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Diterpenes in *Coffea canephora*

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ABSTRACT

The presence of diterpenes in coffee has received a great deal of attention in recent years, due to their physiological effects on human health. Some studies related to kahweol and cafestol contents in *Coffea arabica* are available in the literature; however, information on the impact of genetic variability on the profile of diterpenes in *Coffea canephora* is scarce. This work evaluates the contents of kahweol, cafestol and 16-*O*-methylcafestol in 15 genotypes of *C. canephora*. Coffees corresponded to three cultivars – Diamante ES8112, ES8122 'Jequitibá' and Centenária ES8132 – with different fruit-ripening seasons (early, medium and late). Coffees were grown at two locations in the state of Espírito Santo, the largest *C. canephora* growing region in Brazil, resulting in 30 samples. Kahweol was absent in 70% of the samples and the highest content observed was 14.1 mg 100 g^{-1} in the Jequitibá cultivar. Cafestol was present in all samples and it was the main representative of the diterpene class, with contents varying from 152 mg 100 g^{-1} to 360 mg 100 g^{-1}. Contents of 16-*O*-methylcafestol varied from 26.3 mg 100 g^{-1} to 132 mg 100 g^{-1}. A significant difference among genotypes was observed, and there was an interaction between genotypes and growing site for the three diterpenes studied.

1. Introduction

The main compounds of the unsaponifiable matter of coffee lipids are the diterpenes kahweol and cafestol. The consumption of unfiltered coffee brew has been associated with a possible increase in the levels of seric cholesterol and low density lipoproteins; the effects are transient after withdrawal of the diterpenes and are due mainly to the hypercholesterolemic activity of cafestol (Cano-Marquina et al., 2013; Higdon and Frei, 2006; Urgert et al., 1995, 1996). However, studies have stressed the beneficial effects of diterpene ingestion on health, due to their anti-carcinogenic, anti-inflammatory and antioxidant activities (Cavin et al., 2002; Kim et al., 2006; Gaascht et al., 2015; Higgins et al., 2008; Lee et al., 2007; Muriel and Arauz, 2010; Wang et al., 2012), suggesting that the moderate consumption of coffee reduces the risk or severity of several diseases (Freedman et al., 2012), being associated with a reduction in mortality rate (Ding et al., 2015).

Amongst the most commercially important coffee species, *Coffea canephora* stands out for its vigor, being adapted to regions of low altitude and high temperatures and resistant to hydric stress. A coffee brew with low acidity, with more phenolic, spicy, papery and woody aromas and bitter taste, and high astringency and body is produced with *C. canephora*, which is used directly in the production of instant coffee and in blends with *Coffea arabica* for roasted coffee (Clarke and Macrae, 1985; Williams et al., 1989). Brazil is the second largest grower of *C. canephora* in the world, with a production of 17 million bags of 60 kg in the 2014/2015 harvest (USDA, 2016), concentrated in the states of Espírito Santo (70%) and Rondônia (15%) (CONAB, 2015; MAPA, 2016).

Genetic diversity has contributed to the wide variation in the profile of diterpenes for different coffee species and varieties (Benassi and Dias, 2015; Kitzberger et al., 2013; Speer and Kölling-

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Speer, 2006). Diterpenes have been studied as possible discriminants of the species *C. canephora* and *C. arabica* in roasted coffee blends (De Souza and Benassi, 2012; Speer and Kölling-Speer, 2006). Diterpenes contents in *C. arabica* are well known, but the literature is highly limited and provides conflicting information for *C. canephora*. In general, when compared to C. *arabica*, *C. canephora* stands out because of its low kahweol content (Campanha et al., 2010; De Souza and Benassi, 2012; Speer and Kölling-Speer, 2006) and the presence of a specific diterpene, 16-*O*-methylcafestol (Pettitt, 1987; Speer et al., 1991). In a recent review, Benassi and Dias (2015) studied *C. canephora* from several countries and under different roasting conditions, and found contents of cafestol from 76 to 363 mg 100 g^{-1} and the absence of kahweol or contents lower than 14 mg 100 g^{-1}. As for 16-*O*-methylcafestol, there are little data available, with contents from 100.8 mg 100 g^{-1} to 198.0 mg 100 g^{-1} being reported in roasted coffee (Schievano et al., 2014).

In the breeding program of the Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural (Incaper, Espírito Santo, Brazil), a wide variety of *C. canephora* genotypes has being studied for different agronomic traits (Ferrão et al., 2009). Nine cultivars were developed and recommended for the state of Espírito Santo, out of which eight are clonal and one is seed-propagated. Clonal cultivars are formed by groups of compatible clones of *C. canephora;* each cultivar is formed by at least nine clones used in the plantations. Diamante ES8112, ES8122 'Jequitibá', and Centenária ES8132 are examples of clonal cultivars that present different fruit-ripening seasons (early-maturing, medium-maturing and late-maturing, respectively) (Incaper, 2016a,b,c).

Considering the interest in the study of diterpenes, the lack of data on *C. canephora* matrix, and the importance of Brazil as a grower of this coffee species, the objective of this work was to evaluate the contents of kahweol, cafestol and 16-*O*-methylcafestol in 15 clones (from now on referred to as genotypes) of three *C. canephora* cultivars – Diamante ES8112, ES8122 'Jequitibá' and Centenária ES8132–grown in two locations.

2. Materials and methods

2.1. Reagents, standards and equipment

For extraction of diterpenes and preparation of the mobile phase, potassium hydroxide (KOH) analytical grade (F. Maia, São Paulo, Brazil), ethanol 96% analytical grade (Êxodo Científica, Hortolândia, Brazil), acetonitrile HPLC grade (Fischer Scientific, Bridgewater, NJ) and methyl *tert*-butyl ether (MTBE) HPLC grade (Acrós Organics, Morris Plains, NJ) were used. Kahweol and cafestol (Axxora, San Diego, CA) of 98% purity certified by Alexis Biochemicals (Lausen, Switzerland) and 16-*O*-methylcafestol (Sigma-Aldrich, Saint Louis, MO) of 98.6% purity were used as standards. The water used for the preparation of standards and solutions was obtained by Elga Purelab Option-Q purification system (Veolia Water Technologies, Saint-Maurice, France). Nylon membranes of 0.22 μm were used to filter the mobile phase (Millipore, Billerica, MA) and samples (Whatman, Maidstone, UK).

Chromatographic analyses were performed in an ultra-performance liquid chromatograph Waters Acquity (Waters, Milford, MA) equipped with a flow-through needle injector, a quaternary solvent pumping system, column heater/cooler module and photodiode array detector, controlled by the Empower 3 program.

For color analysis, a Minolta CR-410 colorimeter (Konica Minolta Sensing Inc., Osaka, Japan) was used to obtained the L^* (lightness), and the chromatic coordinates a^* (red-green component) and b^* (yellow-blue component) in the CIE Lab system. The analyses were performed under the conditions of standard illuminant C and 10° observer.

For moisture determination, a gravimetric moisture analyzer MB 45 (Ohaus, Barueri, Brazil) with halogen lamp was used. The analysis was performed at 105 °C, to a constant sample weight.

2.2. Genetic material and preparation

Fifteen genetic materials (genotypes) from *C. canephora* originated from the breeding program of INCAPER (Espírito Santo, Brazil) were studied. These genotypes are agronomic divergent and correspond to three clonal cultivars that have distinct fruit-ripening seasons: Diamante ES8112–early-maturing (genotype 101E, 103E, 105E, 106E, and 108E), ES8122 'Jequitibá' – medium-maturing (genotype 201 M, 202 M, 203 M, 207 M, and 209 M) and Centenária ES8132 – late-maturing (genotype 301 L, 302 L, 303 L, 306 L, and 307 L). Five of the nine genotypes were studied for each cultivar.

Samples were collected from demonstration crops grown under two distinct experimental conditions and at 36 months: a) Experimental Farm of Marilândia – located in the county of Marilândia, in the Northwest of the Espírito Santo state (19° 24′ S, 40° 31′ W), altitude of 104 m, soil of the red-yellow latosol type, dry and hot, with an average annual temperature of 24.2 °C, annual rainfall of 1129 mm; and b) Experimental Farm of Bananal do Norte, located in the county of Cachoeiro do Itapemirim, in the Southern of the Espírito Santo state (20° 75′ S, 41° 29′ W), altitude of 146 m, soil of the red-yellow latosol type, dry and with an average annual temperature of 23.8 °C and annual rainfall of 1086 mm.

Samples (500 g) were collected in the year 2014, during the harvest from May to July. First, the early maturation genotypes (May) were harvested manually followed by those of medium maturation (June) and late maturation (July). Only cherry stage fruit were collected. Coffee were naturally sun-dried, processed and cleaned. Only non-defective and 16 (6.5 mm) sieve-size beans (Brasil, 2003) were selected. The green coffee beans were stored in plastic bags at room temperature until being roasted in October 2014.

Coffees (100 g) were roasted in a Rod Bel (Rod-Bel, São Paulo, Brazil) gas pilot roaster for 17–29 min, at temperatures from 210 °C to 230 °C (Mendes et al., 2001). The diversity in the process was due to differences in size and coffee bean characteristics. The degree of roasting was standardized in order to achieve weight loss of 16.5 ± 0.4 g 100 g^{-1}, similar to the method described by Mendes et al. (2001) for an optimal roasting degree for *C. canephora*.

Samples were ground using a Burr bench grinder GVX 2 (Krups, Shanghai, China). Roast coffee was ground at a fine granulometry (0% retained in sieve size 1.18 mm; 70% retained in sieve size 0.60 mm, and 30% passing a sieve size 0.60 mm), according to ABIC (Brazilian Coffee Roasters Association) (ABIC, 2016). Roast and ground coffees had L^* of 25.3 ± 1.4, a^* of 8.2 ± 0.5, b^* of 10.6 ± 1.9, and moisture of 0.8 ± 0.1 g 100 g^{-1}. Samples were stored in plastic bags and kept at 8 °C until analysis.

2.3. Kahweol, cafestol and 16-O-methylcafestol determination

The extraction was performed as described by Dias et al. (2010). Samples (0.2000 g) were saponified with 2.0 mL of potassium hydroxide (2.5 M) in ethanol (96% v/v) at 80 °C for 1 h. For the extraction of the unsaponifiable matter, 2.0 mL of distilled water and 2.0 mL of MTBE were added. After agitation and centrifugation (3 min at 3000 rpm and room temperature), the organic phase was collected. This last stage of the procedure was repeated three times. Distilled water (2 mL) was added for cleaning and the organic extract was collected and evaporated to dryness in a water bath (70 °C). After resuspension in 4.5 mL of the mobile phase

(45:55, v/v, water: acetonitrile), the extract was filtered. Duplicate independent extractions were performed.

The analysis was carried out according to the method developed and validated by Dias et al. (2010) and modified by Wuerges et al. (2016). The analysis was carried out in a Kinetex 2.6 μm C18 (150 mm × 4.6 mm) (Phenomenex, Torrance, CA) column, at 26 °C, with detection at the maximum wavelength of each diterpene: 230 nm (cafestol and 16-*O*-methylcafestol) and 290 nm (kahweol). Isocratic elution of water:acetonitrile (45:55, v/v) at a flow rate of 1.2 mL min^{-1} and an injection volume of 1.4 μL were applied. The total chromatographic run time was 7 min. Duplicate injections were done.

Identification was based on retention times and UV spectra. Quantification was carried out by external standardization using 6-point analytical curves with triplicate measurements ($r \geq 0.999$, $p < 0.001$) in the concentration range of 2–200 μg mL^{-1}, corresponding to 4.5 mg 100 g^{-1}, and 450 mg 100 g^{-1}, respectively.

Considering the analytical curve parameters (ICH, 2005), a detection limit (DL) of 0.5 μg mL^{-1} was obtained for the three compounds and quantification limits (QL) of 1.4 μg mL^{-1}, 1.6 μg mL^{-1} and 1.5 μg mL^{-1} (corresponding to 3.2 mg 100 g^{-1}, 3.6 mg 100 g^{-1}, and 3.4 mg 100 g^{-1}) for kahweol, cafestol and 16-*O*-methylcafestol, respectively. Diterpene contents were expressed on a dry weight basis (db).

2.4. Statistical treatment

To evaluate the effect of growing site and genetic variability, results were submitted to ANOVA and Tukey Test ($p \leq 0.05$) using the free software SISVAR version 5.6 (SISVAR, 2016). Growing site/experimental farm (main plot) and genotype (subplot) treatments were considered in a split-plot design. If a significant main × subplot interaction ($p \leq 0.05$) was observed, the effect of genotype was independently studied for each experimental farm.

3. Results and discussion

Tables 1, 2 and 3 show the contents of kahweol, cafestol and 16-*O*-methylcafestol, respectively, for 15 different genotypes of *C. canephora* grown at two sites. The total content of diterpenes (the sum of kahweol, cafestol and 16-*O*-methylcafestol) varied from 191 to 415 mg 100 g^{-1}. Cafestol was the main representative, with a contribution of 66%–90% of the total diterpenes.

Considering Diamante, Jequitibá and Centenária cultivars, levels of kahweol varied from absent (below LQ of 3.2 mg 100 g^{-1}) to 5.3 mg kahweol 100 g^{-1} (Table 1). The average content of cafestol for each cultivar varied from 200 mg 100 g^{-1} to 264 mg 100 g^{-1} (Table 2). These values are in agreement with the literature that reports the absence of kahweol or contents below 14 mg of kahweol·100 g^{-1} and from 76 to 363 mg of cafestol·100 g^{-1} for *C. canephora* from several countries and under different roasting conditions (Campanha et al., 2010; De Souza and Benassi, 2012; Lercker et al., 1996; Sridevi et al., 2011).

For kahweol contents, there was a difference ($p < 0.001$) between genotypes but not between growing sites ($p = 0.117$). Considering the average contents for each cultivar, Jequitibá cultivar (medium-maturing) presented higher contents of kahweol; the highest content was observed for genotype 207 M (14.1 mg 100 g^{-1} and 10.3 mg 100 g^{-1}) at the two growing sites. Kahweol was absent in 70% of the 30 samples analyzed (Table 1). There was an interaction between growing site and genotype ($p < 0.001$), showing that kahweol content in each genotype was influenced by the growing site; however, this effect was genotype-dependent.

A similar behavior was observed for cafestol: there was a difference ($p < 0.001$) between genotypes but not between growing sites ($p = 0.149$). In general, higher content of cafestol was observed for the Centenária cultivar (late-maturing); the highest values were observed for genotypes 303L and 306L. An interaction between growing site and genotype also occurred for cafestol ($p < 0.001$). Higher contents of cafestol for genotypes 303L and 306L were observed in Marilândia (around 355 mg 100 g^{-1}) in comparison with those obtained for the same genotypes in Bananal do Norte (around 298 mg 100 g^{-1}) (Table 2). The early-maturing genotypes 101E, 103E and 106E showed the lowest contents of cafestol in Marilândia (165 mg 100 g^{-1}, 173 mg 100 g^{-1} and 178 mg 100 g^{-1}) and genotype 103E showed the lowest content in Bananal do Norte, 152 mg 100 g^{-1} (Table 2).

It is difficult to compare our results with the literature due to the limited data available for *C. canephora*; additionally different bases are still used to express diterpene contents. Speer and Kölling-Speer (2001), studying diterpenes in green *C. canephora*

Table 1
Kahweol content[a] (mg 100 g^{-1}) in *Coffea canephora* genotypes grown at two sites.

Cultivar	Genotypes	Growing Site/Experimental Farm	
		Marilândia	Bananal do Norte
Diamante (early- maturing)	101E	$0.0^{Be} \pm 0.0$	$3.7^{Ae} \pm 0.0$
	103E	$0.0^{Ae} \pm 0.0$	$0.0^{Af} \pm 0.0$
	105E	$0.0^{Ae} \pm 0.0$	$0.0^{Af} \pm 0.0$
	106E	$0.0^{Ae} \pm 0.0$	$0.0^{Af} \pm 0.0$
	108E	$0.0^{Ae} \pm 0.0$	$0.0^{Af} \pm 0.0$
	Mean[b] ± SD (CV%)	0.0 ± 0.0 (0.0)	0.7 ± 1.6 (228.6)
Jequitibá (medium- maturing)	201M	$0.0^{Ae} \pm 0.0$	$0.0^{Af} \pm 0.0$
	202M	$3.8^{Bd} \pm 0.3$	$4.7^{Ad} \pm 0.0$
	203M	$8.4^{Ab} \pm 0.1$	$8.0^{Bb} \pm 0.3$
	207M	$14.1^{Aa} \pm 0.3$	$10.3^{Ba} \pm 0.2$
	209M	$0.0^{Ae} \pm 0.0$	$0.0^{Af} \pm 0.0$
	Mean[b] ± SD (CV%)	5.3 ± 6.0 (113.3)	4.6 ± 4.6 (100.0)
Centenária (late- maturing)	301L	$0.0^{Ae} \pm 0.0$	$0.0^{Af} \pm 0.0$
	302L	$5.0^{Bc} \pm 0.1$	$6.1^{Ac} \pm 0.3$
	303L	$0.0^{Ae} \pm 0.0$	$0.0^{Af} \pm 0.0$
	306L	$0.0^{Ae} \pm 0.0$	$0.0^{Af} \pm 0.0$
	307L	$0.0^{Ae} \pm 0.0$	$0.0^{Af} \pm 0.0$
	Mean[b] ± SD (CV%)	1.0 ± 2.2 (220.0)	1.2 ± 2.7 (225.0)

Means followed by the same capital letter in the same row show no significant difference between growing sites (Tukey, $p \leq 0.05$). Means followed by the same lower case letter in the same column show no significant difference between genotypes (Tukey, $p \leq 0.05$).

[a] Mean (duplicates) ± SD (standard deviation) for each genotype; Zero value corresponds to contents below QL (3.2 mg 100 g^{-1}).

[b] Average content for each cultivar ± SD (standard deviation) and CV (coefficient of variation) between genotypes of the same cultivar.

Table 2
Cafestol content[a] (mg 100 g^{-1}) in *Coffea canephora* genotypes grown at two sites.

Cultivar	Genotypes	Site/Experimental Farm	
		Marilândia	Bananal do Norte
Diamante (early- maturing)	101E	$164.8^{Bg} \pm 8.9$	$234.9^{Ae} \pm 1.4$
	103E	$172.7^{Afg} \pm 2.8$	$151.7^{Bg} \pm 1.5$
	105E	$230.9^{Bc} \pm 1.4$	$242.6^{Ade} \pm 4.6$
	106E	$178.1^{Befg} \pm 0.5$	$235.5^{Ae} \pm 3.2$
	108E	$254.0^{Ab} \pm 0.5$	$252.4^{Acd} \pm 3.1$
	Mean[b] ± SD (CV%)	200.1 ± 39.8 (19.9)	223.4 ± 40.7 (18.2)
Jequitibá (medium- maturing)	201M	$216.7^{Bd} \pm 0.4$	$261.4^{Ac} \pm 4.1$
	202M	$231.3^{Ac} \pm 0.8$	$233.7^{Ae} \pm 5.1$
	203M	$229.7^{Bcd} \pm 3.0$	$245.4^{Ade} \pm 5.8$
	207M	$226.5^{Bcd} \pm 1.7$	$239.0^{Ade} \pm 6.7$
	209M	$227.6^{Acd} \pm 0.8$	$182.0^{Bf} \pm 2.7$
	Mean[b] ± SD (CV%)	226.4 ± 5.7 (2.5)	232.37 ± 29.9 (18.2)
Centenária (late- maturing)	301L	$237.0^{Bc} \pm 1.8$	$275.6^{Ab} \pm 4.0$
	302L	$184.7^{Aef} \pm 1.3$	$174.9^{Bf} \pm 6.0$
	303L	$349.5^{Aa} \pm 0.3$	$296.3^{Ba} \pm 0.1$
	306L	$359.7^{Aa} \pm 1.7$	$300.0^{Ba} \pm 2.6$
	307L	$190.3^{Ae} \pm 3.6$	$178.4^{Bf} \pm 6.1$
	Mean[b] ± SD (CV%)	264.2 ± 85.0 (32.2)	245.0 ± 63.1 (25.8)

Means followed by the same capital letter in the same row show no significant difference between growing sites (Tukey, $p \leq 0.05$). Means followed by the same lower case letter in the same column show no significant difference between genotypes (Tukey, $p \leq 0.05$).
[a] Mean (duplicates) ± SD (standard deviation) for each genotype.
[b] Average content for each cultivar ± SD (standard deviation) and CV (coefficient of variation) between genotypes of the same cultivar.

Table 3
Content[a] of 16-*O*-methylcafestol (mg 100 g^{-1}) in *Coffea canephora* genotypes grown at two sites.

Cultivar	Genotypes	Site/Experimental Farm	
		Marilândia	Bananal do Norte
Diamante (early- maturing)	101E	$26.3^{Bg} \pm 0.7$	$40.8^{Aj} \pm 0.8$
	103E	$35.9^{Bf} \pm 0.7$	$42.0^{Aij} \pm 0.6$
	105E	$34.0^{Bf} \pm 0.2$	$47.1^{Ahi} \pm 1.4$
	106E	$36.3^{Bf} \pm 0.4$	$53.1^{Afg} \pm 0.1$
	108E	$132.1^{Aa} \pm 2.3$	$120.8^{Ba} \pm 0.9$
	Mean[b] ± SD (CV%)	52.9 ± 44.5 (118.9)	60.8 ± 33.9 (55.8)
Jequitibá (medium- maturing)	201M	$34.8^{Bf} \pm 0.4$	$44.2^{Ahij} \pm 2.0$
	202M	$49.3^{Ae} \pm 0.2$	$46.0^{Bhij} \pm 2.1$
	203M	$49.0^{Be} \pm 1.6$	$59.7^{Ae} \pm 1.1$
	207M	$68.1^{Bc} \pm 0.5$	$91.8^{Ab} \pm 3.6$
	209M	$77.7^{Ab} \pm 0.9$	$78.7^{Ac} \pm 0.2$
	Mean[b] ± SD (CV%)	55.8 ± 17.0 (30,5)	64.1 ± 20.7 (32.3)
Centenária (late- maturing)	301L	$48.9^{Be} \pm 0.6$	$59.6^{Ae} \pm 1.1$
	302L	$77.2^{Bb} \pm 0.5$	$83.3^{Ac} \pm 1.3$
	303L	$36.8^{Bf} \pm 0.7$	$48.7^{Agh} \pm 1.5$
	306L	$54.9^{Bd} \pm 0.3$	$58.1^{Aef} \pm 2.0$
	307L	$53.9^{Bde} \pm 0.1$	$65.1^{Ad} \pm 3.6$
	Mean[b] ± SD (CV%)	54.3 ± 14.6 (26.9)	63.0 ± 12.8 (20.3)

Means followed by the same capital letter in the same row show no significant difference between growing sites (Tukey, $p \leq 0.05$). Means followed by the same lower case letter in the same column show no significant difference between genotypes (Tukey, $p \leq 0.05$).
[a] Mean (duplicates) ± SD (standard deviation) for each genotype.
[b] Average content for each cultivar ± SD (standard deviation) and CV (coefficient of variation) between genotypes of the same cultivar.

coffees originating from Vietnam, the Ivory Coast, Indonesia, Zaire, Uganda and New Guinea, reported kahweol contents below 10 mg 100 g^{-1} of unsaponifiable matter and cafestol contents up to 300 mg 100 g^{-1} of unsaponifiable matter. Roos et al. (1997) found kahweol at levels below 8 mg 100 g^{-1} and cafestol contents of 239 mg 100 g^{-1} and 250 mg 100 g^{-1} in two samples of green *C. canephora* coffee from the Ivory Coast. Kahweol was not detected by De Souza and Benassi (2012) in three roasted Brazilian *C. canephora* coffees originating from the states of Rondônia and Espírito Santo. Dias et al. (2010), studying *C. canephora* Apoatã cultivar, did not detect the presence of kahweol in the endosperm of green coffee, and reported cafestol content of 94 mg 100 g^{-1}. Campanha et al. (2010) reported contents from 163 mg to 275 mg of cafestol 100 g^{-1} and the absence of kahweol in two *C. canephora* brazilian coffees, from Rondônia and Espírito Santo states, with different roasting degrees.

Despite the high kahweol values observed for Jequitibá cultivar (Table 1) and of cafestol for the Centenária cultivar (Table 2), no effect was observed on the content of this diterpene related to the fruit ripening season (early-maturing, medium-maturing or late-maturing), since intra-cultivar variations were more significant. Considering the five genotypes of each cultivar, greater variability was observed in the contents of kahweol, with a coefficient of variation (CV%) of up to 228.6%, compared to cafestol (CV of up to 32.2%) (Tables 1 and 2).

The average content of 16-*O*-methylcafestol for the Diamante, Jequitibá and Centenária cultivars varied from 52.9 mg 100 g^{-1} to 64.1 mg 100 g^{-1}, with high variability between the five genotypes in each cultivar (CV from 20.3% to 118.9%). An effect of the fruit-

ripening season (early-maturing, medium-maturing or late-maturing) on the 16-*O*-methylcafestol content was not observed (Table 3).

There were significant differences ($p < 0.001$) between genotypes and growing sites ($p < 0.029$) for the contents of 16-*O*-methylcafestol. For kahweol and cafestol no systematic effect of growing site was observed.

In general, higher contents of 16-*O*-methylcafestol were observed for the coffees grown in Bananal do Norte. The highest contents were observed in genotype 108E (early-maturing) at the two growing sites (132 mg 100 g^{-1} and 121 mg 100 g^{-1}) (Table 3). Two early-maturing genotypes (101E, 103E) and one medium-maturing genotype (201 M) stood out due to their lower contents of 16-*O*-methylcafestol (below 44.2 mg 100 g^{-1}) at the two growing sites. Literature data on 16-O-methylcafestol are scarce and there is no consensus regarding the concentration range. For green *C. canephora* coffees, contents between 1.0 and 5.0 mg 100 g^{-1} were cited by Speer and Kölling-Speer (2006) while Belitz et al. (2009) reported a range from 60 to 180 mg 100 g^{-1}. Contents of 16-*O*-methylcafestol from 102 to 154 mg 100 g^{-1} in green coffees from the Ivory Coast were reported by Roos et al. (1997). For roasted coffee, Schievano et al. (2014) reported contents from 101 to 198 mg 100 g^{-1}. Pacetti et al. (2012) analyzed diterpenes in four roasted *C. canephora* coffees from India, Vietnam and the Ivory Coast, and reported contents of 16-*O*-methylcafestol between 16.2 mg 100 g^{-1} and 2.62×10^4 mg 100 g^{-1} of unsaponifiable matter.

Pettitt (1987) and Speer et al. (1991) described that 16-*O*-metihylcafestol was present only in *C. canephora* at low concentrations and in the *Coffea dewevrei* species. As it is thermally stable, this compound could be used as an indicator of the presence of *C. canephora* species in roasted coffee products (Kemsley et al., 1995). In Germany, the quantification of 16-*O*-methylcafestol is recommended by the norm DIN 10779 (published in 1999) for the evaluation of *C. canephora* coffee percentage in blends with *C. arabica* (Speer and Kölling-Speer, 2006). As Brazil is the second largest producer of this coffee species, the contents of 16-*O*-methylcafestol reported in this study, in which a great number of genotypes were evaluated, can help establish a concentration range of this diterpene in *C. canephora* coffees.

By evaluating the wide range of 16-*O*-methylcafestol contents, from 26.3 to 132 mg 100 g^{-1}, it is suggested that the sole use of this compound content could not be enough to safely estimate the percentage of *C. canephora* in blends with *C. arabica*. A similar observation was made by Schievano et al. (2014). The authors also reported a wide range for 16-*O*-methylcafestol content in roasted coffee of three different origins and pointed that such variation is a problem for *C. canephora* quantification in blends with *C. arabica*, regardless of the analytical method used.

De Souza and Benassi (2012) propose the use of the relationship kahweol/cafestol as an additional tool to estimate the presence of *C. canephora* in blends with *C. arabica*. These authors stated that a kahweol/cafestol ratio above 1.00 is indicative of C. *arabica* and the addition of *C. canephora* coffee should decrease this ratio. In our study, a kahweol/cafestol ratio between 0.00 and 0.06 was obtained; in accordance to De Souza and Benassi (2012) that kahweol/cafestol ratio could be indicative of *C. canephora*. Considering the results, we propose that the combined use of 16-*O*-methylcafestol content with kahweol/cafestol ratio could be useful in the discrimination of coffee species. Further studies would verify if these two parameters could indicate with efficiency the addition of *C. canephora* to *C. arabica* in roasted coffee blends.

4. Conclusions

The diterpenes profile of *C. canephora* genotypes studied here reinforces the wide variation of kahweol and cafestol contents described in the literature and shows a high variability for 16-*O*-methylcafestol content. Cafestol represents the largest proportion of diterpenes in *C. canephora*, being the diterpene with the lowest variability between genotypes of the same cultivar. Kahweol was absent in most genotypes studied.

The fruit-ripening seasons of the cultivars (early-maturing, medium-maturing or late-maturing) do not affect diterpene content, the variation between genotypes of the same cultivar being more relevant. Only contents of 16-*O*-methylcafestol presented a significant influence of growing site, but an interaction between growing site and genotype was observed for all diterpenes.

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