of the presence of another galloyl group attached on dehydroxyxcatechin or dehydroxyepicatechin (Chen et al., 2012). Thus, peak 17 was tentatively identified as digalloyl-dehydroxyxatechin or digalloyl-dehydroxyepicatechin. The MS¹-³ spectra and proposed fragmentation behavior of peak 17 were shown in Figure S9. To our knowledge, this compound was reported in Dianhong for the first time. Peak 27 gave the deprotonated molecular ion at m/z 441 [M-H]- and its deprotonated dimer ion at m/z 883 [2 M-H]-. Its MS² fragment at m/z 289 [M-152-H]- indicated a loss of a galloyl unit, and represented the ion of catechin or epicatechin, similar as that of peak 15. All the MS¹-³ data of peak 27 were highly consistent with those of epicatechin-3-gallate (Kelebek, 2016; Kuhent et al., 2010; Rio et al., 2004).

3.3.5. Flavone C-glycosides in Dianhong

A total of five flavone C-glycosides including one C- O-glycoside were detected. Peak 19 exhibited a [M-H]- ion at m/z 581, and the MS² diagnostic ions at m/z 461 [M-120-H]-, 491 [M-90-H]-, and 371 [M-(120 + 90)-H]- for hexose, along with the ions of [M-(60 + 90)-H]- at m/z 431 and [M-(90+90)-H]- ion at m/z 401, indicating a C-6-hexosyl-C-8-pentoside (Cao et al., 2014). The aglycone-related ion was detected at m/z 371, which was 2 Da higher than that of luteolin. This suggested that the aglycone was dihydro-luteolin, confirmed by the MS³ fragment ion at m/z 209 dissociated from MS² ion at m/z 371. Thus, peak 19 was tentatively identified as dihydroxy-C-6-hexosyl-C-8-pentoside, which is firstly characterized in Dianhong. Peaks 21, 22, 24, and 25 showed the similar fragmentation pattern (Table 2). The presence of the diagnostic ion pairs of [M-120-H]/[M-90-H]- or [M-90-H]/[M-60-H]- in MS² indicated that they might be flavone C-glycosides (Cao et al., 2014). Peak 21 showed the deprotonated ion at m/z 593 [M-H]-, the diagnostic ion pairs of [M-120-H]/[m/z 473, base peak]/[M-90-H]/[m/z 503] in MS², and [M-(120 + 120)-H] (m/z 353, base peak)/[M-(120 + 90)-H]- (m/z 383) in MS³, characteristic of flavone 6,8-di-C-hexosides. The base peak (m/z 353) in the MS³ spectrum corresponded to the aglycone of apigenin (Cao et al., 2014). Thus, peak 21 was identified as apigenin 6,8-di-C-hexoside. Peak 22 exhibited a [M-H]- ion at m/z 551, and MS² fragments at m/z 461 [M-90-H]-, 431 [M-120-H]-, 401 [M-150-H]-, 371 [M-180-H]-, and 491 [M-60-H]-, with the [M-90-H]- ion predominating, indicating the presence of a C-linked pentose unit. The MS³ spectrum of the fragment at m/z 461 produced the prominent ion pair of [M-(90 + 60)-H]- (m/z 401, base peak)/[M-(90 + 90)-H]- (m/z 371), suggesting the presence of another C-pentosyl substitution (Cao et al., 2014). The MS³ fragments (m/z 371 and 209) allowed the aglycone to be deduced as dihydroxideutelin, similar to those of peak 19. Thus, it was plausibly identified as dihydroxideutelin 6,8-di-C-pentoside, which is identified in Dianhong for the first time. The MS¹-³ spectra and the proposed fragmentation pathway of peak 22 were shown in Figure S10. Peak 24 had a [M-H]- ion at m/z 563, which yielded MS² fragments at m/z 443 [M-120-H]- and 473 [M-90-H]- (base peak). The relative intensities of these two ions suggested a C-pentosyl substitution at C-6 (Cao et al., 2014). However, the MS³ spectrum of the fragment at m/z 473 produced the prominent ion pair of [M-(90 + 120)-H]- (m/z 353, base peak)/[M-(90 + 90)-H]- (m/z 383), suggesting the presence of a C-8 hexosyl substitution. The base peak (m/z 353) in MS³ spectrum confirmed apigenin as its aglycone. Thus, peak 24 was identified as apigenin 6-C-pentosyl-8-C-hexoside (Scoparo et al., 2012). Peak 25 showed a deprotonated ion at m/z 593 [M-H]-, and produced MS²
3.3.6. Flavonoid O-glycosides in Dianhong

Peaks 26, 28–33, and 35 were all identified as flavonoid O-glycosides due to their similar MS²-3 fragmentation behaviors. Peak 26 showed a deprotonated ion [M-H]- at m/z 615. Its MS² spectrum displayed a base peak at m/z 463 [M-152-H]- and a minor fragment at m/z 301 [M-(152+162)-H]-, formed by successive losses of a galloyl and a hexosyl unit. Further MS³ fragmentation at m/z 463 led to produce an almost exclusive fragment ion at m/z 301, suggesting an inner hexose attached on the aglycone that was deduced as quercetin by the typical ion of m/z 301. Thus, peak 26 was identified as quercetin galloyl-hexoside (Scoparo et al., 2012). Peaks 28 and 29 showed deprotonated ions [M-H]- at m/z 609 and 465, respectively. The MS² spectra of these two O-glycosides displayed only one abundant Y₁⁺ ion at m/z 301, formed by the corresponding losses of a rutinose and a hexose. This ion at m/z 301 indicated that both of their aglycones were quercetin, which was further confirmed by the MS³ fragments at m/z 151 and 179 formed by retro Diels-Alder fragmentation. Thus, the peaks 28 and 29 were identified as quercetin 3-O-rutinoside (Kelebek, 2016; Rio et al., 2004) and quercetin 3-O-hexoside (Kelebek, 2016; Rio et al., 2004; Scoparo et al., 2012; van der Hooft et al., 2012), respectively. The MS¹-3 spectra and fragmentation pathway of peak 28 were given in Figure S11. Peaks 30–33 had deprotonated ions [M-H]- at m/z 593, 739, 593, and 447. These four peaks yielded aglycone diagnostic ions at m/z 285/284 as the base peak in MS³, suggesting that these compounds were all kaempferol glycosides containing different type and number of saccharide chains. The loss of 308 Da in MS² spectra of peaks 30 and 32 indicated a rhamnoglucoside moiety like rutinoside (1→6 linkage) or neohesperidoside (1→2 linkage) (Kelebek, 2016; Rio et al., 2004). Thus, peaks 30 and 32 were tentatively identified as kaempferol 3-O-rutinoside and kaempferol 3-O-neohesperidoside, respectively, based on their elution sequences on reversed-phase column (Cuyckens & Claeyts, 2002) and the occurrence of [M-120-H]- ion at m/z 473 (Shi et al., 2007) in peak 32. For peak 33, the MS² ions [Y₁⁺]+ (m/z 285) and Y₂⁻ (m/z 284) originated by the neutral loss of 162 Da, together with [Y₂⁻]+ > Y₁⁻ (Qin et al., 2017), suggested that peak 33 was kaempferol 3-O-hexoside (Guo et al., 2018; Kelebek, 2016; Rio et al., 2004). The MS³ spectrum of peak 31 showed a minor fragment ion at m/z 593 [M-146-H]- due to the loss of a terminal rhamnose. The potential loss of 308 Da between the MS² fragments at m/z 593 and 285, could be deduced as a rhamnoglucoside moiety. These fragments were highly consistent with those of kaempferol-3-O-rhamnosyl-hexosyl-rhamnose or kaempferol-3-O-[glucosyl(1→6) rhamnosyl]-4′-O-rhamnose (Guo et al., 2018; Rio et al., 2004; van der Hooft et al., 2012). Thus, peak 31 was tentatively identified as kaempferol 3-O-rhamnosyl-hexosyl-rhamnose. Peak 35 showed the deprotonated ion [M-H]- at m/z 901, along with a minor ion at m/z 755 [M-146-H]-. The MS³ fragments at m/z 609 [M-146-146-H]- and 301 [M-146-146-308-H]- suggested that this compound might be kaempferol O-glycoside containing at least three monosaccharide units. The occurrence of m/z 145 in MS² indicated a coumaroyl group. Thus, peak 35 was tentatively identified as quercetin 3-O-coumaryl-dihamnosyl -hexoside, which is consistent with the data reported by Scoparo et al. (2012).

3.3.7. Alkaloids and other components in Dianhong

Peak 8 had a deprotonated ion at m/z 411 [M-H]- that dissociated to give a base peak MS² ion at m/z 241 [M-170-H]-, indicating the loss of a galloyl unit, and the presence of another main MS² ion at m/z 331 [M-80-H]-, suggesting the loss of a sulfonic acid group (Plouguerne et al., 2013). Based on its fragmentation data (Table 2), peak 8 was tentatively identified as sulfochromene-3-O-gallate, which is firstly reported in Dianhong. Its MS¹-3 spectra and proposed fragmentation pathway were shown in Figure S12. The main peak 9 gave a protonated ion at m/z 181 [M+H]+ as the base peak, and no obvious ion was detected in the negative ion mode, indicating that it was an alkaloid. The MS² spectrum showed fragments at m/z 138 [M+2H]+ (base peak) and 163 [M+18+H]+. A further MS³ fragmentation of the ion at m/z 138 produced ions at m/z 110 and 69. These fragments were highly consistent with those of theobromine (Bartella et al., 2019), a widely occurring alkaloid in various types of tea. Peak 9 was thus identified as theobromine, and it is the only alkaloid detected in Dianhong under our experimental condition. Peak 12 gave a quasi-molecular ion at m/z 387 [M+HCOOH-H]-. And its MS² fragments at m/z 341 [M-H]- and 179 [M-162-H]- (typical masses in the negative mode of caffeoyl), indicated the loss of a hexose. The MS³ fragment at m/z 179 showed three abundant ions at m/z 164, 161 and 146, which were consistent with those of caffeoyl hexoside (Silva et al., 2019). Thus, peak 12 was tentatively characterized as caffeoyl hexoside.

Through the LC-MS analysis of the four Dianhong samples, four main components including catechin, gallic acid, 5-O-galloylquinic acid, and theobromine were found among their 34 common non-VOCs. These components, individually and in combination, significantly affect the taste profile of Dianhong. Several types of non-VOCs have been reported to affect the taste of tea (Yu et al., 2014). The alkaloid, theobromine contributes bitterness to the black tea, whereas catechin contributes both bitterness and astrigency. Gallic acid and 5-O-galloylquinic acid are the main umami-enhancing contributors in fermented tea (Wang et al., 2018).

4. Conclusion

In this study, we profiled the volatile and non-volatile compounds of Dianhong from four main producing areas by a combination of static headspace GC-MS and UPLC-ESI-IT-MS² method. A total of 42 VOCs were detected from four Dianhong samples, with 13 common VOCs accounting for 89.39–94.39% of the corresponding total VOCs. These common VOCs were proposed as important contributors to the characteristic aroma of Dianhong. Thirteen-four non-volatile components including quinic acid derivatives, gallic acid derivatives, catechin derivatives, flavone C-glycosides, flavonol O-glycosides, and alkaloids were successfully characterized in Dianhong, and six of them were reported for the first time.
time in Dianhong. The Dianhong black tea collected from four different producing areas in Yunnan shared almost same profile of non-volatile compounds, differing only in relative contents. The main component was catechin, followed by gallic acid, 5-O-galloylquinic acid, and theobromine. This is the first report on systematic profiling of volatile and non-volatile compounds of Dianhong collected from different producing areas. In summary, Dianhong from different producing areas showed similar profiles of the volatile and non-volatile compounds. However, more comprehensive experiments to explore the relationship between the components and the efficacy in exerting antioxidant and lipid-lowering activities are required for future studies, aided by the principal component analysis (PCA) and the partial least squares-discriminant analysis (PLS-DA).

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