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ORIGINAL ARTICLE

Isolation, Method Development, and Validation of Diterpenes in Coffee Oil: Identification by Multiple Reaction Monitoring Approach with Atmospheric Pressure Chemical Ionization Mode

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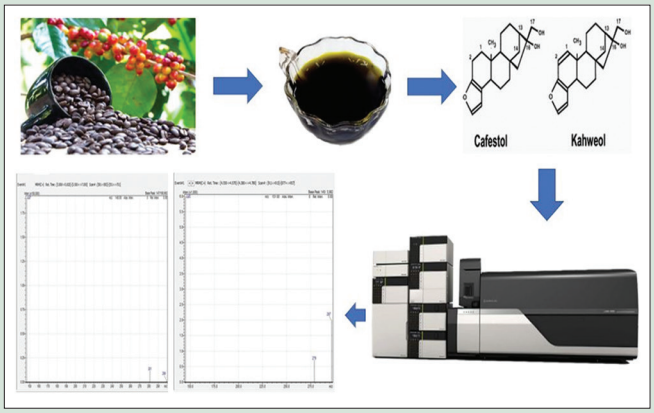
ABSTRACT

Objectives: The aim of the present study is to isolation, identification, and quantification of free diterpenes in coffee oil of *Coffea arabica*. **Materials and Methods:** For the isolation of free diterpenes, saponification reaction step was employed and followed with a liquid-liquid extraction procedure applied. Cafestol and kahweol were identified by multiple reaction monitoring (MRM) of positive ionization (MRM) mode of atmospheric pressure chemical ionization (APCI) mass spectrometry detection. The quantification of free diterpenes was done by high-performance liquid chromatography with diode array detector. The developed method was validated according to the international conference on harmonization guidelines. **Results:** The ion transitions of the precursor to the product ion at $(M + H)^+$ *m/z* 317.20→146.90, 281.00, 299.00 for cafestol and 315.10→144.90, 278.90, 296.90 for kahweol were observed. The proposed method was validated for linearity with excellent correlation coefficient of cafestol and kahweol were found to be 0.9997 and 0.9996, respectively. The intra-day and intermediate precisions and repeatability showed the percentage relative standard deviation was <1%. The recovery rate for cafestol and kahweol were observed to be in the acceptable limit of 95.303%–98.539% and 95.963%–97.174%, respectively. The limit of detection of cafestol and kahweol were found to be 6.81 and 7.35 ppm, respectively. The limit of quantitation (LOQ) was found to be 22.72 and 24.52 ppm, respectively. **Conclusion:** The developed method is simple, rapid, precise, accurate and it is recommended for efficient assays in routine work.

Key words: Atmospheric pressure chemical ionization, cafestol, *Coffea arabica*, kahweol, method validation

SUMMARY

- The objective of the present study is to isolation, identification, and quantification of free diterpenes in coffee oil of *Coffea arabica*. The developed method was validated according to the requirements for the international conference on harmonization guidelines. The quantification of free diterpenes was done by high-performance liquid chromatography with diode array detector. Cafestol and kahweol were identified by multiple reaction monitoring (MRM) of positive ionization (MRM) mode of atmospheric pressure chemical ionization mass spectrometry detection.



Abbreviations Used: LC-MS/MS: Liquid Chromatography-Tandem Mass Spectrometry; ICH: International Conference on Harmonisation; r^2: correlation coefficient; ppm: parts per million; HPLC: High Performance Liquid Chromatography; % RSD: Percentage relative standard deviation; MRM: multiple reaction monitoring; ESI: electrospray ionisation; APCI: atmospheric pressure chemical ionization; PDA: photo diode array; KOH: potassium hydroxide; MTBE: methyl tert-butyl ether; UV: ultraviolet detector; ppm: parts per million; UHPLC: ultra-high-performance liquid chromatography; RP-HPLC: reverse phase-high-performance liquid chromatography; GCMS: Gas chromatography–mass spectrometry; GC-FID: Gas chromatography– flame ionization detector; µm: Micro meter; µl: Micro liter; mm: Milli meter; h: hour; M: molar; L/min: litre per minute; ml: Milli; min: minute; CID: collision induced dissociation; A^0: Angstrom; nm: nano meter; RT: retention time; eV: Electronvolt; V: Volt; °C: Degree Celsius; Kv: Kilovolt; kPa: Kilopascal; DL: desolvation line; µg: Microgram; LOD: limit of detection; LOQ: limit of quantification and USP: United States Pharmacopoeia.

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INTRODUCTION

Coffee is one of the most popular beverages in the world. There are two coffee species popularly known as arabica and robusta having commercial importance according to the international coffee organization.[1] The two coffee species of the most traded agricultural product are *Coffea arabica* (*Arabica coffea*) and *Coffea canephora* (*Robusta coffee*), which correspond to 69% and 31%, respectively, of the world's coffee production. Green coffee bean is the raw coffee bean. The color and content of the coffee bean is changed once it is roasted. Green coffee bean

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extract has an impact on weight loss, normalizing the blood sugar and anti-aging properties. Green coffee oil is widely used in cosmetics[2] and pharmaceutical applications.[3] The coffee oil is rich in diterpenes, mainly cafestol and kahweol are predominantly occurring in coffee oil as esters of fatty acids.[4,5] Recent studies reported that cafestol (182–1308 mg/100 g) and kahweol (0–1265 mg/100 g) are the main diterpenes present in green and roasted coffee bean.[6]

The main phytoconstituents of *C. arabica* are phenolic compounds (such as chlorogenic acids and derivatives), methylxanthines (caffeine, theophylline, and theobromine), diterpenes (cafestol and kahweol), nicotinic acid (Vitamin B_3) and its precursor trigonelline, magnesium and potassium.[7] Diterpenes are a group of terpenoids, derived from a common isoprene precursor, geranylgeranyl diphosphate, via the formation and chemical modification of carbon skeletons. Diterpenes are widely distributed in nature, and it is structurally diverse class of C20 natural compounds, where isoprene units combine in different forms to give an array of diterpenes such as abietane, cembrane, guanacastepene A, quinonoid, jatropha, cafestol, and kahweol types.

Cafestol and kahweol, produced only by plants of the Coffee genus, are of interest due to their physiological effects.[8] Cafestol was found in both *C. arabica* and *C. canephora* plants; however, kahweol was reported to be specific to *C. arabica*.[9] The typical bean of *C. arabica* contains cafestol and kahweol, with individual concentrations ranging from 0.1 to 7 mg/ml in coffee.[10,11] Studies have demonstrated that cafestol and kahweol exhibit a wide variety of pharmacological activities, including anti-inflammatory,[12,13] anti-angiogenic,[12] antioxidant,[14] anti-carcinogenic,[14-16] hepatoprotective,[17] anti-mutagenic,[18] anti-diabetic,[19,20] anti-osteoclastogenic,[21] and anti-tumorigenic properties.[22-24] With multiple bioactivities of cafestol and kahweol reported, the development of potential multi-target drugs with diterpenes should be encouraged.

The objective of the present study is to optimize the saponification reaction and the extraction of diterpenes. In addition, the authors also develop a simple, optimized, characterize, and validated method for quantitative determinations of cafestol and kahweol in green coffee oil.

MATERIALS AND METHODS

Materials

Cafestol and Kahweol were used as reference standards. Cafestol (High-Performance Liquid Chromatography [HPLC] grade 100.0%) and Kahweol (HPLC grade 98.0%) were procured from Sigma-Aldrich (India). All reagents and solvents used were of analytical, HPLC and mass spectrometry (MS) grade. The powdered coffee beans material of *C. arabica* was procured from Vidya Herbs Pvt Ltd, Chikmagalur, India.

Preparation of the extract

Coffee oil of *C. arabica* was prepared by soxhlet extraction of 100 g of green coffee beans powdered raw material using 95% alcohol at 70°C for 3 h in 3 successive batch extractions. The first batch was extracted by adding 500 ml solvent for 3 h, and further two successive extractions were done by adding 500 ml/batch for 1 h. After completion of extraction, the cooled liquid was concentrated by evaporating its liquid contents in rotary evaporator till dryness. The coffee oil yield was obtained 5 g and used for further experiments.

Preparation of standard solution

Accurately weighed 10.0 mg of cafestol and kahweol reference standards were taken separately in 10.0 ml standard volumetric flask and dissolve in methanol to obtain a stock concentration of 1000 parts per million (ppm). Pipetted 5.0 ml of each standard stock solution to 25.0 ml standard volumetric flask and made up with methanol to get a final concentration of 200 ppm each. Filter the standard solution through 0.2 µ nylon syringe filter and inject the solution.

Preparation of sample solution

Saponification, extraction and clean-up procedure

Accurately weighed the appropriate amount of 1 g of sample was transferred into the round bottom flask. 90 ml of 95% ethyl alcohol and 10 ml of 2 M potassium hydroxide (KOH) were added to the flask. The reflux was carried out in a round-bottomed flask at 60°C for 1 h. After the complete saponification, the sample solution was cooled to room temperature and concentrated to dryness using rota evaporator. Dissolve the residue in 50 ml water and directed to liquid-liquid extraction with methyl tert-butyl ether (MTBE), i.e., by adding double the volume of the aqueous phase. Separate the aqueous phase and discarded. Collect the organic phase MTBE and followed by two times wash with water. Concentrate MTBE layer, dry, and dissolve by using 10 ml methanol. Filter the sample solution through 0.22 µ nylon syringe filter and inject the solution.

Preparation of spiked sample solution

Three different volumes (0.6, 0.7, and 0.8 ml) of cafestol and kahweol mix standard solution was added to the sample solution. The standard mix solution was spiked into the samples to determine recovery. Before analysis, the solutions were filtered through 0.20 µm nylon membrane filters.

Diterpene analysis

The diterpene quantification was performed on a Shimadzu LC2030 C Prominence-*i* (Japan) system equipped with a quaternary low-pressure gradient solvent delivery LC2030 pump with high-pressure switching valves, online LC2030 degasser unit, a high sensitivity LC2030 ultraviolet detector, high-speed drive LC2030 autosampler with a 100 µl loop and it accommodates 216 samples at a time with direct access rack system and large capacity column oven. The system was controlled and data was analyzed by LabSolutions software. A separation was carried out in Kinetex XBC-18 column (100 Å, 250 mm × 4.6 mm, 5 µm pore size). The mobile phase consists of isocratic elution with a low-pressure gradient using water: methanol (25:75) with a flow rate of 1.5 ml/min and an injection volume of 10 µl. All solutions were degassed and filtered through 0.45 µm pore size filter. The column was maintained at 25°C throughout analysis, and the photo diode array detector was set at 224 and 289 nm for cafestol and kahweol, respectively. One hundred percent methanol was used as a diluent for assay by HPLC analysis, and the total liquid chromatography (LC) run time was 17 min. Using these chromatographic conditions, it was possible to confirm the peak identification by retention time (RT) of cafestol and kahweol by injection of the corresponding standard separately.

The identity and purity of the peaks were operated using triple quadrupole mass spectrometry (LC-MS/MS-8050, Shimadzu, Japan) equipped with atmospheric pressure chemical ionization (APCI) source operating in positive ionization mode. The separation was carried out in the Kinetex C_{18} column (100 Å, 2.6 µm, 150 mm × 2.1 mm). The mobile phase consists of isocratic elution with a low-pressure gradient using water: methanol (30:70) with a flow rate of 0.3 ml/min in APCI source. The MS chromatographic conditions used in the study as follows: nebulizer gas flow, 3 L/min; drying gas flow, 5 L/min and heating gas flow, 10 L/min. The interface voltage and CID gas pressure were set at 4 Kv and 230 kPa. The interface temperature was maintained at 350°C, while

desolvation line and heat block (interface) temperature were maintained at 200°C in the APCI source. Other parameters were tuned automatically. The MS/MS parameters were optimized by direct injection of 1 µL volumes of mixed standards at 1 ppm. Characterization was established by injecting mixed standard solution of cafestol and kahweol and test sample of coffee oil, respectively, into the tandem mass spectrometry. Cafestol and kahweol identification was made by multiple reaction monitoring (MRM) chromatogram and mass spectrum compared with authentic standards. Peak identification was based on the RT, and the sample ion chromatograms must fully overlap with the standard. The MS data was processed by LabSolutions software.

Validation of the method

The validation of the developed method was done according to the International Conference on Harmonisation (ICH) guidelines.[25] The method is validated for specificity, linearity, repeatability, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), and robustness.

Specificity

Specificity is the ability of a method to discriminate between the study analytes and other components in the sample. In this study, the specificity was demonstrated by running a procedure blank, standard and sample. The chromatographic parameters such as column efficiency and peak symmetry were done to the standard mix (cafestol and kahweol) according to the ICH guidelines.

Linearity

Linearity was determined by different known concentrations of cafestol and kahweol mix standard solution. The standard solutions were injected, and the peak area was measured. For linearity study, six aliquots in the range 0.4–0.9 ml standard stock solution (i.e., 200 ppm) were taken and diluted to 1 ml to obtain concentrations in the range 80–130 ppm. Calibration curve was constructed for cafestol and kahweol by plotting peak areas against concentration and linear regression equations. The correlation coefficient (*R2*) was also computed.

Precision

Precision is a measure of the reproducibility of the whole analytical method. Precision was determined by studying the repeatability, intra- and inter-day (intermediate) precision. The repeatability was determined at a minimum of 6 replicates at 100% test concentration. Intra- and inter-day precision were determined at a minimum of three different concentration levels (100, 110, and 120 ppm) of cafestol and kahweol standards at three replicates. The intermediate precision variations to be studied for different analysts. The precision was expressed as the percentage relative standard deviation (percentage relative standard deviation [% RSD]).

Accuracy

Accuracy is a measure of closeness of test results obtained by a method to the true value. The accuracy of the method was tested by performing the recovery studies at three different levels of standard stock solution added

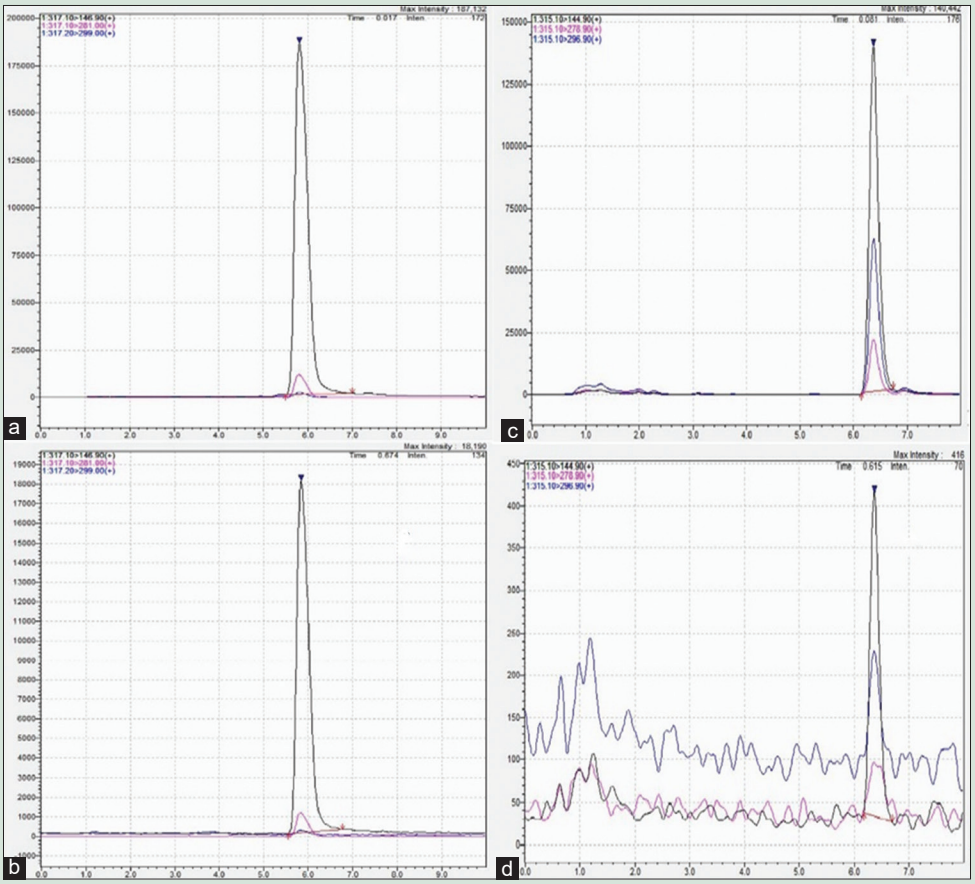

Figure 1: Optimised multiple reaction monitoring chromatogram of cafestol and kahweol. (a) Standard cafestol. (b) Test sample of coffee oil for cafestol. (c) Standard kahweol. (d) Test sample of coffee oil for kahweol

to the samples. The standard stock solution was spiked into the samples to determine recovery. Three different volumes (0.6, 0.7, and 0.8 ml) of standard stock solution were added to the sample solution (200 ppm). Triplicate injections were made with all the spiked samples.

% of recovery = (b − a)/c × 100.

where, "a" is the amount of drug found in the sample before the addition of the standard drug.

"b" is the amount of drug found after the addition of the standard drug.

"c" is the amount of standard drug added.

Limit of detection and limit of quantification

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated under the stated experimental conditions. Quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively determined with accuracy. LOD and LOQ were determined based on the signal to noise ratio response. For LOD, it should not be <3. For LOQ, it should not be <10.

Statistical analysis

Results were expressed as mean ± standard deviation and % RSD. The data were submitted to statistical analysis using excel software.

RESULTS AND DISCUSSION

Diterpenes were extracted and isolated from the coffee oil of *C. arabica*. As described early, cafestol and kahweol occur in the form of fatty acid esters, mainly palmitic and linoleic acid esters.[26] An optimized saponification reaction step was developed with the saponification parameter such as reagent concentration, temperature, reaction time, and the extraction solvent. For the isolation of free diterpenes, saponification reaction step was employed with optimized 2 M KOH solution. After the saponification reaction is followed with a liquid-liquid extraction using various organic solvents such as hexane, diethyl ether, and MTBE studied. The extraction of free forms of cafestol and kahweol is optimized with MTBE extraction solvent to obtaining better recoveries and a neat interface of the compounds between organic and aqueous phases.

For further confirmation of diterpenes, an optimized MRM method was developed using ultra-high-performance LC combined with mass spectrometry (MS/MS). The electrospray ionization positive and negative mode showed a lower response to ionize the diterpenes esters, so that the instrument was operated in the APCI positive mode with the higher response was selected. The diterpenes showed a satisfactory response in the negative mode of APCI. MRM chromatogram of cafestol and kahweol were shown in the [Figure 1]. The ion transitions of the precursor to the product ion were *m/z* 317.20→146.90 ion as a quantifier, 317.20→281.00 ion as a qualifier-1, 317.20→299.00 ion as a qualifier-2 for cafestol and 315.10→144.90 ion as a quantifier, 315.10→278.90 ion

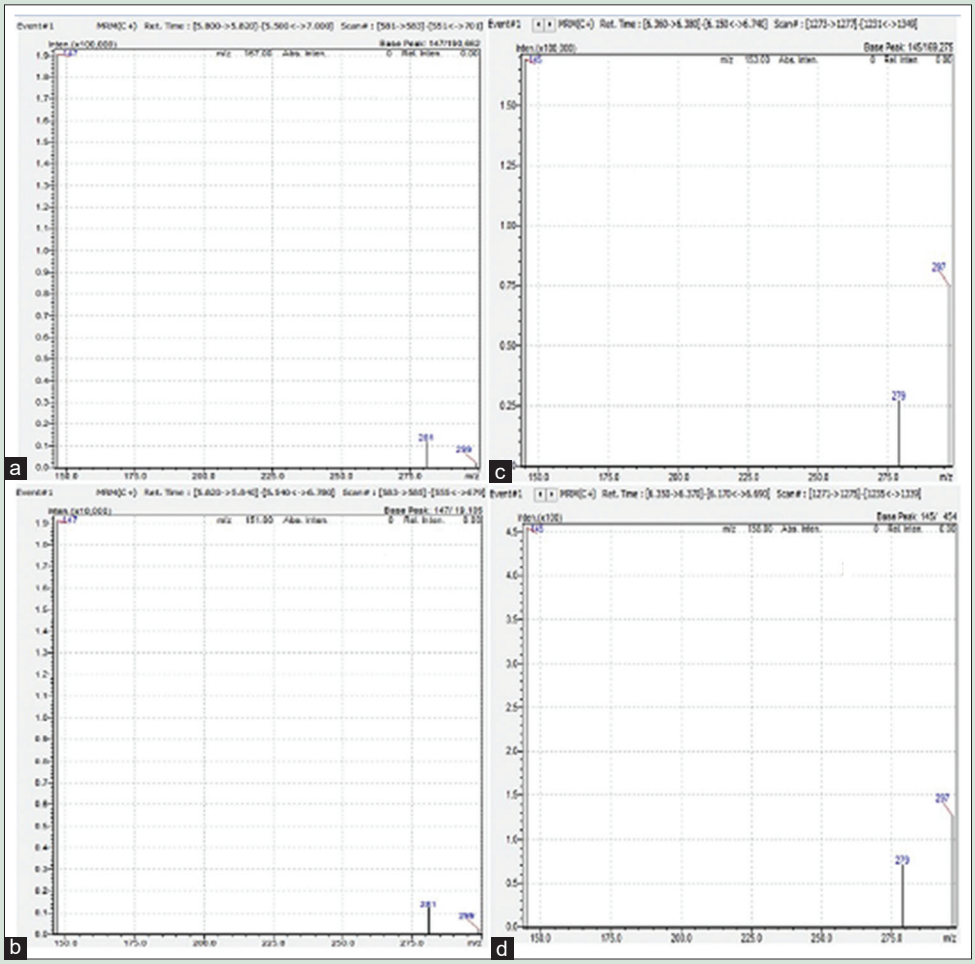

Figure 2: Optimised multiple reaction monitoring spectrum of cafestol and kahweol. (a) Standard cafestol. (b) Test sample of coffee oil for cafestol. (c) Standard kahweol. (d) Test sample of coffee oil for kahweol

as a qualifier-1, 315.10→296.90 ion as a qualifier-2 for kahweol were observed [Figure 2]. In cafestol, more intense fragment was observed at *m/z* 146.90 by the loss of $[M + H\text{-}C_{10}H_{18}O_2]^+$ group from the precursor ion. The other less intense fragments at *m/z* 299.0 $[M + H\text{-}H_2O]^+$ and *m/z* 281 $[M + H\text{-}2H_2O]^+$ were observed for cafestol by the successive loss of one and two molecules of water moiety by dehydration. This mass spectrum is a good match with the cafestol in accordance with other previous reports.[5,17,27,28] Kahweol was showed high intense fragment at *m/z* 144.90 by the removal of $C_5H_2O_3$ $[M + H\text{-}C_5H_2O_3]^+$ group from the precursor ion. Further, less intense fragments were observed at *m/z* at 297 $[M + H\text{-}H_2O]$ + and 279 $[M + H\text{-}2H_2O]$ + for kahweol by the successive loss of one and two molecule H_2O moiety by dehydration from the precursor ion, respectively. A good match with the mass spectrum of kahweol was observed with other previous studies.[5,27] By comparing the ion transitions with authenticate reference standard material and other previous reports, it was clearly identified as cafestol and kahweol.

An reverse phase-HPLC method was developed and validated for the determination of cafestol and kahweol in coffee oil extract. In specificity, the optimization of chromatographic condition was determined by comparing the chromatogram obtained from blank, standard, and sample solutions are summarized in [Figure 3]. The RT of cafestol and kahweol reference standard were found to be 7.349 and 6.667 min, respectively. The test (coffee oil) sample confirms the presence of cafestol and kahweol at 7.339 and 6.667 min, respectively, without any interferences. The chromatogram of the sample solution overlay with the standard solution, so the method is specific. The theoretical plate (United States Pharmacopoeia) 13,182 for cafestol and 13,950 for kahweol was observed. The tailing factor for cafestol and kahweol were observed 1.053 and 1.091, respectively, which indicated column efficiency is satisfactory.

Linearity was evaluated by plotting the peak area against concentration of cafestol and kahweol mix standards in the range 80–130 ppm are summarized in the [Figure 4]. The R^2 of cafestol and kahweol were found to be 0.9997 and 0.9996, respectively. The acceptance criteria for linearity are that the R^2 should not be <0.990.[29] This indicates good fitting of the curve and the method is good linearity corresponds to peak area on concentration. These results are in accordance with other previous reports.[30] Precision of an analytical method expresses the closeness of agreement between a series of measurements that were obtained from multiple sampling of the same homogeneous sample under the prescribed condition. Precision was evaluated based on the % RSD value. The data pertaining to precision are summarized in Table 1. In repeatability, the % RSD of the peak area of test sample concentration (400 ppm) of cafestol and kahweol were found to be 0.080% and 0.082%, respectively. In intra-day precision, the % RSD of peak area at 3 different levels (100, 110, and 120 ppm) of cafestol was found to be 0.023%, 0.011% and 0.040%, kahweol: 0.021%, 0.018%, and 0.032%, respectively. In inter-day (intermediate) precision, the % RSD of peak area at 3 different levels (100, 110, and 120 ppm) of cafestol was found to be 0.112%, 0.133%, and 0.044%, kahweol: 0.083%, 0.112%, and 0.047% respectively. These precision presented % RSD values are <1.0%, so the method was found to be highly precise and reproducible. The recovery of the compounds cafestol and kahweol were determined by spiking the coffee oil with known amounts of mix standard solution. Recovery of each substance was obtained from the calculated amount found and the original amount. Percentage recovery of cafestol and kahweol was calculated from differences between the peak areas obtained for spiked and standard solutions as shown from the data [Table 2]. The average % RSD at three different levels spiked sample of cafestol was found to be 0.044%, 0.082%, and 0.065%, kahweol: 0.012%, 0.094%, and 0.062%, respectively. The average percent recoveries at three different levels (100, 110, and 120 ppm) of cafestol and kahweol were found to be 98.539%, 96.824%, and 95.303%, kahweol: 97.174%, 95.963%, and 96.126%, respectively. The low standard deviation value <1% shows the high accuracy of the method, therefore, this HPLC method can be regarded as selective, accurate and precise. LOD at signal-to-noise ratio (3:1), the smallest concentration of cafestol and kahweol were found to be 6.81 and 7.35 ppm, respectively. The LOQ at signal-to-noise ratio (10:1) for cafestol and kahweol were found to be 22.72 and 24.521 ppm, respectively. Low LOD and LOQ of

Table 1: Precision studies of cafestol and kahweol

Concentration (ppm)	Mean area±SD	RSD (%)
Cafestol		
Intra-day precision (*n*=3)		
100	735,150±68	0.023
110	809,561±93	0.011
120	885,367±358	0.040
Inter-day precision (*n*=3)		
100	742,279±829	0.112
110	802,569±1070	0.133
120	880,739±391	0.044
Repeatability (*n*=6)		
400	1,569,065±1253	0.080
Kahweol		
Intra-day precision (*n*=3)		
100	1,050,699±220	0.021
110	1,151,715±204	0.018
120	1,259,533±404	0.032
Inter-day precision (*n*=3)		
100	1,066,223±887	0.083
110	1,146,590±1288	0.112
120	1,257,439±593	0.047
Repeatability (*n*=6)		
400	2,750,942±2253	0.082

SD: Standard deviation; RSD: Relative standard deviation

Table 2: Results of accuracy

Amount added (ppm)	Mean area (b)±SD	RSD (%)	a	c	Recovery (%)
Recovery for cafestol					
100	2,286,013±996	0.044	1,562,516	734,220	98.539
110	2,346,318±1918	0.082	1,562,516	809,512	96.824
120	2,403,596±1568	0.065	1,562,516	882,529	95.303
Recovery for Kahweol					
100	3,763,720±469	0.012	2,742,863	1,050,541	97.174
110	3,847,893±3067	0.094	2,742,863	1,151,516	95.963
120	3,950,194±2451	0.062	2,742,863	1,255,986	96.126

n=3. SD: Standard deviation; RSD: Relative standard deviation

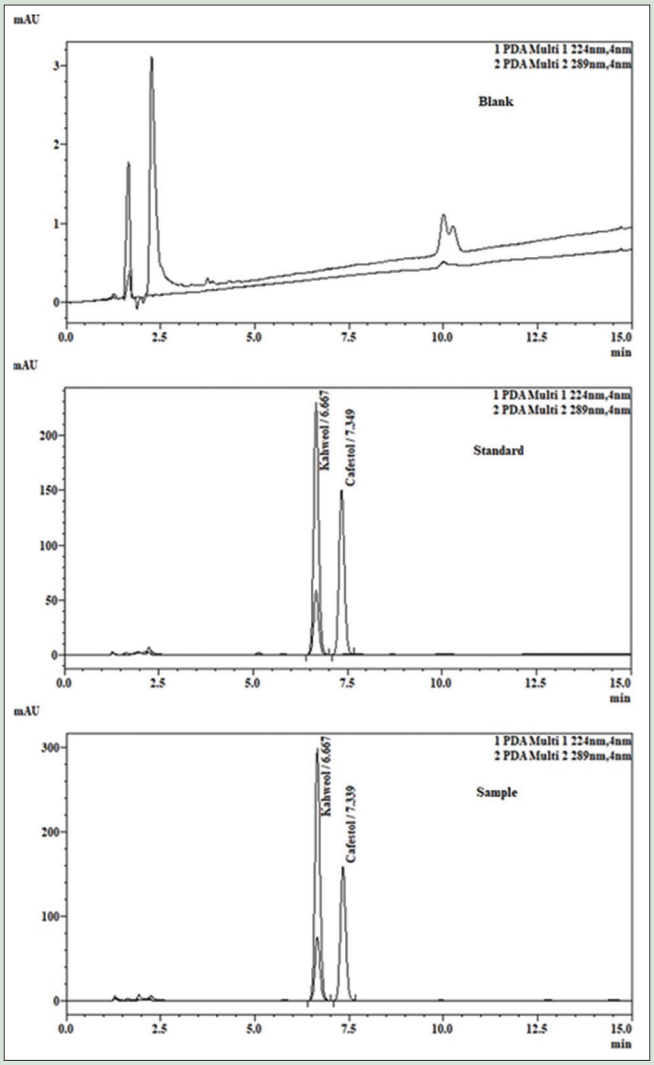

Figure 3: Optimised high performance liquid chromatography chromatograms of blank, standard and sample. The retention time of kahweol and cafestol standard and sample peak were found to be 6.667, 7.349 and 6.667, 7.339 respectively

cafestol and kahweol enable the detection and quantification of cafestol and kahweol in *C. arabica* at low concentrations.

CONCLUSION

The saponification reaction step was optimized, and solvent extraction was successfully applied to isolate free diterpenes from the coffee oil of *C. arabica*. The developed method is simple, precise, specific, selective, and robust. Therefore, the method was proved to be fast, powerful, and simple tool for the quantification of diterpenes in coffee oil without derivatization using Gas chromatography (GC)-MS or GC-flame ionization detector. This optimized method was decreasing the risk of degradation of the free diterpenes and time-saving in sample preparation. The method developed in this study will be useful for food industries. Further exploration is needed to investigate the standardization of individual phytoconstituents in coffee oil of *C. arabica*.

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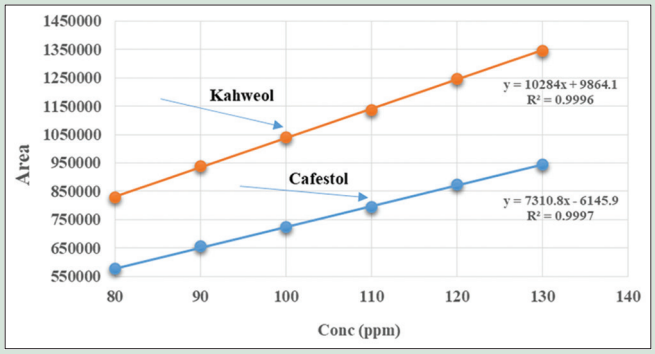

Figure 4: Linearity curve of cafestol and kahweol

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Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Moeenfard M, Alves A. New trends in coffee diterpenes research from technological to health aspects. Food Res Int 2020;134:109207.
2. Alvarez AM, Rodriguez ML. Lipids in pharmaceutical and cosmetic preparations. Grassas Aceites 2000;50:74-96.
3. Follier JF, Plessis S. Use of Coffee Beans oil as a Sun Filter. US Patent,1988. p. 4793990.
4. Gross G, Jaccaud E, Huggett AC. Analysis of the content of the diterpenes cafestol and kahweol in coffee brews. Food Chem Toxicol 1997;35:547-54.
5. Bruce C, Pettitt JR. Identification of the diterpene esters in Arabica and canephora coffees. J Agric Food Chem 1987;35:549-51.
6. Karla LW, Rafael CE, Marcelo CV, Marta de TB. Kahweol and cafestol in coffee brews: Comparison of preparation methods. Rev cienc Argon 2020;51:01-06.
7. Gómez-Ruiz JA, Leake DS, Ames JM. *In vitro* antioxidant activity of coffee compounds and their metabolites. J Agric Food Chem 2007;55:6962-9.
8. Speer K, Speer K. The lipid fraction of the coffee bean. Braz J Plant Physiol 2006;18:201-16.
9. Roos B, Weg G, Urgert R, Bovenkamp P, Charrier A, Katan MB. Levels of cafestol kahweol and related diterpenoids in wild species of the coffee plant coffea. J Agric Food Chem 1997;45:3065-69.
10. Arab L. Epidemiologic evidence on coffee and cancer. Nutr Cancer 2010;62:271-83.
11. Ranheim T, Halvorsen B. Coffee consumption and human health-beneficial or detrimental? Mechanisms for effects of coffee consumption on different risk factors for cardiovascular disease and type 2 diabetes mellitus. Mol Nutr Food Res 2005;49:274-84.
12. Cardenas C, Quesada AR, Medina MA. Anti-angiogenic and anti-inflammatory properties of kahweol a coffee diterpenes. PLOS 2011;6:23407.
13. Kim JY, Jung KS, Lee KJ, Na HK, Chun HK, Kho YH, *et al.* The coffee diterpene kahweol suppress the inducible nitric oxide synthase expression in macrophages. Cancer Lett 2004;213:147-54.
14. Cavin C, Holzhaeuser D, Scharf G, Constable A, Huber WW, Schilter B. Cafestol and kahweol, two coffee specific diterpenes with anticarcinogenic activity. Food Chem Toxicol 2002;40:1155-63.
15. Kim HG, Hwang YP, Jeong HG. Kahweol blocks STAT3 phosphorylation and induces apoptosis in human lung adenocarcinoma A549 cells. Toxicol Lett 2009;187:28-34.
16. Tao KS, Wang W, Wang L, Cao DY, Li YQ, Wu SX, *et al.* The multifaceted mechanisms for coffee's anti-tumorigenic effect on liver. Med Hypotheses 2008;71:730-6.
17. Lee KJ, Jeong HG. Protective effects of kahweol and cafestol against hydrogen peroxide-induced oxidative stress and DNA damage. Toxicol Lett 2007;173:80-7.
18. Lee KA, Chae JI, Shim JH. Natural diterpenes from coffee, cafestol and kahweol induce apoptosis through regulation of specificity protein 1 expression in human malignant pleural mesothelioma. J Biomed Sci 2012;19:60.
19. Mellbye FB, Jeppesen PB, Hermansen K, Gregersen S. Cafestol, a bioactive substance in coffee, stimulates insulin secretion and increases glucose uptake in muscle cells: Studies *in vitro*. J Nat Prod 2015;78:2447-51.

20. Baek JH, Kim NJ, Song JK, Chun KH. Kahweol inhibits lipid accumulation and induces glucose-uptake through activation of AMP-activated protein kinase (AMPK). BMB Rep 2017;50:566-71.
21. Fumimoto R, Sakai E, Yamaguchi Y, Sakamoto H, Fukuma Y, Nishishita K, *et al*. The coffee diterpene kahweol prevents osteoclastogenesis via impairment of NFATc1 expression and blocking of Erk phosphorylation. J Pharmacol Sci 2012;118:479-86.
22. George SE, Ramalakshmi K, Mohan Rao LJ. A perception on health benefits of coffee. Crit Rev Food Sci Nutr 2008;48:464-86.
23. Ren Y, Wang C, Xu J, Wang S. Cafestol and kahweol: A review on their bioactivities and pharmacological properties. Int J Mol Sci 2019;20:???.
24. Kotowski U, Heiduschka G, Seemann R, Eckl-Dorna J, Schmid R, Kranebitter V, *et al*. Effect of the coffee ingredient cafestol on head and neck squamous cell carcinoma cell lines. Strahlenther Onkol 2015;191:511-7.
25. ICH (Q2B) Note for Guidance on Validation of Analytical Procedures. Methodology. Geneva, Switzerland: IFPMA, International Conference on Harmonization; 1996.
26. Kurzrock T, Speer K. Identification of kahweol fatty acid esters in arabica coffee by means of LC/MS. J Sep Sci 2001;24:843-48.
27. Dias RC, Campanha FG, Vieira LG, Ferreira LP, Pot D, Marraccini P, *et al*. Evaluation of kahweol and cafestol in coffee tissues and roasted coffee by a new high-performance liquid chromatography methodology. J Agric Food Chem 2010;58:88-93.
28. Chartier A, Beaumesnil M, de Oliveira AL, Elfakir C, Bostyn S. Optimization of the isolation and quantitation of kahweol and cafestol in green coffee oil. Talanta 2013;117:102-11.
29. ICH (Q2A) Validation of Analytical Procedures: Text and Methodology. Geneva, Switzerland: International Conference on Harmonization; 2005.
30. Fabio junior MN, Francisco radler AN, Fernanda CB, Claudia MR. The occurrence of cafestol and kahweol diterpenes in different coffee brews. Coffee Sci 2019;14:265-80.