This article was published in International Journal of Food Properties, 18(10), 2284-2299, 2015
http://dx.doi.org/10.1080/10942912.2014.933351

Quantification of Diterpenes and their Palmitate Esters in Coffee Brews by HPLC-DAD

Marzieh Moeenfard[1], José Avelino Silva[1], Nuno Borges[2], Alejandro Santos[2], Arminda Alves[1]*

[1]LEPABE, Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

[2]Faculdade de Ciências da Nutrição e Alimentação, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

*Corresponding author: Telephone: +351-225081883; Fax: +351-225081449; E-mail: aalves@fe.up.pt

ABSTRACT

The present paper aimed the optimization of the saponification and extraction of diterpenes from coffee brews using a simple methodology based on HPLC-DAD, as well as to quantify the diterpenes and their palmitate esters content in several coffee brews. Regarding cafestol and kahweol, the best conditions to maximize total extraction yield were achieved through the direct saponification of 2.5 mL of coffee brew along with 3.00 g of potassium hydroxide (KOH) in a water bath (80 ºC). The proposed method consists in two sequential extractions using diethyl ether followed by a clean-up with 5 mL of 2 M NaCl solution prior to HPLC-DAD analysis to eliminate soap and co-eluting interferences. Cafestol and kahweol palmitates also were quantified using the same procedure, but without the saponification step. The proposed methods exhibited high accuracy (average recovery of 85%) and linearity (R^2>0.999) and good precision (%CV<5%) with low limit of detection (less than 0.25 mg/L) and limit of quantification (less than 0.70 mg/L). Comparison of different brewing techniques revealed that capsule coffee with individual cup size of 50 mL contained the highest cafestol (1.85 mg/cup) and kahweol (2.75 mg/cup) concentration. In contrast, the lowest values of diterpenes were found in filter coffee (0.1 mg cafestol and 0.03 mg kahweol/200 mL). The higher and lower values of palmitate esters were also obtained from capsule and filter coffee, respectively. The proposed methods were simple, time saving and require low solvent consumption for quantification of diterpenes and their palmitate esters in various types of coffee brews.

Keywords: Diterpenes, Cafestol, Kahweol, Palmitate esters, HPLC-DAD, Coffee brew.

INTRODUCTION

Nowadays, people all around the world like to drink different types of beverages however one of the most popular beverages tends to be coffee due to its unique sensory properties. The most important commercial coffee species

are *Coffea arabica* and *Coffea canephora* var. *robusta*.[1] Cafestol, kahweol and their related esters are fat-soluble compounds exclusively found in coffee. These compounds are of interest due to their potential impact on human health and their application for discrimination between species.[2] Coffee diterpenes constitute around 1.3-1.9% and 0.2-1.5% (w/w) of green beans of Arabica and Robusta coffee, respectively.[3] Structures of diterpenes quantified in the present study are presented in Figure 1. Even though a large proportion of diterpenes exist esterified with various fatty acids - mainly palmitic acid - free diterpenes occur as minor components.[4] The composition of coffee brew and the level of diterpenes in coffee brews may be influenced by coffee species, roasting degree,[2] and subsequent brewing methods.[5,6,7]

Although elevation of low density lipoprotein and triglycerides may be the consequences of incautious coffee consumption and consequently ingestion of high amounts of diterpenes[8], they are responsible for degradation of toxic substances and may offer protective effects against aflatoxin B1.[9] Moreover, much research has been undertaken and indicated the anti-carcinogenic[10], anti-angiogenic and anti-inflammatory properties[11] of these diterpenes.

Different methodologies may be used for the extraction of cafestol and kahweol.[12,13] Some authors suggested the primary extraction of coffee oil[3,12,14] with subsequent saponification, while others applied direct saponification of beans or brews without prior extraction of coffee lipids.[7,15,16,17] Since diterpenes are relatively polar,[18] their extraction is carried out by organic solvents such as diethyl ether or *tert*-butyl methyl ether.[16,18,19,20] Numerous analytical methodologies have been developed for the determination of diterpenes in coffee samples, mainly, gas chromatography (GC)[5], high performance liquid chromatography (HPLC),[4] or spectrophotometric techniques.[15] Since, mild temperatures is a key parameter for the preservation of sensitive compounds in extracts, application of HPLC is preferred over GC, mainly due to degradation of diterpenes and production of decomposition compounds under high temperatures (200-305 ºC)[5] of the GC system.[20]

The literature describes a variety of techniques for the isolation of diterpene esters in coffee extract fractions like gel permeation chromatography and solid phase extraction.[21] Lam et al.[22] successfully isolated and identified the palmitate esters of kahweol and cafestol. Afterwards, Pettitt,[23] described a method for identification of cafestol and kahweol esters in Arabica and Robusta coffee by HPLC. They could identify five esters of C_{16}, C_{18}, $C_{18:1}$, $C_{18:2}$, and C_{20} for kahweol and only C_{16} for cafestol. In another study, diterpene esters were isolated from coffee by means of gel permeation chromatography and solid phase extraction and were analyzed with LC/MS/DAD, although no quantification of diterpene esters was done in coffee brews.[14]

Most published research papers studied the level of total or free diterpenes in various types of coffee beans or brews using different analytical techniques,[5,6,7,19,20] but little attentions has been given to the quantification of diterpene esters,[24,25] especially palmitate esters. This is an important issue for further studies regarding the health effects, because a large proportion of cafestol and kahweol are esterified with palmitic acid. It has been documented that palmitate esters of cafestol and kahweol are potent chemoprotective compounds that promote glutathione S-transferase activity which, is a major detoxification enzyme and plays significant role in carcinogen detoxification.[22,26] Huber et al.[27] indicated that mixture of cafestol palmitate and kahweol palmitate is a strong

inhibitor of a mutagenic heterocyclic aromatic amine called PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) and palmitate esters of diterpenes could decrease PhIP-induced DNA adduct formation by 54% in the colon.[27] In addition, 80% reduction in activity of N-acetyltransferase, an enzyme involved in carcinogens formation, by a mixture of cafestol palmitate and kahweol palmitate (1:1) has been reported previously.[26] Prevention of the genotoxic effects of PhIP and N-nitrosodimethylamine in a human derived liver cell line by means of cafestol palmitate and a mixture of cafestol and kahweol has been also documented.[28]

The experimental procedure to estimate diterpenes and their palmitate esters contents includes several steps. There are different analytical methods with distinct performances (limits of detection, precision, accuracy and global uncertainty), which may pose difficulties in comparing results of the presence of diterpenes in coffee brews. Although, raw material can be accounted as a factor in the diversity of diterpenes content, the effect of extraction process on this diversity is very prominent and noteworthy.[13]

Few analytical methods for identification of cafestol palmitate and kahweol palmitate in coffee have been reported in literature,[14,22] however, none quantified and compared the amount of these strong chemoprotective compounds in coffee brews. Therefore, development of a simple method to assess the amount of palmitate esters of cafestol and kahweol in coffee brews is of extreme relevance and one of the aims of this study. Methodologies based on application of gel permeation chromatography are accurate, but also time-consuming, expensive and require special skills. To authors' the best knowledge, no work has been published yet, covering the validations of operating conditions regarding diterpene esters extraction by existing techniques.

Finally, it should be stressed that cafestol and kahweol content of coffee brews have been studied regarding classical coffee consumption patterns.[6] Considering the lack of information about the diterpene and specially their palmitate esters concentrations in coffee brews like capsules coffee which are well accepted among consumers, the aim of this study was also to quantify the diterpenes and their palmitate esters content in several coffee brews. Therefore, this study intends to develop and validate the extraction procedure of cafestol and kahweol and to optimize the saponification and extraction of these compounds from coffee brews. Finally, the contents of diterpenes and their related palmitate esters in several types of coffee brews were compared to ensure that the modified methods could be used for quantitative analysis of cafestol, kahweol and their palmitate esters in various types of coffee brews.

MATERIALS AND METHODS

Reagents and equipments

Individual standards of cafestol and kahweol (purity of 98%) and cafestol and kahweol palmitate (purity of 98 %) were purchased from ChromaDex (Irvine, CA, USA). All other reagents or solvents were of analytical or HPLC grade. Solvents were acetonitrile, methanol (HPLC gradient grade) and diethyl ether obtained from VWR (Belgium). Potassium hydroxide powder with purity of approx. 85% was supplied by Merck (Germany) and sodium chloride was purchased from Panreac Quimica (Spain). Filtered water used for HPLC analysis was

prepared by vacuum filtration through 0.45 µm filter membranes. Phase separation was achieved by centrifugation in a Rotofix 32A centrifuge (Hettich, Germany) at ambient temperature. HPLC analysis was carried out in a Merck Hitachi Elite LaChrom (Tokyo, Japan) system equipped with a quaternary pump (L-2130), an L-2200 autosampler and an L-2455 UV/vis spectrophotometry diode array detector. Separation was achieved using a Purospher STAR LichroCART RP 18 end-capped (250 × 4 mm, 5 µm) column attached to a guard column (4 × 4 mm, 5µm) of the same kind. The detection wavelengths were 225 nm for cafestol and cafestol palmitate and 290 nm for kahweol and kahweol palmitate. EZChrom Elite 3.1.6 software was used for data acquisition and analysis.

Preparation of coffee brews

The purpose of the sampling scheme was to obtain different matrices for the verification of the applicability of the proposed methods. However some information about the coffee species used to prepare the blends was not available. Coffee (powder and capsules) and coffee filters were purchased randomly from a local market in Porto, Portugal. Five brewing procedures were evaluated. A total of 15 coffee brews (3 samples for each type) were prepared accordingly to the manufacturers' instructions. Coffee brew were stored at -18 ºC in polypropylene cups for less than two weeks until duplicate analyses of the presence of cafestol, kahweol and their palmitate esters with the proposed methods. The preparation modes were:

- *Capsule coffee*: Extraction of each capsule coffee (blend of Arabica coffees of different origins) was performed using a capsule coffee system (KRUPS XN4006 TERRACOTTA, Germany) at a pressure of 19 bar by hot water (90-95 ºC). Each cup contained 50 mL of coffee brew;
- *Espresso Coffee:* The brew was obtained directly from a local coffee shop in Porto, Portugal, and was transferred to polypropylene test tubes. The individual cup contained 50 mL of espresso coffee;
- *Filter coffee:* 13 g of roast and ground coffee (blend of Arabica and Robusta coffee) were put in a paper filter bag (Nº 2) and extracted with 200 mL of boiling water by means of conventional percolation coffee machine KRUPS Aroma Café 5 (Germany). The brew dripped into a heated pot within 2-5 min.[6] The individual cup size was 200 mL;
- *Instant Coffee:* For this purpose, 2 g of commercial instant coffee powder was extracted with 150 mL of boiled water;[20]
- *Vending coffee:* was obtained from Necta Coffee Vending Machine (Necta Astro Double Brew) to draw a cup of coffee about 50 mL. The brew was poured directly to polypropylene test tubes.

Cafestol and kahweol extraction

The determination of cafestol and kahweol in coffee brews was performed by improved conditions for saponification, extraction and clean-up based on the method described previously by Silva et al.[20] which were by its turn adapted from Dias et al.[19] Frozen coffee samples were defrosted (60 ºC) and stirred in order to obtain a homogeneous solution. Coffee brew (2.5 mL) was transferred to amber glass flask, 3.00 g of potassium hydroxide was added and the flask was placed in a water bath (80 ºC) for 60 min under continuous stirring. After saponification, the whole solution was cooled down immediately and subjected to liquid-liquid extraction with diethyl ether (5 mL). After centrifugation (3000 rpm, 5 min) and collection of the organic phase, the aqueous

phase was re-extracted with organic solvent. The combined extract was then washed with 5 mL of 2 M NaCl solution and centrifuged. The clean organic phase was brought to dryness under N_2 stream and stored at -18 ºC prior to HPLC-DAD analysis.

Extraction of cafestol and kahweol palmitates

Palmitate esters of diterpenes were measured using the same procedure for quantification of total cafestol and kahweol but without the saponification step. For this purpose, heated coffee brew (2.5 ml - 60 ºC) was extracted two times directly (without saponification) with 5 mL of diethyl ether. Since sample preparation was done without saponification, the emulsion had to be broken by centrifugation at 4000 rpm for 10 min. The combined ether phases were cleaned using 5 mL of 2 M NaCl solution. To protect the extract from light, cleaned ether phase was transferred to an amber glass vial and evaporated under N_2 stream. Dried extract was kept at -18 ºC before HPLC-DAD analysis. Due to difficulty in obtaining a neat interface, in each step of extraction and cleaning 0.5 mL of methanol was added, so a neat interface was obtained between ether phase and aqueous phase.

Method optimization for extraction of total cafestol and kahweol

In this paper, optimization of saponification and extraction of diterpenes from coffee brews was done by two sequential procedures using some key parameters. Firstly, type of the incubation bath (ultrasound or water bath), number of extractions using diethyl ether (1, 2, 3 and 4 times) and the amount of 2 M NaCl solution (0, 5, 10 and 15 mL) were optimized and afterwards, because the saponification was still incomplete, the effect of other variables, such as temperature of saponification (80 and 90 ºC), the amount of potassium hydroxide (0.45, 1.00, 3.00 and 5.00 g) and the sample content (5.0 and 2.5 mL) were tested.

HPLC-DAD analysis

Quantitative analysis was performed using external standard calibration curves. Standards were prepared in methanol and calibration curves were constructed for each compound (cafestol, kahweol and their related diterpene esters) by duplicate injections, at 7 and 8 calibration levels (Table 1). The analyzed coffee brews contained diterpenes in very different concentration ranges, which required diluting. Dried extract were reconstituted in an appropriate volume (0.5 - 2.0 mL) of methanol, so diterpenes at very high concentrations, were within the linearity range. For analysis of diterpenes, 20 µL was injected after filtration (0.45 µm Polytetrafluoroethylene membranes, PTFE, VWR, USA). Identification of the analytes was confirmed by retention time and spectrum comparison with standard solutions.

The chromatographic conditions for total cafestol and kahweol was adapted from Dias et al.[19] with slight modification in the flow rate and included: injection volume of 20 µL, isocratic elution of acetonitrile/water (55 / 45); flow rate of 0.8 mL/min during 15 min and detection at 225 nm for cafestol and 290 nm for kahweol. The chromatographic conditions for analysis of the palmitate esters of diterpenes were adopted from Pettitt[23] with

slight adaptations and included: injection volume of 20 µL, isocratic elution of acetonitrile (100%) at a flow rate of 1.2 mL/min during 35 min and detection of cafestol palmitate at 225 nm and kahweol palmitate at 290 nm.

Statistical analysis

To evaluate differences in variation, a two-way ANOVA (effect of extraction time and amount of salt solution) and three-way ANOVA (study of the effect of temperature, the amount of coffee brew and potassium hydroxide) in four replications was performed with a level of significance of 95%. Differences between heating systems were evaluated by Student's *t-test* at four replications with a level of significance of 95%. Data are reported as mean ± standard deviation. All statistical analysis was carried out by MATLAB 7.12.0 software. Graphs were plotted using Microsoft Excel 2007.

RESULTS AND DISCUSSION

Optimization of the type of heating system, the number of extraction and the amount of salt solution

Our first approach was to optimize the type of heating system (water bath or ultrasound bath), the number of extractions by diethyl ether (1, 2, 3 or 4 times) and the amount of salt solution for cleaning the organic phase (0, 5, 10, 15 mL).

Water bath at 80 ºC resulted in higher extracted amount of diterpenes in comparison to ultrasound bath ($p<0.05$) (Figure 2). Nevertheless, differences between water and ultrasound bath at 70 ºC was not significant ($p>0.05$). Figure 2 shows the effect of water and ultrasound bath on the amount of diterpenes. Water bath led to richer extracts in terms of diterpenes. The reproducibility of saponification could be increased by using the water bath instead of ultrasound bath. The fluctuations of temperature in ultrasound bath were responsible for some lack of precision in the results. Therefore, the choice of the heating system mainly depends on the facility to control the temperature and its influence on the diterpenes concentration of the extract. Moreover, it has been reported that, at high temperature of ultrasonication, vapor pressure will be increased and the bubbles generated by ultrasonication would be filled with vapor that may result in reduction of the effects of cavitation of bubbles on the particles and decrease the benefits of ultrasonication.[29] Dias et al.[15] also found that direct hot saponification of coffee samples in water bath at 80 ºC was the most efficient and affordable method for extraction of diterpenes.

After saponification in water bath, the solution was subject to extraction. To increase the extraction yield, successive extraction with diethyl ether was evaluated. One-time extraction of saponified solution using diethyl ether resulted in extracts containing less diterpene content in comparison to 2-4 times extraction ($p<0.05$). Therefore, two successive extractions of the aqueous phase with 5 mL of diethyl ether was found to be adequate to extract cafestol and kahweol. In another study, cafestol and kahweol were quantified from green coffee oil by two consecutive extractions of the aqueous phase with *tert*-butyl methyl ether.[16] Regarding cleanup, differences were negligible ($p>0.05$) between the use of 0 to 15 mL of saline solution for cleaning the ether phase, so 5 mL of 2 M NaCl solution was enough for removing interfering compounds, soap and residue of potassium hydroxide

from the extract. The effect of extraction times and the amount of 2 M NaCl solution on cafestol content of vending coffee, when saponified in water bath at 80 ºC, are shown in Figure 3. The same results were observed for kahweol (data not shown). The advantages of this method are the reduction of costs, as well as the decrease of chemicals consumption. Moreover, it can be considered environmentally friendly. Results of the first step showed that saponification in water bath (80 ºC) followed by two times extraction and subsequently cleaning with 5 mL of 2 M NaCl solution was almost appropriate for extraction of total diterpenes from coffee brews.

Optimization of saponification temperature, the amount of KOH and coffee brew

After extraction in accordance to the conditions described previously, the solution was analyzed by HPLC-DAD using the chromatographic conditions developed for palmitate esters to determine the saponification efficiency. Throughout the experimentation, an extract was injected and the presence or absence of both cafestol palmitate and kahweol palmitate was established by matching their retention times with those obtained for the authentic reference compounds. The presence of palmitate esters peaks meant that there were still some esters, which were not saponified, according to the procedure described above. On the other hand, absence of these peaks implied the completion of the process. Therefore, a second phase of improvement was held and other variables including the temperature of saponification in water bath at 2 levels (80 and 90 ºC), the amount of potassium hydroxide at 4 levels (0.45, 1.00, 3.00 and 5.00 g) and the amount of coffee brew at 2 levels (5.0 or 2.5 mL) were optimized and the efficiency of saponification was examined based on the presence or absence of palmitate esters in the extract.

The results of ANOVA analysis showed that the extracted quantity of cafestol and kahweol can depend greatly on the sample content. The most efficient extraction was obtained by reducing the sample volume from 5.0 mL to 2.5 mL of coffee brew, probably because of the stoichiometric ratio with potassium hydroxide (Figure 4). Significant differences ($p<0.05$) were observed between the use of different amount of potassium hydroxide, both in cafestol and kahweol. With regard to the effects of potassium hydroxide content upon diterpene concentration, kahweol showed some sensitivity to highly basic solutions. Therefore, the optimal condition to maximize total extraction yield both for cafestol and kahweol were 3.00 g of potassium hydroxide. Regarding saponification temperature, Figure 4 denoted that the different temperatures exhibited a soft impact on diterpenes concentration ($p>0.05$). Nevertheless, some authors showed an inverse correlation between high temperature and diterpenes concentration.[30] So, due to some sensitivity of diterpenes, mostly kahweol, to high temperature and energy saving, the bath temperature was adjusted to 80 ºC. Figure 5 shows the comparison of chromatograms obtained with different conditions. As it can be clearly seen, chromatogram (a) includes peaks corresponding to palmitate esters, which are not present in chromatogram (b), proving the complete saponification.

According to our results, the optimization of the parameters of saponification and extraction of diterpenes allowed the improvement of the method to extract cafestol and kahweol from coffee brews. The best conditions to maximize total extraction yield were saponification of 2.5 mL of coffee brew with 3.00 g of potassium hydroxide at 80 ºC in water bath, followed by two sequential extractions using diethyl ether and clean-up with 5 mL of 2 M NaCl solution. In terms of palmitate esters, quantification was done without saponification. Therefore, only

sample content was optimized. Different volumes of coffee brew (2.5 or 5.0 mL) did not have any significant effect ($p>0.05$) on palmitate esters content (data not shown) so the palmitate esters were quantified using the same method for measurement of total diterpenes, but without the saponification procedure.

Method validation

The analytical methods were validated, through the parameters of linearity, limit of detection (LOD), sample quantification limit (LOQ_{sample}), precision and recovery (Table 1). Linear response of the detector was examined for a concentration range of 1-200 mg/L for cafestol and kahweol. Concerning palmitate esters, the linearity of detector was tested by analysis of 7 standards for cafestol palmitate (25-250 mg/L) and kahweol palmitate (25-400 mg/L). The methods exhibited a linear relation between the amount of each analyte and the peak area with correlation coefficients of $R^2 \geq 0.999$ in all studied compounds.

Limits of detection and quantification were calculated based on the signal to noise ratio of three (S/N=3.3) and of ten (S/N=10), respectively. Sample quantification limits were also obtained with a signal to noise ratio of 10 and concentration factor of 5. The detection limits for the analyzed compounds were 0.03 mg/L (cafestol and kahweol), 0.13 mg/L (cafestol palmitate) and 0.21 mg/L (kahweol palmitate). Analysis of diterpenes with HPLC-DAD resulted in quantification limits of 0.08, 0.09, 0.40 and 0.65 mg/L for cafestol, kahweol, cafestol palmitate and kahweol palmitate, respectively, and indicated the sensitivity of HPLC-DAD for quantification of these compounds. Considering the concentration factor, sample quantification limits were measured and the values of 0.02 mg/L (cafestol and kahweol), 0.08 mg/L (cafestol palmitate) and 0.13 mg/L (kahweol palmitate) were achieved. Precision of the analytical method was evaluated through repeatability, which was determined by four independent extracts of the same sample with four injections in the same day (separately for total and esterified diterpenes) and the coefficient of variation (CV%) was reported. For cafestol and kahweol a repeatability of 1.7% and 4.0% was obtained in unspiked and spiked samples, respectively, while the average CV% of cafestol and kahweol palmitate in unspiked samples was 1.7 and 1.6%, respectively, and in spiked sample was 1.0%. Gross et al.[6] presented CV% around 8.6% for cafestol and 8.7% for kahweol using a different analytical method.

The recovery (%) of analytes was determined through detector response obtained for the unspiked extract, compared to the detector response obtained for spike extract.[19] Coffee brews were spiked with a known concentration of cafestol, kahweol and their palmitate esters (about twice the initial concentrations of each related components) and then samples were subject to the entire extraction methods described previously. The possible coelution of sample interferences was discarded by comparing the DAD spectrum of the samples and standards. The proposed methods resulted in good recoveries for cafestol (85%), kahweol (84%), cafestol palmitate (86%) and kahweol palmitate (88%). Recoveries of 97% and 96% has been already described for cafestol and kahweol, respectively.[6] Kurzrock and Speer[14] also indicated cafestol esters recoveries around 94-103 %, but did not specify detailed recoveries for each diterpene ester. A comparison of the method performance against other published methods for total cafestol and kahweol is presented in Table 2. As this is, to the author's best knowledge, the first work that validates an analytical method for diterpene esters, no other published validation parameters were found for cafestol and kahweol palmitate.

Analysis of diterpenes in coffee brews

A profound study of diterpene profiles in coffee brews is necessary in order to clarify the prevailing influence of coffee consumption on populations' health. Cafestol, kahweol and their palmitate esters in different coffee brews were quantified and the results are shown in Table 3. The diterpenes content is attributed to many parameters, mainly the type of coffee beans and the conditions of coffee preparation. It should be stressed that diversity in the methods of diterpene extraction also may influence the final concentrations of cafestol and kahweol in the extract. In this study, various types of coffee brews namely, espresso, capsule, vending, filter and instant coffee were analyzed to verify the applicability of the proposed methods.

A great variability was found in the diterpene content of the analyzed samples. The most abundant diterpene was kahweol, with contents varying from 55.11±1.99 mg/L in capsule coffee to 0.17±0.01 mg/L in filter coffee. When comparing brewing methods, capsule coffee showed higher content of cafestol and kahweol with respect to all other considered techniques. Capsule based systems have become the fastest growing segments of the coffee market as it ensures delivery of coffee of consistent quality at the touch of a simple button. Because of the compact structure of the coffee in the capsule, extraction of compounds is done efficiently. Regarding the diterpenes concentration (mg) per cup (50 mL), the average contents were 1.85 mg for cafestol and 2.75 mg for kahweol in capsule coffee. In another study, analyzing the single-serving size espresso prepared from the commercially available disposable capsules containing 5 g roasted coffee indicated the presence of 0.17 to 0.06 mg cafestol and 0.18 to 0.09 mg kahweol (per cup of 50 mL) in five different commercial prepacked coffee blends.[6]

Under the proposed experimental conditions, mean average contents of 1.33 mg and 1.64 mg per cup of 50 mL was obtained for espresso coffee. Cafestol levels of around 1-2 mg per cup of 25 mL in espresso coffee made in laboratory was reported by Urgert et al.[5] which. The same trend was obtained with previous paper[6] with concentration of 1.0 mg/50 mL both for cafestol and kahweol. Our results were not in accordance with previously published papers.[20] but this differences could be due to using different type of coffee or coffee/water ratio in analyzed samples or may be attributed to the improvements in saponification efficiency.

Vending coffee showed an intermediate diterpenes content (0.73 mg of cafestol and 0.79 mg of kahweol per cup of 50 mL). These findings were in contrast to those reported by Silva et al.[20] for vending coffee. It is important to highlight that coffee beans composition has a crucial effect on the diterpenes content of coffee brews. These differences could be explained by the different operative conditions of the vending machine, diversity in the proportion of coffee species (Arabica/Robusta) in the blend, and extractions methodologies. Instant and filtered coffee are the ones that would least contribute to diterpenes intake as filter brewing technique resulted to be the less extractive among the five studied brewing methods (0.1 mg cafestol and 0.03 mg kahweol/50 mL). These findings were similar to those reported by Urgert et al.[5] and Gross et al.[6] Instant coffee production process has been reported as responsible of diterpenes reduction in coffee extract.[6] The lower diterpenes contents in filter coffee could be explained by filtration of the coffee extract by paper filter which resulted in considerable reduction in diterpenes concentration of coffee beverage.[20]

As already referred, other authors described the presence of diterpene esters in coffee beverage, although no information is given about their contents in various types of coffee brews. Comparison of the cafestol and kahweol palmitates among the five studied coffee brewing procedures revealed that the distribution of palmitate esters of diterpenes was similar to total diterpenes. Capsule coffee contained 28.47 mg/L cafestol palmitate (corresponding to 0.07 mg cafestol palmitate). By considering molar mass of cafestol (316.439 g/mol) and cafestol palmitate (554.430 g/mol) it was found that the concentration of cafestol palmitate (28.47 mg/L) corresponds to 16.25 mg/L cafestol (0.04 mg cafestol). On the other hand, the concentration of cafestol achieved from cafestol palmitate constituted 43.65% of total cafestol. Similarly, by considering molar mass of kahweol (314.420 g/mol) and kahweol palmitate (552.420 g/mol) it was found that 65.17 mg/L kahweol palmitate (0.163 mg kahweol palmitate) present in capsule coffee corresponds to 37.09 mg/L kahweol (0.09 mg kahweol). It means that from total kahweol concentration quantified in capsule coffee, around 67.31% was obtained from kahweol palmitate. More information is shown in Table 3.

By comparing different brews, and considering generally consumed serving size, it could be observed that a cup of capsule coffee contained more total palmitate esters of diterpenes (~4.67 mg/50 mL) than a cup of espresso coffee (~3.09 mg/50 mL) and vending coffee (~1.75 mg/50 mL). As it can be observed in Table 3, lowest amount was detected in filter and instant coffee (below the limit of quantification). Since, palmitate esters of diterpenes are attached to acid palmitic, they may be retained by paper filter more easily than free cafestol and kahweol.

CONCLUSION

The optimized methods proved to be rather inexpensive and time saving for quantification of total cafestol, kahweol and their palmitate esters in coffee brews. The fact that the proposed method does not require special skills regarding sample preparation and analysis step for quantification of palmitate esters of diterpenes, clearly demonstrates it advantages with respect to the existing procedures like gel permeation chromatography which may be a source of unreliability. Therefore, this method can be applied routinely for measurement of these compounds in a large monitoring scheme of coffee brews allowing a high sample throughput. Under the investigated experimental conditions, validation parameters demonstrated that these optimized methods meet all analytical requirements. Recoveries of more than 85%, good precision (less than 5%) as well as high linearity ($R^2 \geq 0.999$) were achieved with regards to cafestol, kahweol and their palmitate esters. Detection limit of 0.03 mg/L was reported both for cafestol and kahweol while limit of quantification equal to 0.08 and 0.09 mg/L, respectively, were obtained for cafestol and kahweol. In terms of related palmitate esters, the limit of detection was 0.13 mg/L for cafestol palmitate and 0.21 mg/L for kahweol palmitate, while quantification limits for cafestol and kahweol palmitate were 0.40 and 0.65 mg/L, respectively. The diterpene contents of coffee brews were highly variable and brewing procedures played a key role in diterpenes extraction of coffee. Capsule coffee produced the most concentrated extract with regards to cafestol (1.85 mg/50 mL), kahweol (2.75 mg/50 mL), cafestol palmitate (1.42 mg/50 mL) and kahweol palmitate (3.25 mg/50 mL) among other brewing techniques. The lowest concentrations were found in filter and instant coffee. Consequently, further investigations should be done in order to know more profoundly how to extract and analyze other diterpene esters simultaneously with palmitate esters.

ACKNOWLEDGMENT

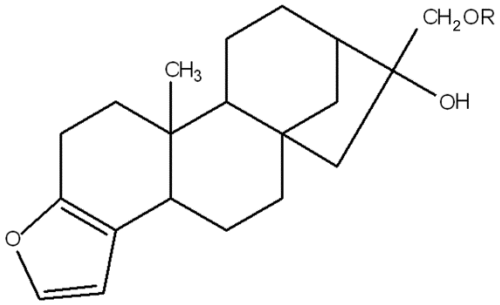
Marzieh Moeenfard would like to thank the Fundação para a Ciência e a Tecnologia (FCT), Portugal, for her financial support (SFRH/BD/79318/2011).

REFERENCES

1. Speer, K.; Hruschka, A.; Kurzrock, T.; Kölling-Speer, I. Diterpenes in coffee. In Caffeinated Beverages; Parliament, T. H., Ho, C. H, Schieberle, P.; American chemical Society, USA, **2000**.
2. Campanha, F.G.; Dias, R.C.; Benassi, M.d. Discrimination of coffee species using kahweol and cafestol: effect of roasting and of defects. Coffee Science, Lavras. **2010**, 5(1), 87-96.
3. Araujo, J.M.A.; Sandi, D. Extraction of coffee diterpenes and coffee oil using supercritical carbon dioxide. Food Chemistry. **2006**. 101, 1087-1094.
4. Speer, K.; Kölling-Speer, I. The lipid fraction of the coffee bean. Brazilian Journal of Plant Physiology. **2006**, 18(1), 201-216.
5. Urgert, R.; Weg, G.; Kosmeijer-Schuil, T.G.; Boverikamp, P.; Hovenier, R.; katan, M.B. Levels of the cholesterol-elevating diterpenes cafestol and kahweol in various coffee brews. Journal of Agricultural and Food Chemistry. **1995**, 43, 2167-2172.
6. Gross, G.; Jaccaud, E.; Huggett, A.C. Analysis of the content of the diterpenes cafestol and kahweol in coffee brews. Food and Chemical Toxicology. **1997**, 35, 547-554.
7. Zhang, C.; Linforth, R.; Fisk, I.D. Cafestol extraction yield from different coffee brew mechanisms. Food Research International. **2012**, 49, 27-31.
8. De Roos, B.; Caslake, M.J.; Stalenhoef, A.F.H.; Bedford, D.; Demacker, P.M.N.; Katan, M.B.; Packard, C.J. The coffee diterpene cafestol increases plasma triacylglycerol by increasing the production rate of large VLDL apolipoprotein B in healthy normolipidemic subjects 1-3. The American Journal of Clinical Nutrition. **2001**, 73, 45-52.
9. Cavin, C.; Holzhauser, D.; Constable, A.; Huggett, A.C.; Schilter, B. The coffee-specific diterpenes cafestol and kahweol protect against aflatoxin B1-induced genotoxicity through a dual mechanism. Carcinogenesis. **1998**, 19(8), 1369-1375.
10. Cavin, C.; Holzhaeuser, D.; Scharf, G.; Constable, A.; Huber, W.W.; Schilter, B. Cafestol and kahweol, two coffee specific diterpenes with anticarcinogenic activity. Food and Chemical Toxicology. **2002**, 40, 1155-1163.
11. Cárdenas, C.; Quesada, A.R.; Medina, M.A. Anti-angiogenic and anti-inflammatory properties of kahweol, a coffee diterpene. PLoS One. **2011**, 6:e23407.
12. Oigman, S.S.; de Souza, R.O.M.A.; dos Santos Júnior, H.M.; Hovell, A.M.C.; Hamerski, L.; Rezende, C.M. Microwave-assisted methanolysis of green coffee oil. Food Chemistry. **2012**, 134, 999-1004
13. Dias, R.C.E.; de Faria, A.F.; Mercadante, A.Z.; Bragagnolo, N.B.; Benassi, M.T. Comparison of extraction methods for kahweol and cafestol analysis in roasted coffee. Journal of the Brazilian Chemical Society. **2013**, 24(3), 492-499.

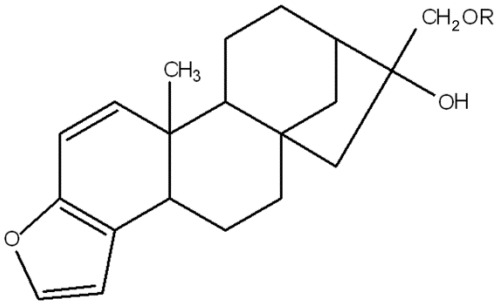
14. Kurzrock, T.; Speer, K. Identification of kahweol fatty acid esters in arabica coffee by means of LC/MS. Journal of Separation Science. **2001**, 24, 843-848.
15. Dias, R.C.E.; Alves, S.T.; Benassi, M.T.; Spectrophotometric method for quantification of kahweol in coffee. Journal of Food Composition and Analysis. **2013**, 31, 137-143.
16. Chartier, A.; Beaumesnil, M.; de Oliveira, A.L.; Elfakir, C.; Bostyn, S. Optimization of the isolation and quantitation of kahweol and cafestol in green coffee oil. Talanta. **2013**, 117, 102–11.
17. Kitzberger, C.S.G.; dos Santos Scholz, M.B.; Pereira, L.F.P.; Vieira, L.G.; Sera, T.; Gonc, J.B.; Silva, A.D.; Benassi, M.T. Diterpenes in green and roasted coffee of Coffea arabica cultivars growing in the same edapho-climatic conditions. Journal of Food Composition and Analysis. **2013**, 30, 52–57.
18. Kurzrock, T.; Speer, K. Diterpenes and diterpene esters in coffee. Food Reviews International. **2001**, 17(4), 433-450
19. Dias, R.C.E.; Campanha, G.F.; Vieira, L.G.E.; Ferreira, L.P.; Pot, D.; Marraccini, P.; Benassi, M.T. Evaluation of kahweol and cafestol in coffee tissues and roasted coffee by a new high performance liquid chromatography methodology. Journal of Agricultural and Food Chemistry. **2010**, 58(1), 88-93
20. Silva, J.A.; Borges, N.; Santos, A.; Alves, A. Method validation for cafestol and kahweol quantification in brews by HPLC-DAD. Food Analytical Methods. **2012**, 5(6), 1404-1410.
21. Speer, K.; Kölling-Speer, I. Lipids. In Coffee: Recent developments. Clarke, R. J.; Vitzthum, O. G. BlackWell Science, UK. **2001**.
22. Lam, L.K.T.; Sparnins, V.L.; Wattenberg, L.W. Isolation and identification of kahweol palmitate and cafestol palmitate as active constituents of green coffee beans that enhance glutathione S-transferase activity in the mouse. Cancer Research. **1982**, 42, 1193-1198
23. Pettitt, B.C. Identification of the diterpene esters in arabica and canephora coffees. Journal of Agricultural and Food Chemistry, **1987**, 35, 549-55.
24. Sehat, N.; Montag, A.; Speer, K. Lipids in the coffee brew. In Proceedings of the 15th ASIC Colloquium (Montpellier), pp. 869-872. ASIC Paris, France. **1993**.
25. Sehat, N.; Niedwetzki, G. Coffee Lipids: Analysis of diterpene 16-O-methylcafestol as an indicator of admixing of coffee, In New techniques and applications in lipid analysis. McDonald, R. E.; Mossoba, M. M.; AOCS Press, USA. **1997**.
26. Huber, W.W.; Teitel, C.H.; Coles, B.F.; King, R.S.; Wiese, F.W.; Kaderlik, K.R.; Casciano, D.A.; Shaddock, J.G.; Mulder, G.J.; Ilett, K.F.; Kadlubar, F.F. Potential chemoprotective effects of the coffee components kahweol and cafestol palmitates via modification of hepatic N-acetyltransferase and glutathione S-transferase activities. Environmental and Molecular Mutagenesis. **2004**, 44, 265-276.
27. Huber, W.W.; McDaniel, L.P.; Kaderlik, K.R.; Teitel, C.H.; Lang, N.P.; Kadlubar, F.F. Chemoprotection against the formation of colon DNA adducts from the food-borne carcinogen 2-amino-1-methyl-6-phenylimidazow4,5-bxpyridine (PhIP) in the rat. Mutation Research, **1997**, 376, 115-122.
28. Majer, B.J.; Hofer, E.; Cavin, C.; Lhoste, E.; Uhl, M.; Glatt, H.R.; Meinl, W.; Knasmüller, S. Coffee diterpenes prevent the genotoxic effects of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and N-nitrosodimethyl amine in a human derived liver cell line (HepG2). Food and Chemical Toxicology. **2005**, 43, 433–441.

29. Abdullah, M. Koc, A.B. Oil removal from waste coffee grounds using two-phase solvent extraction enhanced with ultrasonication. Renewable Energy. **2013**, 50, 965-970.
30. Bertholet, R. Preparation of Cafestol. United States Patent. **1987**, 4(692), 534.



If (R = H) : Free cafestol

If (R = Palmitic acid) : Cafestol palmitate



If (R = H) : Free kahweol

If (R = Palmitic acid) : kahweol palmitate

Figure 1. Structural formulae of the diterpenes.[21]

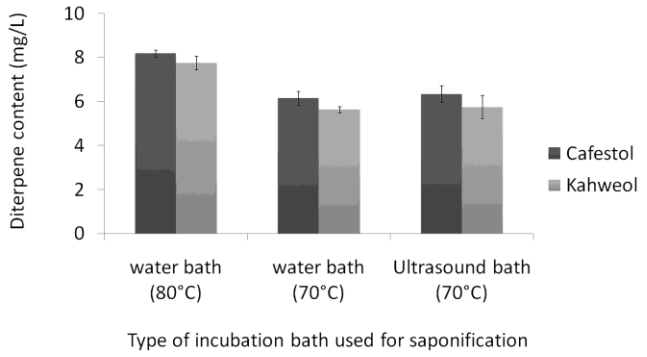


Figure 2. The effect of incubation bath type on diterpenes contents of vending coffee (saponification of 5.0 mL of brew by 0.45 g of KOH followed by 4 times extraction using diethyl ether and cleaning the combined ether phase with 15 mL of 2 M NaCl solution; mean ± standard deviation).

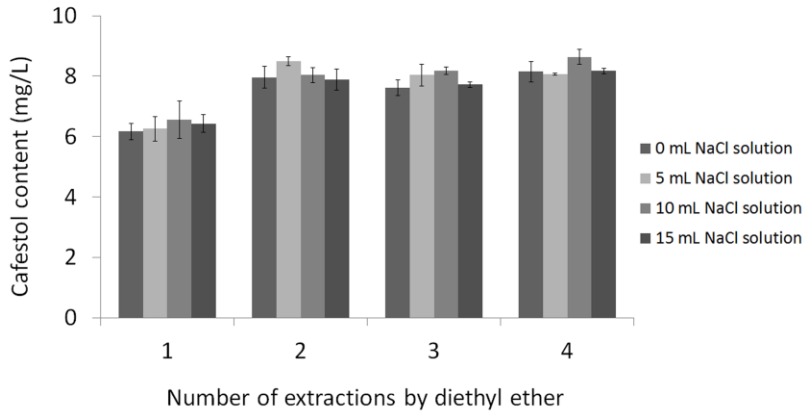


Figure 3. Effect of the number of extractions and the amount of 2 M NaCl solution on cafestol content of vending coffee (saponification of 5.0 mL of brew using 0.45 g of KOH in water bath at 80 ºC during 60 min followed by 1, 2, 3 or 4 times extraction and subsequently cleaning with 0, 5, 10 or 15 mL of 2 M NaCl solution; mean ± standard deviation).

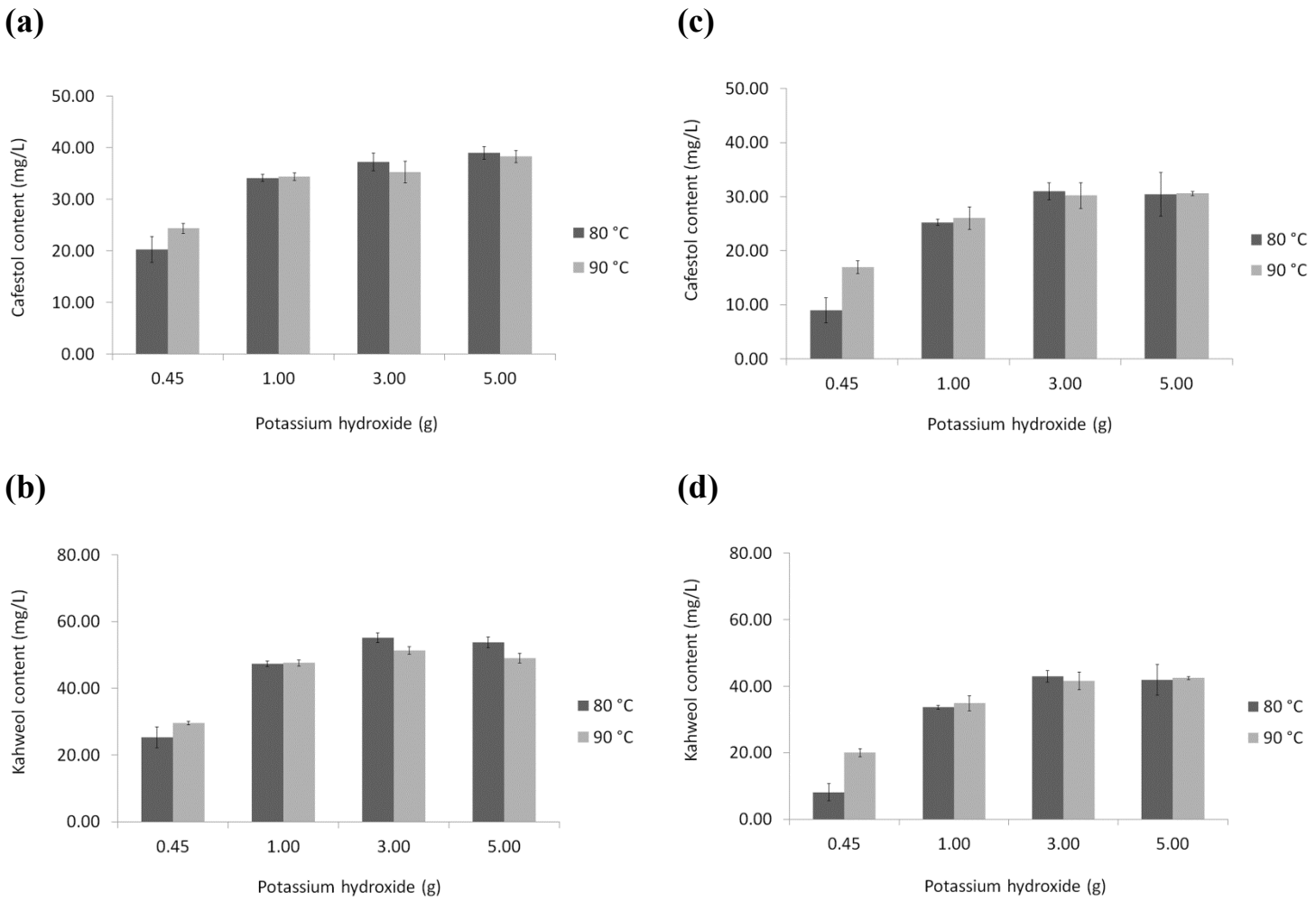
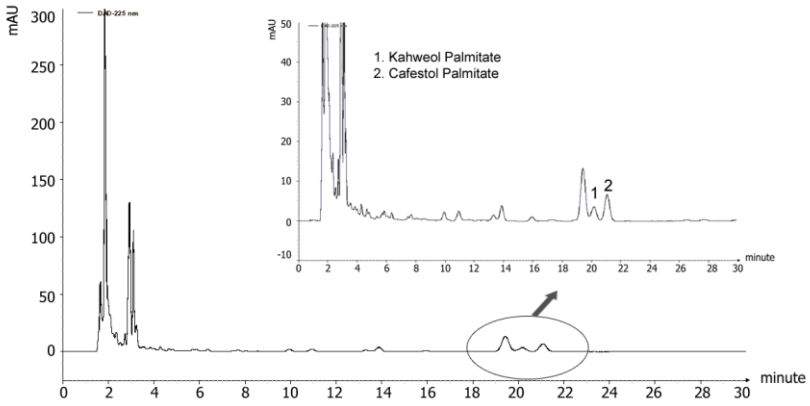


Figure 4. Effect of temperature, sample volume and the amount of KOH on cafestol and kahweol content of 2.5 mL (a,b) and 5.0 mL (c,d) of capsule coffee (saponification of different volumes of brew with different amounts of KOH was done in water bath at 80 and 90 ºC during 60 min; extraction was performed 2 times and the organic phase was cleaned using 5 mL of NaCl solution; mean ± standard deviation).

(a)



(b)

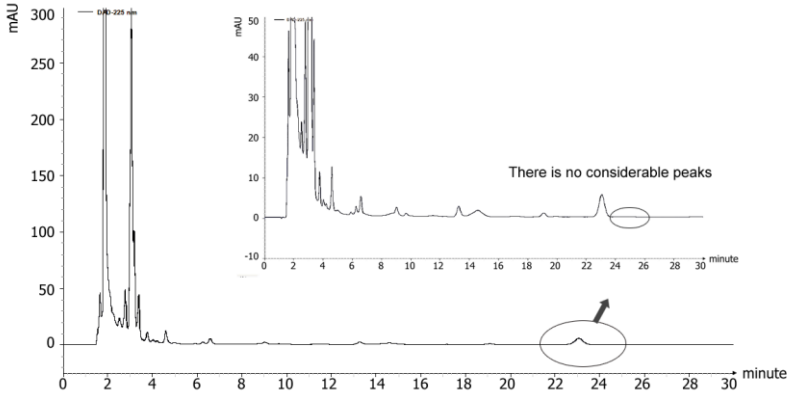


Figure 5. Differentiation of saponification efficiency among samples prepared based on the first and second phase of improvement with chromatographic conditions developed for palmitate esters by HPLC-DAD [(a) 5.0 mL of vending coffee with 0.45 g of KOH and (b) 2.5 mL of vending coffee with 3.00 g of KOH; both were saponified in water bath at 80 °C for 60 min followed by 2 times extraction and cleaning with 5 mL of salt solution].

Table 1. Validation parameters for analysis of cafestol, kahweol and related palmitate esters

	Cafestol	Kahweol	Cafestol Palmitate	Kahweol Palmitate
Linearity range (mg/L)	1-200	1-200	25-250	25-400
R^2	0.999 (N=8)	0.999 (N=8)	0.999 (N=7)	0.999 (N=7)
Limit of detection (mg/L)[a]	0.03	0.03	0.13	0.21
Limit of quantification (mg/L)[b]	0.08	0.09	0.40	0.65
Repeatability (CV%)				
Real sample (n=4)	1.7	1.7	1.7	1.6
Spiked sample (n=4)	4.0	3.9	1.0	1.0
Recovery (%) (n=4)[c]	85.40	84.42	86.70	88.31

N number of calibration curve standards, n number of replicate analysis, R^2 coefficient of determination, CV coefficient of variation.

[a] Calculated from a signal to noise ratio of 3.3.

[b] Calculated from a signal to noise ratio of 10.

[c] Calculated from the average ratio between expected and measured diterpenes concentrations in spiked samples.

Table 2. Performance comparison of the published methods for total cafestol and kahweol

Reference	Linearity range	R^2	Repeatability	Recovery	LOD[b]	LOQ[c]
Urgert e t al., (1995)[5]	0.01-40 mg/ 100 mL	NA[a]	Cafestol, 3.0-6.3% Kahweol, 2.9-5.2%	Cafestol, 102.2 2.3% Kahweol, 100.3 2.5%	NA	NA
Gross et al., (1997)[6]	NA	Cafestol, 0.999 Kahweol, 0.997	Cafestol, 8.6% Kahweol, 8.7%	Cafestol, 97±19% Kahweol, 96±19%	NA	Cafestol and kahweol, 0.05 mg/L
Dias et al., (2010)[19]	50-1000 mg/100 g	Cafestol, 0.99 Kahweol, 0.98	Cafestol and kahweol, <6%	Cafestol, 94% Kahweol, 99%	Cafestol, 3.0 mg/100 g Kahweol, 2.3 mg/100 g	Cafestol, 9.1 mg/100 g Kahweol, 7.1 mg/100 g
Silva et al., (2012)[20]	2.5-250 mg/L	Cafestol, 0.9971 Kahweol, NA	Cafestol, 0.2-2.8% Kahweol, NA	Cafestol, 96-110% Kahweol, NA	Cafestol, 0.01 mg/L Kahweol, NA	Cafestol, 0.04 mg/L Kahweol, NA
Dias et al., (2013)[15]	0.05-0.35 mg/mL	Kahweol, 0.996	Kahweol, <5%	Kahweol, 116%	Kahweol, 5.16 mg/100 g	Kahweol, 17.2 mg/100 g
Our method	1-200 mg/L	Cafestol and kahweol, 0.999	Cafestol and kahweol, 1.7%	Cafestol, 85% Kahweol, 84%	Cafestol and kahweol, 0.03 mg/L	Cafestol, 0.08 mg/L Kahweol, 0.09 mg/L

[a] NA, Not available
[b] Limit of detection
[c] Limit of quantification

Table 3. Analysis of diterpenes in several coffee brews[a]

Brewing method	Cup size (mL)	Cafestol		Kahweol		Cafestol palmitate		Kahweol palmitate		Cafestol obtained from cafestol palmitate[b]			Kahweol obtained from kahweol palmitate[c]		
		mg/L±SD	mg/cup	mg/L±SD	mg/cup	mg/L±SD	mg/cup	mg/L±SD	mg/cup	mg/L±SD	mg/cup	%	mg/L±SD	mg/cup	%
Capsule coffee	50	37.22±1.86	1.85	55.11±1.92	2.75	28.17±1.06	1.42	65.17±1.06	3.25	16.25±0.28	0.81	43.65	37.09±0.60	1.85	67.31
Espresso coffee	50	26.47±0.22	1.33	32.80±0.26	1.64	21.29±0.71	1.07	40.39±1.29	2.02	12.15±0.40	0.61	45.92	22.99±0.07	1.15	70.09
Vending coffee	50	14.55±0.08	0.73	15.69±0.07	0.79	13.20±0.35	0.66	21.85±1.21	1.09	7.53±0.20	0.38	51.79	12.43±0.69	0.62	79.29
Filter coffee	200	0.50±0.09	0.1	0.17±0.01	0.03	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Instant coffee	150	2.03±0.01	0.3	2.50±0.26	0.38	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ

[a] Diterpenes and their palmitate esters were extracted from 2.5 mL of each type of coffee brew via our proposed method for diterpenes and their palmitate esters, (mean ± standard deviation).
[b] Calculated using the molar mass of cafestol (316.439 g/mol) and cafestol palmitate (554.430 g/mol).
[c] Calculated using the molar mass of kahweol (314.420 g/mol) and kahweol palmitate (552.420 g/mol).