Hindawi
Journal of Food Quality
Volume 2018, Article ID 1758381, 14 pages
https://doi.org/10.1155/2018/1758381

Research Article

Time-Related Changes in Volatile Compounds during Fermentation of Bulk and Fine-Flavor Cocoa *(Theobroma cacao)* Beans

Juan Manuel Cevallos-Cevallos [1,2] Laura Gysel,[3] Maria Gabriela Maridueña-Zavala,[1] and María José Molina-Miranda[1]

[1] *Escuela Superior Politecnica del Litoral (ESPOL), Centro de Investigaciones Biotecnológicas del Ecuador (CIBE), Campus Gustavo Galindo, Km 30.5 Vía Perimetral, Apartado 09015863, Guayaquil, Ecuador*
[2] *Escuela Superior Politecnica del Litoral (ESPOL), Facultad de Ciencias de la Vida (FCV), Campus Gustavo Galindo, Km 30.5 Vía Perimetral, Apartado 09015863, Guayaquil, Ecuador*
[3] *Department of Chemistry, Hogeschool Gent, Keramiekstraat 80, 9000 Ghent, Belgium*

Correspondence should be addressed to Juan Manuel Cevallos-Cevallos; jmceva@espol.edu.ec

Received 6 November 2017; Revised 15 March 2018; Accepted 22 March 2018; Published 2 May 2018

Academic Editor: Giuseppe Zeppa

Chocolate is one of the most consumed foods worldwide and cacao fermentation contributes to the unique sensory characteristics of chocolate products. However, comparative changes in volatiles occurring during fermentation of Criollo, Forastero, and Nacional cacao—three of the most representative cultivars worldwide—have not been reported. Beans of each cultivar were fermented for five days and samples were taken every 24 hours. Volatiles from each sample were adsorbed into a solid phase microextraction fiber and analyzed by gas chromatography-mass spectrometry. Aroma potential of each compound was determined using available databases. Multivariate data analyses showed partial clustering of samples according to cultivars at the start of the fermentation but complete clustering was observed at the end of the fermentation. The Criollo cacao produced floral, fruity, and woody aroma volatiles including linalool, epoxylinalool, benzeneethanol, pentanol acetate, germacrene, α-copaene, aromadendrene, 3,6-heptanedione, butanal, 1-phenyl ethenone, 2-nonanone, and 2-pentanone. Nacional cacao produced fruity, green, and woody aroma volatiles including 2-nonanone, 3-octen-1-ol, 2-octanol acetate, 2-undecanone, valencene, and aromadendrene. The Forastero cacao yielded floral and sweet aroma volatiles such as epoxylinalool, pentanoic acid, benzeneacetaldehyde, and benzaldehyde. This is the first report of volatiles produced during fermentation of Criollo, Forastero, and Nacional cacao from the same origin.

1. Introduction

Chocolate is one of the most consumed foods worldwide mostly because of the unique flavor and sensory characteristics of this product. Cacao *(Theobroma cacao)* beans are chocolate's main ingredient and the amount and type of volatile compounds in the beans are considered the most important indicators of cacao quality [1]. In fact, cacao varieties are generally classified as bulk/ordinary or fine-flavor depending on the volatiles that confer the aroma of the beans [2] and the characterization of volatile compounds has been proposed as a mechanism to assess the authenticity of fine-flavor cacao [3].

Bulk cacao is mostly produced in West Africa while fine-flavor cacao is mainly cultivated in Latin American countries. Bulk cacao represents more than 97% of the total cacao production but fine-flavor cacao can be commercialized at premium prices [4]. Around 29,500 cacao clones [5] and more than 10 genetic clusters [6] have been reported, the bulk cacao Forastero and the fine-flavor Criollo, Trinitario, and Nacional being the most representative cultivars worldwide [2]. In addition to the genotype, the flavor potential of raw cacao beans can be affected by the growing conditions of the tree such as climate and soil composition [1]. However, the final flavor profile of the beans is developed during the postharvest stages including fermentation, drying, and roasting. Fermentation is considered a crucial processing step in which flavor precursors are formed [7] and fermentation time has been reported as the key factor in the development of aroma compounds in cacao [8].

Cacao fermentation is characterized by a series of reactions occurring spontaneously after beans have been harvested, placed in containers, and allowed to stand for several days [9]. A 5-day fermentation period is common for the Forastero cultivar while shorter periods of about 2 to 3 days are required for Criollo and Nacional cultivars [10]. During fermentation, volatile flavor compounds are formed through microbial and enzymatic reactions occurring under both aerobic and anaerobic conditions. Compounds such as alcohols, aldehydes, ketones, organic acids, esters, pyrazines, and phenols, among others, are produced during fermentation, contributing to the final flavor profile of chocolate [2]. However, because the generation of volatile compounds can be affected by the cacao cultivar and environmental conditions [2], end products of highly variable quality are often produced [9]. Understanding the dynamics of volatile compounds occurring during fermentation of bulk and fine-flavor cacao has the potential to provide the means for assessing the beans quality during fermentation [11] and contribute to the manufacturing of uniform high quality products. Only few studies have characterized the dynamics of volatile compounds during fermentation of cacao varieties, including one report on the bulk Forastero cultivar [11, 12] and another report on various cacao hybrids [13] but the fine-flavor cultivars Criollo or Nacional were not assessed. A significant amount of knowledge about fermentation is still required to obtain good quality chocolate [14].

The volatile compounds present in Criollo, Forastero, and Trinitario raw beans from the same origin have been characterized [15] but changes occurring during fermentation were not reported. Similarly, comparisons of the volatile profile of dried-fermented beans of Criollo, Forastero, and Trinitario [16] as well as cocoa liquors made from Criollo, Forastero, Nacional, and Trinitario [8] have been carried out but samples were from different origins and time-related changes of volatile compounds during fermentation were not investigated.

To the best of our knowledge, analysis of volatile compounds produced during fermentation of Criollo, Forastero, and Nacional cacao from the same origin cannot be found in the literature. The objective of this research was to characterize the changes in the volatile profile occurring during the fermentation of Criollo, Forastero, and Nacional cacao from Ecuador.

2. Materials and Methods

2.1. Samples and Fermentation Process. Ripen cacao pods of the Forastero, Criollo, and Nacional cultivars were obtained from selected trees growing in the coast of Ecuador. All pods were harvested in mature stage, transported to the lab (about two-hour transport), and processed immediately. Pods were opened with a sterile knife and the beans were manually removed and fermented as described elsewhere [17, 18]. Briefly, 5 Kg of cacao beans was placed in cylindrical plastic containers of about 16 cm diameter and 20 cm height and covered with a plastic film. Holes were drilled at the bottom of each container to allow pulp draining. Spontaneous fermentation was carried out in a greenhouse at ambient temperature (about 35°C) and samples of fermenting beans (about 50 g) were removed daily for five days and stored at −18°C until analyzed (approximately 5 days). Four different fermentation batches were run for each cultivar. Cacao samples were considered fully fermented after 3 (Criollo and Nacional) or 5 (Forastero) days of fermentation [10] but evaluation continued for 5 days for all cultivars.

2.2. Analysis of Volatiles. Volatiles were characterized in each sample by headspace solid phase microextraction (HS-SPME) following the methodology suggested in previous studies [18] with few modifications. Beans from each sample were ground under liquid nitrogen and about 0.5 g of ground material was transferred into 50 mL SPME vials. Samples were then equilibrated at 55°C for 30 min in a water bath. A 50/30 μm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) SPME fiber (Supleco, Bellefonte, PA, USA) placed in a 24 Ga manual holder was exposed to the headspace of each sample for 30 minutes at 55°C. The fiber was then removed from the vial and splitlessly injected into a 7890A GC coupled to a 5975C MS (Agilent Technologies, Santa Clara, CA) with a DB5-MS column of dimensions 30 m × 250 μm × 0.25 μm. The injector temperature was 240°C; the oven was initially held at 70°C and raised to 310°C by a 7°C min−1 increase rate without holding times. Ultrapure helium was used as the carrier gas at 0.8 mL/min. The MS was tuned to maximum sensitivity in electron impact mode, positive polarity, and the total ion current was recorded for a mass range of 40–750 amu. The GC-MS interface was set to 250°C and the scan was recorded with a frequency of 4 s^{-1}. The SPME fiber was conditioned prior to its first use at 270°C for 1 h and at the start of every day at 240°C for 10 min. Data was acquired using ChemStation E.02.02 (Agilent Technologies, Inc.) and volatile compounds were putatively identified by MS spectra matching using both NIST 11 and Wiley 9 databases. Metabolite identity was then confirmed by comparing the linear retention index of each compound with that of the pure standard using our internal database and compounds were quantified by estimating the peak area as suggested for untargeted analysis [19, 20]. The peak area was calculated using the ChemStation software which integrates the total ion current signal intensity (TIC) over the retention time differential in minutes for each metabolite. Preliminary GC-MS runs showed that peaks were too close to each other throughout the chromatogram and the use of internal standards would have interfered with the chromatographic peaks. For this reason, normalization and quality control (QC) techniques not relaying on internal standards were applied, including normalization to the total area [20, 21] and QC by running the same sample after specific intervals [22, 23]. The quality of the headspace

GC-MS runs was assessed by running one selected sample after 5 consecutive sample runs and estimating the variations in retention time and peak areas. Maximum acceptable coefficient of variation was 30% for a given metabolite in QC runs [22, 23]. Aroma descriptors were obtained for each compound detected using the online databases Flavornet [24] and The Good Scents Company [25] as suggested in recent reports [26, 27]. All four fermentation batches were analyzed twice at each fermentation time.

2.3. Statistical Analysis. The peak area of each volatile was obtained and aligned using an in-house alignment protocol. Principal components analysis (PCA) and cluster analysis were carried out to compare to overall volatile profile of each sample. Significant differences in the levels of individual volatile compounds were estimated using t-test at 95% confidence level. All data analyses were carried out using XLSTAT 2015.4 (Addinsoft, Paris).

3. Results and Discussion

3.1. Analysis of Volatile Profile. A total of 121 volatile compounds were detected of which 62 were positively identified including nine organic acids, 12 alcohols, 14 aldehydes and ketones, five esters, 12 hydrocarbons, two amines, two furans, one sulfur, and five others. Table 1 shows the aroma descriptors and the samples in which identified compounds were detected. Results are similar to previous observations in which 136 mases (36 identified) of volatile organic compounds in chocolate [28] as well as 53 identified volatiles in raw cacao beans [15] were reported. The main compound groups detected are in agreement with previous studies reporting volatile acids, alcohols, aldehydes, ketones, esters, hydrocarbons, and furans in beans of Criollo, Forastero, and Trinitario cacao [15].

Before the start of the fermentation, differences in the volatile profile of the three cultivars tested were mostly quantitative (Figure 1), similar to what was observed in previous studies [15]. The predominant volatiles in fresh beans of Criollo cacao were hexanal, pentanal, and 1,3-cyclohexadiene, potentially contributing to the almond and grass aroma notes previously reported for raw beans of this cultivar [15]. Similarly, Nacional cacao showed high amounts of butanal, ethanol, and 5-hepten-2-ol. The high amounts of butanal potentially provide the fruity aroma characteristic of fresh beans of Nacional cacao. Interestingly, the diversity of predominant volatiles observed in fresh beans of Forastero cacao was high as aroma compounds such as 1-phenyl ethenone (flower-like aroma), cyclohexanol (camphoreous aroma), α-copaene (woody aroma), 2-nonanone (fruity aroma), and 2-acetoxytetradecane were detected. Previous reports on volatiles from Forastero cacao products have diverged as this cultivar showed the lowest volatile diversity when compared to Criollo and Trinitario in some studies [15] but other researchers reported a similar number of unique volatiles in Forastero and Criollo cacao liquors [8]. Additionally, liquors made from Forastero cacao showed the highest aroma levels when compared to Nacional and Trinitario liquors [8]. Results suggest that environmental variables can have a significant role in the volatile profile of cacao beans. Other compounds such as thiobis-methane, furan, hexanoic acid, and octacosane were predominant in the three cultivars analyzed (Figure 1).

By the end of the fermentation of Criollo cacao (3 days), predominant amounts of volatiles with floral, fruity, and woody aromas including linalool, epoxylinalool, benzeneethanol, pentanol acetate, germacrene, α-copaene, aromadendrene, 3,6-heptanedione, butanal, 1-phenyl ethenone, 2-nonanone, and 2-pentanone were detected. Results are similar to previous studies reporting fruity and floral aroma characters in dried-fermented Criollo cacao [29]. However, volatiles with potentially undesirable rancid, waxy, or sulfurous aroma including propanoic acid, cyclohexanol, and thiobis-methane, as well as compounds with no database aroma records such as 1-amino-1-ortho-chlorophenyl-2-(2-quinoxaliny)ethene, and undecylamine were among the main compounds in Criollo cacao after a 3-day fermentation period. Normal presence of waxy, rancid, and sulfurous aroma compounds has been reported in cocoa samples from various origins [30] and has been related to inappropriate posthandling of the pods [31] but the reported overall effect of undesirable compounds on sensory scores of fermented Criollo cacao has been low [29]. Similarly, Nacional cacao showed predominant amounts of volatiles with fruity, green, and woody aromas at the end of the fermentation (3 days), including 2-nonanone, 3-octen-1-ol, 2-octanol acetate, 2-undecanone, valencene, and aromadendrene. Results are in agreement with the fruity, floral, and green aroma descriptions of fermented Nacional cacao clones from Ecuador [32]. Similar to the Criollo cultivar, potentially undesirable sulfur and pungent aroma compounds were observed in Nacional cacao, including thiobis-methane and naphthalene, as well as compounds with no database aroma records including 6-undecylamine, 3-fromyl-N-methyl-9-[phenylethynyl]dibenzo[2,3-a:5,6-a](1,4)-thiazine, and 1,3 cyclohexadiene. A 5-day fermentation period of the Forastero cacao yielded predominant amounts of floral and sweet aroma volatiles such as epoxylinalool, pentanoic acid, benzeneacetaldehyde, and benzaldehyde but compounds with potential undesirable rancid, fatty, fishy, cheesy, and acid aroma including butanoic acid, hexanoic acid, pyridine, heptanoic acid, and peracetic acid as well as 1-phenylpent-4-en-2-ol with no aroma record were also among the main compounds found in fermented samples of this cultivar (Figure 1). Results are in agreement with previous studies showing that identical aroma characters with differences in few of the volatiles as pentanoic acid, pyridine, and peracetic acid were not observed in previous reports of the Forastero cultivars [11, 12, 33]. Both pentanoic acid [7] and pyridine [28] have been reported in cacao products made from unidentified cultivars. Differences in cultivars and fermentation practices may affect the formation of various volatiles. In this study, the presence of epoxylinalool and peracetic acid suggests oxidation reactions during

Table 1: Volatile compounds and aroma descriptors found at different sampling stages.

Chemical group	Compound	Aroma descriptor*	Sample**
Acids	Carbamic acid	NF	C0, C2, F0, F1, F2, N1, N2
	Acetic acid	Pungent, stinging sour	C1, C2, C3, C4, C5, F1, F2, F3, F4, F5, N1, N2, N3, N4, N5
	Peracetic acid	Pungent, stinging sour	C4, C5, F4, F5, N4, N5
	Propanoic acid	Pungent, rancid, soy	C1, C2, C3, C4, C5, F2, F3, F4, F5, N2, N3, N4, N5
	Butanoic acid	Rancid, cheese, sweat	C2, C3, C4, C5, F2, F3, F4, F5, N2, N3, N4, N5
	Hexanoic acid	Fatty	C5, F5, N5
	Heptanoic acid	Cheesy	C4, C5, F4, F5, N4, N5
	Pentanoic acid	Sweat	C2, C3, C4, C5, F2, F3, F4, F5, N2, N3, N4, N5
	4-Hexanoic acid	Fatty	C0, F0, N0
Alcohols	Ethanol	sweet	C0, C1, F1, N0, N1
	2-Pentanol	Green	C1, F1, N1
	1-Butanol	Medicine, fruit, wine	N1
	2,3-Butanediol	Fruit, onion	C0, C1, C2, C3, C4, C5, F0, F1, F2, F3, F4, F5, N0, N1, N2, N3, N4, N5
	5-Hepten-2-ol	NF	C0, C1, F0, F1, N0, N1
	Cyclohexanol	Camphoraceous	C0, C1, C2, C3, C4, C5, F0, F1, F2, F3, F4, F5, N0, N1, N2, N3, N4, N5
	2-Heptanol	Citrus	C0, C1, C2, C4, F0, F1, F2, F3, F4, F5, N0, N1, N2, N4, N5
	L-Linalool	Flower, lavender	C0, C1, C2, C3, C4, C5, F0, F1, F2, F3, F4, F5, N0, N1, N2, N3, N4, N5
	Benzeneethanol	Honey, spice, rose, lilac	C0, C1, C2, C3, C4, C5, F0, F1, F2, F3, F4, F5, N0, N1, N2, N3, N4, N5
	Epoxylinalol	Flower	C3, C4, C5, F4, F5, N5
	3-Octen-1-ol	Fruity	C3, F3, F4, N3, N4, N5
	1-Phenylpent-4-en-2-ol	NF	C5, F5
Aldehydes & Ketones	Butanal	Fruity, green, banana/pungent	C0, C1, C2, C3, C4, C5, F0, F1, F2, F4, F5, N0, N1, N2, N3, N4, N5
	2-Pentanone	Fruity	C1, C2, C3, C4, C5, F2, F3, F4, F5, N2, N3, N4, N5
	2-Butanone	NF	C1, C2, C3, C4, C5, F1, F2, F3, F4, F5, N1, N2, N3, N4, N5
	Pentanal	Almond, malt, pungent	C0, C2, F0, N1, N2
	Hexanal	Grass, tallow, fat	C0, C1,
	2-Heptanone	Fruity Floral	C0, C1, C2, C3, C4, C5, F1, F2, F3, F4, F5, N0, N1, N2, N3, N4, N5
	Benzaldehyde	Cherry, candy, almond, burnt sugar	C0, C1, C2, C3, C4, C5, F0, F1, F2, F3, F4, F5, N0, N1, N2, N3, N4, N5
	3,6-Heptanedione	NF	C0, C1, C2, C3, C4, C5, F0, F1, F2, F3, F4, F5, N0, N1, N2, N3, N4, N5
	Benzeneacetaldehyde	Green	C0, C1, C2, C3, C4, C5, F0, F1, F2, F3, F4, F5, N0, N1, N2, N3, N4, N5
	Ethanone, 1-phenyl	Must, flower, almond	C0, C1, C2, C3, C4, C5, F0, F1, F2, F3, F4, F5, N0, N1, N2, N3, N4, N5
	2-Nonanone	Fruity	C0, C1, C2, C3, C4, C5, F0, F1, F2, F3, F4, F5, N0, N1, N2, N3, N4, N5
	Decanal	Soap, orange peel, tallow	C1, C2, F1, F2, F3, N1, N2
	2-Undecanone	Fruity	C1, C2, C3, C4, C5, F1, F2, F3, F4, F5, N1, N2, N3, N4, N5
	Tetramethoxyisoquino(1,2-b)quinazolin-8-one	NF	C0, C1, C2, C3, C4, C5, F0, F1, F2, F4, F5, N0, N1, N2, N3, N4. N5

Table 1: Continued.

Chemical group	Compound	Aroma descriptor*	Sample**
Esters	2-Pentanol acetate	Bananas	C0, C1, C3, C4, F0, F1, F3, F4, N0, N1, N4
	2-Heptanol, acetate	NF	C0, C1, C2, C3, C4, C5, F0, F1, F2, F3, F4, F5, N0, N1, N2, N3, N4, N5
	2-Octanol, acetate	Fruity	C0, F0, F1, N0, N3
	2-Octenyl-acetate	NF	F1, N4
	Benzoic acid, pentyl ester	Floral	C0, C1, C2, C3, C4, C5, F0, F1, F2, F3, F4, F5, N0, N1, N2, N4, N5
Hydrocarbons	2,4-Diacetoxypentane	NF	C0, F0, N0, N1
	1,3-Cyclohexadiene	NF	C0, N3
	Naphthalene	Pungent, dry, tarry	C0, C1, C2, C3, C4, C5, F0, F1, F2, F3, F4, F5, N0, N1, N2, N3, N4, N5
	2-Acetoxytetradecane	NF	C0, C1, C2, C3, C4, F0, F1, F2, F3, F4, F5, N0, N1 N2, N3, N4, N5
	Nonacosane	NF	C2, C3, C4, C5, F2, F3, N2, N4, N5
	α-Copaene	Wood, spice	C0, C1, C2, C3, C4, C5, F0, F1, F2, F3, F4, F5, N0, N1, N2, N4, N5
	Germacrene	Wood	C3, F3
	Valencene	Green oil	C3, N3
	Trimethylacenaphthylene	NF	F1, F2, F3, F4, F5, N4
	Aromadendrene	Wood	C3, N3
	Octacosane	NF	C0, F0, N0
	Octane	Alkane	C1, C2, C3, C4, C5, F1, F2, F3, F4, F5, N2, N4, N5
Amines	Pyridine	Fishy	C4, C5, F4, F5, N4, N5
	6-Undecylamine	NF	C3, N3
Furans	Trimethyltetrahydrofuran	NF	C0, C1, N1
	Furan	NF	C0, F0, N0
Sulfur	Methane, thiobis-	Sulfurous	C0, C3, C4, F0, F3, F4, N0, N3, N4
Others	Carbon dioxide	NF	C3, C4, C5, F3, F4, F5, N3, N4, N5
	1-Amino-1-ortho-chlorophenyl-2-(2-quinoxaliny)ethene	NF	C0, C1, C2, C3, C4, C5, F0, F1, F2, F3, F4, F5, N0, N1, N2, N3, N4, N5
	3-Fromyl-N-methyl-9-[phenylethynyl]dibenzo[2,3-a:5,6-a](1,4)-thiazine	NF	C0, C1, C2, C3, F0, F2, N0, N1, N2, N3
	Caffeine	NF	C1, C2, C3, C4, C5, F5, N1, N4
	2-Methil-5H-dibenz[b,f]-azepine	NF	C0, C1, C2, C3, F0, F1, F2, F3, N1, N2, N3

*NF = not found. **First digit represents the cultivar (C = Criollo, F = Forastero, and N = Nacional) and the second digit the fermentation time in days.

fermentation. Oxidation reactions commonly occur during fermentation of cacao [7].

Heatmap analyses revealed two main sample clusters, including one group with samples from 0 to 2 fermentation days and another group with samples from 3 to 5 fermentation days. No clustering of cacao cultivars was observed in heatmap analysis (Figure 1). Heatmaps are tools used for visualization of metabolite levels and are commonly built using average values with no consideration of the variation among samples. Other multivariate tools including PCA take into account the variance of the samples, allowing additional clustering. In this study, the PCA run using the overall volatile profile of each sample showed partial clustering of the raw beans from the different cacao cultivars. However, a more notorious clustering was observed when fermented beans of the three cultivars were compared. The main volatiles responsible for the clustering included organic acids such as propanoic, butanoic, and heptanoic acids; alcohols such as linalool, benzeneethanol, 2,3-butanediol, cyclohexanol, 2-heptanol, 2-octanol, and 3-octen-1-ol; aldehydes and ketones such as 1-phenyl ethenone, 2-undecanone, and butanal; esters like 2-pentanol acetate, hydrocarbons such as nonacosane and 1,3 cyclohexadiene, among others (Figure 2). In general, fresh beans of cacao are considered bitter and astringent but aroma compounds are developed after fermentation. The more notorious clusters observed in fully fermented

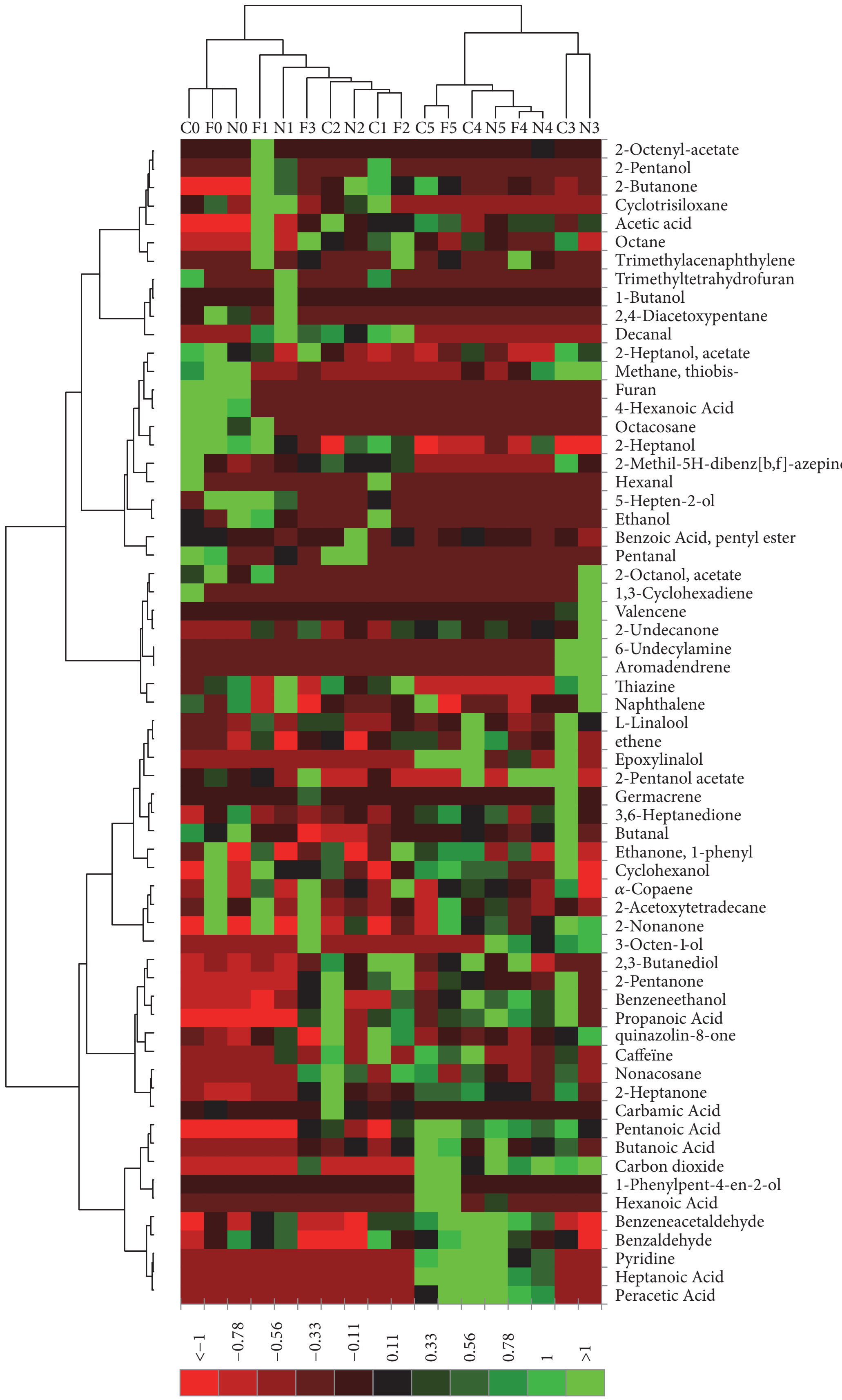

Figure 1: Heatmap of the cluster analysis of identified volatile compounds. Sample codes are as in Table 1.

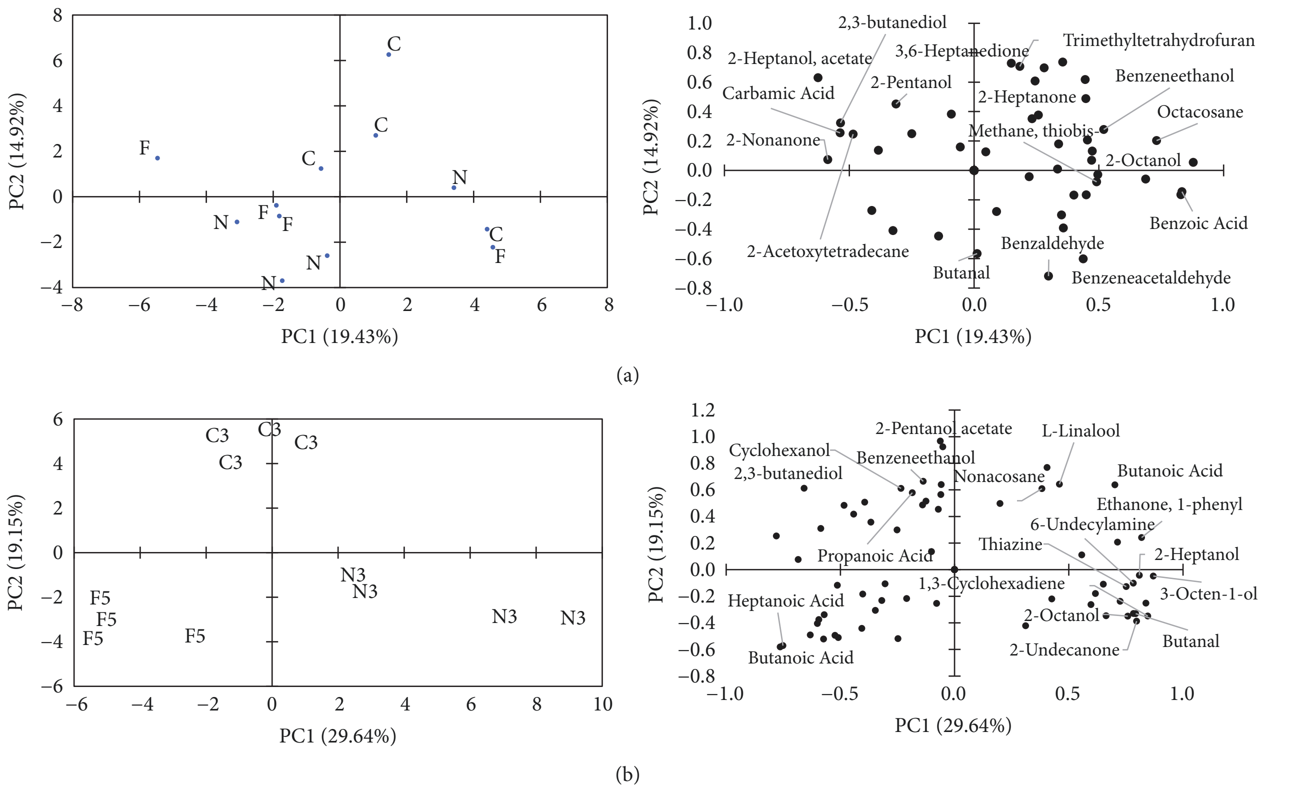

Figure 2: Principal components analysis score and loading plots of fresh (a) and fully fermented (b) beans. Sample codes are as in Table 1. Values in parenthesis represent the percentage of variance explained by each component.

beans suggest cultivar-dependent production of volatile compounds and characteristic volatile profiles of fermented beans. To the best of our knowledge, this is the first report of volatile profiling of fermented beans of Criollo, Forastero, and Nacional cacao cultivars.

3.2. Analysis of Individual Volatiles. Statistical t-test analysis of individual metabolites showed significant differences in 28 identified compounds, including four organic acids, six alcohols, four esters, and eight hydrocarbons, among others. Figures 3, 4, 5, 6, and 7 show the dynamics of the acid, alcohol, ester, hydrocarbon, and other volatiles, respectively, showing significant differences between cultivars (significant volatiles).

Among the significant volatiles, no organic acids were detected in any cacao cultivar at the start of the fermentation. Small chain fatty acids such as propanoic and butanoic acids were produced from day 2 whereas big chain fatty acids like heptanoic and hexanoic acids were produced from day 5 in all cacao cultivars. Production of significant organic acids during cacao fermentation has been reported to start from day two and has been attributed to the metabolism of sugars from the pulp [30]. The Criollo cacao produced significantly higher amounts of organic acids including propanoic and butanoic acids when compared to Nacional and Forastero cultivars at day 3 of fermentation. Chocolate made from Criollo cacao has been reported to have more acidic tones than chocolate produced from other cultivars [34]. At the end of a regular fermentation period for fine-flavor cacao (3 days), the Nacional cacao showed the lowest amounts of all acids. Similarly, after a 5-day fermentation, the levels of hexanoic acid were the lowest ($p < 0.05$) in Nacional cacao but no significant differences were observed in the other acids (Figure 3). Results correlate with the weak acid aroma character reported for liquors produced from Nacional cacao [32].

Among significant alcohols, only linalool and benzeneethanol were present in all cultivars at the start of the fermentation. The presence of both alcohols has been reported in fresh beans of Criollo, Forastero, and Trinitario [15]. Alcohol production was observed from days 1, 2, 3, and 5 for cyclohexanol, benzeneethanol, 3-octen-1-ol, and 1-phenylpent-4-en-2-ol, respectively. Alcohol production during the first fermentation days of cacao beans has been previously observed [12] and is probably associated with microbial and enzymatic activities occurring during fermentation. Samples from Criollo cacao produced significantly higher amounts of cyclohexanol than the Nacional cacao and significantly increased quantities of benzeneethanol, linalool, and epoxylinalool were observed in Criollo cacao when compared to the other two cultivars after a 3-day fermentation. Benzeneethanol, linalool, and epoxylinalool potentially provide the honey-like and flowery aroma characters reported in Criollo [29]. The Forastero cacao produced significantly higher amounts of 1-phenylpent-4-en-2-ol and epoxylinalool than the Nacional cacao after a 5-day fermentation (Figure 4).

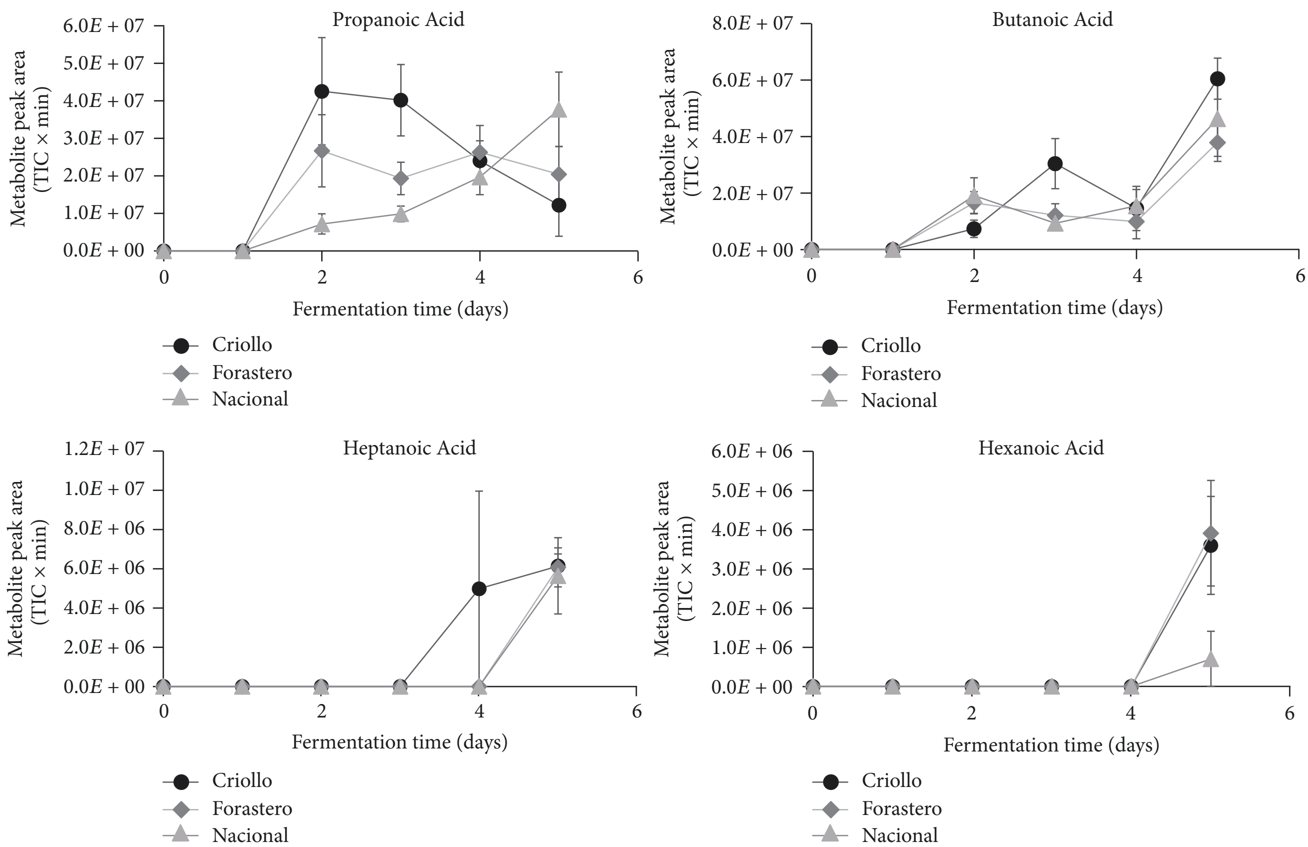

Figure 3: Levels of significant organic acids in Criollo, Nacional, and Forastero beans at different fermentation times. Levels of each metabolite are presented as peak area (total ion current times the differential retention time for each metabolite in minutes).

At the start of the fermentation, the Forastero cultivar showed the highest levels of significant esters including 2-pentanol acetate, 2-heptanol acetate, and 2-octanol acetate. The presence of esters have been reported in fresh beans of Criollo, Forastero, and Trinitario cultivars [15]. In general, production of esters was the highest at 2–4 fermentation days, decreasing afterwards. Except for the significant esters, most esters decreased when increasing fermentation time in all cultivars tested (Figure 1). In general, the total concentration of esters has been reported to decrease during the fermentation of Forastero cacao [12] but certain individual esters such as 2-pentanol acetate have been reported to increase as the fermentation progressed, probably due to yeast activity [33]. The Nacional cacao yielded the lowest amounts of 2-pentanol acetate but the highest amounts of 2-octanol acetate ($p < 0.05$) after 3 days of fermentation, potentially contributing to the fruity and floral aroma notes previously observed in liquors of this cultivar [32]. Similarly, the Nacional cacao produced the highest levels of benzoic acid pentyl ester at day 2 but the levels were negligible after day 3 (Figure 5).

Hydrocarbons have been considered among the important contributors to the aroma of cacao beans [15, 35]. At the start of the fermentation, the Forastero cacao showed the highest levels of α-copaene ($p < 0.05$) while Nacional cacao presented the lowest levels of octacosane ($p < 0.05$). Results are different to previous reports showing higher amounts of α-copaene in fresh beans of Criollo when compared to Forastero [15] suggesting potential differences in the cacao genotype and environmental growth conditions in the different regions the studies took place. No significant differences were observed when comparing the rest of hydrocarbons at time zero. Nacional cacao showed the lowest levels of α-copaene and nonacosane but the highest levels of valencene and 1,3 cyclohexadiene at day 3 of fermentation potentially contributing to the green aroma described for liquors of this cultivar [32]. Similarly, at day 3, Forastero cacao showed the lowest levels of aromadendrene but the highest levels of trimethylacenaphthylene. Additionally, Forastero cacao showed significantly higher amounts of germacrene than Nacional cacao at day 3. However, the levels of aromadendrene, valencene, germacrene, 1,3 cyclohexadiene, and octacosane were negligible after 4 days of fermentation in Forastero cacao (Figure 6). Results are in agreement with previous studies in which hydrocarbons were not reported among the volatile compounds found during fermentation of Forastero [11, 12, 33]. Results suggest that hydrocarbons are one of the main contributors to the aroma differentiation of fermented fine-flavor cacao.

At the start of the fermentation, the levels of benzaldehyde and 2-nonanone were the highest ($p < 0.05$) in Nacional and Forastero beans, respectively, but no significant differences were observed after 1 day of fermentation. Previous studies

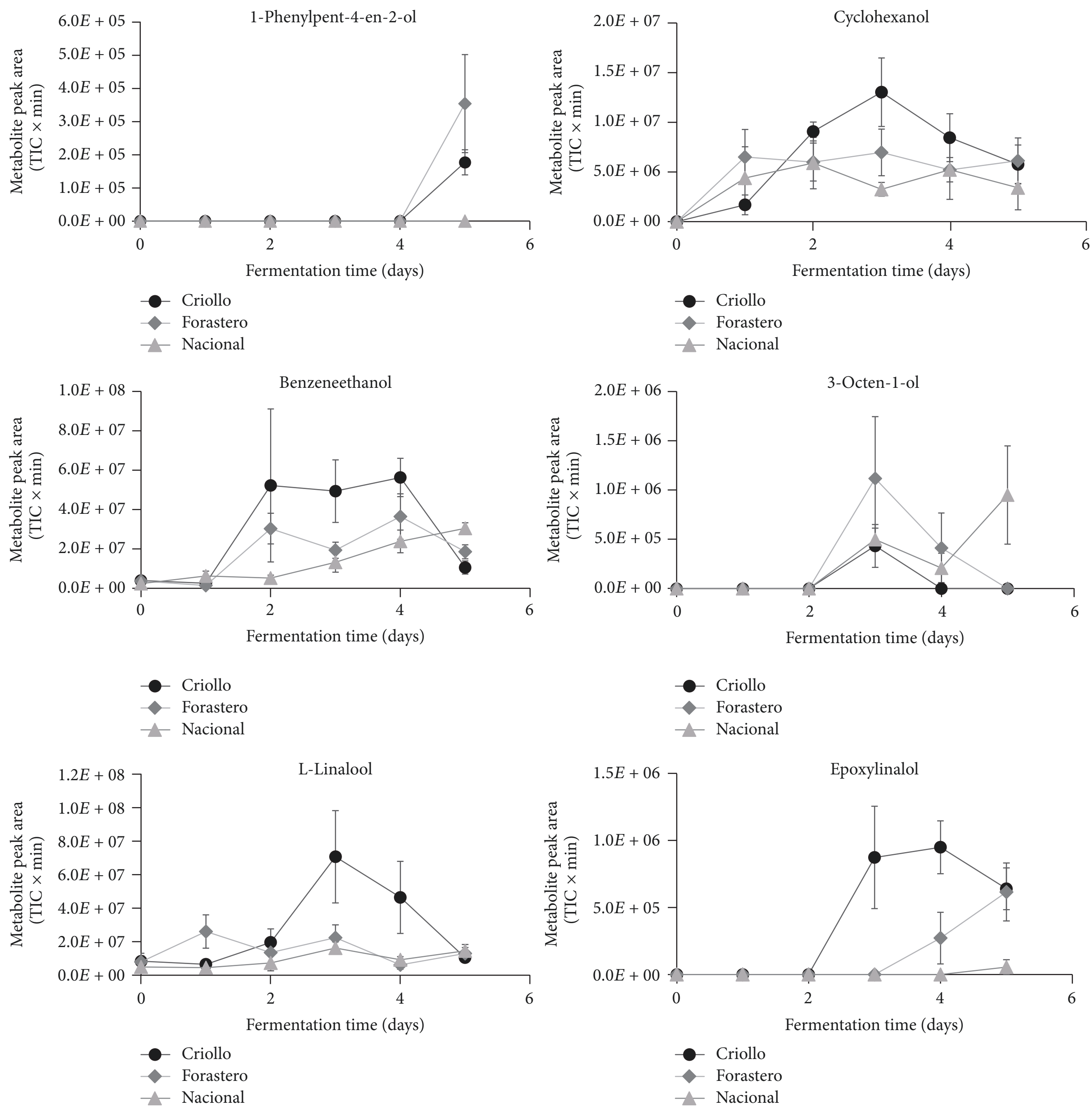

Figure 4: Levels of significant alcohols in Criollo, Nacional, and Forastero beans at different fermentation times. Levels of each metabolite are presented as peak area (total ion current times the differential retention time for each metabolite in minutes).

reported no benzaldehyde and low amounts of 2-nonanone in fresh Forastero beans, suggesting differences in the cacao genotype as well as growth and fermentation conditions [15] in the different regions where the studies took place. The levels of 1-phenyl ethenone were significantly higher in Criollo cacao when compared to the other cultivars at day 3 of fermentation and potentially contribute to the flowery/almond-like aroma character previously reported for the Criollo cultivar [29]. Similarly, the Nacional cacao produced significantly higher amounts of butanal than the other cultivars at day 3, potentially contributing to the fruity aroma reported for this cultivar [32]. The levels of 3-fromyl-N-methyl-9-[phenylethynyl]dibenzo[2,3-a:5,6-a](1,4)-thiazine were negligible in Forastero cacao ($p < 0.05$) from day 3 onwards. Levels of 6-undecylamine were only detected at day 3 of fermentation in Criollo and Nacional cultivars (Figure 7).

Cacao fermentation is a key processing step for chocolate production. Various reports have characterized the volatile compounds in cacao and chocolate produced from different cultivars and different origins as Forastero cacao is predominantly produced in Africa while Criollo and Nacional can be found in South America. Additionally, genetic variations

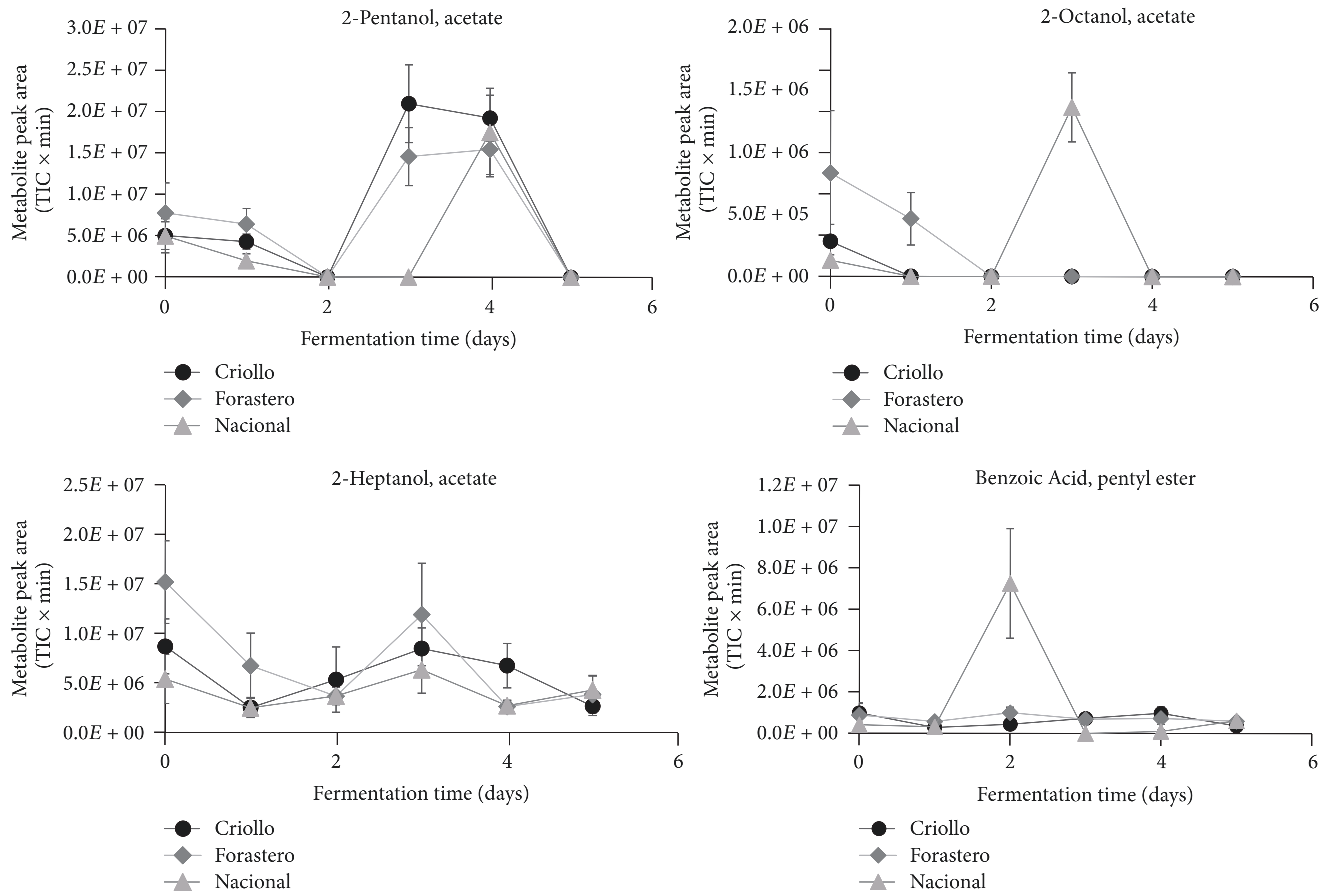

Figure 5: Levels of significant esters in Criollo, Nacional, and Forastero beans at different fermentation times. Levels of each metabolite are presented as peak area (total ion current times the differential retention time for each metabolite in minutes).

within cultivars have been reported in different countries due to locally adapted varieties [34, 36]. Also, different environmental growth and fermentation conditions have been reported to affect the final flavor profile of the beans as cocoa batches of the same cultivar but from different countries have shown significant differences in various flavor attributes [34]. Therefore, further research is needed to assess the effect of environmental conditions in the aroma of cacao products and fully characterize the main commercial cultivars. To the best of our knowledge, this is the first fermentation report of Criollo, Forastero, and Nacional cultivars from the same origin.

4. Conclusions

This work characterized for the first time the dynamics of volatile compounds during fermentation of Criollo, Nacional, and Forastero cacao beans from the same origin.

The volatile profile of fresh beans of each cultivar allowed a partial PCA grouping of the samples, but a complete grouping was observed at the end of the fermentation. Fermentation of Criollo cacao was characterized by the production of volatiles with floral, fruity, and woody aromas including linalool, epoxylinalool, benzeneethanol, pentanol acetate, germacrene, α-copaene, aromadendrene, 3,6-heptanedione, butanal, 1-phenyl ethenone, 2-nonanone, and 2-pentanone. Similarly, fermentation of Nacional cacao produced volatiles with the characteristic fruity, green, and woody aromas including 2-nonanone, 3-octen-1-ol, 2-octanol acetate, 2-undecanone, valencene, and aromadendrene. The fermentation of Forastero cacao yielded predominant amounts of floral and sweet aroma volatiles such as epoxylinalool, pentanoic acid, benzeneacetaldehyde, and benzaldehyde. Volatiles with undesirable aroma descriptors were also produced during the fermentation of all tested cultivars but the impact on the overall aroma of fermented beans is expected to be low. Fine-flavor cultivars were characterized by the presence of significant amounts of hydrocarbons such as valencene, aromadendrene, germacrene, 1,3 cyclohexadiene, and octacosane while fermented beans of the Forastero bulk cacao showed negligible levels of these volatiles.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Laura Gysel carried out the experimental design and ran part of the SPME-GCMS analyses guided by Juan Manuel

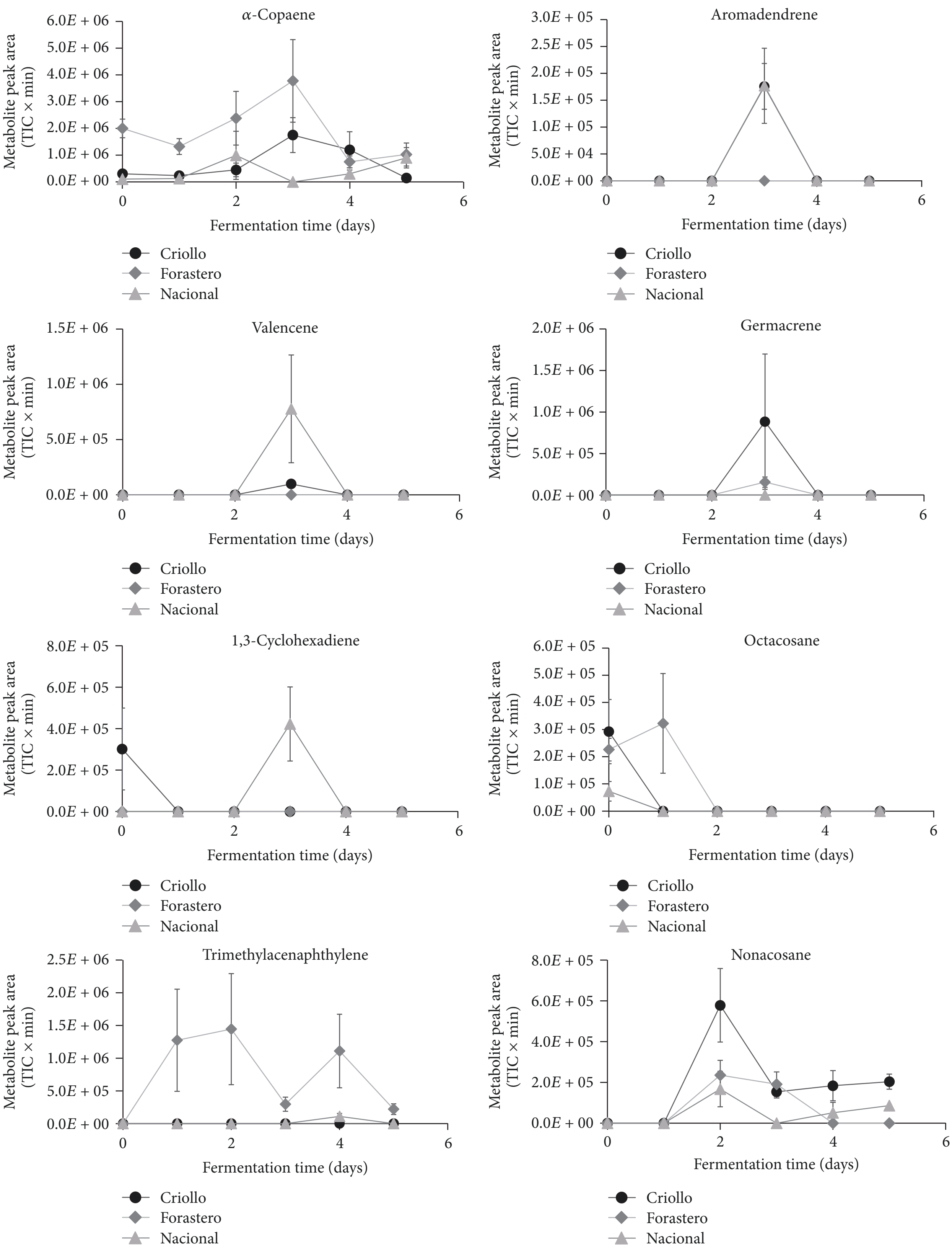

Figure 6: Levels of significant hydrocarbons in Criollo, Nacional, and Forastero beans at different fermentation times. Levels of each metabolite are presented as peak area (total ion current times the differential retention time for each metabolite in minutes).

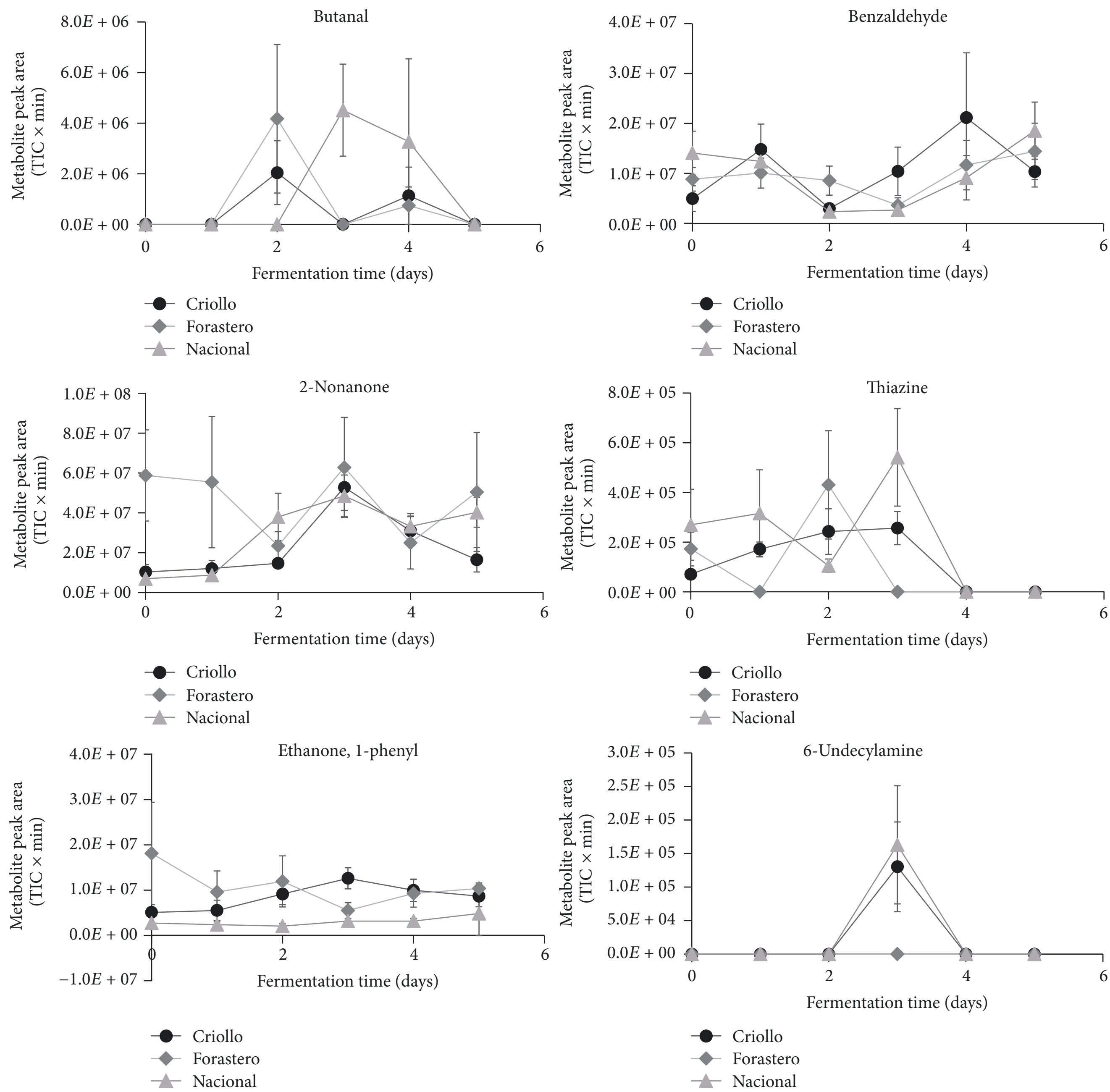

Figure 7: Levels of significant volatiles classified as others in Criollo, Nacional, and Forastero beans at different fermentation times. Levels of each metabolite are presented as peak area (total ion current times the differential retention time for each metabolite in minutes).

Cevallos-Cevallos. Gabriela Maridueña ran SPME-GCMS analyses and carried out peak alignment. María José Molina-Miranda carried out compound identification and statistical analysis. The manuscript was written by Juan Manuel Cevallos-Cevallos.

Acknowledgments

The authors want to thank Andres Ochoa for providing invaluable help in the tree selection for this study. This research was funded by a VLIR-NETWORK grant to JMC. Access to cacao samples were granted by the National Authority under Permit no. MAE-DNB-2017-0266-O.

References

[1] J. E. Kongor, M. Hinneh, D. V. de Walle, E. O. Afoakwa, P. Boeckx, and K. Dewettinck, "Factors influencing quality variation in cocoa (Theobroma cacao) bean flavour profile—a review," *Food Research International*, vol. 82, pp. 44–52, 2016.

[2] A. C. Aprotosoaie, S. V. Luca, and A. Miron, "Flavor chemistry of cocoa and cocoa products-an overview," *Comprehensive*

Reviews in Food Science and Food Safety, vol. 15, no. 1, pp. 73–91, 2016.

[3] F. Amores, D. Butler, and G. Ramos, "Chemical, Physical and Organoleptic Parameters to Establish the Difference Between Fine and Bulk Cocoa," https://icco.org/projects/projects-home/10-projects/144-study-the-chemical-physical-and-organoleptic-parameters-to-establish-the-difference-between-fine-and-bulk-cocoa.html, 2007.

[4] S. Kooij van der, "Market study of fine flavour cocoa in 11 selected countries – revised version Pre – read of revised version Overview of the main revisions. R Trop Inst, 1–109," 2013.

[5] C. J. Turnbull and P. Hadley, "International cocoa germplasm database," vol. 20, CRA Ltd/ICE Futur Eur Reading, UK, 2017, http://www.icgd.reading.ac.uk.

[6] J. C. Motamayor, P. Lachenaud, and J. W. da Silva e Mota, "Geographic and genetic population differentiation of the amazonian chocolate tree (Theobroma cacao L)," *PLoS ONE*, vol. 3, pp. 1–8, 2008.

[7] E. O. Afoakwa, A. Paterson, M. Fowler, and A. Ryan, "Flavor formation and character in cocoa and chocolate: a critical review," *Critical Reviews in Food Science and Nutrition*, vol. 48, no. 9, pp. 840–857, 2008.

[8] C. Counet, C. Ouwerx, D. Rosoux, and S. Collin, "Relationship between procyanidin and flavor contents of cocoa liquors from different origins," *Journal of Agricultural and Food Chemistry*, vol. 52, no. 20, pp. 6243–6249, 2004.

[9] L. De Vuyst and S. Weckx, "The cocoa bean fermentation process: from ecosystem analysis to starter culture development," *Journal of Applied Microbiology*, vol. 121, no. 1, pp. 5–17, 2016.

[10] A. Caligiani, A. Marseglia, B. Prandi, G. Palla, and S. Sforza, "Influence of fermentation level and geographical origin on cocoa bean oligopeptide pattern," *Food Chemistry*, vol. 211, pp. 431–439, 2016.

[11] J. Rodriguez-Campos, H. B. Escalona-Buendía, I. Orozco-Avila, E. Lugo-Cervantes, and M. E. Jaramillo-Flores, "Dynamics of volatile and non-volatile compounds in cocoa (Theobroma cacao L.) during fermentation and drying processes using principal components analysis," *Food Research International*, vol. 44, no. 1, pp. 250–258, 2011.

[12] J. Rodriguez-Campos, H. Escalona-Buendía, S. Contreras-Ramos, I. Orozco-Avila, E. Jaramillo-Flores, and E. Lugo-Cervantes, "Effect of fermentation time and drying temperature on volatile compounds in cocoa," *Food Chemistry*, vol. 132, no. 1, pp. 277–288, 2012.

[13] C. L. Ramos, D. R. Dias, M. G. D. C. P. Miguel, and R. F. Schwan, "Impact of different cocoa hybrids (Theobroma cacao L.) and S. cerevisiae UFLA CA11 inoculation on microbial communities and volatile compounds of cocoa fermentation," *Food Research International*, vol. 64, pp. 908–918, 2014.

[14] A. McShea, E. Ramiro-Puig, S. B. Munro, G. Casadesus, M. Castell, and M. A. Smith, "Clinical benefit and preservation of flavonols in dark chocolate manufacturing," *Nutrition Reviews*, vol. 66, no. 11, pp. 630–641, 2008.

[15] X. Qin, J. Lai, L. Tan et al., "Characterization of volatile compounds in Criollo, Forastero, and Trinitario cocoa seeds," *International Journal of Food Properties*, vol. 20, no. 10, pp. 2261–2275, 2016.

[16] P. D. Tran, D. Van de Walle, N. De Clercq et al., "Assessing cocoa aroma quality by multiple analytical approaches," *Food Research International*, vol. 77, pp. 657–669, 2015.

[17] V. T. T. Ho, J. Zhao, and G. Fleet, "The effect of lactic acid bacteria on cocoa bean fermentation," *International Journal of Food Microbiology*, vol. 205, pp. 54–67, 2015.

[18] V. T. T. Ho, J. Zhao, and G. Fleet, "Yeasts are essential for cocoa bean fermentation," *International Journal of Food Microbiology*, vol. 174, pp. 72–87, 2014.

[19] J. M. Cevallos-Cevallos, R. García-Torres, E. Etxeberria, and J. I. Reyes-De-Corcuera, "GC-MS analysis of headspace and liquid extracts for metabolomic differentiation of citrus huanglongbing and zinc deficiency in leaves of "Valencia" sweet orange from commercial groves," *Phytochemical Analysis*, vol. 22, no. 3, pp. 236–246, 2011.

[20] J. M. Cevallos-Cevallos, D. B. Futch, T. Shilts, S. Y. Folimonova, and J. I. Reyes-De-Corcuera, "GC-MS metabolomic differentiation of selected citrus varieties with different sensitivity to citrus huanglongbing," *Plant Physiology and Biochemistry*, vol. 53, pp. 69–76, 2012.

[21] Y. Wu and L. Li, "Sample normalization methods in quantitative metabolomics," *Journal of Chromatography A*, vol. 1430, pp. 80–95, 2016.

[22] M. G. Maridueña-Zavala, A. Freire-Peñaherrera, J. M. Cevallos-Cevallos, and E. L. Peralta, "GC-MS metabolite profiling of Phytophthora infestans resistant to metalaxyl," *European Journal of Plant Pathology*, vol. 149, no. 3, pp. 563–574, 2017.

[23] B. Warth, A. Parich, C. Bueschl et al., "GC–MS based targeted metabolic profiling identifies changes in the wheat metabolome following deoxynivalenol treatment," *Metabolomics*, vol. 11, no. 3, pp. 722–738, 2015.

[24] H. Arn and T. E. Acree, "Flavornet: a database of aroma compounds based on odor potency in natural products," *Developments in Food Science*, vol. 40, no. C, p. 27, 1998.

[25] The Good Scents Company, The Good Scents Company Information System. 1, 2015.

[26] S. Rice and J. A. Koziel, "Characterizing the smell of marijuana by odor impact of volatile compounds: an application of simultaneous chemical and sensory analysis," *PLoS ONE*, vol. 10, no. 12, Article ID e0144160, 2015.

[27] E. J. Krüsemann, W. F. Visser, J. W. Cremers, J. L. Pennings, and R. Talhout, "Identification of flavour additives in tobacco products to develop a flavour library," *Tobacco Control*, vol. 27, no. 1, pp. 105–111, 2017.

[28] V. Acierno, S. Yener, M. Alewijn, F. Biasioli, and S. Van Ruth, "Factors contributing to the variation in the volatile composition of chocolate: botanical and geographical origins of the cocoa beans, and brand-related formulation and processing," *Food Research International*, vol. 84, pp. 86–95, 2016.

[29] F. Frauendorfer and P. Schieberle, "Changes in key aroma compounds of Criollo cocoa beans during roasting," *Journal of Agricultural and Food Chemistry*, vol. 56, no. 21, pp. 10244–10251, 2008.

[30] J. S. Bonvehí, "Investigation of aromatic compounds in roasted cocoa powder," *European Food Research and Technology*, vol. 221, no. 1-2, pp. 19–29, 2005.

[31] A. Vázquez-Ovando, L. Chacón-Martínez, D. Betancur-Ancona, H. Escalona-Buendía, and M. Salvador-Figueroa, "Sensory descriptors of cocoa beans from cultivated trees of Soconusco, Chiapas, Mexico," *Food Science and Technology*, vol. 35, no. 2, pp. 285–290, 2015.

[32] F. Luna, D. Crouzillat, L. Cirou, and P. Bucheli, "Chemical composition and flavor of Ecuadorian cocoa liquor," *Journal of Agricultural and Food Chemistry*, vol. 50, no. 12, pp. 3527–3532, 2002.

[33] P. C. Aculey, P. Snitkjaer, M. Owusu et al., "Ghanaian cocoa bean fermentation characterized by spectroscopic and chromatographic methods and chemometrics," *Journal of Food Science*, vol. 75, no. 6, pp. S300–S307, 2010.

[34] R. Saltini, R. Akkerman, and S. Frosch, "Optimizing chocolate production through traceability: a review of the influence of farming practices on cocoa bean quality," *Food Control*, vol. 29, no. 1, pp. 167–187, 2013.

[35] D. Kadow, J. Bohlmann, W. Phillips, and R. Lieberei, "Identification of main fine or flavour components in two genotypes of the cocoa tree (Theobroma cacao L)," *Journal of Applied Botany and Food Quality*, vol. 86, pp. 09–98, 2013.

[36] O. Sounigo, R. Umaharan, Y. Christopher, A. Sankar, and S. Ramdahin, "Assessing the genetic diversity in the International Cocoa Genebank, Trinidad (ICG,T) using isozyme electrophoresis and RAPD," *Genetic Resources and Crop Evolution*, vol. 52, no. 8, pp. 1111–1120, 2005.