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From solvent extraction to the concurrent extraction of lipids and proteins from green coffee: An eco-friendly approach to improve process feasibility

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ABSTRACT

The production of green coffee oil by mechanical pressing of green coffee beans has been precluded by low extraction yields, which generates a protein-rich byproduct (cake) containing variable amounts of lipids. Subsequent utilization of the cake requires the removal of the residual cake oil by solvent extraction. An eco-friendly extraction strategy, using water, enzymes, and mechanical treatments, was evaluated to concurrently extract lipids and proteins from green coffee flour, without the use of harsh solvents. Among the enzymatic treatments evaluated, the use of 0.5% alkaline protease led to higher protein (62.2%) and oil (47.7%) extractability in a shorter time (30 min). This enzymatic treatment was optimized with respect to solids-to-liquid ratio (SLR) (1:17.5–1:7) and concentration of enzyme (0.1–0.9% w/w). Although optimum extraction conditions (1:17.5 SLR and 0.1% enzyme) achieved high protein (70%) and oil (48%) extractability and reduced enzyme use by 80%, a higher water usage was required. Therefore, a two-stage countercurrent extraction was developed to reduce water usage in the process. The countercurrent extraction strategy not only reduced the amount of water used in the process by 60% but promoted higher protein (72%) and oil (58%) extractability, compared with the single-stage process (62.2 and 47.7%, respectively).

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1. Introduction

Coffee beans are a well-known commodity with a significant impact on the world economy. According to the International Coffee Organization (ICO), the world coffee production in 2018/2019 was 170.2 million bags (~10.2 million tons) (International Coffee Organization, 2019). Coffee beans are primarily used to produce coffee, a brewed beverage from roasted and ground beans that ranks among the three most popular beverages (alongside water and tea) (Van den Brandt, 2018). However, the unique composition of coffee beans, which includes the presence

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of several health-promoting compounds (i.e., alkaloids, phenolic acids, diterpenes, tannins, lipids, vitamin precursors, among others) (Gaascht et al., 2015), has attracted growing interest of the pharmaceutical and cosmetic industries (Hussein, 2020; Oliveira et al., 2006).

Coffee beans contain approximately 17% lipids, 11% proteins, 60% carbohydrates, 4.2% minerals, besides minor compounds such as caffeine (1.3%), trigonelline (2.0%), diterpenes (1.2%), and chlorogenic acids (up to 7.9%) (Farah, 2012). The oil extracted from green coffee beans is known as green coffee oil (GCO) and is considered a natural source of several bioactive compounds (e.g. diterpene esters, fatty acids, unsaponifiable matter) with desirable biological properties such as antioxidant and cancer-preventive properties (Hussein, 2020). Moreover, numerous skin benefits have been attributed to GCO, such as protection from solar radiation; aid in the regeneration/restructuring of the corneal layer lipids and the protective lipids responsible for skin hydration; and greater lubrication properties (Barrera-Arellano et al., 2005; Esquivel and Jiménez, 2012; Ferrari et al., 2010; Wagemaker et al., 2011). Such benefits have been attributed to the presence of essential fatty acids, unsaponifiable materials, and compounds such as sterols, alkaloids, tocopherols, carotenes, triterpene alcohols, as well as linoleic fatty acids (omega 6) (Barrera-Arellano et al., 2005; Esquivel and Jiménez, 2012; Pereda et al., 2008; Speer and Kölling-Speer, 2006)

Because extraction conditions might have a strong impact on the physicochemical properties of the extracted oil, green coffee oil is commonly extracted by mechanical expression of unroasted green coffee at low temperatures (Çakaloğlu et al., 2018; Hussein, 2020; Turatti, 2001). While the lack of use of flammable solvents and the use of low temperature unquestionably benefit the environment and the composition/stability of the extracted oil, low extraction yields are often achieved by this strategy compared with solvent extraction (Uitterhaegen and Evon, 2017).

Supercritical CO_2 has been employed for recovering oil from green coffee beans (Cornelio-Santiago et al., 2017). Although this technique has achieved good oil extraction yields (5.95–7.60 g oil/100 g coffee) with improved oil quality (Cornelio-Santiago et al., 2017; Baldino et al., 2021), it still presents some limitations due to the complexity of the equipment that operates at elevated pressures and the high power consumption that increases operation costs (Khawli et al., 2019).

There is limited information in the literature with respect to oil extraction yields from coffee beans by mechanical expression, especially for unroasted coffee beans. Industrially, to obtain 2 kg of oil by cold pressing, 60 kg of green coffee beans are required (Almeida, 2015), which demonstrates the low process yield (~3.3%, based on the weight of coffee beans and ~20% based on the amount of oil in the coffee beans) and the consequent production of a byproduct (cake) that still contains a significant amount of lipids (~ 8%) (Mayanga-Torres et al., 2017). On the other hand, higher extraction yields (~10.5% based on the amount of starting material) have been reported for roasted coffee beans, which may facilitate the cell wall disruption and release of oil during the mechanical pressing of the beans. However, protein denaturation and degradation of bioactive compounds have been reported (Turatti, 2001). The low extraction yields achieved by mechanical pressing lead to the need to further extracting the residual oil in the cake with neurotoxic solvents (e.g. hexane), a practice that has raised several environmental and health concerns (de Souza et al., 2020a).

Aqueous-(AEP) and enzymatic-aqueous extraction processes (EAEP) are eco-friendly extraction approaches that have the potential to address the low oil extractability from the mechanical pressing of green coffee without the use of neurotoxic and flammable solvents. Additional advantages of these processes include ease of scalability, reduced processing costs, and short operation time, which in turn lead to reduced energy consumption and CO_2 emissions. Besides, the possibility of enzyme recycling can significantly reduce processing costs (Chemat et al., 2020, 2019). The reduced environmental impact of AEP and EAEP could help meet the sustainable goals of the chemical and food industries.

AEP/EAEP can also benefit from the use of upstream mechanical treatments to break down the food matrix and facilitate the release of the intracellular compounds, water, and if needed, enzymes. This approach brings a unique opportunity, which is the concurrent extraction of lipids and proteins from the matrix (De Moura et al., 2011a) with reduced effluent production, in agreement with the bio-refinery concept (Chemat et al., 2019). In the AEP, solubilization and diffusion of proteins in the medium result in a more porous structure that favors the "washing" of the lipids into the aqueous medium. The use of enzymes in the EAEP can further enhance the extraction of lipids and proteins by either disrupting the cell wall integrity (carbohydrases), hydrolyzing proteins into more soluble peptides (proteases), and/or hydrolyzing the oleosin membrane surrounding the lipid bodies (proteases) (De Moura and Johnson, 2009; Rosenthal et al., 2001; Souza et al., 2019).

Common challenges associated with the AEP/EAEP are related to maximizing the extractability of both lipids and proteins; to recover the extracted oil which although extracted, is commonly entrapped in an emulsion that needs to be broken down to free the lipids for subsequent utilization, and to reduce process water usage without reducing extraction yields (De Moura et al., 2011; De Moura and Johnson, 2009; Dias et al., 2020). While low solids-to-liquid ratios (SLR, ~1:10) have been successfully used to achieve high extractability of lipids and proteins from soybeans (De Moura et al., 2009) and almonds (Almeida et al., 2019; Souza et al., 2019), high extractability comes at the expense of a high water usage that leads to the production of a high volume of a slurry that needs to be centrifuged to separate the lipid (cream) and protein-rich fractions (skim) from the spent solids (insoluble) (De Moura and Johnson, 2009). The use of multistage countercurrent extraction approaches has been shown to be effective to minimize water usage without compromising extraction yields. Therefore, this approach can be used to reduce the amount of slurry that needs to be centrifuged to separate the extracted compounds from the solids (De Moura and Johnson, 2009).

Although this environmentally friendly extraction approach (AEP/EAEP) has been widely used to extract lipids and proteins from several food matrices such as soybeans (De Moura Bell et al., 2013; De Moura et al., 2009, 2011; De Moura and Johnson, 2009), rapeseed (Huang et al., 2012; Sari et al., 2013), peanuts (Jiang et al., 2010; Li et al., 2016), and almonds (Almeida et al., 2019; Dias et al., 2020; Souza et al., 2019), its application to green coffee beans has not been assessed. There are no studies in the literature describing the effects of aqueous and enzyme-assisted extraction parameters on the extractability of lipids and proteins from green coffee beans.

Because the extraction conditions employed strongly impact extraction yields and might lead to structural modifications in the extracted compounds that can alter their functional properties, the present study was undertaken to assess the effects of type and amount of enzyme, solids-to-liquid ratio (SLR), and reaction time on the extractability of lipids and proteins and on the physicochemical properties of the extracted protein from unroasted green coffee flour. Importantly, we determined the efficiency of a multi-stage countercurrent extraction process to decrease water usage in the process without loss in extractability. The present work evaluated (i) the effects of different enzymatic treatments on oil and protein extractability and their impact on the solubility and physicochemical properties of green coffee flour protein extracts; (ii) selected the best enzymatic treatment and further optimized important extraction parameters (solids-to-liquid ratio and enzyme concentration), and (iii) developed a two-stage countercurrent extraction to reduce water usage in the process without loss in extraction efficiency.

2. Material and methods

2.1. *Raw material*

Brazilian arabica green coffee beans were acquired from Brazilian Cerrado (Genuine Origin, USA). Four commercial enzymes were evaluated in the enzyme-assisted aqueous extraction process: (i) Alkaline protease (AP), an endoprotease from *Bacillus licheniformis* (625,034 DU/g, optimum conditions: pH 7.5–10.5, 40–70 °C), was provided by Danisco (Rochester, NY, USA); (ii) Neutral Protease 2 million (NP), a bacterial

neutral endoprotease from *Bacillus subtilis* (2,000,000 PC/g, optimum conditions pH 5.5–9.0, 30−70 °C); (iii) cellulase (C) from *Trichoderrma reesei*, with multiple cellulolytic activities (endo and exo-cellulase, β-glucosidase, β-glucanase, hemicellulose, pectinase, and xylanase) (200,000 CU/g, optimum conditions: pH 4.0–6.5, 45−70 °C); and (iv) hemicellulase (H) from *Aspergillus Niger* (600,000 HCU/g, optimum conditions: 2.0–8.0, 25−90 °C) were kindly provided by BIO-CAT (Troy, VA, USA). The other chemicals used were of analytical grade.

2.2. *Green coffee milling*

Green coffee beans were ground in a Vitamix blender (VM0103, Cleveland, OH, USA) at maximum speed and the flour was subsequently sieved in an 850 μm sieve (# 20), with a minimum recovery of 75%. The coffee flour contained 9.4% lipids, 12.7% proteins, and 5.3% moisture, which were determined according to the methods described in Section 2.3.

2.3. *Proximate analysis and amino acid composition*

The starting material (coffee flour) and all fractions generated by the extraction process (cream — lipid-rich fraction, insolubles — spent solids, and skim — protein-rich fraction) were analyzed for dry matter, oil, and protein contents. Solids, oil, and protein content were measured according to the AOCS method 925.09, AOCS method 989.05 (AOAC, 1990), and Dumas combustion method (Vario MAX cube, Elementar Analysensysteme GmbH, Langenselbold, Germany), respectively. The amino acid composition was determined by ion-exchange chromatography (Biochrom 30 Amino Acid Analyser, Cambourne, Cambridge, UK) in the Amino acid laboratory, UC Davis. The protein conversion factor (5.24) was determined according to the amino acid composition of the flour (Supplementary material — Table S1).

2.4. *Enzyme screening for the single-stage extraction of green coffee flour*

The use of enzymes with different specificities to assist the simultaneous extraction of lipids and proteins from green coffee flour (EAEP) was evaluated (Fig. 1A). The aqueous extraction process (AEP), without the addition of enzyme, was used as the control. Overall, extractions were carried out by dispersing 50 g of green coffee flour into 500 mL of water (1:10 solids-to-liquid ratio, SLR). Reaction time and slurry pH varied according to the process (AEP or EAEP). For the AEP (without enzyme use), extractions were performed at pH 7.0 and 9.0. The slurry was kept at 50 °C under a constant stirring of 120 rpm for 60 min.

For the EAEP, four commercial enzymes were evaluated alone or in combination: alkaline protease (AP), neutral protease (NP), cellulase (C), and hemicellulase (H). The effectiveness of using a pre-treatment with carbohydrases, prior to the use of proteases, was evaluated. For the EAEP, 0.5% of enzyme (weight of enzyme/weight of flour, w/w) was added to the slurry, determining the selection of the slurry pH. The following enzyme combinations were evaluated: (i) 0.5% NP at pH 7.0 for 30 and 60 min; (ii) 0.5% AP at pH 9.0 for 30 and 60 min; (iii) (0.25% C + 0.25% H) at pH 5.6 for 30 min followed by the addition of 0.5% NP at pH 7.0 for 30 min; and (iv) (0.25% C + 0.25% H) at pH 5.6 for 30 min followed by the addition of 0.5% AP at pH 9.0 for 30 min (Fig. 1A). All reactions were performed at 50 °C. Extraction conditions for each enzyme were selected based on the manufacturer's recommendations.

After the extraction, the slurry was centrifuged (4000×*g*, 30 min, 4 °C; Beckman Coulter, Allegra X-14 R, Brea, CA, USA) to remove the insoluble fraction from the liquid fraction (cream + skim) (Fig. 1A). The latter was transferred to a separatory funnel and refrigerated at 4 °C overnight to separate the lipid-rich cream from the protein-rich skim (Fig. 1A). The starting material (coffee flour) and the fractions obtained (cream, insoluble, and skim) were analyzed for oil and protein contents. The distribution of extracted lipids and proteins in each fraction (cream, skim, and insoluble) was calculated according to Eqs. (1) and (2), respectively. Total oil (TOE) and total protein (TPE) extraction yields were also determined as described in Eqs. (3)–(4), respectively. AEP and EAEP were carried out in triplicates.

$$\text{Oil in the fractions } (\%) = \left(\frac{\text{Oil in the fraction (g)}}{\text{Oil in the coffee flour (g)}}\right) \times 100\% \quad (1)$$

$$\text{Protein in the fractions } (\%) = \left(\frac{\text{Protein in the fraction (g)}}{\text{Protein in the coffee flour (g)}}\right) \times 100\% \quad (2)$$

$$\text{TOE } (\%) = \left[100 - \left(\frac{\text{Oil in the insoluble fraction (g)}}{\text{Oil in the coffee flour (g)}}\right)\right] \times 100\% \quad (3)$$

$$\text{TPE } (\%) = \left[100 - \left(\frac{\text{Protein in the insoluble fraction (g)}}{\text{Protein in the coffee flour (g)}}\right)\right] \times 100\% \quad (4)$$

2.5. *Tailoring enzyme use and solids-to-liquid ratio in the single-stage EAEP*

Upon the selection of the enzyme resulting in higher oil and protein extractability (alkaline protease, AP), the individual and simultaneous eff ;ects of SLR (1:17.5−1:7) and concentration of enzyme (0.1−0.9%, w/w) were evaluated to better elucidate the extraction mechanisms of lipids and proteins from coffee flour and to identify optimum extraction conditions for increased extractability in the single-stage EAEP (Table 2). To accomplish this goal, a central composite rotatable design (CCRD) with two independent variables, four axial points (α = ±1.41, obtained by interpolation), and three repetitions in the central point (average of levels −1 and +1) was used (Table 2). Optimum experimental conditions suggested by the regression models were validated in triplicate and the relative error between experimental and predicted values was calculated.

2.6. *Effects of extraction methods on the physicochemical and functional properties of green coffee proteins*

2.6.1. *Color analysis*

The color of the skim fractions obtained from the enzyme screening (Section 2.4) was measured using a ColorFlex spectrophotometer (Hunter Lab ColorFlex 45/0, CX2478, Reston,

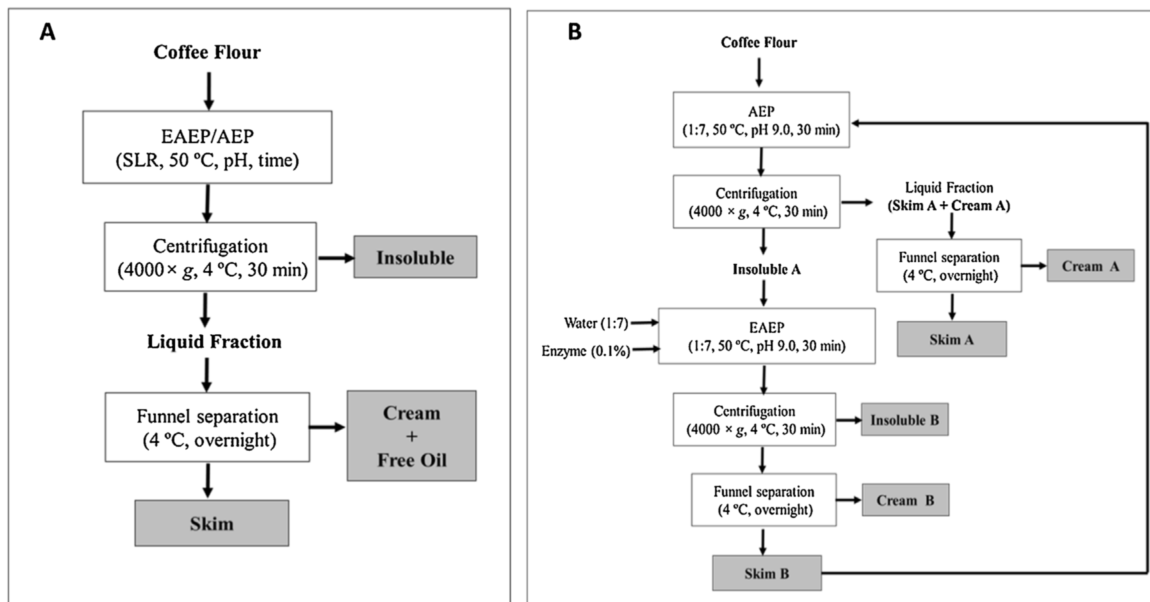

Fig. 1 – Process flow diagram for the single-stage AEP and EAEP (A) and two-stage countercurrent EAEP (B).

VA, USA), with reflectance mode and CIELab scale. The colorimeter uses as a reference system D65 as illuminant and a 10 ° observer angle. The parameters L* (lightness), a*, and b* (chromaticity parameters) were recorded at least in triplicate.

2.6.2. *Molecular weight distribution of green coffee proteins*

The molecular weight distribution of AEP and EAEP skim proteins obtained in the enzyme screening (Section 2.4) was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein separation was carried out onto a precast gel (12% acrylamide, CriterionTM TGX Precast Gels, Bio-Rad, Hercules, CA, USA), where each well was loaded with 30 μg of protein. Samples consisted of a 1:1 v/v mixture of skim and Laemmli solution containing β-mercaptoethanol (Bio-Rad, Hercules, CA, USA). Samples were vortexed and denatured at 95 °C for 5 min (Laemmli, 1970). Electrophoretic separation of proteins was performed with the use of a Tris–HCl running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) (Bio-Rad, Hercules, CA, USA) at 200 V, and room temperature for 1 h. A low molecular range (14.4–97.4 kDa) weight standard was used as a protein marker (Bio-Rad, Hercules, CA, USA). The relative abundance and distribution of proteins were evaluated using a Gel DOCTM EZ Imager system and Image Lab software (Bio-Rad, Hercules, CA, USA).

2.6.3. *Solubility of green coffee proteins*

AEP and EAEP skim protein solubility was measured as previously described (Rickert et al., 2004), with some modifications. At first, freeze-dried skims (Labconco, Kansas, Missouri, USA) were dispersed in deionized water to achieve a 1% solution (w/v). The pH of a 10 mL 1% (w/v) dispersion was adjusted to 4.0 and 9.0 using 0.5 N HCl or 0.5 N NaOH solutions. The dispersions were stirred at 150 rpm for 1 h, and then centrifuged at 10,000×*g* for 10 min at 20 °C. The protein content of the freeze-dried powders and the supernatant was measured using the Dumas combustion method (Section 2.3, nitrogen conversion factor of 5.24). Skim samples were analyzed in triplicate for each pH. Solubility (%) was calculated as follows (Eq. (5)):

$$\text{Solubility}\ (\%) = \frac{\text{Protein in the supernatant at the different pH values}}{\text{Total protein content from freeze dried skims at natural pH}} \times 100 \quad (5)$$

2.6.4. *Surface hydrophobicity (H_0) of green coffee proteins*

Protein surface hydrophobicity of the liquid skim fractions was determined using 1-anilino-8-naphthalenesulfonate (ANS) as a fluorescence probe as described by Zhang et al. (2013), with some modifications. Briefly, skim fractions were diluted into 0.01 M sodium phosphate buffer solution (pH 7.0) to achieve protein concentrations from 0.022 to 0.22 mg mL^{-1}. Then, 1.25 μL of ANS (8.0 mM in 0.01 M phosphate buffer, pH 7.0, solution) was added to 250 μL of skim into a 96-Well plate. A SpectraMax iD5 Multi-Mode Microplate Readers (Molecular Devices, San Jose, California, USA) was used to measure fluorescence intensity at an excitation wavelength of 390 nm and an emission wavelength of 470 nm (both with a slit width of 5 nm). Protein hydrophobicity was calculated as the slope of the fluorescence intensity vs. protein concentration plot by linear regression analysis. Measurements were replicated six times for each sample.

2.6.5. *Determination of the degree of protein hydrolysis (DH)*

The o-phthaldialdehyde (OPA) method was used to determine the degree of protein hydrolysis (Nielsen et al.,2001). The standard solution consisted of a 0.9516 meqv/L L-serine solution and distilled water was used as the reaction control. A sample background was also prepared by replacing the OPA reagent with distilled water to eliminate potential color interference of the samples. Proteins were quantified by using the Dumas combustion method (Section 2.3, nitrogen conversion factor of 5.24). The following equations were used to calculate the degree of hydrolysis (Eqs. (6)–(7)):

$$h = \frac{\text{Serine}\,NH_2 - \beta}{\alpha} \quad (6)$$

$$DH\ (\%) = 100 \times \frac{h}{h_{total}} \quad (7)$$

where α, β and h_{tot} values correspond to 1.0, 0.4 (Nielsen et al., 2001), and 7.84 (calculated based on the amino acid composition of coffee beans), respectively.

2.7. *Development of a two-stage countercurrent enzyme-assisted extraction process (EAEP)*

To overcome the loss in extractability that often arises when the amount of water used during the extraction is reduced, a two-stage countercurrent extraction was developed (Fig. 1B) and compared with the single-stage EAEP of green coffee flour. Each two-stage countercurrent EAEP run was performed sequentially with three fresh samples of coffee flour. Each coffee flour sample was subjected to two extractions in batch mode, with the skim obtained from the second extraction being recycled to the next first extraction, where fresh incoming coffee flour was used (Fig. 1B).

For the first extraction, coffee flour was extracted through the AEP (without enzyme addition) at 50 °C, pH 9.0, 1:7 solids-to-liquid ratio (SLR), under constant stirring at 120 rpm for 30 min. Afterward, the slurry was centrifuged at 4000 × *g* for 30 min at 4 °C to separate the insoluble fraction (Insoluble A) (Fig. 1B) from the liquid phase, which was subsequently separated into skim A and cream A by the use of a separatory funnel. The insoluble fraction obtained from the first extraction (Insoluble A) was then subjected to a second extraction, with the addition of fresh water and enzyme. Insoluble A was dispersed into water to achieve 1:7 SLR and the dispersion pH was adjusted to 9.0. Alkaline protease was added at a concentration of 0.1% enzyme (weight of enzyme/weight of flour, w/w) and the mixture was stirred at 120 rpm for 1 h at 50 °C. Following the second extraction, the slurry was centrifuged to separate the second insoluble (Insoluble B) from the liquid fraction, which was separated into cream B and skim B. The skim B was recycled to the next first extraction, where the second batch of fresh coffee flour was used. The two-stage extraction above described was repeated for the second and third sample of fresh coffee flour, thus representing a complete two-stage countercurrent run. Only fractions (insoluble, skim, cream) obtained from the extraction of the third sample, after enzyme had been adequately recycled in both extractions, were analyzed and used to calculate oil and protein mass balances according to equations previously described (Eqs. (1)–(4)).

2.8. *Statistical analyses*

Extraction, physicochemical, and functional measurements were performed at least in triplicate and the results were expressed as the mean ± standard deviation (SD) of the replicates. Analysis of variance (ANOVA) was followed by Tukey tests to identify significant differences within the measurements (Statistica® version 13.3, TIBCO Software Inc., Palo Alto, CA, USA) at the level of confidence of $p < 0.05$. The experimental design (R^2 and the F test to assess the regression significance) was evaluated by the Protimiza Experimental Design Software (http://experimentaldesign.protimiza.com.br).

3. Results and discussion

3.1. *Effectiveness of enzymatic hydrolysis in the single-stage EAEP of green coffee flour*

The use of enzymes to assist the extraction of several food matrices is an efficient strategy to increase the overall extractability of lipids and proteins as well as to produce protein extracts with unique functional and biological properties (Almeida et al., 2019; De Moura et al., 2009; de Souza et al., 2020b; Dias et al., 2020). To identify the most effective enzymatic strategy in the EAEP of green coffee flour, the use of proteases, alone or following the use of carbohydrases, was evaluated with respect to oil and protein extractability and protein functionality (Fig. 2). Despite the extraction methods employed (AEP or EAEP), protein and oil extraction yields above 55 and 43% were achieved in the single-extraction process, respectively (Fig. 2A and B).

Enzyme use and pH selection had a significant effect on protein extractability (Fig. 2A). When not using enzyme (AEP), protein extraction yields increased from 55.1 to 59.6% when the slurry pH increased from 7.0 to 9.0. Although the comparison of our results with the literature is difficult because of the lack of studies using green coffee, the results presented herein are in agreement with the literature, in which alkaline conditions increase protein extractability due to greater biomass cell wall degradation (Jarpa-Parra et al., 2014; Sari et al., 2013).

The use of an alkaline protease (AP) or a neutral protease (NP) led to a small but significant increase ($p < 0.05$) in protein extractability (higher TPE) compared to the control (AEP). Protein extraction yields increased from 59.6% (AEP at pH 9.0) to 62.2% and 61.3% when AP was used at 30 and 60 min, respectively, and from 55.1 (AEP at pH 7.0) to 59.2% and 61.1% when enzyme NP was used at 30 and 60 min, respectively. As observed in Fig. 2A, alkaline protease AP achieved similar protein extractability to ones observed with the use of neutral protease NP, but at shorter reaction times (30 min instead of 60 min). The use of a mixture of cellulase + hemicellulose (C + H) before the use of proteases (NP or AP), resulted in a minimum or no significant increase in protein extractability (Fig. 2A) when compared with the use of proteases alone. The use of C + H (30 min) followed by NP (30 min) increased protein extractability from 59.2 to 61.1% when using NP alone at 30 min, but was not higher when compared with NP alone at 60 min (61.1%). It is well known that the use of carbohydrases and proteases promotes protein extractability by hydrolysis of the plant cell wall or proteins, respectively (Jung et al., 2006). In the first case, an increase in extraction yields is associated with the breakdown of the plant cell wall, which makes the intracellular content available for extraction (Rosset et al., 2014; Vergara-Barberán et al., 2015). Proteases, conversely, partially hydrolyze the proteins and oleosins (structural proteins of plant oil bodies), causing a reduction in molecular size and, consequently, increasing the solubility and dispersion of protein hydrolysates and oil in the medium (Pojić et al., 2018; Sari et al., 2013). Fig. 3 shows the potential extraction mechanisms involved in the aqueous and enzyme-assisted extraction processes in the present work.

Similar protein yields were observed among the skim fractions generated by the AEP (pH 9.0) and EAEP, except when using pretreatment with cellulase + hemicellulase (C + H) followed by alkaline protease (AP). Although the use of C+H

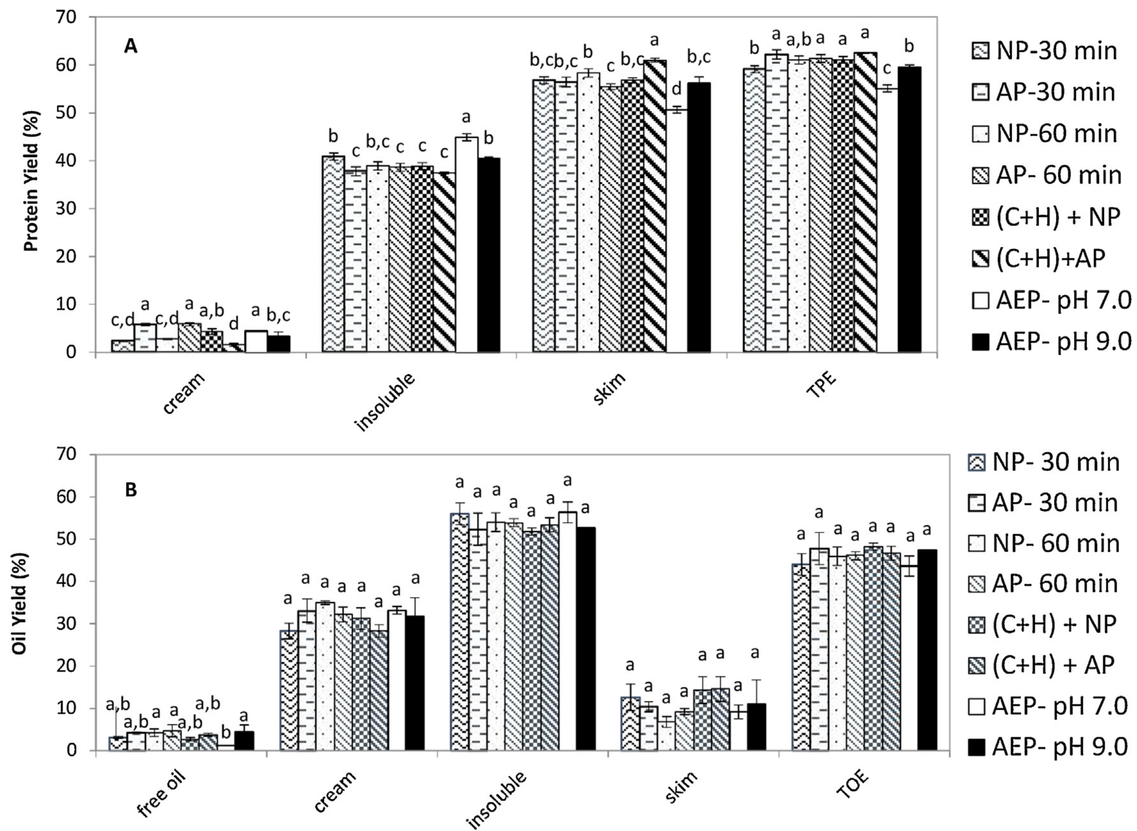

Fig. 2 – Protein (A) and oil (B) distribution among the fractions and total extraction yields when using different enzymes and pH values, at 50 °C and 1:10 solids-to-liquid ratio, in the single-stage AEP and EAEP.

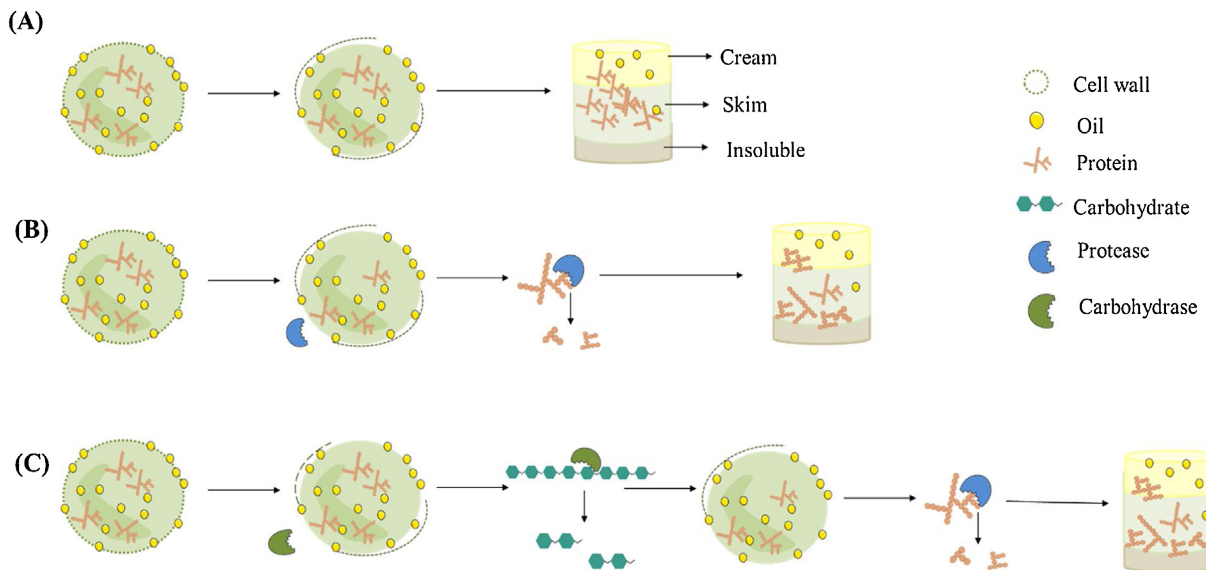

Fig. 3 – Schematic diagram showing the extraction mechanisms of the aqueous extraction process (A), enzyme-assisted extraction process with protease (B), and enzyme-assisted extraction process with a carbohydrase pretreatment followed by extraction with protease (C).

followed by AP did not increase overall protein extractability (1.0% enzyme use), compared with the other enzymatic strategies that had reduced enzyme use (0.5%), a higher protein yield (61%) was observed in the skim generated by this strategy, leading to a reduced amount of protein in the cream. Although not the scope of this work, the amount and type of protein in the cream fraction can significantly impact the release and subsequent utilization of the oil entrapped in the cream emulsion (Dias et al., 2020).

Oil extractability and distribution of the extracted oil in the fractions were not statistically different for the AEP and EAEP (Fig. 2B). Moreover, a suitable distribution of the extracted components (more oil in the cream or preferably in the free oil fraction, and more proteins in the skim) was observed. Based on the amount of oil and protein extracted, more than 65% of the extracted oil is present in the cream and free oil fractions, while more than 90% of the extracted protein is present in the skim fraction. Such distribution profile is important to maximize the recovery of the extracted oil from the cream and

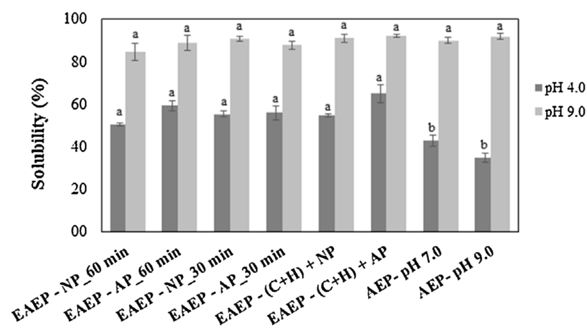

Fig. 4 – Solubility of green coffee proteins at pH 4.0 and 9.0 for different extraction conditions. Different letters within the same pH indicate statistical differences ($p < 0.05$).

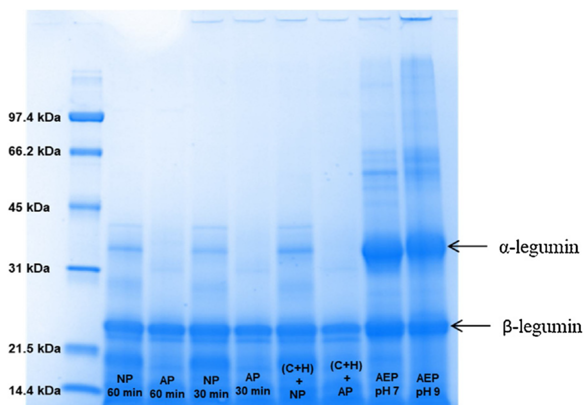

Fig. 5 – SDS-PAGE gel electrophoresis for AEP and EAEP proteins for different extraction conditions. NP-60 min (0.5% neutral protease at pH 7.0 for 60 min and 50 °C); AP-60 min (0.5% alkaline protease at pH 9.0 for 60 min and 50 °C); NP-30 min (0.5% neutral protease at pH 7.0 for 30 min and 50 °C); AP-30 min (0.5% alkaline protease at pH 9.0 for 30 min and 50 °C); (C+H) + NP (0.25% cellulose and 0.25% hemicellulase at pH 5.6 for 30 min followed by the addition of 0.5% NP at pH 7.0 for 30 min); (C+H) + AP (0.25% cellulose and 0.25% hemicellulase at pH 5.6 for 30 min followed by the addition of 0.5% AP at pH 9.0 for 30 min).

to produce skim proteins with reduced oil content. The latter could favor protein solubility, thus opening up a wide range of potential applications for the extracted protein (Souza et al., 2019).

Because of the lack of information about the simultaneous extraction of lipids and proteins from coffee beans by the AEP and EAEP, the comparison of our data with the literature becomes challenging. However, previous studies using the EAEP for oil-bearing materials such as almonds and peanuts have showed increased extractability of lipids and proteins when using enzymes to assist the extraction. The use of protease in the EAEP increased almond oil and protein extractability from 48.2 to 50% and 70 to 75%, respectively (Souza et al., 2019). For peanuts, an improvement from 30.6 to 79.3% and from 68.5 to 71.4% was achieved for oil and protein extractability in the EAEP, respectively (Jiang et al., 2010).

Considering the impact of extraction time and amount of enzyme on protein and oil extraction yields, our results indicate that high extractability can be achieved by the use of 0.5% of AP (62.23% protein extraction and 47.7% oil extraction) or NP (59.17% protein extraction and 44% oil extraction) at shorter extraction time (30 min) (Fig. 2A). However, in addition to high extractability, the impact of the enzyme used to assist the extraction on the physicochemical properties of the extracted protein, which might significantly alter its functionality, should also be considered during an enzyme screening.

3.2. *Effects of the extraction methods on the physicochemical properties of the skim proteins*

Considering that enzymatic hydrolysis can also affect protein functionality, skim proteins were characterized with respect to protein solubility (Fig. 4), molecular weight (Fig. 5), degree of hydrolysis, surface hydrophobicity, and color (Table 1).

3.2.1. *Surface hydrophobicity of green coffee proteins*

Protein surface hydrophobicity decreased from 1205 to 693 when the slurry pH increased from 7.0 to 9.0 in the absence of enzymes (AEP treatments) (Table 1). Protein surface hydrophobicity (H_0) is related to the tendency of protein molecules to aggregate (Wagner et al., 2000), so a higher H_0 may decrease protein solubility, thus resulting in low extraction yields. Higher protein extractability at pH 9.0 compared with pH 7.0 could be attributed to the presence of more hydroxyl groups and unfolding of the protein structure, which could promote higher electrostatic repulsion between protein molecules and higher affinity with water (Jarpa-Parra et al., 2014); and could thus explain the reduced hydrophobicity at pH 9.0.

Protein hydrolysis can promote a decrease in molecular weight, an increase in the number of ionizable groups, besides exposing or hiding hydrophobic groups. Thus, it can change the physicochemical properties of the protein and their environmental interactions, the extent of which depending on the type of enzyme chosen and reaction conditions (Tavano, 2013). As described in Table 1, protein surface hydrophobicity was significantly affected by the type of enzyme and reaction pH. At pH 7.0, protein surface hydrophobicity was reduced from 1205 to 1073–978 when neutral protease NP was used during the extraction (30–60 min). However, the use of carbohydrases (C+H) followed by neutral protease (NP) did not change protein surface hydrophobicity in relation to the control (AEP-pH 7.0). Yet, protein surface hydrophobicity increased from 693 to 871–895 when alkaline protease was used during the extraction compared to the AEP-pH 9.0. A further increase in surface hydrophobicity was observed by the use of carbohydrases (C+H) followed by alkaline protease (1195). Overall, surface protein hydrophobicity was higher for proteins extracted by neutral protease compared with the ones extracted by alkaline protease (Table 1). Considering that surface hydrophobicity is related to protein unfolding during denaturation and to the level of hydrophobic amino acids exposed at the protein surface (Zayas, 1997), different enzymes will likely generate different hydrolysates (i.e., exposing distinct hydrophobic sites on the protein surface) according to each enzyme specificity.

Moreover, it was noted that increased extraction time (30 vs. 60 min) did not significantly affect protein hydrophobicity for both proteases, which indicates that no significant additional changes occurred in protein structure after 30 min. Such observation also agrees with the degree of hydrolysis of the proteins (Table 1) and extractability results (Fig. 2A), where no significant changes in protein extraction yields were observed after 30 min.

3.2.2. *Solubility of green coffee proteins*

Protein solubility is a critical functional property due to its influence on other properties such as emulsification, gelation,

Table 1 – Physicochemical properties of the AEP and EAEP skim proteins from green coffee flour.

Treatments	H_0	DH	L*	a*	b*
NP-30 min	1073.4 ± 66.9^{b}	$18.1 \pm 3.9^{b,c}$	$3.02 \pm 0.58^{b,d}$	-2.62 ± 0.76^{a}	1.29 ± 0.35^{d}
AP-30 min	$894.6 \pm 29.2^{c,d}$	13.5 ± 2.7^{c}	1.84 ± 0.14^{d}	-2.43 ± 0.30^{a}	$1.41 \pm 0.22^{c,d}$
NP-60 min	$978.0 \pm 51.8^{b,c}$	22.4 ± 2.9^{b}	$2.91 \pm 0.15^{b,d}$	-2.82 ± 0.25^{a}	$1.39 \pm 0.19^{c,d}$
AP-60 min	870.8 ± 39.2^{d}	12.4 ± 1.2^{c}	2.38 ± 0.25^{d}	$-3.09 \pm 0.59^{a,b}$	$1.79 \pm 0.31^{c,d}$
(C+H) + NP	1194.6 ± 36.8^{a}	31.1 ± 3.4^{a}	4.72 ± 0.33^{b}	-4.37 ± 0.29^{b}	$2.78 \pm 0.36^{b,c}$
(C+H) + AP	$921.6 \pm 70.8^{c,d}$	$18.2 \pm 1.9^{b,c}$	2.17 ± 0.09^{d}	$-3.19 \pm 0.22^{a,b}$	$1.64 \pm 0.16^{c,d}$
AEP- pH 7.0	1205 ± 90.3^{a}	0.0 ± 0.0^{d}	12.39 ± 0.93^{a}	-8.98 ± 0.43^{d}	5.48 ± 1.25^{a}
AEP- pH 9.0	693.3 ± 32.1^{e}	0.0 ± 0.0^{d}	$3.89 \pm 0.25^{b,c}$	-5.87 ± 0.65^{c}	3.44 ± 0.41^{b}

Different letters in the same columns indicate statistical differences ($p < 0.05$) among samples.

and foaming (Idris et al., 2003) since those properties usually require the protein to be soluble in the relevant medium (Wouters et al., 2016). Solubility is highly related to the amino acid composition, the content of polar and nonpolar groups, as well as molecular weight and conformation (Zayas, 1997). Thus, to understand how AEP and EAEP treatments affect the extracted protein solubility, solubility was measured at pH 4.0 (close to the isoelectric point of green coffee proteins) and pH 9.0 (Fig. 4). Regardless of the extraction pH (7.0 vs. 9.0) and extraction method (AEP vs. EAEP), green coffee protein solubility was higher (> 81%) at alkaline pH (pH 9.0), compared with pH 4.0 (~50%). While alkaline media usually increase protein solubility by causing dissociation and disaggregation of proteins, acidic media are known to promote association between molecules, since attractive forces predominate close to the isoelectric point (pI) of the protein (Zayas, 1997).

Moreover, it was observed that at pH 4.0, enzymatic treatments significantly increased the solubility of the extracted proteins when compared to the control (AEP), for both neutral (from 43 to 51%) and alkaline proteases (from 35 to 56%). These results indicate that EAEP improves the solubility of the extracted protein in acidic media, likely due to the presence of more soluble and smaller peptides (>DH) (Table 1), and increased release of ionizable amino and carboxyl groups (Tavano, 2013). Previous studies also reported similar findings for soy (De Almeida et al., 2014), sunflower (Yust et al., 2003), almond (Souza et al., 2019), and peanut proteins (Jamdar et al., 2010). The higher solubility of the hydrolysates in acidic media is of key importance for the development of food formulations or other applications involving acidic pH.

3.2.3. *Molecular weight distribution and degree of hydrolysis of green coffee proteins*

Protein molecular weight distribution of extracts from the AEP and EAEP was characterized by SDS-PAGE (Fig. 5). Fractions above 45 kDa and two main protein bands around 33.6 kDa (~40.75% of relative abundance) and 23.3 kDa (~19.55% of relative abundance) were observed for proteins obtained from the AEP (no enzyme use) (AEP-pH 7.0 and AEP-pH 9.0). The two main bands could be attributed to the α and β fragments of legumin, the major coffee storage protein under reducing conditions (Acuña et al., 1999) (Fig. 5). Coffee proteins are known for containing 11S storage proteins (Acuña et al., 1999; Montavon et al., 2003), which provide amino acids and nitrogen for seed germination (Montavon et al., 2003). The fragments of legumin have a molecular mass of around 55 kDa in the absence of a reducing agent; however, in the presence of such agent, two polypeptides constituted of subunits α and β appear with molecular masses around 33 and 24 kDa. Such behavior is expected for legumin-like proteins, considering the subunit of 11S legumins contains acidic and basic chains linked via disulfide bonds (Acuña et al., 1999).

DH (Table 1) and SDS-PAGE (Fig. 5) demonstrated that the use of enzymes to assist the extraction led to a higher degree of protein hydrolysis and the formation of smaller peptides. While neutral protease treatments hydrolyzed the protein bands with MW above 45 kDa, in addition to the partial hydrolysis of the 33 kDa band (α unit of coffee legumin), alkaline protease completely hydrolyzed such bands previously present in both controls (AEP-pH 7.0 and AEP-pH 9.0). Such differences illustrate the distinct proteolytic activities of both proteases on coffee proteins, which might lead to changes in protein characteristics (e.g. different H_0) and functionality considering the different sizes and types of peptides produced by each treatment. The formation of smaller peptides for EAEP treatments, for example, resulted in increased protein solubility as observed at pH 4.0 (Fig. 4) and in changes in the EAEP protein hydrophobicity when compared to the AEP (Table 1). Our results are in agreement with the literature, where the exploitation of different enzymatic treatments (Flavourzyme 1000 L, Novozym FM 2.0 L, and Alcalase 2.4 L FG) has shown to produce soy hydrolysates with different functionalities and amino acid composition due to each enzyme specificity (Hrčková et al., 2002).

3.2.4. *Color analysis*

Color is an important parameter that can affect the application of the extracted protein in the final product and, therefore, was evaluated (Table 1). All skim fractions showed a greenish color (-a*) and both the addition of enzymes and extraction pH affected the color parameters of the skims (Table 1). Enzyme-assisted extractions contributed to the darkening of the skim (<L*), reduced the green (>a*) and the yellow values (<b*). Neutral extraction pH (pH 7.0), on the other hand, resulted in the production of lighter skims (>L*), with increased green (<a*) and yellow (>b*) colors. Such differences promoted by AEP and EAEP on color parameters can be explained due to the extraction/release of compounds in the medium and/or degradation of carotenoids pigments (e.g. chlorophylls and lycopene) and anthocyanins at different extents (Vieira, 2015).

Considering that chlorophylls are susceptible to chlorophyllase degradation (Damodaran et al., 2008), the color changes observed after the enzymatic treatments are likely due to the different pH values used during the extraction and release of compounds in the skim fractions such as peptides and carbohydrates after enzymatic activities. Anthocyanins, for example, are natural pigments highly influenced by pH. They present a blue color under alkaline conditions (Jackma and Yada, 1987), which can explain the lower b* values found for the AEP skim (pH 9.0) compared to AEP skim (pH 7.0).

Table 2 – Experimental design for optimizing total oil (TOE) and protein (TPE) extraction yields while varying solids-to-liquid ratio (SLR) and amount of alkaline protease. Extractions were carried out at pH 9.0, for 30 min at 50 °C.

Experiment#	Variables (coded and real values[1])		Responses	
	SLR	Enzyme (%) (w_{enzyme}/w_{flour})	TOE (%)	TPE (%)
1	−1 (1:14.3)	−1 (0.216)	52.40	65.71
2	1 (1:7.7)	−1 (0.216)	34.43	61.14
3	−1 (1:14.3)	1 (0.784)	45.86	68.52
4	1 (1:7.7)	1 (0.784)	38.87	61.25
5	−1.41(1:17.5)	0 (0.500)	39.49	69.48
6	1.41(1:7)	0 (0.500)	39.95	61.79
7	0 (1:10)	−1.41 (0.100)	49.63	65.98
8	0 (1:10)	1.41 (0.900)	47.54	64.77
9	0 (1:10)	0 (0.500)	44.02	65.16
10	0 (1:10)	0 (0.500)	48.19	65.95
11	0 (1:10)	0 (0.500)	45.96	65.91

[1] Real values are presented inside the parenthesis.

Additionally, chlorophylls are well-known for their instability towards lower pH values at high temperatures due to the conversion of chlorophyll to pheophytin and pheophorbide, resulting in changes from bright green to olive-yellow color (Koca et al., 2007). The potential application of green coffee proteins will likely require the development of strategies to mask its green color to avoid rejection by consumers. In that view, further studies to investigate the potential removal of coloring compounds from the protein extracts or strategies to mask their color is warranted.

3.3. Experimental design and validation

Because the use of alkaline protease resulted in higher protein extraction yields in shorter reaction time and considering that TOE, color, and protein solubility were similar to the other enzymatic treatments, the use of alkaline protease AP (30 min) was selected for additional processing optimization. An experimental design was carried out to optimize the eff ;ects of solids-to-liquid ratio (1:17.5−1:7) and amount of enzyme (0.1%–0.9% (w/w)) with respect to the extraction of lipids and proteins from green coffee flour (Table 2). Considering the costs associated with the amount of enzyme used in the process and the centrifugation of high volumes of slurry to separate the extracted compounds, optimization studies are crucial for large-scale applications of the EAEP.

Protein extraction yields varied from 61.1 to 69.5% and were significantly influenced only by solids-to-liquid ratio ($p < 0.05$). According to the predictive model (Y_{TPE} = 65.06 – 2.84*SLR) obtained by regression analysis (Fcal (55.5) > $Ftab_{(1,9)}$ (5.12) and R^2 = 86.04%), maximum extraction yield of 69.5% could be obtained at lowest SLR (1:17.5). Because the amount of enzyme used in the EAEP did not significantly affect protein extractability, within the range studied ($p > 0.05$), the minimum concentration of enzyme (0.1%, w/w) was selected for subsequent experiments. The reduction in the amount of enzyme used from 0.5 to 0.1% represents an 80% reduction in enzyme use in the process. Considering that TPE had a negative correlation with SLR ($p < 0.05$), the most diluted condition (1:17.5 SLR) was used for the validation of the predictive model. Higher protein extractability is usually favored in more diluted systems because protein solubilization and diffusion into the aqueous phase are enhanced (Souza et al., 2019).

Despite the lack of studies detailing the effects of extraction parameters on coffee protein extractability, the use of diluted mediums (lower SLR) has improved the overall extractability of proteins from almond cake (Souza et al., 2019), soybeans (De Moura and Johnson, 2009), and peanuts (Rhee et al., 1972)

Total oil extraction (TOE) varied from 34.4% to 52.4% (Table 2). However, the observed differences in TOE were not statistically significant at 95% of confidence, indicating that neither SLR nor enzyme concentration significantly affected TOE. Therefore, for TOE, each variable could be used in its lowest value (less enzyme and less water).

To validate the optimum extraction conditions to maximize both protein and oil extractability, extractions were performed in triplicate at 1:17.51 SLR and 0.1% (w/w) of enzyme. A control was also performed at the same SLR but in the absence of enzyme (Table 3).

Protein and oil extraction yields of 70 and 48% were achieved during the experimental validation, respectively. Oil extraction yields were within the range observed in the experimental design (34.4–52.4%) and protein extraction yields were in close agreement with the value predicted by the regression model (70 vs. 69%) (Table 3). The low relative error between predicted and observed values (1.57%) indicates that the regression model is accurate and reliable to predict protein extractability of green coffee proteins within the range of parameters evaluated. Moreover, adequate optimization of enzyme use (0.1% of AP) and SLR (1:17) resulted in an 11.7% increase in protein extraction compared to the extraction without enzyme use (AEP, control) (Table 3). These results evidence the effectiveness of using alkaline protease in the EAEP of green coffee. TOE was not significantly affected ($p > 0.05$) by the addition of alkaline protease (0.1%, w/w), in agreement with the experimental design (Table 2). Although processing optimization improved protein extractability when compared to the initial conditions for AP (70.1 vs 65.7%) and reduced the overall use of enzyme in the process by 80%, while maintaining oil extractability constant (48 vs 46%), the selected conditions required the use of more water during the extraction (1:17.5 vs 1:10 SLR). Because the centrifugation of high volumes of effluent is costly, a countercurrent extraction approach was subsequently evaluated to minimize water usage without a reduction in the overall extractability of oil and protein.

3.4. Two-stage countercurrent extraction of green coffee flour

To decrease the amount of water used during the single-stage extraction of oil and protein from green coffee flour, without reducing extraction yields, a two-stage countercurrent extrac-

Table 3 – Total oil (TOE) and protein (TPE) extraction yields for the validation of the predictive model.

	Enzyme (%) (w_{enzyme}/w_{flour})	SLR	TOE (%)	TPE (%)
Control	0	1:17.51	41.52 ± 4.46^a	62.79 ± 1.18^b
Experimental validation (Alkaline protease)	0.1	1:17.51	48.13 ± 3.57^a	70.15 ± 1.32^a
Predicted value (Regression model)	0.1	1:17.51	–[1]	69.06

Different letters in the same columns indicate statistical differences ($p < 0.05$) among samples.
[1] There was no model generated for this response.

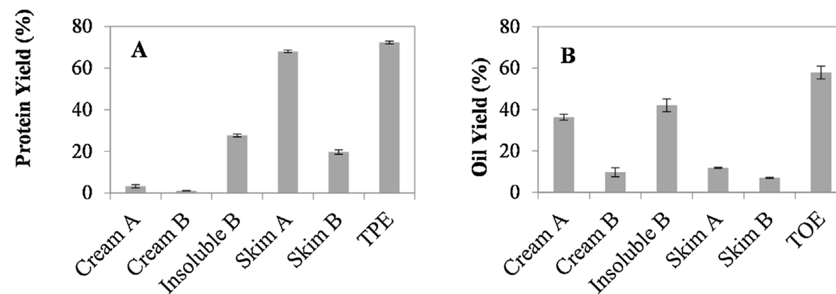

Fig. 6 – Extraction yields and distribution of proteins (A) and oil (B) for the two-stage countercurrent extraction of green coffee flour (0.1% of alkaline protease (w/w), 30 min, 50 °C, and 1:7 SLR). Fractions A and B refer to fractions from the 1st and 2nd extraction stage, respectively.

Table 4 – Comparison of total oil (TOE) and protein (TPE) extraction yields for all extraction conditions evaluated.

Treatments	SLR	Enzyme (%) (w/w)[1]	TOE (%)	TPE (%)
Screening (Initial condition)[2]	1:10	0.5	46.06 ± 2.08^b	65.67 ± 0.45^c
Optimized condition (Validation)	1:17.5	0.1	48.13 ± 3.57^b	70.15 ± 1.32^b
Two-stage countercurrent	1:7	0.1	57.90 ± 3.10^a	72.33 ± 0.70^a

[1] Weight of enzyme/weight of flour.
[2] Central points in the experimental design.

tion was carried out by using previously optimized conditions (0.1% alkaline protease, 30 min, 50 °C); except by the SLR, which was increased from 1:17 to 1:7 (Fig. 6).

The effects of using a two-stage countercurrent extraction process on protein and oil extraction yields from green coffee flour are shown in Fig. 6A and B. By recycling the skim from the second extraction (skim B) into the next first extraction (where it contacted fresh incoming green coffee flour) and by adding fresh water and enzyme only in the second extraction, where it contacted the first insoluble fraction (oil and protein depleted fraction) (Fig. 1B), 72% of the proteins were extracted, from which 94% was present in the skim phase and about 4% was present in the cream fraction (Fig. 6A). Moreover, a total oil extraction yield of 58% was achieved, from which 63 and 17% were extracted in the first and second extraction stages, respectively (Fig. 6B). Despite the high oil extractability achieved, future evaluation of strategies to break down the cream emulsion to release the oil entrapped for further utilization is warranted.

The two-stage countercurrent extraction was effective in reducing the amount of water used in the process without loss in oil and protein extractability compared with the optimized single-stage extraction (Table 4). As a matter of fact, higher extraction yields were achieved by the two-stage countercurrent enzyme-assisted extraction process.

Processing optimization of the single-stage extraction resulted in similar oil extraction yields (48 vs. 46%) and slightly higher protein extraction yields (70 vs. 66%) compared with the screening experiments (Section 3.1). Although the use of enzyme was reduced from 0.5 to 0.1%, higher protein extraction yields in the optimized process were achieved at the expense of higher water usage (1:17 vs. 1:10). Conversely, the two-stage countercurrent extraction process significantly increased TOE yields from 46-48% to 58% and TPE yields from 66–70% to 72% while reducing the amount of water used in the process from 1:17–1:10 to 1:7 (Table 5). The use of a countercurrent extraction strategy was able not only to overcome the commonly observed reduction in extractability when using higher SLR (reduced amount of water) (Table 2) but also increased oil and protein extractability by 26 and 10% compared with the single-stage (initial conditions), respectively (Table 4).

Table 5 – Effects of enzyme recycling on total oil (TOE) and protein (TPE) extraction yields for the two-stage countercurrent enzyme-assisted aqueous extraction of coffee flour.

	%TOE	%TPE
Insoluble-B1	55.65 ± 1.87	75.76 ± 0.81
Insoluble-B2	53.62 ± 0.79	74.46 ± 0.90
Insoluble-B3	55.72 ± 1.24	75.03 ± 0.18

Our results are in agreement with previous studies that reported higher oil and protein extraction yields when using a two-stage EAEP for soybeans (oil and protein extraction yields of 98 and 92%, respectively) (De Moura and Johnson, 2009) and for dehulled yellow mustard flour (Tabtabaei and Diosady, 2013), in which 91.8 and 86.5% of protein and oil extraction yields were achieved.

Moreover, the two-stage countercurrent process reduced the amount of water used in the process by ~60%, compared

with the optimized single-stage EAEP (Table 3). Such reduction is of key importance considering the centrifugation costs of a high volume of aqueous effluent generated when high amounts of water are used. Besides, evaporation or concentration of the extracted protein becomes more expensive in highly diluted streams. Therefore, reduction of water usage without loss in extractability is necessary to reduce environmental issues and improve the commercial viability of the process (De Moura and Johnson, 2009). Although beyond the scope of this work, the evaluation of scaling up the two-stage countercurrent extraction of green coffee flour is warranted to identify possible pitfalls associated with the extraction and recovery of green coffee oil and protein at pilot- and industrial scales.

Oil and protein composition of the insoluble fractions from each sample from each countercurrent run (a complete countercurrent run involved the consecutive extraction of 3 samples to enable adequate enzyme recycling) was evaluated (Table 5). Even though we expected to reach steady-state extraction after extraction of the third sample, because three trials are required to adequately complete enzyme recycling within the fractions, TOE and TPE were relatively constant since the extraction of the first sample.

4. Conclusions

The simultaneous extraction of protein and oil from green coffee by the enzyme-assisted extraction process was successfully developed and optimized for higher extraction yields, higher protein solubility, and reduced enzyme and water usage during the EAEP of green coffee. Within the enzymatic treatments evaluated, the use of 0.5% alkaline protease led to higher oil (48%) and protein (62%) extractability at shorter extraction time (30 min) and to the production of proteins with higher solubility when using 1:10 SLR. Adequate processing optimization resulted in high extraction yields (48% oil and 70% protein) and 80% reduction in enzyme use in the single-extraction process. However, increased extraction yields were achieved at the expense of higher water usage (1:17.5 SLR). A two-stage countercurrent extraction process was successfully developed to reduce water usage while improving extraction yields. The two-stage countercurrent extraction increased oil and protein extraction yields to 58 and 72%, respectively. Importantly, the two-stage countercurrent EAEP reduced the amount of water used in the process by 60% compared with the single-stage EAEP. The results presented herein (enzyme and water use reduction and improved protein extractability and solubility) are key to the development of a more competitive process to produce green coffee proteins and lipids for subsequent industrial applications. Moreover, future cream demulsification and protein functional studies are required to enable the recovery of the extracted oil and to identify potential applications of the extracted proteins.

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Declaration of competing interest

The authors declare that they have no known conflict of interest to disclose.

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Flávia Souza Almeida: Conceptualization, Methodology, Data curation, Formal analysis, Writing - review & editing. **Fernanda Furlan Goncalves Dias:** Methodology, Data curation, Writing - review & editing. **Ana Carla Kawazoe Sato:** Conceptualization, Supervision, Writing - review & editing. **Juliana Maria Leite Nobrega de Moura Bell:** Conceptualization, Data curation, Supervision, Funding acquisition, Writing - review & editing.

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Appendix A. Supplementary data

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