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Research Article

Nutritional, Bioactive Compounds, and Antioxidant Properties of Temanggung's Robusta and Arabica Coffee By-products: A Comparative Chemometric Analysis

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Abstract
Coffee by-products, including coffee husk and coffee pulp, have attracted increasing interest as a source of nutrition and bioactive compounds. This study aimed to investigate the different nutritional composition and bioactive compounds of coffee by-products obtained from arabica and robusta coffee processed in Temanggung, Indonesia. Four samples were evaluated: CHA (dried coffee husk arabica), CHR (dried coffee husk robusta), CPA (dried coffee pulp arabica), and CPR (dried coffee pulp robusta). Macronutrients, minerals, Total Polyphenol Content (TPC), Total Flavonoid Content (TFC), and antioxidant activity were measured. In addition, untargeted high-resolution mass spectrometry (HRMS) was employed to characterize the putative annotated bioactive compounds. The results showed that macronutrients, except fat content, differed significantly among samples, and minerals also differed significantly among samples. Principal Component Analysis (PCA) revealed a segregation among the samples, with a total PC of 83.8% in explaining the differentiation. In addition, HRMS analysis putatively annotated 84 bioactive compounds, which, in PCA, explained 77.1% of the total variance and showed distinct clustering among samples. Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) further identified differences in annotated metabolites related to antioxidant activity. Overall, this study shows that coffee husk and pulp contain valuable nutritional and bioactive compounds and may represent promising raw materials for valorizing coffee by-products and developing value-added functional ingredients.

Keywords: Bioactive compounds, Chemometrics, Coffee husk, Nutrition, Valorization

Applied Science and Engineering Progress, Vol. 20, No. 4, 2027, 8360

1 Introduction

Worldwide, coffee is the third most consumed beverage, after water and tea. The two major coffee varieties used as beverages are *Coffea arabica* (arabica) and *Coffea canephora* (robusta). The two species show differences in bioactive compounds and sensory properties [1]. However, this industry generates a significant amount of waste, accounting for around 40-50% of the cherry fruit [2]. The coffee husk (CH), a remaining waste of the fruit, has gained interest and has been processed into different types of by-products such as biochar, ceramics, functional drinks, and other products [3]. The coffee husks are processed using dry and wet processes. Both by-products offer potential as sources of nutritional and bioactive compounds because their characteristics differ, allowing different applications.

The characteristic diversity of coffee husks is influenced by species, post-harvest handling, and geographical origin [4]. Indonesia is renowned for its coffee beverage industry, specifically Temanggung, which has a distinct coffee industry, producing robusta and arabica coffee beans [5]. Arabica and robusta CHs show a similar nutritional composition, such as proteins (8-11%), minerals (3-7%), lipids (0.5-3%), and total carbohydrates (58-85%), as well as crude fiber fractions such as cellulose, hemicellulose, and lignin [1], [6]. However, notable differences emerge between the two species, with arabica coffee containing higher levels of carbohydrates and ash, which can be used as a proxy for its total mineral content [6]. A study shows that CH has higher amounts of potassium, calcium, phosphorus, and magnesium, which differ between arabica and robusta CHs [7].

CHs also differ by species, showing the diversity of bioactive compounds in robusta and arabica coffee. A study reveals that chlorogenic acid and its isomers are abundantly found in CH extracts, along with several other phenolic compounds such as gallic acid, caffeic acid, and tannins [8], which might provide antibacterial and anti-diabetic effects [9]. Robusta seed and husk contain caffeine and higher levels of chlorogenic acids, as well as proanthocyanidins and coumarins, than arabica. Arabica seed and its husk, on the other hand, retain a comparatively higher trigonelline content, and its husk flour has been studied as a valuable source of trigonelline, phenolic compounds, and caffeine [10]-[13]. Although some studies highlighted their differences, advanced analytical methods, such as high-resolution mass spectrometry (HRMS) for the comprehensive profiling of secondary metabolites and chemometric-assisted analysis, in arabica and robusta Temanggung coffee husk and coffee pulp have not been fully explored.

This study, thus, investigated the nutritional, bioactive compound, and antioxidant activity of different coffee husk by-product species of robusta and arabica from Temanggung, Indonesia. In addition, a chemometric approach was used to analyze the clustering of robusta and arabica coffee by-products.

2 Materials And Methods

2.1 *Materials*

Farmers in Temanggung, Jawa Tengah, Indonesia supplied arabica and robusta coffee by-products, which were planted at >1200 Meters Above Sea Level (MASL) and 600-800 MASL, respectively. The coffee by-product samples were obtained by processing coffee berries, which were then processed using different methods. The dry method involves drying the whole coffee cherry before mechanically removing the dried fruit layers from the coffee bean (Supplementary Figure 1). The resulting dried coffee husk (CH) comprises the outer skin (exocarp), fleshy pulp (mesocarp), and the dry, papery parchment layer surrounding the bean. In contrast, the wet method mechanically removes the outer skin, yielding pulp from coffee cherries before fermentation and drying (Supplementary Figure 1). In this study, we processed wet-method coffee pulp into dried wet coffee pulp (CP). Thus, CH and CP differ in their processing origins and anatomical composition, which may contribute to differences in their nutritional, phytochemical, and metabolomic profiles. All samples were subsequently powdered and sieved to a particle size of >100 mesh. This study had 4 samples (CHA, CPA, CHR, and CPR): dried coffee husk arabica, dried coffee husk robusta, dried coffee pulp arabica, and dried coffee pulp robusta, respectively, which were extracted in triplicate on three independent occasions. Chemical reagents were purchased from Merck (Germany), including methanol (liquid chromatography grade), gallic acid, carbonic acid, Folin-Ciocalteu, potassium acetate, and 2,2-diphenyl-1-picryl-hydrazil-hydrate (DPPH). In addition, chemicals such as nitric acid (HNO_3), quercetin, and aluminum chloride were bought from Sigma (Germany). All standards used, including magnesium

(Mg), manganese (Mn), sodium (Na), zinc (Zn), aluminum (Al), calcium (Ca), copper (Cu), iron (Fe), and potassium (K) in the mineral analysis, were also bought from Sigma (Germany).

2.2 *Ethanolic extraction*

An extraction with 80% ethanol at a 1:10 mass-to-solution ratio was performed for 40 minutes in a sonicator (JP SeleCPA, Spain) at 40 Hz [14]. After centrifuging for 20 minutes at 10,000 rpm, the solution was dried using a vacuum oven. The yield was calculated using the weight of dried extract divided by the weight of raw material multiplied by 100, as indicated in Equation (1) below.

$$Yield(\%) = \frac{Weight\ dried\ extract\ (g)\ x\ 100\%}{Weight\ plant\ sample\ (g)} \quad (1)$$

The dried extracts were stored at 20 °C in a freezer until analysis.

2.3 *Nutritional and mineral analysis*

Nutritional analysis measured protein, fat, and carbohydrate. All analyses were performed as described in Standar Nasional Indonesia number SNI 01-2891-1992. Fat was determined by hexane extraction, and protein by Kjeldahl analysis. Moreover, carbohydrates were calculated by difference as: 100 − (protein + fat + ash). In addition, crude fiber and moisture content were analyzed using acid-base extraction and the gravimetric method, respectively.

Mineral analysis was performed using ICP-MS, analyzing Mg, Mn, Na, Se, Zn, Al, Ca, Cu, Fe, and K [15]. Approximately 0.200–0.300 g of homogenized sample was accurately weighed into a microwave digestion vessel, then 8 mL of concentrated HNO_3 was added. Samples were digested using a microwave digestion system at 190°C for 30 min. After cooling, the digests were quantitatively transferred to 50-mL volumetric flasks and diluted to volume with ultrapure water. The solutions were homogenized and filtered through a 0.2-µm membrane before ICP-MS analysis. A procedural blank containing the digestion reagents but no sample was processed using the same digestion procedure. Lastly, a standard curve for each mineral, ranging from 10 to 1000 ppm, was prepared to calculate the mineral content in the samples.

2.4 *Total phenolic content*

Twenty µl of sample/blank/standard was added to the well according to Nasori's method [16]. After that, 50 µl of Folin-Ciocalteu solution was added to the well and incubated for 30 minutes. Lastly, 50 µl of sodium bicarbonate was pipetted and reincubated for 90 minutes. An ELISA reader at 780 nm was used for the reading. The concentration of the samples (mg GAE/g extract) was calculated using a gallic acid standard curve spanning 20-740 ppm.

2.5 *Total flavonoid content*

A method by Rahayu was used to calculate the total flavonoid content [17]. 50 µl of sample/blank/standard and 50 µl of methanol were added to the well. After that, 10 µl of $AlCl_3$ and 100 µl of methanol were added to the well. The samples were read at 410 nm using an ELISA reader. A standard curve for quercetin, ranging from 10 to 500 ppm, was prepared to calculate the TFC in the extracts, expressed as mgQE/g extract.

2.6 *Bioactive compounds analysis using Ultra-performance liquid chromatography-high resolution mass speCPRometry (UPHLC-HRMS)*

Ultra-performance liquid chromatography-high resolution mass speCPRometry (UPHLC-HRMS) was used to analyze the bioactive compounds in the extract using a UHPLC Quadrupole-Orbitrap High Resolution Mass SpeCPRometer (Thermo Scientific, USA). The chromatography step used a Thermo Scientific Accucore Phenyl-Hexyl analytical column (100 mm × 2.1 mm ID × 2.6 µm). MS-grade water with 0.1% formic acid (A) and MS-grade methanol with 0.1% formic acid (B) were used as solvents and delivered at 0.3 mL/min in a gradient. Five percents of phase B gradually increased to 90% over a period of 16 minutes. The concentration was maintained for 4 minutes before being decreased to 5% B by the 25-minute point. The column was kept at 40 °C, with an injection volume of 3 microliters. For untargeted screening, we employed full MS/dd-MS2 acquisition mode in both positive and negative ionization modes. The sheath, auxiliary, and sweep gases were set at 32, 8, and 4 arbitrary units, respectively, with nitrogen used for each. The spray voltage was set at 3.30 kV, with a capillary temperature of 320 °C and an auxiliary gas heater temperature of 30 °C. Scans were conducted over a mass range of 66.7–1000 m/z, with

Applied Science and Engineering Progress, Vol. 20, No. 4, 2027, 8360

resolutions of 70,000 for full MS and 17,500 for dd-MS2 in both ionization modes. The system was operated using Xcalibur 4.4 software (Thermo Scientific, Bremen, Germany). The instrument was tuned and calibrated weekly using Thermo Scientific Pierce ESI ion calibration solutions (Waltham, MA) for both ESI-positive and ESI-negative modes, ensuring consistent mass accuracy (<5 ppm), ion transfer, ion isolation, and sensitivity throughout the analysis [18]. The annotated putative compounds for statistical analysis were selected using an mzCloud best match of over 90, and the relative intensity of each compound was calculated as the ratio of the compound ion intensity to the total ion intensity (annotated and unannotated compounds), multiplied by 100%, as indicated in Equation (2) below.

$$Relative\ Intensity\ (\%) = \frac{\text{Ion intensity of annotated compound } x\ 100\%}{\text{Total intensity of all of the compounds (annotated or unannotated)}} \quad (2)$$

2.7 *Antioxidant activity*

The DPPH (Sigma-Aldrich, Germany) method was used to determine the extracts' antioxidant activity using vitamin C as a positive control [19]. Each well received 100 µL of sample or a positive control, along with 100 µL of 0.2 mM DPPH solution. The plate was read at 517 nm after 30 minutes of dark incubation. Antioxidant activity was evaluated by determining percentage inhibition, then calculating the IC50 value, which represents the sample concentration needed to inhibit 50% of the DPPH free radical, as shown in Equation (3) below.

$$\text{Percent Inhibition}(\%) = \frac{\text{Blank absorbance} - \text{Sample absorbance}}{\text{Blank absorbance}} \quad (3)$$

2.8 *Statistical analysis*

The PAST software was used to analyze differences in macronutrients and minerals, TPC, TFC, and antioxidant activity among the extracts using ANOVA, after the test of normality and Levene's test appropriate for the ANOVA test [20]. This was then followed by the Tukey test for calculating the differences between the samples. Next, Principal Component Analysis (PCA) was performed in MetaboAnalyst 6.0 for nutritional composition and untargeted bioactive compounds [21]. In addition, MetaboAnalyst 6.0 was used for OPLS-DA analysis of antioxidant activity across extracts, with IC50 values less than 100 ppm indicating very strong/strong activity and between 100 and 200 ppm indicating moderate or weak activity, as categorized in the study [22].

3 Results and discussions

3.1 *Macronutrient and mineral compositions*

The macronutrient and micronutrient compositions of dried coffee husks and coffee pulp prepared from arabica and robusta coffee are presented in Table 1. Results were on a dry basis; water content was 7.48%, 7.98%, 9.66%, and 7.04% for CHA, CPA, CHR, and CPR, respectively. This study reported significant differences (p-value < 0.001) for all measured parameters except fat content. Carbohydrates were the major macronutrient among the samples, ranging from 74.00–78.80 g/100 g dry basis (db), with CPA at the highest level (78.80 g/100 g) and CHR at the lowest (74.00 g/100 g db). Protein content ranged from 12.00 to 14.25 g/100 g, with CHR exhibiting the highest concentration. A study found that arabica and robusta CH had fat (1.20 vs. 1.78%), protein content (8.98 vs. 8.95%), and a higher carbohydrate content (78.02 vs. 73.43%) [6], showing a similar pattern in this study. Ash content ranged from 7.53 to 11.12 g/100 g db (p-value < 0.001). A study found that, compared with arabica CH, robusta CH had lower ash (7.36 vs. 9.16%) [6]. Crude fiber values ranged from 40.12 to 42.43 g/100 g db, with CHR being the only different sample (p-value < 0.001).

Supplementary Table 1 shows the analytical performance of the mineral analysis using ICP-MS over a concentration range of 10 to 1000 µg/L. Linearity was close to 1 for nine elements, with determination coefficients (R^2) ranging from 0.9976 (K) to 1.0000 Cu, indicating a strong correlation between measured signal intensity and analyte concentration across the calibration range. LOD values ranged from 0.08 ppm (Fe) to 29.31 ppm (Se), while LOQ values ranged from 0.26 ppm (Fe) to 5.3 ppm (Na), reflecting the wide variation in instrumental sensitivity among the elements analyzed. In particular, this study found significant differences in all analyzed minerals, including Mg, Mn, Na, Al, Ca, Cu, Fe, and K (p-value < 0.001). This study also showed that robusta outperformed arabica in K, Ca, Mg, Na, and Cu content. However, no studies compared the mineral content of arabica and robusta CHs. In

Table 1: Macronutrients and minerals in 100 g (db) of CHA, CPA, CHR, and CPR.

Macro-and micronutrients	Mean±SD				*p*-value
	CHA	CPA	CHR	CPR	
Carbohydrate (g)	78.39±0.55[a]	78.80±0.52[a]	74.00±0.53[b]	78.34±0.51[a]	<0.001
Protein (g)	13.32±0.18[b]	12.63±0.18[c]	14.25±0.18[a]	12.00±0.20[d]	<0.001
Fat (g)	0.76±0.19	0.79±0.17	0.64±0.17	0.65±0.14	0.621
Crude fiber (g)	42.43±0.28[a]	42.31±0.28[a]	40.12±0.23[b]	41.89±0.23[a]	<0.001
Ash (g)	7.53±0.18[c]	7.79±0.17[c]	11.12±0.18[a]	9.01±0.17[b]	<0.001
Magnesium (mg)	1709.30±6.93[b]	1701.13±10.70[b]	2692.94±6.35[a]	1463.28±43.99[c]	<0.001
Manganese (mg)	4.37±0.03[c]	5.52±0.04[b]	11.09±0.04[a]	3.92±0.01[d]	<0.001
Sodium (mg)	27.23±0.21[b]	12.61±0.45[d]	79.10±1.20[a]	16.37±0.19[c]	<0.001
Zinc (mg)	0.79±0.02[d]	1.38±0.01[a]	1.31±0.01[b]	1.04±0.03[c]	<0.001
Aluminum (mg)	42.53±0.11[a]	19.16±1.39[c]	41.70±0.35[a]	24.72±0.31[b]	<0.001
Calcium (mg)	359.84±1.58[d]	377.10±5.69[c]	737.94±5.42[a]	424.35±5.11[b]	<0.001
Copper (mg)	2.17±0.04[c]	1.97±0.03[d]	6.01±0.05[a]	3.23±0.09[b]	<0.001
Iron (mg)	21.14±0.14[b]	24.98±0.73[a]	21.60±0.07[b]	24.36±0.10[a]	<0.001
Potassium (mg)	5063.32±254.66[b]	5146.98±152.85[b]	6425.91±164.07[a]	6402.34±239.30[a]	<0.001

Different letters indicate significant differences among samples at **p**-value<0.05.

addition, the most abundant minerals found were potassium (5,063.32 to 6,425.91 mg/100 g), magnesium (1,463.28–2,692.94 mg/100 g), and calcium (359.84–424.35 mg/100 g). This study aligns with Nguyen et al 2025, showing potassium as the highest mineral content [23]. The mineral analysis showed that Mg, K, and Ca were present at relatively high concentrations compared with the other elements analyzed. These findings are nutritionally relevant because Mg, K, and Ca are essential minerals involved in numerous physiological functions, including muscle and nerve function, electrolyte balance, bone development, and cellular metabolism [24]. However, these minerals' nutritional contribution should not be inferred from concentration alone, because their actual dietary contribution depends on intake and bioavailability.

This study also found that aluminum concentrations were high in the samples, particularly in CH rather than CT. The relatively high Al concentration in CH samples may be linked to environmental and processing-related factors, including accumulation from soil, water, or agricultural inputs. In contrast, CT samples showed half the aluminum content of CH. Because aluminum is not an essential mineral, higher consumption should be considered for safety. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) established a provisional tolerable weekly intake (PTWI) for Al of 2 mg/kg body weight/week, based on the potential health effects of long-term exposure [25]. Therefore, potential dietary exposure to Al from coffee by-products should be considered in relation to the amount consumed, consumption frequency, and the extent of Al transfer into the prepared beverage.

A further analysis using Principal Component Analysis (PCA) was performed to evaluate the variation in macro- and mineral composition among the four coffee by-product samples (Figure 1). PCA is an unsupervised statistical analysis used as an exploratory tool to evaluate the overall distribution of samples and identify natural clustering patterns without prior knowledge of class membership [26]. The first two principal components explained 83.8% of the total variance, with PC1 and PC2 accounting for 65.3% and 18.5%, respectively. This high cumulative variance indicates that the PCA model adequately captured the nutritional differences among the samples. The score plot showed clear separation among the four sample groups, indicating distinct nutritional profiles between coffee species and processing forms. CHA was on the positive side of

Applied Science and Engineering Progress, Vol. 20, No. 4, 2027, 8360

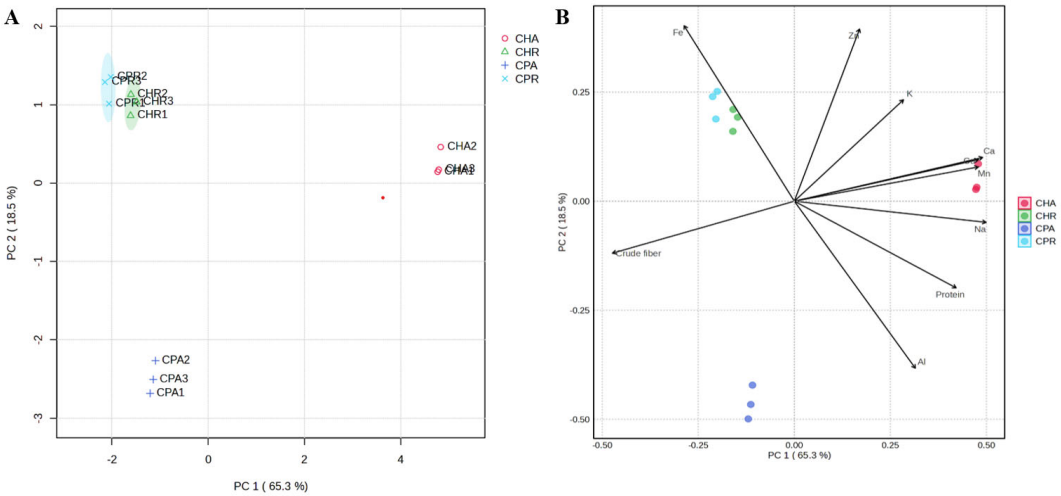


Figure 1: The unsupervised analysis using Principal Component Analysis of nutritional composition in different coffee husk wastes, namely CHA, CPA, CHR, and CPR. CHA, CHR, CPA, and CPR correspond to dried coffee husk arabica, dried coffee husk robusta, dried coffee pulp arabica, and dried coffee pulp robusta, respectively.

PC1, whereas the other samples were on the negative side, suggesting that CHA had a markedly different nutrient composition. CPA separated distinctly from the other samples along PC2, highlighting compositional differences between CPA and the other coffee by-products. In contrast, CHR and CPR grouped closely together, reflecting greater similarity in their overall nutritional profiles than the CHA samples.

The loading plot offered additional insight into the variables driving sample separation. CHA correlated with elevated levels of potassium, magnesium, calcium, manganese, sodium, phosphorus, and aluminum, in line with the mineral data in Table 1. Minerals such as iron were linked to CHR and CPR, indicating that the coffee pulp process did not alter these minerals and distinguished them from robusta by-products. Crude fiber content was the key factor in distinguishing the CPA from other samples.

3.2 *Yield, total polyphenol content, and total flavonoid content*

Based on Figure 2, the extraction yield, total phenolic content (TPC), and total flavonoid content (TFC) differed significantly among the coffee husk-derived samples (p-value < 0.05), indicating that both coffee species and processing methods influenced the recovery of bioactive compounds. CPA showed the highest extraction yield (approximately 32%), followed by CPR (approximately 20%), CHA (approximately 19%), and CHR (approximately 5%) (Figure 2A). The substantially higher yield from the coffee pulp samples suggests that the extraction process released water-soluble constituents from the coffee husk matrix. In contrast, the low yield of CHR may indicate a higher proportion of insoluble structural components or lower extractability of its chemical constituents under the extraction conditions employed.

TPC values also differed significantly (Figure 2B). CPR exhibited the highest phenolic content (approximately 95 mg GAE/g extract), followed by CHA (approximately 55 mg GAE/g extract), CHR (approximately 45 mg GAE/g extract), and CPA (approximately 30 mg GAE/g extract). The remarkably high TPC of CPR indicates that coffee pulp is a particularly rich source of phenolic compounds. This research aligns with previous findings showing that TPC in robusta waste was higher than in Arabica [6], [27]. Robusta contains higher levels of polyphenols such as caffeine, chlorogenic acids, rutin, quercetin, caffeic acid, and unique markers (roemerine, cajanin), while Arabica is rich in diverse chlorogenic acid derivatives, procyanidins, catechin, and methylxanthines [10-12]. However, this interpretation should be made cautiously because the Folin–Ciocalteu assay used to determine TPC is not specific to phenolic compounds,

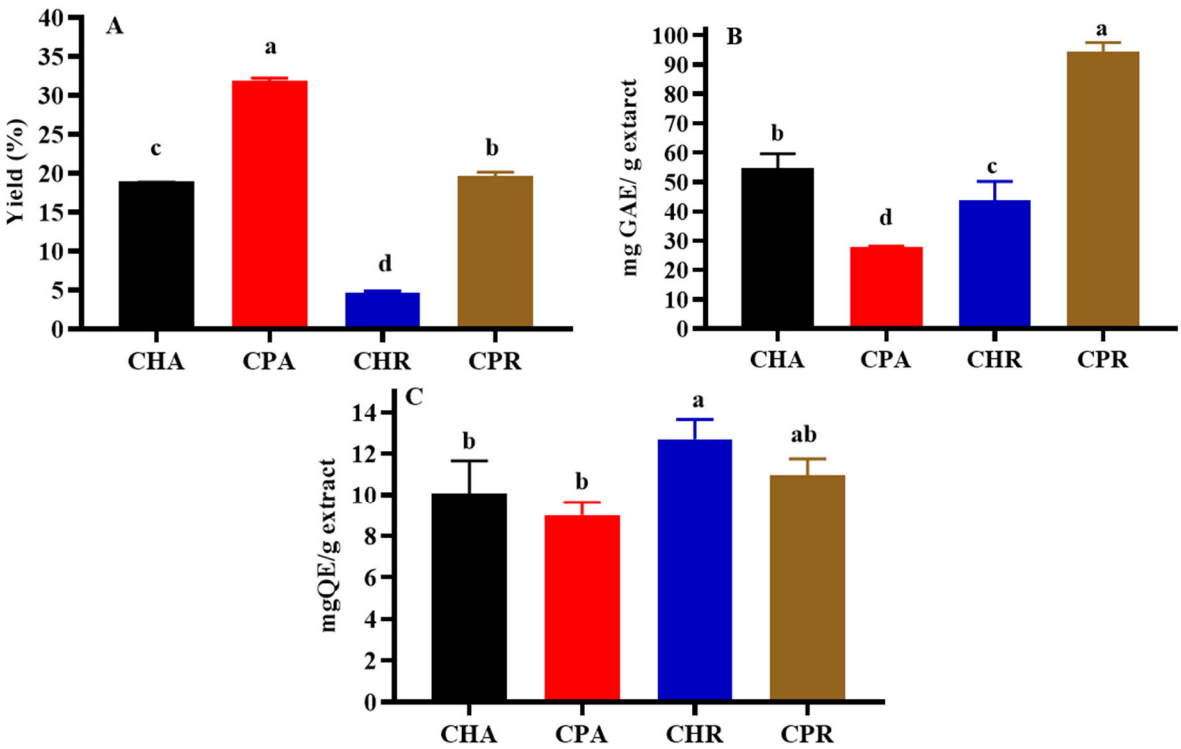


Figure 2: The yield (A), total phenolic content (B), and total flavonoid content (C) among the samples, namely CHA, CPA, CHR, and CPR, which correspond to dried coffee husk arabica, dried coffee husk robusta, dried coffee pulp arabica, and dried coffee pulp robusta, respectively. Different letters show significant differences among the samples at p-value < 0.05.

as it measures the sample's reducing capacity by reducing the phosphomolybdic/phosphotungstic reagent and can therefore respond to other reducing substances present in the matrix [28].

Figure 2C shows the TFC values, ranging from 9 to 13 mg QE/g extract. CHR had the highest TFC, followed by CPR, CHA, and CPA. CHR showed the highest flavonoid content, followed by CPR, while CHA and CPA exhibited lower but statistically comparable values. The study found that robusta was superior to arabica, showing a value of 0.83 to 4.07 mg QE/g [29]. Moreover, the study also found that robusta coffee husk increased its TFC when fermented with *Leuconostoc mesenteroides* [6].

3.3 *HRMS Bioactive compounds and chemometric-assisted analysis*

The compound-discovery software detected 732 molecular features, of which 434 were assigned compound annotations, while 298 remained unannotated (Figure 3). Among the annotated compounds, 93 achieved mzCloud library match scores >90% and were considered putatively annotated. Nine of these compounds were not recognized as naturally occurring compounds and were therefore excluded from further analysis. The remaining 84 putatively annotated compounds were included in the subsequent statistical analysis (Supplementary tables 2 and 3). Consequently, 84 annotated compounds were retained for both unsupervised Principal Component Analysis (PCA) and supervised Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) of antioxidant activity (Supplementary Material).

The PCA was performed to explore variations in the bioactive compound profiles among the coffee husk-derived samples. The first two principal components explained 77.1% of the total variance, with PC1 and PC2 accounting for 55.6% and 21.5%, respectively (Figure 4). The relatively high cumulative variance indicates that the PCA model effectively

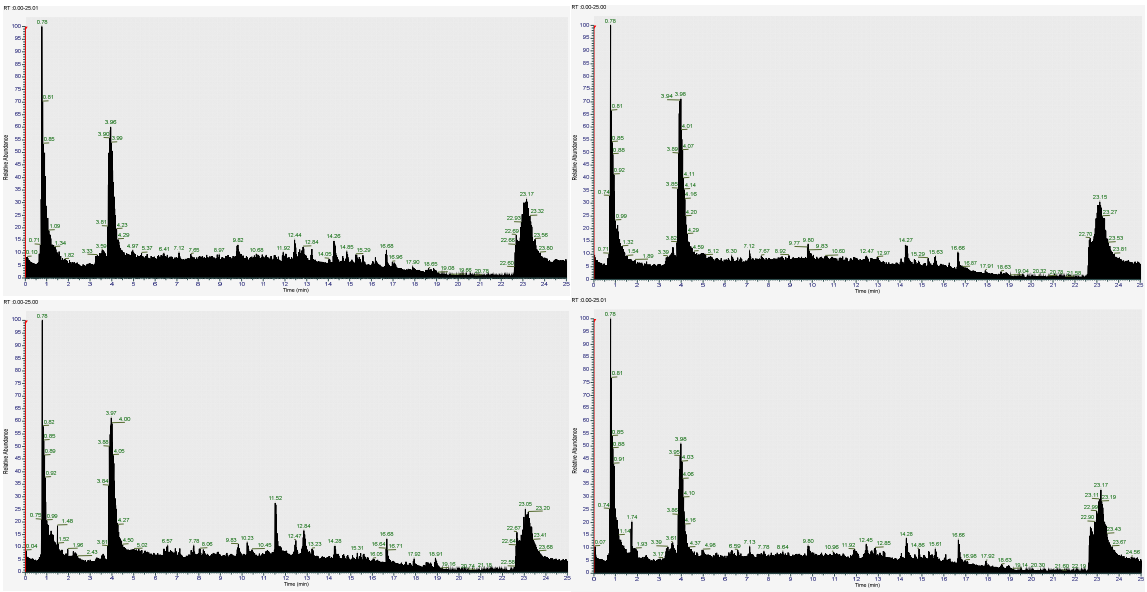


Figure 3: Total ion chromatograms (TIC) of CHA (A), CPA (B), CHR (C), and CPR (D), which correspond to dried coffee husk arabica, dried coffee husk robusta, dried coffee pulp arabica, and dried coffee pulp robusta, respectively.

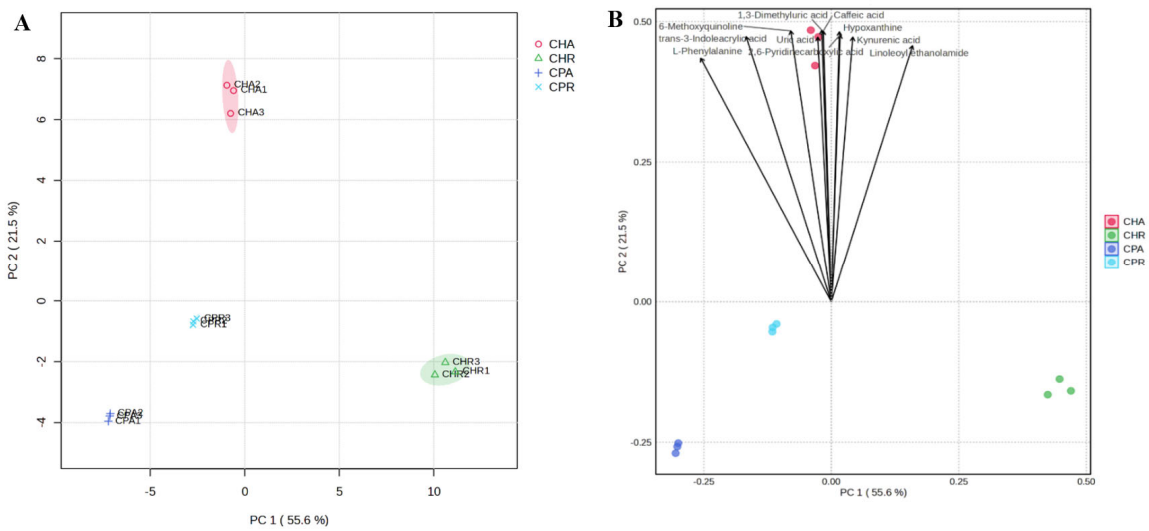


Figure 4: Principal component analysis (PCA) of annotated bioactive compounds in coffee husk-derived samples. (A) PCA score plot showing the distribution of CHA, CHR, CPA, and CPR samples based on their annotated bioactive compound profiles. (B) PCA biplot illustrating the annotated compounds contributing most strongly to sample discrimination.

summarized the differences in chemical composition among the samples. The score plot revealed a clear separation of the four sample groups, suggesting that both coffee species and processing method influenced the bioactive compound composition. CHA was positioned in the positive region of PC2, while CHR appeared in the positive region of PC2. In contrast, CPA and CPR clustered in the negative regions.

This pattern suggests that the process significantly altered their metabolite profile. Biplot analysis reveals that phenolic acids and related metabolites, such as caffeic acid, methoxycoumarin, 1,3-dimethoxyuric acid, kynurenic acid, and trans-3-indoleacrylic acid, exhibited strong positive loadings on PC2 and were closely linked to the CHA samples.

3.4 *Antioxidant activity*

Based on the IC50 values, CPR (33.80 ± 1.91 ppm) exhibited the strongest antioxidant activity, followed by CHA (64.07 ± 2.43 ppm), CHR (103.28 ± 3.34 ppm), and CPA (175.73 ± 6.62 ppm) (Figure 5). As expected, vitamin C showed the highest antioxidant activity among all tested samples (5.13 ± 0.03 ppm), serving as a positive control. This finding is consistent with previous studies reporting differences in antioxidant activity between robusta and arabica coffee by-products. A study observed that robusta coffee husk has higher potency as an antioxidant than arabica coffee husk, showing IC50 values of 33.95 and 44.94 ppm, respectively [27]. Similarly, a study also reported higher antioxidant capacity for robusta than arabica spent coffee ground when extracted with water and ethanol, with values of 396.6 vs 326.6 mMAAE/g and 460.8 vs 366.6 mMAAE/g, respectively [30]. Although these values use different antioxidant metrics, which should not be directly compared quantitatively, both studies indicate a similar trend: greater antioxidant activity or capacity in robusta-derived by-products than in arabica.

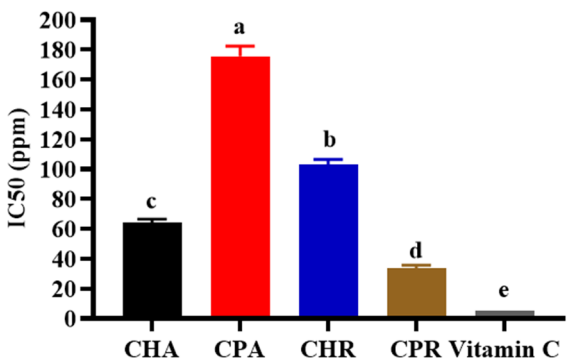


Figure 5: Antioxidant activity (IC50) against DPPH for CHA, CPA, CHR, and CPR, corresponding to dried coffee husk arabica, dried coffee pulp arabica, dried coffee husk robusta, and dried coffee pulp robusta, respectively. Different letters show significant differences between the samples.

To explore annotated metabolites associated with differences in antioxidant activity, OPLS-DA was performed using the metabolite profile data and samples categorized according to their DPPH radical scavenging activity [31]. The OPLS-DA score plot displayed a distinct separation between samples with very strong/strong and moderate/weak antioxidant activities (Figure 6A). However, CTR and DHA samples were classified as very strong/strong, whereas CTA and DHR samples were classified as moderate/weak. Thus, the antioxidant-activity category was completely confounded with sample type, and the observed separation may reflect differences among sample types rather than a metabolite pattern specifically associated with antioxidant activity. Therefore, the OPLS-DA results should be interpreted as exploratory and hypothesis-generating rather than definitive evidence of a general metabolite signature associated with antioxidant activity.

The model was further assessed using a permutation test with 1,000 permutations (Figure 6B). The original model showed R^2Y and Q^2 values of 0.993 and 0.982, respectively. Only 9 of the 1,000 permuted models achieved values comparable to or greater than those of the original model, corresponding to permutation *p*-values of 0.009 for both R^2Y and Q^2. These findings indicate that the observed class separation was unlikely to have arisen solely from random class assignment. Nevertheless, permutation testing cannot completely rule out overfitting or establish the model's generalizability to an independent sample. Therefore, the OPLS-DA model and associated VIP metabolites are exploratory and hypothesis-generating and require confirmation in larger, independently validated sample sets.

VIP analysis was then used to identify the annotated compounds that contributed most to sample discrimination between the two antioxidant categories (Figure 6C). However, because antioxidant-activity category was confounded with CH and CP sample type, these VIP metabolites may also represent annotated metabolites that differentiate the underlying sample types. The top ten metabolites were neochlorogenic acid, 4,5-dicaffeoylquinic acid, 7-hydroxy-6-methoxy-2H-chromen-2-one, (1S,3R,4R,5R)-1,3,4-trihydroxy-5-{[(2E)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoyl]oxy}cyclohexane-1-carboxylic acid, palmitic acid, L-phenylalanine, trans-3-indoleacrylic acid, chlorogenic acid, 6-methoxyquinoline, and fraxetin. Several of these

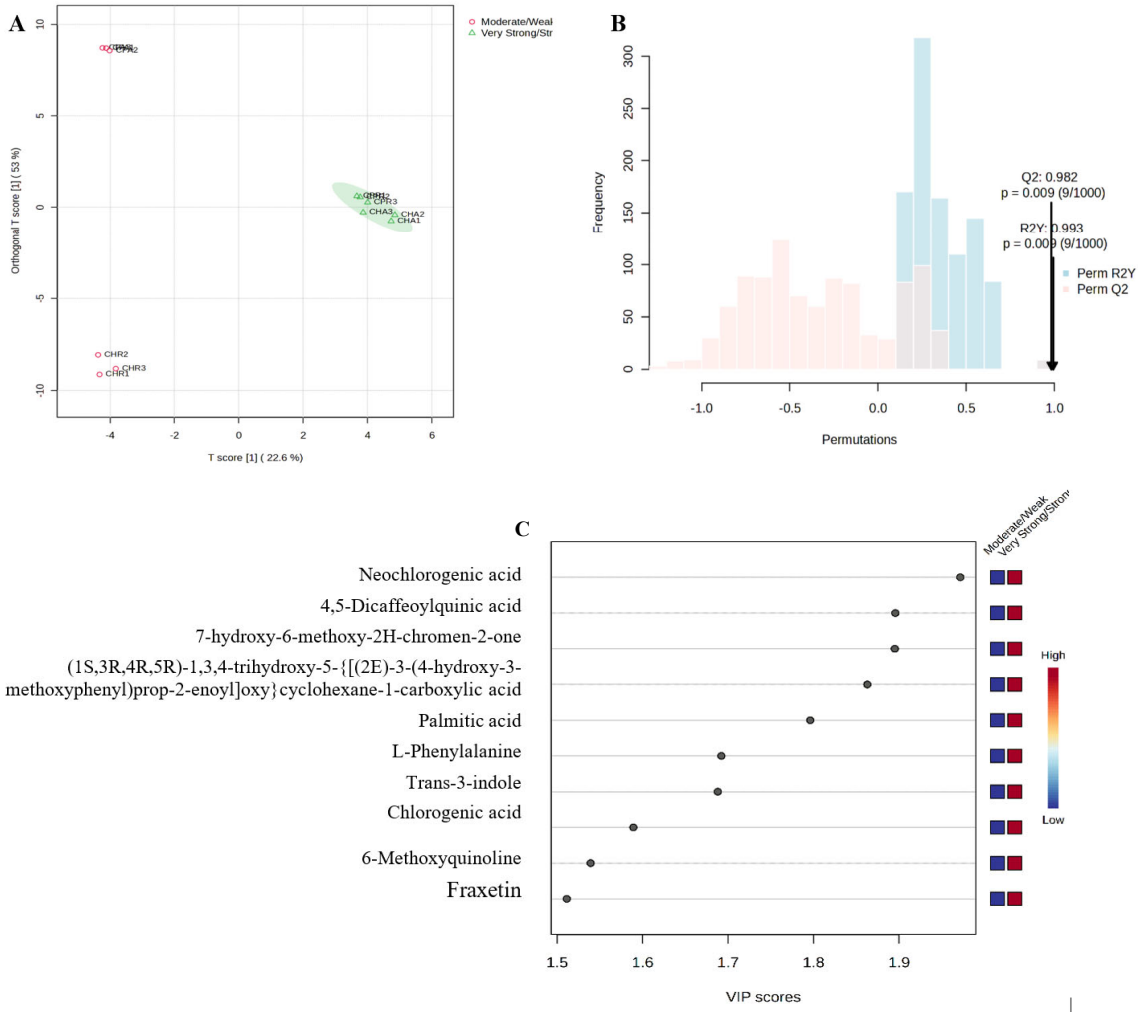


Figure 6: Orthogonal partial least squares discriminant analysis (OPLS-DA) of bioactive compounds associated with antioxidant activity in arabica and robusta coffee husk and coffee pulp. Samples were classified into very strong to strong and moderate to weak antioxidant activity. (A) OPLS-DA score plot showing discrimination between antioxidant activity classes; (B) permutation test (1,000 permutations) validating the robustness of the OPLS-DA model; and (C) the top 10 compounds ranked by variable importance in projection (VIP) scores contributing to antioxidant activity differentiation.

compounds, particularly chlorogenic acid and its derivatives, have been associated with antioxidant activity since they can donate their hydrogen atoms [32]. Chlorogenic acid and its derivatives have been reported to exhibit radical scavenging activity because their multiple hydroxyl groups boost electron-donating ability [33]. Coumarin-related compounds, such as fraxetin and 7-hydroxy-6-meth oxy-2H-chromen-2-one, might also increase antioxidant activity through their redox-active structure [34]. However, because sample type and antioxidant-activity category were completely confounded, the VIP metabolites cannot be conclusively attributed to antioxidant activity alone. Accordingly, these compounds are candidate metabolites associated with the observed differences in antioxidant activity, and their contribution should be further validated using larger, independently collected sample sets, targeted quantitative analysis, and compound-specific antioxidant assays.

A limitation of the Total Ion Chromatogram (TIC)-normalized UHPLC-HRMS approach is that relative intensities are semi-quantitative and may be influenced by highly abundant metabolites, adducts, isotopes, and background signals; therefore, they should not be interpreted as absolute concentrations. In addition, the small number of biological replicates relative to the 84 metabolite variables and the complete confounding between sample type and antioxidant-activity category limit the interpretation of the OPLS-DA and VIP results. Although permutation testing indicated non-random class separation, it cannot exclude overfitting or establish generalizability to independent samples. Thus, these findings are exploratory and hypothesis-generating and require validation using larger, independently designed sample sets and targeted quantitative analyses. The relatively high aluminum content and other potential food-safety concerns, along with the use of *in vitro* antioxidant assays, also warrant further evaluation before proposing food- or health-related applications.

4 Conclusions

The present study showed substantial differences in the nutritional composition, mineral content, phytochemical profiles, metabolite composition, and in vitro antioxidant capacity of coffee by-products from different species and processing methods. Untargeted UHPLC-HRMS putatively annotated several candidate metabolites associated with the observed antioxidant-activity patterns, providing a basis for further investigation of their potential bioactive properties. However, the OPLS-DA and VIP findings are exploratory and should not be considered confirmatory biomarkers because of the limited sample size, sample-type confounding, and lack of independent validation. Further studies incorporating targeted quantification, food-safety assessment, bioaccessibility, toxicity, realistic serving sizes, and in vivo evaluation are needed before these by-products can be considered for functional ingredients or health-related applications.

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Author Contributions

Conceptualization, G.K.A., and P.A.; Methodology, G.K.A., A.S.N., and M.D.R.; Software, G.K.A.; Validation, A.S.N.; Formal Analysis, D.P., F.E., and M.N.A.; Investigation, D.P., M.M., D.R., M.D.R., A.S.N, and B.B.; Resources, B.B., and G.A.; Writing – Original Draft Preparation, G.K.A.; Writing – Review & Editing, All Authors; Visualization, M.N.A.; Supervision, B.M., G.K.A., and P.A.; Funding Acquisition, G.K.A.

Conflicts of Interest

The authors declare no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used Grammarly to edit and refine the wording of the text. In addition, ChatGPT was used to edit and create the graphical abstract. After using this service, the authors reviewed and edited the content as needed and took full responsibility for the publication's content.

Appendix A. Supplementary data

Supplementary data to this article can be found online here.

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