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ARTICLE

Evaluation of Kahweol and Cafestol in Coffee Tissues and Roasted Coffee by a New High-Performance Liquid Chromatography Methodology

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A reverse phase high-performance liquid chromatography (HPLC) method was developed for the simultaneous quantification of kahweol and cafestol in tissues of fresh fruits, leaves, and roasted coffee beans. The best resolution was obtained with isocratic elution of acetonitrile/water (55/45% v/v) and UV detection. A single sample preparation method carried out by direct saponification and extraction with organic solvent was standardized for all matrices. Good recovery (average of 99% for kahweol and 94% for cafestol), repeatability, and linearity were obtained. Detection limits of 2.3 and 3.0 mg/100 g were observed for kahweol and cafestol. The HPLC method was effective in quantifying these diterpenes in the different coffee matrices. The endosperm and perisperm of *Coffea arabica* cv. IAPAR 59 showed elevated amounts of kahweol as compared to the pericarp and leaves. On the other hand, cafestol was detected in all samples except in leaves from *Coffea canephora* cv. Apoatã.

KEYWORDS: *Coffea arabica*; *Coffea canephora*; diterpenes; HPLC; validation

INTRODUCTION

Coffee is one of the main agricultural export products of developing countries. The two species of economic significance in the coffee trade are *Coffea arabica* (arabica coffee) and *Coffea canephora* (robusta coffee), which correspond to 69 and 31%, respectively, of the world's coffee production. Brazil is the largest green coffee producer and exporter, besides being the second largest consumer of coffee in the world. In Brazil, 81% of the commercial roasted coffee are blends of the species *C. arabica* and *C. canephora* (*1*).

Many studies have related the consumption of coffee beverages with its effects on human health, and several of these studies have focused on the lipid composition of these products (*2*−*5*). Besides the triacylglycerols, sterols, and tocopherols, which are typical components of common edible vegetable oils, coffee oil contains unsaponifiable pentacyclic diterpene alcohols of the kaurene family. Kahweol and cafestol, diterpenes produced only by plants of the *Coffea* genus, are of interest due to their physiological effects and their potential use as discriminants between species (*4*, *6*). Cafestol was found in both *C. arabica* and *C. canephora* plants; however, kahweol was reported to be specific to *C. arabica* (*7*).

The coffee diterpenes are mainly esterified with various fatty acids. Up to 14 derivatives of cafestol and 12 of kahweol have been described (*8*). These compounds are partially stable during roasting (*6*, *9*); however, they can undergo dehydration and form dehydroderivatives in addition to other decomposition products such as kahweal, cafestal, isokahweol, and dehydroisokahweol (*4*).

The physiological action of kahweol and cafestol has been reported to be both desirable and undesirable in terms of human health. These diterpenes may induce the degradation of toxic substances and provide protective action against aflatoxin B1 (*3*). Furthermore, anticarcinogenic, antioxidant, and anti-inflammatory properties as well as hepatoprotector effects have been reported (*3*, *10*−*12*). The anticarcinogenic potential warrants consideration regarding future application in foods and in the pharmaceutical industry. The use of coffee oil in sunblock has been patented due to its capacity to function as a cosmetic (*13*) and because it has anti-inflammatory properties (*14*). On the other hand, a hypercholesterolemic action has been reported (*10*, *15*, *16*), attributed mainly to cafestol (*2*, *5*, *6*).

It is therefore relevant to study the metabolic pathways of kahweol and cafestol in the coffee plant. For this, a rapid and practical methodology is required to evaluate the amount of these compounds in the plant and fruit tissues during their development. This information may help to elucidate the metabolic pathways of cafestol and kahweol and allow for the identification of the genes coding for the enzymes involved in the biosynthesis of these diterpenes (*17*). Furthermore, the diterpene content could be used both in the taxonomic classification of *Coffea* species and

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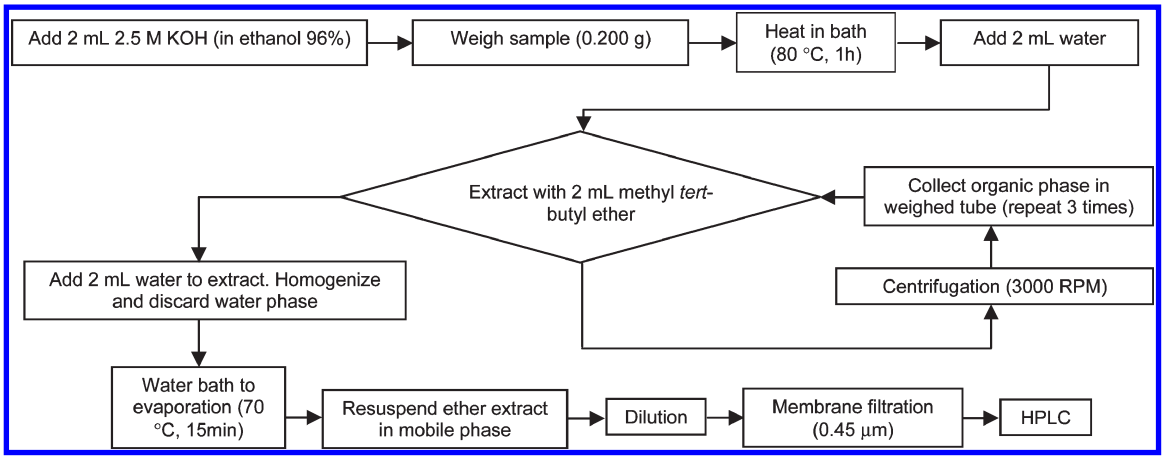

Figure 1. Flowchart of the extraction of the unsaponifiable material.

also as a tool for the discrimination of arabica and robusta coffee species present in the roasted and ground products.

The diterpene content of a coffee beverage is strongly dependent on the method of preparation. In espresso coffee, there can be 5−10 times more diterpenes than in filtered coffee (*6*, *18*). In leaves of *C. arabica*, a maximum content of 11 mg of cafestol/100 g was described (*19*). In mature beans, a maximum concentration of 740 mg/100 g was reported for both kahweol and cafestol (*6*−*8*, *20*). Despite the availability of data on green coffee, no studies have been undertaken regarding the distribution of diterpenes during fruit development or in different tissues of the fresh fruit (pericarp, perisperm, and endosperm).

Different methodologies have been investigated to identify and quantify kahweol and cafestol. The use of Raman spectroscopy (*20*) and gas chromatography (GC) (*6*, *7*, *18*, *21*, *22*) has been reported for the analysis of the beverage and fresh fruits. Spectrophotometric techniques have also been used for kahweol analysis in roasted coffee (*9*, *23*). However, the technique of high-performance liquid chromatography (HPLC) has proven very effective (*16*, *18*, *21*, *24*), as it allows for analysis without derivatization of the sample, avoiding thermal degradation of the lipid components (*25*). In general, a reverse phase is employed, and binary mixtures of water and organic solvents [acetonitrile (ACN), isopropanol, and methanol] (*8*, *16*, *21*, *24*) are used as the mobile phase. However, there is still considerable disagreement regarding the extraction procedure. The use of Soxhlet and organic solvents (*n*-hexane, diethyl ether, or petroleum ether) is the common methodology for lipid extraction and has been applied in the extraction of kahweol and cafestol with subsequent saponification (*4*, *20*−*22*). Some studies have used direct saponification with KOH in alcohol (methanol or ethanol) and extraction of unsaponifiable material using organic solvents (*n*-hexane, diisopropyl ether, and methyl *tert*-butyl ether) (*6*, *9*, *23*). Direct saponification has been described as a rapid and efficient alternative for the extraction of other unsaponifiable compounds, avoiding the formation of artifacts (*25*).

Methods for the analysis of kahweol and cafestol have been developed and applied to specific products, but there are just a few studies that have described the validation of these techniques. To the best of our knowledge, there is no available method that allows the evaluation of these diterpenes in different matrices, such as roasted coffee, leaves, and fruit tissues. Therefore, the objective of this study was to simultaneously determine kahweol and cafestol in roasted coffee, in different tissues of the fresh fruit (pericarp, perisperm, and endosperm), and in the leaves of the coffee plant. To that end, an analytical methodology by HPLC was developed and validated.

MATERIALS AND METHODS

Material. The fresh coffee fruits and the leaves from *C. arabica* cv. IAPAR-59 and *C. canephora* cv. Apoatã species were obtained from the Instituto Agronômico do Paraná (IAPAR, Londrina, Brazil). The collection of the fruits was carried out at different times (days after bloom, DAB) to obtain enough sample mass for the validation of the method for each tissue (∼5.0 g).

The perisperm is present mainly at the beginning of the ripening phase and up to 150 DAB. The endosperm is present in a greater proportion during the riper phases of the fruit (between 150 and 240 DAB). The pericarp is present in the fruit at all of the ripening stages (*26*, *27*). To obtain a greater sample mass, the perisperm was dissected from *C. arabica* fruits at 83 DAB and at 120 DAB from *C. canephora*. The endosperm and pericarp were separated from the whole fruits of *C. arabica* e *C. canephora* harvested at 240 and 214 DAB, respectively. In addition, mature leaves (third pair on the plagiotropic branch) were also collected.

After harvest, the fruits and leaves were immediately frozen in liquid nitrogen to avoid oxidation and stored at −80 °C until analysis. Fruit tissues (perisperm, endosperm, and pericarp) were separated. Each tissue was ground independently in the presence of liquid nitrogen with a pestle and mortar. The samples were weighed in a test tube, taking care to avoid the elevation of temperature (just before the complete evaporation of liquid nitrogen).

The roasted and ground coffee samples of *C. arabica* cv. IAPAR-59 and *C. canephora* cv. Apoatã were also obtained from mature beans harvested at the IAPAR experimental station. The coffee beans were roasted in a roaster (Rod-Bel, São Paulo) until reaching a weight loss of around 17%, considered a medium roast degree. Grinding was carried out after roasting (sieve size ABNT 20), and a blend with 30 wt % *C. canephora* added to *C. arabica*, the most usual for Brazilian commercial coffee, was then prepared.

Extraction of Kahweol and Cafestol and Preparation of Samples. A procedure for the extraction of the diterpenes, originally developed for spectrophotometric determination of kahweol in roasted coffee (*9*, *23*), was tested. It consisted of direct saponification with subsequent separation of the unsaponifiable fraction. A solution of 2.5 M potassium hydroxide (Synth, Diadema, Brazil) in ethanol (Merck, Darmstadt, Germany) was used for the saponification step. The saponification was followed by extraction with methyl *tert*-butyl ether (Vetec, Duque de Caxias, Brazil) and clean up with purified water (Milli-Q, Millipore purification system, Billerica, MA) as detailed in **Figure 1**. The ether extract was resuspended in the mobile phase, filtered using a nylon membrane filtration (0.45 μm Millipore), and injected into the chromatograph. Temperatures of the saponification (60, 70, and 80 °C) and solvent evaporation (60 and 70 °C) steps were tested.

Chromatographic Evaluation. The analysis was carried out in a Shimadzu Liquid Chromatograph (Kyoto, Japan), with a quaternary system of solvent pumping (model LC10 ATvp), online degasser DGU-14 Avp, a CTO-10 ASvp column oven model, and a Rheodyne injection valve with a 20 μL loop. The system was coupled to a Shimadzu model SPD-M10 Avp, UV/vis spectrophotometry diode array detector (spectral scan

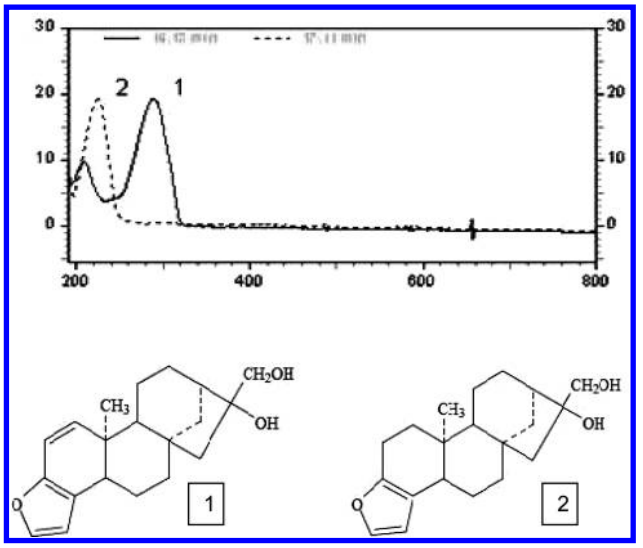

Figure 2. Spectrum (190−800 nm) of kahweol (1) and cafestol (2) standards.

from 190 to 800 nm) connected to a PC through an interface (SCL-10 Avp) for data acquisition and integration. The mobile phases were filtered in a Millipore vacuum filtration system through 0.45 μm membranes.

For the identification and quantification of the compounds and validation of the method, cafestol and kahweol standards (Axxora, San Diego, CA) certified by Alexis Biochemicals (Lausen, Switzerland) were used. The purity of the standards was 98%. The identification was based on retention time comparison and coelution with the authentic standards. The quantification was carried out by external standardization.

The studied chromatographic conditions were chosen based on available literature (*10*, *19*, *24*). Two Waters Spherisorb columns (250 mm L × 4.6 mm i.d., 5 μm) were tested as follows: ODS 1, carbon loading of 7% (w/w) and nonend-capped; and ODS 2, carbon loading of 12% (w/w) and end-capped. Mixtures of water with organic modifiers, ACN (Carlo Erba, Duque de Caxias, Brazil), and methanol (Merck), were studied as mobile phases in isocratic elution (water:methanol mixtures of 25:75, 20:80, 15:85, and 10:90; water:ACN mixtures of 50:50, 45:55, 40:60, and 30:70) and linear gradient elution (ACN/water, 50% ACN until 15 min, and 60% ACN until 20 min). Flow rate (0.6, 0.7, 0.8, 0.9, 1.0, 1.1, and 1.2 mL/min) and sample dilution (1:5 and 1:6) were tested in addition. The best wavelength of detection for each compound was also evaluated (**Figure 2**).

Validation of the Analytical Methodology. Reliability of the method was tested for linearity, precision, sensitivity, and recovery (*28*). The linearity was determined by analysis of the standard mixtures in six concentrations (between 50 and 1000 mg of kahweol or cafestol per 100 g), in triplicate. The concentration range was determined based on previous analysis and literature data (*6*−*8*, *20*). The coefficient of determination (R^2) and significance level (p) of the calibration curves were determined.

The recovery tests were carried out in duplicate. The kahweol and cafestol standards were added to the samples (0.200 g) before analysis, in an amount of approximately 50% of the initial content (0.01, 0.01, and 0.05 mg for the perisperm, endosperm, and pericarp of *C. canephora*; 0.04 mg for the leaves of *C. arabica*; and 0.40 mg for the roasted coffee).

To determine precision, five different extracts of the same sample (0.20 g) were analyzed using the same analytical method (extraction and analysis conditions), in the same equipment at the same time (intraday repeatability). The repeatability study was carried out for each of the matrices.

The values for limit of detection (LOD) and limit of quantification (LOQ) were estimated using eq 1 (*28*, *29*).

$$\mathrm{LOD, LOQ} = c \cdot (\mathrm{S/N}) \cdot N/H \quad (1)$$

where S/N is the signal/noise ratio (for LOD, S/N = 3.3, and for LOQ, S/N = 10), c = sample concentration, N = noise value when the blank is analyzed (10 measurements), and H = value of the signal when the sample is analyzed.

RESULTS AND DISCUSSION

Extraction of Diterpenes and Sample Preparation. The original extraction procedure of direct saponification (**Figure 1**) was adapted after preliminary tests with standards of kahweol and cafestol and all of the matrices under study (pericarp, perisperm, and endosperm from fresh fruits, roasted coffee beans, and leaves from *C. arabica* and *C. canephora*). The temperature of the baths was adjusted to 80 °C for 1 h for the saponification step and 70 °C for the solvent evaporation. The centrifugation time was set at 2 min. In the case of leaves, it was verified that an additional clean up step to remove pigments from the extract was not required. Despite the large number of eluted substances at the beginning of the chromatogram, the efficiency of the chromatographic system was not affected, since the peaks of kahweol and cafestol had a chromatographic profile and separation efficiency similar to the results with other matrices (**Figure 3**).

It should be noted that to avoid oxidation of the compounds and enzymatic browning, special care was required to always carry out the maceration and weighing of the fruit tissues and leaves at low temperature, as described above. Increased repeatability was observed when the leaf and fresh fruit tissue samples were weighed directly in the KOH solution, rather than in the tube prior to adding the saponificating solution (**Figure 1**).

Definition of Chromatographic Conditions. For the definition of a standard procedure, the resolution of cafestol and kahweol in all of the matrices was evaluated, as well as the possibility for their simultaneous determination. As verified in other studies (*16*, *21*, *24*), the greatest difficulty was the adequate separation of kahweol and cafestol peaks, due to their very close retention times, adversely affecting the efficiency of the chromatographic process. For the various combinations of conditions tested, the differences regarding the separation of the peaks of interest were small, even using a diode array detector that allowed the detection in different wavelengths. This is probably due to the structural similarity of the two compounds, as only one double bond in the kaurene ring differentiates kahweol from cafestol (**Figure 2**).

The best condition for identification and quantification was obtained using a Spherisorb ODS 1 column (250 mm L × 4.6 mm i.d.; 5 μm spherical particles; carbon loading of 7%, not end-capped) under controlled temperature condition (25 °C). As a mobile phase gradient was not required, a simpler isocratic elution (0.9 mL/min) with an ACN/water mixture (55/45%; v/v) could be applied and resulted in well-defined peaks and good separation (**Figure 3**). The maximum absorption wavelength for kahweol (290 nm) and cafestol (230 nm) was also used, obtained from the spectrum of each compound in the diode array detector (**Figure 2**). The methodology had a relatively short run time (20 min), with a retention time of around 16 min for kahweol and 17 min for cafestol (**Figure 3**).

Validation of the Analytical Methodology. The calibration curves were linear in the range studied (50−1000 mg/100 g) with a high correlation (**Table 1**). A good recovery for kahweol was observed for all samples: 94 (perisperm), 101 (pericarp), 95 (endosperm), 97 (leaves), and 109% (roasted coffee). Satisfactory results were also observed for cafestol: 99 (perisperm), 88 (pericarp), 93 (endosperm), 82 (leaves), and 106% (roasted coffee). For the cafestol esters, a recovery varying from 94 to 103% has been described (*8*). Kolling-Speer et al. (*30*), determining free diterpenes in green and roasted coffee, reported a cafestol recovery of 80.5% (for standard addition of around 50% of the initial sample value) but did not report the recovery for kahweol.

Coefficients of variation (CV) below 6% were observed for the two compounds, with the exception of the kahweol from perisperm (**Table 2**). For the determination of free cafestol, a CV of

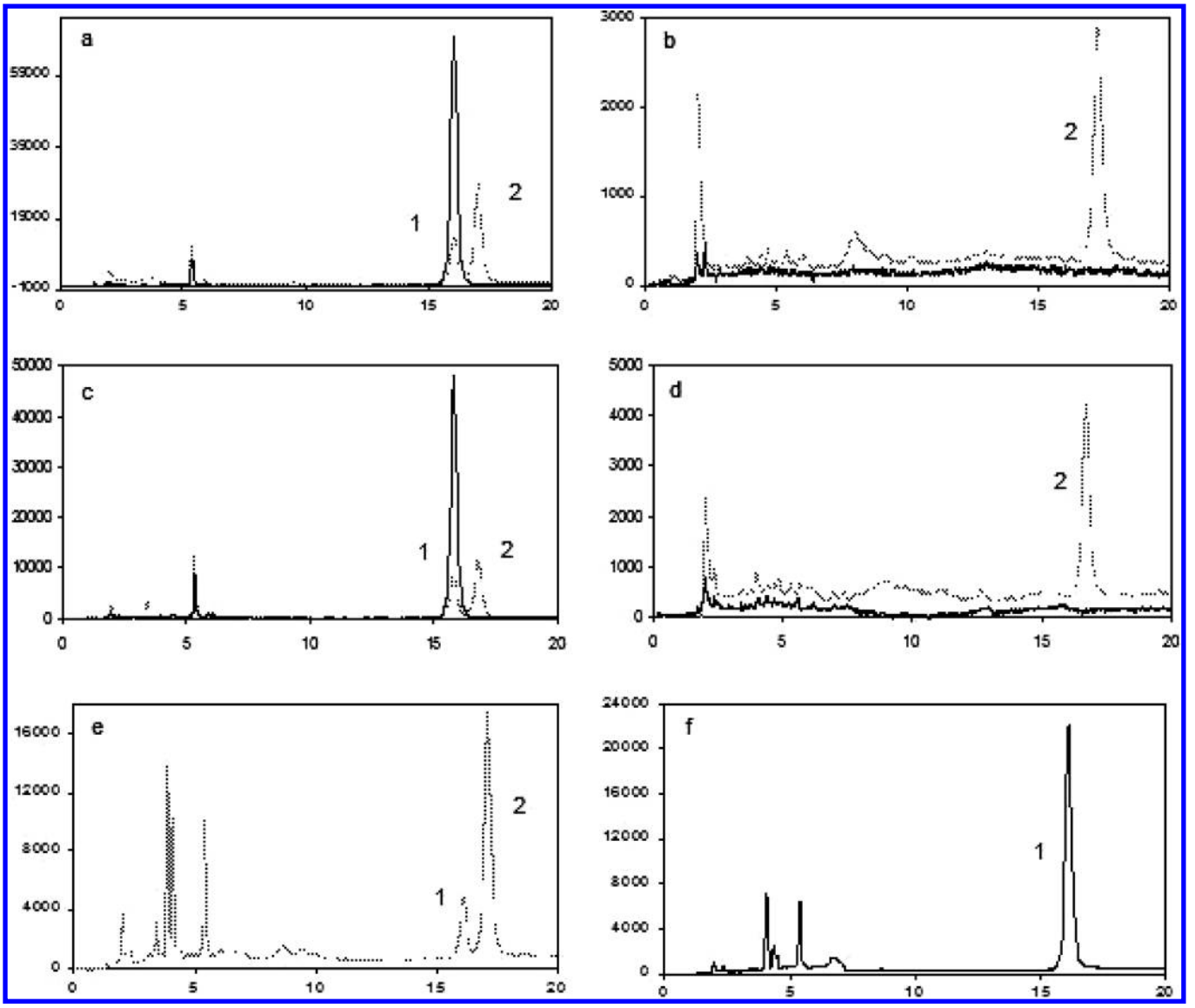

Figure 3. Typical chromatograms of the endosperm (**a**), pericarp (**b**), perisperm (**c**), and leaf (**d**) of *C. arabica* and roasted coffee (70:30 blend of *C. arabica*: *C. canephora*) (**e** and **f**). Detection was at 230 (- - -) and 290 nm (—). Kahweol (1) and cafestol (2) peaks.

Table 1. Parameters of the Linear Regression of the Calibration Curves for the Diterpenes

parameters	kahweol	cafestol
equation[a]	$y = 0.00051991x + 79.5875$	$y = 0.000477975x + 12.3132$
R^2; p	0.98; <0.001	0.99; <0.001
range (mg/100 g sample)	50−1000	50−1000

[a] y = concentration in mg/100 g sample; x = peak area.

2% has been reported, but the repeatability observed for kahweol was not given (*30*). Through the method development process, it was observed that there was a difference between the stability of the two diterpenes during the extraction and analysis, as observed by the higher repeatability in the quantification of cafestol. Because kahweol has an additional double bond in its structure as compared with cafestol (**Figure 2**), it tends to be more susceptible to addition and hydrogenation reactions. It has been reported that kahweol, in its pure form, has low stability in the presence of heat and light (*4*).

The diterpene content in the samples analyzed was greater than the LODs and LOQs of the method (**Table 2**). For kahweol, a LOD of 2.3 mg/100 g sample and a LOQ of 7.1 mg/100 g, respectively, were obtained. For cafestol, the results were similar, 3.0 and 9.1 mg/100 g sample, respectively.

The highest concentrations of kahweol were observed in the endosperm (589 mg/100 g) and perisperm (516 mg/100 g) of *C. arabica* cv. IAPAR-59 (240 and 83 DAB, respectively). The species *C. canephora* cv. Apoatã did not show a detectable amount of kahweol in the tissues analyzed. For cafestol, the highest concentration (304 mg/100 g) was detected in the endosperm of *C. arabica* 83 DAB (**Table 2**).

The kahweol content in the leaves of *C. arabica* has been reported to be negligible (< 0.01 mg/100 g), while the concentration of cafestol has been described as around 55.0 mg per 100 g for the leaves of *C. arabica* and 4.9 mg per 100 g for the leaves of *C. canephora* (*19*).

In mature dry beans (mainly endosperm) of *C. arabica*, kahweol contents already reported in the literature varied between 100 and 736 mg of kahweol/100 g (*6*, *8*, *20*). Cafestol contents were also divergent, ranging from 150.0 to 370.0 (*6*) and from 300 to 700 mg/100 g (*20*). In dry beans of *C. canephora* (endosperm), values below 10.0 mg kahweol/100 g and in the range of 270.0−670.0 mg cafestol/100 g (*6*, *7*) have been reported.

Table 2. Diterpene Contents for the Separate Fruit Tissues, Leaves, and Roasted Coffee [a]

diterpene	sample	perisperm[b]	endosperm[c]	pericarp[c]	leaf	roasted coffee[d]
kahweol	*C. arabica*	516 ± 68 (13)	589 ± 31 (5)	ND[e]	ND	446 ± 8 (2)
	C. canephora	ND	ND	ND	ND	
cafestol	*C. arabica*	132 ± 4 (3)	304 ± 11 (3)	49 ± 2 (5)	45 ± 2 (4)	323 ± 14 (4)
	C. canephora	109 ± 4 (3)	94 ± 3 (3)	36 ± 2 (6)	ND	

[a] Mean (mg/100 g of fresh weight) for five replicates ± standard deviation (coefficient of variation, %). [b] 83 and 120 DAB for *C. arabica* and *C. canephora*. [c] 240 and 214 DAB for *C. arabica* and *C. canephora*. [d] Blend of *C. arabica*:*C. canephora* (70:30). [e] ND, not detected.

In another study (*20*), less variation and lower contents of cafestol in *C. canephora* dry beans were observed (100 and 300 mg/100 g). The wide range of diterpene concentrations reported in the literature is probably due to the methodologies applied for the extraction and quantification of cafestol and kahweol. However, the edaphic−climatic conditions and the use of different cultivars of *C. arabica* and *C. canephora* could also explain the considerable variability shown in the different reports.

Whatever the reasons for these discrepancies, our results (**Table 2**) clearly indicate that cafestol and kahweol accumulate in both perisperm and endosperm tissues. This accumulation occurs at the same time as the synthesis of reserve proteins and complex polysaccharides (*31*, *32*). As described for the metabolism of sucrose (*26*), this suggests that the conversion of cafestol to kahweol occurs in the perisperm and that these diterpenes can be transferred from one tissue to another. The evaluation of the accumulation of these diterpenes during ripening should allow biochemical and genomic analysis of the metabolism of kahweol and cafestol during the development of the coffee fruit.

In a sample of roasted coffee (blend of *C. arabica* and *C. canephora*, 70:30), the kahweol content was higher than that of cafestol (446 and 323 mg/100 g), and these results are comparable with those described in the literature. Values close to 650 mg kahweol/100 g and 600 mg cafestol/100 g sample have been reported for dark-roasted *C. arabica* coffees (*6*). In a sample of medium-roasted *C. canephora*, there was no detection of kahweol, and 250 mg of cafestol per 100 g sample has been reported (*33*).

In conclusion, a chromatographic methodology for the simultaneous separation and quantification of kahweol and cafestol in different samples (fruit tissues, leaves, and roasted coffee) of *C. arabica* and *C. canephora* was successfully developed. The best conditions for separation and quantification were obtained using a reverse phase column Spherisorb ODS-1, ACN/H_2O as mobile phase, and UV detection. An extraction procedure using direct saponification with subsequent separation of the unsaponifiable fraction and clean up with water was standardized for all of the matrices. For fresh fruit tissues (pericarp, endosperm, and perisperm) and leaves, there is a need to employ liquid nitrogen at the beginning of the extraction (separation of the tissues, maceration, and weighing) to avoid oxidation of the material. Adequate repeatability, recovery, and linearity were observed. The LODs were lower than the lowest concentrations found in the samples and allowed for a reliable quantification. Kahweol was present only in the endosperm and perisperm of *C. arabica* cv. IAPAR-59. Cafestol was detected in all samples except for the leaves of *C. canephora* cv. Apoatã.

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