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Bioaccessibility and anti-inflammatory activity in Caco-2 cells of phytochemicals from industrial by-products of coffee (*Coffea arabica* L.)

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ABSTRACT

Coffee by-products are rich in nutrients and bioactive compounds in free soluble form and bound to cell wall components. These compounds undergo chemical changes during gastrointestinal digestion, affecting their bioaccessibility and bioactivity. This study is the first to investigate coffee by-products from industrial wet processing to evaluate the impact of simulated gastrointestinal digestion on their phytochemical composition and subsequent anti-inflammatory activity in Caco-2 cells. Digestion significantly reduced the stability and solubility of main compounds; however, digested bioaccessible by-products still exhibited anti-inflammatory properties, reducing IL-6, IL-8, and TNF-α levels. Correlation analysis identified rutin, quercetin-3-glycoside, caffeine and 5-caffeoylquinic acid as strongly linked to cytokine suppression, suggesting key roles and possible synergies. These results highlight the potential of coffee by-products as functional ingredients targeting intestinal inflammation. Future work should confirm *in vivo* efficacy, optimize extraction at scale, and address regulatory requirements to support industrial application and promote circular economy benefits.

1. Introduction

The world production of coffee (*Coffea arabica* L. and *Coffea canephora Pierre*) in 2022/2023 was 171.3 million bags of 60 kg each, with an estimated daily intake of 3.5 billion cups of coffee worldwide (International Coffee Organization, 2023; Santos et al., 2021). Coffee beans undergo various processing methods before they are ready for human consumption Among them, the wet processing method consists of placing the coffee cherries in a water tank to eliminate impurities, such as green and mature beans. Afterwards, a depulper is used to remove the skin and pulp (coffee husk). Then, the coffee mucilage is removed either through a fermentation process using enzymes, or with a machine called desmucilaginator. Finally, the coffee beans are dried and the leftover parchment is removed (Esquivel & Jiménez, 2012; Santos et al., 2021). As a consequence of this process, considerable amounts of by-products are generated during the industrial manufacture (Cañas et al., 2023; Hikichi et al., 2017). Several efforts have been carried out to valorize coffee by-products in various applications to reduce the waste, including the generation of biodiesel, their use as substrate and fertilizer, and the extraction of specific compounds such as fiber, carbohydrates, flavonoids, anthocyanins, procyanidins, tannins, caffeine, chlorogenic acid, and other organic compounds (Díaz-Gómez et al., 2023). The widespread cultivation of coffee has led to a global challenge in managing coffee by-products, posing a significant concern for the community. Consequently, modern agricultural sustainability principles have encouraged the implementation of circular economy practices aiming to convert waste into valuable resources, thus addressing this urgent issue on a global scale (Magoni et al., 2018).

Coffee consumption is associated with several health benefits. A recent meta-analysis reported a 10 % lower risk of type-2 diabetes with each additional cup of coffee consumed (Henn et al., 2025). Similarly, an umbrella review of meta-analysis concluded that consuming 3–4 cups of coffee per day is linked to a 15 % reduction in the relative risk of cardiovascular diseases (Poole et al., 2017). While these effects have traditionally been attributed to bioactive compounds present in brewed coffee, recent interest has turned toward coffee by-products, such as husk, pulp and mucilage, which also possess high levels of phytochemicals with functional value, yet are usually discarded and underutilized

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(Gemechu, 2020). Potential applications of these industrial by-products in the food and pharmaceutical industry have been explored, including their use as antioxidants, natural preservatives and functional ingredients (Bondam et al., 2022; Cañas et al., 2023; Kasapoğlu et al., 2023). To exert these health benefits in humans, however, phytochemicals must first be released from the plant matrix and solubilized in the gastrointestinal tract (bioaccessibility), then absorbed into the bloodstream and delivered to target tissues (bioavailability), where they can exert biological effects (bioactivity) (Gonçalves et al., 2019; Hu et al., 2023). Simulated gastrointestinal digestion is a widely used model to mimic the human digestion of food. When combined with intestinal epithelial cellular models such as differentiated Caco-2 cells, it enables the assessment of the bioaccessibility and *in vitro* bioactivity of dietary phytochemicals (Cañas et al., 2023; Kondrashina et al., 2023). This approach has been applied to study coffee by-products, which are rich in bioactive (poly)phenols. For example, Abreu et al. (2024) reported that both free and bound (poly)phenols extracted from organic coffee husk (100 % Arabica, Typica variety) remained bioaccessible after *in vitro* digestion, and showed antioxidant and anticoagulant potential. Similarly, studies on coffee pulp flour and aqueous extracts indicated that the food matrix could influence the release and stability of phenolics during digestion, with caffeine and protocatechuic acid emerging as major bioaccessible compounds (Cañas et al., 2022). In addition to their antioxidant activity, coffee-derived compounds have demonstrated anti-inflammatory properties. Extracts from coffee pulp and leaves have been shown to reduce pro-inflammatory cytokine secretion in various cell models, including Caco-2 and U937 cells (Chen et al., 2019; Magoni et al., 2018; Mei & Chen, 2023), with (poly)phenols and mangiferin identified as a potential key bioactives. Building on this background, the aim of the present study was to determine the (poly)phenolic profile of two coffee by-products, namely husk and mucilage, from the industrial processing of *Coffea arabica* L., both before and after simulated gastrointestinal digestion, and to evaluate their anti-inflammatory activity in a Caco-2 cell model of inflammation.

2. Materials and methods

2.1. Chemicals

All solvents, salts, acids and alkali were obtained from Carl Roth GmbH (Karlsruhe, Germany), unless stated differently. Ultrapure water was obtained using a Barnsted EasyPure water filter (Thermo Scientific, Ohio, USA). Amberlite XAD7HP® was obtained from Sigma Aldrich (Schnelldorf, Germany).

2.2. Coffee material

The selected coffee by-products, husk and mucilage, were obtained from a mix of coffee varieties, specifically Caturra rojo and Catuaí rojo (*Coffea arabica* L.). They were obtained from the wet processing of coffee beans and kindly donated by a commercial coffee mill located in Tres Ríos, Cartago, Costa Rica. Coffee husk was separated from the coffee cherry using a depulper (DCV 306, Penagos Equipment, Colombia), followed by the removal of mucilage using a desmucilaginator (DCV 306, Penagos Equipment, Colombia). Three batches of each coffee by-product were collected on three different days (between February and March 2023). The samples were lyophilized (DCV 306, Penagos, Colombia), then pulverized with a coffee grinder and vacuum sealed in plastic. The samples were weighted at 300 g each and stored in plastic containers at −30 °C to ensure preservation until use.

2.3. Obtention of a (poly)phenol enriched extract (PEE)

A (poly)phenol-enriched extract was prepared from the coffee husk and mucilage following the procedure described by Jiménez-Aspee et al. (2016), with some modifications. A mixture of food-grade ethanol (EtOH) and acetic acid were used in order to obtain an extract prepared with GRAS (generally recognized as safe) ingredients (Code of Federal Regulations, 2024), with a proportion of EtOH:H_2O:acetic acid (70:29:1, *v*/v). Coffee husk and mucilage (5 g each) were extracted five times with the mentioned solvents, in a 1:10 lyophilized coffee by-product:solvent ratio, under sonication (10 min each, 35 kHz). The remaining residue was flushed with N_2 and stored at −20 °C for further bound phenolic compounds extraction (described in Section 2.4). The combined extracts were vacuum filtered and then dried under reduced pressure at 37 °C. Afterwards, extracts were dissolved in 100 mL of ultrapure water, vacuum filtered through a Whatman N°1 filter paper and the soluble fraction was loaded into a glass column (60 cm long, 3 cm diameter) packed with 200 cm^3 of Amberlite XAD7HP. The column was washed with 500 mL of H_2O and left to dry completely before desorbing the compounds with EtOH:acetic acid (99:1). The extracts were evaporated, by using a rotary evaporator (Laborota 4001, Heildolph, Schwabach, Germany), under reduced pressure at 37 °C, lyophilized and stored at −80 °C until chromatographic analysis.

2.4. Bound phenolic compounds extraction

The extraction of bound phenolics was carried out according to the method described for beans by Ross et al. (2009). The solid residue left from the free phenolic extraction (Section 2.3) was flushed with N_2 for 1 min and then vortexed for 30 s. Then, it was mixed with 20 mL of 2 mol/L NaOH supplemented with 13.4 mmol/L EDTA and 2 % *w/v* ascorbic acid. The samples were incubated in a water bath at 30 °C overnight at 100 rpm. At the end of the incubation, the samples were centrifuged at 4696 x*g* for 15 min and 4 °C. The supernatant was recovered into a 100 mL glass container and acidified to pH 2.00 ± 0.05 using 2.5 mL of 37 % HCl. The sample was filtered to remove precipitated NaCl and then bound phenolics were recovered by liquid:liquid extraction using 25 mL of diethylether:EtOAC (1:1, *v*/v), for a total of three times. The recovered organic phase was evaporated at a rotary speedvac (RVC 2–25 CDplus, Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germanz), for 4 h at 10 mBar and stored at −20 °C until chromatographic analysis.

2.5. Methylxanthine extraction

The extraction of caffeine, theobromine and theophylline from coffee husk and mucilage was carried out according to the method proposed by Srdjenovic et al. (2008). Briefly, 1 g of freeze-dried coffee husk or mucilage were weighted into a Falcon tube and 40 mL of boiling water was added. The tubes were placed in ultrasonic water bath at 35 kHz for 30 min at 70 °C. The extracts were then filtered through Whatman N°1 filter paper to remove particulate matter. An aliquot of 10 mL of this filtered solution was adjusted to pH 8.0 using 0.1 mol/L NaOH and subjected to clean-up by solid-phase extraction. The Supelclean™ LC-18 SPE cartridges (500 mg bed weight, 6 mL volume) were pre-conditioned with 2 × 5 mL of MeOH, followed by 2 × 5 mL of ultrapure water and 2 × 5 mL of ultrapure water at pH 8.0. Then, the samples were passed through the cartridges, washed with 5 mL of ultrapure water and dried under vacuum. Methylxanthines were recovered from the SPE cartridge using 2 × 5 mL of $CHCl_3$. The solution was evaporated under vacuum and the residue was stored at −20 °C until analysis.

2.6. Identification of (poly)phenols by HPLC-DAD-MS

The mass spectrometric analyses of the free and bound phenolics from coffee husk and mucilage were carried out using an Agilent 1290 Infinity Ultra Performance Liquid Chromatography, equipped with an G4212 diode array detector, a G4226 autosampler, a G4220 binary pump, a G1316 column oven, and a Q-Exactive Plus Mass Spectrometer (Thermo Fischer Scientific, Waltham, MA, USA). Compounds were separated on a Kinetex PFP 100 Å (2.1 × 150 mm; 1.8 µm, Phenomenex)

maintained at 40 °C. *mobile* phases were water (A) and methanol (B), each acidified with 0.1 % formic acid and delivered at a flow rate of 0.3 mL/min. The following steps were applied for the gradient elution: at 0–3 min, 98 %A, 2 %B; at 3 min: 98 %A, 2 %B; at 33 min, 20 %A, 80 %B; at 35 min, 0 %A, 100 %B; at 39 min, 0 %A, 100 %B; at 40 min, 98 %A, 2 %B; at 44 min, 98 %A, 2 %B. UV spectra was recorded from 190 to 640 nm, with scan steps of 2 nm. Mass spectrometry settings used for both polarities were scan range: 100–1400 *m/z*; spray voltage, 4200 V (positive) and 3500 V (negative); sheath gas flow, 60; auxiliary gas flow; 20; and sample heater temperature, 380 °C. The system was operated with XCalibur 4.0 software, and the same software was used for data analysis.

2.7. Quantification of main (poly)phenols and methylxanthines by HPLC-DAD

A Nexera XR HPLC system (Shimadzu, Kyoto, Japan) equipped with a DGU-403 degassing unit, two LC-40D XR solvent delivery modules, a SL-40C XR autosampler, a CTO-40C column oven, and an SPD-M40 photo diode array detector was used.

For (poly)phenols, separation was carried out in a Kinetex PFP 100 Å C18 column (5 µm, 250 × 4.6 mm, Phenomenex) set at 40 °C. The mobile phase consisted of H_2O:formic acid:acetonitrile (885:85:30 *v*/v, mobile phase A) and H_2O:formic acid:acetonitrile (415:85:500 v/v, mobile phase B), at a constant flow rate of 0.8 mL/min. An elution gradient was used as follows: at 0 min, 98 %A, 2 %B; at 1.2 min, 98 %A, 2 %B; at 10.8 min, 95 %A, 5 %B; at 60 min, 70 %A, 30 %B, at 61 min, 0 %A, 100 %B, at 62 min, 0 %A, 100 %B; at 63 min, 98 %A, 2 %B and at 68 min, 98 %A, 2 %B. Chromatograms were recorded for 68 min following an injection of 20 µL of sample. The chromatographic peaks were registered at 190–800 nm and integrated with the LabSolutions software platform. Calibration curves were built using the following standards: gallic acid, quercetin-3-glycoside, quercetin-3-rutinoside, 3-Caffeoylquinic acid (3-CQA), 5-Caffeoylquinic acid (5-CQA), mangiferin, *p*-Coumaric acid, ferulic acid, cyanidin-3-glucoside, and cyanidin-3-rutinoside. Analytical parameters for the individual calibration curves can be found in the Supplementary Table S1.

For methylxanthines, separation was carried out in an Luna C8 column (3 µm, 150 × 4.6 mm, 100 Å, Phenomenex) set up at 25 °C. *mobile* phases used were 0.1 % THF in water (pH 8.0, A) and acetonitrile (B) in a proportion of 9:1 (A:B), isocratic for 10 min at a flow rate of 0.8 mL/min, with an injection volume of 20 µL. The analytes were detected at 273 nm. The analytical parameters for the caffeine calibration curve can be found in the supplementary Table S1.

2.8. In vitro gastrointestinal digestion of coffee by-products

The static *in vitro* digestion of coffee husk and mucilage was carried out following the INFOGEST 2.0 protocol described by Brodkorb et al. (2019). The simulated salivary fluid (SSF) gastric fluid (SGF) and intestinal fluid (SIF) were prepared accordingly (Brodkorb et al., 2019) and warmed up to 37 °C before use. To calculate the stability, solubility and bioaccessibility of main compounds, the mol of each compound obtained at the end of the digestion process was expressed as the percent of total mol present in the non-digested sample using previously described equations (Mieres-Castro et al., 2022).

Briefly, 1 g of freeze-dried coffee husk and mucilage were weighted (in triplicate each batch) in 50 mL Falcon tubes and mixed with 0.8 mL of SSF. Then, an aliquot of $CaCl_2(H_2O)_2$ (44.1 mg/mL) was added to achieve a total concentration of 1.5 mM in SSF, followed by the addition of α-amylase (75 U/mL, from *Aspergillus oryzae*) and ultrapure water to a final volume of 2 mL. The samples were incubated at 37 °C, for 2 min in a linear shaker at 95 rpm. Afterwards, the SGF solution was added to the oral bolus and the pH was adjusted to 3.00 ± 0.05 with 0.10 mol/L HCl. Then, an aliquot of $CaCl_2(H_2O)_2$ (44.1 mg/mL) solution was added to achieve a total concentration of 0.15 mM in SGF, followed by porcine pepsin solution (2000 U/mL) and ultrapure water to a final volume of 10 mL. The samples were incubated in a linear shaker at 37 °C, for 2 h and 180 rpm. Subsequently, the SIF solution was added, and the pH was adjusted to 7.00 ± 0.05 using 0.10 mol/L NaOH. After this, a bile solution (10 mmol/L) and $CaCl_2(H_2O)_2$ (44.1 mg/mL) solution to reach a concentration of 0.6 mmol/L in SIF were added. Finally, a pancreatin solution (100 U/mL of trypsin) was added followed by ultrapure water to a final volume of 20 mL. The samples were flushed with nitrogen gas to displace oxygen and to generate a semi anaerobic environment. The samples were incubated at 37 °C, for 2 h and 180 rpm in a linear shaker. To stop the enzymatic digestion, an aliquot of protease inhibitor Pefabloc® (Merck, Darmstadt, Germany) was added to a final concentration of 0.5 mmol/L to inhibit residual protease activity.

After vortexing, an aliquot of 1 mL was recovered and the digestive stability of the main (poly)phenolic compounds was calculated. Then, the Falcon tubes were centrifuged (4696 xg, 1 h, 4 °C) and a 1 mL aliquot of the supernatant was recovered and analyzed by HPLC to determine the solubility of the compounds. Finally, the supernatants were filtered (Filtropure S, 0.2 µm, Sarstedt, Nümbrecht, Germany) to separate mixed micelles from the larger lipid emulsions. The micellar fraction corresponds to the bioaccessible fraction of the digestion, and the percent bioaccessibility was calculated. All aliquots were stored at −80 °C until HPLC-DAD and HPLC-MS analysis. Before injection, samples were diluted with mobile phase by a factor of 1:1 for the HPLC-DAD and 1:10 for the HPLC-MS.

After completion of the intestinal phase and sampling for stability, solubility and bioaccessibility, the remaining intestinal digesta was prepared for cell culture experiments in accordance with the recommendations of Kondrashina et al. (2023) to ensure biocompatibility. These post-digestion steps included the adjustment of the pH of the bioaccessible fraction to 7.00 ± 0.05 and the dilution in cell culture medium to decrease bile salt concentrations to non-toxic levels.

2.9. Total phenolic content

The Folin–Ciocalteu method described by Singleton and Rossi (1965) was used to determine the total phenolic content of digested samples of coffee husk and mucilage. A stock solution of gallic acid standard (2.5 mg/mL) was prepared in MeOH and a calibration curve was performed with concentrations between 50 µg/mL and 300 µg/mL. Aliquots of 40 µL of the blank (MeOH), samples and dilutions of the calibration curve were taken and mixed with 160 µL of ultrapure water in Eppendorf tubes of 2 mL. Then, 50 µL of each were taken into 2 mL Eppendorf tubes, mixed with 500 µL of 10 % Folin–Ciocalteu Reagent, vortexed and incubated for 5 min at room temperature, protected from the light. Then, 500 µL of 10 % *w/v* Na_2CO_3 and 800 µL of ultrapure water were added and mixed in a vortex. After 1 h incubation at room temperature, protected from light, the samples were vortex and 200 µL were transferred into a 96-well plate. The absorbance was measured at 725 nm on a microplate reader (Synergy HT, Biotek, Winooski, VT, USA). The TPC was expressed as grams of gallic acid equivalents (GAE) per gram of PEE or mL of digested solution.

2.10. Cell culture

Human Caco-2 Cells clone TC7 (ATCC®, HTB-37TM) were grown in 75 cm^2 Falcon Flaks using high glucose Dulbecco's Modified Eagles's Medium (DMEM) supplemented with 10 % Fetal Bovine Serum (FBS), 1 % sodium pyruvate, 1 % non-essential-amino acids and 1 % penicillin/streptomycin. Cells were incubated in a humidified atmosphere with 5 % CO_2 at 37 °C in an incubator (Hera Cell 150, ThermoElectron Corporation, Langenselbold, Germany). After confluence, cells were detached with 0.025 % trypsin-EDTA and passaged or seeded. Medium was changed every two days, and the cells were maintained for 21 days post-confluence. After 21 days of differentiation, cells form a monolayer with morphological and functional characteristics that resemble the brush border of small intestine (Fleet et al., 2003).

2.11. Cytotoxicity assay

The evaluation of the effect of *in vitro* digested coffee husk or mucilage on the Caco-2 cell viability was determined by means of the dimethylthiazol diphenyl tetrazolium bromide (MTT) reduction assay. For this purpose, Caco-2 cells were used after 21 days of differentiation in 48-well plates. The cells were treated with the *in vitro* digested samples, previously dissolve in medium not supplemented (Dulbecco's Modified Eagles's Medium (DMEM) high glucose), at three different concentrations, in duplicate, (25, 5 and 1 μg GAE/mL). After 16 h of incubation (37 °C, 5 % CO_2), the treatments were removed, and each well was washed once with PBS. The PBS was removed, and a MTT solution (0.5 mg/mL) was added to each well followed by 2.5 h of incubation (37 °C, 5 % CO_2). The MTT solution was removed, and the resulting formazan was dissolved in 5 % HCl in EtOH. The plate was incubated 10 min at room temperature on a rotation shaker at 220 rpm in the dark. The absorbance at 580 nm (reference at 660 nm) was measured in a microplate reader (Synergy HT). Percentual viability was calculated regarding the absorbance obtained from formazan generated in the wells only treated with cell culture medium (100 % viability).

2.12. Anti-inflammatory capacity of digested coffee husk and mucilage

The anti-inflammatory capacity of digested coffee husk and mucilage was evaluated in a Caco-2 cell model of inflammation described by Melillo De Magalhães et al. (2012). Briefly, Caco-2 cells were seeded in 12-well plates at a density of 100.000 cells/well and grown for 21 days in supplemented medium. Medium was changed the day before the experiment to avoid studying cells under starved conditions. On day 22, the cells were washed two times with 1 mL of PBS and then submitted to the different treatments: basal control (5 % FBS in high-glucose DMEM), chyme control (blank of digestion in 5 % FBS in high-glucose DMEM), or the digested samples diluted at 25 μg GAE/mL (prepared also in 5 % FBS in high-glucose DMEM). The samples were incubated overnight at 37 °C and 5 % CO_2. The next morning, a pro-inflammation medium was prepared in 5 % FBS DMEM-high glucose with 100 ng/mL of human recombinant interferon gamma (rHuINF-gamma, Biomol GmbH, Hamburg, Germany), 100 ng/mL of recombinant human interleukin 1ß (IL-1ß, Elabscience, Houston, TX, USA) and 2 μg/mL of LPS from *Salmonella typhimurium* (L-6511, Sigma Aldrich, St. Louis, MO, USA). The pre-incubated cells were washed two times with 1 mL of PBS and then incubated with the pro-inflammation medium with or without samples (final concentrations: 25 μg GAE/mL for the samples, 50 ng/mL IL-1ß and INF-γ, and 1 μg/mL of LPS). Cells without pre-treatment nor stimuli were included as basal control, while cells without pre-treatment but treated with pro-inflammation medium were used as inflammation controls. After adding the respective treatments, cells were incubated for 24 h at 37 °C and 5 % CO_2. At the end of the incubation period, medium was recovered and divided into 200 μL aliquots for storage at −80 °C until determination of inflammation markers by enzyme-linked immune assay as described in the following section.

2.13. Quantification of pro-inflammatory cytokines

The concentrations of pro-inflammatory cytokines released into the cell culture medium were measured using ELISA kits obtained from Elabscience (Houston, TX, USA) following the manufacturer instructions. Interleukin 8 (IL-8) was determined after 1:100 dilution in sample buffer using the high-sensitive human IL-8 ELISA Kit (*E*-HSEL-H0004). Interleukin 6 (IL-6) was determined without dilution using the high-sensitive human IL-6 ELISA kit (E-HSEL-H0003). Tumor Necrosis Factor Alpha (TNF-α) was determined after 1:4 dilution in sample buffer using the Human TNF-alpha ELISA Kit (E-EL-H0109).

2.14. Statistical analyses

All results are presented as mean values ± standard deviation from three independent experiments, except for cell culture where four independent experiments were carried out. For the ELISA measurements, samples were assayed in technical duplicates on the plate ($n = 6$ for each lot). Normal distribution of the results was tested using the D'Agostino Pearson omnibus normality test. One-way analysis of variance (ANOVA) with Tukey's multiple comparison post-hoc test was applied to compare mean values among lot numbers. The statistical differences between the inflammation control and the pre-treatment samples was determined using ANOVA followed by Dunnett's multiple comparison tests, with a level of significance set at $p < 0.05$. To explore potential relationships between compound bioaccessibility and anti-inflammatory activity, Spearman's rank correlation coefficients (ρ) were calculated between the % bioaccessibility of each compound and the % reduction of IL-6, IL-8 and TNF-α secretion in Caco-2 cells for the corresponding digested samples. The choice of Spearman's correlation was based on its non-parametric nature, which does not assume normality and is suitable for monotonic but non-linear relationships. Correlation strength was interpreted as weak ($|\rho| < 0.3$), moderate ($0.3 \leq |\rho| < 0.7$), or strong ($|\rho| \geq 0.7$), and statistical significance was set at $p < 0.05$. All analysis and graphics were done using the GraphPad Prism software version 10.2.3. (GraphPad Software LLC, Boston, MA, USA).

3. Results and discussion

3.1. Content and composition of phytochemicals from coffee husk and mucilage before digestion

A total of 33 compounds were identified in the free (poly)phenol extract of coffee husk, while for the mucilage only 27 compounds were identified (Table 1). In the bound (poly)phenol extract, a total of 18 compounds were identified for both the husk and mucilage (Table 1). In the methylxanthines extract, three compounds were identified (Table 1). Representative chromatograms of each extract are presented in Figs. S1, S2 and S3. The identification and quantification of main compounds are detailed in the following paragraphs. Three batch numbers representing different production batches were considered for the analyses. Single batch analysis might not represent the full range of variability, while testing multiple batches ensures that the findings are generalizable and not biased by an outlier batch. The variability of concentrations of the diverse compounds that were quantified, as well as the difference in content reported in literature, could be attributed to the different characteristics of the raw materials (cultivar place, weather conditions, ripeness grade), post-harvest management, processing conditions, extraction methods and analytical bias for identification and quantification (Rodríguez-Durán et al., 2014).

3.1.1. Phenolic acids

Phenolic acids were detected in the free and bound extracts obtained from coffee husk and mucilage. In the free phenolic extract, 14 phenolic acids were identified in the husk and 12 in the mucilage, while in the bound phenolic extract, 14 phenolic acids were identified in the husk and 15 in the mucilage (Table 1). The identification of the compounds is detailed in the following paragraphs.

5-Caffeoylquinic acid (**9**, 5-CQA) and 3-caffeoylquinic acid (**20**, 3-CQA) were identified and quantified in both coffee by-products. They presented a $[M-H]^-$ base peak at *m/z* 353.0881 amu and *m/z* 353.0876 amu, respectively, and shared a common ion fragment at *m/z* 191.05 amu which is formed by the cleavage of the ester bond between the quinic and caffeic acid moieties (Zhang et al., 2018). 5-CQA exhibited two more ion fragmentations at *m/z* 179.0341 amu and 135.0440 amu, which is the loss of quinic moiety and the loss of a carbon dioxide moiety, respectively (Zhang et al., 2018). Regarding 3-CQA, it presented an ion fragmentation at *m/z* 173.0445 amu corresponding to dehydrated

Table 1
Retention time (R_t), UV/VIS absorption maxima, molecular mass and MS fragmentation of free and bound (poly)phenols and alkaloids in coffee husk and mucilage extracts, as well as the presence of identified compounds after *in vitro* digestion.

Peak	Rt (min)	λ_{max} (nm)	Precursor ion	Mode	Error (ppm)	Molecular Formula	MS/MS fragmentation	Tentative identification	Husk			Mucilage		
									Free	Bound	After digestion	Free	Bound	After digestion
1	1.04	232	138.0550	+	−0.165	$C_5H_8O_2N$	94.0657	Trigonelline	X		X			
2	4.02	280	169.0134	−	0.280	$C_7H_5O_5$	125.0232	Gallic acid*		X	X		X	
3	5.68	296	171.0291	−	0.270	$C_7H_7O_5$	127.0388	Dehydroshikimic acid		X			X	
4	6.40	276	167.0341	−	0.245	$C_8H_7O_4$	123.0439	Vanillic acid		X			X	
5	6.75	274	181.0498	−	0.225	$C_9H_9O_4$	135.0441	3-hydroxy-(3-hydroxyphenyl) propionic acid		X			X	
6	7.42	260	315.0722	−	1.112	$C_{13}H_{15}O_9$	153.0183	Protocatechuic acid hexoside	X			X		
7	7.52	260	153.0184	−	0.195	$C_7H_5O_4$	109.0283	Protocatechuic acid	X		X	X		
8	8.12	272	181.0718	+	−0.212	$C_7H_9O_2N_4$	138.0662	Theobromine	X		X	X		X
9	9.64	320	353.0881	−	1.391	$C_{16}H_{17}O_9$	191.0553, 179.0341, 135.0440	5-caffeoylquinic acid	X		X	X		X
10	9.73	280	137.0234	−	0.039	$C_7H_5O_3$	108.0204	*p*-hydroxybenzoic acid		X	X		X	X
11	10.13	268	181.0719	+	−0.052	$C_7H_9O_2N_4$	124.0507	Theophyllin	X		X			
12	10.55	276	577.1356		1.567	$C_{30}H_{25}O_{12}$	451.1027, 289.0719	(epi)-catechin-(epi)-catechin dimer 1	X					
13	10.55	338	183.0290	−	0.190	$C_8H_7O_5$	124.0154	Methylgallate		X			X	
14	11.12	268	447.1142	−	0.883	$C_{18}H_{23}O_{13}$	271.0846	Dihydroxybenzoic acid hexoside pentoside	X					
15	11.45		153.0184	−	0.255	$C_7H_5O_4$	125.0233, 107.0126	Gentisic acid		X			X	
16	11.80	344	197.0445	−	0.470	$C_9H_9O_5$	125.0232	Syringic acid		X			X	
17	12.06	276	289.0718		1.145	$C_{15}H_{13}O_6$	245.0816	(epi)-catechin	X			X		
18	12.30	276	577.1357	−	1.627	$C_{30}H_{25}O_{12}$	451.1027, 407.1771, 289.0719	(epi)-catechin-(*epi*)-catechin dimer 2	X			X		
19	12.89	270	195.0876	+	−0.022	$C_8H_{11}O_2N_4$	138.0389	Caffeine*	X	X	X	X	X	X
20	12.94	320	353.0876	−	0.941	$C_{16}H_{17}O_{19}$	191.0553, 173.0445	3-caffeoylquinic acid*	X		X	X		X
21	13.10	320	367.1030	−	0.641	$C_{17}H_{19}O_9$	193.0498, 173.0446	Feruloylquinic acid 1	X					
22	13.22	310	179.0341	−	0.265	$C_9H_7O_4$	135.0440	Caffeic acid*	X	X	X	X	X	X
23	13.45	270	865.1992	−	1.839	$C_{45}H_{37}O_{18}$	713.1500, 577.1340, 451.1042, 407.0771. 287.0561	B-type procyanidin trimer	X			X		
24	13.49	270	863.1834	−	1.610	$C_{45}H_{35}O_{18}$	711.1357, 451.1039, 411.0721 289.0717	A-type procyanidin trimer	X			X		
25	14.63	270	289.0720		1.355	$C_{15}H_{13}O_6$	245.0815	(epi)-catechin	X			X		
26	15.17	320	337.0931	−	1.346	$C_{16}H_{17}O_8$	191.0553	p-coumaroylquinic acid	X		X	X		X
27	15.41	360	423.0917	+	−0.518	$C_{19}H_{19}O_{11}$	303.0497	Mangiferin*	X		X	X		X
28	15.87	317	163.0392	−	0.229	$C_9H_7O_3$	119.0490	*p*-coumaric acid*	X	X	X		X	X
29	16.28	520	449.1077	+	−0.138	$C_{21}H_{21}O_{11}$	287.0548	Cyanidin-3-glucoside*	X			X		
30	16.35	320	367.1036	−	1.251	$C_{17}H_{19}O_9$	191.0190	Feruloyl quinic acid 2	X		X	X		X
31	16.88	520	595.1645	+	−1.197	$C_{27}H_{31}O_{15}$	287.0549	Cyanidin-3-rutinoside*	X			X		
32	17.19	324	465.1041	−	0.308	$C_{21}H_{21}O_{12}$	303.0514	Taxifolin hexoside	X			X		X
33	17.50	360	625.1405	−	0.634	$C_{27}H_{29}O_{17}$	301.0365	Quercetin dihexoside	X		X	X		X
34	19.22	320	193.0501	−	0.595	$C_{10}H_9O_4$	133.0284	Ferulic acid*	X	X	X	X	X	X
35	19.41	320	515.1196	−	1.168	$C_{25}H_{23}O_{12}$	353.0882, 191.0554, 173.0444	Dicaffeoylquinic acid 1	X			X		
36	20.14	310	151.0391	−	0.119	$C_8H_7O_3$	92.0254	2-hydroxy-2-phenylacetic acid		X			X	
37	20.15	360	609.1459	−	0.859	$C_{27}H_{29}O_{16}$	301.0350	Quercetin rutinoside*	X	X	X	X	X	X

(*continued on next page*)

Table 1 (*continued*)

Peak	Rt (min)	λ_{max} (nm)	Precursor ion	Mode	Error (ppm)	Molecular Formula	MS/MS fragmentation	Tentative identification	Husk			Mucilage		
									Free	Bound	After digestion	Free	Bound	After digestion
38	20.23	360	463.0877	–	0.578	$C_{21}H_{19}O_{12}$	301.0352	Quercetin-3-O-ß-D-glucoside*	X	X	X	X	X	X
39	20.55	320	515.1203	–	1.898	$C_{25}H_{23}O_{12}$	353.0882, 173.0444. 135.0441	Dicaffeoylquinic acid 2	X		X	X		X
40	21.62	347	447.0931	–	0.952	$C_{21}H_{19}O_{11}$	285.0404	Kaempferol glucoside*	X	X	X	X	X	X
41	22.28	328	515.1203	–	1.898	$C_{25}H_{23}O_{12}$	353.0879, 191.0554, 173.0446	Dicaffeoylquinic acid 3	X			X		
42	25.49	360	301.0357	–	1.413	$C_{15}H_9O_7$	151.0026	Quercetin	X	X		X	X	
43	27.43	366	285.0407	–	1.316	$C_{15}H_9O_6$	164.9986	Kaempferol*		X			X	

* compound identified by matching of retention time and mass spectra with commercial standards.

quinic moiety (Zhang et al., 2018). The content was found between 9.09 and 13.48 mg/100 g for 5-CQA and 128.27–285.82 mg/100 g for 3-CQA in coffee husk, while for coffee mucilage the range values were 1.19–2.77 mg/100 g for 5-CQA and 15.69–46.79 mg/100 g for 3-CQA (Table 2). The presence and contents of these compounds are consistent with the findings in coffee pulp and peels of diverse *Coffea arabica* L. varieties (Esquivel et al., 2020) as well as in coffee mucilage (Chaves-Ulate et al., 2023; Viñas et al., 2012). In addition, other quinic acid derivatives were tentatively identified in the samples such as feruloylquinic acids (**21** and **30**), or *p*-Coumaroylquinic acid (**26**), based on literature about the fragmentation patterns of these hydroxycinnamic acids (Clifford et al., 2003).

Ferulic acid (**34**) presented a $[M-H]^-$ base peak at *m/z* 193.0501 amu and the MS^2 spectra showed a loss of 133 amu, characteristic loss of CO_2 and -CH_3 (Sinosaki et al., 2020). This phenolic acid was found in the range of 0.50–0.66 mg/100 g and 0.28–0.47 mg/100 g for coffee husk and mucilage, respectively (Table 2). Caffeic acid (**22**) was identified by the $[M-H]^-$ signal at *m/z* 179.0341 amu, *p*-Coumaric acid (**28**) was identified by the $[M-H]^-$ signal at *m/z* 163.0392 amu, while protocatechuic acid (**7**) was identified by the $[M-H]^-$ signal at *m/z* 153.0184 amu (Cañas et al., 2022). All these phenolic acids presented a neutral loss of 44 amu, indicating the CO_2 loss. Caffeic acid was in the range of 1.33–2.95 mg/100 g in husk and 0.27–0.43 mg/100 g in mucilage, while *p*-Coumaric acid was in the range of 1.08–1.52 mg/100 g and 0.32–0.77 mg/100 g, in husk and mucilage, respectively (Table 3). These three hydroxycinnamic compounds have been also found in Caturra variety of *Coffea arabica* L. pulps, specifically, in acidified (HCl 1 %) water extracts at considerably higher concentrations of 19.81 mg/100 g of pulp for caffeic acid, 7.95 mg/100 g of pulp for *p*-Coumaric acid and 7.26 mg/100 g of pulp for ferulic acid (Delgado et al., 2019).

Compounds **35**, **39** and **41** were characterized by the presence of the $[M-H]^-$ signal at *m/z* 515.1 amu, and the MS^2 base peak at *m/z* 353.08 amu, indicative of dicaffeoylquinic acids. These compounds have been identified previously as 3,4-di-*O*-Caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid, in peels and pulp of diverse varieties of *Coffea arabica* L. (Esquivel et al., 2020). However, in the present study, we were not able to assign the identity of each isomer to specific peaks. The content of dicaffeoylquinic acids corresponds to the sum of all individual peaks and it was in the range of 18.89–25.25 mg/100 g and 8.30–17.36 mg/100 g for coffee husk and mucilage, respectively (Table 2).

3.1.2. Flavonols and xanthonoids

Rutin (quercetin-3-rutinoside) (**37**) was identified and quantified in both coffee by-products. It presented a parent ion $[M-H]^-$ at *m/z* 609.1459 amu and the neutral loss of 308 amu, characteristic of the neutral loss of hexose+rhamnose, leading to the MS^2 ion at *m/z* 301.0350, in agreement in the flavonol quercetin (Cañas et al., 2022; Zhang et al., 2018). Rutin was quantified in the range of 9.24–11.04 mg/100 g in coffee husk, while in mucilage the content ranged between 2.59

Table 2
Chromatographic quantification of free and bound (poly)phenols and alkaloids from coffee husk and mucilage (*Coffea arabica* L.) extracts.

Compound	Coffee Husk			Coffee Mucilage		
	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3
Hydroxycinnamic acids						
5-Caffeoylquinic acid	12.43 ± 0.01^a	9.26 ± 0.00^b	13.48 ± 0.36^c	2.77 ± 0.08^a	2.29 ± 0.01^a	1.19 ± 0.07^b
3-Caffeoylquinic acid	285.82 ± 4.16^a	128.27 ± 6.65^b	201.85 ± 26.26^c	49.12 ± 10.75^a	43.98 ± 6.01^b	18.04 ± 2.74^c
Ferulic acid	0.50 ± 0.07^a	0.50 ± 0.05^a	0.66 ± 0.22^a	0.47 ± 0.08^a	0.33 ± 0.07^b	0.28 ± 0.03^b
Caffeic acid	2.95 ± 0.50^a	1.72 ± 0.06^b	1.33 ± 0.19^b	0.55 ± 0.15^a	0.42 ± 0.10^{ab}	0.38 ± 0.13^b
p-Coumaric acid	1.08 ± 0.08^a	1.26 ± 0.01^b	1.01 ± 0.03^c	0.77 ± 0.01^a	0.32 ± 0.00^b	0.49 ± 0.01^c
Dicaffeoylquinic acid	21.52 ± 0.04^a	20.06 ± 0.13^b	25.25 ± 2.55^b	15.10 ± 2.61^a	8.30 ± 2.88^b	8.45 ± 0.59^b
Xanthonoid						
Mangiferin	6.20 ± 1.07^a	4.36 ± 0.58^{ab}	4.95 ± 0.76^b	3.11 ± 0.26^a	1.95 ± 0.07^b	1.50 ± 0.04^c
Flavonols						
Rutin	11.04 ± 0.10^a	9.62 ± 0.15^b	9.24 ± 0.21^c	3.51 ± 0.07^a	2.59 ± 0.10^b	2.61 ± 0.02^b
Quercetin-3-glucoside	6.36 ± 0.11^a	4.73 ± 0.07^b	4.75 ± 0.03^b	2.16 ± 0.02^a	1.74 ± 0.03^b	1.78 ± 0.04^b
Anthocyanins						
Cyanidin-3-glucoside	0.23 ± 0.01^a	0.24 ± 0.01^a	0.39 ± 0.01^b	0.08 ± 0.00^a	0.06 ± 0.02^a	0.12 ± 0.00^b
Cyanidin-3-rutinoside	1.01 ± 0.04^a	1.03 ± 0.02^a	1.92 ± 0.05^b	0.29 ± 0.02^{ab}	0.24 ± 0.07^b	0.33 ± 0.01^a
Alkaloid						
Theobromine	1.88 ± 0.48^a	2.58 ± 0.10^{ab}	3.13 ± 0.36^b	2.06 ± 0.16^a	1.36 ± 0.36^b	1.60 ± 0.13^b
Theophylline	0.82 ± 0.21^a	1.65 ± 0.04^b	2.85 ± 0.34^c	2.31 ± 0.19^a	0.71 ± 0.11^b	1.66 ± 0.15^c
Caffeine	400.05 ± 14.03^a	434.53 ± 10.79^a	480.53 ± 65.55^a	239.61 ± 45.62^a	223.66 ± 49.78^a	228.27 ± 42.18^a

The results are expressed as means ± SD ($n = 3$) within each row within husk and mucilage not sharing a superscript letter (a–c) are significantly different (Tukey's test, $p < 0.05$). All results are expressed as mg corresponding compound / 100 g of dry sample.

Table 3
Chromatographic quantification of bound phenolics from coffee husk and mucilage (*Coffea arabica* L.) extracts.

Compound	Coffee Husk			Coffee Mucilage		
	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3
Gallic acid	0.44 ± 0.01^{a}	0.26 ± 0.02^{b}	0.18 ± 0.01^{c}	0.45 ± 0.01^{a}	0.49 ± 0.01^{a}	0.31 ± 0.04^{b}
3-Caffeoylquinic acid	31.4 ± 1.25^{b}	31.8 ± 1.70^{b}	47.3 ± 8.61^{a}	14.8 ± 0.71^{a}	17.2 ± 0.13^{b}	8.92 ± 0.94^{c}
Ferulic acid	0.72 ± 0.01^{b}	0.86 ± 0.05^{b}	3.19 ± 0.34^{a}	0.88 ± 0.18^{a}	0.58 ± 0.05^{b}	0.49 ± 0.06^{b}
Caffeic acid	0.11 ± 0.002^{b}	0.09 ± 0.01^{b}	0.15 ± 0.01^{a}	0.19 ± 0.01^{b}	0.16 ± 0.03^{b}	0.25 ± 0.03^{a}
p-Coumaric acid	0.53 ± 0.002^{b}	0.47 ± 0.03^{b}	0.92 ± 0.07^{a}	0.43 ± 0.02^{a}	0.59 ± 0.06^{b}	0.39 ± 0.04^{a}
Dicaffeoylquinic acid	1.80 ± 0.061^{b}	2.13 ± 0.17^{b}	3.80 ± 0.50^{a}	1.26 ± 0.10^{a}	1.49 ± 0.22^{a}	0.73 ± 0.09^{b}
Caffeine	2.77 ± 0.08^{a}	1.95 ± 0.13^{b}	2.19 ± 0.02^{c}	1.46 ± 0.02^{a}	5.56 ± 0.84^{b}	2.96 ± 0.18^{c}

The results are expressed as means ± SD (n = 3) within each row within husk and mucilage not sharing a superscript letter (a–c) are significantly different (Tukey's test, $p < 0.05$). All results are expressed as mg corresponding compound / 100 g of dry sample.

and 3.51 mg/100 g. The literature indicates that rutin was identified and quantified in peels of diverse varieties of *Coffea arabica* L. (Esquivel et al., 2020) as well as in ethanolic extracts of coffee mucilage (Chaves-Ulate et al., 2023). Peak **38** presented a precursor ion $[M-H]^-$ at *m/z* 463.0877 amu and a fragment ion at *m/z* 301.0352 amu, indicating the neutral loss of an hexose. The compound was identified as quercetin-3-O-ß-D-glucoside by matching with reference compound. This compound was quantified in coffee husk and mucilage, within range values between 4.73 and 6.36 mg/100 g and 1.74–2.16 mg/100 g, respectively. Similar MS spectra was obtained for compound **32**, with a difference of 2 amu in the $[M-H]^-$ and MS^2 peaks, indicating that the compound could be taxifolin hexoside (Hashim et al., 2013). In addition, compound **33** presented the same MS^2 peak at *m/z* 301.0365 amu, but the $[M-H]^-$ signal was found at *m/z* 625.1405 amu. The neutral loss of 324 amu is indicative of two hexoses, and the compound was tentatively identified as quercetin dihexoside (Geng et al., 2014). Compound **42** presented the $[M-H]^-$ base peak at *m/z* 301.0357 amu and was identified as quercetin (Wang & Morris, 2005). Compound **40** presented the $[M-H]^-$ peak at *m/z* 447.0931 amu and the MS^2 signal at 285.0407, corresponding to kaempferol glycoside (Kiselova-Kaneva et al., 2022).

Mangiferin (1,3,6,7-tetrahydroxyxanthone-C2-ß-glycoside) was identified and quantified in the husk and mucilage. Peak **27** showed the $[M + H]^+$ base peak at *m/z* 423.0917 amu and the MS^2 spectra showed the neutral loss of 120 amu, characteristic loss of C-glycosides (Trevisan et al., 2016). The compound was quantified in the husk within the range of 4.36–6.20 mg/100 g, while in the mucilage the content ranged between 1.5 and 3.0 mg/100 g (Table 3). The literature indicates the presence of mangiferin mainly in coffee leaves from Brazil and Costa Rica (Mei & Chen, 2023), while only some studies have reported the presence in the fruits of *C. pseudozanguebariae*, *C. arabica* and *C. canephora* (Campa et al., 2012). In the study of Campa et al. (2012), the content of mangiferin ranged from 0.0 to 1.13 % *w*/w, and with contents influenced by the stage of development and maturation of the perisperm or endosperm.

3.1.3. Flavan-3-ols and proanthocyanidins

Compounds **17** and **25** presented the same $[M-H]^-$ base peak at *m/z* 289.0720 amu and the MS^2 peak at *m/z* 245.0815 amu, indicative of catechin or *epi*catechin (Lin et al., 2014). The exact identity of both isomers could not be assigned. Related compounds were tentatively identified based on the *m/z* 289 signal. Compound **12** and **18** presented the same $[M-H]^-$ base peak at *m/z* 577.1357 amu and the MS^2 signals at *m/z* 451 and 289 amu, pointing to (epi)-catechin dimers (Lin et al., 2014). Compound **23** presented a $[M-H]^-$ base peak at *m/z* 865.1992 amu and the MS^2 signals at *m/z* 577, 451, 407 and 287 amu, characteristic of a B-type procyanidin trimer (Lin et al., 2014). On the other hand, compound **24** presented a $[M-H]^-$ base peak at *m/z* 863.1834 amu and the MS^2 signals at *m/z* 451, 411, and 289 amu, characteristic of a A-type procyanidin trimer (Lin et al., 2014). Monomeric (−)-epicatechin has been identified in a methanol extract prepared from fresh coffee pulp (Arabica variety of *Coffea arabica* L.), while (+)-catechin was identified as the terminal unit after thiolytic depolymerization of proanthocyanidins present in the same extract (Ramirez-Coronel et al., 2004). In a Typica variety of *Coffea arabica* L., A-type procyanidin trimer was identified in coffee pulp (Wong-Paz et al., 2021), while in methanolic extracts from coffee leaves of unknown variety a C-type procyanidin trimer was identified (Ngamsuk et al., 2019).

3.1.4. Anthocyanins

The HPLC-DAD-ESI-MS^n analysis in the positive ion mode and UV detection at 520 nm revealed the presence of two main peaks, namely peaks **29** and **31,** in both coffee husk and mucilage extracts (Fig. S1). Both peaks were characterized by MS^2 signal at *m/z* 287.0549 amu, in agreement with cyanidin (Esquivel et al., 2020). Peak **29** showed the neutral loss of 162 amu, while peak **31** showed the neutral loss of 308 amu, in agreement with hexoside and rutinoside, respectively. The identity of both compounds was confirmed by matching retention time with authentic standards and were assigned as cyanidin-3-*O*-ß-glycoside (**29**) and cyanidin-3-O-β-rutinoside (**31**). Both compounds were quantified in husk and mucilage (Table 3). The content of cyanidin-3-*O*-β-glycoside in husk ranged from 0.29 to 0.31 mg/100 g, while in mucilage the content was found between 0.06 and 0.12 mg/100 g. The content of cyanidin-3-*O*-β-rutinoside ranged from 1.01 to 1.92 mg/100 g in husk, and from 0.24 to 0.33 mg/100 g in mucilage. Our results agree with literature, where both anthocyanins have been reported in coffee husk among five different varieties, with cyanidin-3-*O*-β-rutinoside as the main anthocyanin (Prata & Oliveira, 2007). Esquivel et al. (2020) reported that anthocyanins were present at quantifiable levels only in the peels and not in the pulps of the coffee varieties Anaranjado, Arabica, Catuaí, Caturra Rojo. Cyanidin-3-*O*-ß-rutinoside was the main anthocyanin in the range of 1.02–9.07 mg/100 g dry weight, while cyanidin-3-*O*-ß-glycoside was found in the range of 0.05–0.23 mg/100 g dry weight (Esquivel et al., 2020), which are in a similar range to our findings.

3.1.5. Alkaloids

Purine and pyrimidine alkaloids have been identified in coffee and their presence plays important roles in the acceptance of final products for the consumers. Trigonelline (**1**) was identified in the husk, but not in the mucilage, by its $[M + H]^+$ base peak at *m/z* 138.0548 amu and the MS^2 signal at *m/z* 94.0657 amu which represents the loss of CO_2 (Wood et al., 2002). The compound plays an important role for the formation of furans, pyrayzine, alkyl-pyridines and pyrroles during coffee roasting, and it is involved in the bitterness, flavor and aroma of the beverage (Samanidou, 2015). The compound was below the quantification limit and therefore its content is not reported. The purine alkaloids theobromine (**8**) and theophylline (**11**) were identified by the $[M + H]^+$ base peak at *m/z* 181.0718 and 181.0719, corresponding to the molecular formula of $C_7H_9N_4O_2$. Theophylline was identified by the MS^2 characteristic ion at *m/z* 124.0507 amu, while theobromine presented the characteristic MS^2 ion at *m/z* 138.0662 amu (Mendes et al., 2019). Both

compounds were quantified after the targeted methylxanthines extraction, and their content is reported as caffeine equivalents. The content of theobromine ranged from 1.88 to 3.13 mg/100 g in the husk, while in the mucilage the content was between 1.36 and 2.06 mg/100 g. Theophylline ranged from 0.82 to 2.85 mg/100 g in the husk, while the content in the mucilage ranged from 0.71 to 2.31 mg/100 g. Caffeine (**19**) was identified based on the $[M + H]^+$ base peak ion at *m/z* 195.0876 amu and the MS^2 signal at 138.0389 (Mendes et al., 2019), as well as by matching with a commercial standard. Caffeine was the main phytochemical present in the husk and mucilage, with contents ranging from 400 to 480 mg/100 g and 223–239 mg/100 g, respectively (Table 2). Our results agree with the literature, where both caffeine and theobromine have been identified and quantified in coffee husk. In literature, caffeine was extracted with ethanol, while theobromine was obtained with dichloromethane from an unknown variety of coffee husk, and their contents were 12.96 g/100 g extract for caffeine and 0.075 g/100 g extract for theobromine (Andrade et al., 2012). Trigonelline has also been detected and quantified in coffee husk of the Bourbon Arabicas variety. The authors extracted Trigonelline with a mixture of MeOH and acetone (50 % *v*/v and 70 %v/v, respectively) and its concentration was 0.54 g/100 g (Cangussu et al., 2021).

3.2. Bound phenolics

Simple phenolic acids including gallic acid (**2**), dehydroshkimic acid (**3**), vanillic (**4**), 3-hydroxy-(3-hydroxyphenyl)-propionic acid (**5**), *p*-hydroxybenzoic acid (**10**), methylgallate (**13**), gentisic acid (**15**), syringic acid (**16**) and 2-hydroxy-2-phenylacetic acid (**36**) were identified in the bound phenolic extracts from husk and mucilage. The tentative identification of these compounds was based on fragmentation patterns and molecular formula. Kaempferol (**43**) was identified in the bound phenolic extract by the base peak signal at *m/z* 285.0407 amu (Merghany et al., 2024), while kaempferol glucoside (**40**) was identified at *m/z* 447.0931 amu (Pascoal et al., 2015). The compound of quercetin (**42**) was identified based on the $[M + H]^-$ base peak ion at *m/z* 301.0357 amu (Merghany et al., 2024). All these compounds were present at concentrations that were below the quantification limits.

Table 3 shows the concentration of compounds present in the bound phenolic extract that were above the analytical limits of quantification. The major compound was 3-CQA in both by-products within the range of 14.81–47.32 mg/100 g for coffee husk and 8.92–17.17 mg/100 g for mucilage. The alkaline hydrolysis is performed for the extraction of compounds that are covalently bound to the matrix, nevertheless, it leads to losses of phenolic compounds such as hydroxy compounds (Rodríguez-Durán et al., 2014). The compounds of caffeic acid, ferulic acid and *p*-Coumaric acid have been identified and quantified in spent coffee by alkaline hydrolysis, with concentrations of 156.27, 17.41 and 2.60 μmol/g lyophilized spent coffee extract, respectively (Monente et al., 2015). Moreover, caffeic acid was the only bound phenolic detected in lyophilized coffee pulp (unknown variety) (Da Silveira et al., 2020) but in the study of Rodríguez-Durán et al. (2014) chlorogenic acid, caffeic acid, ferulic acid, *p*-Coumaric acid and caffeic acid were identified and quantified in a Costa Rican cultivar (unknown variety) at different stages of maturity. To the best of our knowledge, this is the first study that investigates the presence of bound phenolics in industrial residues of coffee production.

3.3. Digestive stability, solubility and bioaccessibility of (poly)phenols from coffee husk and mucilage during in vitro digestion

The bioaccessibility of phytochemicals from coffee by-products was assessed *in vitro* following the INFOGEST-2 protocol (Brodkorb et al., 2019). The effect of gastrointestinal digestion is an important parameter to evaluate, considering the potential of different industry waste products as a source of bioactive compounds for circular economy. These studies are important because they can predict which phytochemicals are stable, soluble and accessible for a potential absorption at the intestinal level as well as their possible biological functions (Kasapoğlu et al., 2023).

The stability, solubility and bioaccessibility of main compounds present in the by-products is calculated comparing the contents obtained before and after gastrointestinal digestion the compounds 3-CQA, 5-CQA, caffeic acid, ferulic acid, coumaric acid, dicaffeoylquinic acid, rutin, quercetin-3-glycoside, caffeine and mangiferin were found as main compounds after *in vitro* digestion and the results are depicted in Fig. 1 for each batch number. Significant differences were observed between batch numbers for most of the compounds. Regarding (poly) phenols, 3-CQA was the least stable compound, with an average stability of 10.3 ± 4.9 % for the husk, and 9.2 ± 2.9 % for the mucilage (Fig. 1A). The low stability of 3-CQA has been previously observed (Burgos-Edwards et al., 2019). On the other hand, the most stable (poly)phenol compound was dicaffeoylquinic acid, with an average stability of 113.9 ± 29.7 % for husk, and 106.0 ± 30.8 % for mucilage (Fig. 1F). Caffeine was stable throughout the gastrointestinal digestion, with average stabilities of 177.0 ± 27.6 % for husk and 191.5 ± 10.9 % for mucilage (Fig. 1D).

After centrifugation, the supernatant obtained contains the soluble fraction of compounds that would remain after the intestinal step. Flavonols presented higher solubility compared to phenolic acids, with average values of 27.3 ± 1.0 % and 30.2 ± 7.3 % for rutin and quercetin glycoside in the husk, and 73.4 ± 26.2 % and 54.6 ± 18.4 % for the same compounds in the mucilage, respectively (Fig. 1I and J). Among phenolic acids, dicaffeoylquinic showed the highest solubility, with average values of 98.1 ± 11.9 % for husk, and 105.2 ± 33.5 % for mucilage (Fig. 1F). Caffeine was the most soluble compound during gastrointestinal digestion, with average solubilities values of 170.2 ± 30.3 % for husk and 187.4 ± 18.0 % (Fig. 1D).

The soluble fraction was further filtrated toto remove mixed micelles with particle size >0.22 μm, in order to retain those compounds that could potentially pass through the small intestine epithelium. Caffeine was the most bioaccessible compound with a % bioaccessibility range value of 138.79–166.87 % for coffee husk and 185.63–204.34 % for mucilage (Fig. 1D). No significant difference were observed among the batches within the same by-product or between husk and mucilage. Caffeine is present mainly in free form but may also associate non-covalently with matrix components such as polysaccharides or proteins, limiting its extractability prior to digestion (Santana Andrade et al., 2022). The acidic and enzymatic conditions during simulated gastrointestinal digestion can disrupt these interactions, releasing additional caffeine into the soluble fraction and leading to apparent bioaccessibility values above 100 % (Cañas et al., 2022). While small deviations could arise from analytical variability, the reproducible >100 % values across batches indicate digestion-mediated release is the more probable explanation. The high chemical stability of caffeine is explained by its hydrophilic and lipophilic properties, as well as its resistance to pH changes and digestive enzymes (Patil et al., 2022). This stability likely contributes to its persistence and efficient recovery. Our results are consistent with previous findings in coffee pulp flour and coffee pulp extract (*Coffea arabica* L. from a wet processing method and biomass from Nicaragua) with % bioaccessibility values of 82.2 and 84.9 %, respectively (Cañas et al., 2022).

Regarding the hydroxycinnamic acids, Fig. 1F shows that dicaffeoylquinic acid was the second compound with the highest % bioaccessibility values in the range of 71.24–111.57 % for coffee husk and 70.12–139.59 % for mucilage, with significant differences obtained among batch numbers. The third compound with % bioaccessibility greater than 100 % was *p*-Coumaric acid with range values of 77.43–100.10 % and 84.08–111.01 % for coffee husk and mucilage, respectively (Fig. 1E). Hydroxycinnamic acids can be covalently linked to the polysaccharides of the cell wall by ester bonds. We identified and quantified *p*-Coumaric acid in the bound fraction of phenolics from husk and mucilage (Table 3). Therefore, the high percentual bioaccessibility

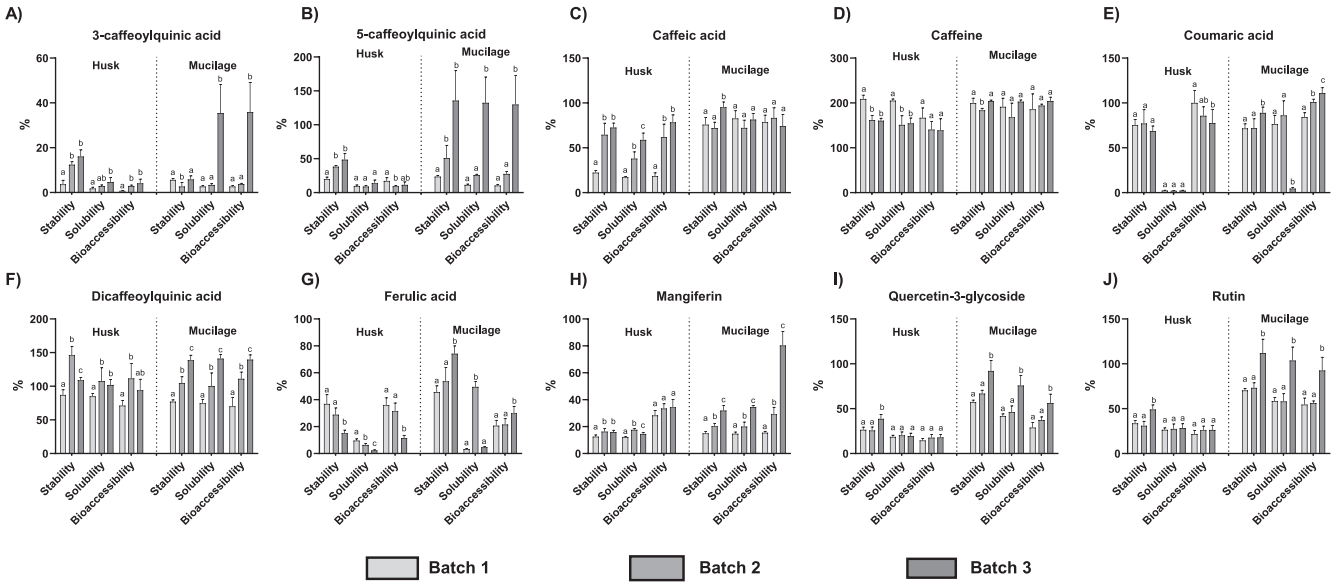

Fig. 1. Percentage values of stability, solubility and bioaccessibility of main phytochemical compounds present in coffee by-products after *in vitro* gastrointestinal digestion. Bars labelled with different lower-case letters indicate significant differences at $p < 0.05$ according to Tukey's test. Results are presented as mean values ± S·D (n = 3).

might be associated with the release of the compound from the matrix by enzymes that have degraded the cell wall (Cañas et al., 2022). Another reason could be because the deprotonation of phenolic compounds from the food matrix increases when neutral or slightly alkaline pH is used during *in vitro* digestion (Corona-Leo et al., 2021). The intestinal bioaccessibility of dicaffeoylquinic acid is consistent with the study of Cañas et al. (2022) which reported for 3,5-dicaffeoylquinic acid % bioaccessibility values of 78.9 % and 97.7 %, for coffee pulp flour and coffee pulp extract, respectively.

The % bioaccessibility values of the other hydroxycinnamic compounds were for caffeic acid 18.55–78.33 % for coffee husk and 74.10–83.32 % for mucilage (Fig. 1C); for ferulic acid 11.54–35.84 % and 20.72–21.56 % for coffee husk and mucilage, respectively (Fig. 1G); for 5-Caffeoylquinic acid 9.23–17.24 % for coffee husk and 10.41–129.95 % for mucilage (Fig. 1B), and for 3-Caffeoylquinic acid 0.767–4.70 % and 2.66–3535.93 % for coffee husk and mucilage, respectively (Fig. 1A). The lower % bioaccessibility of the hydroxycinnamic compounds during *in vitro* gastrointestinal digestion could be because to these (poly)phenols are more unstable than caffeine and, as a result, are more prone to degradation by digestive enzymes and changes in pH (Patil et al., 2022). When compared to other studies, the % bioaccessibility of phytochemicals from coffee husk such as caffeic acid and chlorogenic acid have been investigated. The % bioaccessibility of caffeic acid was 11.09 % and 10.68 % for extractable polyphenols and for hydrolysable non-extractable polyphenols, respectively. Regarding chlorogenic acid, the % bioaccessibility was 6.78 % and 44.76 % for extractable polyphenols and for hydrolysable non-extractable polyphenols, respectively (Abreu et al., 2024).

The % bioaccessibility of the flavonols quercetin-3-glycoside and rutin are depicted in Fig. 1I and J, respectively. In both cases, in the bioaccessibility of these compounds was higher in the mucilage compared to the husk, with range values of 56.37–92.55 % for mucilage *versus* 21.67–26.34 % for coffee husk, for rutin (Fig. 1J), and for quercetin-3-glycoside were 28.96–56.36 % for mucilage and 14.55–17.98 % for coffee husk (Fig. 1I). A previous study did not find rutin at the end of *in vitro* digestion of coffee pulp extract. However, in the same study, rutin showed a % bioaccessibility of 58.4 % when coffee husk flour was digested (Cañas et al., 2022). A possible explanation of the low % bioaccessibility of these two flavonoids compared to caffeine, dicaffeoylquinic acid and *p*-Coumaric acid could be due to most of the flavonoids exist in their glycoside form in the food matrix, which means that for their absorption in the intestinal phase, the sugar moiety must be eliminated in order to release the aglycone form and permeate through the intestinal phase or membrane cells (Cañas et al., 2022).

Mangiferin showed no significant differences in % bioaccessibility between batches of coffee husk, nevertheless, there were significant differences between batches in the mucilage. The % bioaccessibility range values were 28.33–34.34 % for coffee husk and 15.49–80.26 % for mucilage (Fig. 1H). The low bioaccessibility of mangiferin can be attributed to their degradation during gastrointestinal digestion, as reflected by its low stability (Fig. 1H). Roa-Tort et al. (2024) studied the % bioaccessibility of mangiferin and other compounds present in mango peel (Kent® variety) ethanol extracts. Authors reported that the % bioaccessibility of mangiferin was higher when the extracts were microencapsulated through spray drying (99.95 %) and spout-fluid bed drying (69.98 %).

Significant differences in stability, solubility, and bioaccessibility were observed among the three batches of coffee husk and mucilage. These variations are likely linked to differences in raw material characteristics, including coffee cherry maturity, cultivar, and environmental conditions during growth, as well as processing factors such as the time elapsed between harvest and pulping, fermentation duration, and drying conditions. Such factors can modulate the initial phytochemical profile, the proportion of compounds bound to cell wall components, and the composition of other matrix constituents, thereby influencing both compound degradation and release during digestion. Understanding these sources of variability will be essential for developing standardized extraction and processing strategies for industrial applications.

While simulated gastrointestinal digestion coupled to intestinal Caco-2 cell models are widely used to study bioaccessibility and intestinal bioactivity, both have inherent limitations that should be considered when extrapolating to human physiology. For *in vitro* digestion, the absence of a resident microbiota means that microbial metabolism, which can significantly and further alter the chemical profile and bioactivity of phytochemicals in the small and large intestine, is not captured (Guerra et al., 2012). Incorporating microbiota in future digestion models could provide a more accurate prediction of *in vivo* bioaccessibility. Similarly, Caco-2 cells, although representative of human enterocytes, differ from the intestinal epithelium *in vivo* in terms

of epithelial cell diversity, enzyme expression, and transporter profiles (Kalungwana et al., 2023). These differences may influence the uptake and metabolism of bioactive compounds. To bridge this gap, co-culture systems with mucus-producing HT-29 cells, or complementary *ex vivo* approaches, such as using mouse or human intestinal tissues, could provide more physiologically relevant data (Kondrashina et al., 2023).

3.4. Anti-inflammatory capacity of bioaccessible phytochemicals from coffee husk and mucilage in Caco-2 cells

Caco-2 cells differentiate into mature enterocytes when cultured for at least 21 days (Van De Walle et al., 2010). Prior to assessing anti-inflammatory activity, the potential cytotoxic effects of the bioaccessible fractions of digested coffee husk and mucilage were evaluated at concentrations of 0–50 μg GAE/mL for 24 h. No significant differences in cell viability were observed compared with untreated controls (Fig. S4). Similar findings have been reported for digested coffee extracts from green, light, and dark roasted *Coffea arabica* (Brazil Cerrado) and *Coffea canephora* (India cherry), which showed no toxicity toward Caco-2 cells at 50 μg/mL after 12 h incubation, despite containing chlorogenic acids and caffeine (Grzelczyk et al., 2024). These results confirmed that the digested extracts were non-cytotoxic under the tested conditions and could be used for subsequent anti-inflammatory assays.

Inflammation was induced by exposing differentiated Caco-2 cells to a pro-inflammatory cocktail of IL-1β (25 ng/mL), IFN-γ (50 ng/mL), and LPS from *Salmonella typhimurium* (1 μg/mL). Preliminary experiments (data not shown) confirmed that a 24 h stimulation period resulted in robust cytokine induction, with IL-6, IL-8, and TNF-α levels increasing by 28-, 5-, and 1.5-fold, respectively, compared with untreated controls (Fig. 2), consistent with an inflammatory phenotype (Melillo De Magalhães et al., 2012).

The pre-incubation of Caco-2 cells with 25 μg GAE/mL of the bioaccessible fraction from coffee husk or mucilage for 24 h prior to inflammatory stimulation significantly reduced IL-8 secretion for both by-products, with coffee husk showing greater inhibition (44–73 %) compared with mucilage (11–50 %) (Fig. 2B). IL-6 reduction was only significant for coffee husk batch 1 (23 %, Fig. 2A), while TNF-α suppression was observed exclusively for coffee husk across batches (13–16 %, Fig. 2C). These batch-specific differences may be linked to variations in raw material composition (*e.g.*, maturity, environmental conditions) or post-harvest processing parameters, which can influence the phytochemical profile and bioaccessibility.

The presence of chlorogenic acids (5-CQA, 3-CQA, dicaffeoylquinic acids), mangiferin, and caffeine in our digested samples may explain the observed anti-inflammatory effects, as these compounds have been reported to modulate inflammatory responses through NF-κB inhibition, antioxidant activity, and cytokine suppression. For instance, digested coffee extracts from green and roasted *Coffea spp.* beans reduced IL-6 (10–30 %) and TNF-α (10 %) secretion in LPS-stimulated RAW 264.7 macrophages (Grzelczyk et al., 2024). Similarly, undigested coffee leaf extracts containing 3,5- and 4,5-dicaffeoylquinic acids and mangiferin reduced TNF-α and IL-6 in Caco-2/U937 co-cultures (Mei & Chen, 2023). Mangiferin has shown strong cytokine suppression *in vitro*, reducing TNF-α and IL-6 by >80 % in LPS-stimulated macrophages (Jeong et al., 2014), while caffeine has been reported to attenuate IL-6 and TNF-α expression *in vivo* in rats through NF-κB downregulation (Li et al., 2011).

Beyond individual effects, synergistic interactions among bioaccessible phytochemicals likely contribute to the overall bioactivity of digested coffee husk and mucilage. Correlation analysis revealed that rutin, quercetin-3-glucoside, caffeine, and 5-CQA had the strongest negative associations with IL-8 and TNF-α secretion (Spearman $\rho = -0.56$ to -0.77, $p < 0.001$; Supplementary Table S2). These findings suggest that the anti-inflammatory effects arise from the combined action of multiple bioaccessible compounds, rather than from a single molecule. Furthermore, the coffee by-product matrix itself may enhance

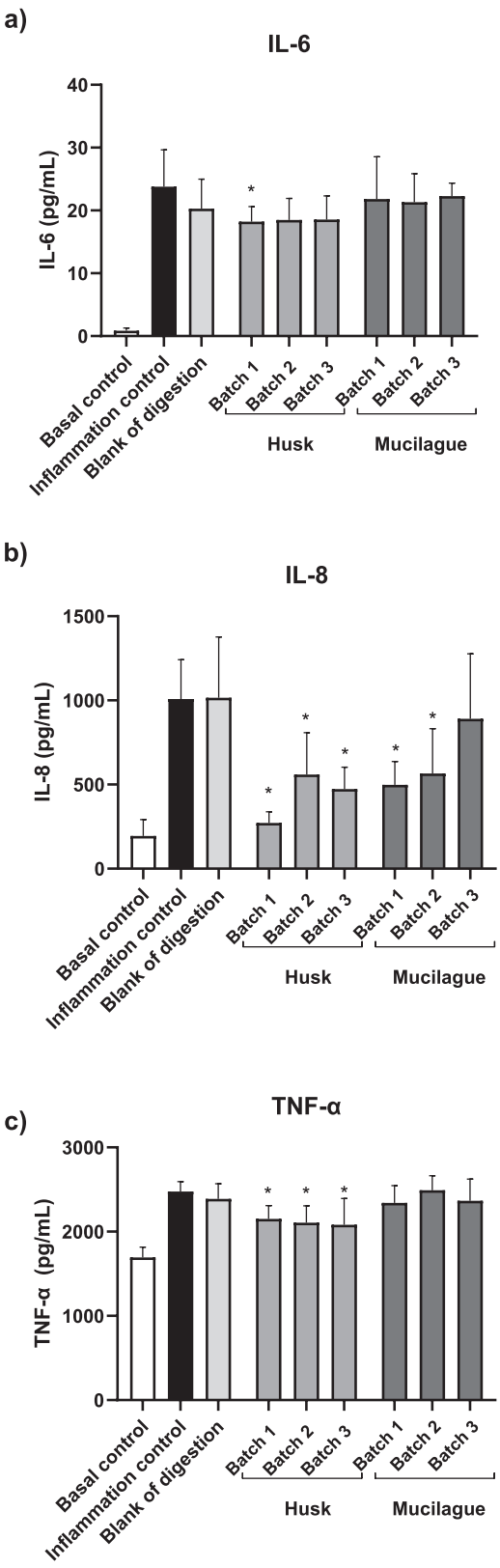

Fig. 2. Effect of *in vitro* gastrointestinal digested coffee by-products (*Coffea arabica* L.) on the reduction of cytokines secretion: A) IL-6; B) IL-8 and C) TNF-α in human Caco-2 cells (clone TC7) stimulated with interleukin IL-1ß, INF-γ and LPS. The results are expressed as means ± SD (n = 3). The symbol (*) on the bars shows significant differences of each treatment compared to the inflammation control. (Tukey's *t*-test; $p < 0.05$).

compound stability and solubility during digestion *via* non-covalent interactions between phenolics, fibers, and proteins. Such matrix effects could explain bioaccessibility values exceeding 100 % for certain compounds (*e.g.*, dicaffeoylquinic acid, p-coumaric acid, caffeine), reflecting release from bound forms or enhanced solubilization under gastrointestinal conditions.

4. Conclusions

The phytochemical profile of extracts from coffee husk and mucilage (*Coffea arabica* L.), the two major by-products of coffee processing, revealed a rich composition of phenolic acids, flavonoids, and alkaloids with anti-inflammatory potential. Simulated gastrointestinal digestion showed that while certain (poly)phenols degraded, while others remained highly bioaccessible. The digested extracts were non-toxic to Caco-2 cells and effectively reduced the release of pro-inflammatory cytokines, particularly IL-8, in a model of intestinal inflammation. These findings support the potential of coffee husk and mucilage as sources of bioactive compounds for nutraceutical or functional food applications, contributing to both human health and the circular economy. However, extrapolating these results to *in vivo* contexts requires caution, as systemic absorption, metabolism, and microbiota-mediated transformations may alter the bioactivity of the identified compounds. Future studies in animal models or human trials are needed to verify efficacy, determine pharmacokinetics, and identify active metabolites. The present work is also limited by the use of a single epithelial cell model, absence of dose–response curves, and inability to directly assign activity to individual compounds, as well as by batch-to-batch variability whose origins warrant further investigation. Translating these results into industrial use will require addressing practical considerations, including optimization of extraction processes to maintain compound stability at scale, regulatory compliance and safety assessments for coffee by-products in target markets, and economic feasibility in terms of processing costs, raw material availability, and potential market positioning. Future research should also explore formulation strategies that preserve bioactivity, ensure reproducibility across production batches, and meet consumer and industry requirements.

CRediT authorship contribution statement

Milena Jiménez-Gutiérrez: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Christian Zielinski:** Methodology, Investigation, Formal analysis. **Patricia Esquivel:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis, Conceptualization. **Jan Frank:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization. **Andrea Irías-Mata:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Felipe Jiménez-Aspee:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2025.107008.

Data availability

Data will be made available on request.

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