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Natural Intervarietal Hybrids of *Coffea canephora* Have a High Content of Diterpenes

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Abstract: The present investigation characterized the diterpene profile of *Coffea canephora* coffees, which are natural intervarietal hybrids of Conilon and Robusta. The impact of genetic diversity and environment on these compounds was also evaluated. Five genotypes (clones 03, 05, 08, 25, and 66) from six growing sites in the State of Rondônia in the western Amazon (Alto Alegre dos Parecis, São Miguel do Guaporé, Nova Brasilândia do Oeste, Porto Velho, Rolim de Moura, and Alto Paraíso) were analyzed. The contents of kahweol, cafestol, and 16-*O*-methylcafestol in light-medium roasted coffees were assessed by UPLC. Data were analyzed by ANOVA and Tukey's test ($p \leq 0.05$). The contents of cafestol and 16-*O*-methylcafestol ranged from 96 to 457 mg 100 g^{-1} and 75 to 433 mg 100 g^{-1}, respectively. As for kahweol, from absence up to contents of 36.9 mg 100 g^{-1} was observed. The diterpene profile was dependent on genetics, growing site, and the interaction between these factors. A higher variability was observed for kahweol contents. The natural intervarietal hybrid coffees stood out for their high contents of diterpenes and increased frequency of kahweol presence (77% of the samples).

Keywords: kahweol; cafestol; 16-OMC; Rondônia; UPLC; Conilon; Robusta

1. Introduction

Brazil is the largest global green coffee producer and exporter, and the second-largest grower of the *Coffea canephora* species [1,2]. *C. canephora* coffee is of great relevance for the State of Rondônia, in the Brazilian Amazon region; research has allowed an increase in yield and beverage quality, with the cultivation of clones and the development of new varieties adapted to the edaphoclimatic conditions of the region [3,4]. Clones cultivated in Rondônia have become the genetic basis for renewing coffee plantations across the western Amazon [5,6].

The botanic varieties Conilon and Robusta are the most commercially cultivated *C. canephora* coffees. Conilon has shrublike growth, early flowering, and drought resistance. Robusta has larger fruits (of late maturity), better beverage quality, higher vigor, and greater disease tolerance [6–8]. Brazil produces Conilon and Robusta commercially [3]; however, due to Robusta's greater demand for water, the cultivation of this variety has been restricted to the Amazon region, which offers a favorable climate with abundant rainfall throughout the year [9]. As for the two varieties grown in Rondônia, hybridization occurred naturally in the field. Many years of study allowed the identification of genotypes with hybrid characteristics that have stood up for good beverage quality and yield [10–13].

Coffee is one of the most widely consumed beverages globally due to its stimulant effect and desirable flavor, as well as health benefits that are attributed to the large contents

and diversity of its bioactive components [14–16]. Among them, we highlight the diterpenes kahweol, cafestol, and 16-*O*-methylcafestol (16-OMC), which are compounds with positive physiological effects associated with anticarcinogenic, anti-inflammatory, and antioxidant activities. They are also related to increased serum cholesterol levels (notably attributed to cafestol) [17–22].

Compared to other roasted coffee bioactive compounds, there is a lack of information on diterpenes in the literature. Most investigations are related to *Coffea arabica,* and there is no consensus regarding the profile of diterpenes in *C. canephora.* Cafestol, usually described as the main diterpene of the *C. canephora* species, varies in a wide range (76 to 363 mg 100 g^{-1} of roasted coffee); for kahweol, its absence or presence at low contents (up to 20 mg 100 g^{-1}) [19,23] was reported. For 16-OMC, a 10-fold variation was described (from 26 to 223 mg 100 g^{-1}) [19], and some authors did not report its presence [24–26]. For a long period, 16-OMC was described as exclusive to *C canephora* and suggested as a species marker in blends with *C. arabica* (which has increased market value) [27]. However, the wide range of 16-OMC content described in the literature for *C. canephora* [28,29], as well as recent reports on traces of the compound in *C. arabica* [30–32], emphasize the importance of studies on the efficiency of 16-OMC as a species discriminator. In addition, even though the diterpene profile is highly affected by genetic diversity [32–34], many works have not adequately identified the *C. canephora* variety used, and there is no information in the literature on intervarietal hybrids of Conilon and Robusta.

Thus, the objective of this research was to characterize *Coffea canephora* coffees, natural intervarietal hybrids of Conilon and Robusta, regarding the content of kahweol, cafestol, and 16-*O*-methylcafestol. The study also evaluated the impact of genetic diversity and environment on the composition of these diterpenes.

2. Materials and Methods

2.1. Reagents, Standards, and Equipment

The following chemicals were used for the extraction of compounds and preparation of the mobile phase: potassium hydroxide analytical grade (KOH; purity ≥ 99%) (F. Maia™, Belo Horizonte, Brazil), ethanol 96% analytical grade (Alphatec™, São José dos Pinhais, Brazil), methyl *tert*-butyl ether (MTBE) HPLC grade (purity ≥ 99%, Merck™ KGaA, Darmstadt, Germany), acetonitrile HPLC grade (Merck™, Darmstadt, Germany). We used 0.45 µm nylon membranes and 0.22 µm syringe filters (Merck Millipore™, Tullagreen, Ireland). Kahweol and cafestol (Axxora™, San Diego, CA, USA) with 98% purity certified by Alexis Biochemicals (Lausen, Switzerland), and 16-*O*-methylcafestol (16-OMC) (Sigma-Aldrich™, Saint Louis, MO, USA), with 98.6% purity, were used as standards.

The water used to prepare solutions was obtained with an Elga Purelab Option-Q™ purification and filtration system (Veolia Water Solutions & Technologies™, High Wycombe, UK). A Supelcosil LC-18 column (150 × 3 mm, 3 µm) (Supelco Park™, Bellefonte, PA, USA) was used.

Analysis was performed in a Waters Acquity ultra-efficient liquid chromatograph–UPLC (Waters™, Milford, CT, USA) equipped with an automatic sample injector, a quaternary solvent pumping system, a column heater/cooler module, and a diode array detector, controlled by the Empower 3 program.

The following equipment was also used: gas pilot roaster with 300 g capacity (Palini & Alves Máquinas Agrícolas™, Espírito Santo do Pinhal, Brazil); coffee grinder Krups GVX 2 (Krups™, Xangai, China); Minolta CR-410 colorimeter (Konica Minolta Sensing Inc., Osaka, Japan) with illuminant D65 and diffused illumination/viewing angle of 0°; MB 45 moisture analyzer (Ohaus™, Barueri, Brazil); MX-S vortex (Phox Suprimentos Científicos™, Colombo, Brazil); water bath (Marconi Equipamentos para Laboratórios Ltda, Piracicaba, Brazil), and 5804 R centrifuge (Eppendorf™, Hamburg, Germany).

2.2. Material

Coffea canephora clonal coffees, natural hybrids of the Conilon and Robusta varieties, were provided by Embrapa Rondônia, Brazil. The five currently most cultivated genotypes in Rondônia—clones 03, 05, 08, 25, and 66—were studied. These unregistered clones were selected by the coffee growers themselves due to their good agronomic traits and beverage quality [10]. Each clone was grown in six different growing sites—Nova Brasilândia do Oeste, São Miguel do Guaporé, Alto Alegre dos Parecis, Rolim de Moura, Porto Velho, and Alto Paraíso—in the State of Rondônia in the western Amazon, Northern Brazil. The climate in the region is Aw (Köppen classification), defined as tropical humid, with a rainy season (October to May) in the summer and a dry season in the winter [10].

In this way, a total of 30 samples with diversity in genetics and growing sites were studied. For the agronomic traits, the plants differed in size, yield, vigor, resistance to disease, maturity (fruit-ripening seasons), and fruit size (Table 1). Growing sites differed regarding the location in the State of Rondônia (Forest Zone, Jamari Valley, Madeira Mamoré, and the Guapore Valley), altitude (86 to 381 m), average temperature (23.1 to 26 °C), and annual rainfall (1735 to 2302 mm) [35].

Table 1. Characteristics of the main clones of *C. canephora* grown in the State of Rondônia.

	Genotypes				
	Clone 03	**Clone 05**	**Clone 08**	**Clone 25**	**Clone 66**
Plant size	Medium	Medium	Medium	Medium	Short
Main features	High production per branch	Disease resistance	Vigor and high yield	High yield	High yield
Maturity	Intermediate [1]	Intermediate/late [2]	Intermediate [1]	Intermediate [1]	Early [3]
Fruit size	Medium	Medium	Large	Large	Small
Presence in field	80%	41%	89%	89%	63%

Source: [6]. [1] May. [2] The end of May/beginning of June. [3] April.

Coffees were harvested (approximately 300 g per sample) and processed between April and June in 2018. Harvesting was manual and selective to obtain only ripe fruit at the cherry stage. Coffees were naturally sun-dried and processed. Green coffee was stored in plastic bags at 8 °C until roasting.

Roasting conditions were based on the information described by Mori et al. [28] for Brazilian Conilon coffees: from 7 to 24 min at temperatures from 210 to 230 °C. The diversity in the process was due to differences in size and coffee bean characteristics. The degree of roasting was standardized to achieve weight loss of approximately 16%, described by Mendes et al. [36] as optimal for *C. canephora*.

Roasted beans were packed in plastic bags and kept under refrigeration (8 °C) until grinding. Coffee beans were ground at fine granulometry: 3% of coffee particles were retained in sieve size #20 (0.850 mm mesh opening); 57% were retained in sieve size #40 (0.420 mm mesh opening), and 40% were retained in the bottom pan. Ground coffees were stored (8 °C) for a maximum of two months until the analyses.

For color characterization, the ground coffee samples were evaluated in triplicate. Values of 31.2 ± 1.4 for lightness and 39.3 ± 4.0 for hue indicated a light-medium roast degree. Moisture was determined at 105 °C for 7 min, and the average value was 2.1 ± 0.2 g 100 g^{-1}.

2.3. Diterpene Analysis

The extraction was carried out according to Dias et al. [37]. Samples (0.200 g) were saponified with 2.0 mL of potassium hydroxide 2.5 mol L^{-1} in ethanol 96% v/v at 80 °C for 1 h. After adding 2.0 mL of water, the extraction step was repeated 3 times, by adding 2.0 mL of MTBE and collecting the organic phase after shaking and centrifugation (2 min; 3000 rpm; room temperature). For cleaning, 2 mL of water was added. The organic extract was collected and evaporated to dryness (70 °C). After resuspension with 4.0 mL of the mobile phase, the extract was filtered. Genuine duplicate extraction was performed.

Chromatographic analysis was performed based on Mori et al. [28]. Isocratic elution with mobile phase water:acetonitrile (45:55, *v/v*) at a flow rate of 0.7 mL min^{-1}, and detection at 290 nm (for kahweol) and 230 nm (for cafestol and 16-OMC) was applied. The injection (3 µL) was made in duplicate. Identification was based on retention times and UV spectra. Quantification was carried out by external standardization using 6-point analytical curves with triplicate measurements ($r = 0.99$, $p < 0.01$) in the concentration ranges of 1 to 200 µg mL^{-1} for kahweol, 50 to 300 µg mL^{-1} for cafestol, and 2 to 400 µg mL^{-1} for 16-OMC. Considering the analytical curve parameters, limits of detection (LD) of 0.8, 2.0, and 0.6 µg mL^{-1} were obtained for kahweol, cafestol, and 16-OMC, respectively. Limits of quantification (LQ) of 2.4, 6.1, and 1.9 µg mL^{-1} (corresponding to 5.2 mg 100 g^{-1}, 13 mg 100 g^{-1}, and 4.2 mg 100 g^{-1}) were also defined. The results were expressed as mg 100 g^{-1} on a dry basis (db). The total contents of diterpenes (i.e., the sum of the three compounds) and the ratio of the contents of cafestol and kahweol were also calculated.

2.4. Data Analysis

To evaluate the effect of environment (growing site) and genetic variability (genotype/clone), the results were submitted to ANOVA and Tukey's test ($p \leq 0.05$) using the free software Rstudio™ version 1.2.5033. Growing site (main plot) and genotype (sub-plot) were considered as treatments in a split-plot design. If a significant main X subplot interaction ($p \leq 0.05$) was observed, the effect of genotype was independently studied for each growing site.

3. Results

The sum of kahweol, cafestol, and 16-OMC contents (total diterpenes content) varied from 192 to 742 mg 100 g^{-1} (Figure 1). These results stood out from those reported in the literature for the increased content and wide variation. Total diterpenes contents from 163 to 505 mg 100 g^{-1} were reported for *C. canephora* coffees [24–26,28,29].
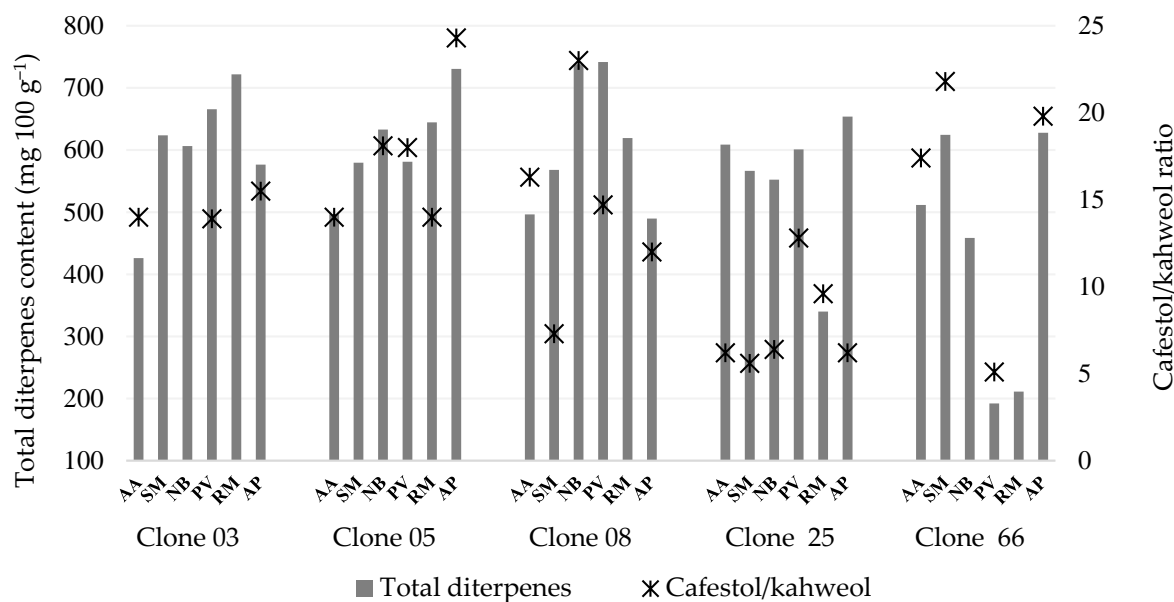

Figure 1. Total diterpene content (mg 100 g^{-1}) and cafestol/kahweol ratio for roasted *C. canephora* intervarietal hybrids cultivated in different growing sites. (Growing site AA: Alto Alegre dos Parecis; SM: São Miguel do Guaporé; NB: Nova Brasilândia do Oeste; PV: Porto Velho; RM: Rolim de Moura; AP: Alto Paraíso.

The results showed great variability in total diterpene content, both among genotypes and growing sites (Figure 1). São Miguel do Guaporé stood out as the growing site with less variation among genotypes (CV of 5%); an opposite behavior was observed for Porto Velho (38%) and Rolim de Moura (44%). The lowest and highest total diterpenes values

were obtained in Porto Velho for clones 66 and 08, respectively. Clone 66 stood out for the highest variation in diterpenes content in different growing sites (CV of 44%) compared to the other genotypes studied (CV of 13 to 20%).

For the three diterpenes studied, a difference among genotypes ($p < 0.001$) and growing sites ($p < 0.001$) was observed, and an interaction occurred between the genotype and the growing site ($p < 0.001$). Thus, the contents of kahweol, cafestol, and 16-OMC in each genotype were influenced by the growing site; however, this effect was genotype-dependent.

For kahweol, clone 25 stood out for the presence of this diterpene in all growing sites, with contents from 17.4 to 41.6 mg 100 g^{-1}. For the other clones, from absence (below LQ of 5.2 mg 100 g^{-1}) up to 36.9 mg 100 g^{-1} were observed. Importantly, the presence of kahweol was noticed for 77% of the intervarietal hybrid coffees studied (Table 2).

Table 2. Kahweol contents * (mg 100 g^{-1}) for roasted *C. canephora* intervarietal hybrids cultivated in different growing sites.

Growing Sites	Genotypes				
	Clone 03	Clone 05	Clone 08	Clone 25	Clone 66
Alto Alegre dos Parecis	17.3 [aB] ± 0.1	17.5 [bcB] ± 0.1	18.0 [dB] ± 0.2	39.0 [bcA] ± 0.3	17.6 [bB] ± 0.1
São Miguel do Guaporé	0.0 [bD] ± 0.0	0.0 [dD] ± 0.0	36.9 [aB] ± 1.0	38.3 [cA] ± 1.3	17.7 [bC] ± 0.1
Nova Brasilândia do Oeste	0.0 [bD] ± 0.0	18.4 [abC] ± 0.1	19.5 [cB] ± 0.2	39.7 [bA] ± 0.3	0.0 [cD] ± 0.0
Porto Velho	18.0 [aD] ± 0.1	17.0 [cE] ± 0.1	23.7 [bA] ± 0.1	21.9 [dB] ± 0.3	19.0 [aC] ± 0.1
Rolim de Moura	0.0 [bC] ± 0.0	18.4 [abA] ± 0.1	0.0 [eC] ± 0.0	17.4 [eB] ± 0.1	0.0 [cC] ± 0.0
Alto Paraíso	17.2 [aC] ± 0.1	18.8 [aB] ± 0.1	18.1 [dBC] ± 0.1	41.6 [aA] ± 0.5	18.8 [aB] ± 0.1

* Mean (genuine duplicates) ± SD (standard deviation); zero value corresponds to contents below LQ (5.16 mg 100 g^{-1}). Means followed by the same capital letter in the same row showed no significant difference between genotypes (Tukey, $p \le 0.05$). Means followed by the same lowercase letter in the same column showed no significant difference between growing sites (Tukey, $p \le 0.05$).

The literature reported from absence [24,26] to low content of kahweol [38–40] in *C. canephora.* Mori et al. [28] noticed the presence of kahweol in 30% of 30 Conilon samples (15 genotypes in two growing sites). Finotello et al. [29] reported the presence of kahweol in 28% of commercial *C. canephora* coffees from several countries in Asia and Africa (39 samples). In general, a range from the absence of kahweol to 20 mg 100 g^{-1} was reported for *C. canephora* coffees of the Conilon variety and for those that had no identification of the variety [24–26,28,29]. The exception was the research of Sridevi et al. [41], who reported elevated contents, up to 313 mg of kahweol 100 g^{-1}. Thus, the *C. canephora* intervarietal hybrids studied showed not only a higher frequency of the kahweol occurrence, but also higher values overall (Table 2) than those described in the literature.

All clones presented kahweol when grown in Alto Alegre dos Parecis, Alto Paraíso, and Porto Velho; the last growing site also stood out for the lowest variation in kahweol contents among genotypes (CV of 14%). Rolim de Moura was the growing site with the highest kahweol variation among genotypes (CV of 137%). Clone 25 showed the higher kahweol values in four of the six growing sites studied (Alto Paraíso, Alto Alegre dos Parecis, Nova Brasilândia do Oeste, and São Miguel do Guaporé), as well as the lowest variability among growing sites (CV of 32%); clone 03 was the most affected by the environment (CV of 110%) (Table 2).

Values in the range of 96 to 457 mg 100 g^{-1} were observed for cafestol, the highest content being for clone 05 grown in Alto Paraíso, and the lowest for clone 08 grown in Porto Velho (Table 3). The literature reported from 76 to 363 mg of cafestol 100 g^{-1} for Conilon or *C. canephora* with no identification of variety [23–26,28,29,41]. It is important to highlight that 17% of the samples in this study showed cafestol contents above the highest value reported in the literature (Table 3).

High variability in cafestol content among genotypes was observed on each growing site (CV from 24 to 39%), except for Alto Alegre dos Parecis (CV of 12%). Clones 03 and 66 were, respectively, the least (CV of 6%) and the most (CV of 46%) affected by the environment. The results showed that the variation in cafestol content was, in general, lower than that observed for kahweol (Tables 2 and 3).

Table 3. Cafestol contents * (mg 100 g^{-1}) for roasted *C. canephora* intervarietal hybrids cultivated in different growing sites.

Growing Sites	Genotypes				
	Clone 03	Clone 05	Clone 08	Clone 25	Clone 66
Alto Alegre dos Parecis	242 [bB] ± 5	245 [cB] ± 2	293 [cA] ± 19	243 [bcB] ± 2	306 [bA] ± 1
São Miguel do Guaporé	258 [abB] ± 11	243 [cBC] ± 2	268 [cB] ± 14	216 [cC] ± 13	387 [aA] ± 4
Nova Brasilândia do Oeste	275 [abC] ± 0	333 [bB] ± 23	448 [aA] ± 14	253 [abC] ± 9	277 [bC] ± 13
Porto Velho	251 [bC] ± 7	306 [bB] ± 9	348 [bA] ± 7	281 [abBC] ± 9	96 [dD] ± 2
Rolim de Moura	288 [aB] ± 2	258 [cB] ± 5	369 [bA] ± 32	167 [dC] ± 9	136 [cC] ± 6
Alto Paraíso	268 [abC] ± 4	457 [aA] ± 14	216 [dD] ± 7	258 [abC] ± 18	374 [aB] ± 3

* Mean (genuine duplicates) ± SD (standard deviation). Means followed by the same capital letter in the same row showed no significant difference between genotypes (Tukey, $p \leq 0.05$). Means followed by the same lowercase letter in the same column showed no significant difference between growing sites (Tukey, $p \leq 0.05$).

Novaes et al. [42] reported that a cafestol/kahweol ratio above 1.2 was associated with *C. arabica* beverages with pleasant aroma and flavor. For *C. arabica* coffees from quality contests, Barbosa et al. [43] also associated an increase in the ratio value with the rise in cup quality. No information regarding the relation between the cafestol/kahweol ratio value and beverage quality for *C. canephora* is available in the literature. Although calculation was not possible for samples without kahweol, cafestol/kahweol values from 5 to 24 were obtained for the others. Variation among genotypes and growing sites was observed, but overall, clone 05 stood out for its higher cafestol/kahweol ratio values (Figure 1). It is interesting that Dalazen et al. [10] reported that clone 05 has a great potential for beverage quality in different environments.

The content of 16-OMC ranged from 75 to 433 mg 100 g^{-1}; the lowest and the highest values were observed for clones 66 and 03, respectively, both grown in Rolim de Moura (Table 4). There were few data and many divergences in the literature regarding the16-OMC content in *C. canephora*. Studies have reported contents from 1 to 154 mg 100 g^{-1} for green coffees [27,44,45], and from absence to 223 mg 100 g^{-1} roasted coffees [23–26,28,29,41]. It is worth emphasizing that 77% of the samples studied presented contents above the highest value cited in the literature (Table 4), evidencing a trend towards increased 16-OMC contents for the natural intervarietal hybrids.

Table 4. The 16-*O*-methylcafestol contents * (mg 100 g^{-1}) for roasted *C. canephora* intervarietal hybrids cultivated in different growing sites.

Growing Sites	Genotypes				
	Clone 03	Clone 05	Clone 08	Clone 25	Clone 66
Alto Alegre dos Parecis	167 [fD] ± 4	233 [dB] ± 2	185 [cCD] ± 3	326 [bA] ± 1	188 [bC] ± 4
São Miguel do Guaporé	365 [cA] ± 4	337 [bB] ± 20	263 [bD] ± 14	312 [bcC] ± 12	220 [aE] ± 1
Nova Brasilândia do Oeste	330 [dA] ± 6	281 [cB] ± 8	270 [bBC] ± 2	259 [dC] ± 12	181 [bD] ± 10
Porto Velho	397 [bA] ± 5	258 [cdD] ± 0	370 [aB] ± 24	298 [cC] ± 5	77 [cE] ± 1
Rolim de Moura	433 [aA] ± 7	367 [aB] ± 2	250 [bC] ± 1	155 [eD] ± 3	75 [cE] ± 5
Alto Paraíso	291 [eB] ± 5	255 [dC] ± 0	255 [bC] ± 7	354 [aA] ± 10	235 [aC] ± 6

* Mean (genuine duplicates) ± SD (standard deviation). Means followed by the same capital letter in the same row showed no significant difference between genotypes (Tukey, $p \leq 0.05$). Means followed by the same lowercase letter in the same column showed no significant difference between growing sites (Tukey, $p \leq 0.05$).

A higher influence of the environmental on the 16-OMC content was observed for clone 66 (CV of 43%), and a smaller influence was observed for clone 05 (CV of 18%) (Table 4). The lowest variability among the genotypes in a growing was observed for Alto Paraíso (CV of 17%), and the highest for Rolim de Moura (CV of 57%) (Table 4). In general, the variation in 16-OMC content was intermediate compared to that observed for cafestol (the lowest) and kahweol (the highest) (Tables 3 and 4).

Regarding the potential to identify the presence of *C. canephora* in blends with *C. arabica* coffees, in this study, the contents of 16-OMC in *C. canephora* hybrids were approximately 30 to 170 times higher than those recently reported for *C. arabica* (a maximum

of 2.6 mg 100 g^{-1}) [30–32]. However, the wide range of values observed corroborated the conclusions reached by several authors [28,29,31] that the high variability of 16-OMC content made it difficult to achieve accurate quantification of *C. canephora* in commercial roasted coffee blends using only this compound as an indicator.

4. Conclusions

The *C. canephora* coffees studied here, natural intervarietal hybrids of Conilon and Robusta, stood out for their higher frequency of the kahweol occurrence and elevated diterpene content. The profile of diterpenes depended on genetics and growing site and the interaction between these two factors. Among the compounds, kahweol showed a higher variability.

Clone 25 stood out for its high kahweol content and the presence of this diterpene in all growing sites. Clone 66 merits mention for the greater influence of the growing site on the diterpene profile. Greater diversity in the diterpene profile was observed among genotypes at Rolim de Moura. In general, the cultivation in Alto Paraíso allowed hybrid coffees with increased diterpenes content; the cultivation of clone 03 in Rolim de Moura and clone 08 in Porto Velho also showed good potential.

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