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Rwandan *Coffee arabica* has caffeine increasing with altitude, but undetectable phytomelatonin

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ABSTRACT

Altitude affects plants' constituents. The presence of caffeine and melatonin in many plants, including *C. arabica,* has been widely reported. However, a possible variation of their quantity in Rwandan *C. arabica* samples across different altitudinal locations is not yet known. This study sought to provide this information. C. *arabica* samples from Kayonza, Ruhango, Gakenke, Gatsibo, Nyagatare, and Karongi districts, covering different altitude ranges in Rwanda, were obtained from the National Agriculture Export Development Board (NAEB). Analytical techniques like Thin-Layer Chromatography (TLC), High-Performance Liquid Chromatography (HPLC), and Gas Chromatography-Mass Spectrometry (GC–MS) were used. *C. arabica* samples from higher altitudes yielded higher caffeine content, while instant coffee samples showed a broad caffeine range. However, all the techniques detected no phytomelatonin in the *C. arabica* samples collected from different altitudinal locations in Rwanda following two extraction methods, even though melatonin supplements from pharmaceutical outlets manufactured in the USA (110 %), Canada (106 %), Denmark (102 %), and Belgium (2.8 %) had detectable melatonin. Rwandan *C. arabica* has no phytomelatonin but shows increased caffeine content with increasing locational altitude. Further studies to analyze the precursors and enzymes involved in the phytomelatonin biosynthetic pathways in plants are needed to unravel this mystery.

1. Background

Coffee arabica and *Coffee robusta* are the two coffee species that exist (López-Froilán et al., 2016), with the former being the most adored, accounting for over 70 % of the world's coffee production and plantation (Ali et al., 2022). Its high consumption is due to many health benefits linked to its constituent phytochemicals, including amino acids, polyphenols, alkaloids, hormones, and sesquiterpenoids; while caffeine, quinic acid derivatives, caffeic acid, ferulic acid, p-coumaric acid, melatonin, and serotonin are reported as its most readily available components (Yamagata, 2018). Furthermore, the components of coffee include proanthocyanidins, cinnamaldehydes, and cinnamic acids, which have been shown to possess antibacterial, antioxidant, antidiabetic, anticancer, and cardio-protective qualities (Saeed et al., 2019). Because phenolic chemicals can scavenge free radicals in the biological system, they are primarily responsible for coffee's antioxidant qualities (Komes et al., 2009). Apart from *C. arabica*, caffeine is also present in other plants like tea, cocoa, and cola nut (Alagbonsi et al., 2016a). Caffeine, for instance, is known to increase blood glucose (Patil, 2012; Alagbonsi et al., 2016a) while also stimulating the cardiac muscle, central nervous system, and respiratory system (Sati et al., 2023).

Melatonin, a metabolite of serotonin from the primary amino acid precursor tryptophan, is an environment-friendly bioactive molecule with little or no toxic effect on living things, and thus, it is a safe natural dietary supplement (Ruddick et al., 2006). It is a native neuro-hormone synthesized and released into the brain interstitial, cerebrospinal fluid (CSF), and circulation by the pineal gland (Geoffriau et al., 1998), though little is also secreted from the extra-pineal tissues (e,g. eye, intestine, skin, platelet, and bone marrow). It peaks at night under normal environmental conditions, transmits the information about "darkness", and is suppressed by light (Cheng et al., 2021). Nevertheless, it can also be gotten from external sources as a supplement, either in the form of synthetic melatonin or phytomelatonin derived from vegetables, seeds, fruits, and nuts (Alagbonsi et al., 2016b). It was initially documented to

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modulate the biological clock and seasonal breeding in living organisms, but further studies have revealed its other pharmacological effects, including antioxidant (Olayaki et al., 2018; Omeiza et al., 2021; Maliki et al., 2021; Nopparat et al., 2017), anti-inflammatory, anxiolytic, anti-depressant, anti-convulsant, and anti-apoptotic activities (Vivien-Roels, 1993).

Melatonin has been identified in various animals and plants. For instance, it has been identified in phototrophic unicell (*Gonyaulax polyedra*) and many non-vertebrates like crustaceans, mollusks, planarians, cnidarians, and insects (Vivien-Roels, 1993). Its presence in many medicinal herbs and plants has been reported. For instance, it has been identified in *C arabica*'s bean (6,800 ng/g), *C. robusta's* bean (5,800 ng/g), black pepper's leaf (1,093 ng/g), Wolf berry's fruit (530 ng/g), white radish' bulb (485 ng/g), white mustard's seed (189 ng/g), black mustard's seed (129 ng/g), curcuma's root (120 ng/g), almond's seed (39 ng/g), sunflower's seed (29 ng/g), cucumber's seed (11–80 ng/g), strawberry's fruit (11.3 ng/g), tomato's fruit (2.5 ng/g), corn's seed (1.4 ng/g), rice (1 ng/g), just to mention a few among so many plants (Salehi et al., 2019). In plants, it increases the capacity for stress tolerance, serves as a crop bio-stimulating agent and pathogen repeller, and stimulates growth (Salehi et al., 2019). Even though they both have the needed enzymatic systems to synthesize melatonin, its levels in plants are higher than those found in animals. Moreover, plants can synthesize tryptophan (the precursor of melatonin) while animals cannot, as they only rely on food to get this precursor. Thus, tryptophan from plant sources—including coffee—is necessary for the synthesis of melatonin in all animals, including humans. However, there is presently no study that has either confirmed the presence of melatonin in Rwandan coffee or reported its quantity therein.

There have been reports of how caffeine and melatonin differently affect the sleep-wake cycle in humans. For instance, agonists of adenosine receptors elicit an increase in melatonin secretion by causing accumulation of cyclic adenosine monophosphate via activation of the A2 receptor in the pineal gland, while caffeine, being an antagonist of the adenosine receptors, prevents melatonin rise in the pineal gland and reduces sleep quality (Shilo et al., 2002). As both compounds have been reported in coffee samples, it is important to know how altitude affects their concentrations. Moreover, the availability of substandard pharmaceutical products in the markets poses significant risks to public health, as such products often fail to meet required safety and efficacy standards, leading to ineffective treatment and prolonged illness. These substandard products may contain incorrect quantities of bioactive components, resulting in adverse health outcomes, including drug resistance and increased mortality (Haji et al., 2022). As melatonin products are available in the Rwandan market as dietary supplements, there is a need to conduct a quality assurance study to determine if they comply with the United States Pharmacopeia (USP). Presently, there is no clear information on whether the quantities of caffeine and melatonin differ in *C. arabica* samples sourced from different altitudinal locations, which would guide farmers and consumers about their choice and serve other quality assurance purposes. Thus, this study sought to identify and quantify caffeine and melatonin in *C. arabica* sourced from different altitudinal locations in Rwanda.

2. Materials and methods

2.1. Sample collection

The study utilized both qualitative and quantitative analytical methods using Thin-layer chromatography (TLC) and Gas Chromatography-Mass Spectrometry (GC–MS) from the Biotech Laboratory of the University of Rwanda, Huye campus, and the Ultra High-Performance Liquid Chromatography (UHPLC) from the Rwandan Forensic Institute (RFI), Kigali. The research employed a purposive sampling technique, collecting 500 g of green (unroasted) *C. arabica* samples from the National Agricultural Export Development Board (NAEB), which had already collected them from each of the six coffee plantations across three agroecological zones in Rwanda, including Virunga/Volcano (high altitude region, HAR) with an altitude between 3000 and 5500 m, Lake Kivu Shores and Central Plateau (middle altitude region, MAR) with an altitude between 2000 and 3000 m, and Akagera/Eastern Plateau (low altitude region, LAR) with an altitude between 500 and 2000 m Specifically, the samples were collected from HAR (Gakenke [3,807 m]), MAR (Ruhango [2,206 m] and Karongi [2,133 m]), and LAR (Gatsibo [1,472 m], Kayonza [1,568 m], and Nyagatare [1,410 m]). Furthermore, melatonin supplements available in the Rwandan pharmaceutical outlets, whose details are presented in Table 1 below, were purchased.

2.2. Sample preparation

About 5 mg of melatonin standard powder, dissolved in 50 mL of methanol, was filtered into HPLC vials using 0.45 µm filters. Twenty samples of melatonin from Denmark (209.8 mg), Belgium (54.50 mg), USA (22.88 mg), and Canada (106 mg) were ground into powder, and each was transferred into a 10 ml volumetric flask in duplicate. Following the addition of 5 ml of mobile phase, the flask was sonicated for 5 min, mechanically shaken for 10 min, and then combined and diluted with mobile phase to volume. One milliliter of the stock solution, which had been previously filtered using a 0.45 µm filter, was put into a ten-milliliter volumetric flask. This solution was then passed through a 0.45 µm filter with a finer porosity, with the first 2 ml of the filtrate being discarded and the clear filtrate being used. Two sets of samples were made. Solutions that were blank, standard, and samples were injected.

2.3. Extraction of melatonin from coffee samples

Two methods of extraction were used for the *C. arabica* samples: maceration and solid-liquid phase (SPE). For extraction method 1 (maceration), about 1 g of *C. arabica* beans from each district mentioned earlier was placed in a 25 ml conical flask, then extracted with 10 ml acetonitrile at 4 °c for 24 h in the dark. The resulting solution was placed into a centrifuge at 12,000 rpm and 4 °c for 10 min. The supernatant was collected and dried under nitrogen. The evaporated sample was dissolved with 1 ml of 0.1 M HCl, followed by extraction with 10 ml of diethyl ether. The ether phase was collected and dried under nitrogen gas and reconstituted in 1000 µL H_2O/MeOH (50:50 v/v) for analysis. For the extraction method 2 (SPE), a vortex mixer was used to stir 5 g of dried *C. arabica* bean powder and 7 mL of 5 % trifluoroacetic acid for 5 min. The mixture underwent a 10 min centrifugation at 10,000 rpm and 4 °C. The resulting supernatant was gathered and subjected to SPE via a cartridge filter. About 7.5 mL of 80 % methanol was used to wash the cartridge. The washing solution was mixed with the passing solution. After being dried down completely with nitrogen, the mixture was reconstituted in 0.5 mL of 50 % methanol.

2.4. Analytical methods

2.4.1. Thin layer chromatography (TLC)

For TLC analysis, 1 g of powdered *C. arabica* sample was weighed and

Table 1
Different brands of melatonin with their respective countries of origin.

Country of Origin	Manufacturer	Quantity obtained	Lot/Batch No.
Belgium	Kruidvat BVBA/SPRL	500 tablets	10993268
Danemark	Pharma Nord	40 tablets	2351672
USA	Natural organics 9500	90 tablets	47626
CANADA	Jamieson natural sources	30 Capsules	5484197

added to a 10 mL volumetric flask. Methanol was added to reach the desired volume, and the solution was sonicated for 30 min. The standards of caffeine and melatonin were also separately prepared with methanol as the vehicle. The TLC silica gel 60 f2 54 paper was used, and a capillary tube of 10 μL was used. A starting line was drawn approximately 1 cm from the bottom of the plate. Migration areas were divided, and samples were spotted on the plate using a 10 μL capillary pipette. The mobile phase used was composed of 25:3.4:2.5 of ethyl acetate, methanol, and water for melatonin analysis, but 100:13.5:10 of the same solvents for caffeine analysis. The saturation time was 40 min, and the migration distance was 10 cm, and 40 μl was spotted on the plates, then detected using a UV lamp at wavelength 254 nm. Staining with revelation reagents (potassium iodide and iodine in ethanol, and hydrochloric acid mixed with ethanol) was performed, and observations including spot sizes and colors were documented.

2.4.2. High-performance liquid chromatography (HPLC)

2.4.2.1. Identification and quantification of melatonin. An Agilent Technology 1290 Infinity II HPLC system (Wilmington, DE, USA) with a diode array detector, a fluorescence detector, an auto-sampler, a solvent degasser, and a column oven was used for this study. Both data (collection, recording, and treatment) and instrument operation were managed by Agilent Open LAB software. For separation, a Poros hell 120 EC-C18 (2.7 μm, 2.1 × 150 mm) column was utilized. A 10 μl injection volume was used for all HPLC tests. The mobile phase used was composed of water and methanol (80:20 %v/v) at a flow rate of 0.5 mL min^{-1}. The analytical period, including re-equilibration time, was 12 min, with the column oven being conditioned at 30 °C. λ_{max} values were obtained by using a DAD detector to scan at 254 nm.

Verification and integration of HPLC was performed by considering several characteristics defined by the International Council on Harmonization (ICH) (ICH, 2014). Preliminary tests for verification before quantification activities were performed to ensure the reliability of the analytical method as well as the results to be obtained. The specificity of an analytical method is the capacity to identify the intended component (s) in the presence of additional components (i.e., excipients) that may be anticipated to be present in the sample. The solutions of the melatonin standard, melatonin formulations, and the blank were injected into the HPLC apparatus to determine their chromatograms. The retention time of the standard, samples, and blank solution was evaluated. An analytical method is considered linear if it can yield test findings that are proportionate to the analyte concentration in samples falling within a specified range, either directly or through a well-defined mathematical transformation. The linearity between peak area and concentration was investigated using a calibration curve constructed from six standard solutions of melatonin at 30, 60, 90, 100, 120, and 150 μg/ml concentrations each in triplicate. These concentrations were prepared using methanol and water (80:20) as diluent. Methanol was sonicated for 10 min before being mixed, and the coefficient of correlation was evaluated.

The precision of an analytical method is defined as the degree of agreement between individual test results when the technique is applied repeatedly to multiple samples of a homogeneous population. It is common practice to express an analytical process' precision in terms of either standard deviation or relative standard deviation. The method's precision was proven by achieving repeatability, which was accomplished by injecting six different melatonin sample preparations at 0.1 mg/ml concentrations and evaluating the relative standard deviation. According to ICH, the relative standard deviation must be <2 %. The measure of the closeness to the true value was verified by evaluation of the recovery of un-spiked and spiked samples through standard addition. In triplicate, the recovery studies were assessed using three concentrations: 0.08, 0.10, and 0.12 mg/ml corresponding to the levels 80 %, 100 %, and 120 % with the un-spiked sample at 60 % as recommended by ICH.

The quantity (%) of melatonin in the samples was calculated using the USP formula below:

ASSAY $= (r_u/r_s) \times (C_u/C_s) \times 100$; $r_{u:}$ represents the peak response from the sample solution $r_{s:}$ represents the peak response from the standard solution

$C_{s:}$ represents the known concentration of melatonin in the standard solution (mg/ml)

$C_{u:}$ represents the estimated concentration of melatonin in the sample solution (mg/ml)

2.4.2.2. Identification and quantification of caffeine. For UHPLC analysis, green coffee beans were ground, and 1 g of powdered *C. arabica* sample was transferred to a 250 mL volumetric flask. Deionized water was added, and the flask was placed in a boiling water bath for 20 min. After cooling, the solution was filtered through a 0.45 μm filter. Instant coffee samples underwent a similar process with a 0.5 g sample weight. A standard stock solution of caffeine was prepared by dissolving 10 mg of caffeine in a 10-ml volumetric flask, filled with a 24 % methanol in water solution, and sonicated for dissolution. Standard solutions with varying caffeine concentrations were prepared and used to create a calibration curve. UHPLC analysis employed a mobile phase of deionized water and 50 % methanol in water (50:50 volume ratio) and utilized an "InfinityLab Poroshell 120 EC-C18" column (4.6 mm internal diameter, 100 mm length, 2.7 μm particle size) with isocratic flow. The UHPLC system included a solvent reservoir, quaternary pump, autosampler, and columns. The analysis involved injecting 10 μl of blank and standard solutions, followed by sample extracts. The UHPLC system was flushed between batches.

Our initial UHPLC analysis failed to identify caffeine effectively. Despite using 2 g of Gakenke green coffee powder and a 0.005 g caffeine standard with a 5 min hot water extraction and 10 min sonication, the chromatograms likely showed significant peak tailing. This tailing suggested the chosen conditions, including a flow rate of 0.4 mL/min, 1 % formic acid in water/methanol (50/50) mobile phase, UV detection wavelength to 276 nm, pressure of 600 bar and 25 °C temperature, were not ideal for caffeine separation on the InfinityLab Poroshell 120 EC-C18 column. To address this, the experimental conditions were adjusted. The temperature was increased to 30 °C, the injection volume increased to 10 μL, and the flow rate lowered to 0.1 mL/min. Additionally, the isocratic mobile phase of just methanol and water (50/50), set the UV detection wavelength to 272 nm, and used a pressure of 800 bar. These modifications aimed to improve peak shape and allow for better detection of caffeine in subsequent analyses.

2.4.3. Gas chromatography-mass spectrometry (GC–MS)

The phytochemicals present in the *C. arabica* samples were detected using GC–MS. The data were analyzed using the National Institute of Standards and Technology (NIST) software after the oven's temperature, gas flow rate, and electron gun were configured. The GC–MS analysis was performed using a DB-5 MS capillary column (30 m × 0.25 mm × 0.25 μm), a 5977B mass detector, and a 7693 GC–MS system (Agilent Technologies, Atlanta, GA, USA). The system includes a 30 m HP-5 MS column (5 % phenyl methyl siloxane), an inert XL EI/CI mass spectrometer (5975C), and a gas chromatograph. For GC–MS detection, a 70 eV electron ionization device was used. For a 1 μl injection volume (splitless mode), the injector and mass transfer line temperatures were set to 230 °C and 250 °C, respectively. The carrier gas consisted of 1.25 mL per minute of 99.99 % helium. The oven was set to operate at 35 °C for five minutes, 10 °C/min to 280 °C for ten minutes, and 50 °C/min to 285 °C for 30 min during its seventy-minute operation. The GC–MS ran for a total of seventy minutes.

3. Results

3.1. TLC identified caffeine in Rwandan green C. arabica beans from different altitudinal locations

Using TLC, we initially confirmed the presence of caffeine in the *C. arabica* samples from the six altitudinal locations. The TLC study showed the presence of caffeine in the *C. arabica* samples sourced from all six districts, irrespective of their altitude (**Fig. S1, supplementary file**).

3.2. Establishment of the caffeine calibration curve for HPLC determination of caffeine

Following TLC confirmation, we went further to determine the quantity of caffeine in the *C. arabica* samples from the six locations using UHPLC to enable us to compare them concerning their altitudes. First, we established a calibration curve with a standard caffeine of known concentrations. Various caffeine concentrations were investigated to establish a robust calibration curve with a high correlation coefficient. Seven standard levels were prepared, ranging from 20 ppm to 140 ppm, to encompass the anticipated range of caffeine content in the samples. The 20 ppm standard resulted in an undetectable peak, while the 120 ppm and 140 ppm standards exhibited broadened peaks due to their excessively high concentrations. These high-concentration peaks also yielded a lower correlation coefficient, indicating a less ideal fit for the calibration curve. Thus, we rejected the chromatograms with caffeine concentrations of 20 ppm, 120 ppm, and 140 ppm [**See Fig. S2 (a–c) in the Supplementary file**]. Next, a new set of standard solutions was prepared with a narrower concentration range, focusing on 40 ppm to 100 ppm (see Supplementary file), for analysis the following day. This refined range provided a more accurate representation of the expected caffeine content and a stronger calibration curve's correlation coefficient ($R^2 = 0.99648$) for caffeine samples' quantification (**See Fig. S3 to S9 in the supplementary file**).

3.3. Caffeine content in Rwandan C. arabica samples increases with altitude

The chromatogram and the spectrum of the 60 ppm caffeine standard used for analytical purposes (**Fig. S5a in the supplementary file**) were comparable to that of the caffeine identified in the *C. arabica* samples (**Figs. S10–S15 in the supplementary file**). The similarity of the peaks in the graphs of the standard and the *C. arabica* samples suggests that the samples contain caffeine, like the standard. The chromatograms of the *C. arabica* samples displayed the separation of caffeine and other chemicals over time. The standard's caffeine peak eluted at 4.79 min. The chromatograms of *C. arabica* samples from Gakenke (4.863), Ruhango (5.157), Karongi (4.823), Gatsibo (4.814), Nyagatare (4.804), and Kayonza (4.819) peaked at comparable times, though some slightly later peaks were observed (especially for Ruhango), possibly due to interference from other components of the *C. arabica beans.* However, it was very similar to the standard's spectrum, confirming the identified compound as caffeine.

Table 2 summarizes the caffeine content in the Rwandan *C. arabica* across different altitude ranges. The UHPLC analysis provided more precise quantification, revealing caffeine levels ranging from 0.9385 to 1.701 % (w/w) in green *C. arabica* beans. Specifically, the results showed that *C. arabica* beans grown at higher altitudes of Virunga (Gakenke) had significantly higher caffeine concentrations (68.038 ppm) compared to those grown at medium (Ruhango [47.875 ppm], Karongi [44.534 ppm]) and low altitudes (Akagera, including Kayonza [37.535 ppm], Gatsibo [45.185 ppm], and Nyagatare [44.190 ppm]).

Table 2
Caffeine content in green coffee beans at various altitudes in Rwanda.

RetTime	Type	Category of area's altitude	Amount (ppm)	% (w/w)
4.863	Gakenke green coffee	HAR	68.038	1.701
5.157	Ruhango green coffee	MAR	47.875*	1.19675
4.823	Karongi green coffee	MAR	44.534*	1.11325
4.814	Gatsibo green coffee	LAR	45.185*	1.1295
4.804	Nyagatare green coffee	LAR	44.190*	1.10475
4.819	Kayonza green coffee	LAR	37.535*	0.9385

HAR, high altitude region; LAR, low altitude region; MAR, medium altitude region.
*$p < 0.05$ vs. HAR using one-way analysis of variance.

3.4. Instant coffee samples in the Rwandan market show varying caffeine content

The chromatogram and the spectrum of the 60 ppm caffeine standard used for analytical purposes (**Fig. S5a in the supplementary file**) were also comparable to that of the caffeine identified in the instant coffee samples obtained from Rwandan pharmaceutical stores (**Figs. S16–S21 in the supplementary file**). More importantly, Table 3 illustrates the caffeine content among the six instant coffee brands sourced from different Rwandan pharmaceutical outlets. The analysis of instant coffee samples using UHPLC revealed a broad range of caffeine content, from 0.8096 to 3.207 % (w/w). The variations in caffeine content among the instant coffee samples highlight inconsistencies in product formulation and quality control. For instant coffee, the caffeine content ranged significantly, as Gorilla instant coffee had the highest caffeine content at 64.138 ppm (3.207 % w/w), followed by Dormans (19.659 ppm), Inganzo (19.474 ppm), Easyway (19.381 ppm), and Gicumbi (16.595 ppm). Huye Mountain instant coffee had the lowest caffeine content, at 16.192 ppm (0.8096 %¦w/w).

3.5. Verification of analytical methods for melatonin

We confirmed the specificity of this approach to the analysis of melatonin since the prepared standard and sample solutions produced responses with the same retention time of 3.88 min when injected into the HPLC, while the blank solution did not produce any reaction. Also, the spectra for the standard and sample were identical but different from the spectrum of the blank whose matrix had no interference with the active pharmaceutical ingredient (API) (**Fig. S22 in the Supplementary file**). Using a calibration curve made from six standard solutions of melatonin at 30, 60, 90, 100, 120, and 150 μg/ml concentrations in triplicate, the linearity between peak area and concentration was examined. The responses of peak areas vs. analyte concentrations were plotted on the calibration curves, which were then fitted using least

Table 3
Caffeine content distribution in instant coffee samples.

RetTime	Brand of Instant coffee samples in pharmaceutical outlets	Amount (ppm)	% (w/w)
4.955	Gorilla instant coffee	64.138	3.207
5.156	Dormans (Kenyan) instant coffee	19.659*	0.983
5.167	Inganzo instant coffee	19.474*	0.9735
5.175	Easyway instant coffee	19.381*	0.969
5.141	Huye Mountain Instant coffee	16.192*	0.8096
5.127	Gicumbi instant coffee	16.595*	0.8295

*$p < 0.05$ vs. Gorilla instant coffee using one-way analysis of variance.

squares linear regression.

The calibration curve was used to ascertain this, and R^2 was assessed to see if it approached 1. The obtained R^2 for this scenario was 0.997 (See **Fig. S23 in the Supplementary file**).

The precision of the method was verified by obtaining a repeatability parameter with a relative standard deviation (RSD) of 1.58 %, and this was obtained by injecting six individual sample preparations of melatonin at 0.1 mg/ml. The RSD is not up to 2 %, which is the maximum acceptable value. Thus, the analytical method used in this study is precise (**Table S1 in the Supplementary file**). By calculating recovery, one can compare the experimental and theoretical amounts to assess the correctness of the analytical approach. Unspiked samples with 60 %, and spiked samples with 80 %, 100 %, and 120 % concentrations were prepared in triplicate to determine the recovery. The recovery studies were assessed using three concentrations: 0.08, 0.10, and 0.12 mg/ml. The accepted recovery, according to the USP, is between the range of 90 % to 110 %. In this study, the recovery for 0.08 mg/ml was 98.46 %, that of 0.10 mg/ml was 103.18 %, and that of 0.12 mg/ml was 90.22 %. These show that our analytical method passed the accuracy criteria (**Table S2 in the Supplementary file**).

3.6. TLC detected no melatonin in Rwandan C. arabica

The initial identification of melatonin in the Rwandan *C. arabica* samples was done using TLC. As shown in the TLC plate in Fig. S24, no melatonin was detected in the coffee samples from Kayonza, Nyagatare, Ruhango, Gakenke, Gatsibo, and Karongi. Similarly, no melatonin was detected in the formulation from Belgium. However, melatonin was detected in the formulation sourced from Denmark.

3.7. HPLC detected varying quantities of melatonin in melatonin formulation supplements in the Rwanda market

About 0.1 mg/ml of melatonin standard and samples were eluted on the HPLC in quadruplicates and their retention times and peak responses were recorded (**Table S3 of the Supplementary file**). The chromatograms of the samples are very similar to that of the melatonin standard (**Figs. S25–S28 in the Supplementary file**). The average retention time of the melatonin samples from the USA (3.907 min), Denmark (3.912 min), Canada (3.899 min), and Belgium (3.90 min) were calculated, and they are all comparable to that of the melatonin standard (3.911 min) within the acceptable difference of $\pm$ 0.2. The samples from the USA (1.14×10^4), Denmark (1.08×10^4), Canada (1.13×10^4), and Belgium (0.0297×10^4) also had a peak response similar to that of the melatonin standard (1.06×10^4). Having confirmed the presence of melatonin in the four formulations in the market, their quality was further determined by determining the percentage of melatonin in them. The USA product had the highest quantity (107.8 %), followed by those from Canada (106.6 %) and Denmark (101.9 %). However, the Belgium product had as low as 2.8 %, which falls below the acceptable range of 90 % - 110 % using the USP guideline. Thus, the product from Belgium failed the quality assessment test while the other three products from the USA, Denmark, and Canada proved to be of high quality (Table 4).

3.8. HPLC detected no melatonin in Rwandan C. arabica samples

Using 2 different methods of extraction (maceration and solid extraction), two types of *C. arabica* extracts were made. HPLC analysis of both extracts did not identify melatonin in the *C. arabica* samples from all the locations irrespective of their altitude, as the peaks of identified compounds were not comparable to that of melatonin as shown in **Figs S29–S34 in the Supplementary file**.

3.9. GC–MS identified no melatonin in Rwandan C. arabica samples from different altitudes

The GC–MS method could not identify melatonin in the *C. arabica* sample from Rwanda, as the retention times of the identified compounds were not comparable with that of melatonin (**Fig. S35 of the Supplementary file**).

4. Discussion

Adaptation of plants to their environment determines their growth and development throughout their lifetime, and plants have created means to survive under many challenging conditions (Arnao et al., 2022). Altitude and geographical location have been established to influence the type and number of active constituents in plants via modulation of soil nutrients, precipitation, and temperature, all of which affect the secondary metabolite contents and biological activities of plants (Munné-Bosch et al., 2016). This study aimed to identify and quantify the levels of caffeine and melatonin in Rwandan *C. arabica* samples from different altitudinal locations. Caffeine and melatonin standards were used for analytical purposes, while instant coffee and melatonin supplements commercially available in Rwandan markets were also analyzed for quality control purposes.

The TLC analysis of green *C. arabica* samples in this study yielded an Rf value of 0.53 for caffeine. This finding aligns with the results from Azady et al. (2021), who found Rf values between 0.4 and 0.6 for caffeine in tea samples using a similar solvent system. This consistency suggests that TLC is a reliable method for identifying caffeine across different sample types. The UHPLC results for green *C. arabica* coffee beans in this study (0.9385–1.701 % w/w) are also comparable to those reported by Atlabachew et al. (2021) for green coffee bean extracts (0.63–2.20 % w/w) in Ethiopia, demonstrating UHPLC's capability to produce accurate and reproducible measurements suitable for detailed research. However, the caffeine content in instant coffee samples varied considerably, ranging from 0.8096 % to 3.207 % w/w. This variability is likely due to differences in processing methods, as highlighted by previous studies that noted that different methods of processing instant coffee can significantly influence its chemical characteristics, including caffeine content (Vareltzis et al., 2020).

The caffeine content in green coffee beans varied significantly across different altitudes, as a significant difference between the caffeine content from various altitudes was observed. For instance, high-altitude regions, such as Gakenke, had the highest content of caffeine (1.701 % w/w); Karongi and Ruhango, representing medium-altitude regions, showed caffeine contents of 1.11325 % and 1.19675 % w/w respectively; while low-altitude regions like Kayonza, Nyagatare, and Gatsibo

Table 4
Assessment of melatonin content in commercially-available melatonin supplements using HPLC.

	Melatonin standard	USA melatonin product	Denmark melatonin product	Canadian melatonin supplement	Belgian melatonin supplement
Concentration analyzed (mg/ml)	0.1	0.1	0.1	0.1	0.1
Peak response	1.06×10^4	1.14×10^4	1.08×10^4	1.13×10^4	297.1831525
Retention time (min)	3.911	3.907	3.912	3.899	3.90
Melatonin present in the sample	100	107.8	101.9	106.6	2.8
Assessment verdict	–	Pass	Pass	Pass	Fail

exhibited the lowest caffeine contents ranging from 0.9385 % to 1.1295 % w/w. This significant variation supports the hypothesis that altitude influences caffeine content in coffee beans. These findings align with the research by Hagos et al. (2018), which documents a trend of increasing caffeine content with altitude. It also agrees with the previous reports that demonstrated increase in tea's caffeine content as altitude increases (Nyirahabimana & Uwimana, 2017). Moreover, a variation in various coffee samples sourced from the four provinces of Rwanda had been reported (Jean D'amour et al., 2021).

Cooler temperatures and potentially greater stress conditions at higher altitudes might lead to higher caffeine synthesis in coffee plants as a defensive mechanism. This correlation implies that geographical origin and altitude are crucial determinants of caffeine content in coffee beans, impacting both the quality and biochemical composition of the coffee. Our study is also comparable with the previous observation in India that phytochemicals like phenol and flavonoids and bioactive constituents like gallic acid, ascorbic acid, and quercetin are higher in *Taxus wallichana* from higher altitudes than those from lower altitudes. The authors also reported that *T. wallichana* from higher altitudes has a stronger anti-oxidant capacity than those from lower altitudes (Adhikari et al., 2022). Altitude has also been shown to affect the quantity and quality of essential oils in various plants, including *Thymus kotschyanus* (Lamiaceae), *Teucrium hyrcanicum* (Lamiaceae), *Cymbopogon olivieri* (Poaceae), *Mentha piperita* (Lamiaceae), *Tanacetum polycephalum* (Asteraceae), *Ziziphora clinopodioides* (Lamiaceae) and *Lavandula angustifolia* (Lamiaceae) (Demasi et al., 2018). There is also a Libyan report of some compounds (e.g. α-terpinolene, allo-ocimene, viridiflorine, α-cedrene oxide, caryophllene oxide, β-oplopenone, caryophyllenol, valerenol, 2-tetra-butyl-4-methyl-phenol) detected in *Satureja thymbra* L. at an altitude of 156 m but not detected in the same plant at an altitude of 661 m above sea level. This report also highlighted the presence of dehydro-p-cymene, 2,3,5,6-tetramethyl,3,4-diethyl phenol, cembrene, *p*-anisaldehyde, 1,2-diethyl-3,4-dimethyl-benzene, ethyl-tetra-methyl-cyclo penta-diene, 4, methoxy-6-methyl-2(3′, 5′-dimethoxy benzyl) benzoic acid, 1,2,3,1′,2′,3′-hexamethyl-bicyclopentyl 2,2′-diene, 2-hydroxy-3,4,6,7-tetra methoxy phenanthrene, 1.8-dimethoxy-3-methylanthracene,9,10-dione, and benzie-α-pyrone in *S. thymbra L* from 661 m, but absent in that from 156 m (Khalil et al., 2020).

Melatonin, being an amphipathic molecule, is recognized as a new plant hormone that stimulates many biochemical and physiological processes in plants, including growth, photosynthesis, germination, flowering, fruiting, and improvement of plant tolerance to both biotic and abiotic stress (Wang et al., 2020). Some of these stressors include drought, waterlogging, severe temperatures, salt, alkalinity, chemical contaminants in soils (such as heavy metals, pesticides, and others), UV radiation, and their combinations (Arnao & Hernández-Ruiz, 2021). While melatonin is used to refer to the one either derived from animals or created chemically, phytomelatonin refers to a plant-derived melatonin Arnao and Hernández-Ruiz (2018). In *C. arabica* and *C. canephora*, serotonin and phytomelatonin have been detected and quantified with HPLC (Ramakrishna et al., 2012). This study reported that the phytomelatonin and serotonin concentrations in *C. canephora* in green beans (5.8 ± 0.8 μg/g, 10.5 ± 0.6 μg/g dry weight), roasted beans (8.0 ± 0.9 μg/g, 7.3 ± 0.5 μg/g dry weight), and brewed sample (3.0 ± 0.6 μg/g, 4.0 ± 0.7 μg/g dry weight). The authors also reported the concentrations of phytomelatonin and serotonin in *C. arabica*'s green beans (6.8 ± 0.4 μg/g, 12.5 ± 0.8 μg/g dry weight), roasted beans (9.6 ± 0.8 μg/g, 8.7 ± 0.4 μg/g dry weight), and brewed samples (3.9 ± 0.25 μg/g, 7.3 ± 0.6 μg/g dry weight). Lower levels of phytomelatonin have been reported in coffee samples at nanogram (Jeszka-Skowron et al., 2023) and picogram (Kocadagili et al., 2014) concentrations using LC-MS. In addition to the LC technique, melatonin has also been identified in pharmaceutical formulations and human plasma using GC–MS (Nùñez-Vergara et al., 2001). In this study, we investigated whether Rwandan *C. arabica* samples sourced from various altitudinal locations have phytomelatonin, considering the well-established significance of this molecule in plant ecophysiology.

To achieve our aim of confirming the presence of phytomelatonin in the Rwandan *C. arabica* from different altitudes, we used three different analytical methods including TLC, HPLC, and GC-MC to identify melatonin from the samples following two different methods of extraction. Surprisingly, we confirmed that the *C. arabica* samples separately extracted with both methods had no detectable phytomelatonin. In other to ascertain that the lack of detection of phytomelatonin in our lab was not an experimental error in the analytical process, we sourced melatonin supplements (pills) commercially available in the Rwandan pharmaceutical stores for quality assurance and quality control purposes, in addition to the standard melatonin compound obtained for analytical purpose. We also verified the specificity, linearity, precision, and accuracy of our analytical methods using the standard analytical melatonin compound. Using HPLC for identification and quantification of melatonin in the supplements, we found that melatonin supplements from the USA had the highest quantity (107.8 %), followed by Canadian (106.6 %) and Denmark (101.9 %) samples. However, the Belgium sample had as low as 2.8 %, which falls below the acceptable range of 90 % - 110 % using the USP guideline, while the others passed the analytical test. Confirmation of the presence and quantity of caffeine in the supplements using HPLC and TLC, while the same procedures and GC–MS did not detect any phytomelatonin in the *C. arabica* samples, provide convincing evidence that our finding was not based on analytical error. It strongly establishes the absence of phytomelatonin in Rwandan *C. arabica* from different altitudes. It is thus assumed that a different melatonin-independent ecophysiological mechanism may exist for *C. arabica*'s survival where melatonin can't be synthesized in the plant.

Different pathways, all of which start from tryptophan, have been established for the biosynthesis of phytomelatonin in plant, and the enzymes that drive these pathways include tryptophan decarboxylase (TDC), tryptophan hydroxylase (TPH), tryptamine 5-hydroxylase (T5H), serotonin N-acetyltransferase (SNAT), N-acetylserotonin methyltransferase (ASMT), and caffeic acid O-methyltransferase (COMT) (Back et al., 2016). The process starts with the conversion of l-tryptophan to tryptamine by TDC or to 5-hydrotryptophan by T5H, both of which are converted to serotonin by T5H and TDC, respectively. Serotonin is then converted to N-acetylserotonin by SNAT, followed by the formation of melatonin via the action of hydroxyindole-O-methyltransferase (HIOMT) and ASMT. Alternatively, serotonin can be converted to 5-methoxytryptamine by ASMT, followed by the formation of melatonin via the action of SNAT. While our preliminary study reports the absence of melatonin in Rwandan *C. arabica* from different altitudes, it is limited by a lack of data on the presence or otherwise of the precursors of phytomelatonin and their biosynthetic enzymes. Our next study will assess and quantify, if present, the levels of the enzymes and the precursors in the phytomelatonin synthetic pathways. However, the strength of our study lies in its novelty, being the first study reporting the absence of phytomelatonin in *C. arabica* samples from various altitudinal locations in Rwanda.

In conclusion, this study confirms the hypothesis that high altitude favors more caffeine concentration. It also establishes the fact that phytomelatonin is absent in *C. arabica* sourced from different altitudinal locations in Rwanda. The implications of our study are multifaceted. Firstly, the quantification of caffeine content in coffee beans from different altitudes can guide consumers who are sensitive to caffeine or prefer certain caffeine levels in their coffee. For instance, individuals seeking higher caffeine content might opt for beans from higher altitudes like Gakenke. Secondly, the significant variation in caffeine content due to altitude highlights the importance of geographical indicators in coffee labeling and marketing. Producers and marketers can leverage this information to differentiate their products and cater to specific consumer preferences. Thirdly, the absence of phytomelatonin but the presence of caffeine in varying concentrations according to the altitude makes Rwandan coffee more suitable as a dietary product to induce wakefulness. This will not be the case in coffee samples from other sources that may contain both compounds, as melatonin may induce sleep and attenuate the potency of caffeine to induce wakefulness. From a research perspective, our findings suggest several avenues for further investigation. One area worth exploring is the impact of specific agricultural practices on caffeine and melatonin synthesis in coffee plants at different altitudes. Additionally, comparing caffeine and melatonin content in coffee from different regions worldwide could help build a global comprehensive profile of these compounds in coffee, contributing to a deeper understanding of how environmental and genetic factors interact to influence their synthesis.

Ethical approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

Details about the raw data of this study are presented in the Supplementary file attached to this paper.

Conflict of interest

The authors declare that they have no conflict of interest related to the research, financial relationships, or personal affiliations. No research grants, honoraria, or financial support from any external agencies or organizations have been received that could influence the outcome of this work. All data collection, analysis, and manuscript preparation have been carried out independently of any commercial, advisory, or consulting engagements. There are no intellectual property rights, financial holdings, or positions on any advisory boards related to this research, nor do any non-financial interests or personal beliefs affect the objectivity of this manuscript.

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CRediT authorship contribution statement

James Rurangwa: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Francoise M. Twambaze:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Vedaste Kagisha:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Abdullateef Isiaka Alagbonsi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest related to the research, financial relationships, or personal affiliations. No research grants, honoraria, or financial support from any external agencies or organizations have been received that could influence the outcome of this work. All data collection, analysis, and manuscript preparation have been carried out independently of any commercial, advisory, or consulting engagements. There are no intellectual property rights, financial holdings, or positions on any advisory boards related to this research, nor do any non-financial interests or personal beliefs affect the objectivity of this manuscript.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.focha.2025.100992.

Data availability

The data are contained in the supplementary files attached in the submission of this manuscript.

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