Green coffee extracts rich in diterpenes – Process optimization of pressurized liquid extraction using ethanol as solvent

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A B S T R A C T

In this study, a pressurized liquid extraction (PLE) was optimized to obtain green coffee extract rich in cafestol and kahweol diterpenes. The effects of temperature (T) and static time (St) (time of contact between the solvent and the matrix during each cycle or batch) on global yield, cafestol and kahweol concentrations and antioxidant activity were studied. The greatest green coffee extract global yield (9.78%) was obtained at 70 °C and 8 min St. PLE at 74 °C and 6 min St presented the highest concentration of cafestol (20.08 g/kg extract) in the green coffee extract, whereas, the highest kahweol concentration (25.16 g/kg extract) was obtained at 60 °C and 6 min St. The extracts showed high DPPH antioxidant activity, particularly after PLE at 50 °C and 8 min St (EC50 1.56 mg/mL of extract). This extract also presented a high total phenolic content (35.99 mg GAE/g of extract).

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1. Introduction

Coffee is currently, the second most important commodity in the world (Ortiz et al., 2015), considered one of the most valuable products and Brazil is one of the largest coffee producers worldwide, being the second largest coffee consumer behind the United States ABIC (2016). Coffea arabica (Arabica coffee) and Coffea canephora (Robusta coffee) are the main species produced in Brazil, which has a wide territorial coverage of coffee plantations with diverse cultivation conditions and genetic variability. Studies on coffee beans and green coffee oil have become more evident due to increased coffee consumption and dissemination of beneficial health effects associated with green coffee oil and its active ingredients (Kitzberger et al., 2013; Baeza et al., 2014).

Coffee contains a complex mixture of nutrients, such as carbohydrates, lipids, vitamins, minerals, and nitrogen compounds and is particularly rich in bioactives, like caffeine, cafestol and kahweol diterpenes, and chlorogenic acid (Akash et al., 2014). Furthermore, because of its bioactive compounds, green coffee oil has been promoted as a raw material for applications in cosmetics and pharmaceuticals, mainly due to its antioxidant activity (Chartier et al., 2013).

Information about its bioactive constituents has motivated investigations to determine its chemical composition. For example, Martín et al. (1998) evaluated the concentration of chlorogenic acid, caffeine, trigonelline, amino acids and polyphenols in 41 samples of Arabica and Robusta coffee beans and found that the two varieties had different concentrations of caffeine and free amino acids. The discrimination between the two species was also analysed in relation to their tocopherol concentration because the tocopherols (α, β, γ and σ) are lipophilic compounds with high antioxidant properties. The results indicated that the tocopherol concentration can be used to differentiate the species, showing that the Arabica coffee contains a higher concentration of these compounds, specifically β-tocopherol, than Robusta (Alves et al., 2009).

Green coffee oil is rich in phenolic compounds, particularly in chlorogenic acids (Perrone et al., 2008), whose high antioxidant potential has been demonstrated (Naidu et al., 2008). This oil is usually used as an emollient in the cosmetics industry due to its fatty acid content and its ability to block sunlight. It is an ingredient...
added to creams used to treat ageing and other skin-related diseases (Chiari et al., 2014).

In conventional extraction processes, the use of organic solvents may have a negative environmental impact, depending on the type and quantity of the solvents used. However, when using pressurized liquid extraction (PLE) processes, the amount of solvent used is much lower than the conventional processes (Oliveira et al., 2014). Although the overall environmental impact of an industrial extraction cycle is not easily estimated, it is known that energy costs are high and the waste that is produced is not always recycled at all stages of the process (Chemat et al., 2012).

The challenges posed by the competitiveness of the market and the globalised environment require advanced technological innovations that minimise the use of organic solvents and the production of waste that has negative impacts on the environment and human health. In this context, the technique of extraction with pressurized liquid or with supercritical CO2 (Chemat et al., 2012; Wang and Weller, 2006) is attractive.

The most widely used solid-liquid extraction methods for obtaining extracts rich in bioactive compounds are classified as “traditional” and “new”. Traditional solid-liquid extraction techniques are essentially based on the diffusion and osmosis processes, whereas the new processes under solvent boiling and reflux and/or ultrasonic agitation. The most modern methods of extraction are supercritical fluid extraction (SFE), PLE and microwave-assisted extraction (MAE). The traditional methods of extraction are performed at atmospheric pressure, while in the new techniques, it is possible to change both the temperature and pressure conditions (Vandenbussche et al., 1999).

PLE, also known as accelerated solvent extraction (ASE), was first described in 1995 (Carabias-Martínez et al., 2005). It was initially developed for use in laboratory analysis but was seen as a plausible technological innovation to be used on a larger scale. Studies about this process aim to optimise the factors that directly influence the extraction. The process variables normally studied are the temperature, the static time, the number of cycles or batch (i.e. the number of times that the solvent passes through the extraction matrix when the process occurs in batch) and the amount of solvent used (Oliveira et al., 2014; Mustafa and Turner, 2011). Minimising the extraction time avoids a possible thermal degradation. Thus, this method is widely used as an extraction technique to identify components present at low concentrations and with photosensitive and thermosensitive characteristics (De Oliveira et al., 2014).

PLE presents an attractive technique to fractionate natural products because it is a rapid extraction, with relatively low solvent consumption (Carabias-Martínez et al., 2005). Process parameters may be adjusted to increase the selectivity of the method for a particular group of compounds. Currently, PLE has been successfully used in the extraction of thermally sensitive phytochemicals from various plant sources (De Oliveira et al., 2014), such as olive leaves (Xynos et al., 2014), pitanga seeds (Oliveira et al., 2014), blackberry (Machado et al., 2015), waxy barley (Benito-Román et al., 2015) and even green coffee (Belandria et al., 2016). This technique has recently been used to extract active compounds from plants and foods, such as milk and eggs (Prestes et al., 2013), veterinary drugs from animal organs, and to determine pesticide residues in soil.

Parameters, such as diffusivity, fixed bed porosity, extraction process parameters and physicochemical properties of the solvent used in PLE may directly influence the global yield and antioxidant activity of the extracts (Colivet et al., 2016). The advantages of PLE are the high solubility of the analytes in solvents at high temperatures, which provides a high rate of diffusion of the solute to the matrix and rupture of the strong solute-matrix interaction involving van der Waals bonds, and hydrogen and dipole-dipole attractions between solute molecules and active sites in the matrix. Additionally, the polarity of the solvent can be chosen from a wide variety, according to the respective matrix used and the type of solute to be extracted (Ong et al., 2000).

In the PLE optimization to obtain extracts with high global yields or enriched in compounds of interest, many process variables can be studied. In this study, the PLE process was optimized considering the temperature (T) used and the static time (St) in which the solvent stays in contact with the matrix during each cycle or batch. The other process variables (volume of the solvent, pressure, number of cycles) were kept constant. The main objective of the study was to evaluate the influence of the T and St variables on the green coffee extract global yield, and cafestol and kahweol concentrations, and to determine the in vitro antioxidant activity of these extracts.

2. Materials and methods

2.1. Raw material

The raw material used was coffee beans (Coffea arabica), cultivar Catuai Amarelo, from the August 2013 harvest, produced in the region of Jau, Torrinha and Dois Corregos, Sao Paulo/Brazil (22°25′34″S and 48°10′09″W; 802 m above sea level; average temperature of 295.15 K). The beans, harvested at a mature stage, were sun-dried and then peeled.

At the High-Pressure Technology and Natural Products Laboratory (ITAPPN/FZEA/USP), Pirassununga, Sao Paulo/Brazil, sound beans were selected, while any beans showing defects were discarded. The selected beans were dried in a forced air oven at 50°C for 48 h and then ground.

The grain moisture was determined using a gravimetric method (AOAC, 1995). The beans were ground in a knife-type mill (Marconi, Piracicaba, BR), and then stored in a freezer (Frost Free, Brastemp, BR) at −18°C.

The granulometry of the ground green coffee beans was measured in a set of Tyler standard series sieves and stirred for 5 min so that the particles were evenly distributed in each of the sieves. The mean particle diameter was calculated by Eq. (1).

\[ d_{\text{ng}} = \log_{10} \left[ \frac{1}{n} \sum_{j=1}^{n} \left( \frac{w_i \log d_i}{\sum_{j=1}^{n} w_i} \right) \right] \]  

where, \( d_i = (d_{i,i+1})^{0.5} \). Where \( d_i \): nominal opening of the i-th sieve (mm); \( d_{i,i+1} \): nominal opening of the sieve greater than the i-th sieve (mm); \( w_i \): mass of the material retained in the i-th sieve.

2.2. Real and apparent density and porosity of the fixed bed

The real density of the ground green coffee beans was determined by the staff at the Analytical Centre of the Institute of Chemistry, State University of Campinas (UNICAMP), using a gas pycnometer (Quantachrome Ultrayp 1200e, FL, USA) and an analytical balance (Quimis, model QI-AS, USA). The equipment used helium gas to measure the volume. The actual density of the solid particles was determined by the gas displacement technique.

The apparent density was determined empirically by the ratio between the total mass of ground coffee beans packed in the fixed extractor and its volume of 34 cm³. The green coffee mass was packed in the extractor and weighed 10 times in an analytical balance (SHIMADZU AUY220, Tokyo, JP) and the apparent density
calculation was represented as the average of these eleven tests.

The porosity of the extractor bed was calculated by the relation between the real and the apparent densities, using Eq. (2).

$$e = 1 - \frac{\rho_a}{\rho_r}$$

(2)

where: \(\rho_a\) = apparent density; \(\rho_r\) = real density.

2.3. Pressurized liquid extraction (PLE)

PLE consisted of weighing 22 g of dried and ground green coffee beans and packing them in the 34 cm³ capacity fixed bed extractor. Ethanol (EtOH, 99.3%, Ciclo Farma, Serrana, BR) was used as a solvent. This solvent is considered GRAS (generally recognised as safe). The total solvent volume used per extraction 30 mL, corresponding to approximately 80% (27.2 mL) of the extractor capacity volume (V) in each extraction cycle or batch. In this experiment, 3 cycles (C) were used and the fixed operating pressure was 10.30 MPa. These experiments were made in an ASE™ 150 equipment, (Dionex, Sunnyvale, USA), in which the sample was packed in a fixed bed in a vertical position.

In this study, the extraction temperature (T) and static time (St) (defined as the time the sample was in contact with the solvent in each cycle) were considered as independent variables. In a previous study by this same research group, it was demonstrated that among these two variables on the green bean coffee extract global yield, cafestol and kahweol concentrations and antioxidant activity of the extracts, were studied. A complete central rotational design (CCRD) was applied. The levels of the coded variables are presented in Table 1.

2.4. Acid value (AV)

The AV was determined using oleic acid as the standard. Thus, the influences of the process on the AV of oleic acid provided information about the state of preservation and the quality of the extracted oil.

Briefly, to determine the AV of oleic acid (%), 2 g of the extract was mixed with a solution of ethyl ether (Ecibra, São Paulo, BR) and EtOH (Panreac chemistry, Barcelona, Spain) at 2:1 v:v. Three drops of phenolphthalein indicator (Synth, São Paulo, BR) were added to the solution and then titrated with 0.01 M sodium hydroxide (Haloquímica, São Paulo, BR) until the appearance of a pink colour and its permanence in the solution for 30 s (Zenebon et al., 2008). The AV result was calculated from the volume of the sodium hydroxide solution used in the titration (Eq. (3)).

$$AV(\%) = \frac{v \times f \times 0.01 \times 28.2}{P}$$

(3)

where v: volume of sodium hydroxide used in the titration (mL); f: factor of the solution; P: sample weight (g).

2.5. Quantification of cafestol and kahweol diterpenes

The extracts obtained by PLE were saponified and esterified according to the method developed by this research group (Chartier et al., 2013). Briefly, 100 mg of green coffee extract, 8.25 g potassium hydroxide (Exodo Científica, Hortolandia, BR) and 100 mL methanol (Merck, Darmstadt, GE) were placed in a flask and ultrasonicated (ultrasonic bath) for 10 min. Next, the solution was placed in a thermal bath (Marconi, Piracicaba, BR) at 70 °C for 1 h. The solution was dried over Na2 before 2 mL tert-butyl methyl ether (MTBE) (Sigma-Aldrich, St. Louis, USA) and 2 mL demineralised water (MilliQ Direct Q3, Billerica, Massachusetts, USA) were added. Finally, the solution was centrifuged at 3000 rpm for 5 min (Thermo Jouan BR4i, Thermo Fisher Scientific, Waltham, USA). The organic phase (supernatant) was collected and a further 2 mL of MTBE was added to the solution. This procedure was repeated 2 times, to remove all cafestol and kahweol and other unsaponifiables present in the sample. The collected organic phase was washed with 2 mL of demineralised water and again centrifuged. After washing, the collected organic phase was dried under an N2 flow at room temperature using a concentrator (Tecnal, TE-019, Piracicaba, BR).

The unsaponifiable fractions (100 mg of diluted sample in 3 mL of MTBE) were injected (5 µL) into a gas chromatograph (GC-FID 10 Plus, Shimadzu, Kyoto, JP) equipped with a RTX-5MS (5% diphenyl/95% polydimethylsiloxane) capillary column (30 m × 0.25 mm, 0.25 µm) (Restek, USA). The conditions of analysis were based on those suggested by Chartier et al. (2013) and De Oliveira et al. (2014). The oven temperature gradient started at 70 °C/2.5 min then increased at 40 °C/min to 200 °C/10 min; 6 °C/min to 235 °C and 30 °C/min to the final temperature of 303 °C/7 min. Thus, the

| Test | T (C) | St (min) | GY (%) | Acid index | Cafestol (g/kg of oil) | Kahweol (g/kg of oil) | EC50 (µg/mL) | TPC | mg GAE/g extract | mg GAE/100 g
|------|-------|----------|--------|------------|-----------------------|----------------------|--------------|-----|-----------------|----------------
| 1    | −1    | −1       | 50     | 4          | 7.08 ± 0.11            | 7.85 ± 0.12          | 11.12 ± 0.12 | 36.5 ± 0.5 | 24.63 ± 0.4    | 3.48 ± 0.2 |
| 2    | 1     | −1       | 70     | 4          | 6.60 ± 0.14            | 6.64 ± 0.14          | 7.92 ± 0.14 | 51.2 ± 0.4  | 24.79 ± 0.3    | 3.76 ± 0.3 |
| 3    | −1    | 1        | 50     | 8          | 6.97 ± 0.14            | 17.71 ± 0.14         | 21.28 ± 0.14 | 1.7 ± 0.2  | 35.99 ± 0.3    | 5.16 ± 0.3 |
| 4    | 1     | 1        | 70     | 8          | 9.78 ± 0.15            | 18.44 ± 0.15         | 21.09 ± 0.15 | 8.7 ± 0.0  | 35.18 ± 0.3    | 3.60 ± 0.3 |
| 5    | 0     | 0        | 60     | 6          | 7.45 ± 0.11            | 19.43 ± 0.11         | 25.16 ± 0.11 | 74.9 ± 2.5 | 24.76 ± 0.3    | 3.32 ± 0.3 |
| 6    | 0     | 0        | 60     | 6          | 7.98 ± 0.15            | 17.52 ± 0.15         | 20.3 ± 0.15 | 78.1 ± 6.8 | 24.51 ± 0.3    | 3.07 ± 0.3 |
| 7    | 0     | 0        | 60     | 6          | 7.22 ± 0.13            | 11.36 ± 0.13         | 14.61 ± 0.13 | 45.3 ± 0.4 | 20.28 ± 0.2    | 2.81 ± 0.1 |
| 8    | −2    | 0        | 46     | 6          | 7.23 ± 0.12            | 6.58 ± 0.12          | 9.15 ± 0.12 | 11.9 ± 0.0 | 26.27 ± 0.3    | 3.63 ± 0.3 |
| 9    | +2    | 0        | 74     | 6          | 8.78 ± 0.13            | 20.08 ± 0.13         | 23.9 ± 0.13 | 12.3 ± 0.3 | 27.96 ± 0.3    | 3.18 ± 0.3 |
| 10   | 0     | −2       | 60     | 3          | 7.21 ± 0.15            | 7.94 ± 0.15          | 11.20 ± 0.15 | 138.7 ± 0.0 | 17.02 ± 0.2    | 2.36 ± 0.2 |
| 11   | 0     | +α       | 60     | 9          | 8.60 ± 0.14            | 12.27 ± 0.14         | 15.72 ± 0.14 | 47.9 ± 0.2 | 23.58 ± 0.3    | 2.74 ± 0.3 |
| SFE  | −     | −        | −      | −          | 8.78 −                 | 60.4 ± 0.14          | 46.9 ± 0.14 | 17.681 ± 3.5 | −             | −            |

*a Central points of CCRD.

b Effective concentration at 50%.

c For 100 g of green coffee beans (dry basis).

d Extraction conditions (time of 6 h, static time of 20 min, 30 MPa and 70 °C). Temperature (T), Static time (St).
total run time was 25 min. Nitrogen (Linde Gases, Hamburg-Wilhelmshurg, GE) was used as a carrier gas at a flow rate of 1.20 mL/min. The GC interface temperature was 300 °C.

The quantification of cafestol and kahweol diterpenes was made by external normalisation. This method compares the area of the analytes present in the sample with the areas obtained from the same substance in standard solutions of known concentrations made with certified analytical standards. The pure cafestol and kahweol solutions (Santa Cruz Biotechnology, California, USA) were diluted in MTBE at 90, 150, 300, 600, 900, 1,200, 1,500 and 1,800 μg/mL.

2.6. Quantification of total phenolic content (TPC)

The TPC was spectrophotometrically determined using the Folin-Ciocalteu reagent, according to Singleton and Rossi (1965). Briefly, 1 mL of the green coffee extract solution, diluted to 1,000 μg/mL in methanol (Panreac, Barcelona, ES), was pipetted into test tubes and 5 mL of the Folin-Ciocalteu reagent (Haloquímica, São Paulo, BR) diluted in distilled water (1:10, v:v) was added. After 10 min, 4 mL of aqueous 7.5% analytical grade anhydrous sodium carbonate (Synth, Diadema, BR) was added. This mixture remained undisturbed at room temperature for 2 h in the absence of light. Finally, the absorbance was determined at 760 nm (Biospectro, SP-22, Curitiba, BR). A blank was similarly prepared, with 1 mL of methanol replacing the extract. Gallic acid (10, 20, 40, 60 and 80 μg/mL) was prepared in methanol to prepare the calibration curve. The TPC was expressed as mg of gallic acid equivalents (GAE)/g of extract and mg of GAE/100 g of green coffee beans.

2.7. 2,2-Diphenyl-1-picrylhydrazyl free radical (DPPH*) antioxidant capacity

The stable DPPH* free radical produces a violet solution in EtOH. In the presence of antioxidant molecules, the radical is reduced to the yellow diphenylpicrylhydrazine (Mensor et al., 2001). In a test tube, 3.9 mL of the methanolic solution (JT Baker, Pennsylvania, USA) containing 6 × 10⁻³ M DPPH* (Sigma-Aldrich, Darmstadt, GE) was added to 0.1 mL of the extracts at various concentrations (1.5, 3, 10, 30, 100 and 300 μg/mL). The absorbance of these solutions was determined at 515 nm (Biospectro, SP-22, Curitiba, BR). All tests were performed in triplicate and accompanied by a control (the DPPH* solution without extract). The decrease in the optical density reading of the extracts with added DPPH* radical was read against the blank (99.6% EtOH), thus, establishing the percentage of DPPH* discoloration, which indicates the antioxidant activity of the extracts obtained (Brand-Williams et al., 1995).

The DPPH* free radical sequestering was expressed as a percentage antioxidant activity (AA) according to Eq. (4).

\[
AA\% = 100 - \left( \frac{(Abs_{sample} - Abs_{blank})}{Abs_{control}} \right) \times 100 \quad (4)
\]

where AA (%): antioxidant activity; Abs_{sample}: sample absorbance, Abs_{blank}: blank absorbance, Abs_{control}: control absorbance.

The EC₅₀ values were calculated from the linear regression of the % AA curves obtained for all extract concentrations. The % AA and EC₅₀ for all extracts were obtained considering the mean value of triplicate assays.

2.8. Statistical analysis

The optimization of the PLE process was evaluated by response surfaces analysis. The dependent variables obtained as responses, such as extraction global yield, AV, antioxidant activity and the quantification of the bioactives cafestol and kahweol, were analysed considering the complete central rotational design (CCRD) with two independent variables (T and St). Statistical analyses and response surfaces generated by the significant models were done using the Statistica v.12.0 program (USA).

3. Results and discussion

3.1. Raw material characteristics

The oven-dried and ground green coffee beans had a moisture content of 4.13% (±0.10). The comminuted green coffee bean particles had an average diameter of 0.88 mm, which was maintained for all PLE. This parameter is important because it is directly related to the internal mass resistance. Therefore, to increase the extraction global yield, a reduction in the particle size may increase the surface area of the solid/solvent contact, thereby decreasing the distance that the solute travels within the porous particle to the surface (Oliveira et al., 2010). Consequently, the particle size of oil-rich vegetable matrices is an important consideration in extraction.

The mean value of the empirically measured apparent density was 0.40 g/cm³ for the ground green coffee beans, and the real density was 1.35 ± 0.01 g/cm³, thus, the bed porosity was 0.30. Porosity is a geometric property related to the particles that compose a fixed bed. It is defined as the ratio of the void volume to the total volume of the bed and, the ratio (1-ε) represents the volume fraction of the particles in the bed. So, the porosity of the fixed bed is an important factor to be determined because it indicates the packaging efficiency. An ε = 0.3, indicates that there are few voids in the extractor.

3.2. Green coffee extract global yield

Green coffee beans present an amount of oil of 7.57 g/100 g of coffee beans and the profile of fatty acid has 14:0 (M), 16:0 (P), 18:0 (S), 18:1n-9 (O), 18:2n-6 (L), 20:0 (A), 18:3n-3 (Ln), and 22:0 (Be). The triacylglycerol profile, statistically determined in function of the fatty acid content, presents PSL, PLL, PLP, PLO, PLA, POP, SLL, POS, SLA, SLO, POA, TTO, LLA, LLI, PLL, OLA, SOA, PLnP, and SLBe (Cornelio-Santiago et al., 2017).

The global yield of the green coffee extracts quite oil from PLE ranged from 6.60 to 9.78% (Table 1). When the influence of the process variables (T and St) on the global yield of extracts obtained via PLE was evaluated in the statistical analysis of the first-order model (tests 1–7, Table 1), the main effects of T and St and of their interaction were observed. The variables and the interaction between them positively influenced the global yield (Y) (p ≤ 0.05), as visualised in the Pareto diagram (Fig. 1A). The significant effect of the interaction between the variables (T × St) was positively higher than the effect of the individual variables. In the studied temperature range, the static time (St) was the second variable that showed the greatest positive influence on the global yield (Fig. 1A).

By means of the F test, the first-order model (Eq. (5)) for the global yield of the extract obtained via PLE was significant and, therefore, predictive because F_{calc} was higher than F_{tab} (Table 2). Thus, the generated linear model has the capacity to explain 95% of the data variation. The response surface generated (Fig. 1B) by this first-order model, shows that the best green coffee extract global yields obtained by PLE were achieved when high temperatures were used for longer contact times between the solvent and the matrix packed in the fixed bed extractor. The optimized region in this experiment indicates the direction to adopt other ranges of T and St in the study of PLE process optimization i.e. those that lead to higher temperatures and longer contact times of the matrix with the solvent so that better global yields can be achieved. In the statistical analysis of the coefficients that would compose a
quadratic model analysed by analysis of variance (ANOVA), it was verified that the quadratic or second-order regression coefficients were not significant.

\[ Y = 7.583 + 0.583 \times T + 0.768 \times St + 0.823 \times T \times St \]  \hspace{1cm} (5)

Previously, other processes have been applied to obtain green coffee extracts, such as supercritical extraction, which globally yielded 8.08% (De Oliveira et al., 2014). Supercritical extraction in this optimized condition reproduced in this paper generated a yield of 8.78% (Table 1). A comparison of the data verified that the PLE presents greater efficiency because this process uses relatively shorter extraction times to obtain similar global yields.

Ethanol (EtOH) was selected as the extraction solvent, based on its polarity. Polar solvents promote extracts rich in compounds of medium and high polarity. Also, it has been reported that diterpenes and phenolics compounds may have a high affinity for, and greater solubility in EtOH when a PLE process is used (Chassagnez et al., 1997).

### 3.3. Influence of the process variables on the acid value (AV)

The determination of the acid value (AV) was carried out to evaluate if the T and St influenced the extract composition and thereby, interfered with the quality of the final product.

The green coffee bean extracts obtained through PLE presented AVs for oleic acid between 0.1097 and 0.1549% (Table 1). Brazilian legislation limits the AV for refined vegetable oils to 0.3% BRASIL (2006). Thus, the green coffee bean extract very oilily obtained by this technique has a low AV and is in accordance with current legislation. This value indicates that both the temperature range used and the variation in contact with the solvent (EtOH) did not interfere with the quality of the extract. A great advantage of PLE is that it is performed in a nitrogen atmosphere, thus, the absence of oxygen contributes to the quality of the extract.

In the statistical analysis of the influence of T and St on the AV of the extracts, none of these variables or the interaction between them influenced the AVs, as shown in the Pareto diagram (Fig. 2A), with 95% confidence level.

### 3.4. Influence of the process variables on the diterpenes concentration

The quantification of diterpenes was performed in the extracts obtained via PLE as a function of the studied process variables (T and St). The diterpenes concentrations ranged from 6.58 to 20.08 7.92–25.16 g/kg of extract for cafestol and kahweol, respectively (Table 1). The analysis of the main effects (T and St), and the interaction between them (T \times St) indicates that none of the variables influenced the concentrations of kahweol (Fig. 2B) and cafestol (Fig. 2C) in the extracts, as shown in the Pareto diagrams, with 95% confidence level.

Although it was not possible to generate predictive models, the
The highest cafestol and kahweol concentrations were obtained under the conditions described by the central points of the CCRD (60 °C and 6 min) and under condition 9 (74 °C and 6 min) (Table 1). The conditions of tests 3 and 4 also presented high concentrations of the diterpenes.

According to Araujo and Sandi (2006) and Oliveira et al. (2014), moderate temperatures (70 °C) exert a positive influence on the extraction of diterpenes (cafestol and kahweol) for supercritical CO2 extraction technique. In this study, when pressurized ethanol was used as a solvent, it was observed that the highest concentrations of cafestol and kahweol were obtained at a temperature slightly above 70 °C (Table 1), indicating that a small increase in the temperature (4 °C) extracts more diterpenes. In a supercritical extraction process that used ethanol as a polarity modifier, the higher temperature also positively interfered in the extraction of diterpenes (Barbosa et al., 2014), indicating that this process receives interference from the co-solvent. Similar behaviors were observed between these two processes as a function of the use of the same polar solvent.

In another study, also using polar solvent, pressurized methanol (MeOH) in a dynamic PLE process at 100 °C, the cafestol concentration ranged from 185 to 461 mg/100 g of green coffee beans and, for kahweol, ranged from 325 to 690 mg/100 g of green coffee beans. In this case, the temperature acts positively on the result (Belandria et al., 2016). In the comparison of these experiments it was noted that the solvent and the temperature act in different ways in the extraction of diterpenes from coffee beans as a function of its polarity.

This finding can be related to the synergism between the polarity of pressurized EtOH and MeOH and high temperatures that may aid in the greater solubilisation of the diterpenes and consequent enrichment of the extracts with these compounds. However, when supercritical CO2 extraction was used, even at low temperatures, diterpene concentrations were higher.

For the green coffee extract from supercritical CO2 which was reproduced in this study, 70 °C and 30 MPa (De Oliveira et al., 2014), 42 g of kahweol/kg of green coffee oil and 21 g of cafestol/kg of green coffee oil were obtained.

3.5. Antioxidant activity and TPC

The antioxidant activity of green coffee extract is associated with its rich composition of free radical scavengers, such as tocopherols, linoleic acid and chlorogenic acid (Sandi, 2003). The green coffee oil extracted with supercritical CO2 had a low DPPH• antioxidant activity, with an EC50 of 32.71% at 10,000 µg/g. The supercritical CO2 extracts contain compounds of low polarity, like lipids. Thus, the extracts obtained by supercritical CO2 extraction are essentially rich in diterpenes because cafestol and kahweol are normally esterified to the fatty acids of the green coffee oil, namely palmitic, linoleic and stearic, among others (De Oliveira et al., 2014; Sandi, 2003), but are not rich in more polar

![Fig. 2. Pareto diagrams showing the effects of the variables T and St on the acid value (A); on cafestol (B) and kahweol (C) content; on the total phenol content (TPC) (D) in the green coffee extract obtained by PLE.](image)
compounds that act as antioxidants.

However, low concentrations of green coffee extract obtained by PLE (Table 1) are necessary to inhibit the DPPH• radical. The EC50 ranged from 1.56 to 138.77 μg/mL. The best antioxidant activity (the best EC50) occurred when the conditions of test 3 (50 °C and 8 min) were used.

Analysis of the first-order effects of the studied variables (T and St) on the DPPH• antioxidant activity of the extracts (tests 1–7, Table 1), verified that these variables did not influence this response. In contrast, ANOVA showed that the coefficients constituting the quadratic model were significant both for T and for the interaction of T × St because the value of calculated test F (Fcal) was higher than tabulated F (Ftab) (Table 2). The regression coefficient of the model (Eq. (6)) was 0.84.

\[
EC_{50} = 64.12 + 2.82 \times T - 33.0 T^2 - 25.75 \times St + 7.58 \\
\times St^2 - 1.94 \times T \times St
\]

(6)

The response surface (Fig. 3) generated by the quadratic model (Eq. (6)) shows that for the studied temperature (T) range, the lowest St values generated extracts with relatively high DPPH• antioxidant activities. Relatively high antioxidant activities were also obtained for intermediate temperature values (Fig. 3), indicating that low temperatures are not sufficient to extract antioxidant compounds with high efficiency and high temperatures may degrade them during the process.

The TPC (Table 1) ranged from 17.02 to 35.99 mg GAE/g extract, which corresponds to 2.36–5.16 mg GAE/100 g of green coffee beans (dry basis).

Analysis of the main effects of the variables T and St on the TPC of the green coffee extracts revealed there was no significant (with 95% confidence level), as shown in the Pareto diagram (Fig. 2D). Although there is no model describing the influence of the variables on the TPC in the extracts, individual evaluation of the experimental values (Table 1) suggests that the static time (St) presented a positive influence on the TPC.

In this experimental design (CCRD) the triplicate error at the central point is distributed between the other assays. In the specific case of extraction yield (Table 1) the deviations between tests 5, 6 and 7 are low, and this influences the model goodness of fit (Fig. 1). In the case of other responses (acidity value, diterpene concentration, and TPC), such deviations are not low (Table 1), and this may have influenced the outcome of the process variables having no significant effects (Fig. 2).

The lack of fit of the model to the experimental points that describes the influence of the process variables on the antioxidant activity through the EC50 can also be a product of this deficiency of homogeneity in the results of the central point. Due to the analytical error that can occur in the chemical analyzes, there is always the concern in repeating analyzes to confirm the result. But the error may be intrinsic to the extraction process, the lack of homogeneity of the grains, for example, could even provide high yield but low concentration of the components under study. In the case of data inconsistency, the extraction must be repeated in order to confirm the analytical results.

A comparison of the results obtained by PLE and SFE showed that PLE extracted more phenolic compounds due to the affinity of these polar compounds for EtOH. The solubility of these compounds in supercritical CO2 is relatively lower because CO2 has a greater power of solubilisation for apolar compounds (De Oliveira et al., 2014; Roesler et al., 2007).

4. Conclusions

The influence of two important variables, temperature (T) and static time (St) of PLE on different responses by means of a CCRD allows evaluating the behaviour of a new extraction using a reduced number of experiments. Higher extraction global yields were attained when high temperatures (T) and contact time (St) were applied in the process, whereas test 4 (70 °C and 8 min) provided the highest extract global yield.

However, these conditions (70 °C and 8 min) did not generate the extracts with the highest concentrations of diterpenes and phenolic compounds, and antioxidant activity. No influence of the process variables (T and St) on the concentration of the bioactives cafestol, kahweol and TPC was observed in the statistical analysis. The antioxidant activity was higher for the extracts obtained under the conditions of shorter contact time (St) and intermediate temperatures (T), within the studied ranges.

Extracts from green coffee obtained by PLE showed high DPPH• antioxidant activity and high TPC. If the objective of this research was to extract green coffee oil rich in active compounds, the results presented in this study indicate the feasibility of the process optimization for PLE technique. Results from the PLE process optimization could encourage the large-scale use of PLE in the food, cosmetics and pharmaceutical industries.

Specifically, for green coffee oil rich in diterpenes may be a potential product to chemopreventive studies and as raw material for the development of new drugs based on the bioactives (cafestol, kahweol, phenolic compounds).

The cafestol and kahweol concentrations in the green coffee oil extracted with supercritical CO2 corroborated the usefulness of SFE in obtaining these diterpenes from coffee beans. Particularly for the DPPH• antioxidant activity, however, the green coffee oil extracted by PLE presented the best results.

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