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Physicochemical, Antioxidant and Mineral Composition of Cascara Beverage Prepared by Cold Brewing

Running title: Brewing Condition and Quality of Cascara Beverage

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SUMMARY

Research background. Cascara, the dried husk of coffee cherries, has gained attention as a potential beverage due to its unique flavor profile and potential health benefits. Traditionally, cascara has been prepared using hot brewing methods. However, recent interest in cold brewing techniques has led to investigations into how temperature affects the functional properties of cascara beverages.

Experimental approach. Colour (CIEL*a*b*), total dissolved solids, and titratable acidity were determined in cascara beverages prepared at 5, 10, 15 and 20 °C. Phenolics and flavonoids

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content, and antioxidant properties were evaluated using spectrophotometric methods. Caffeine, chlorogenic acid and melanoidins were quantified by HPLC. Mineral composition was determined by ICP-MS. The results were compared with hot-brewed cascara beverage.

Results and conclusions. Cold brewing led to significantly higher levels of total phenolic compounds (ranging from 309 to 354 mg GAE/L), total flavonoids (11.8–13.6 mg QE/L), and caffeine (123–136 mg/L) compared to the hot-brewed cascara beverage sample ($p<0.05$). Temperature had a noticeable impact on most variables, although the effect appeared random. Specifically, caffeine content ($p<0.01$) and copper levels ($p<0.001$) were highest in beverages prepared at 20 °C and decreased with decreasing brewing temperature. Multivariate analysis showed that minerals (As, Co, Mn, Sn, Mg, Ca), hue and phenolic content contributed to the first principal component, which primarily distinguished the hot-brewed samples. Antioxidant-related variables, total titratable acidity and Se were the main contributor to the second principal component, facilitating the separation of the samples brewed at 5 °C.

Novelty and scientific contribution. To our knowledge, this is the first study to suggest that the temperature impacts the functional properties of cascara beverage prepared using the cold brewing method. Experimental evidence supports the existence of a direct proportionality between caffeine content, copper levels, and brewing temperature.

Keywords: cascara; cold brewing; temperature effect; caffeine

INTRODUCTION

Coffee is one of the beverages consumed most frequently, with a worldwide production of more than 105 million tons annually (*1*). During the production of coffee beans, a lot of waste material is produced. The presence of caffeine, polyphenols, and tannins in coffee by-products creates environmental challenges in coffee-producing countries when it is improperly disposed (*2*). Furthermore, its use as animal feed is limited due to similar factors. Coffee by-products are considered a sustainable source of beneficial bioactive compounds, including nutrients and other important substances.

Cascara, the main by-product of the coffee industry, has garnered increased attention in the past decade due to its revalorization (*3*). This unique beverage boasts tea-like qualities and

a distinct coffee aroma. Typically, cascara is created by drying the coffee pulp in the sun for 4 to 5 days, resulting in a blackish brown colour. Beyond its use in beverage, cascara offers a potential source of phenolic compounds with antioxidant properties, which could positively impact human health (*3–5*). Additionally, it serves as a rich source of minerals, including potassium, calcium, magnesium, sodium, and iron (*6*).

Cascara is currently used for composting purposes in coffee-producing nations. This by-product has also been suggested for various applications, including the production of biofuel, enzymes, biosorbents, and animal feed (*7*). Furthermore, there are proposals for its application in the food industry as an enriching agent for functional foods, such as gluten-free bread *(8)*, yoghurt *(9)* and alcoholic beverages (*10*) due to its antioxidant properties, low fat, low sugar, and high fibre content.

Cold brewing is a method of preparing beverages by steeping plant material in cold water for an extended period, typically between 8 and 24 hours. Despite its popularity, there is still a dearth of precise information on the impact of cold brewing on the quality of cascara beverages. Recently, Abduh *et al.* (2023) conducted the first and only comparative study, evaluating the quality of cascara prepared using hot and cold brewing methods (*11*). To provide a well-informed comparison, we will draw on established processes used in the preparation of coffee beverage (*12–15,17–19*).

When coffee is brewed in cold water, the resulting drink exhibits distinct characteristics compared to its hot-brewed counterpart. Cold-brewed coffee tends to be less acidic, less bitter, and notably sweeter (*6,17*). Additionally, it contains lower levels of caffeine and brown compounds than its hot-brewed counterpart (*14,17,18*). However, the specific effects of cold brewing on cascara infusion have not been unexplored. Several investigations were carried out to examine the effect of low temperatures (*i.e.* <25 °C) on the final quality of coffee beverages (*12–15,17,18*). Exploring the physical and chemical parameters of cascara beverages under this preparation technique is intriguing. Coffee cherries, rich in simple sugars and free amino acids, undergo a combination of drying processes that can lead to the formation of various Maillard reaction products (*4*). In particular, dried coffee husks have recently been deemed safe by the European Commission in terms of acrylamide content (*20*). However, the impact of cold brewing on the content of furfural and 5-HMF, both substances of interest, within cascara infusion remains an

open question. The European Food Safety Authority (EFSA) has established an Acceptable Daily Intake (ADI) value of 0.5 mg/kg body mass for furfural due to its potential liver toxicity. Additionally, EFSA provides a maximum allowable level of 10 mg/kg for 5-HMF in nonalcoholic beverages (*21*). Although these guidelines apply to other contexts, their relevance to cold-brewed cascara deserves further investigation (*22,23*).

While several studies have explored the cold brewing of coffee, there is notable lack of research specifically focused on cold cascara preparation. To date, only one study has compared hot and cold brewing methods for cascara (*11*). This current study aims to fill this gap by investigating the impact of various brewing temperatures on the quality of cascara beverages, and sets a new benchmark in the field of beverage science.

MATERIALS AND METHODS

Chemicals

The following substances (Sigma-Aldrich, St. Louis, MO, USA) were used to determine antioxidant activity: DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS (2,2´-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), TPTZ (2,4,6-tri(2-pyridyl-S-triazin)), and Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid). Total phenolic content (TPC) was examined using Folin-Ciocalteu´s phenol reagent (2N) with gallic acid (purity≥98 %) as standard. Phenolic compounds (quercetin hydrate, chlorogenic acid), melanoidins (furfural and 5-(hydroxymethyl)furfural) and caffeine were at least 95 % pure purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide, sodium carbonate, hydrochloric acid, aluminium chloride hexahydrate, ferric chloride tetrahydrate, sodium acetate, potassium persulfate, and potassium chloride were obtained from Lach-ner, s.r.o. (Neratovice, Czech Republic) with ACS grade. Methanol, acetonitrile (both gradient grade, Honeywell, NC, USA), and deionised water (Mili-Q system, Merck, Darmstadt, Germany) were used for the preparation of mobile phases in HPLC analysis.

The preparation of a cascara beverage

The dried coffee cherry husks were purchased from a local store, vacuum sealed and kept at room temperature until further analysis. The sample originates from Hacienda Sonora (Costa

Rica) and was harvested in 2022. For cold brewing (CB), 9.0 g of coffee cherry husks were transferred to 300 mL of pre-tempered distilled water in a glass baker covered with a watch glass and macerated at 5, 10, 15, and 20 °C (CB5, CB10, CB15, and CB20, respectively) for 24 h under constant stirring (200 rpm) using IKA™ Plate RCT digital (IKA-Werke GmbH & Co. KG, Staufen, Germany). Subsequently, the solids were removed by Whatman™ filter paper (Grade 1, Whatman, Maidstone, Great Britain). For comparison purposes, hot brewed cascara beverage (HB) was prepared by mixing 9.0 g of cascara and 300 mL of boiled distilled water. Coffee cherry husks were macerated for 8 min and stirred at 90 s intervals with a glass rod (each stirring lasted 5 s). The temperature of the mixture decreased to 70±3 °C during maceration and the beverage was cooled in an ice bath. Each sample was prepared in triplicate and each of them was analyzed at least twice. After removal of solids by filtration, the liquid samples were used to determine colour, total titration acidity, and total dissolved solids. For further analysis, the samples were kept in small aliquots at -25 °C.

Colour, total titratable acidity and total dissolved solids of cascara beverages

The colour was measured in reflectance mode on a 50 mm path quartz cuvette using an UltraScan VIS spectrophotometer (Hunter Associates Laboratory, Reston, VA, USA). The CIELAB colour space was applied to describe L^* [dark (0) to light (100)], a^* [red (+) to green (-)], and b^* [yellow (+) to blue (-)]. The hue angle ($h°$) and the chroma (C^*_{ab}) of the colour were also determined. Total titratable acidity (TTA) was determined using a titration method. A 50.0 mL aliquot of cascara brew was titrated with a 0.1 M NaOH solution to a pH of 8.1 using the pH glass electrode HC 103 (Theta 90, Prague, Czech Republic) (*24*). The results were expressed as milliliters of NaOH per liter. Total dissolved solids (TDS) of HB and CB cascara beverages were measured according to the protocol of Moreno *et al.* (*25*). The °Brix of each beverage was measured using an AR3/AR4 refractometer (Mettler-Toledo, Greifensee, Switzerland) followed by conversion to TDS using the equation:

$$\text{TDS (w/\%)} = 0.87 \times \text{Brix} \qquad /1/$$

Determination of total phenolic (TPC), and total flavonoid (TFC) contents

The TPC was estimated using the Folin-Ciocalteu´s reagent with a procedure adopted from our previous study (*26*). The absorbance was monitored after 30 min at 765 nm (Shimadzu UV-2600, Kyoto, Japan) and the results were expressed as mg of gallic acid per liter of beverage (*γ*(phenols as GAE)/(mg/L)). The ability of aluminum chloride to form complexes with flavonoids in an acidic environment was used for the determination of TFC using a protocol described in our previous work (*26*). The formation of Al^{3+}-flavonoid complexes was determined at 425 nm and the results were expressed as quercetin equivalent (*γ*(flavonoids as QE)/(mg/L)).

Antioxidant properties of cascara beverages

The radical scavenging assays using stable DPPH and ABTS radicals were adopted from our previous study (*26*). The absorbance of the samples was monitored at 734 and 517 nm for the ABTS and DPPH assays, respectively. The results were expressed as equivalent antioxidant capacity of Trolox (TEAC in mg Trolox/L). The ferric reducing antioxidant power (FRAP) was determined after the reaction of the sample with TPTZ solution and $FeCl_3$ in an acid environment (*27*). FRAP values were expressed in milligrams of Trolox per liter ((*γ*(activity as Trolox)/(mg/L)).

Determination of the total melanoidin content

The melanoidin content in both the HB and CB cascara beverages was observed spectrophotometrically at 420 nm in a 10.0 mm path-length quartz cuvette. Melanoidins were quantified at the caramel (E-150d) calibration curve in the range of 0.19–6.66 g/L, and the results were expressed in equivalent milligrams of caramel melanoidins per liter of beverage (*γ*(caramel)/(mg/L)) (*4*).

HPLC analysis of target compounds

Caffeine and chlorogenic acid isomers (chlorogenic, neochlorogenic, and cryptochlorogenic) were analysed using a liquid chromatography system consisting of two high-pressure pumps LC-20ADXR, a degassing unit DGU-20A3R, an autosampler SIL-20ACXR, a photodiode array detector SPD-M30A (all Shimadzu, Kyoto, Japan), and an LCO 102 column thermostat (Ecom, Prague, Czech Republic). Separation was carried out on the YMC-Triart C18 chromatographic column (150 × 3 mm, 3 μm particle size) with the mobile phase composed of

deionised water acidified with formic acid 0.3 % (by volume) of formic acid (solvent A) and methanol (solvent B). The optimal gradient program was as follows: 0 min–10 % B, 6 min–33 % B, 9 min–33 % B, 13 min–55 % B, and 18 min–90 % B, and 20 min–10 % B. The mobile phase flow rate, the injection volume, and the column temperature were set to 0.4 mL/min, 2.0 µL, and 30 °C, respectively. Detection wavelengths of 273 and 325 nm were used for the monitoring of caffeine and chlorogenic acid isomers, respectively (*28*).

The Agilent 1200 Series liquid chromatography system was used for the determination of furfural and 5-HMF in cascara beverages (Agilent Technologies, Santa Clara, CA, USA) by adopting a method of Czerwonka *et al.* with some modifications (*23*). Separation was carried out on a Luna® Omega Polar column (150 × 3 mm, 3 µm particle size) with the mobile phase composed of deionised water (solvent A) and acetonitrile (solvent B), both acidified with 0.1 % (by volume) acetic acid. The optimal gradient program was as follows: 0 min–0 % B, 8 min–8 % B, 8.1 min–100 % B, 12 min–100 % B, 12.1–0 % B, and 17 min–0 % B. The mobile phase flow rate, the injection volume, and the column temperature were set to 0.5 mL/min, 15.0 µL, and 40 °C, respectively. Both compounds were monitored by detection wavelength of 280 nm.

The quantitative analysis of all chlorogenic acid isomers was performed using a standard of chlorogenic acid (5-O-caffeoylquinic acid) and are present as their sum. Caffein, furfural and 5-HMF were quantified using corresponding standards. Seven calibration solutions of chlorogenic acid and caffeine were prepared by sequential dilution of their stock methanolic solutions (1.0 g/L) with 20% (by volume) aqueous methanol to concentrations ranging from 5 to100 mg/L for caffeine, and 0.5–10 mg/L for chlorogenic acid and six calibration solutions (0.1–10.0 mg/L) of furfural and 5-HMF were prepared by dilution of their stock solutions in 50% (by volume) aqueous methanol. All samples were filtered through a 0.45 µm PTFE syringe filter prior to injection.

Mineral composition of cascara beverages

The multi-element quantification of major (K, Ca, P, Mg and Na) and trace (Fe, Mn, Cu, Zn, Se, Ni, Co, Cr, Al, As, Cd and Pb) elements was performed using the Agilent 7900 ICP-MS (Agilent Technologies, Inc., Santa Clara, CA, USA). The instrument was configured with standard nickel cones, a glass concentric nebulizer (MicroMist, 400 µL/min), a Peltier-cooled quartz spray chamber (2 °C), and a quartz torch with a 2.5-mm internal diameter. A low-pulsation, 10-roller

peristaltic pump with three separate channels ensured accurate delivery of both samples and internal standard (ISTD). To mitigate polyatomic interferences, the instrument incorporated an octopole-based collision cell, employing kinetic energy discrimination (KED) in either standard helium ("He") or high-energy helium ("HE He") mode. The collision cell parameters for both modes were manually adjusted, and the instrument's MassHunter software automatically tuned it at startup to enhance sensitivity across elements with varying mass-to-charge ratios (low, middle, and high). Consistent plasma and ion lens tuning parameters were maintained for all collision cell modes (refer to Varrà *et al.* (*29*) for detailed information).

The determination of analyte concentrations involved the construction of calibration curves encompassing multiple elements. These curves were established through the analysis of calibration solutions containing standards at five distinct concentrations. The concentration ranges for various elements were as follows: 0 to 100 µg/L for Fe, Mn, Cu, Zn, Se, Ni, Co, Cr, Al, As, Cd and Pb; and 0 to 10 mg/L for K, Ca, P, Mg and Na. Linear calibrations with a coefficient of determination exceeding 0.999 were successfully achieved for all analyzed elements. To account for potential instrumental drift and matrix effects, a 200 µg/L Rh ISTD was simultaneously introduced and mixed with the samples. The employed method underwent comprehensive validation utilizing certified reference standards. The outcomes of this rigorous validation process are detailed in the reference by Varrà *et al*. (*29*).

Statistical analysis

Each brewing was performed in triplicate and results were expressed as the mean with standard deviation. Homogeneity of variance and normal distribution of the data were assessed using Box's M test for variance homogeneity and Shapiro-Wilk's test for normal distribution. Analysis of variance (ANOVA) was applied to study the effect of brewing temperature for CB samples. A *post-hoc* Duncan test was used for multiply pairwise comparison among means. Pearson’s correlation (r) analysis was used to calculate the pairwise correlation coefficient matrix with the corresponding significance test p-values, between pairs of variables. In all cases, a p-value lower than 0.05 was selected as the statistical significance threshold.

Hidden patterns in the data were unveiled through the application of multivariate analysis. A selection of the most informative variables was performed beforehand to simplify the final

multivariate models. Neighborhood component analysis (NCA) was applied for this purpose *(29)*. Elemental differences between the investigated groups of cascara samples were assessed using principal component analysis (PCA). Hierarchical cluster analysis (HCA) was additionally applied to the same input data used for PCA, namely a matrix consisting of the normalised yields obtained from each measurement. With respect to HCA, an agglomerative hierarchical algorithm was used, which progressively combines pairs, by measuring Euclidean distances among the clusters. The method of average linkage was selected, as it provided the highest cophenetic correlation (0.98). All the statistical analyses were carried out using the software packages MATLAB® R2022b (*30*) and Statistica 14.0 (*31*).

RESULTS AND DISCUSSION

The effect of brewing temperature on dissolved solids, titratable acidity, and colour of cascara beverage

The results are summarized in Table 1. TDS values were highest for the CB20 sample (0.38±0.10) % and decreased with decreasing temperature, just as reported for the coffee beverage (*13*). They found a higher TDS after brewing at 30 °C and the lowest at 4 °C with respect to other variables such as coffee-water ratio, mesh size, and extraction time. In our research, the TDS values were not significantly different in cascara samples brewed at 15 to 5 °C and were similar to those observed in the hot brewed sample (0.19±0.05) %. Hot water is known to be more effective in the dissolution of chemical substances and/or the breakdown of tea polymers, increasing the turbidity of solutions and hence the total soluble solid content (*33*). Despite this fact, Zhao *et al*. (2022) found that hot (85 °C for 25 min) and cold brews (4 °C for 9 h) of *Apocynum venetum* tea had a similar level of solid content (*34*). Furthermore, room temperature brewed tea (25 °C for 3 h) showed a significantly lower total soluble solid in their research.

Table 1

The TTA values decreased from (34.1±1.0) to (30.7±0.4) mL/L with the decrease in the brewing temperature ($p<0.001$). The HB sample showed acidity similar to those prepared at 5–15 °C in our study. Generally, hot water extraction is more efficient in dissolving and transferring

some acidic species, as demonstrated in the case of Columbian or arabica coffee infusions (*14,18*).

In our study, the cold-brew method was supported by constant stirring, which was probably more effective for the release of substances of an acidic nature. As for the colour, the HB cascara beverage differed in parameters *L** and *b** from those prepared in cold water (Table 1). The final beverages prepared at 20, 15 and 10 °C were darker and less yellow. The sample prepared at 5 °C showed a high red colour (*a**=53.4±1.8), yellow (*b**=109.9±0.2), and lightness (*L**=80.9±3.4). Recently, the colour of the cascara beverage was attributed to the presence of melanoidins that develop during the drying of cherry pulp (*4*). This was also confirmed in our experiment, where the melanoidins content was negatively associated with the colour values *L** and *b** for the HB and CB samples brewed at 20, 15, and 10 °C. In other words, the more melanoidins, the darker ($r=-0.745$; $p<0.01$) and less yellow ($r=-0.621$; $p<0.05$) beverage.

Antioxidant properties, phenolic and caffeine content in cascara beverage

Generally, both the total phenolic content and the total flavonoid content were higher ($p<0.05$) in the cold-brewed cascara beverage samples compared to the hot-brewed samples (Fig. 1a). While the TPC for the CB cascara was 309–354 mg GAE/L, the hot-brewed sample yielded (265±52) mg GAE/L. Heeger *et al*. (*2*) determined a similar total phenolic content (283 mg GAE/L) in a cascara drink by extracting 1.0 g of coffee cherry pulp in 24 mL of hot water at 85 °C for 15 min. There is insufficient data comparing the TPC of hot-brewed and cold-brewed cascara beverages. Abduh *et al*. (*11*) suggested in their preliminary experiment that TPC was higher in hot-brewed cascara compared to cold-brewed, though specific numerical values were not provided. Numerous studies have investigated the effects of hot and cold brewing methods on various beverages, but their outcomes are inconsistent and vary widely (*12–16*). For example, total phenolic content was higher in hot brew (92 °C for 3 min) than in cold brew (5 °C, 16 h) coffee (*12*). This is usually explained by an increase in the solubility of some phenolics leading to an increase in their concentration in the final beverage. On the other hand, French press and cold brew (room temperature for 12 h or 4 °C for 24 h) produced coffee beverage with similar level of TPC (*13*). It should be noted that the extraction efficiency of phenolic substances from ground coffee beans is a complex process that involves not only the temperature of the water (or the

brewing method), but also the brewing time, grinding size, the origin of the beans and the degree of roasting (*13,15*). In a recent study by Muzykiewicz-Szymańska *et al*. (*15*), a higher content of polyphenols was observed in unroasted coffee bean infusion prepared with cold water (4 °C for 24 h) than those using hot brewing methods (combinations of 86 and 95 °C for 4 or 10 min). Non-fermented and partially fermented teas had higher TPC when brewed at low temperature (4–6 °C for 16 h) compared to brews prepared with boiled water (*16*). However, in their study, the opposite trend was observed in fermented teas. They proposed that non-fermented tea leaves contain low molecular weight water-soluble phenolics that are easily extractable in cold water. On the contrary, fermentation results in the formation of polymeric products of higher molecular weight that are more soluble in hot water. Similarly, the total flavonoid content was higher in all samples of the CB cascara beverages (11.8–13.6 mg QE/L) than in hot-brewed cascara beverage ((7.6±2.1) mg QE/L). There are two potential explanations for this phenomenon. Coffee husk is usually dried in the sun and, as described for Cornelian cherries, the contribution of free phenolics was more dominant in sun-dried samples compared to fresh samples (*35*). It is also necessary to mention the possible overestimation of TPC values due to the reaction of melanoidins with Folin-Ciocalteu´s reagent (*28*). As described below, the CB samples had a higher content of total melanoidins than those of the HB cascara samples. The effect of temperature on both TPC and TFC during cold brewing has not been proven ($p>0.05$). Among the phenolic compounds, protocatechuic acid, pyrocatechol, and chlorogenic acid were the most abundant in five cascara samples, as Pua *et al*. (*5*) observed. The chlorogenic acid content ranged from 31.6 to 256 mg/L which was considerably higher than that found for both HB and CB cascaras in our study, *i.e*. 8.4–13.2 mg/L (Fig. 1b).

This discrepancy can be attributed to the low solid-liquid ratio. We prepared cascara in a ratio of 3:100, while Pua *et al*. (*5*) applied more dried coffee husk (1:10). Additionally, both HB and CB cascara beverages had similar levels of chlorogenic acid ($p>0.05$). Regarding the caffeine content, the CB cascaras had a significantly higher amount (123–136 mg/L) compared to the HB sample ($p<0.01$). The effect of water temperature on the chlorogenic acids and caffeine content in cascara beverage has not yet been described. However, there are some studies that compare the contents of these substances in cold and hot coffee drinks (*6,14,28)*. The high solubility of neochlorogenic acid in water was probably responsible for facilitating its extraction at low (21–25

°C, 24 h) and high (98 °C for 6 min) temperatures, as demonstrated in Arabica coffee brews (*36*). They also found that the caffeine content was higher for long maceration at low temperature than for fast and hot extraction, which was attributed to the diffusion limitation of caffeine across the larger-radius particles. This was similar to our study. Furthermore, the decrease in temperature from 20 °C to 5 °C resulted in a decrease in the caffeine content from 136 to 123 mg/L ($p<0.01$) in cascara beverages. The extraction efficiency of both chlorogenic acid and caffeine from coffee beans depends on a number of factors such as water temperature, extraction time, particle size, coffee origin, and degree of roasting. For example, coarse grinding and higher extraction temperature (15 °C) favoured the caffeine content of Arabica coffee brew, while fine particles extracted at 5 °C were more effective for Robusta coffee (*6*). A higher caffeine content in a coffee drink prepared at 25 °C than at 15 °C and 5 °C was also found in the study by Maksimowski *et al*. (*19*), but only for a coffee at one roast level. This shows that the extraction of caffeine and phenolics from the plant matrix is a complex process. More studies are needed to reveal the reasons for the decrease in caffeine content with decreasing temperature.

The antioxidant properties of the HB cascara sample were 165 and 75 mg Trolox/L in terms of the ABTS and DPPH assays, respectively (Fig. 1c). While the ABTS method resulted in similar TEAC values for samples prepared at 20 °C and 15 °C, a further decrease in the brewing temperature caused a significant decrease in antioxidant activity ($p<0.01$). On the contrary, the DPPH radical scavenging activity was lower for cascara beverage samples brewed at 20 °C and 15 °C compared to the HB counterpart, although the differences were not significant at $p>0.05$. A decrease in soak temperature to 10 and 5 °C gave the cascara beverage with significantly higher $TEAC_{DPPH}$ values ($p<0.01$). Therefore, a negative correlation was observed between $TEAC_{DPPH}$ and $TEAC_{ABTS}$ ($r=-0.685$, $p<0.01$) in this study. It is known that the ABTS assay is more sensitive to hydrophilic and lipophilic compounds, while the DPPH radical scavenging assay is suitable for the analysis of hydrophobic compounds (*37*). They found a negative association between the ABTS and DPPH radial scavenging ability in the Umbelliferae plant. In a study by Yi *et al.* (*38*), some phenolics (chicoric, chlorogenic and 3,5-dicaffeoylquinic acids) determined in *Lactuca indica* L. extracts showed a negative correlation coefficient for ABTS. Although the correlation coefficients were not significant at $p>0.05$, it was weakly positive between TPC and DPPH ($r=0.254$) and weakly negative between TPC and ABTS ($r=-0.445$). FRAP values were higher for

samples prepared at 15 and 10 °C (42.54–43.59 mg Trolox/L) than at 20 and 5 °C with respective values of (32±7) and (31±9) mg Trolox/L. However, the means were not different due to the high standard deviations ($p>0.05$). Hot brewing gave a cascara beverage with significantly lower FRAP value ($p<0.01$).

Figure 1

Melanoidin content in hot- and cold-brewed cascaras

Although the total melanoidin content was (3339±307) mg of caramel/L for the HB cascara beverage, cold brewing resulted in significantly higher levels ranging from (4879±396) mg of caramel/L to (5142±242) mg of caramel/L ($p<0.05$) (Table S1). Iriondo-DeHond *et al*. (*4*) first reported the occurrence of melanoidins in the coffee cascara at a level of 1480 mg of caramel/L. However, three times less instant coffee husk powder was used to prepare the drink in their study. Temperatures below 20 °C did not influence the melanoidin content in cascaras in our research. 5-HMF and furfural made up only a small proportion of the total melanoidin content. We have only observed 0.16–0.20 mg/L and 0.91–0.97 mg/L of furfural and 5-HMF, respectively. This is in agreement with the study conducted in coffee pulp distillate (*22*). The authors presented that the furfural and 5-HMF content was below the detection limit, which was 0.17 mg/L and 0.11 mg/L, respectively. Generally, hot brewing resulted in higher brown compounds or 5-HMF content in coffee beverages than cold brewing (*17,18*). Our investigation exhibited that both compounds did not show any significant trend with temperature, despite some significant differences provided by the pairwise comparison test. The total melanoidin content was only positively associated with 5-HMF (r = 0.658; $p<0.01$) but not with furfural. Both cascara beverages HB and CB can be considered safe due to the low 5-HMF content (*21*).

Mineral content of coffee cascara beverages

The content of the elements in the HB and CB cascaras is presented in Table 2. Potassium, phosphorus, magnesium, calcium, and sodium were found in the range 507–594, 29.2–36.9, 6.4–12.0, 7.3–20.1, and 0.72–1.29 mg/L, respectively. Among trace elements, manganese (102–140 µg/L), iron (112–143 µg/L), and copper (64.1–111 µg/L) occurred at the highest concentration in all samples. Zinc, at a concentration of (42±2) µg/L was found only in HB cascara beverages,

while in CB cascara brews were below the detection limit. Cold brewing for 24 h improved the extraction of all minerals, resulting in their significantly higher content compared to the hot brewed sample. Although the effect of temperature during cold brewing was found to be significant in most minerals, no trend in mineral contents was evident with the brewing temperature. The exception is the decrease in the copper content from (111±3) to (79.7±0.75) μg/L when the temperature decreased, and the lowest level of K ((552±5) mg/L) at 5 °C. It was previously demonstrated that the coffee making technique had an impact on the level of mineral content (*39)*. For example, the highest level of Mn was found in Aeropress coffee, and the lowest level was found in the coffee from the French press. Cu was below the detection limit in Aeropress and drip brews, but espresso coffee contained 85 μg/L of copper, which is similar to our study. Regarding the daily intake recommendation, cascara brews can serve as a source of some minerals. For potassium, adequate intakes (ADI) were established to be 3,4 and 2,6 g per day for adult men and adult female, respectively (*40*). When consuming two cups a day (approximately 360 mL), the daily limit is reached by 6.3 % (male) and 8.2 % (female). Considering the reference daily intake (RDA) for Cu, which amount is 900 μg/day, two cups of cascara beverage prepared at 20 °C can deliver up to 4.4 % of the daily requirement for this mineral (*41*). Manganese is involved in the formation of bones and is a co-factor in many enzymes. It is recommended to ensure ADI levels at 2.3 and 1.8 mg for men and women (*39*), which would only be satisfied at 2.2 and 2.8 % by drinking two cups of CB cascara brew (prepared at 10 °C), respectively. The same volume of cascara beverage provided approximately 1 % or less of the RDA or ADI for other minerals determined in this study. Instant cascara powder has been recognised as a “source of potassium and magnesium” because it represented at least 15 % of the daily recommendation (Regulation (EC) No 1925/2006) (*4,42*). Cascara beverages prepared at low temperature (particularly at 10 °C) can be considered a “source of potassium” only if a larger volume is consumed per day (860 mL for men, 660 mL for women). The aluminium and tin content was higher in the CB cascara samples, but their content peaked at different temperatures (15 °C for Al, 20 °C for Sn). On the other hand, cold brewing reduced arsenic or lead concentrations.

Table 2

Multivariate analysis of hot and cold brewed cascara samples

Prior to building the final model, a selection of the most informative variables was performed using neighborhood component analysis (NCA), allowing us to simplify the multivariate models while retaining key information. The NCA results (see Fig. S1) highlighted specific analytes, with the exception of TFC, melanoidins (TMC, furfural, 5-HMF), and Al, as significant contributors to the dataset. These analytes were incorporated into the development of the PCA model designed to discriminate HB samples from CB samples, as well as CB samples prepared at different temperatures. The first two principal components (PCs) captured 71 % of the total variability in the analyte profiles (Fig. 2). Importantly, the variables As, Co, Mn, Sn, Mg, Ca, hue, and TPC showed strong contributions to the first principal component (PC1), which was primarily responsible for differentiating HB samples. On the other hand, the characteristics of L^*, a^*, C_{ab}, $TEAC_{ABTS}$, $TEAC_{DPPH}$, TAA, and Se were the main contributors to PC2, leading to the separation of CB5 samples. The significance of these results lies in the fact that specific elemental and phenolic components play critical roles in defining the unique analyte profiles of HB and CB samples. For example, the contributions of heavy metals (As, Co, Mn, and Sn) to PC1 suggest that these elements might be important discriminators for HB samples, potentially due to differences in soil composition or cultivation conditions between HB and CB sources. Meanwhile, the influence of antioxidant-related variables (TEAC, TAA) in PC2 for CB5 samples highlights the potential impact of different temperature treatments on the antioxidant properties of CB, aligning with previous studies showing that temperature can significantly affect the antioxidant profile of coffee beans (*12-15*). More detailed information can be depicted in Fig. S2, which visualizes the contributions of each variable to the principal components as a heat map. This figure clearly shows the relative influence of individual analytes across the different principal components, allowing for a more nuanced understanding of their roles in distinguishing between HB and CB samples.

Moreover, hierarchical cluster analysis (HCA) produced a similar grouping of samples as PCA, with five distinct clusters emerging naturally from the data (Fig. S3). This reinforces the robustness of the PCA model and suggests that the discriminating features identified by PCA are consistent across different multivariate approaches. The agreement between PCA and HCA further underscores the reliability of our feature selection process and the stability of the identified variables in distinguishing the different sample groups. The dendrogram of hierarchical clustering analysis (HCA) (Fig. S3) and the accompanying heatmap visually represent the analyte

concentrations in HB and CB samples prepared at different temperatures. This clustergram plays an important role in confirming the natural separation between the sample groups and highlighting the distinct analyte profiles across the different brewing conditions. The heatmap allows for a clear visualization of the relative concentration levels of key analytes, emphasizing patterns that may not be immediately evident from PCA alone. The dendrogram further validates the results from PCA by showing the hierarchical relationships between samples, with clear divisions based on temperature and preparation method. This consistency across different multivariate techniques highlights the robustness of the overall data analysis and strengthens the conclusions drawn about the influence of temperature on the analyte profiles of HB and CB samples.

To fully appreciate the broader relevance of these findings, future studies could further investigate how these key variables, especially metal elements and antioxidant-related compounds, correlate with the sensory properties or health benefits of HB and CB samples. Additionally, expanding the analysis to larger datasets or different geographical regions could provide further validation of these discriminating factors and enhance the generalizability of our model.

CONCLUSIONS

Cascara drinks prepared at low temperatures had different colour, a higher content of total phenolics and flavonoids compared to the hot-brewed sample. Higher antioxidant activity was demonstrated in all cold-brewed samples only in the case of FRAP. Cold brewing also resulted in a beverage with a higher content of caffeine and minerals, especially K, P, Mg, Ca, Mn, and Cu. The total melanoidin content and 5-HMF were also higher in the cold-brewed samples, but the concentrations were considered safe for human health.

The temperature during the cold brewing process affected the chemical composition of the cascara beverage. Brewing at 20 °C (room temperature) has been shown to be highly effective. This method results in a beverage with a high caffeine content, which is the main reason for its consumption. Additionally, brewing at this temperature eliminates the need for a cooling medium, thereby ensuring safe energy consumption. Moreover, cold-brewed cascara can be an intriguing addition to the non-alcoholic beverage market. Its unique flavour profile, high caffeine and copper content make it a compelling alternative to traditional caffeinated drinks. This innovative product

could appeal to consumers seeking new and exciting beverage options. Future research could explore the long-term benefits of regular cold-brew cascara consumption, as well as its potential applications in functional beverages.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIALS

All supplementary materials are available at: www.ftb.com.hr/

AUTHORS´ CONTRIBUTION

S. Muriqi, L. Červenka design the study. S. Muriqi, P. Česla prepared the samples and performed the chemical analysis. L. Česlová performed HPLC analysis of caffeine. M. Kašpar and S. Řezková performed HPLC analysis of melanoidins. J. Patočka determined mineral content. L. Husáková provided statistical analysis. S. Muriqi, L. Červenka and H. Velichová wrote a manuscript draft. L. Červenka and L. Česlová revised the manuscript.

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Table 1. Total dissolved solids (TDS), total titratable acidity (TTA), and colour of cascara beverages

Parameter	Hot brewed	Cold brewed at				p†
		20 °C	15 °C	10 °C	5 °C	
TDS (*w*/%)	(0.19±0.05)^{b}	(0.38±0.10)^{a}	(0.27±0.05)^{b}	(0.20±0.03)^{b}	(0.26±0.01)^{b}	*
TTA (*φ*/(ml/L))	(31.9±1.8)^{bc}	(34.1±1.0)^{a}	(32.8±0.20)^{b}	(33.1±0.4)^{ab}	(30.7±0.4)^{c}	**
*L**	(63.1±1.8)^{b}	(45.3±4.7)^{c}	(45.9±4.4)^{c}	(37.7±1.6)^{d}	(80.9±3.4)^{a}	**
*a**	(26.6±1.9)^{bc}	(28.6±0.7)^{b}	(29.3±1.2)^{b}	(26.0±0.95)^{bc}	(53.4±1.8)^{a}	**
*b**	(82.2±1.3)^{b}	(70.5±5.5)^{c}	(71.7±5.2)^{c}	(60.2±2.1)^{d}	(109.9±0.2)^{a}	*
h°	(72.1±0.9)^{a}	(67.9±1.2)^{b}	(67.8±1.2)^{b}	(66.7±0.8)^{b}	(64.1±0.8)^{c}	***
C^*_{ab}	(86.4±1.8)^{b}	(76.1±5.4)^{c}	(77.5±5.1)^{c}	(65.6±2.1)^{d}	(122.2±0.9)^{a}	***

Mean ± standard deviation (*N*=3). Different small letters in superscript indicate statistical differences in row using Duncan´s multiply pairwise test; ($p<0.05$)

† probability factor indicating the significance of cold brewing temperature on the variables, * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$)

Table 2. Selected elements in cascara beverage

Element	Hot brewed	Cold brewed at				p†
		20 °C	15 °C	10 °C	5 °C	
		γ(element)/(mg/L)				
K	(507±1.3)^{d}	(577±11)^{b}	(573±5)^{b}	(594±8)^{a}	(552±5)^{c}	**
P	(29.2±0.61)^{c}	(35.4±0.24)^{a}	(35.7±0.67)^{a}	(36.9±1.5)^{a}	(33.3±0.77)^{b}	**
Mg	(6.40±0.02)^{c}	(10.6±1.1)^{b}	(10.2±0.17)^{b}	(12.0±0.63)^{a}	(11.7±0.36)^{ab}	*
Ca	(7.31±0.20)^{d}	(16.6±1.9)^{b}	(12.9±0.89)^{c}	(19.9±1.7)^{a}	(20±1)^{a}	***
Na	(0.80±0.01)^{c}	(1.12±0.14)^{a}	(0.72±0.02)^{c}	(1.07±0.08)^{a}	(1.29±0.22)^{a}	**
		γ(element)/(μg/L)				
Fe	(112±0.8)^{b}	(133±4)^{a}	(112±6)^{b}	(130± 29)^{a}	(143±3)^{a}	NS
Mn	(102±0.4)^{c}	(135±3)^{ab}	(121±10)^{b}	(140± 4)^{a}	(127±2)^{b}	*
Cu	(64.1± .81)^{d}	(111±3)^{a}	(98±7)^{b}	(90± 9)^{bc}	(79.7±0.75)^{c}	**
Co	(0.586±0.001)^{a}	(0.424±0.001)^{e}	(0.474±0.001)^{c}	(0.537±0.001)^{b}	(0.436±0.001)^{d}	***
As	(0.67±0.03)^{a}	(0.19±0.01)^{c}	(0.23±0.04)^{bc}	(0.18±0.03)^{c}	(0.25±0.02)^{b}	NS
Se	(0.32±0.01)^{b}	(0.41±0.03)^{a}	(0.208±0.008)^{d}	(0.35±0.01)^{b}	(0.26±0.03)^{c}	***
Cr	(0.112±0.003)^{d}	(0.18±0.01)^{b}	(0.15±0.01)^{c}	(0.150±0.005)^{c}	(0.36±0.01)^{a}	***
Sn	(0.23±0.01)^{e}	(1.797±0.001)^{a}	(0.972±0.001)^{c}	(0.766±0.001)^{d}	(1.008±0.001)^{b}	***
Al	(0.11±0.01)^{b}	(0.18±0.02)^{a}	(0.20±0.01)^{a}	(0.18±0.02)^{a}	(0.191±0.002)^{a}	NS
Cd	(0.24±0.02)^{b}	(1.20±0.02)^{a}	(0.10±0.03)^{c}	< LOQ	(0.25±0.03)^{b}	ND
Pb	(3.67±0.15)^{a}	(0.31±0.28)^{b}	< LOQ	< LOQ	< LOQ	ND
Ni	(2.54±0.06)	< LOQ	< LOQ	< LOQ	< LOQ	ND
Zn	(42.0 ± 2.0)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ

Mean ± standard deviation (*N*=3). Different small letters in superscript indicate statistical differences in row using Duncan´s multiply pairwise test (p<0.05); LOQ, limit of quantification;
† probability factor indicating the significance of cold brewing temperature on the variables, * (p<0.05), ** (p<0.01), *** (p<0.001); NS, not significant; ND, not determined;

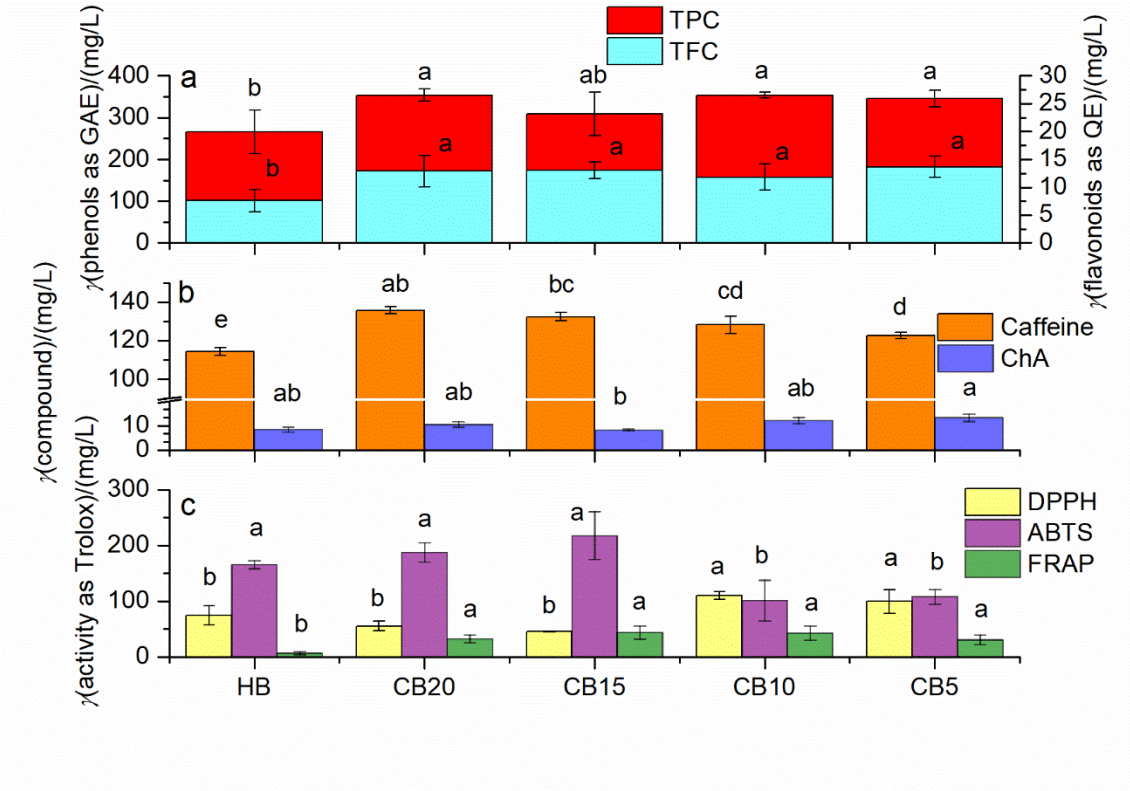

Fig. 1. Phenolics and antioxidants in cascara beverages prepared by hot (HB) and cold brewing at 20, 15, 10 and 5 °C (CB20, CB15, CB10, and CB5). A) total phenolic (TPC) and total flavonoid (TFC) content; B) chlorogenic acid and caffeine content; C) antioxidant activity. Mean ± standard deviation (N=3). Different small letters above the bars for each individual variable indicate a significant difference at $p<0.05$

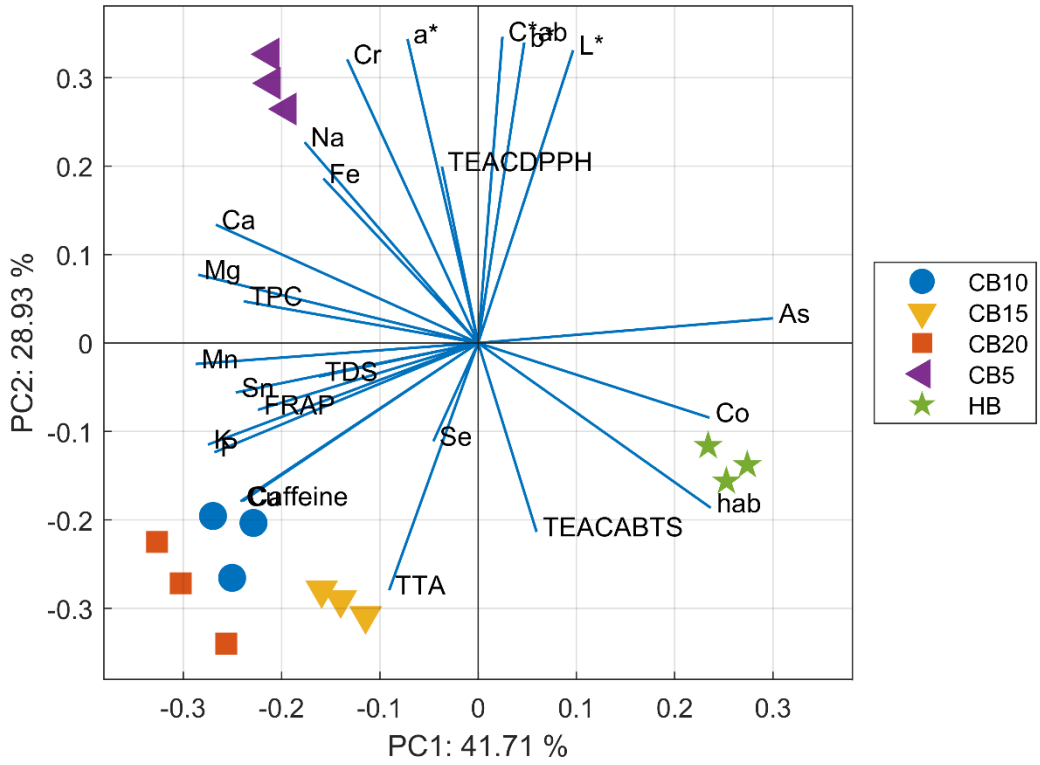

Fig. 2. Biplot illustrating the principal components analysis of analyte profile in the cascara beverage samples prepared by hot (HB) and cold brewing at 5, 10, 15, and 20 °C (CB5, CB10, CB15, and CB20)

SUPPLEMENTARY MATERIAL

Table S1. The effect of brewing methods on the content of melanoidins in cascara samples

γ(compound) /(mg/L)	Hot brewed	Cold brewed at				p†
		20 °C	15 °C	10 °C	5 °C	
5-HMF	(0.91±0.03)^{b}	(0.95±0.01)^{a}	(0.93±0.04)^{ab}	(0.96±0.04)^{a}	(0.97±0.02)^{a}	NS
Furfural	(0.18±0.01)^{ab}	(0.16±0.04)^{b}	(0.19±0.01)^{a}	(0.20±0.01)^{a}	(0.19±0.01)^{a}	NS
TMC (10^3)	(3.40±0.30)^{b}	(4.9±0.4)^{a}	(5.1±0.3)^{a}	(5.1±0.4)^{a}	(5.12±0.08)^{a}	NS

Mean ± standard deviation (N=3). 5-HMF, 5-hydroxymethylfurfural; TMC, total melanoidin content
γ(melanoidins as caramel)/(mg/L); different small letters in superscript indicate statistical differences in row using Duncan´s multiply pairwise test ($p<0.05$)
† NS, the effect of cold brewing temperature on variables was not significant

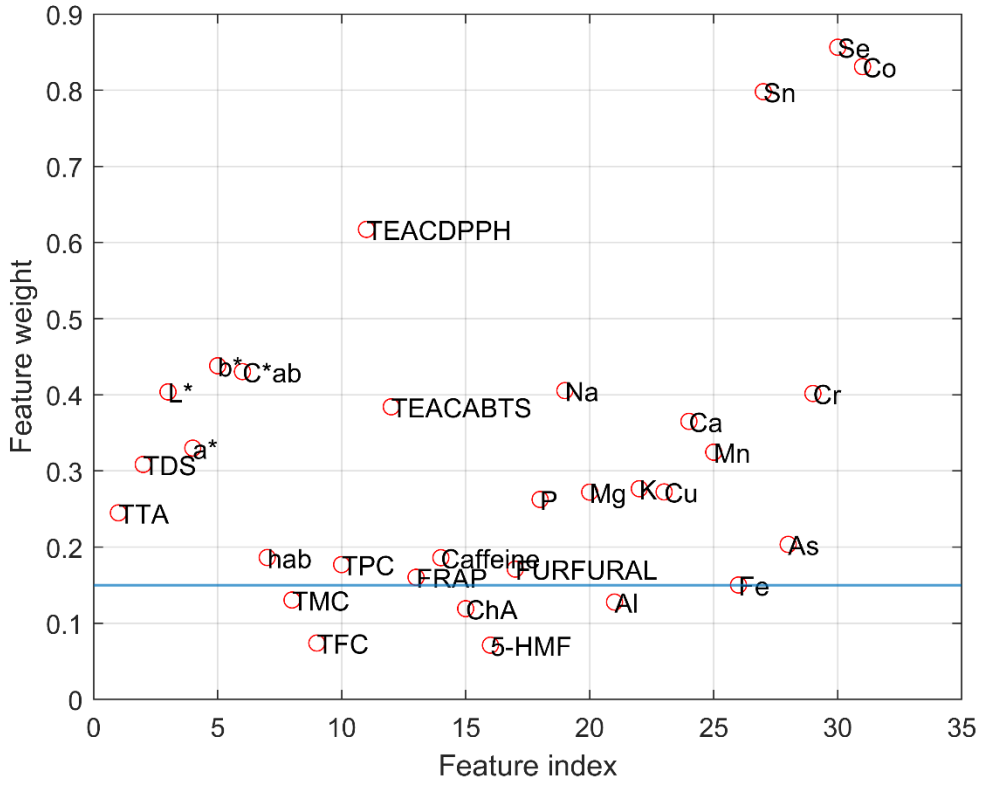

Fig. S1. Plot of the feature weights obtained from the neighbourhood component analysis (NCA) feature selection algorithm for PCA classification: a relative threshold of the weights of the irrelevant features was set <0.15

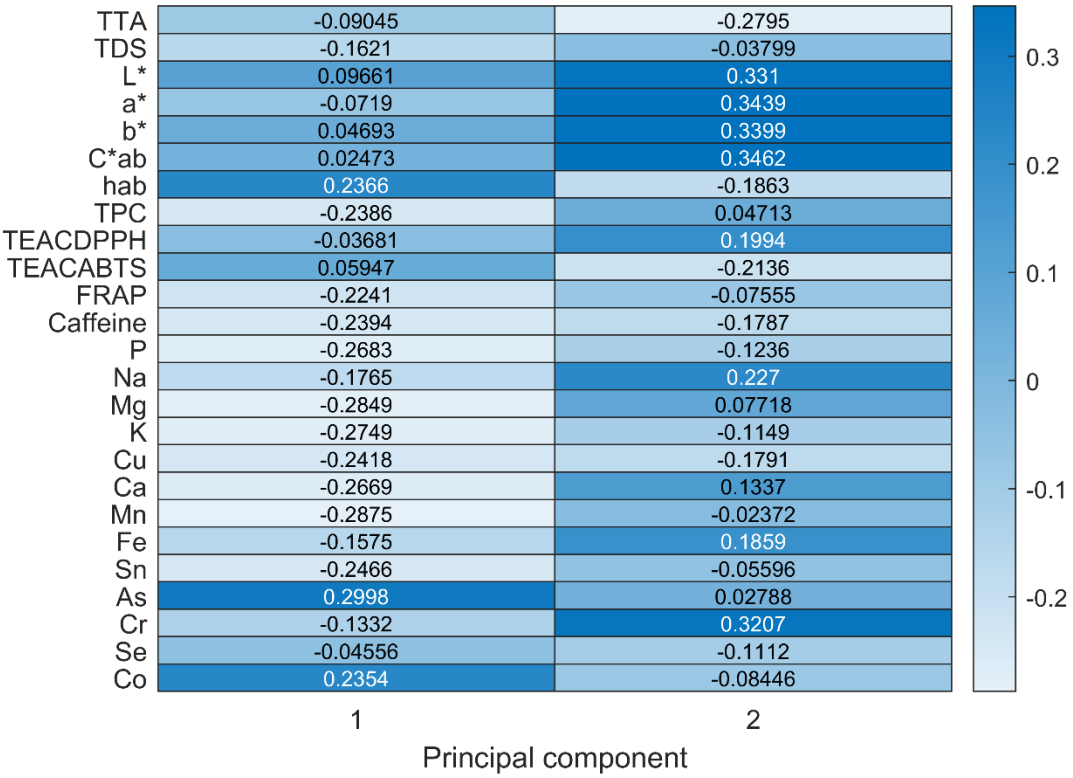

Fig. S2. Heat map of the analyte loadings on the two components

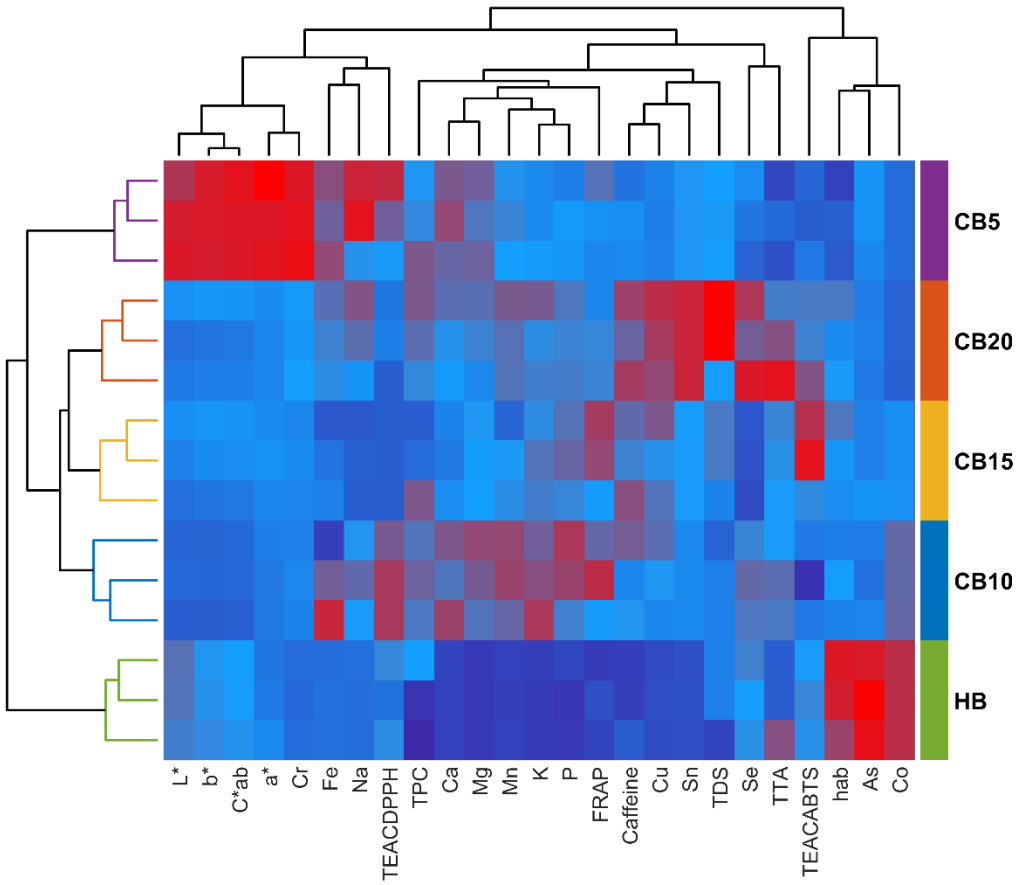

Fig. S3. Dendrogram of hierarchical clustering analysis (HCA) and heatmap of analyte concentrations of cascara samples/species across various preparation temperature; HB, hot-brewed, CB5, CB10, CB15, and CB20, cold-brewed at 5, 10, 15, and 20 °C