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# Microwave-assisted methanolysis of green coffee oil

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ABSTRACT

Optimisation of a microwave-assisted methanolysis was performed to obtain cafestol and kahweol directly from green coffee oil (*Coffea arabica*). A two-factor (the methanolysis period and temperature), three-level, factorial experimental design ($3^2$) was adopted. The methanolysis procedure was performed under microwave irradiation, using closed vessel and accurate fast responding internal fibre-optic temperature probe. The effects on the responses were measured by HPLC. After 3 min of microwave irradiation (hold time) at 100 °C, with 500 mg of green coffee oil, a yield higher than 99% was obtained. The yield of this reaction is 26% after 2 h when working under conventional heating. The methods described in the literature lead to long reaction times, poor yields and formation of side products. The microwave-assisted technique proved to be faster, avoided undesired side products and gave better conversion, when compared to conventional heating process.



## 1. Introduction

Cafestol (1) and kahweol (2) (Fig. 1) are two examples of naturally-occurring furan diterpenes in the lipid fraction of coffee (Bengis & Anderson, 1932; Dias et al., 2010; Djerassi, Wilfred, Visco, & Lemin, 1953; Haworth & Johnstone, 1956; Haworth, Jubb, & McKenna, 1954; Lam, Sparnins, & Wattenberg, 1982). Of the two most significant species in the coffee trade, *Coffea arabica* L. (Arabica) contains about 0.6% of cafestol (1) and 0.3% of kahweol (2) while *Coffea canephora* Pierre (Robusta) contains mostly cafestol (1) (0.2%) and 16-*O*-methylcafestol (0.15%), not present in Arabica coffee (De Roos et al., 1997; Nackunstz & Maier, 1987; Pettitt, 1987; Ratnayake, Hollywood, O'Grady, & Stavric, 1993). Coffee furan diterpenes are mainly present in the esterified form, with 26 different fatty acids, and only small amounts are in the free form (Fig. 1) (Kurzrock & Speer, 2001).

The amount of diterpenes present in the coffee brew depends on the way coffee is prepared. The highest content of diterpenes was found in boiled, unfiltered coffee brews, while in drip-filtered coffee brews they are negligible (Martín, Pablos, González, Valdenebro, & León-Camacho, 2001).

Cafestol and kahweol have been described to be both desirable and undesirable in human health. They are anticarcinogenic (Butt & Sultan, 2011; Cavin et al., 2002; Hatzold, 2012; IARC, 1991; Kim, Hwang, & Jeong, 2009; Lam et al., 1982; Lee, Choi, & Jeong, 2007; Tao et al., 2008), antioxidant (IARC, 1991; Lee & Jeong, 2007) and anti-inflammatory (Bertholet, 1987) and showed hepatoprotective effects (Lee et al., 2007). On the other hand, a hypercholesterolaemic action has been reported, attributed mainly to cafestol (Arnesen, Forde, & Thelle, 1984; Butt & Sultan, 2011; Hatzold, 2012; Urgert, Schulz, & Katan, 1995; Weusten-Van Der Wouw et al., 1994), and they are also responsible for increasing low-density lipoprotein (Urgert & Katan, 1997).z

Green coffee oil is obtained by mechanical cold pressing or extraction with solvents, such as hexane, but these traditional methods are labour intensive and time-consuming, and sometimes require large quantities of solvents (Araujo & Sandi, 2006). Other procedures involve supercritical fluid extraction method (SFE) (Araujo & Sandi, 2006; De Azevedo et al., 2008) and high-speed countercurrent chromatography (HSCCC) (Scharnhop & Winterhalter, 2009).

The extraction of the unsaponifiable material from green coffee beans is commonly performed by Soxhlet apparatus (Araujo & Sandi, 2006; Bertholet, 1987), with subsequent basic hydrolysis in methanol, to obtain cafestol and kahweol in their free form (Araujo & Sandi, 2006; Bertholet, 1987; Grollier & Plessis, 1988; Hartman & Lago, 1973). Analysis and purification of the diterpenes have been mainly carried out by HPLC (Gross, Jaccaud, & Huggett, 1997; Hartman & Lago, 1973; Kolling-Speer, Strohschneider, & Speer, 1999).

The most critical step of the whole process is the hydrolysis. The furan moiety of these diterpenes is labile, sensitive to acids, bases and oxidants, a problem associated with the heating procedure commonly used to obtain the free diterpenes. Furthermore, kahweol is quite unstable in the free form, which highlights the importance of developing a more efficient and faster isolation method. Cafestol has been synthesised in many steps, being practically unfeasible (Corey, Wess, Xiang, & Singh, 1987). These difficulties have led to a restricted commercial availability of those furan diterpenes.

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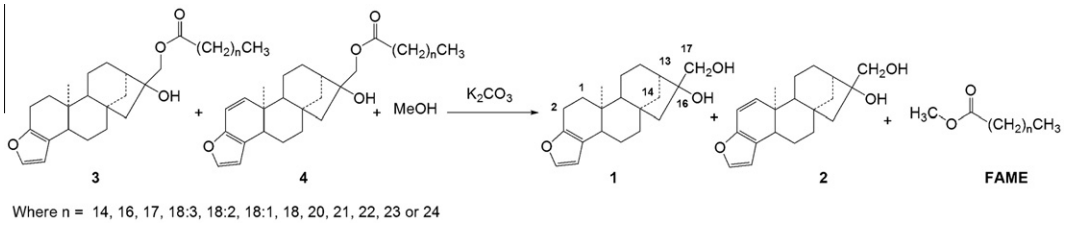


**Fig. 1.** Arabica green coffee oil methanolysis under conventional heating (fatty acid methyl esters (FAME)).

In the field of organic chemistry, microwave irradiation proved to be a powerful method to enhance chemical processes. In many instances, the use of sealed-vessel high-temperature microwave processing was able to dramatically reduce reaction times, consume less solvent, increase yields, reduce side reactions and improve reproducibility. Microwaves are known to be a more efficient heating method than traditional thermal processes. Reactions that require long reflux times can sometimes be carried out in a few hours or minutes in dedicated microwave irradiation equipment (Kappe, 2004). A significant number of reports have described microwave-assisted hydrolysis reactions and have shown them to be better than conventional heating (Cheng & Wu, 2011; Richel, Laurent, Wathelet, Wathelet, & Paquot, 2011).

In the present study, a new method to obtain cafestol and kahweol was developed by a microwave-assisted protocol, through the methanolysis of the natural fatty acid furan diterpene derivatives present in green coffee oils (*C. arabica*).

## 2. Materials and method

### 2.1. Chemicals

Methanol (HPLC grade), hexane and ethyl acetate were purchased from Tedia (Rio de Janeiro, Brazil). Deionised water (Type I, 18 mΩ cm), filtered through a 0.45-μm pore size filter (Millipore, Bedford, MA) was used as an HPLC solvent. Potassium carbonate ($K_2CO_3$) was obtained from Vetec (Duque de Caxias, Brazil).

### 2.2. Sample preparation

Brazilian commercial green coffee beans (*C. arabica*) were provided as a gift from Grão Mestre Café (Rio de Janeiro, Brazil). The beans were ground in a hammer mill grinder and sieved to obtain particles with diameters ranging from 0.297 to 0.59 mm. Thirty grams of the powder were transferred into a Soxhlet apparatus and extracted with 300 mL of hexane at 90 °C for about 16 h, in triplicate, according to the procedure developed by Araujo and Sandi (2006). The extract was filtered and the solvent removed using a rotary evaporator to yield 8.8% of oil.

### 2.3. Procedure

#### 2.3.1. Conventional heating

The procedure used to hydrolyse the diterpenes used 500 mg of green coffee oil which were treated with 3 mL of anhydrous methanol in the presence of 50 mg of $K_2CO_3$. The round-bottomed flask was then immersed in a preheated oil bath, heated under reflux on a hotplate and held at constant temperature (90 °C), using an electronic probe to regulate the oil temperature. After 2 h, the sample vial was removed from the oil bath and allowed to cool slowly at room temperature. The contents of the reaction flask were transferred into a separating funnel and rinsed with distilled water and ethyl acetate. The organic phase was dried over sodium sulphate, the drying agent was filtered off and the solvent removed by rotary evaporator to yield 2.7% of oil. The vial containing the oil was stored for later analysis at 4 °C. These procedures were conducted in triplicate.

#### 2.3.2. Microwave heating

The methanolysis was carried out using a closed-vessel single-mode microwave system (Monowave™ 300; Anton Paar GmbH, Graz, Austria), using standard Pyrex vessel (10 mL capacity). The reaction was performed at a fixed temperature internally measured by a ruby thermometer. The pressure in the microwave vessel during reaction achieved 6 bar under the best conditions. The microwave irradiation equipment was operated in temperature control mode. Five hundred milligrams of Arabica green coffee oil were treated with 3 mL of methanol (see Section 2.4). The highest yield obtained for the hydrolysed coffee oil was 10.4%. The methanolysis efficiency was determined by using the sum of cafestol and kahweol HPLC chromatographic peak areas, on the basis of the largest area being 100%. After heating time, the hydrolysed oil which dissolved in methanol was removed and the solid catalyst filtered in a paper filter. The solution was refrigerated at 4 °C for later HPLC analysis. Analyses were performed in duplicate, and the data were presented as mean ± standard deviation (SD) values. To determine repeatability, five different oils (500 mg) of the same sample were analysed using the same analytical method (hydrolysis conditions), in the same equipment at the same time (intraday repeatability).

### 2.4. Experimental design and statistical analysis

A two-factor, three-level, full-factorial design ($3^2$ FFD; Morgan, 1991) was used to analyse the response pattern and establish a model. The two independent variables used in the study were methanolysis time: 1, 3 and 5 min ($X_1$); temperature: 80, 90 and 100 °C ($X_2$), while the dependent variable was the total yield of the target compounds (as a recovery measurement obtained by HPLC analysis). Nine experiments were conducted to optimise the reaction conditions. The reactions were carried out in the presence of methanol (3 mL) and $K_2CO_3$ (0.05 g). The factors, experimental and predicted data obtained are shown in Table 1.

The results of each design were analysed by using the software Statistica™ Version 7 (Statsoft, Tulsa, OK). Both linear and quadratic effects of each variable (factors) under study, as well as their interaction and significance, were evaluated by analysis of variance.

A statistically significant multiple regression relationship between the independent variables ($X_1$ and $X_2$) and the response variable ($Y$) was established. A second order polynomial model was fitted to evaluate the yield (response variable, $Y$) as a function of independent variables ($X$) and their interactions:

$$Y = B_0 + B_1X_1 + B_2X_2 + B_{12}X_1X_2 + B_{11}X_1^2 + B_{22}X_2^2 + \epsilon$$

where $B_0$ is the constant coefficient, $B_1$ and $B_2$ are the linear coefficients, $B_{11}$ and $B_{22}$ are the quadratic coefficients, $B_{12}$ is the interaction coefficient of the model and $\varepsilon$ is the random error.

**Table 1**
Experimental matrix of the two-level factorial design ($3^2$) with the value of the methanolysis time ($X_1$) and temperature ($X_2$) for each reaction of Arabica green coffee oil.

| Run No. | $X_1$ | $X_2$ | Observed yield[a] % | Predicted yield (%) | Observed yield[a] (g/kg) | Predicted yield % (g/kg) |
|---|---|---|---|---|---|---|
| 1 | 1 (−1) | 80 (−1) | 15.6 ± 0.1 | 12.4 | 1.4 ± 0.0 | 1.2 |
| 2 | 1 (−1) | 90 (0) | 33.6 ± 0.9 | 35.8 | 3.2 ± 0.1 | 3.3 |
| 3 | 1 (−1) | 100 (+1) | 41.6 ± 1.5 | 42.6 | 3.8 ± 0.1 | 4.0 |
| 4 | 3 (0) | 80 (−1) | 72.1 ± 0.5 | 75.3 | 6.7 ± 0.0 | 7.0 |
| 5 | 3 (0) | 90 (0) | 90.7 ± 0.2 | 93.1 | 8.4 ± 0.0 | 8.6 |
| 6 | 3 (0) | 100 (+1) | 99.6 ± 0.7 | 94.4 | 9.2 ± 0.1 | 8.7 |
| 7 | 5 (1) | 80 (−1) | 83.2 ± 0.7 | 83.3 | 7.8 ± 0.1 | 7.7 |
| 8 | 5 (1) | 90 (0) | 100 ± 1.6 | 95.6 | 9.2 ± 0.1 | 8.9 |
| 9 | 5 (1) | 100 (+1) | 87.0 ± 0.2 | 91.4 | 8.0 ± 0.0 | 8.4 |

$X_1$, microwave period (min); $X_2$, temperature (°C).
The coded values are indicated in the parentheses.
[a] The values represent the means ± standard deviations, (n = 2).

### 2.5. Semi-preparative and analytical HPLC analyses

All material was analysed using an Agilent 1100 Series HPLC system, with degasser G1379A, quaternary pump G1311A, manual injector G1328B and a Rheodyne 7725i injection valve. Both the semi-preparative column (250 mm × 9.4 mm) and the analytical column (150 mm × 4.6 mm) were Zorbax Eclipse XDB-C18 columns (5 μm) and were kept at room temperature during the analysis. The mobile phase was methanol: water (85:15, v/v). The flow rates were 3 mL/min (200-μL loop) for semi-preparative isolation and 0.7 mL/min (5-μL loop) for analytical control (solutions were at 0.5 mg/mL). Detection was performed at 220 nm with UV detector G1314A. In order to avoid chemical interference, the solvent was carefully monitored in the UV. In the case of semi-preparative purification, all fractions obtained were submitted to rotary evaporator and freeze-dried for later analysis. All analytical HPLC determinations were conducted in duplicate.

### 2.6. NMR

$^1$H and $^{13}$C spectra were measured on a Bruker AMX 200 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) at 200 and 75 MHz, respectively. Solvent was chloroform-d (Merck, Darmstadt, Germany).

### 2.7. Quantification of cafestol (1) and kahweol (2)

A standard solution was used to quantify the mixture of free diterpenes in the hydrolysed green coffee oils. The standard (a cafestol/kahweol mixture) was initially obtained from basic methanolysis of the green Arabica coffee oil by conventional heating, with the aid of HPLC semi-preparative isolation of 1 and 2. The purity of the isolated compounds was estimated by $^1$H NMR analysis. A stock solution of the cafestol/kahweol mixture (7.7 mg/mL) was diluted in methanol to construct the calibration curve with 6 different concentrations, prepared on the same day as the injections (1–56 μg/mL). All determinations were conducted in duplicate.

### 2.8. LC-HRESIMS and MS/MS analyses

Analyses and peak identification by LC–HRESIMS and MS/MS were performed on a Waters Alliance HT 2795 HPLC system coupled to a QTOF Micro (Waters, Manchester, UK) mass spectrometer equipped with an ESI source. The analyses were carried out using the same column and isocratic elution described for the analytical HPLC method, with addition of 0.1% formic acid in the mobile phase. The column eluent was split at a ratio of 5:1. LC–MS TIC chromatograms were recorded between $m/z$ 90 and 1000 in positive ion mode, and the mass spectrometer parameters were maintained the same in all analyses. The nebulisation gas was set to 500 L/h at 140 °C, the cone gas set to 50 L/h, and the source temperature set to 100 °C. The capillary voltage and cone voltage were 4000 and 30 V, respectively. The QTOF acquisition rate was set to 1.0 s, with a 0.4 s inter-scan delay. Analytes were acquired using LockSpray to ensure mass accuracy. For MS/MS experiments, argon was employed as the collision gas at collision energies ($E_{lab}$) varying between 15 and 25 eV.

## 3. Results and discussion

Initially, the hydrolysis method was carried out with anhydrous methanol and allowed to stand overnight at ambient temperature with agitation, as recommended by Bertholet (1987). No apparent modification was observed in the oil, which required heating at about 90 °C in reflux (as proposed by Scharnhop and Winterhalter (2009)). Free cafestol and kahweol were isolated from green Arabica coffee oil by conventional reflux with methanol/$K_2CO_3$, purified by semi-preparative HPLC and confirmed by NMR and HRESIMS in accordance with the literature (Scharnhop & Winterhalter, 2009). The methyl esters of fatty acids were removed under the same semi-preparative HPLC conditions.

Later, the experiments were focused on establishing the optimum microwave irradiation conditions for the green coffee oil methanolysis, with respect to reaction time and temperature. The hydrolysis method typically requires heating under reflux conditions from 80 to 90 °C (Dias et al., 2010; Scharnhop & Winterhalter, 2009). According to Bertholet (1987) and De Lucia et al. (2009) a mild procedure should be used in order to avoid any thermal decomposition of kahweol.

Due to the explorative nature of the present work, the samples were heated at temperatures ranging from 60 to 120 °C, for a maximum of 9 min under microwave irradiation. When hydrolysis was carried out at lower temperatures for longer periods or at higher temperatures for shorter periods, the yields were low, showing that time and temperature are important parameters in the reaction and suggests that their interaction is also relevant. The ideal working range seems to be from 80 to 100 °C, with heating time of about 5 min. The experiments were then optimised. Results are shown in Table 1.

The identities of the diterpenes in the oil extracts were assigned by co-chromatography with standards in HPLC. The conventional heating technique was also conducted to compare its performance in obtaining the free diterpenes (Bertholet, 1987). The reflux showed lower yield of free cafestol and kahweol (around 25%) and 2 h were necessary for a complete conversion of the diterpene esters into the free compounds.

In order to provide a statistical model to identify trends in high yield for the target compounds, a two-factor three-level full-factorial design ($3^2$ FFD; Morgan, 1991) was used. Response surface methodology (RSM) was used to study the effect of free diterpenes

**Table 2**
Analysis of variance (ANOVA) of the regression parameters evaluated for diterpene esters methanolysis from Arabica green coffee oil.

| Factors/interactions | SS | df | MS | *F* | *p* |
|---|---|---|---|---|---|
| Microwave time ($X_1$) | 5363 | 1 | 5363 | 163.0 | 0.0010 |
| Microwave time ($X_1^2$) | 1504 | 1 | 1503 | 45.70 | 0.0066 |
| Temperature ($X_2$) | 549.7 | 1 | 549.7 | 16.71 | 0.0265 |
| Temperature ($X_2^2$) | 135.7 | 1 | 135.7 | 4.13 | 0.1352 |
| $X_1X_2$ | 122.5 | 1 | 122.5 | 3.72 | 0.1492 |
| Error | 98.7 | 3 | | | |
| Total *SS* | 7774 | 8 | | | |
| $r^2$ | 0.99 | | | | |
| Adjusted $r^2$ | 0.97 | | | | |
| MS residual | 32.9032 | | | | |

SS, sum of square; df, degree of freedom; MS, mean square; *F*, *F* ratio.

yield after methanolysis. The developed regression model for the relationship between dependent variable and the coded values of independent variables of microwave period ($X_1$) and temperature ($X_2$) and their interaction is given in equation Eq. (1) for total free diterpenes yield:

$$Y = -841.622 + 80.984X_1 + 16.616X_2 - 0.277X_1X_2 - 6.855X_1^2 - 0.082X_2^2 \quad (1)$$

The adequacy of the model was evaluated by the coefficient of determination $r^2$ and adjusted $r^2$ values. The analysis of variance (ANOVA) shown in Table 2 indicates a good model fitting with an $r^2$ value of 0.99 and adjusted $r^2$ among linear and quadratic of the total model. The adjusted $r^2$ is a measurement of the amount of variation about the mean explained by the model and $r^2$ is defined as the ratio of the explained variations to the total variation and is a measurement of degree of fit. Guan and Yao (2008) reported that $r^2$ should be at least 0.80 for a good model fit. The linear variables namely microwave time ($p < 0.005$) and temperature ($p < 0.05$) showed significant fit. Microwave time ($p \leqslant 0.01$) significantly affected the free diterpenes yield in a quadratic manner. The interaction between microwave period and time ($X_1X_2$) and the quadratic variable ($X_2^2$) showed a lack of fit ($p > 0.1$).

Microwave time and temperature were investigated over the range of 1–5 min and 80–100 °C, respectively. The 3D response surface and the 2D contour plots presented in Fig. 2 show the effect of the independent variables and their interaction on free diterpenes yield. The maximum yield was obtained at 100 °C after 3 min of reaction. The 3D response surface provided an indication of the robustness of the method, since small variations around the best point do not significantly change the diterpene yields. The main goal of the response surface is to hunt efficiently for the optimum values of the variables, such that the response is maximised (Tanyildizi, Ozer, & Elibiol, 2005).

Although the probabilities level for the quadratic variable ($X_2^2$) and interaction ($X_1 \cdot X_2$) showed $p$ value > 0.05, the elliptical contours observed in the 2D contour plots, especially in a working range of 90 and 100 °C, are a result from the perfect interaction between the independent variables (Muralidhar, Chirumamila, Marchant, & Nigam, 2001) that are being considered in the model.

By comparing the two methods, the reactions under microwave irradiation (9.2 ± 0.1 g/kg, corresponding to 99.6%) presented a much better result rather than conventional heating (2.3 g/kg, corresponding to 25.9%) for the free diterpenes obtained by methanolysis (Table 1), using reduced times. Another remarkable aspect was that highest temperatures afforded higher yields in the microwave irradiation optimised conditions. In general, other authors described an inverse correlation between the temperature and the free diterpenes concentration, mainly due to degradation products (Bertholet, 1987). No degradation products were observed by ESI-MS-TOF for the microwave irradiation experiments. This behaviour can be explained due to the fast heating and cooling of the reaction under microwave irradiation which cannot be achieved under conventional heating.

A typical HPLC chromatogram of green Arabica coffee oil before and after microwave irradiation is shown in Fig. 3. Table 3 presents the assigned structures for HPLC chromatographic peaks of Fig. 3 by LC–HRESIMS in positive ion mode, all previously reported in Arabica coffee (Alonso-Salces, Guillou, & Berrueta, 2009; Kurzrock & Speer, 2001; Scharnhop & Winterhalter, 2009). The mass errors were lower than 5 ppm confirming the molecular formulas. The results obtained in tandem mass spectrometry studies of *m/z* 195, 315 and 317 corroborate with the assigned structures in accordance with literature data: caffeine (Alonso-Salces et al., 2009) ($E_{lab}$ 25 eV: 195 → 138, 110), cafestol (Scharnhop & Winterhalter, 2009) ($E_{lab}$ 18 eV: 317 → 299, 281, 147, 133) and kahweol (Scharnhop & Winterhalter, 2009) ($E_{lab}$ 18 eV: 315 → 297, 279, 149, 131). The conditions clearly improve cafestol and kahweol concentrations; methylated fatty acids can be seen at the end of chromatogram in Fig. 3(B), due to methanolysis. Furthermore, the literature

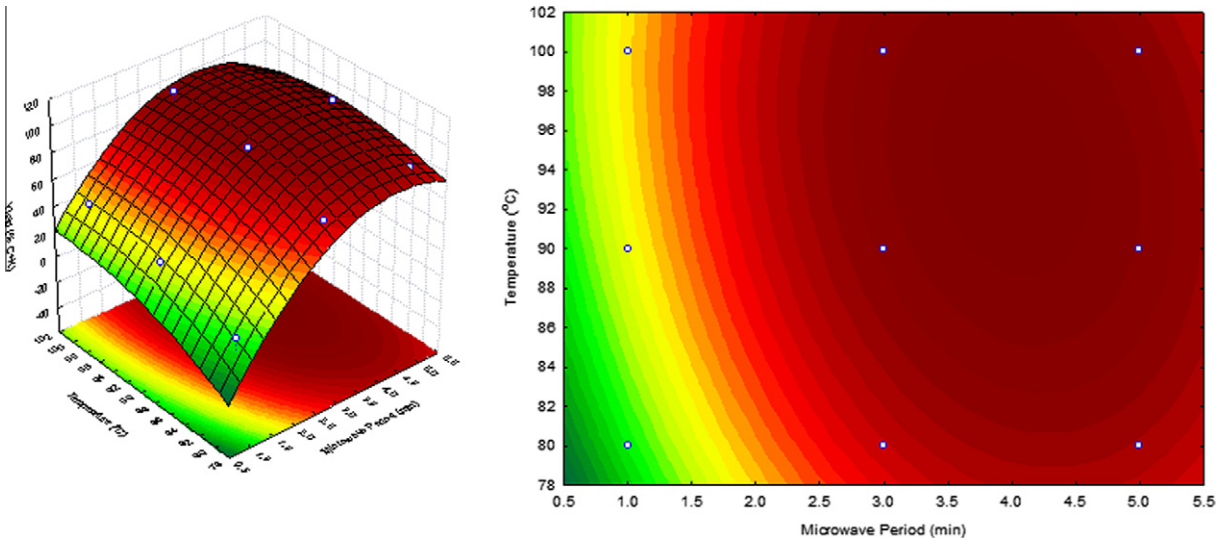


**Fig. 2.** 3D response surface and 2D contour plots showing the effects of microwave period and temperature on free diterpenes yield after methanolysis.

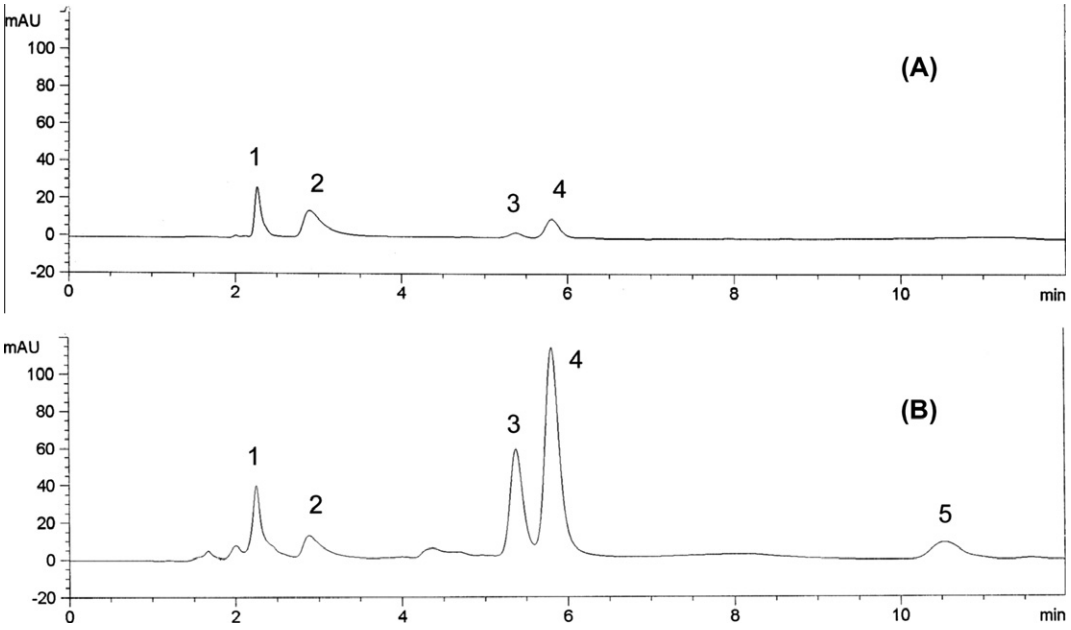


**Fig. 3.** HPLC chromatograms of the unsaponified green coffee oil extracted with Soxhlet apparatus in hexane before (A), and after 3 min of methanolysis under microwave irradiation (B), both at 0.5 mg/mL.

**Table 3**
Identification of HPLC chromatographic peaks of green Arabica coffee oil by high-resolution mass spectrometry.

| Peak[a] | RT (min) | Compound | Molecular formula | Calculated mass | Experimental mass | Error (ppm) |
|---|---|---|---|---|---|---|
| 1 | 2.2 | Caffeine | $C_8H_{11}N_4O_2^+$ | 195.0877 | 195.0875 | −1.03 |
| 2 | 2.8 | n.a. | n.a. | n.a. | 331.1935 | n.a. |
| 3 | 5.4 | Kahweol | $C_{20}H_{27}O_3^+$ | 315.1955 | 315.1959 | 1.27 |
| 4 | 5.8 | Cafestol | $C_{20}H_{29}O_3^+$ | 317.2111 | 317.2109 | −0.63 |
| 5 | 10.7 | Methyl linoleate | $C_{19}H_{32}O_2K^+$ | 331.2034 | 331.2031 | −0.91 |
| | | Methyl linoleate | $C_{19}H_{34}O_2K^+$ | 333.2190 | 333.2196 | 1.80 |

n.a., not assigned.
[a] Peak numbers refer to Fig. 3.

highlights the need for anhydrous methanol, which microwave heating showed to be unnecessary, greatly simplifying the methodology and costs (Bertholet, 1987).

Quantification of cafestol and kahweol was accomplished with the external standard method as response factor for the HPLC, obtained by linear regression of known concentrations versus peak area. Linearity was observed for a concentration range of 1–56 μg/mL, with a 5% confidence level and a *r* correlation coefficient for cafestol and kahweol higher than 0.99. Coefficients of variation (CV) below 7% were observed for the mixture of free diterpenes.

## 4. Conclusion

A fast and improved method to obtain a mixture of cafestol (1) and kahweol (2) from green Arabica coffee oil was successfully developed. The microwave-assisted protocol proved to be simple, fast, enabled the use of higher reaction amounts and can be carried out at higher temperatures. The rapid speed of reaction avoided the development of undesired products and increased product yield. In addition, the microwave-assisted method required no clean-up procedure when compared to conventional heating.

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