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Identification, semi-quantification, and dynamics of volatile organic compounds during spontaneous fermentation of two cocoa varieties from northern Peru, using HS-SPME/GC-MS techniques

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Abstract

During spontaneous fermentation, an important process through which cocoa beans pass, volatile organic compounds (VOCs) and non-VOCs are generated and act as precursors of aromas and flavors that influence the chocolate. In the north of Peru, cocoas of different varieties and qualities are grown, and one of the most valued is the fine aroma native cocoa (*Cacao Fino de Aroma* (CFA)) compared with the Castro Naranjal 51 Collection (CCN-51) variety. In this investigation, the VOCs generated during the spontaneous fermentation of CFA and CCN-51 varieties, from the department of Amazonas, Peru, were analyzed. For the extraction of VOCs, the headspace (HS) and solid-phase microextraction (SPME) techniques were used, while gas chromatography coupled to a mass spectrophotometer (GC-MS) was employed for their subsequent separation and identification. The spontaneous fermentation process lasted for 156 h, and a total of 122 VOCs were identified in 2 varieties. The dynamics of 20 VOCs were assessed, and the principal component analysis of 22 VOCs was performed to compare the profiles of VOC of the 2 cocoa varieties. In conclusion, the CFA variety presented higher concentrations of floral, fruity, and sweet aromatic VOCs that positively influence the quality of the chocolate.

Keywords: fine aroma cocoa; volatile organic compounds; chocolate.

Practical Application: The CFA presented a promising profile of VOC to be used in the fine chocolate industry.

1 INTRODUCTION

Cocoa (*Theobroma cacao* L.) is a fruit that contains a considerable number of large seeds, which are fermented and dried to be used as raw material for chocolate. The FAO has records of annual cocoa production since 1961, and the largest cocoa producers were Ghana, Nigeria, and Brazil in that decade. Peru entered the top of the first 20 countries in 1981, and then, it has been increasing its production, nationally and internationally (FAO, 2022) since 2000, due to the increase in chocolate consumption (Sánchez et al., 2019). Countries in Asia and Africa, such as Indonesia, the Ivory Coast, and Ghana, are the main producers of the Trinitario and Forastero varieties of bulk cocoa (ICCO, 2023). Latin American countries produce varieties of high-quality cocoa, such as Criollo, originally from Venezuela, fine aroma native cocoa (or *Cacao Fino de Aroma*, CFA) from Peru, and National cocoa from Ecuador (ICCO, 2023; Rottiers et al., 2019b).

Peru has native and foreign varieties of cocoa, and in the north of the country, in Amazonas, San Martín, and Cajamarca, 9.4% of native fine aroma cocoa is grown (which the inhabitants of these areas call "Criollo"); 53.3% of Trinitario in Junín; and 37.3% of Forastero in Cuzco and Ayacucho, according to the Ministry of Agrarian Development and Irrigation of Peru (2020). However, of all of them, the most expensive—at the same time more susceptible to pests and diseases—are the fine aroma native cocoas (Ascrizzi et al., 2017) as they obtain great scores in the chocolate tasting, like the one produced in Amazonas from Perú (Castro-Alayo et al., 2019).

To obtain the chocolates, the cocoa beans are subjected to three important post-harvest processes: fermentation, drying, and roasting. Fermentation and drying are usually carried out in the same places where they are harvested, or in nearby places appropriate for these processes (ICCO, 2023) because fermentation

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depends on the cocoa pulp and the microorganisms that are spontaneously introduced during the harvest activity.

During fermentation, VOCs are generated, as well as precursors of aromas and flavors characteristic of chocolates. The precursors, free amino acids, short-chain peptides, and reducing sugars are formed by increasing the acidity during this process, while the VOCs will be mainly formed during roasting through the Maillard reaction. VOCs confer pleasant aromas, such as fruity, floral, and sweet, and also unpleasant aromas, such as astringent and bitter (Hamdouche et al., 2019; Rodriguez-Campos et al., 2011, 2012; Torres-Moreno et al., 2021).

VOCs have been studied comparing different varieties of cocoa from different countries, such as between the Nacional and CCN-51 cocoa from Ecuador (Rottiers et al., 2019b), between hybrid cocoas from Brazil (Moreira et al., 2018) and between Forastero, Trinitario y Chinese Criollo (Qin et al., 2017). In these studies, VOCs such as 2-heptanol, 2-phenylacetaldehyde, and 1-phenylethanone were identified, which are present in fine aroma cocoas and provide floral, fruity, and caramel aromas. Other VOCs confer higher concentrations of rancid and bitter aromas, such as isobutyric acid, 2,3-butanedione, and acetoin. For this reason, it could be said that VOCs are quality indicators.

Therefore, the objective of this investigation was to analyze the dynamics of the VOCs of two varieties of cocoa beans from the Amazonas of Peru: the CFA and the CCN-51, during the entire spontaneous fermentation process. To achieve this, the VOCs were extracted by solid-phase microextraction (SPME) technique and headspace (HS), and its separation, identification, and semi-quantification were developed by gas chromatography, coupled to a mass spectrophotometer (GC-MS).

2 MATERIALS AND METHODS

2.1 Sampling place

The CFA and CCN-51 cocoa fruits were harvested in the province of Utcubamba, Amazonas, Peru. Approximately 600 kg of cocoa beans together with their respective pulp, of each variety, were transported to the *Central de Productores Agropecuarios de Amazonas* (CEPROAA) cooperative, located in the Cajaruro district, Utcubamba Province, where fermentation of the cocoa beans took place, with a total duration of 156 h.

2.2 Temperature and pH recording

The temperature and pH of the cotyledon (also called cocoa bean or bean) and pulp were measured every 12 h. To measure the temperature, a mercury thermometer was used, which was introduced to the center of the fermentation mass and left to rest for 5 min. For the pH measurement, a pH meter (Hanna, HI98100 Checker® *Plus*, Romania) was used. To determine the pH of the cotyledon, 15 g of cotyledons were taken and mixed with 135 g of distilled water. Then, 20 g of cocoa pulp was taken and mixed with 20 g of distilled water to measure the pH of the cocoa pulp (Crafack et al., 2013).

2.3 Sampling

The samples were taken every 12 h, from 0 to 156 h, the time that the spontaneous fermentation process lasted. After that, 200 g of cocoa beans of each variety were taken, properly packaged, and stored in a tank with liquid nitrogen until the samples were transported to the UNTRM Plant Physiology and Biotechnology Laboratory (where the VOC analysis was performed). The samples were then stored in a freezer at -20°C.

2.4 VOC extraction and identification by HS-SPME and GC-MS

SPME was used for the extraction and the GC-MS technique for the separation and identification of VOCs (Moreira et al., 2018).

Twenty cocoa beans were shelled with liquid nitrogen, and then crushed in an automatic mill intermittently for approximately 20 s; the size of the particles of the crushed beans was standardized using an 850-μm sieve, and the same was done for each of the samples of the two varieties and the 14 fermentation times.

For VOC extraction, 4 g of crushed beans were deposited in 20 mL vials, adding 5 L of the internal standard 4-methylpyridine (0.5 μg/μL of 4-methylpyridine diluted in methanol) (Rottiers et al., 2019a), and then, the vial was hermetically capped. Three vials were prepared for each sample. The SPME fiber used was composed of the following polymers: divinylbenzene, carboxene, and polydimethylsiloxane (DVB/CAR/PDMS). The equilibrium time of the sample was 15 min at 60°C, and then the SPME fiber was exposed, within the vial, in HS for 30 min at 60°C (Rodriguez-Campos et al., 2011, 2012).

In the Agilent gas chromatograph, model 7890B, equipped with a mass detector, model MSD 5977B, and a DB–5MS UI (60 m × 0.25 mm × 1.0 μm) capillary column (Agilent Technologies®). The initial temperature of the oven was programmed at 50°C for 5 min with a gradient of 4°C/min until 250°C. The carrier gas was helium, with a flow of 1.1 mL/min. The ionization source and the quadrupole detector were kept at 280 and 150°C, respectively. The injector was used in splitless mode and was kept at 250°C. Under these conditions, the SPME fiber was introduced into the chromatograph injector, with a desorption time of 5 min. In total, the process lasted for 55 min.

For the identification of the VOCs, two comparison criteria were used: the mass spectra of the VOCs of each sample with the NIST 2017 library (National Institute of Standards and Technology) and the retention indices calculated from the VOCs of each sample, after injection of the n-alkanes standard (C_{10}–C_{40}) under the same analytical conditions, with the retention index found in the literature.

2.5 Semi-quantification of the identified VOCs

The semi-quantification was achieved due to the addition of the internal standard of 4-methylpyridine, of known concentration (0.5 μg/μL), to each sample. The calculation was performed using Equation 1 (Wang et al., 2022):

$$[X\ of\ the\ VOC] = \frac{[Internal\ Standard] * \bar{X}Area\ of\ the\ VOC}{\bar{X}Area\ of\ the\ Internal\ Standard} \quad (1)$$

Where:

[Internal Standard]: Known concentration of the internal standard;

XA*rea* of the Internal Standard: Internal standard area;

[X of the VOC]: Relative concentration of the VOC of interest;

XA*rea* of the VOC: Average area of the VOC of interest.

2.6 Analysis of data

The physicochemical measurements of temperature and pH, taken during the fermentation process, were performed in Excel, and the averages and standard deviations of the pH of the cotyledon and pulp were calculated.

The data obtained by the GC-MS were analyzed using MassHunter Unknowns Analysis, MassHunter Quant 10.1, MassHunter Qual 10.0, and the NIST 2017 library, which were used to process, purify, and identify the VOCs of the analyzed samples. After the identification of the VOCs, they were extracted and organized in tables using Excel. The tables included the averages of the areas, standard deviations, and relative concentrations, which were calculated in order to organize the data (separated by variety and fermentation time). Additionally, the graph of the VOC dynamics was drawn, to achieve the principal component analysis (PCA), considering an accumulated percentage greater than 60% of the variability explanation of the relative concentrations. For the final analysis, the R software (R Development Core Team, 2020) was also used. Moreover, significant differences were determined between the relative concentrations of VOCs between the CFA and CCN-51 varieties using the Wilcoxon non-parametric test, at a 95% confidence interval.

3 RESULTS AND DISCUSSION

Peru produces varieties of native and foreign cocoa in Amazonas of Peru, San Martín, Piura, Cusco, Ucayali, Junín, Huánuco, and Cajamarca. The common characteristics of these beans are their pleasant aromas and flavors, but they differ in their genetics, climatic conditions where the cocoas are grown, and, due to these circumstances, their agronomic management (García Carrión, 2010). In Amazonas, specifically in the provinces of Bagua and Utcubamba, 136 cocoas were characterized between 2011 and 2013, but at the morphological level of fruits and cocoa beans (Altini et al., 2016), rather than at a molecular characterization level. In recent years, native fine-aroma cocoa from Amazonas has been investigated at the molecular (Bustamante et al., 2022) and microbiological levels, mainly in the fermentation stage (Ayala Tocto, 2022). It is an important natural and native resource and is being introduced into national and international markets for high-end cocoa beans (ICCO, 2023).

3.1 Temperature and pH changes in the spontaneous fermentation process

The cocoa fermentation process is characteristic of its change in the microbiota. The activity of these microorganisms generates metabolites and different conditions, such as the increase in the temperature of the cocoa mass and changes in the pH of the cotyledon and pulp, which lead to the death of the embryo and the production of volatile, non-volatile, and semi-VOCs through a chain of biochemical and chemical reactions (Ardhana & Fleet, 2003; Pereira et al., 2012). However, the changes may depend on the genetics of each cocoa variety (Torres-Moreno et al., 2021). Figure 1A shows that, under the same environmental and harvest conditions, the temperature of the fermentation mass of the CFA variety increases at a faster rate than that of the CCN-51 variety as time passes. When performing the Student's t-test to compare the fermentation temperature between the two varieties, the p-value was 0.1886; therefore, there were no statistically significant differences. However, the small difference in temperatures between the varieties may be due to genetic factors, as in a study carried out with varieties from Ecuador (Nacional and CCN-51), which are very different genotypically. Differences were also found in VOCs, with aromas and pleasant flavors related more to the National variety than to the CCN-51 variety (Rottiers et al., 2019b). On the contrary, the pH of the cotyledon and the pulp also changed as the fermentation time increased (Figures 2B and 2C), but no statistically significant differences were found between both varieties when performing the Student's t-test (p-value of pulp pH equal to 0.613 and p-value of cotyledon pH, 0.542). Despite this, a tendency is observed, in which the cotyledon pH decreases faster in the CFA variety than in the CCN-51 variety (Figure 1B). The pulp contains high amounts of citric acid. Hence, at the beginning of fermentation, its pH was low: 3.86 in the CFA variety and 3.43 in the CCN-51 variety. Also, it increases as the microorganisms metabolize the nutrients in the pulp: sugars, organic acids, and pectin, among others (Ardhana & Fleet, 2003), until at the end of fermentation, the pulp pH of the CFA variety was 4.22 and of the CCN-51 variety was 4.28 (Figure 1C). Regarding the pH of the cotyledon, its initial values were 6.27 in the CFA variety and 6.59 in the CCN-51 variety, because the pH closest to neutral favors seed germination. However, during fermentation, the organic acids are generated by large families of microorganisms, such as acetic acid and lactic acid bacteria, and they are introduced by permeability until they reach the cotyledon and acidify it (Schwan & Wheals, 2004). They reach a pH of 4.29 in the CFA variety and 4.33 in CCN-51. Furthermore, acidification generates biochemical reactions that favor the production of flavors and aromas typical of chocolate, decreasing the concentrations of bitter and astringent compounds (Bonvehí, 2005).

3.2 Identification of VOCs generated during the spontaneous fermentation process

At different fermentation times, 122 VOCs were identified (data not shown), from 0 to 156 h, in the CFA and CCN-51 varieties. The VOCs present in both varieties were 61, which are compounds that provide sweet, fruity, and citrus pleasant aromas, such as 2-heptanol, benzaldehyde, and 2-nonanone. These VOCs at the beginning of fermentation had low relative concentrations but were present throughout the fermentation, as in the Criollo, Forastero, and Nacional varieties of Ecuador (Cevallos-Cevallos et al., 2018).

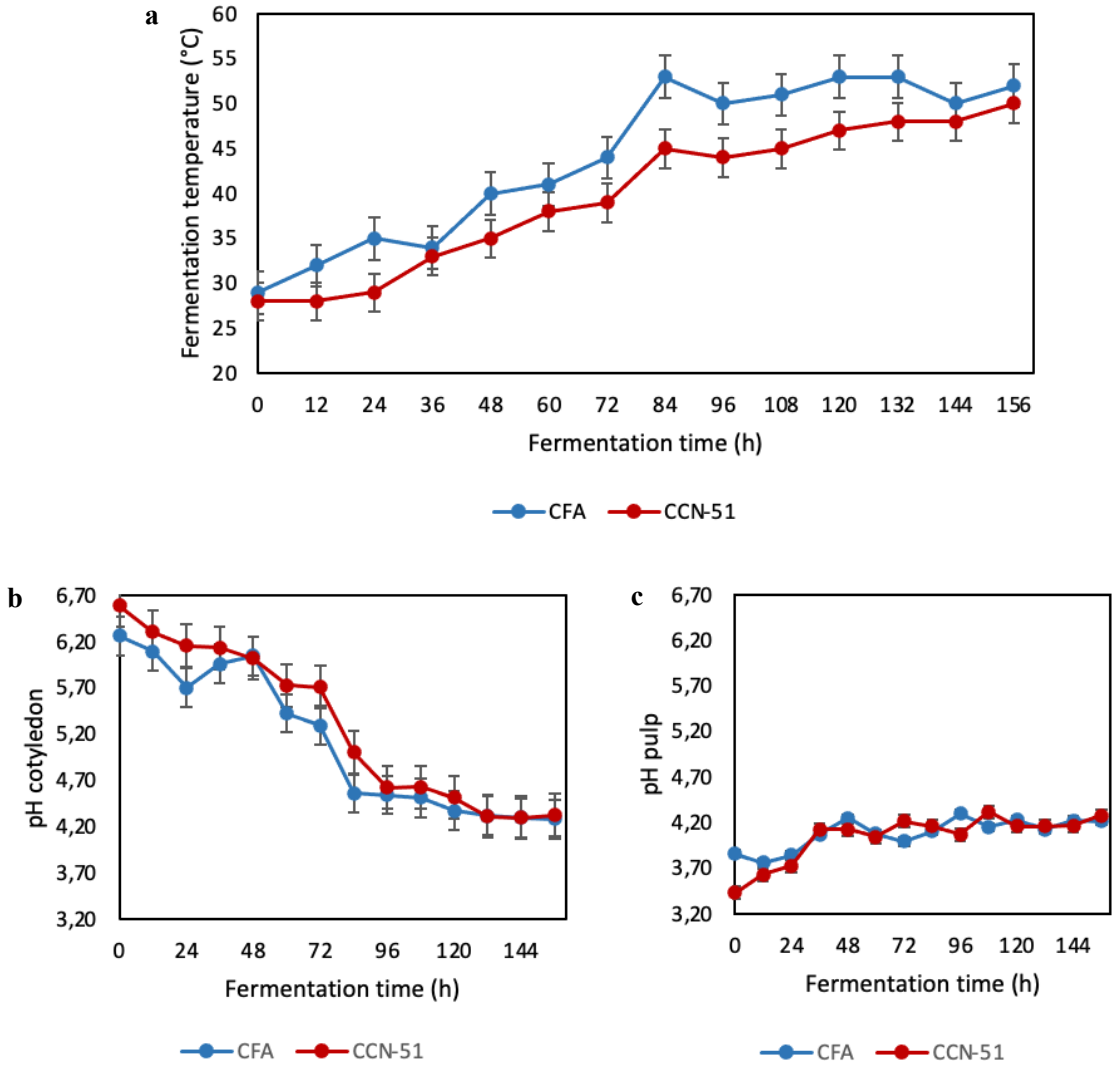

Figure 1. Behavior of the (A) fermentation temperature and (B) the pH of the cotyledon and (C) of the pulp throughout the fermentation time between the CFA and CCN-51 varieties. The bars represent the standard error.

The concentrations of the volatile compounds vary as the fermentation time increases. They can disappear, decrease, or increase, either during the fermentation, drying, and/or roasting process (Frauendorfer & Schieberle, 2008; Owusu et al., 2012; Utrilla-Vázquez et al., 2020). The formation of new compounds derived from VOCs also occurs, which decreases due to the degradation of fatty acids, cotyledon proteins, and microbial activity (Afoakwa et al., 2008).

The three most abundant compounds in both varieties were 2-heptanol, which provides sweet and citrus aromas (Rodriguez-Campos et al., 2012); benzeneacetaldehyde, which provides aromas of honey and floral (Rottiers et al., 2019a); and phenylethyl alcohol, which confers aromas of honey, rose, floral, and caramel (Frauendorfer & Schieberle, 2006; Rodriguez-Campos et al., 2011). The 2-heptanol and benzaldehyde were some of the compounds present in all fermentation hours that were analyzed, while phenylethyl alcohol was not detected only at 0 h fermentation of the CCN-51 variety.

3.2 Individual comparison of 20 VOCs of CFA and CCN-51 varieties

To compare the behavior of relative concentrations, it is necessary to have the largest amount of data in both varieties. Therefore, the 20 most representative VOCs with the greatest presence were chosen. VOC dynamics were performed using scatter plots of relative concentrations over fermentation time.

3.2.1 Alcohols

During spontaneous fermentation, 16 VOCs from the functional group of alcohols were identified. Figure 2 shows the dynamics of phenylethyl alcohol, 2-heptanol, 2-nonanol, and (S)-alpha-methyl-benzenemethanol, where a tendency of higher relative concentration is observed in the CFA variety with respect to CCN-51.

Alcohols are produced mainly at the beginning of fermentation by the metabolism of yeasts, which are involved in

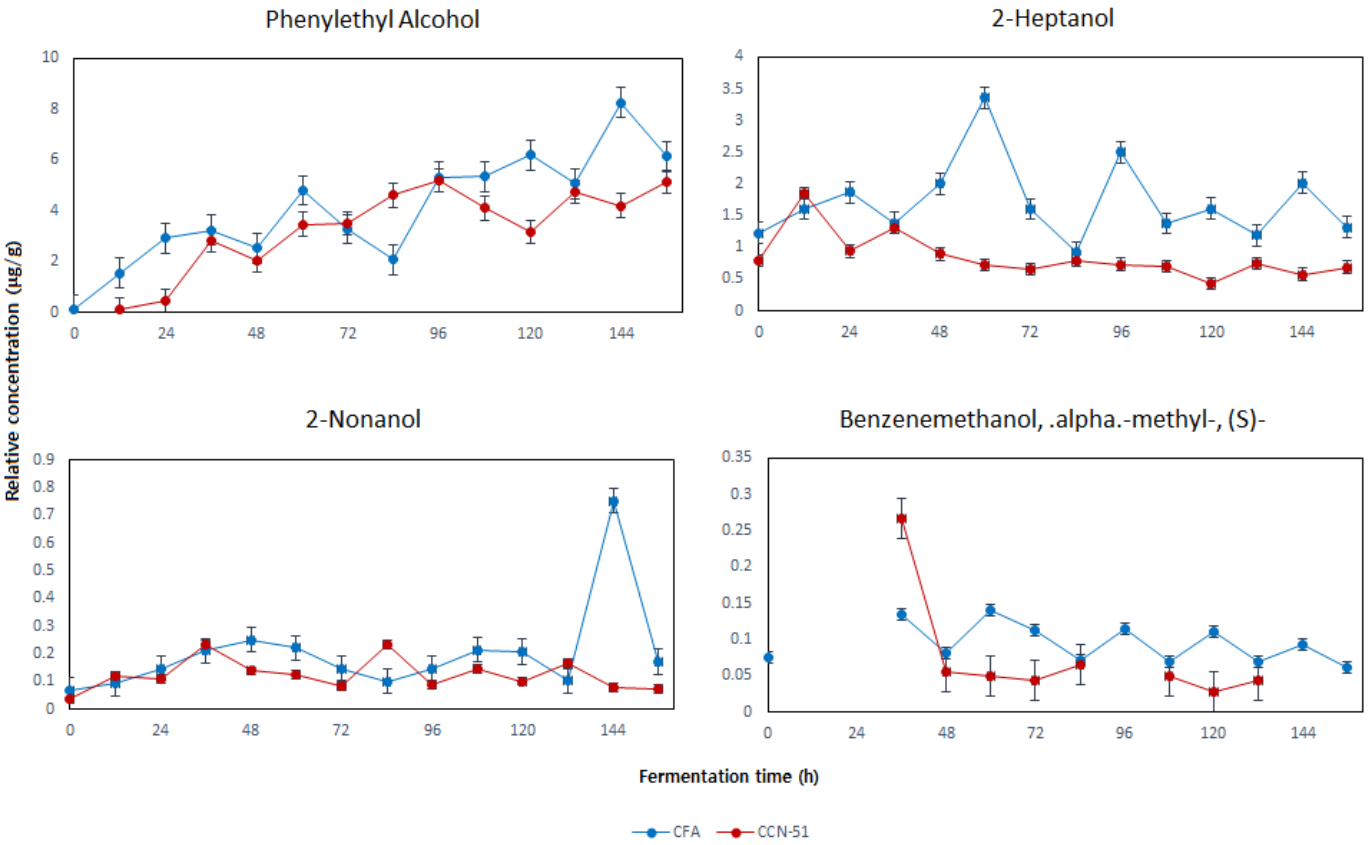

Figure 2. Dynamics of the relative concentrations of alcohols generated in cocoa beans during the spontaneous fermentation process. The bars represent the standard error.

alcoholic fermentation (Schwan & Wheals, 2004). Phenylethyl alcohol and 2-heptanol provide citrus, floral, and sweet aromas (Rodriguez-Campos et al., 2012; Utrilla-Vázquez et al., 2020), which were present in both varieties, but predominated in the CFA (Figure 2). In addition, the increase in phenylethyl alcohol plays an important role, as it is the precursor of phenylacetaldehyde, a compound that was reported in the drying stage (Rodriguez-Campos et al., 2011). On the contrary, alcohols such as 2-pentanol and 2-heptanol are endogenous to fine aroma cocoa genotypes, that is, the cocoa beans already contain it before fermentation (Kadow et al., 2013; Rottiers et al., 2019a), and during fermentation, their concentration decreases due to the esterification of alcohols, an activity in which acetic bacteria contribute, forming esters (Rodriguez-Campos et al., 2012; Rottiers et al., 2019a). In addition, the decrease in alcohols, in the case of 2-heptanol and (S)-alpha methyl benzenemethanol, is also due to the increase in the fermentation temperature and the volatilization of these compounds (Bastos et al., 2019). The 2-nonanol provides fruity and citrus aromas and presents a constant relative concentration in both varieties, except for a higher concentration in the CFA variety at 144 h of fermentation. Although in this case a great graphic difference was not noticeable, in a comparative study of fermented and dried native cocoa beans hybrid with CCN-51 from Ecuador, the 2-nonanol concentrations were higher in the CCN-51 variety in relation to the native fine aroma hybrid cocoa beans (Rottiers et al., 2019b).

3.2.2 Esters

Twenty esters were identified in the cocoa beans in both varieties during the 156 h of spontaneous fermentation. The esters started with low concentrations and increased as the fermentation time increased. Like the alcohols, the esters are generated by the metabolism of the yeasts, which metabolize acids found in the cocoa pulp (Bastos et al., 2019). Likewise, acetic acid bacteria abound toward the end of fermentation, when aeration increases (Beckett, 2008), mainly due to the removal of cocoa beans starting at 60 h and every 24 h until the end of fermentation. These bacteria esterify alcohols, which increases the concentrations of esters, and therefore, produce fruity flavors and aromas; 3-methyl-1-butanol acetate and 2-heptanol acetate compounds provide these characteristics (Smit et al., 2005). In the CFA variety, the ethyl ester of benzoic acid and 2-heptanol acetate, which confer fruity and floral aromas, presented higher relative concentrations until the last hours of fermentation, in comparison with the CCN-51 variety (Figure 3). On the contrary, CCN-51 contained higher concentrations of octanoic acid ethyl ester, which provides fruity and floral aromas, between 60 and 96 h of fermentation, as in bulk cocoa beans Forastero, in which the highest concentration of esters was reported between 48 and 96 h of fermentation (Cevallos-Cevallos et al., 2018).

Other factors that influence the presence of esters in cocoa beans are the fermentation temperature and the removal of beans for a homogeneous fermentation (Perestrelo et al.,

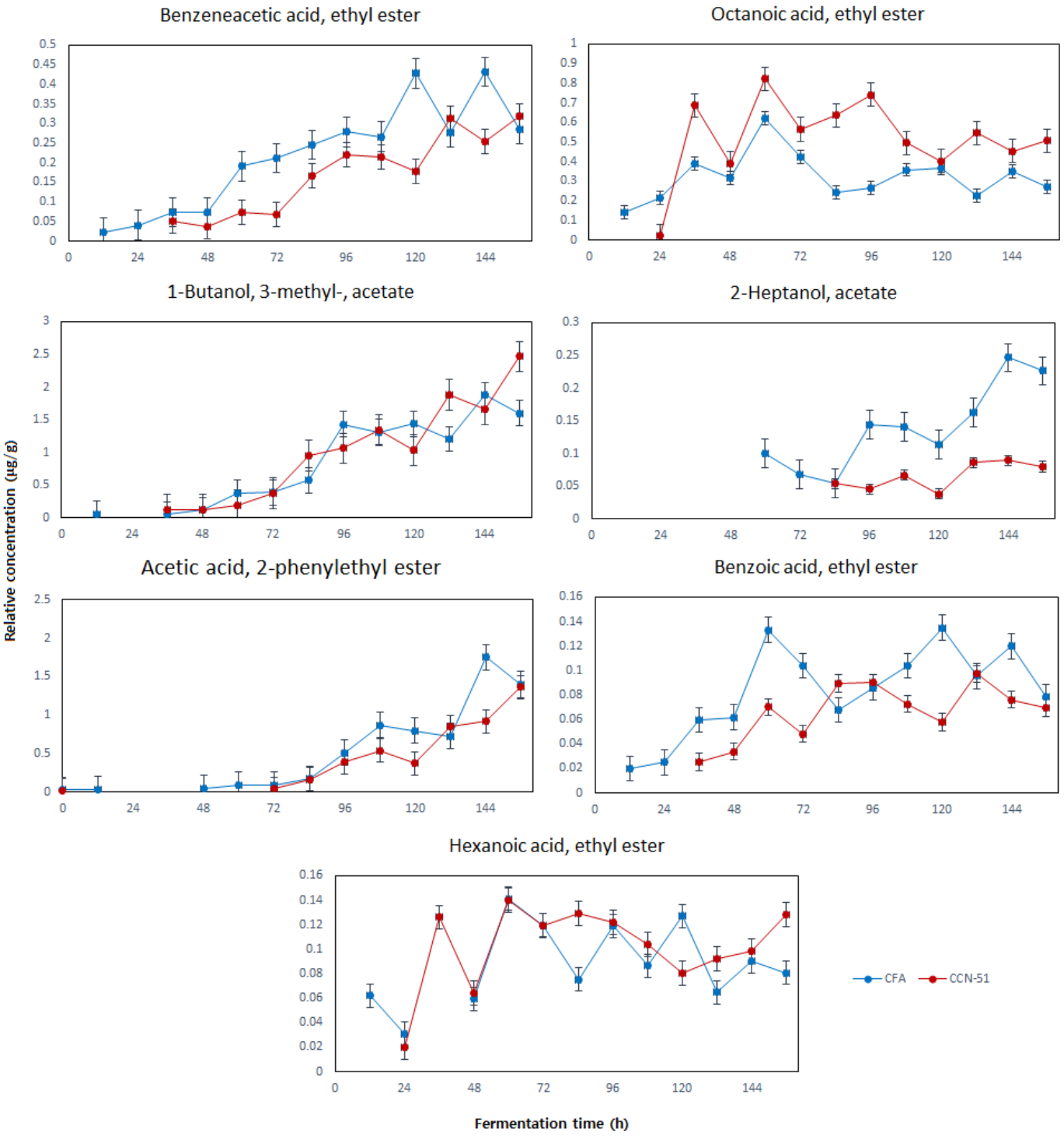

Figure 3. Dynamics of the relative concentrations of esters generated in cocoa beans during the spontaneous fermentation process. The bars represent the standard error.

2006). The acetic and lactic acids, produced by the bacteria during fermentation, cause the beans to become permeable, which consequently provoke the decomposition of aroma and flavor precursors and the death of the cotyledon. In addition, it is assumed that permeability allows the migration of aromatic compounds from the pulp toward the seed tissue, which is considered a reservoir of cocoa aromatic compounds (Chetschik et al., 2018; Eskes et al., 2007).

3.2.3 Aldehydes

Six VOCs from the functional group of aldehydes were identified, of which nonanal, benzeneacetaldehyde, and benzaldehyde contribute to the fruity, sweet, honey, and floral pleasant aroma (Rottiers et al., 2019a). Benzaldehyde also provides bitter aromas (Bonvehí, 2005). The 2-heptanal and methional provide fermented and potato aromas, respectively. No records

of 2-propenal aromas have been found. The relative concentrations of aldehydes, such as benzeneacetaldehyde, are quite high with respect to the other VOCs of the other functional groups, except for the group of ethers. This could be because aldehydes participate in the degradation of lipids, which are abundant components in cocoa beans (Bryant & McClung, 2011). Figure 4 shows the dynamics of three aldehydes: benzeneacetaldehyde, benzaldehyde, and methional. In all three cases, the relative concentrations were higher in the CFA variety, the same way as the study that compared VOCs of the Criollo, Forastero, and Nacional varieties from Ecuador, where the Nacional variety (a fine aroma cocoa variety from Ecuador) presented higher concentrations of benzaldehyde than CCN-51 during the fermentation process (Cevallos-Cevallos et al., 2018). Aldehydes could be considered good quality indicators because they provide fruity and floral aromas to the final product.

3.2.4 Ketones

Ten VOCs from the functional group of ketones were identified, which are also known to be generated by yeast metabolism according to Guehi et al. (2010). Out of these ten VOCs, four are responsible for providing fruity, floral, coconut, almond, and sweet aromas. These four VOCs are 2-butanone (Rottiers et al., 2019a), 2-heptanone, 2-nonanone (Kadow et al., 2013), and acetophenone (Bonvehí, 2005). Figure 5 shows the behavior over time of 2-heptanone, acetophenone, and 2-nonanone, which are ketones present in all the samples during the fermentation stage and in both varieties, as well as a clonal variety from Brazil called TSH565 (Trinidad Selected Hybrids 565), resistant to *Moniliophthora perniciosa* (Bastos et al., 2019). Also in an analysis of Forastero cocoa from Mexico, the presence of ketones was reported during cocoa fermentation and drying, even when the cocoa beans were subjected to different fermentation and drying treatments (Rodriguez-Campos et al., 2012). Even these VOCs persisted and increased during the roasting process (Bonvehí, 2005). The 2-heptanone and acetophenone in the CFA variety presented higher relative concentrations with respect to the CCN-51, which could provide better organoleptic characteristics to the CFA. Finally, the concentrations of 2-nonanone differ among the varieties, and, in this case, the relative concentrations were low and constant, but higher concentrations were found in Criollo cacao from Ecuador after fermentation (compared with the Forastero variety) (Cevallos-Cevallos et al., 2018). In another study, also comparing hybrid cocoas of the Nacional variety with CCN-51, higher concentrations of 2-nonanone were reported in the CCN-51 variety (Rottiers et al., 2019b). However, these results cannot be taken as contradictory, because, in the last-mentioned study, dried cocoa beans were analyzed, which suggests that, after the drying process, ketones were more concentrated, like other VOCs, due to the dehydration of cocoa beans.

3.2.5 Terpenes and terpenoids

There were nine terpenes identified, with descriptions of floral, sweet, citrus, herbal, and spicy aromas, and three terpenoids, all with descriptions of pleasant aromas, such as citrus, refreshing, sweet, and even nutty notes. The terpenes trans-beta-ocimene and beta-myrcene presented higher relative concentrations in the CFA variety, specifically at 60 h. The decrease is

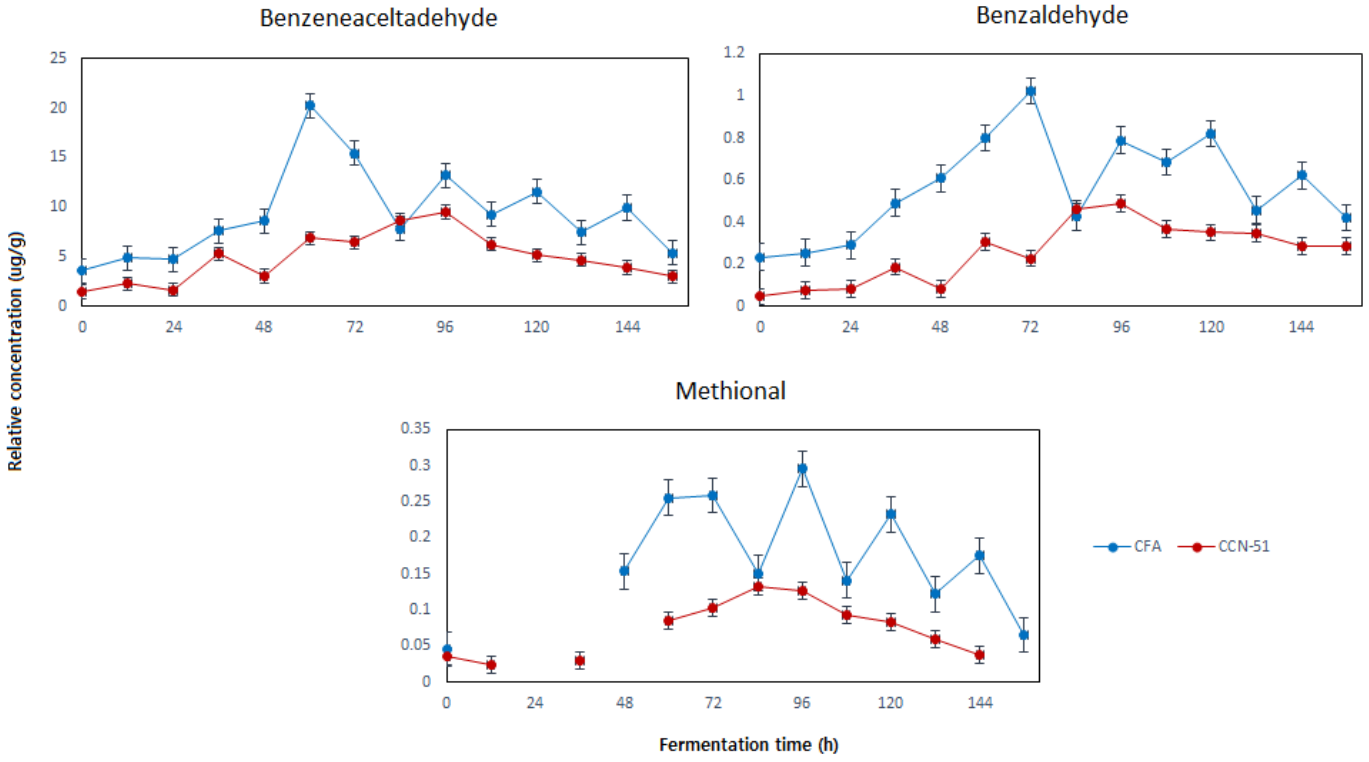

Figure 4. Dynamics of the relative concentrations of aldehydes generated in cocoa beans during the spontaneous fermentation process. The bars represent the standard error.

until the end of fermentation regarding CCN-51, as occurred in similar studies, in which a greater amount of these volatile compounds was reported in fine aroma cocoa beans (hybrids of the Nacional variety) (Rottiers et al., 2019b). Beta-myrcene produces spicy and balsam aromas and is usually found in fine aroma cocoas (Kadow et al., 2013; Rottiers et al., 2019b), whose compound could be an indicator of characteristics of fine cocoas and not necessarily contribute positively to the aroma of the final product. On the contrary, compounds such as beta-ocimene, which confers a floral aroma, only occurred in the CFA variety, but in a few fermentation times. Linalool was identified only at 12 and 24 h of fermentation, which provides floral aromas similar to roses, as well as sweets and citrus. Unlike different studies (Aprotosoaie et al., 2016; Ardhana & Fleet, 2003; Rottiers et al., 2019a), which presented linalool in the different postharvest stages, in this investigation, the presence of linalool concentration was low.

3.2.6 Ethers

During the fermentation process, four ethers were identified: dimethyl ether, isobutyl ether, methyl nonyl ether, and n-butyl ether. Of these, the most abundant ether was dimethyl ether, which presented higher relative concentrations compared with all the other VOCs identified at all times in both varieties. However, it is not a volatile compound registered in studies carried out with cocoa. The presence of dimethyl ether could be from the interaction of cocoa beans with fermentation boxes. It should be mentioned that the NIST library also refers to dimethyl ether as wood ether. It could have also been due to the dehydration of methanol (Pelaez-Fernandez, 2016), which is a diluent of the internal standard 4-methylpyridine, or even produced during spontaneous fermentation (Packiyasothy et al., 1981), at some stage of sample preparation, forming the dimethyl ether. Furthermore, some ethers can be produced by the action of microorganisms (George & Häggblom, 2008) that, in this case, could be participating in the fermentation.

3.3 Principal component analysis

PCA was performed to search for clusters and visualize the relationships between the identified VOCs and the CFA and CCN-51 cocoa varieties (in the R statistical software). The 22 VOCs (Table 1) were considered for the PCA, of which 8 VOCs were present in both varieties during all the fermentation hours analyzed: 2-heptanol, 2-heptanone, 2-nonanol, 2-nonanone, acetophenone, benzaldehyde, benzeneacetaldehyde, and styrene. The 14 VOCs were present in more than 50% of the samples (in both varieties). The other 100 VOCs were not considered due to their absence in many fermentation times; consequently, they did not contain sufficient information on the relative concentration variable to perform the PCA.

Likewise, an exploratory analysis was carried out including the dispersion diagram with the purpose of knowing the behavior of the relative concentration variable of the 22 VOCs. Higher VOC concentrations were observed in the CFA variety compared with the CCN-51 variety throughout the fermentation time. Using the Wilcoxon test (non-parametric test), a p-value was obtained = 3.898 $e10^{-6}$, compared with a 5% level of significance. Therefore, it is concluded that there is a statistically significant difference between the CFA and CCN-51 varieties.

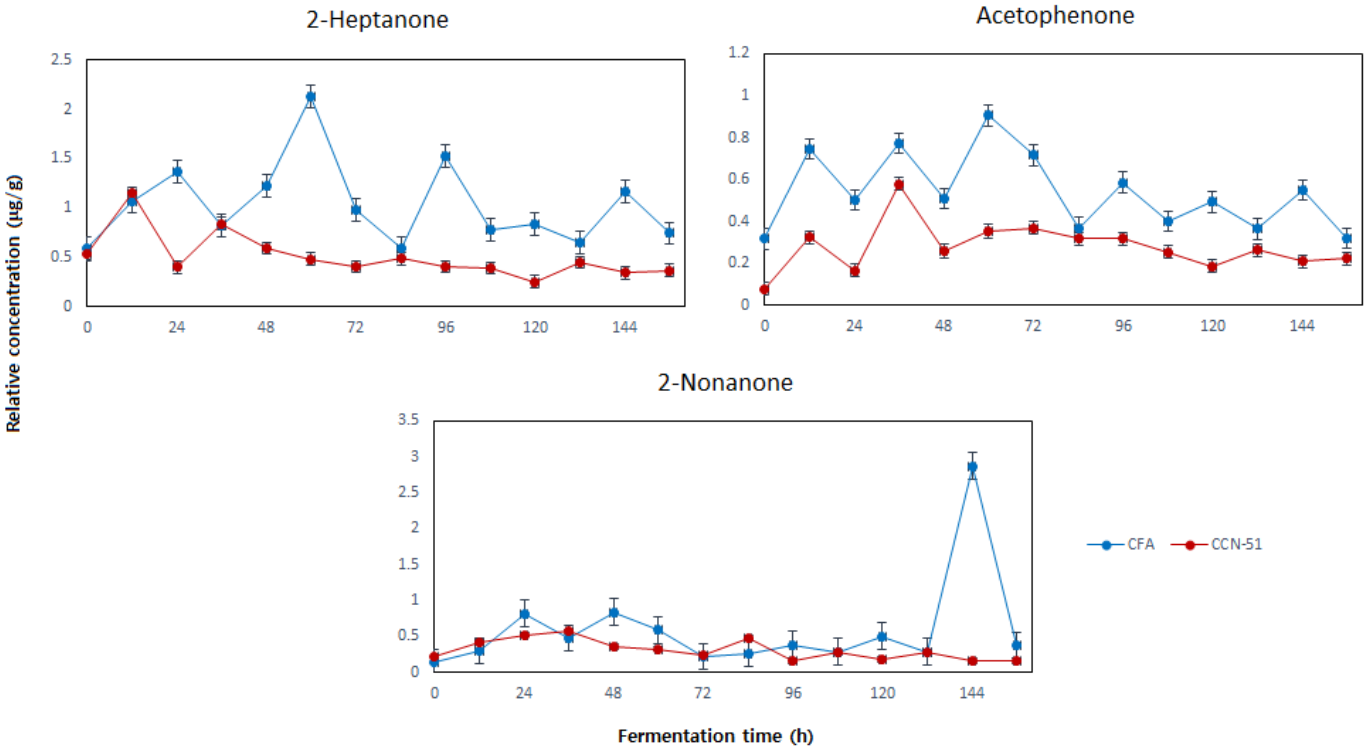

Figure 5. Dynamics of the relative concentrations of ketones generated in cocoa beans during the spontaneous fermentation process. The bars represent the standard error.

Figure 6 shows the PCA graph (after data standardization by square root transformation of the relative concentration variable), in which the study of the proportion of explained variance shows that component 1 explains 44.9% of the total variance of the data and component 2 explains 23.4%. In total, the accumulated percentage of variance in the first two components is 68.3%.

In component 1, the VOCs that positively influence are benzoic acid ethyl ester, phenylethyl alcohol, benzaldehyde, benzeneacetic acid ethyl ester, and benzeneacetaldehyde, of which the VOCs that confer floral aromas to the cocoa beans are phenylethyl alcohol and benzeneacetaldehyde, while the VOC that provides fruity aroma is benzeneacetic acid ethyl ester and the VOC that imparts both aromas is the benzoic acid ethyl ester. On the contrary, benzaldehyde provides a bitter and sweet aroma at the same time. In addition, Figure 6 shows that component 1 groups the VOCs that provide fruity, floral, and sweet aromas between 60 and 144 h of fermentation, whose compounds appeared with higher relative concentrations in the CFA variety.

For component 2, the VOCs that have large negative influences are 2-heptanone, 2-heptanol, trans-beta-ocimene, beta-myrcene, and acetophenone; 2-heptanone confers floral aroma; acetophenone confers floral and fruity; 2-heptanol provides sweet-citrus aroma; trans-beta-ocimene provides sweet-herbal aroma; and beta-myrcene provides balsam-spicy aroma. The VOCs trans-beta-ocimene and beta-myrcene were present in higher concentrations between 12 and 60 h of fermentation and 2-heptanone and 2-heptanol were present between 60 and 96 h of fermentation in the CFA variety. In general, the component 2 groups, on its negative axis, are the VOCs that abound between 12 and 96 h of fermentation in the CFA variety.

Table 1. VOCs identified during spontaneous fermentation of CFA and CCN-51 varieties used for PCA.

Name of VOCs	RT (min)	RI calculated	RI—Library NIST 2017	Variety and fermentation time (h)*	Aroma description	Aroma description reference
Alcoholes						
2-Nonanol	32.46	1,085	1,102	C0-C156, F0-F156	Fruity, citrus	(Rottiers et al., 2019a)
Phenylethyl alcohol	33.53	1,115	1,116	C12-C156, F0-F156	Honey, spice, rose, lilac, floral, candy	(Rodriguez-Campos et al., 2011) y (Frauendorfer & Schieberle, 2006)
2-Heptanol	23.29	835	900	C0-C156, F0-F156	Sweet, citrus	(Rodriguez-Campos et al., 2012) y (Kadow et al., 2013)
Benzenemethanol, .alpha.-methyl-, (S)-	31.00	1,045	1,055	C36-C84, C108-C132, F0, F36-F156		
Aldehydes						
Benzaldehyde	26.91	934	962	C0-C156, F0-F156	Bitter, sweet, almond, cherry	(Bonvehí, 2005; The Good Scents Company, 2021)
Benzeneacetaldehyde	30.41	1,029	1,045	C0-C156, F0-F156	Honey, floral	(Rottiers et al., 2019a)
Methional	23.70	846	907	C0, C12, C36, C60-C144, F0, F48-F144	Potato	(Afoakwa et al., 2009)
Ketones						
2-Heptanone	22.81	822	891	C0-C156, F0-F156	Fruity, coconut, cheesy, floral	(Kadow et al., 2013)
2-Nonanone	32.09	1,075	1,092	C0-C156, F0-F156	Fruity, fresh, sweet	(Kadow et al., 2013)
Acetophenone	31.61	1,062	1,065	C0-C156, F0-F156	Floral, almond, sweet	(Bonvehí, 2005)
Esters						
2-Heptanol, acetate	29.42	1,002	1,045	C84-C156, F60-F156	Fruity	(Kadow et al., 2013)
Benzeneacetic acid, ethyl ester	38.33	1,246	1,246	C36-C156, F12-F156	Fruity, sweet	(Rodriguez-Campos et al., 2012)
Octanoic acid, ethyl ester	36.08	1,184	1,196	C24, C156, F12-F156	Fruity, floral	(Bonvehí, 2005)
Hexanoic acid, ethyl ester	27.84	959	1,000	C24-C156, F12, F24, F48-F156	Fruity, apple, banana	(Bonvehí, 2005)
Benzoic acid, ethyl ester	35.69	1,173	1,171	C36-C156, F12-F156	Fatty, floral, fruity	(Bonvehí, 2005)
1-Butanol, 3-methyl-, acetate	21.90	797	876	C36-C156, F12, F36-F156	Banana, fruity	(Rottiers et al., 2019a)
Acetic acid, 2-phenylethyl ester	39.00	1,264	1,258	C0, C72-C156, F0, F12, F48-F156	Honey, floral	(Rodriguez-Campos et al., 2012)
Ethers						
Dimethyl ether	5.18	340	324	C12-C156, F0-F156		
Terpenes						
Beta-myrcene	27.69	955	991	C0-C48, C72, C84, C108, C144, C156, F0-F60	Spicy, balsam	(Kadow et al., 2013)
Trans-beta-ocimene	30.06	1,025	1,049	C0-C36, C72, F0-C156	Sweet, herbal	(Kadow et al., 2013)
Others						
Styrene	23.42	839	893	C0-C156, F0-F156	Sweet , balsam, floral	(The Good Scents Company, 2021; Yao et al., 2021)
Glycerin	25.94	907	967	C48, C84-C156, F24, F36, F60, F84-F156		

*The first letters represent the varieties (C = CCN-51 and F = fine aroma cocoa), numbers, and the hours of fermentation in which the compounds were detected.

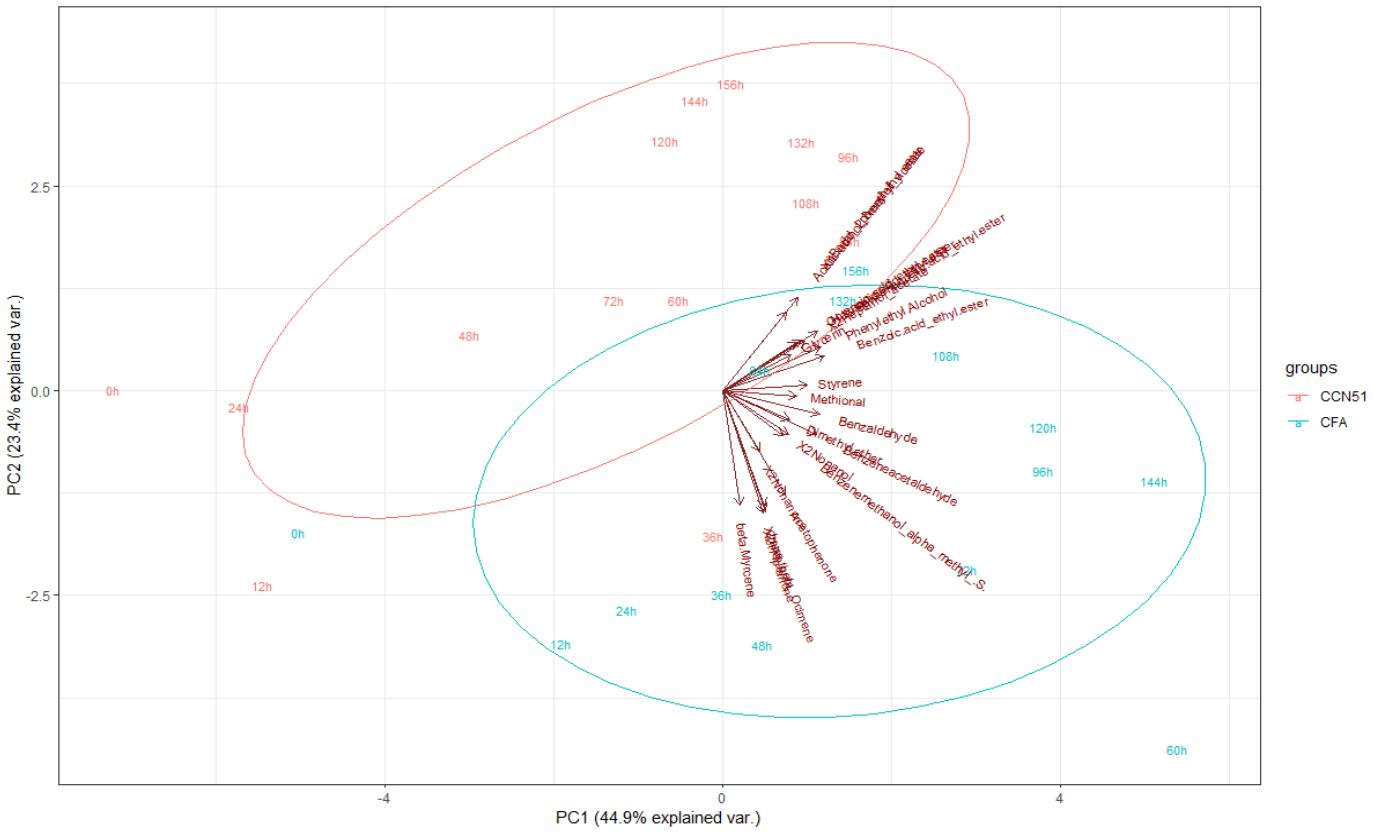

Figure 6. PCA of relative VOC concentrations and fermentation times, grouped by variety.

The higher relative concentrations of VOCs that provide fruity, floral, and sweet aromas to the CFA variety can be mainly attributed to its genetics (Acierno et al., 2016; Rottiers et al., 2019b) as the conditions of the fermentation process were the same. In addition, the geographical location of the cocoa plantations from which the pods were extracted was from the same province, and it is likely that the microorganisms responsible for fermentation were similar, as the farmers use the same harvesting tools for different varieties.

4 CONCLUSION

The dynamics of 20 VOCs with greater abundance and presence between the CFA and CCN-51 varieties were compared during 156 h of the spontaneous fermentation process. The VOCs with the highest relative concentrations in the CFA variety, with respect to CCN-51, were benzoic acid ethyl ester, 2-heptanol acetate, benzeneacetaldehyde, benzaldehyde, 2-heptanol, 2-heptanone, acetophenone, 2-nonanone, and trans-beta-ocimene. All of these provide fruity, floral, and sweet aromas. For CCN-51, there was a higher relative concentration of octanoic acid ethyl ester, 3-methyl-1-butanol acetate, and hexanoic acid ethyl ester, which are compounds that provide fruity aromas to cocoa beans. In addition, the PCA demonstrated that the VOCs generated in each variety of cocoa are related to quality. The VOCs that impart fruity, sweet, and floral aromas, found in higher concentrations in CFA, provide better quality to the cocoa beans.

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