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Spectrophotometric method for quantification of kahweol in coffee

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ABSTRACT

The diterpene kahweol, a component of the unsaponifiable matter of coffee oil, has anticarcinogenic and antioxidant properties. Kahweol is specific to *Coffea arabica*, so it can be used to discriminate between coffee species. Chromatographic or infrared techniques are usually required for the evaluation of kahweol. The objective of this study was to develop a methodology to quantify kahweol based in colorimetric reactions and spectrophotometric measurements. The best extraction conditions were achieved by direct saponification of roasted and ground coffees, extraction with MTBE, cleaning the extract with water, reaction of the extract with KI, dilution with HAc 50% and absorbance analysis at 620 nm for quantification. This method demonstrated good precision (RSD below 5%) considering different extractions and intraday repeatability. Linearity was also observed (R^2 = 0.996, $p \leq 0.05$), with low limits of detection (5.16 mg 100 g^{-1}) and quantification (17.2 mg 100 g^{-1}). Kahweol contents were assessed at similar levels to those obtained using a standard HPLC methodology, and a good recovery was observed (116%).

1. Introduction

The production and marketing of coffee has become an important economic activity as the popularity of this aromatic beverage has spread around the world. More than 80 species of the genus *Coffea* L. (Rubiacea) are known. The most important species are *Coffea arabica* (arabica) and *Coffea canephora* (robusta), which correspond to 63% and 37% of global production, respectively (ICO, 2011).

Arabica coffee has greater acceptability, better cup quality and a higher price than the robusta species. The green beans of the two species are distinctive: arabica is light green and oval in shape, whereas robusta is brown and more rounded. However, it is impossible to visually distinguish the species after roasting and grinding. As they belong to the same genus, few physical and chemical differences enable the detection and/or quantification of the addition of robusta coffee to arabica coffee (González et al., 2001; Kemsley et al., 1995).

The unsaponifiable matter content is approximately 12 g 100 g^{-1} of coffee oil, with diterpene kahweol present as a primary component (Lago, 2001). The level of kahweol has been cited as a possible discriminating factor between coffee species in blends of *C. arabica* and *C. canephora*, as this compound is considered to be specific to arabica coffee and is relatively stable at processing temperatures (Dias et al., 2011; Campanha et al., 2010; Kemsley et al., 1995).

The contents of diterpenes in coffee products have been investigated because of the physiological action of these compounds in human health. Some diterpenes, including kahweol, may induce the degradation of toxic substances and provide protective action against aflatoxin B1 (González et al., 2001). In addition, anti-inflammatory, anticarcinogenic and antioxidant properties, as well as hepatoprotection, have been reported (Muriel and Arauz, 2010; Kim et al., 2006, 2009; Nkondjock, 2009; Lee et al., 2007; Lee and Jeong, 2007; Cavin et al., 2002). The evaluation of kahweol in the coffee plant may help to elucidate the metabolic pathways and enzymes involved in the biosynthesis of this diterpene in the plant and fruit tissues during their development (Dias et al., 2010; Vieira et al., 2006).

Abbreviations: HAc, glacial acetic acid; MTBE, methyl *tert*-butyl ether; DS, direct saponification; DAB, days after bloom; ACN, acetonitrile..

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There are two general ways of extracting the unsaponifiable matter: direct saponification (DS) or the extraction of lipids followed by saponification. DS has been reported as the most rapid and efficient alternative, as it avoids the formation of artifacts (Saldanha et al., 2006). DS is considerably faster and demands lower amounts of solvent in comparison with the method using the pre-extraction of lipids (Mariutti et al., 2008).

Wurziger (1985) and Wurziger et al. (1979) described a method for differentiating coffee species (green and roasted beans) by reacting the petroleum ether extract with potassium iodide and hydrochloric acid in glacial acetic acid. KI and HCl react with the double bond of the kahweol structure. In an acidic medium, the reaction products absorb at 620 nm. The difference determined from the absorbance measurements at 290 nm (maximum wavelength, λ_{max}, for kahweol) and 620 nm (λ_{max} for the reaction products) was due to the high level of kahweol that is in arabica coffee and nearly absent in robusta coffee. However, the method was empirical and only semi-quantitative, and there is no mention of its later use.

The literature describes a variety of techniques based, in general, on analyzing various compounds for differentiating coffee species. High performance liquid chromatography (HPLC) stands out (Scharnhop and Winterhalter, 2009; Araújo and Sandi, 2006; Kurzrock and Speer, 2001a; Castillo et al., 1999; Pettitt, 1987), but techniques based on Raman spectrometry (Wermelinger et al., 2011; Keidel et al., 2010; Ribeiro et al., 2010; Rubayiza and Meurens, 2005) and gas chromatography (GC) have also been reported (Guerrero et al., 2005; Castillo et al., 1999; Urgert et al., 1995).

The detection of the fraudulent or accidental addition of robusta to arabica coffees is of interest to the cooperatives of coffee producers, coffee industry and regulatory authorities. However for the coffee-producing countries, chromatographic or infrared techniques are relatively expensive and not always available for routine analyses, since depend on equipment and a trained technician. In this study, the principles of the Wurziger technique were used to develop a methodology for quantification of kahweol based on reactions involving the unsaponifiable matter. The extraction steps were adapted, and the reaction conditions were standardized. After the development of the methodology, a validation step was performed to ensure that the results are reliable and to enable the method to be used routinely for the quantitative analysis of kahweol in roasted and ground coffees.

2. Materials and methods

2.1. Reagents and equipment

The reagents used on the method development were potassium hydroxide, KOH (analytical grade, Synth, Diadema, Brazil), ethanol (purity ≥99.5%; Merck, Darmstadt, Germany); methyl *tert*-butyl ether, MTBE (analytical grade; Vetec, Duque de Caxias, Brazil), sodium thiosulfate, $Na_2S_2O_5$ (anhydrous, analytical grade; Merck, Darmstadt, Germany), purified water (Milli-Q® purification system, Millipore, Billencia, USA), acetic acid, HAc (glacial; Merck, Darmstadt, Germany), hydrochloric acid, HCl (36.5–38.0%; Merck, Darmstadt, Germany), potassium iodide, KI (≥99.5%; Icasa SA., Monterrey, Mexico), hexane (purity ≥99.0%; Merck, Darmstadt, Germany), diethyl ether (purity ≥99.5%; Merck, Darmstadt, Germany). A kahweol standard (Axxora, San Diego, USA) of 98% purity, certified by Alexis Biochemicals (Lausen, Switzerland), was used to develop and validate the method. Water purified and acetonitrile, ACN (HPLC grade; Carlo Erba, Duque de Caxias, Brazil), used as mobile phase of HPLC analysis were filtered in a Millipore vacuum filtration system through 0.45 μm membranes.

A UV–vis GBC Cintra 20 spectrophotometer with a detection range from 190 to 1000 nm and a slit aperture of 2 nm was used for the absorbance measurements. A colorimeter (Color-guide portable BYK-Gardner, USA) with 45/0 geometry and D65 light source was used in color characterization of samples.

For HPLC analysis a Shimadzu liquid chromatograph equipment (Kyoto, Japan) was used, with a quaternary system of solvent pumping (LC10 ATvp model), online degasser DGU-14 Avp, aCTO-10ASvp column oven model, and a Rheodyne injection valve with a 20 μL loop. The system was coupled to a Shimadzu model SPDM10 Avp, UV–vis spectrophotometry diode array detector (spectral scan from 190 to 800 nm; sensitivity of 0.8×10^{-8} UA), connected to a PC through an interface (SCL-10Avp).

2.2. Material

The samples of green coffees *C. arabica* cv. IAPAR 59 and *C. canephora* cv. robusta of known origin (Paraná/Brazil and Rondônia/Brazil respectively) were supplied by the Instituto Agronômico do Paraná (IAPAR, Londrina, Brazil). The cherry fruits were sundried and the green coffee beans (at least 300 g per each sample) were processed. Roasting was performed using a pilot-type Rod Bel roaster with a capacity of 3.6 kg h^{-1} at a maximum temperature of 230 °C. The samples were ground to a granulometry of around 0.5 mm, stored in plastic bags, conditioned in a cold chamber (10 °C), and analyzed in the sequence. The samples were characterized as a medium roast degree according to their color (in triplicate). For the arabica coffee, a lightness (L^*) of 22.0 was observed, and an L^* 30.3 was observed for the robusta. A blend of the species robusta and arabica (70/30 w/w) was used for the recovery test.

For the accuracy test, commercial coffees from the local market (supplied by Companhia Iguaçu de Café Solúvel®, Cornélio Procópio, Brazil) were also used. These products were labeled as "traditional", indicating a blend of arabica and robusta coffees. Their color (L^* of 18.2, 17.6 and 19.1 for samples 1, 2 and 3, respectively) indicated a medium to dark roast degree.

Fruit tissues and coffee plant leaves from *Coffea arabica* cv. IAPAR 59, supplied by the Instituto Agronômico do Paraná, were also studied. Each tissue was dissected from fruits at different ripening stages (days after bloom, DAB) to obtain enough sample weight for analysis. The perisperm was obtained from fruit collected 83 DAB, and the endosperm and pericarp were obtained from fruit collected 240 DAB (Geromel et al., 2006). Leaves of young and mature *C. arabica* coffee trees were also evaluated. The fruit and leaves were conditioned in closed plastic tubes, immediately frozen in liquid nitrogen to avoid oxidation, and stored at −80 °C until analysis. After the tissue separation, each sample was macerated with the aid of a mortar and pestle in the abundant presence of liquid nitrogen, and analyzed in the sequence.

2.3. Method development

The extraction of kahweol and the conditions for the colorimetric reactions were based on the Wurziger (1985) method. To obtain a quantitative extraction, the technique and possible extraction solvents were studied, and the conditions most specific for kahweol used by authors working with chromatographic techniques were tested (Urgert et al., 1995). As the original method lacks details, standardization tests were conducted to determine the quantities and form for the addition of the reagents.

A full spectrum UV–vis (200–800 nm) was ordered after direct saponification and the reaction between kahweol and a colorimetric reagent, HCl or KI. The absorbance measurements were normalized after the reaction as a function of the initial

sample weight to eliminate variability due to weighing differences.

Dilution tests were performed with the products of the colorimetric reaction with KI and HCl prior to measuring the absorbance. Different solvents (H_2O, glacial acetic acid (HAc), and a solution of H_2O:HAc 50:50 v/v) and proportions (1:1–1:5) were tested. The best dilution conditions were established for each colorimetric reagent (HCl or KI) considering the spectrogram profiles and a greater absorbance at the peak of interest.

The robustness of the methodology was evaluated using a fractional factorial design (2^{6-3}). The variables chosen were the following: the sample weight (0.20 and 0.25 g), the saponification time (45 min and 60 min), the centrifugation time (1 min and 2 min), the volume of water for cleaning the extract (1 mL and 2 mL), the KI volume (0.1 mL and 0.2 mL) and the reaction time (5 min–30 min). The experimental design was obtained using the Statistic 6.0 Program (Statsoft, 2006), revealing the effects and significance level for each variable.

2.4. Method validation

The reliability of the method was tested for linearity, precision, sensitivity, and accuracy, as previously suggested (AOAC, 2003; Ribani et al., 2004).

The linearity was determined by the analysis of standard solutions in six concentrations (between 0.05 and 0.35 mg of kahweol per mL). The coefficient of determination (R^2) and significance level (p) of the calibration curve were obtained.

The limit of detection (LOD) and the limit of quantification (LOQ) were estimated using Eqs. (1) and (2):

$$\mathrm{LOD} = 3\,s/S \tag{1}$$

$$\mathrm{LOQ} = 10\,s/S \tag{2}$$

where s is the standard deviation of the blank (10 measurements) and S is the slope of the calibration curve.

To verify the precision of the method, six extracts (0.200 g) were sequentially taken from the same sample in the same analysis conditions (repeatability in different extractions). The intraday repeatability was also studied, evaluating the same sample extract (0.200 g) in five reading repetitions.

The accuracy was estimated by recovery and comparison with a reference method.

A blend of robusta:arabica roasted coffee (70:30 w/w) was used for the recovery test. The kahweol standard was added to the samples (0.200 g) in two levels: approximately 18% and 35% of the initial kahweol content (0.25 and 0.50 mg).

The results obtained by the proposed methodology were compared with those of a reverse phase liquid chromatography method (Dias et al., 2010). All tests were conducted in duplicate, and samples of commercially roasted coffee were used. This approach assumes that the uncertainty of the reference method is known. The HPLC analyses were conducted using the same extraction process, Spherisorb ODS-1 column, isocratic elution (ACN:H_2O 55:45), and UV detection (290 nm). The kahweol concentration data were submitted to analysis of variance (one way ANOVA, Tukey's test for $p \leq 0.05$) considering the method (spectrophotometric and chromatographic) as the source of variation (Statistic 6.0–Statsoft, 2006).

2.5. Applicability to other samples

In addition to roasted coffee, fruit tissues and coffee plant leaves were studied to test the applicability of the spectrophotometry method to other coffee matrices.

3. Results and discussion

3.1. Method development

The use of Soxhlet in the extraction technique presented by Wurziger et al. (1979) made the extraction onerous and slow. A quantitative extraction of the lipid fraction would require refluxing the sample for 6–8 h with a large volume of solvent and sample weight. Wermelinger et al. (2011) verified that kahweol is not stable after extraction by 5 h in Soxhlet. Furthermore, interferent compounds that could influence the reaction yield might also be extracted when extracting the full lipid fraction. Because only the unsaponifiable fraction of coffee lipids (which contains kahweol) is of interest, the method of extraction proposed by Urgert et al. (1995) for quantifying the coffee diterpenes by GC was modified for the extraction of unsaponifiable matter.

With regard of the solvent used for the extraction, Kurzrock and Speer (2001b) and Wermelinger et al. (2011) suggested that because diterpenes are relatively polar, methyl *tert*-butyl ether (MTBE) (dipole moment: 1.32 D) and diethyl ether (1.15 D) should be used in preference to non-polar solvents, such as the petroleum ether (~0.05 D) proposed by Wurziger et al. (1979) or hexane (0.08 D).

Preliminary tests were conducted with hexane, diethyl ether and MTBE. The mean absorbance values at 620 nm were five times greater for diethyl ether and MTBE than for hexane, which confirms that more polar solvents are more efficient. The solvent MTBE was selected for its operational effectiveness in the extraction and reduced volatility compared with diethyl ether.

Three steps of solvent extractor additions were sufficient to ensure a quantitative operation (Fig. 1). However, it was difficult to completely avoid the extraction of some pigments and, likely, other substances with an affinity for the solvents. The extract presented a dark color, which could interfere with the spectrophotometric measurements. By introducing an additional step, cleaning with water, a clear ether extract was obtained. Analysis of the discarded aqueous phase demonstrated that these steps did not result in a significant loss of kahweol.

The use of hydrochloric acid or potassium iodide as colorimetric reagents (suggested by Wurziger 1985; Wurziger et al., 1979) was evaluated. Because the presence of iodine in solution should eventually interfere with the absorption measurements, the addition of a reducing agent of iodine solution, sodium thiosulfate ($Na_2S_2O_5$), was included to clear the extracts before the absorbance measurements (Fig. 1) to improve the reaction step.

Roasted and ground arabica and robusta coffees and a kahweol standard were used for the tests in order to compare matrices with different kahweol contents. The best conditions were defined as a dilution of 1:2 extract:HAc for the HCl reaction and a dilution of 1:2 extract:HAc 50% (water:HAc 50:50) for the KI reaction. Spectrograms for the standard of the kahweol can be observed in Fig. 2. A visual differentiation between the species was clear after the reactions; with HCl, the colors of the extracts varied from dark green (arabica) to salmon (robusta), and with KI, they varied from blue–green (arabica) to yellow/orange (robusta).

For the roasted coffee blends, some absorbent compounds were observed from the reaction with HCl, especially between 260 and 400 nm. For the KI reaction, there was no interference in the region of analytical interest, and the spectra for the samples and the standards were similar, demonstrating the specificity of the colorimetric reaction of KI with kahweol (Fig. 3).

Comparing the spectra (200–800 nm), the best results were observed with a KI reactant and dilution with HAc 50%, which produces a clearer extract and a better-defined peak at 620 nm than that obtained with HCl (Fig. 2). In addition, due to the increased danger of using HCl, KI was chosen as the reagent color reaction standard.

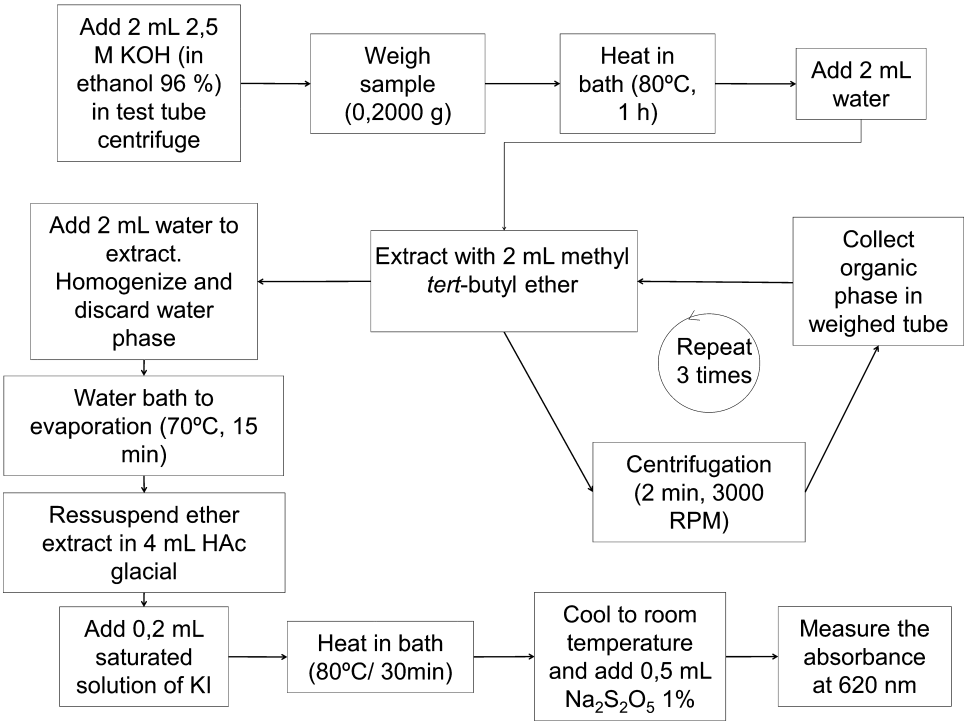

Fig. 1. Flowchart for the extraction and colorimetric reaction to spectrophotometric analysis of kahweol.

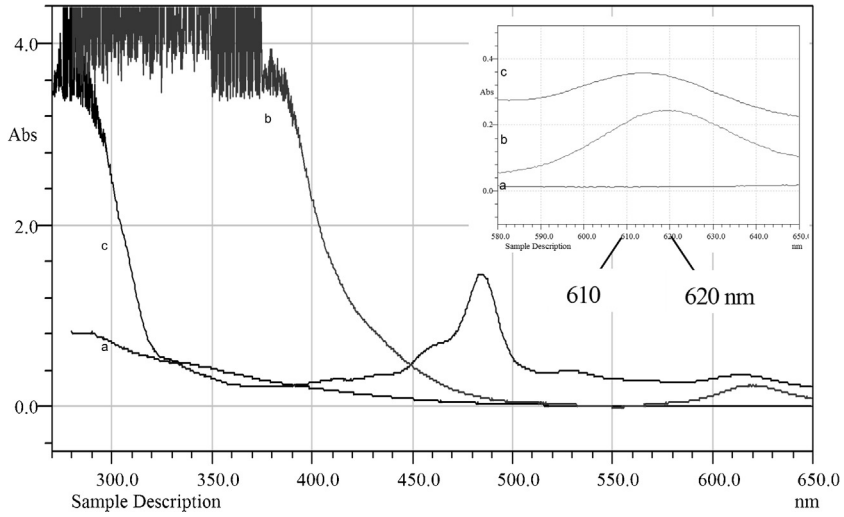

Fig. 2. Spectrograms of standard of kahweol: without reaction and dilution (a), reaction with KI and dilution 1:2 with HAc 50% (b) and reaction with HCl and dilution 1:2 with HAc glacial (c). The detail is the absorbance peak of reaction product.

The spectrum profile is characteristic for the product of the colorimetric reaction between kahweol and KI in the detection range, as observed in both the spectra for the standard and the samples (Figs. 2 and 3).

In the robustness test, the variables of sample weight ($p = 0.699$) and centrifugation time ($p = 0.915$) were not significant. Better results were observed for the saponification time of 1 h ($p = 0.047$) and the reaction time of 30 min ($p = 0.069$). The main effects ($p < 0.001$) were obtained for the volume of H_2O for cleaning the extract and the volume of KI. A greater volume of H_2O (2 mL) facilitated the solubilization of possible interferents that could react with KI and reduce its availability. A greater volume of KI (0.2 mL) increased the efficiency of the reaction.

Thus, the final sequence of the extraction and colorimetric reaction for kahweol was direct saponification with KOH, extraction with methyl *tert*-butyl ether, clean with water, reaction with KI, dilution with HAc 50%, addition of $Na_2S_2O_5$ solution and the absorbance measurement (Fig. 1).

3.2. Validation

The calibration curve presented a range of concentrations from 0.05 to 0.35 mg of kahweol per mL of solution. The maximum value corresponds to 700 mg of kahweol per 100 g of sample based on the range more usually described in the literature. The minimum value was defined according to a previous study (Dias et al., 2010) that demonstrated the impossibility of achieving an accurate reading of the absorbance below this limit (that corresponds to 50 mg of kahweol per 100 g of sample).

The calibration curve revealed linearity over the wide concentration range studied, with a coefficient of determination

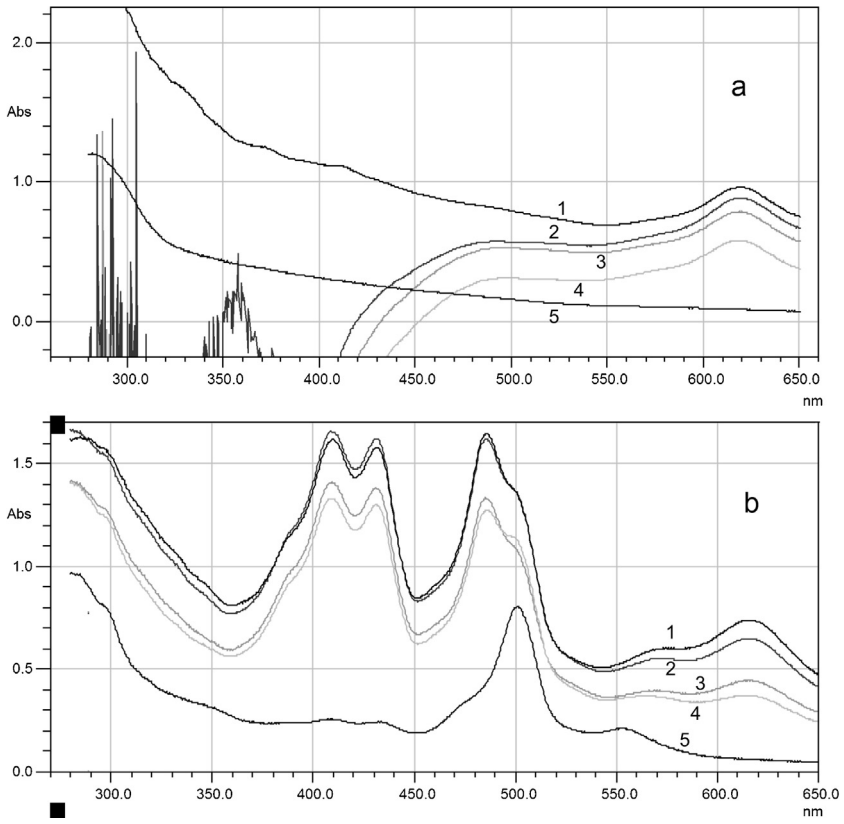

Fig. 3. Spectrograms obtained from colorimetric reaction of KI (a) or HCl (b) with unsaponifiable matter of samples of coffee – medium roast degree. Numbers indicates samples: arabica 100% (1); conilon 20% (2); conilon 30% (3); conilon 50% (4); conilon 100% (5).

of 0.996 ($p \leq 0.05$) (Eq. (3)).

$$y = 1.6264x - 0.3278 \quad (3)$$

where y is the absorbance value and x is the amount of kahweol.

The sensitivity of the method was estimated from the calibration curve. The LOD and LOQ values were 5.16 mg 100 g^{-1} and 17.2 mg 100 g^{-1}, respectively. Dias et al. (2010) used an HPLC method for the analysis of kahweol in roasted coffee and reported an LOD of 2.3 mg 100 g^{-1} of sample and an LOQ of 7.1 mg 100 g^{-1}, indicating that the sensitivity of the spectrophotometric methodology presented in this study is comparable to that of an HPLC method. None of the other chromatographic (Araújo and Sandi, 2006; Kurzrock and Speer, 2001b; Urgert et al., 1995; Frega et al., 1994) or spectroscopic (Kemsley et al., 1995) methods reported in the literature for the quantification of kahweol reported the sensitivity parameter.

Considering the usual contents of kahweol in roasted coffees, the sensitivity of the proposed method is adequate. The kahweol level depends on the geographic origins and coffee species, so a wide range is reported: from small traces in robusta coffee to up to 1096 mg 100 g^{-1} in arabica coffee (Campanha et al., 2010; Kitzberger et al., 2013). De Souza et al. (2010) reported mean values of 415 ± 19 mg of kahweol per 100 g of sample for 28 commercial Brazilian roasted coffees, likely blends of arabica and robusta. The lowest level reported for arabica coffee was 50 mg 100 g^{-1} (Kurzrock and Speer, 2001a; Lago, 2001; Urgert et al., 1995; Frega et al., 1994). For commercial samples, the lowest level of kahweol was 100 mg 100 g^{-1} (De Souza et al., 2010), almost 6 times greater than the LOQ of the spectrophotometric method developed in this work.

Tests of the repeatability of different extractions (RSD of 1.98) and the intraday repeatability (RSD of 3.61) demonstrated satisfactory precision of the method. According to the Health Surveillance Report (2002), acceptable levels for the coefficients of variation for repetitions with the same sample should be less than 15%.

The mean recovery was close to 116% for the two levels of addition of the standard of kahweol. Despite the indication that the methodology would overestimate the concentrations, the recovery results are satisfactory when taking into consideration that the matrix of roasted coffee is relatively complex; there are many components and interactions. The spectrophotometric methodology developed here uses a preliminary colorimetric reaction; thus, the analyte is not directly evaluated but the product of the reaction between the analyte and the reagent (indirect analysis). Consequently, the spectrophotometric results were not expected to be as accurate and precise as those obtained using a direct evaluation methodology, such as chromatography.

The accuracy was also evaluated by a comparison between the performance of the proposed method (spectrophotometric) and a reference HPLC method (Dias et al., 2010) for three commercial

Table 1
Comparison of the results for kahweol levels in samples of commercial coffees using different methodologies.

Samples	Concentration[a] (mg 100 g^{-1} ± SD)	
	Spectrophotometry	HPLC
1	$395^{A} \pm 2$	$388^{A} \pm 3$
2	$427^{A} \pm 6$	$399^{A} \pm 12$
3	$418^{A} \pm 23$	$481^{A} \pm 24$

[a] Average of two replicates ± standard deviation, coefficient of variation <5% for all replicates. Same letter in the line indicates no significant difference between means (Tukey, $p \leq 0.05$).

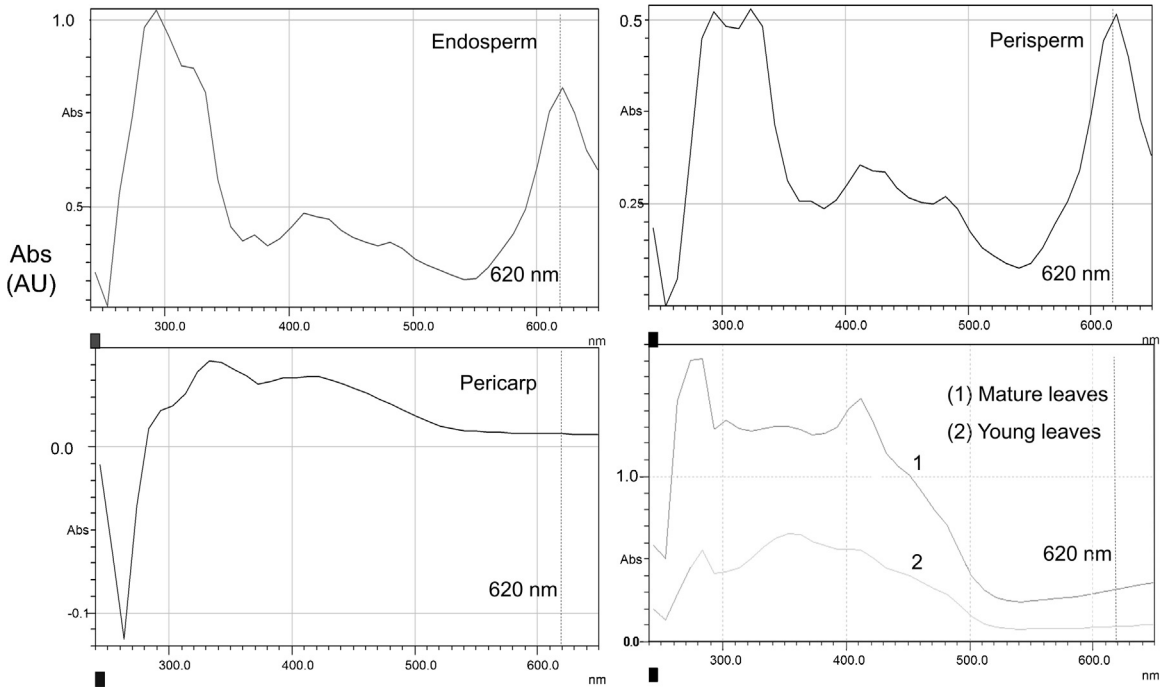

Fig. 4. Typical spectrograms of tissues of green beans of *C. arabica* and leaves.

coffees. A positive result was obtained; no significant difference in the kahweol contents was observed, demonstrating that the methods are in agreement (Table 1).

3.3. Applicability to other samples

In preliminary tests with the tissues of fresh coffee fruit (endosperm, perisperm, and pericarp) and young and mature coffee tree leaves, the method demonstrated potential for use with these matrices. It is noteworthy that it is not necessary to modify the procedure used for roasted coffee. The spectrograms indicated no interference in the λ region, where the reaction product absorbs with the greatest intensity (620 nm).

The endosperm and perisperm produced similar spectra, with compounds absorbing below 400 nm and intense peaks at 620 nm, which indicates the presence of kahweol in these tissues (Fig. 4). For the pericarp, no peak was detected in the region of interest, indicating that kahweol is not present in this tissue. Similar spectrum profiles were observed for the leaves of the arabica species collected in different periods, with compounds that absorb between 200 and 500 nm, but no peaks were observed in the analysis region. This result is in agreement with Kölling-Speer and Speer (1997), who reported traces of kahweol in the leaves of *C. arabica* (<0.01 mg 100 g^{-1}).

Dias et al. (2010) reported that the endosperm and perisperm of fresh *C. arabica* seeds revealed levels of kahweol greater than 500 mg 100 g^{-1} sample, but the compound was not detected in the pericarp or leaves of this species.

4. Conclusions

A spectrophotometric method for the analysis of kahweol by the direct saponification of roasted coffee, extraction with MTBE, cleaning of the extract with water, reaction of the extract with KI, and quantification at 620 nm was proposed. The methodology presented high-precision (RSD less than 5%) and linearity (R^2 = 0.996, $p \leq 0.05$) over a wide concentration range, with a limit of detection of 5.16 mg 100 g^{-1} and a limit of quantification of 17.2 mg 100 g^{-1}. The method was accurate, with good recovery (116%), and the results were similar to those obtained by a reference HPLC methodology.

Acknowledgments

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