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# Targeted Quantification of Bioactive Compounds from *Anacardium humile* and *Anacardium occidentale* by HPLC-MS/MS and Evaluation of Antioxidant Capacity

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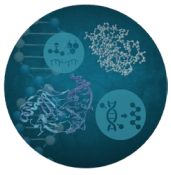
Although *Anacardium humile* and *Anacardium occidentale* fruits are widely consumed in Brazil, quantitative data on their bioactive compound content remain scarce. This study aimed to develop and validate a high-performance liquid chromatography mass spectrometry (HPLC-MS/MS) method for the targeted quantification of 12 metabolites and to assess their antioxidant potential. Twenty-four mature fruit samples were collected from individual trees and purchased from open-air markets in Goiás and the Federal District over three different weeks (during September and October) and were analyzed using a validated method ($r > 0.99$; relative standard deviation (RSD) < 12.84%; recovery 88-111%). Ascorbic acid was the predominant compound in both species, although notable interspecific differences were observed for catechin, isoquercetin, and gallic acid. Principal component analysis (PCA) confirmed distinct chemical profiles between species. Antioxidant activity varied significantly, with the effective concentration ($EC_{50}$) values ranging from 65.67 to 381.94 g fruit *per* g 2,2-diphenyl-1-picrylhydrazyl (DPPH) for *A. occidentale* and from 85.37 to 227.62 for *A. humile*. These findings underscore the importance of robust analytical techniques for characterizing the complex phytochemistry of native Brazilian fruits.



**Keywords:** *Anacardium humile*, *Anacardium occidentale*, metabolites quantification, HPLC-MS/MS, cashew fruit, "*cajuzinho do Cerrado*"

## Introduction

*Anacardium occidentale* L., popularly known as cashew or caju, belongs to the Anacardiaceae family and is extensively cultivated in tropical countries such as Brazil, Vietnam, India, Ivory Coast, Nigeria, and Tanzania.[1-3] The nuts are of considerable economic importance,[4,5] and the cashew apples can be consumed fresh or processed into a variety of products, including juices, liqueurs, sweets, and jams.[6,7] Although *A. occidentale* is the most widely recognized species, the genus *Anacardium* comprises 20 species distributed across tropical regions, nine of which occur in Brazil.[2,8] Among the lesser-known species, *Anacardium humile* St. Hil - popularly known as "*cajuzinho do Cerrado*" or bushy cashew - stands out for its abundance and wide distribution in the Brazilian Cerrado.[7,9]

*A. humile* holds considerable potential for commercial use and can be consumed fresh or processed by the food and pharmaceutical industries. However, this species has not yet been domesticated and is currently exploited only through extractive practices.[10] Its fruits offer significant health benefits due to their nutritional and medicinal properties. *A. humile* stem extracts have been reported to regulate blood glucose levels,[11] while leaves are traditionally used to treat gastrointestinal disorders, such as ulcers and gastritis.[12] Furthermore, *A. humile* demonstrates antifungal,[13] antioxidant, and antiglycation activities, as well as the capacity to inhibit the α-amylase enzyme.[14]

The phytochemical composition of both cashew species, *A. occidentale* and *A. humile*, has been investigated, revealing that they are promising sources of bioactive compounds, including carotenoids, alkaloids, and polyphenols.[6,12-16] A comprehensive study on *A. humile*, conducted by the authors using high-performance liquid chromatography mass spectrometry (HPLC-MS/MS), identified the presence of ascorbic acid, quercetin, catechin,

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rutin, and other related compounds.[9] For example, ascorbic acid (vitamin C) is an antioxidant compound that plays a key role in the biosynthesis of carnitine, collagen, and neurotransmitters.[17,18] Quercetin, on the other hand, has been associated with anti-asthmatic, anti-carcinogenic, anti-hypertensive, and anti-diabetic properties.[19,20]

Species of the *Anacardium* genus are renowned for their diverse and abundant polyphenolic profiles. Numerous studies[2,9,21-24] have demonstrated that leaves, bark, fruits, and nuts-particularly those of *A. occidentale*-contain substantial levels of these compounds. Nonetheless, quantitative data on the key phenolic constituents potentially responsible for the reported biological activities remain limited, and the metabolic comparison between *A. humile* and the more widely cultivated *A. occidentale* is still poorly understood. To advance these approaches and move toward a comprehensive *Anacardium* metabolomic fingerprint, robust analytical techniques are essential. In this regard, mass spectrometry emerges as a pivotal tool, offering high sensitivity and selectivity, and proving highly effective in the annotation of plant metabolites.[9,25-30]

In this study, we report the development and validation of a selective and sensitive HPLC-MS/MS method for the quantification of key bioactive metabolites in *A. humile* and *A. occidentale*. In addition, the antioxidant activity of both species was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. These findings offer new insights into the chemical diversity within the Anacardiaceae family, support the distinction between *A. humile* and the conventionally cultivated *A. occidentale*, and support future studies exploring the nutritional benefits and bioactive potential of Brazilian native fruits.

## Experimental

### Materials and reagents

HPLC-grade acetonitrile and methanol were purchased from Tedia Company (Fairfield, USA). Formic acid (99%) was purchased from Sigma-Aldrich (St. Louis, USA). Quercetin (QUER, ≥ 95%), rutin (RUT, 95%), catechin (CAT, ≥ 98%), ferulic acid (FER, 99%), kaempferol (KAM, ≥ 96%), caffeic acid (CAF, ≥ 98%), chlorogenic acid (CGA, ≥ 95%), and epicatechin (EPI, ≥ 96%) were acquired from Sigma. Cyanidin 3-glucoside chloride (CYA, 99.55%) was obtained from PhytoLab GmbH & Co. KG. Isoquercetin (ISO, 94.16%) was purchased from HWI Analytik. Gallic acid (GAL, ≥ 98%) and ascorbic acid (ASC, 99%) were acquired from Vetec. The internal standard (IS) 6-acetylmorphine-$d_3$ (99.8%) was acquired from Cerilliant®. Ultrapure water was produced using a water purification system (Gehaka Master A&D TOC, São Paulo, SP, Brazil) with a resistivity of 18.2 MΩ cm.

### Cashew fruits collection

Fruits were collected from multiple locations across the state of Goiás, Brazil, to ensure broad geographic representation. A representative image of one collected fruit (Figure 1a) and a map indicating the geographic origin of each sample, including all municipalities in Goiás and the Federal District (Figure 1b), are presented in Figure 1. The plant material is registered in the Sistema Nacional de Gestão do Patrimônio Genético (SisGen) under code A1D88F2.

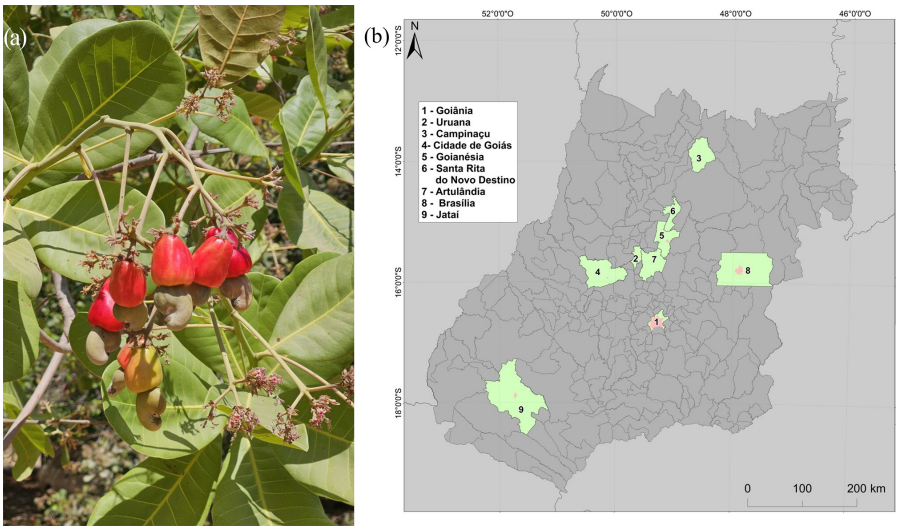


**Figure 1.** (a) Representative image of an *A. humile* fruit. (b) Geographic distribution of sampling sites for *A. humile* and *A. occidentale* fruits in the state of Goiás and the Federal District, Brazil. *A. humile* fruits were collected from 14 individual trees across eight municipalities, whereas *A. occidentale* fruits were obtained from seven trees located in Jataí and Goianésia, as well as from open-air markets in Goiânia over three different weeks to enhance sample representativeness.

Samples of *A. humile* were collected from fourteen individual trees across eight municipalities. Specifically, one tree was sampled in each of the following locations: Goiânia, Uruana, Campinaçu, Cidade de Goiás, Artulândia, and Brasília (Federal District). In Goianésia, fruits were harvested from three different trees, while in Santa Rita do Novo Destino, five trees were sampled. From each tree, three fruit samples were collected, all harvested at the fully mature stage and presenting a uniform degree of ripeness.

For *A. occidentale*, fruits were collected from seven trees distributed across two regions of Goiás: the southwestern region (Jataí) and the central region (Goianésia). As with *A. humile*, three mature-stage fruits were sampled *per* tree. Additionally, to enhance the representativeness and chemical diversity of *A. occidentale* samples, fruits were also purchased from open-air markets in Goiânia over several consecutive weeks.

### Extracts preparation

After thorough washing with tap water, cashews were freeze-dried for 48 h, ground using a high-speed mixer, and stored at –80 °C until extraction. For the preparation of the extracts, 20 mg of lyophilized cashew peduncle were extracted with 1 mL of methanol containing 500 ng $mL^{-1}$ of IS, following a previously published study.[9] The samples were vortexed for 1 min, sonicated for 30 min, and then left to stand at 2-8 °C for 12 h. Subsequently, they were centrifuged at 14,000 rpm for 30 min at 4 °C. The resulting supernatant (organic phase) was collected and stored under refrigeration for analysis. For metabolite analyses and quantification, the extracts were diluted in mobile phase (0.1% formic acid in water: methanol, 50:50, v/v) at a 1:15 (v/v) ratio and transferred to autosampler vials.

### Standard solution preparation

For LC-MS/MS quantification, stock solutions of CAF, GAL, CAT, QUER, CGA, ISO, FER, EPI, CYA, RUT, KAM, ASC, and IS were prepared in methanol at 1.0 mg $mL^{-1}$. Working solutions were prepared in the mobile phase (0.1% formic acid in water:methanol, 50:50, v/v) and grouped according to distinct concentration ranges, described in Table 1. Group 1 (CAF, CGA, FER, and QUER) had a linear range of 0.25-500 ng $mL^{-1}$; group 2 (EPI, ISO and KAM) had a linear range of 5-640 ng $mL^{-1}$; group 3 (CAT, CYA, GAL and RUT) had a linear range of 10-640 ng $mL^{-1}$; and group 4 (ASC) had a linear range of 100-1,000 ng $mL^{-1}$. The IS was prepared in methanol and incorporated into the working solution at a concentration of 500 ng $mL^{-1}$, followed by dilution with the mobile phase (1:15, v/v). All solutions were transferred to injection vials for LC-MS/MS analyses.

### Quantification analyses by LC-MS

Quantification analyses were performed using a high-performance liquid chromatography (HPLC) system (ExionLC™; Sciex, Singapore) coupled to a 4500 QTrap mass spectrometer equipped with an electrospray ionization (ESI) source (TurboIonspray), operating in both positive and negative ionization modes. Chromatographic separation was achieved on an ACE Generix C18 column (150 × 4.6 mm, 5 µm particle size) fitted with Phenomenex guard cartridges (4 × 3 mm), maintained at 40 °C. The autosampler temperature was set to 15 °C. Two distinct chromatographic methods were employed for analyte quantification. Method 1 utilized 0.1% formic acid in water as mobile phase A and methanol as mobile phase B for the quantification of phenolic compounds and flavonoids. The gradient elution commenced at 15% B, held for 2 min, followed by a linear increase to 85% B over 8.5 min, maintained at 85% B until 11 min, then returned to the initial conditions (15% B) over 0.1 min and held for an additional 5 min, totaling a 16-min runtime. The flow rate was set at 1.0 mL $min^{-1}$, and the injection volume was 10 µL. Method 2, applied for ascorbic acid quantification, employed 0.1% formic acid and 10 mM ammonium formate in water as mobile phase A and methanol as mobile phase B. Separation was performed under isocratic conditions with a 70:30 (A:B, v/v) composition at a flow rate of 1.0 mL $min^{-1}$ and an injection volume of 10 µL. The total analysis time for this method was 4 min.

Ion source parameters were as follows: curtain gas (CUR, nitrogen) was 20 psi, and ion source gas pressures (GS1 and GS2) were 60 and 40 psi, respectively. The temperature of the electrospray source was 600 °C, collision gas (CAD) set to medium, ion source voltage of +4,500 V (positive mode) and –4,500 V (negative mode). Optimization of MS/MS parameters was performed through direct infusion experiments using individual standard solutions at 500 ng $mL^{-1}$. In Q1 scan mode, declustering potential (DP) values were determined for protonated ($[M + H]^+$), and/or deprotonated ($[M - H]^-$) precursor ions, by applying voltage ramps ranging from 0 to 150 V. The DP value corresponding to the highest ion intensity was selected. Subsequently, product ion scan experiments were conducted using collision energy (CE) values ranging from 0 to 50 V to identify the most intense and efficiently generated fragment ions, with the CE value yielding the maximum response chosen for each precursor ion. Additional instrumental parameters, including entrance

potential (EP) and collision cell exit potential (CXP), were also optimized. The final values selected were those that provided the highest signal intensity, thereby enhancing ion transmission and overall method sensitivity. The optimized values for DP, CE, EP, and CXP are presented in Table 1. System control was carried out using Analyst® 1.7.2 (Sciex, Singapore).

### Validation of the method

The methods employed for the quantification of the analytes were validated based on the criteria established by Resolution RDC No. 166, dated July 24, 2017, from Anvisa, Brazil,[31] and the International Guidelines from the European Union document SANTE/11312/2021[32] were also followed. In accordance with these guidelines, the following performance parameters were evaluated: linearity, matrix effect, limits of detection (LOD) and quantification (LOQ), accuracy, and precision (repeatability and intermediate precision).

Linearity was assessed by constructing calibration curves in the mobile phase (water with 0.1% formic acid: methanol, 50:50, v/v) across eight concentration levels, each prepared in triplicate (n = 3). Curves were generated by plotting the ratio of the analyte peak area to that of the internal standard against the corresponding analyte concentration. Due to differences in analyte ionization efficiencies, they were grouped according to appropriate concentration ranges. Linearity was evaluated based on the correlation coefficient (r) and the statistical significance of the slope, determined via analysis of variance (ANOVA) applied to three independent calibration sets. Linearity was considered acceptable when r exceeded 0.99 and the calculated *F*-value surpassed the critical *F*-value at the 95% confidence level, considering the relevant degrees of freedom.

**Table 1.** Optimized HPLC-MS/MS parameters for the quantification of bioactive metabolites in *A. humile* and *A. occidentale*

| Linear range / (ng mL$^{-1}$) | Compound | Acronym | $t_R$ / min | Precursor ion (*m/z*) | Product ion (*m/z*) | DP / V | CE / V | CXP / V |
|---|---|---|---|---|---|---|---|---|
| 0.25-500 | caffeic acid (–) | CAF | 6.77 | 179 | 134[a] | –60 | –32 | –9 |
| | | | | | 107 | | –30 | –7 |
| | chlorogenic acid (–) | CGA | 6.06 | 353 | 191[a] | –65 | –26 | –13 |
| | | | | | 135 | | –48 | –9 |
| | ferulic acid (–) | FER | 8.05 | 193 | 178[a] | –60 | –17 | –15 |
| | | | | | 134 | | –24 | –9 |
| | quercetin (–) | QUER | 9.80 | 301 | 151[a] | –100 | –28 | –11 |
| | | | | | 179 | | –24 | –9 |
| 5-640 | epicatechin (–) | EPI | 6.61 | 289 | 109[a] | –90 | –28 | –9 |
| | | | | | 245 | | –20 | –9 |
| | isoquercetin (–) | ISO | 8.41 | 463 | 300[a] | –100 | –36 | –11 |
| | | | | | 301 | | –30 | –11 |
| | kampherol (+) | KAM | 10.55 | 287 | 15[a] | 86 | 43 | 10 |
| | | | | | 185 | | 37 | 12 |
| 10-640 | catechin (–) | CAT | 5.52 | 289 | 245[a] | –90 | –20 | –9 |
| | | | | | 203 | | –24 | –15 |
| | cyanidin (+) | CYA | 6.43 | 449 | 287[a] | 90 | 24 | 10 |
| | | | | | 213 | | 61 | 19 |
| | gallic acid (–) | GAL | 2.63 | 169 | 125[a] | –95 | –10 | –11 |
| | | | | | 79 | | –32 | –14 |
| | rutin (+) | RUT | 8.34 | 611 | 303[a] | 56 | 29 | 10 |
| | | | | | 465 | | 19 | 14 |
| 100-1,000 | ascorbic acid (+) | ASC | 1.58 | 177 | 95[a] | 55 | 16 | 10 |
| | | | | | 85 | | 19 | 10 |
| – | 6-acetylmorphine-$d_3$ (+) | IS | 5.57[b] | 331 | 165[a] | 30 | 49 | 12 |
| | | | 2.52[c] | | 211 | | 35 | 14 |

[a]Style quantitative ion; [b]method 1; [c]method 2. (–) Negative ionization mode; (+) positive ionization mode; HPLC-MS/MS: high-performance liquid chromatography mass spectrometry; CE: collision energy; CXP: collision exit potential; DP: declustering potential; IS: internal standard; $t_R$: retention time.

To evaluate the matrix effect (ME), calibration curves were constructed in both the solvent and fortified matrix, each at eight distinct concentration levels and prepared in triplicate (n = 3). The presence of matrix effects was assessed by comparing the slopes of the calibration curves obtained from analyte solutions in the solvent and fortified matrix, as described in equation 1. In this context, $S_{mat}$ denotes the slope of the curve obtained in the fortified matrix, while $S_{sol}$ refers to the slope of the curve prepared in the solvent. The matrix effect was considered insignificant when the relative variation between slopes was less than ± 20%.

$$\text{Matrix effect (\%)} = \left( \frac{S_{mat}}{S_{sol}} - 1 \right) \times 100 \quad (1)$$

The limit of quantification (LOQ) was determined based on the calibration curves by calculating the ratio between the standard deviation (σ) of the responses from ten blank samples (solutions containing only solvent) and the slope of the calibration curve (S), as described in equation 2. The limit of detection (LOD) was calculated as one-third of the LOQ value.

$$\text{LOQ} = \frac{10 \times \sigma}{S} \quad (2)$$

Accuracy was evaluated by recovery experiments at three quality control levels: low (LQC), medium (MQC), and high (HQC), as described in equation 3, where $C_{found}$ is the concentration of the analyte in the fortified sample, $C_{original}$ is the concentration in the unfortified sample, and $C_{spike}$ is the concentration of the analyte added. The analytes were grouped according to their fortification levels as follows: CAF, FER, QUER, and CGA were spiked at 25, 50, and 100 ng $mL^{-1}$; EPI, ISO, KAM, CAT, CYA, GAL, and RUT at 80, 160, and 320 ng $mL^{-1}$; and ASC at 250, 400, and 600 ng $mL^{-1}$. These concentration levels were selected to represent low, medium, and high points within the linear range previously established for each analyte. Each concentration level was analyzed in triplicate (n = 3), and recovery values within the range of 80 to 120% were considered acceptable.

$$\text{Recovery (\%)} = \left( \frac{C_{found} - C_{original}}{C_{spike}} \right) \times 100 \quad (3)$$

Precision was assessed at MQC level for all analytes. Repeatability was determined under the same experimental conditions using six independently prepared replicates. Intermediate precision was evaluated under the same conditions, but performed on different days and by different analysts, using the same number of replicates. Precision was expressed as relative standard deviation (RSD), with values less than or equal to 15% considered satisfactory. To further assess intermediate precision, a one-way ANOVA was conducted at a 95% confidence level. A *p*-value greater than 0.05 was interpreted as the absence of statistically significant differences among replicate sets.

After validating the proposed analytical methods, a total of 72 fruit samples-comprising both *A. occidentale* and *A. humile*-were analyzed.

### Determination of antioxidant capacity by DPPH assay

To evaluate the antioxidant capacity of the samples, the method based on the scavenging of the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical was employed, according to previously described protocols,[33,34] with modifications. A stock solution of DPPH• (0.06 mM) was prepared in methanol, and 250 µL of this solution were transferred to 96-well microplates. Then, 50 µL of the extracts at different concentrations (0.25 to 2.5 mg $mL^{-1}$) were added. The mixture was kept in the dark for 30 min, and the absorbance was subsequently measured at 515 nm using an EnSpire® microplate reader (PerkinElmer). A calibration curve was prepared in parallel using standard DPPH• solutions (10-60 µM). Antioxidant activity was expressed as the effective concentration ($EC_{50}$), in grams of fruit *per* gram of DPPH• (g fruit *per* g DPPH•). All determinations were performed in triplicate.

### Statistical and chemometric analysis

All statistical analyses and data visualization were performed using in-house Python 3 scripts. The libraries NumPy[35] and pandas[36] were used for efficient data manipulation and analysis; Matplotlib[37] was employed for figure generation and visualization; SciPy supported statistical analysis, including ANOVA; and scikit-learn[38] provided additional tools for machine learning and statistical modeling. ANOVA was applied at the fruit level (n = 72) to test for significant differences ($p < 0.05$) in metabolite concentrations between *A. humile* and *A. occidentale*.

Principal component analysis (PCA) was conducted to explore patterns in metabolite concentration profiles. Prior to PCA, data were mean-centered and scaled to unit variance to mitigate the dominance of high-variance metabolites. Two PCA models were evaluated: (*i*) using all quantified metabolites (n = 12), and (*ii*) using only metabolites that showed significant differences in ANOVA.

In both cases, the score plots exhibited a similar visual tendency of separation between species; however, the model based on ANOVA-significant metabolites showed higher cumulative variance explained by PC1 + PC2. For clarity, only the ANOVA-filtered PCA is presented in the main text, while the complete PCA is provided in the Supplementary Information (SI) section (Figure S1).

## Results and Discussion

### Development and optimization of LC-MS/MS methods

The chromatographic separation and detection performance of the developed methods were evaluated through representative chromatograms, as illustrated in Figure 2. Optimized MS/MS spectra for all quantified compounds are presented in Figure S2 (SI section). For method 1, chromatograms acquired in both negative ion mode (Figure 2a) and positive ion mode (Figure 2b) demonstrated effective resolution and clear peak definition for phenolic compounds and flavonoids under gradient elution conditions. The method provided well-resolved peaks within a total runtime of 16 min, confirming its suitability for the simultaneous quantification of these analytes. Method 2, optimized for ascorbic acid quantification, yielded a concise chromatogram under isocratic conditions (Figure 2c), with a total analysis time of 4 min. Overall, the chromatograms confirmed the efficacy of the selected mobile phases, gradient profiles, and instrument settings for the detection of the analytes.

The optimization of detection parameters significantly enhanced both the sensitivity and selectivity for each target analyte. The optimized values for DP, CE, EP, and CXP are listed in Table 1. The selection of precursor and product ions with the highest signal intensities enabled the establishment of highly sensitive transitions in multiple reaction monitoring (MRM) mode, ensuring both quantitative accuracy and qualitative confirmation. A detailed evaluation of the instrumental parameters indicated that excessively high DP values could compromise the integrity of the target ions, whereas appropriately tuned CE values maximized fragmentation efficiency without sacrificing selectivity.[39] For each analyte, the two most intense product ions were selected - one for quantification and one for confirmation, contributing to the robustness of the method. The impact of EP and CXP parameters was also considered; optimal adjustments of these potentials resulted in improved signal stability and overall analytical performance. These findings underscore the importance of meticulous parameter optimization to achieve optimal performance in mass spectrometry-based analytical methods.

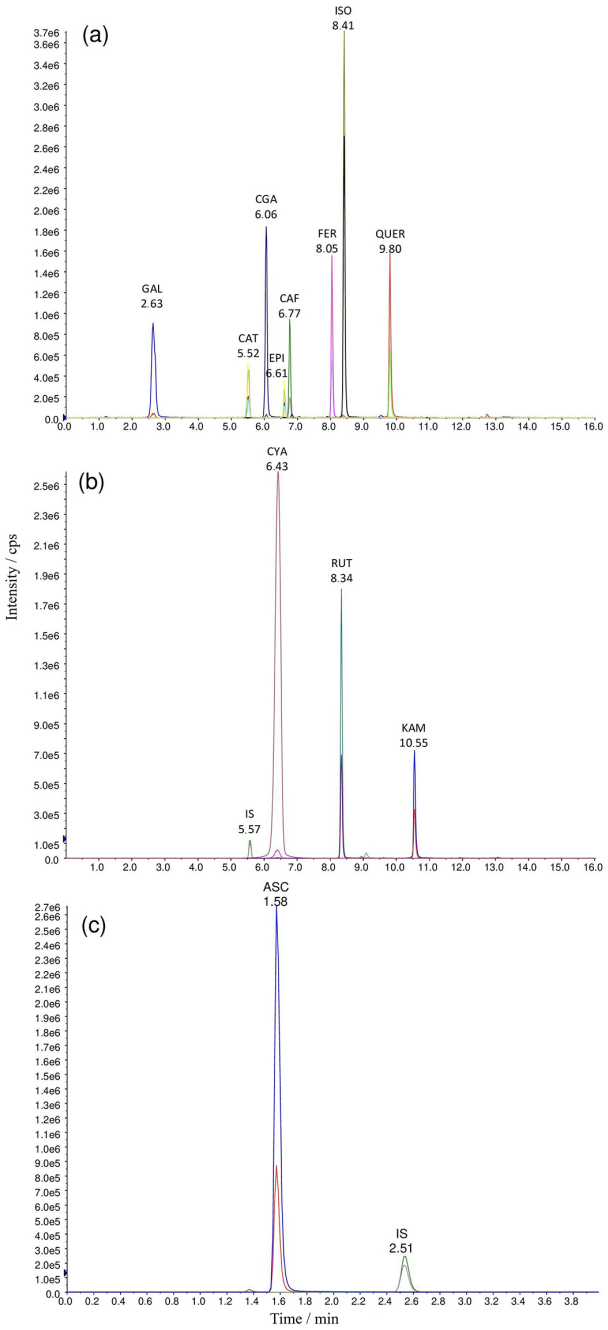


**Figure 2.** Chromatograms obtained: (a) negative ion mode, method 1; (b) positive ion mode, method 1; (c) method 2.

### Methods validation

Linearity was evaluated by constructing calibration curves for the analytes in the solvent at eight concentration levels prepared in triplicate, grouped into appropriate ranges according to the expected response (Table 1). The linear regression was performed by plotting the ratio of the analyte to internal standard peak areas against the analyte concentration. The Pearson correlation coefficients (r), linear regression, and $F_{cal}$ are described in Table 2. The

r values ranged from 0.99823 to 0.99980, demonstrating excellent linearity across all concentration ranges evaluated. In addition to correlation analysis, the statistical significance of the slope was assessed by ANOVA applied to the triplicate curves. For all analytes, the calculated *F*-values exceeded the critical *F*-value of 4.30 (n = 8, triplicate curves; 95% confidence level), confirming that the variation in response was significantly associated with analyte concentration and that the regression models were statistically valid. These results confirm the linearity of the method according to the guidelines used in this work.

Matrix effects were assessed by comparing the slopes of calibration curves prepared in solvent and in matrix-matched fortified samples. The matrix effect was expressed as the percentage difference between these slopes, as calculated using equation 1. Negative values indicated signal suppression, while positive values reflected ionization enhancement. According to commonly accepted analytical criteria, matrix effects were classified as low (< ± 20%), moderate (± 20% to ± 50%), or high (> ± 50%). As shown in Table 2, signal suppression was observed for most analytes, except for CGA and FER, which exhibited ionization enhancement. Nevertheless, all analytes showed matrix effect values within the ± 20% range, indicating that matrix influence was negligible and did not compromise the analytical performance, *per* established validation guidelines.

The LOQs were calculated based on the calibration slope of the curve and the standard deviation of ten analytical blank samples, according to equation 2. The LODs were estimated as one-third of the corresponding LOQs. The calculated values are presented in Table 2. For method 1, LOQs ranged from 0.25 to 10 ng mL$^{-1}$. In the case of ascorbic acid quantification using method 2, the LOQ was established at 100 ng mL$^{-1}$. The LOD and LOQ values obtained in this study are consistent with those reported in the literature. In a study evaluating 36 phenolic compounds in red fruits, Mustafa *et al.*[40] reported LODs ranging from 0.4 to 3.3 mg mL$^{-1}$ and LOQs from 1.2 to 10 ng mL$^{-1}$. For instance, Freitas *et al.*[41] determined phenolic compounds in *Anacardium othonianum* Rizz and reported an LOQ of 10 ng mL$^{-1}$. These findings indicate that our proposed methods exhibit analytical performance parameters comparable to those of previously published studies.

To evaluate the accuracy of the developed analytical methods, cashew samples were fortified at three concentration levels-LQC, MQC, and HQC-with three replicates *per* level. Accuracy was assessed based on analyte recovery. The results, summarized in Table 3, demonstrated recoveries ranging from 88 to 111%. Mean recoveries for each analyte at all concentration levels fell within the acceptable range recommended by regulatory guidelines.

### Method application in real samples

Method precision was evaluated in terms of repeatability and intermediate precision. Repeatability was assessed by

**Table 2.** Method performance parameters: linearity, matrix effect, LOQ, and LOD for the quantification of target analytes

| Compound | r | Linear regression | $F_{cal}$ | ME / % | LOQ / (ng mL$^{-1}$) | LOD / (ng mL$^{-1}$) |
|---|---|---|---|---|---|---|
| Range 1: 0.25-500 ng mL$^{-1}$ | | | | | | |
| CAF | 0.99979 | y = 0.0177x + 0.0023 | 15,461.43 | –0.41 | 0.2 | 0.09 |
| CGA | 0.99940 | y = 0.0318x + 0.0715 | 5,722.99 | 0.15 | 0.1 | 0.04 |
| FER | 0.99979 | y = 0.0124x + 0.0085 | 11,071.57 | 18.1 | 0.6 | 0.2 |
| QUER | 0.99823 | y = 0.0542x + 0.3627 | 3,678.92 | –15.8 | 0.2 | 0.1 |
| Range 2: 5-640 ng mL$^{-1}$ | | | | | | |
| EPI | 0.9995 | y = 0.0010x + 0.0002 | 5,506.1 | –5.95 | 4.9 | 1.6 |
| ISO | 0.9996 | y = 0.0423x + 0.0702 | 4,431.49 | –8.58 | 2.4 | 0.8 |
| KAM | 0.9998 | y = 0.0049x + 0.0146 | 4,548.49 | –5.29 | 2.3 | 0.8 |
| Range 3: 10-640 ng mL$^{-1}$ | | | | | | |
| CAT | 0.9978 | y = 0.0086x – 0.0032 | 1,883.11 | –1.10 | 2.8 | 0.9 |
| CYA | 0.9979 | y = 0.0539x + 0.1398 | 2,675.43 | –0.82 | 4.1 | 1.4 |
| GAL | 0.9986 | y = 0.0281x + 0.0794 | 2,846.03 | –19.94 | 8.6 | 2.8 |
| RUT | 0.9974 | y = 0.0145x + 0.0392 | 2,226.25 | –1.32 | 3.3 | 1.1 |
| Range 4: 100-1000 ng mL$^{-1}$ | | | | | | |
| ASC | 0.9984 | y = 0.0116x + 0.0882 | 2,203.13 | –16.91 | 94.5 | 31.2 |

ME: matrix effect; LOD: limit of detection; LOQ: limit of quantification; CAF: caffeic acid; CGA: chlorogenic acid; FER: ferulic acid; QUER: quercetin; EPI: epicatechin; ISO: isoquercetin; KAM: kaempferol; CAT: catechin; CYA: cyanidin 3-glucoside chloride; GAL: gallic acid; RUT: rutin; ASC: ascorbic acid.

**Table 3.** Mean recovery values obtained from accuracy assays performed at three concentration levels within the validated linear range: low (LQC), medium (MQC), and high (HQC). Precision was evaluated at the MQC level in terms of repeatability (intra-day) and intermediate precision (inter-day), expressed as relative standard deviation (RSD). ANOVA was applied to assess variability in intermediate precision, with *p*-values > 0.05 indicating no statistically significant difference between replicate sets

| Analyte | Accuracy - recovery / % | | | Precision - RSD / % | | |
|---|---|---|---|---|---|---|
| | LQC | MQC | HQC | Repeatability | Intermediate | ANOVA |
| | 25 ng mL$^{-1}$ | 50 ng mL$^{-1}$ | 100 ng mL$^{-1}$ | 50 ng mL$^{-1}$ | 50 ng mL$^{-1}$ | *p*-value |
| CAF | 97 | 101 | 98 | 7.08 | 5.67 | 0.21 |
| CGA | 93 | 97 | 102 | 9.29 | 8.07 | 0.18 |
| FER | 100 | 96 | 96 | 10.13 | 8.60 | 0.98 |
| QUER | 111 | 110 | 107 | 7.70 | 6.60 | 0.14 |
| | 80 ng mL$^{-1}$ | 160 ng mL$^{-1}$ | 320 ng mL$^{-1}$ | 160 ng mL$^{-1}$ | 160 ng mL$^{-1}$ | *p*-value |
| CAT | 88 | 103 | 106 | 11.69 | 8.85 | 0.42 |
| CYA | 88 | 104 | 105 | 7.47 | 5.64 | 0.89 |
| EPI | 95 | 102 | 102 | 9.19 | 7.50 | 0.33 |
| GAL | 90 | 106 | 110 | 12.84 | 8.78 | 0.28 |
| ISO | 106 | 106 | 105 | 9.02 | 3.49 | 0.22 |
| KAM | 101 | 101 | 96 | 7.31 | 3.72 | 0.20 |
| RUT | 89 | 97 | 99 | 9.53 | 7.27 | 0.48 |
| | 250 ng mL$^{-1}$ | 400 ng mL$^{-1}$ | 600 ng mL$^{-1}$ | 400 ng mL$^{-1}$ | 400 ng mL$^{-1}$ | *p*-value |
| ASC | 97 | 93 | 89 | 3.57 | 4.12 | 0.17 |

CAF: caffeic acid; CGA: chlorogenic acid; FER: ferulic acid; QUER: quercetin; EPI: epicatechin; ISO: isoquercetin; KAM: kaempferol; CAT: catechin; CYA: cyanidin 3-glucoside chloride; GAL: gallic acid; RUT: rutin; ASC: ascorbic acid.

analyzing six replicates at the MQC level. RSD values ranged from 3.57 to 12.84%, remaining within the acceptable limits defined by current regulatory guidelines.[32] Intermediate precision was determined across different days and analysts, using the same concentration level (MQC) and number of replicates as in the repeatability study. All RSD values were below 20%, complying with established acceptance criteria.

The results, expressed as RSD, were subjected to statistical comparison using *F*-test analysis, as summarized in Table 3. The corresponding *p*-values were used to evaluate the statistical significance of the observed variations. A significance level of 5% ($p < 0.05$) was adopted. *p*-values greater than 0.05 indicated that the variations across days and analysts were not statistically significant, thereby confirming the reliability and robustness of the analytical method.

Overall, the validated analytical methods demonstrated robust performance characteristics, including high linearity, minimal matrix interference, satisfactory sensitivity, and acceptable accuracy and precision, in accordance with internationally recognized guidelines.[31,32] These results confirm the reliability and reproducibility of the proposed methods, supporting their applicability in routine quality control of cashew-derived products, as well as in target metabolomic investigations requiring precise quantification of phenolic compounds and vitamin C. The methodological rigor and analytical reliability achieved make these approaches suitable tools for both regulatory and research settings, contributing to the advancement of analytical capabilities in complex plant matrices.

After optimization and validation, the analytical methods were applied to 72 individual fruits from 24 trees (three fruits *per* tree), comprising both *A. humile* and *A. occidentale*. Metabolite concentrations were expressed as mg *per* 100 g of lyophilized cashew. All statistical analyses (ANOVA, heatmap, boxplots, and PCA) were performed at the fruit level (n = 72). Table 4 reports tree/lot-level summaries as mean ± standard deviation (SD) across the three fruits from each tree/lot. Each tree/lot was assigned a unique code indicating species and collection site, with "AH" representing *A. humile* and "AO" representing *A. occidentale*. Codes were structured as species_locality_tree (e.g., AH_01_1), where the first element refers to the species, the second to the collection site, and the third to the sampled tree; codes labeled "M" denote market lots. Detailed information regarding collection sites is provided in the SI section (Table S1). Figure 3 shows the distribution of ASC alone due to its notably higher concentrations.

Most targeted bioactive compounds were successfully quantified, except for CGA and KAM, which were below the LOQ and therefore considered absent. The

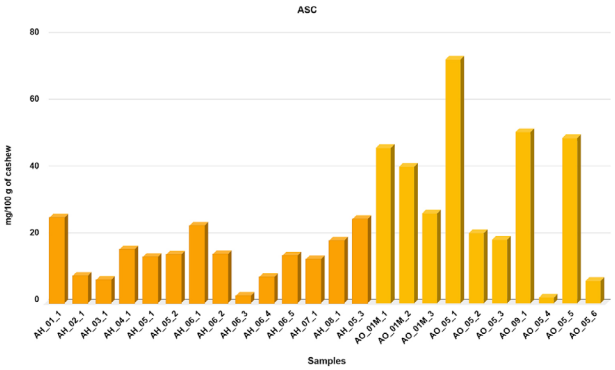


**Figure 3.** Ascorbic acid (ASC) concentrations in *A. occidentale* and *A. humile*. Values are expressed as mg *per* 100 g of lyophilized cashew.

concentrations of the quantified compounds ranged from 0.02 to 76.49 mg *per* 100 g of lyophilized cashew (Table 4).

Among *A. humile* individual fruits, ASC was generally the predominant compound, except in AH_02_1 and AH_03_1, where GAL was more abundant. AH_06_3 exhibited the highest CAT content, while AH_08_1 had the highest concentration of CYA. The distribution of RUT, FER, and EPI was relatively uniform across these fruits. Similarly, in *A. occidentale* individual fruits, ASC was the most abundant compound in most cases, particularly in AO_01M_1, AO_01M_2, AO_05_1, AO_09_1, and AO_05_5. Notably, ISO was present at higher concentrations in AO_05_1 and AO_09_1. GAL was also relatively abundant in AO_01M_1, AO_01M_2, AO_01M_3, and AO_05_3.

As expected, ASC was the most abundant compound in both species, ranging from 7.78 to 25.57 mg *per* 100 g in *A. humile* and from 19.05 to 72.98 mg *per* 100 g in *A. occidentale*. Our findings also revealed that yellow-skinned *A. occidentale* fruits (AO_01M_3, AO_05_3, and AO_05_4) exhibited lower ASC concentrations, except for sample AO_05_5, suggesting that peel color may not be a reliable predictor of ASC content.

**Table 4.** Concentrations summarized *per* tree/lot for *A. occidentale* and *A. humile* (mg *per* 100 g of lyophilized cashew; mean ± SD of three fruits *per* tree/lot.). The statistical analysis was performed at the fruit level (n = 72)

| Sample | Concentration / (mg *per* 100 g of lyophilized cashew) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ASC | CAF | CAT | CYA | EPI | FER | GAL | ISO | QUER | RUT |
| AH_01_1 | 25.57 ± 1.65 | < LOQ | 4.93 ± 0.36 | < LOQ | < LOQ | < LOQ | 1.99 ± 0.09 | 1.31 ± 0.13 | 0.06 ± 0.02 | 4.85 ± 0.49 |
| AH_02_1 | 8.18 ± 0.32 | 0.14 ± 0.00 | 3.16 ± 0.05 | < LOQ | 0.61 ± 0.04 | < LOQ | 9.14 ± 0.87 | 0.52 ± 0.02 | 0.29 ± 0.03 | 2.40 ± 0.12 |
| AH_03_1 | < LOQ | 0.12 ± 0.00 | 5.53 ± 0.30 | < LOQ | < LOQ | < LOQ | 18.40 ± 2.34 | < LOQ | 0.07 ± 0.01 | < LOQ |
| AH_04_1 | 16.06 ± 0.50 | 0.04 ± 0.01 | 3.60 ± 0.35 | < LOQ | < LOQ | < LOQ | 2.62 ± 0.19 | 1.49 ± 0.14 | 0.03 ± 0.01 | 1.31 ± 0.05 |
| AH_05_1 | 13.79 ± 1.49 | 0.03 ± 0.01 | 6.55 ± 2.09 | < LOQ | 0.57 ± 0.08 | < LOQ | 1.37 ± 0.46 | 1.56 ± 0.39 | 0.10 ± 0.03 | 2.93 ± 0.98 |
| AH_05_2 | 14.50 ± 0.72 | < LOQ | 5.31 ± 0.75 | < LOQ | 0.58 ± 0.04 | < LOQ | 1.13 ± 0.06 | 0.65 ± 0.17 | < LOQ | 1.03 ± 0.21 |
| AH_06_1 | 23.19 ± 0.93 | < LOQ | 2.83 ± 0.93 | 2.15 ± 0.65 | 0.36 ± 0.32 | < LOQ | 0.63 ± 0.55 | 1.64 ± 0.65 | 0.02 ± 0.03 | 4.03 ± 1.75 |
| AH_06_2 | 14.75 ± 0.05 | 0.05 ± 0.01 | 11.98 ± 4.04 | 0.53 ± 0.92 | 1.03 ± 0.37 | 0.04 ± 0.01 | 6.48 ± 3.07 | 2.64 ± 2.21 | 0.27 ± 0.19 | 5.54 ± 4.02 |
| AH_06_3 | < LOQ | 0.03 ± 0.00 | 18.15 ± 0.89 | < LOQ | 1.55 ± 0.28 | 0.02 ± 0.02 | 10.08 ± 1.71 | 1.90 ± 0.13 | 0.12 ± 0.01 | 0.97 ± 0.12 |
| AH_06_4 | 7.79 ± 0.53 | < LOQ | 5.35 ± 0.55 | 4.21 ± 0.68 | 1.07 ± 0.02 | < LOQ | 1.98 ± 1.45 | 1.39 ± 0.09 | 0.03 ± 0.02 | 1.33 ± 0.13 |
| AH_06_5 | 14.23 ± 0.58 | < LOQ | 4.91 ± 0.44 | < LOQ | < LOQ | < LOQ | 1.05 ± 0.10 | < LOQ | < LOQ | 0.98 ± 0.22 |
| AH_07_1 | 13.22 ± 2.60 | 0.02 ± 0.01 | 4.70 ± 0.79 | 0.82 ± 0.73 | 1.35 ± 0.18 | < LOQ | 1.13 ± 0.05 | 1.35 ± 0.57 | 0.03 ± 0.00 | 2.27 ± 0.77 |
| AH_08_1 | 18.69 ± 2.97 | 0.03 ± 0.00 | 10.49 ± 2.53 | 43.82 ± 5.35 | 1.83 ± 0.11 | 0.03 ± 0.03 | 5.31 ± 0.96 | 3.03 ± 0.45 | 0.12 ± 0.01 | 2.94 ± 0.52 |
| AH_05_3 | 25.23 ± 0.40 | 0.04 ± 0.00 | 4.84 ± 0.35 | 2.87 ± 0.53 | 3.78 ± 0.48 | < LOQ | 1.56 ± 0.04 | 0.69 ± 0.14 | < LOQ | < LOQ |
| AO_01M_1 | 46.46 ± 8.33 | 0.02 ± 0.02 | < LOQ | < LOQ | 0.56 ± 0.16 | < LOQ | 4.20 ± 1.74 | 6.39 ± 4.97 | 0.11 ± 0.08 | < LOQ |
| AO_01M_2 | 40.97 ± 6.11 | 0.02 ± 0.02 | 0.69 ± 0.62 | < LOQ | 0.59 ± 0.07 | < LOQ | 7.19 ± 5.72 | 8.76 ± 5.49 | 0.14 ± 0.10 | < LOQ |
| AO_01M_3 | 26.86 ± 0.79 | 0.07 ± 0.01 | 2.02 ± 0.07 | < LOQ | 0.93 ± 0.19 | < LOQ | 14.74 ± 0.71 | 9.85 ± 3.16 | 0.47 ± 0.11 | < LOQ |
| AO_05_1 | 72.98 ± 3.12 | 0.02 ± 0.02 | 2.41 ± 1.15 | < LOQ | 0.51 ± 0.45 | < LOQ | 1.77 ± 0.31 | 16.66 ± 8.31 | 0.45 ± 0.04 | < LOQ |
| AO_05_2 | 20.94 ± 1.75 | 0.06 ± 0.00 | 1.96 ± 0.14 | < LOQ | 0.47 ± 0.06 | 0.05 ± 0.04 | 1.92 ± 0.18 | 2.96 ± 0.18 | 0.05 ± 0.00 | < LOQ |
| AO_05_3 | 19.05 ± 0.21 | 0.04 ± 0.01 | 4.59 ± 0.82 | < LOQ | < LOQ | 0.03 ± 0.02 | 4.78 ± 0.42 | 5.97 ± 0.68 | 0.13 ± 0.01 | < LOQ |
| AO_09_1 | 51.31 ± 1.37 | 0.04 ± 0.01 | 6.17 ± 0.36 | < LOQ | 4.04 ± 0.18 | 0.00 ± 0.00 | 4.45 ± 0.16 | 16.10 ± 1.53 | 0.24 ± 0.03 | < LOQ |
| AO_05_4 | < LOQ | 0.08 ± 0.01 | 4.01 ± 0.09 | < LOQ | 1.18 ± 0.03 | 0.04 ± 0.04 | 3.22 ± 0.19 | 5.04 ± 0.14 | 0.18 ± 0.00 | < LOQ |
| AO_05_5 | 49.51 ± 0.64 | 0.05 ± 0.00 | 3.25 ± 0.10 | < LOQ | 1.36 ± 0.07 | 0.05 ± 0.04 | 1.75 ± 0.17 | 3.01 ± 0.72 | 0.06 ± 0.00 | < LOQ |
| AO_05_6 | < LOQ | 0.06 ± 0.01 | 5.91 ± 0.57 | < LOQ | 0.62 ± 0.06 | 0.01 ± 0.02 | 4.63 ± 1.02 | 3.75 ± 0.32 | 0.18 ± 0.04 | < LOQ |

KAM was below the LOQ in all analyzed cases. For CGA, only sample AH_08_1 was above the LOQ, with a value of 0.04 ± 0.01. CAF: caffeic acid; CGA: chlorogenic acid; FER: ferulic acid; QUER: quercetin; EPI: epicatechin; ISO: isoquercetin; KAM: kaempferol; CAT: catechin; CYA: cyanidin 3-glucoside chloride; GAL: gallic acid; RUT: rutin; ASC: ascorbic acid; LOQ: limit of quantification.

Despite the nutritional and antioxidant relevance of ASC, studies employing highly selective techniques-such as HPLC-MS/MS-for its quantification in cashew remain limited. Conventional methods, including titration, spectrophotometry, and electrochemical assays, are still widely used due to their simplicity and cost-effectiveness. Among these, titrimetric methods are routinely applied for the analysis of fruit and juices, such as the AOAC official method based on the reduction of 2,6-dichlorophenolindophenol.[42-44] Several studies using titration have consistently reported elevated ASC levels in cashew. For instance, Akinwale[45] measured 203.5 mg *per* 100 mL of ASC in cashew juice, substantially higher than values typically reported for other tropical fruits. Assunção and Mercadante[6] reported high ASC contents ranging from 106-121 mg *per* 100 g in red and yellow cashew varieties.

When comparing our results with those obtained using titrimetric approaches, some discrepancies in ASC content were observed. These differences may be attributed to the fact that titrimetric methods are susceptible to overestimation due to interference from other reducing substances, and may present limitations in endpoint determination, particularly when applied to intensely colored fruit matrices.[46-48] Notably, our results showed strong agreement with those obtained using more selective chromatographic techniques, such as HPLC-UV. For example, Borges *et al.*[23] reported an ASC content of 7.5 mg *per* 100 g in *A. humile* using HPLC-UV, which is consistent with our findings. This convergence reinforces the reliability of selective analytical methods for accurate ASC quantification. Overall, these findings highlight the importance of employing highly specific techniques, such as HPLC-MS/MS, to ensure accurate assessment of ASC content in cashew species. Such approaches not only enhance compositional studies but also enable more precise evaluation of their antioxidant potential and nutritional value.

CAT was the second most abundant compound in *A. humile*, while ISO was identified as the second most abundant in *A. occidentale* fruits. This profile is consistent with, or even exceeds, previously reported values. Freitas *et al.*[41] quantified CAT in yellow, red, and orange *A. humile* using HPLC-UV and reported concentrations ranging from 5.46 to 6.75 mg $kg^{-1}$, equivalent to 0.55-0.68 mg *per* 100 g. In our study, CAT concentrations ranged from 3.16 to 18.15 mg *per* 100 g in *A. humile* and from 1.03 to 6.17 mg *per* 100 g in *A. occidentale*, indicating a richer composition in our fruits. Conversely, Bataglion *et al.*[49] reported no detectable levels of CAT in cashew and other tropical fruits, which contrasts with our findings. However, their reported GAL concentrations (14.85 mg *per* 100 g) were consistent with our results, in which GAL ranged from 0.86 to 18.40 mg *per* 100 g in *A. humile* and from 1.71 to 14.74 mg *per* 100 g in *A. occidentale*.

An ANOVA was performed to compare the distribution of each metabolite between the two cashew species, *A. occidentale* and *A. humile*, at the fruit level (n = 72). The analysis revealed statistically significant differences ($p < 0.05$) for five metabolites: QUER, CAT, RUT, ISO, and ASC. These findings suggest that the concentrations of these compounds differ significantly between the species, potentially reflecting species-specific metabolic fingerprints or distinct bioactive profiles. Given that these metabolites are known for their biological functions and antioxidant properties,[9,50-55] the observed differences imply variations in the bioactive composition and, consequently, in the potential health benefits offered by each species.

To illustrate the relative variation in metabolite concentrations, a heatmap and boxplots of the key metabolites were constructed (Figure 4). In the heatmap, yellow and purple indicate higher and lower metabolite concentrations, respectively. *A. occidentale* individual fruits exhibited significantly higher concentrations of QUER, ISO, and ASC. Conversely, *A. humile* fruits showed a predominance of CAT and RUT, with RUT observed exclusively in *A. humile* (Figure 4b), suggesting potential species specificity.

Principal component analysis (PCA), presented in Figure 5, illustrates the distribution of fruits from both species based on the first two principal components (PC1 and PC2), which together account for 73.88% of the total variance when PCA is built with ANOVA-significant metabolites. PC1 explains 48.32% of the variance, while PC2 contributes 25.56%. The score plot reveals a species-related grouping pattern, with *A. humile* (red circles) predominantly occupying the left region of the score space and *A. occidentale* (blue circles) in the right, although some overlap persists. A PCA including all quantified metabolites (n = 12) exhibited a similar species-related pattern, but a lower cumulative variance explained (PC1 + PC2 = 40.84%), reflecting the influence of less informative variables. For clarity, the main text reports the PCA based on ANOVA-significant metabolites (PC1 + PC2 = 73.88%), with the full PCA provided in the SI section (Figure S1).

Despite the apparent separation, a slight overlap of points in the central region of the PCA plot (Figure 5) suggests that some fruits may share compositional features, or that the variables are partially correlated. Moreover, within the present sampling design, no consistent geographic pattern was evident relative to the interspecific differences. This suggests that the variations are more likely

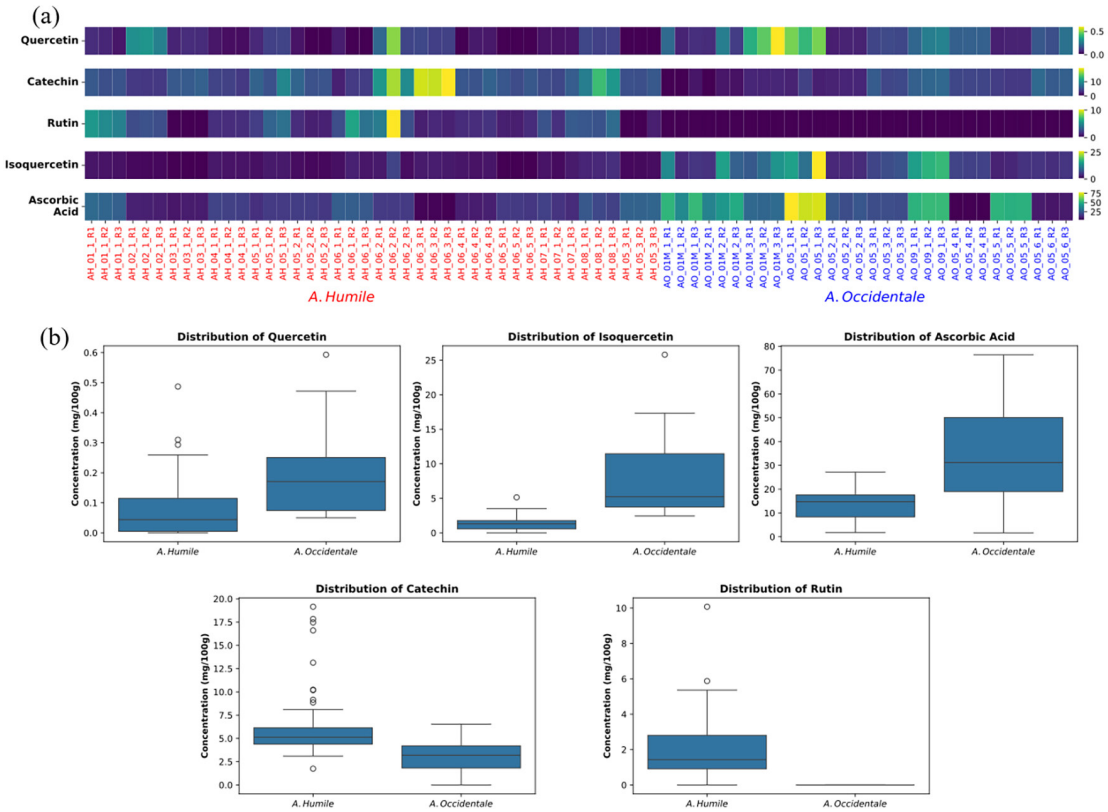


**Figure 4.** Bioactive compound content profiles in *A. humile* and *A. occidentale*. (a) Heatmap with the predominance of bioactive compounds (QUER, CAT, RUT, ISO, and ASC) in cashew fruits from different locations. (b) Boxplots with the distribution of these compounds across the two cashew species.

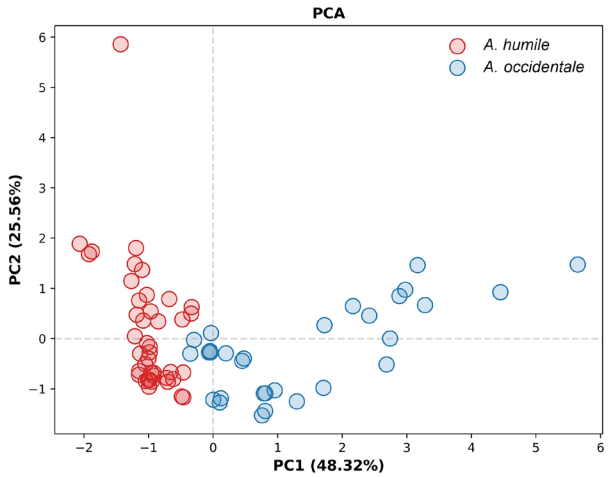


**Figure 5.** PCA score plot of individual fruits (n = 72) based on standardized concentrations of ANOVA-significant metabolites. Points are colored by species - *A. humile* (red) and *A. occidentale* (blue). A PCA including all quantified metabolites is shown in Figure S1.

attributable to intrinsic species-specific characteristics rather than to local environmental conditions.

These results highlight the distinct metabolite profiles of *A. humile* and *A. occidentale*, with marked differences in the concentration and distribution of key bioactive compounds. While ASC was the most abundant analyte in both species, *A. occidentale* exhibited significantly higher ASC levels, suggesting a greater potential contribution to its antioxidant capacity. Conversely, *A. humile* fruits demonstrated a more diverse distribution of phenolic compounds, including higher relative concentrations of CAT and CYA in specific cases. The variability observed among individual fruits highlights the combined influence of species-specific characteristics and environmental factors on metabolite composition. The application of HPLC-MS/MS enabled highly sensitive and selective quantification, effectively overcoming the limitations of conventional analytical techniques and allowing for more accurate comparisons with previously published data.

Overall, the results offer valuable insights into the chemical composition of cashew species and underscore the importance of advanced chromatographic methods for reliable metabolite profiling in complex fruit matrices, further highlighting the nutritional and functional potential of native Brazilian fruits.

## Antioxidant capacity assessed by the DPPH assay

The antioxidant capacity of the samples was evaluated using the DPPH• radical scavenging assay, a widely recognized and validated method for assessing antioxidant activity in various plant extracts and natural products.

Table 5 presents the results obtained for the antioxidant activity of *A. humile* and *A. occidentale* extracts, expressed as effective concentration ($EC_{50}$) in units of grams of fruit *per* gram of DPPH• (g fruit *per* g DPPH•). The $EC_{50}$ value represents the concentration of extract required to reduce the initial DPPH• radical content by 50%, serving as an inverse indicator of antioxidant potency. The antioxidant activity of the samples revealed significant variability between the *A. occidentale* and *A. humile* samples.

**Table 5.** Antioxidant capacity of *A. humile* and *A. occidentale* extracts determined by the DPPH• assay, expressed as $EC_{50}$ ± SD (n = 3)

| Species | Sample | $EC_{50}$ ± SD / (g fruit *per* g DPPH•) |
|---|---|---|
| *A. humile* | AH_01_1 | 89.75 ± 0.007 |
| | AH_02_1 | 227.62 ± 0.024 |
| | AH_03_1 | 107.58 ± 0.007 |
| | AH_04_1 | 138.07 ± 0.007 |
| | AH_05_1 | 133.63 ± 0.008 |
| | AH_05_2 | 170.73 ± 0.008 |
| | AH_06_1 | 152.39 ± 0.005 |
| | AH_06_2 | 159.40 ± 0.002 |
| | AH_06_3 | 145.48 ± 0.004 |
| | AH_06_4 | 97.47 ± 0.024 |
| | AH_06_5 | 125.16 ± 0.026 |
| | AH_07_1 | 118.11 ± 0.030 |
| | AH_08_1 | 85.37 ± 0.006 |
| | AH_05_3 | 95.60 ± 0.006 |
| *A. occidentale* | AO_01M_1 | 75.77 ± 0.002 |
| | AO_01M_2 | 92.77 ± 0.019 |
| | AO_01M_3 | 103.54 ± 0.008 |
| | AO_05_1 | 65.67 ± 0.009 |
| | AO_05_2 | 110.43 ± 0.012 |
| | AO_05_3 | 121.70 ± 0.006 |
| | AO_09_1 | 82.39 ± 0.003 |
| | AO_05_4 | 381.94 ± 0.003 |
| | AO_05_5 | 82.56 ± 0.006 |
| | AO_05_6 | 133.34 ± 0.006 |

$EC_{50}$: effective concentration; DPPH: 2,2-diphenyl-1-picrylhydrazyl; SD: standard deviation.

Among the *A. occidentale* samples, AO_05_1 exhibited the highest antioxidant capacity, with an $EC_{50}$ of 65.67 g fruit *per* g DPPH•, indicating strong radical scavenging activity. Samples AO_01M_1 (75.77), AO_09_1 (82.39), and AO_05_5 (82.56) also showed notable antioxidant performance, with $EC_{50}$ values in a similar range. In contrast, sample AO_05_4 demonstrated the weakest antioxidant activity ($EC_{50}$ = 381.94 g fruit *per* g DPPH•), requiring nearly six times the extract concentration of AO_05_1 to achieve comparable DPPH• neutralization. This pronounced variability in antioxidant efficiency among samples likely reflects differences in the concentration and composition of phenolic compounds, such as isoquercetin, gallic acid, and ascorbic acid, underscoring the contribution of these bioactive metabolites to the antioxidant potential of *A. occidentale.*

Within the *A. humile* group, sample AH_08_1 exhibited the highest antioxidant capacity, with an $EC_{50}$ of 85.37 g fruit *per* g DPPH•. Samples AH_01_1, AH_03_1, AH_05_1, and AH_06_4 showed similar $EC_{50}$ values, ranging from 89.75 to 103.54 g fruit *per* g DPPH•, indicating a relatively homogeneous antioxidant profile among these extracts. In contrast, sample AH_02_1 demonstrated a considerably lower antioxidant capacity ($EC_{50}$ = 227.62 g fruit *per* g DPPH•), suggesting the need for a much higher extract concentration to achieve equivalent radical scavenging activity. These variations in antioxidant potential appear to correlate with the quantified levels of specific bioactive compounds, particularly ascorbic acid, catechin, and cyanidin, reinforcing the relevance of these metabolites to the antioxidant profile of *A. humile* fruits.

Variations in edaphoclimatic conditions, such as soil composition, rainfall, altitude, and solar radiation, directly affect the biosynthesis and accumulation of secondary metabolites, particularly phenolic compounds and ascorbic acid, which are major contributors to antioxidant activity.[56-58] Additionally, genotypic variability among populations of *A. humile* from different regions may result in distinct metabolic profiles, as previously suggested by phytochemical and metabolomic studies on native Brazilian flora. Differences in harvest maturity stage and post-harvest handling, including time elapsed between collection and processing, exposure to oxygen and light, and storage temperature, may further contribute to the degradation or transformation of bioactive compounds prior to analysis.[59-61] Furthermore, methodological variations in extraction protocols, such as solvent composition, solid-to-liquid ratio, extraction time, and temperature, can significantly impact the recovery efficiency of antioxidant constituents and, consequently, the measured $EC_{50}$ values.

## Conclusions

This paper reports the chemical characterization and antioxidant potential of *Anacardium humile* and *Anacardium occidentale* fruits using a validated LC-MS/MS method for the targeted quantification of 12 bioactive compounds. The method overcame limitations of less selective approaches, enabling reliable quantification and revealing interspecific differences in key metabolites, particularly catechin, isoquercetin, and gallic acid. Principal component analysis supported the distinction between species, and antioxidant activity showed considerable variation across samples. By combining advanced

analytical techniques with chemometric analysis, this study provides a methodological framework for profiling native Brazilian fruits and contributes data that may support their use in nutritional and functional foods research.

### Supplementary Information

Supplementary data (sampling location data for *Anacardium occidentale* and *Anacardium humile* fruits, the principal component analysis (PCA) of all quantified metabolites, and the optimized MS/MS spectra of all quantified compounds) are available free of charge at https://jbcs.sbq.org.br/ as a PDF file.

### Data Availability Statement

All data are available in the text.

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### Author Contributions

Naiara R. L. de Oliveira, Hugo G. Machado, Lucas S. Machado, and João V. B. Assis were responsible for formal analysis, data curation, and investigation; Gabriel F. dos Santos, Gesiane S. Lima, and Nerilson M. Lima for conceptualization, visualization, and writing (original draft, review, and editing); Andrea R. Chaves for writing (review, and editing); Boniek Gontijo for funding acquisition, visualization and writing (review, and editing).

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