Optimization of the extraction process of phenolic compounds from native bee pollen from the Amazon using water with ultrasound assisted through experimental design

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***Abstract*— This study aimed to study physicochemical composition and to achieve the best condition of the extraction of phenolic and flavonoid compounds from the pollen of the Amazonian stingless bee *Scaptotrigona depilis* using aqueous solution as solvent with assisted ultrasound (UAE) aiming at green chemistry. To determine the best condition of the extraction a 2^2 factorial design with three replicates at the central point was used, the factors studied were extraction time (5-15 minutes) and solid-liquid ratio (2 – 10%w/v). The responses were total phenolic content (TPC), total flavonoids content (TF) and the antioxidant activity (AA) through the ABTS method. Regarding the physicochemical composition, pollen showed to be a source of proteins (25.14 g/100 g). Statistical analysis revealed that the extraction time and the solid-liquid ratio significantly influenced the TF and AA responses ($p<0.1$). The optimization, based on the Harrington desirability function (d=0.57), indicated optimal conditions of 12 minutes and a solid-liquid ratio of 8% (w/v), resulting in experimental values of 30.02 mg GAE/L for TPC, 5.1 mg QE/L for TF, and 3.10 µmol TE/L for AA. The mean relative errors between the experimental and predicted values were less than 10% for TF and AA, validating the reproducibility of the model. Although the obtained values of bioactive compounds and antioxidant activity were lower than those reported in the literature with hydroalcoholic solvents, the sustainable approach using aqueous extracts demonstrated potential for efficient extraction of bioactive compounds. This study highlighted the potential of SBB pollen as a natural Amazon resource with nutritional and health values. UAE with water solvents proved to be an efficient alternative in the bioactive compound extraction while reducing organic solvent usage and environmental impact. In addition, the valorization of this resource can encourage the conservation of SBB and the sustainable management of hives, promoting ecological and socioeconomic benefits for local communities.**

***Keywords*—Green chemistry, native bee, natural products, optimization, sustainability.**

I. INTRODUCTION

Meliponiculture, the process of raising and managing stingless bees, is an activity that provides income for family farming [1]. It involves a reduced investment, a favorable financial return, and sustainability [2].

The Amazon ecosystem is suitable for meliponiculture due to the variety of bee species, the tropical climate, the abundant flora that provides nectar, pollen, and resin, and the more uniform flowering throughout the year [3].

Pollen is the male gametophyte present in flowers, serving as a means of reproduction. Several species of insects, including bees, use pollen as a source of protein, vitamins, and minerals [4]. Social bees collect pollen from plants and store it inside the hive, thus ensuring a food supply for the entire community.

As in the diet of bees, pollen has been highlighted as a rich source of nutrients for the human diet. Bee pollen, due to its nutritional qualities and because it is seen as a natural product,

is currently used as a food supplement [4].

The nutritional value of pollen varies according to the plant species, environmental conditions, drying process, temperature and storage period [5]. It includes nutrients such as carbohydrates, proteins, amino acids, lipids, vitamins, and minerals. In addition, it contains carotenoids, flavonoids, and phytosterols, which justify its use as an alternative food, and/or dietary supplement [6].

In addition to its nutritional qualities, pollen has gained scientific attention for its bioactive properties, including antibacterial, antifungal, anti-inflammatory, immunomodulatory, and anti-cariogenic activity. It also performs antioxidant functions and prevents the harmful activity of free radicals [6].

The experimental design optimizes analytical processes and methods, increasing efficiency and reducing execution time. One challenge of this approach occurs in multi-residue methods, which generate multiple responses with different recovery rates [7],[8]. To this end, the Desirability Function, proposed by Harrington, allows us to find the optimum point of a set of variables simultaneously, with maximum analytical efficiency [8]. Integrating this strategy with validation procedures and uncertainty calculations is essential to ensure the quality and reliability of laboratory analyses.

There are many techniques for obtaining extracts of bioactive compounds from biological matrices. Still, these techniques have some bottlenecks, such as high energy costs, reagent costs, and toxic reagents such as methanol [9]. Thus, pure water is a promising approach and part of green chemistry.

Green chemistry is a sustainable approach for the chemical industry that seeks to reduce or eliminate the use of toxic and environmentally hazardous substances [10].

The ultrasound-assisted extraction technique is essential and can be considered an environmentally friendly technology for obtaining polyphenols from various matrices [11]. Ultrasonic extraction has high reproducibility, requires fewer solvents, facilitates handling, eliminates large volumes of remaining water, and the extracts obtained have greater purity [12]. Two mechanisms determine the increase in extraction efficiency and the reduction in extraction time: ultrasonic cavitation and the mechanical mixing effect [11].

The ultrasonic extraction process involves the following methods: detexturing, fragmentation, erosion, effect, sonoporation, and local shear stress [13]. Cell rupture occurs when cavitation bubbles separate from the cell surface due to an increase in molecular speed caused by ultrasonic waves propagating in the solvent [11], [14].

In this context, the objective of this article was to initiate the process of extracting phenolic and flavonoid compounds from the pollen of the native Amazonian bee *Scaptotrigona depilis* using water as a solvent by assisted ultrasound. The aim was to obtain bioactive compounds using experimental design and desirability function for a better production response.

II. MATERIAL AND METHODS

Sample collecting

Pollen samples from *Scaptotrigona depilis* (Figure 1-a) were purchased from a local market in Curuça-PA. Approximately 450 g of pollen were acquired for analysis. Although an expert did not perform confirmation of the botanical origin, the species *S. depilis* is not considered a single-flower bee, which indicates a probable multifloral origin of the pollen, a fact that may contribute to the diversity of the phenolic compound profile identified. Access to the genetic heritage was duly registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN), under number AA08398. After collecting, the sample was stored in hermetically sealed polyethylene containers and transferred to the Animal Nutrition Laboratory at the Federal Rural University of the Amazon. The sample was milled to standardize the particle size and remove the lumps naturally formed by this type of sample. After this step, they were vacuum-packed and frozen at -18ºC until the analysis time.

Physicochemical characterization

Pollen was characterized according to the protocol of the Official Methods of Analysis of the Association of Official Analytical Chemists [15], where the following analyses were determined: moisture no. 925.09 of AOAC (using the factor 6.25 to convert nitrogen) [15], lipids no. 925.38 of AOAC [15], crude protein no. 920.87 of AOAC [15], ash no. 923.03, AOAC [15]. Carbohydrates were calculated by difference: carbohydrates = 100 – (g ash + g total protein + g total lipids). Total energy value (TEV) was calculated using the equation: VET (kcal) = 4 x (g of protein + g of carbohydrate) + 9 × (g of total lipids). All determinations were performed in triplicate and tabulated, and means and standard deviations were calculated, considering a coefficient of variation of up to 10%; data were expressed on a dry basis in g 100/g of pollen.

Color analysis

The color analysis was performed at the Animal Nutrition Laboratory – LABNUTAN. The readings of the L*, a* and b* values were taken directly on the pollen surface, using a CM 600d colorimeter (Konica Minolta®) in the CIEL*a*b* color system. The light source used in this work was D65. The Hue angle ($H_{a,b}$) is the value in degrees corresponding to the three-dimensional color diagram: 0º (red), 90º (yellow), 180º (green), and 270º (blue), and it was calculated by the numerical values of a* and b* converted to the Hue angle as in **Eq. 1** [16]:

$$H_{a.b} = \tan^{-1}\left(\frac{b*}{a*}\right) \quad \text{Eq. 1}$$

Optimization of the extraction of bioactive compounds

The runs were carried out from previous experiments, which were considered preliminary tests to choose the minimum and maximum extraction conditions.

A 2^2 linear experimental design was used (considering the curvature) with 2 independent variables, namely extraction time (min), and solid-liquid ratio percentage (w/v), with 4 trials plus 3 repetitions at the central point, constituting a total of 7 trials. The dependent variables (responses) were the content of total phenolic compounds (TPC), total flavonoids (TF), and antioxidant activity for ABTS assay (AA). **Table 1** presents the levels of each study variable.

TABLE I.
LEVELS OF EACH STUDY VARIABLE.

Code	Levels		
	-1	0	+1
X_1	5.00	10.00	15.00
X_2	2.00	6.00	10.00

Note: X_1 = Extraction time (min); X_2 = Solid-liquid ratio ((%) w/v).

For the tests, the amount of defatted sample was weighed with the solid-liquid ratio and residence time according to the experimental design. The tests were performed in a 2 mL Eppendorf tube subjected to a SolidSteel 3.8 L ultrasonic tank, 40Khz 35°C Fixed (Figure 1-b). After completion of the reactions, the liquid fraction was separated from the solid fraction by centrifugation in SIGMA centrifugation at 10,000 g at room temperature, which was discarded; the liquid fraction was stored in amber 1,5 mL Eppendorf tube to be analyzed in terms of total phenolic compounds (TPC), total flavonoids (TF) and antioxidant activity by ABTS.

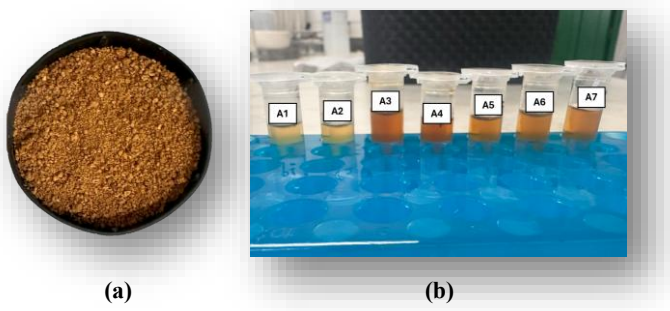


Fig. 1. (a) Pollen from *Scaptotrigona depilis* (b) Experimental trials of planning 2^2 for extraction of bioactive compounds from pollen from *Scaptotrigona depilis.*
Source: Own authorship.

Analysis of bioactive compounds

Total phenolic compounds (TPC), Total flavonoids (TF) and ABTS method Antioxidant activity

TPC followed the methodology of Singleton & Rossi [17], modified and adapted for microplates, where a reaction is performed in a 96-well microplate, in which 25 µL of extract will be inserted, followed by the addition of 125 µL of Folin-Ciocalteu reagent at 10% (v/v), the absorbance reading was made using the spectrophotometer (Thermo Fisher Scientific Oy, Multiskan Go-SN-1530-8001397, Finland. The absorbance reading was performed at a wavelength of 765 nm. The concentration of TPC in the extracts was determined from the equation of the straight line obtained in the standard curve of gallic acid (Sigma, 99% purity) (y=0.0059x+0.0929 R^2 = 0.9996) and expressed in mg of Gallic acid or GAE/g of sample.

FT were quantified according to the method described by [18], with modifications and adapted for microplates 115 µL of sample extract and 115 µL of 2% $AlCl_3$ solution were added. The absorbance was then measured at 425 nm. FT contents were calculated from a quercetin standard curve (y=0.02x-0.0047, R^2 = 0.9987) and expressed in (mg QE/100g).

The AA of the samples against the free radical ABTS•+ (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) was evaluated according to the methodology proposed by Rufino et al. [19], with modifications and adapted for the microplates. The ABTS•+ cation was produced by the reaction of a stock solution of 7 mM ABTS (>98%, Sigma-Aldrich, Brazil) with 140 mM potassium persulfate (Neon, Brazil), and the samples were read at 734 nm. Results were expressed in Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, 97%, Sigma, Brazil) (y = -0.0002x+0.7055, R^2 = 0.9902) and expressed in micromoles of Trolox equivalent (TE) per gram of extract (µmol TE/g of pollen).

According to Nicolson [20], pollen is well known for its antioxidant capacity, which makes this product help prevent several diseases, as it protects cells from oxidizing agents such as free radicals. These antioxidants present in pollen are chemical products capable of blocking or minimizing the oxidation of other molecules, interrupting reactions that can lead to harmful health conditions. According to the same author [20], these characteristics have been related to the high content of polyphenols in the sample. Polyphenols, such as flavonoids found in bee pollen, can inactivate reactive oxygen species (ROS), preventing their mutagenic action.

Analysis of phenolic compounds by high-performance liquid chromatography (HPLC)

A liquid chromatograph (Shimadzu, Japan) with an LC10AT controller, a DGU-14A pump coupled to a Rheodyne manual injector, and a UV/vis detector with SPD-M10A VP diode array was used. The compounds will be analyzed at a wavelength of 254 to 280 nm. The software used was Real Time. The column used was the C18 XBridge BEH of 150 x 4.6 mm and 3.5 µm particles (Waters - Ireland) in reverse phase. The injection volume was 20 µL, and the mobile phase gradient was: Solvent A, 0.1% TFA in MilliQ water; Solvent B, 0.1% TFA in 90% acetonitrile and 10% MilliQ water. Two HPLC-grade standard solutions of gallic acid and quercetin (Sigma-Aldrich) were injected to suggest the identification of the pollen extract peaks. The total run time was 35 minutes. The retention time of the standard substances was used as a criterion for identifying the molecules in the sample.

Statistical analysis

The results of the physical-chemical composition parameters were tabulated and calculated as mean, standard deviation, and coefficient of variation.

The experimental design's responses (TPC, TF, and AA) underwent analysis of variance, with the F regression performed to validate these data and generate response surfaces.

The analysis was performed using Statistic software (Version 7), and the confidence level adopted was 90% ($p < 0.1$).

Pearson's correlation was also calculated for TPC, TF, and AA. This measures the strength and direction of the linear relationship between continuous variables using the coefficient r, which varies from -1 to 1.

Optimization by the desirability tool

The calculation of the overall desirability function followed the Harrington methodology, with the classification as shown in Table 2 [8] .

TABLE II
CLASSIFICATION OF DESIRABILITY ACCORDING TO HARRINGTON METHODOLOGY.

Meaning of the desirability function value criterion (d)	Value
0.80 – 1.00	Very acceptable
0.63 – 0.79	Acceptable
0.37 – 0.62	Satisfactory
0.20 – 0.36	Not acceptable
0.0 –0.19	Very not acceptable

III. RESULTS AND DISCUSSION

Physicochemical characterization

The results of the physicochemical characterization of pollen samples from *Scaptotrigona depilis* are shown in Table 3.

TABLE III
PHYSICOCHEMICAL CHARACTERIZATION OF POLLEN SAMPLES FROM SCAPTOTRIGONA DEPILIS.

Determination	Mean ± Standard Deviation	Coefficient of variation (%)*
Moisture (g/100 g)	7.50± 0.76	10.13
Ash (g/100 g)	3.00 ± 0.18	6.00
Total Lipids (g/100 g)	19.29 ± 0.60	3.11
Total Proteins (g/100 g)	25.14 ± 0.65	2.58
Total Carbohydrates *(g/100 g)	52.57	-
Total energy value (TEV) kcal/g	484.45	-

The results are expressed on a dry basis (where total dry matter is equal to 92.5 g/100 g).
*Carbohydrates were calculated by the difference "100 - (total lipids + total proteins + total ash).
*** Calculated as the quotient between the standard deviation and the mean multiplied by 100. This work adopted a coefficient of variation of up to 10% to validate the replicates performed.**

It is noted that pollen has a high protein content in its composition, which makes it an interesting food for human consumption. These proteins are sources of essential amino acids, important for maintaining the body.

Proteins are essential components of all living cells and are involved in nearly every physiological process. They play an important role in immunological reactions. They serve as catalysts in chemical reactions involving enzymes or hormones in living things, aid in tissue regeneration, and, in conjunction with nucleic acids, are crucial for growth and multiplication processes [21], [22].

The fundamental building blocks of proteins are amino acids, which are joined by peptide bonds to form high molecular weight polymers (above 10,000). The quantity and kind of amino acid residues, as well as the arrangement of these molecules within the molecule, define the characteristics of a protein [21], [22].

Pollen can be seen as a valuable nutritional supplement for human nutrition, as it contains essential amino acids that play an important role in functions such as nutrient absorption, gene expression, among others [20].

Oliveira et al. [23] found high protein content in pollen from native bees in the Amazon, with an average value of 33.72 g/ 100g, 39.56 g/100 g, and 26.98 g/100 g, for the species *Melipona flavolineata*, *Melipona fasciculata,* and *Scaptotrigona* sp., respectively.

Souza et al. [24] found an average protein value of 19.5±3.3 g/100 g for several species of stingless bees, with the species *Melipona seminigra* presenting an average protein value of 23.8±0.3 g/100 g.

For moisture, lipids and ash, Souza et al. [24] found mean values of 36.9±11.1, 4.00±11.1, and 2.1±0.4 for stingless bees, respectively.

Perusso [22] found average percentage values for the bee species *Scaptotrigona* spp of 22.56 g/100 g for protein content, 21.15 g/100 g for lipids, 13.68 g/100 g of moisture, and 2.41 g/100 g of ash.

Color analysis

Color analysis in food is a very important parameter because color is one of the first attributes consumers perceive, playing a fundamental role in food product acceptance and quality assessment. In addition, color is also related to natural pigments present in food.

The a* values indicate the position on the scale, which goes from green for negative values to red for positive values. In contrast, b* indicates the position on the scale from blue for negative values and yellow for positive values. The L* parameter indicates luminosity and ranges from black (0) to white (100).

The results for pollen color analysis were 10.90±0.98, 28.17±0.54, and 44.58±0.35 for parameters a*, b*, and L*, respectively.

Based on the values obtained for the chromatic parameters a* (10.90 ± 0.98) and b* (28.17 ± 0.54), the Hue angle was calculated, resulting in 68.85°. This indicates a $H_{a,b}$ approaching the yellow range, which is compatible with the visual coloration observed for the analyzed pollen.

The a* parameter indicates that the pollen reflects a more reddish hue, since positive values indicate a tendency towards red coloration. The b* parameter indicates that the sample has a predominantly yellow hue since positive b values indicate a yellowish hue. This agrees with pollen samples, as these generally tend to present such characteristics. Pollen presented an L* value that indicates intermediate coloration in terms of luminosity, with the sample being neither too light nor too dark.

Machado [25] We found color values a* (1.03 to 12.98), b* (31.51 to 63.18), and L* (42.75 to 72.35), and we also found a correlation of these parameters with the chemical composition and biological properties of bee pollen of the species *Apis mellifera*.

To complement the color analysis, a digital optical microscope was used to show the appearance of the pollen magnified 1300 times (Fig. S1), allowing a better view of its color and luminosity.

Phenolic compounds, flavonoids, and antioxidant activity

The results for TPF in tests 1 to 7 of the experimental design ranged from 2.49 to 3.83 mg GAE/g of extract; about TF, the values were from 0.63 to 0.87 mg QE/ g of extract, while the AA values by ABTS were between 21.47 and 85.27 µmol of TE/g of extract. Table 4 presents the Pearson correlation data treatment performed for the analyses of bioactive compounds from *Scaptotrigona depilis* pollen and indicates that for TPC and AA by ABTS, the correlation was strong (0.83) and positive, indicating that AA increases as the TPC concentration increases, about TF the correlation was positive, but weak (0.22), indicating that TF alone does not influence the increase in AA.

TABLE IV
PEARSON CORRELATION COEFFICIENTS FOR THE BIOACTIVES OF *SCAPTOTRIGONA DEPILIS* POLLEN.

	TPC mg GAE/g	TF mg QE/g	AA µmol TE/g
TPC mgGAE /g	1		
TF mgQE/g	- 0,243545527	1	
AA µmol TE/g	**0,829630902**	0,216715259	1

In the current research, we observed that TPC was highly correlated with antioxidant activity, supporting their protective function in the redox protection of pollen extract. On the other hand, TF was poorly correlated, suggesting that other phenolics, excluding flavonoids or even other reducing capacity compounds, may be the culprits behind the obtained result.

Phenolic compounds are derived from secondary plant metabolites and are made up of flavonoids and nonflavonoids. Flavonoids comprise two aromatic rings joined by oxygen and exist in the form of glycosides. Nonflavonoids include phenolic acids, i.e., benzoic and cinnamic acids, stilbenes, tannins, and lignins [26]. Thus, the phenolic compounds in the pollen sample may be predominantly associated with non-flavonoid phenolic compounds

Also, we should consider that other compounds with reducing capacity, such as proteins (especially peptides with exposed amino acid residues like tryptophan, tyrosine, and cysteine), free amino acids [27], vitamin C, carotenoids, and others, could contribute to the antioxidant activity in pollen extracts [28].

Barbosa et al.[29] found for the Jataí bee (*Tetragonisca angustula* Latreille), which is also a stingless bee. It has TPC values of 12.25 mg GAE/g, TF of 2.59 mg QE/g, and ABTS of 119.65 µmol TE/g sample. These authors used hydroalcoholic solutions to produce these extracts.

Lira et al. [30], studied the antioxidant activity of solutions and several species of bees using solvents such as methanol, ethanol, and hydroalcoholic solutions as extracting solutions. They found an average value of 8.92 µMol TE/g of sample for the Jataí bee species.

Carpes et al. [31] reported values of 3.6 to 10.9 mg GAE/g of bee pollen from Alagoas, Brazil, for TCP. The authors evaluated the TPC content for different proportions of ethanol extraction solution: water, and they found that the proportions that proved to be most efficient were 60, 70, and 80% ethanol.

Pessoa [32] found AA values of 13.68 ± 2.52 µg TE/mL for extracts of pollen from the stingless bee of the species *Hymenoptera Apidae* using ultrasound-assisted maceration using a 70% hydroethanolic mixture as solvent and the ABTS method. The present study's values in µg TE/mL ranged from 1.82 to 3.41 µg TE/mL. The present study's values in µg TE/mL ranged from 1.82 to 3.41 µg TE/mL. The values were close to those obtained by the author but were still lower.

According to Nicolson [20], pollen is well known for its antioxidant capacity, which helps prevent several diseases, as it protects cells from oxidizing agents such as free radicals. These antioxidants in pollen are chemical products capable of blocking or minimizing the oxidation of other molecules, interrupting reactions that can lead to harmful health conditions. According to the same author [20], these characteristics have been related to the high content of polyphenols present in the sample. Polyphenols such as flavonoids in bee pollen can inactivate reactive oxygen species (ROS), preventing their mutagenic action.

The present study explored the use of ultrasound-assisted water extraction to extract bioactive compounds from the pollen of *Scaptotrigona depilis*, a native Brazilian Amazon bee species. Even though the bioactive compound yields were inferior to those reported in the literature, the results still indicate the excellent potential of water-based extraction. This approach aligns with green chemistry principles because it does not use any poisonous solvents and is a low-energy, eco-friendly alternative. Considering the specificity of this Amazonian species, it is possible to optimize the process even further, rendering it a sustainable and valuable resource for both local industries and broader applications in the formulation of environmentally friendly products.

Optimization of the extraction of bioactive compounds

The statistical analysis results of the process variables for extracting bioactive compounds from pollen are presented in the Pareto chart in **Fig. 2 (a, b, and c)**, obtained with 90% confidence. Statistically significant effects are located to the right of the significance threshold for $p < 0.1$.

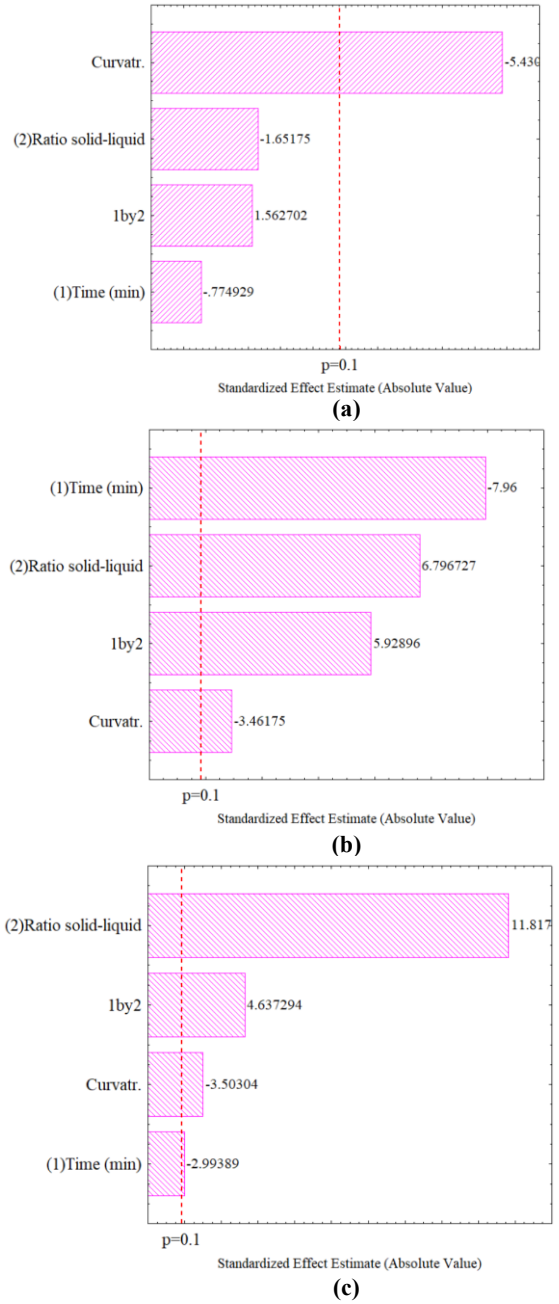


Fig. 2. Pareto chart of effects estimate for (a) TPC mg GAE/L;(b) TF mg QE/L and (c) AA µmol TE/mL.

When analyzing the Pareto chart, we can observe that no variable was significant for extracting TPC (Fig. 2-a); only the curvature that its relationship with the process variables can explain may have a more direct influence on the response. For TF (Fig. 2-b) and AA (Fig. 2-c), all independent variables and curvature had statistical significance at the 90% confidence level. The extraction time negatively influenced the TF response, indicating that the increase in this variable implies a decrease in the response. The same was observed for AA, while the solid-liquid ratio had a positive effect; that is, an increase in this variable causes an increase in the response of TF and AA.

After Pareto analysis, ANOVA variance analysis and Fisher's F test were performed for TF and AA, as shown in **Table 5**.

TABLE V
ANALYSIS OF VARIANCE (ANOVA) AND F TEST FOR THE DESIGN OF EXPERIMENTS OF ULTRASOUND-ASSISTED EXTRACTION OF *SCAPTOTRIGONA DEPILIS* POLLEN.

Source of variation	**TF (mg QE/L)**				
Effects	**QS**	**Df**	**QM**	**$F_{calculated}$**	**$F_{tabulated}$**
Regression	18.2697	3	6.0899	10.35	5.39
Residue	1.7655	3	0.5885		
Lack of fit	1.51295	1	1.512953		
Pure error	0.25250	2	0.126251		
Total	20.03519	-	-		
% variation explained	91.19%	-	-		
Maximum % explainable variation	98.74%	-	-		
	AA (µmol TE/mL)				
Regression	1.5824	3	0.5275	11.92	5.39
Residue	0.1328	3	0.0443		
Lack of fit	0.114147	1	0.114147		
Pure error	0.018604	2	0.009302		
Total	1.715190	6	-		
% variation explained	92.26%	-	-		
Maximum % explainable variation	98.92%	-	-		

Through the ANOVA analysis, we observed that the calculated $F_{3,3}$ value for the regression was 10.35 for TF and 11.92 for AA, values greater than the tabulated value of 5.39 ($F_{calculated}>F_{tabulated}$), a criterion for a model to be considered significant and capable of explaining a significant part of the variability in the data.

The explained variation was 91.19 and 92.26% for TF and AA, respectively, indicating that the models explain a large part of the observed variation for TF and AA. However, a percentage for TF of 7.55% and for AA of 6.66 could be explored, indicating that there is room to improve the fit, either by including new variables, transforming existing variables, or adjusting the experimental methodology to reduce pure error.

The mathematical models for the variables TF and AA are presented according to **Eq. 2** and **3**:

$$\mathbf{Y_2=9.65-0,60*X_1-0,23*X_2+,053*X_1*X_2+0}$$

Eq. 2.

$$\mathbf{Y_3 = 2,86-,096*\ X_1+,03065625*\ X_2+,01118125*\ X_1*X_2+0}$$

Eq. 3.

Note: X_1 = Extraction time (min); X_2 = Solid-liquid ratio ((%) w/v). Y_2 = TF mg QE/L; Y_3 = AA µmol TE/mL.

The response surface methodology (RSM) was applied to generate the graphs in **Fig.3** (a and b), which will be discussed below.

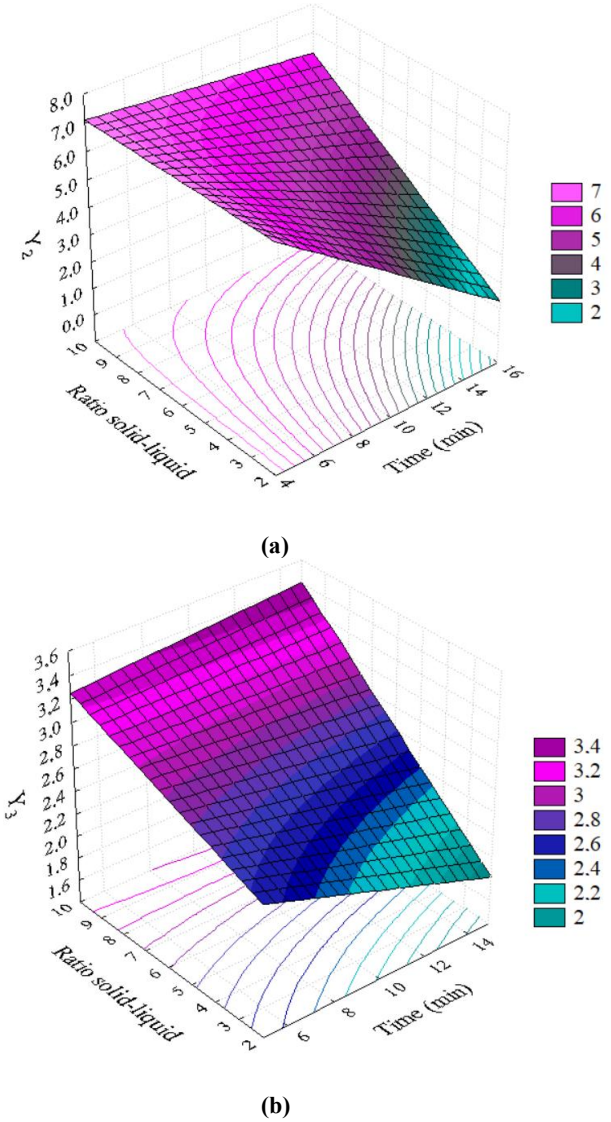


Fig. 3. Response surface for Y_2 – TF mg QE/L and (a) and Y_3 - AA µmol TE/mL. (b).

Fig. 3 (a) shows that for the TF response to increase, the time must be increased up to 10 min at any solid-liquid ratio. This behavior indicates that, up to this point, the effect of ultrasound intensifies over time, probably due to the gradual increase in cavitation or agitation efficiency generated by the ultrasonic waves. Cavitation may have a more pronounced effect as the exposure time increases, promoting greater interaction between the solid particles and the liquid.

Fig. 3 (b) demonstrates that the solid-liquid ratio must be increased to observe a further increase in the response. This suggests that greater contact between the solid particles and the liquid medium is required for ultrasound to be more effective in mass transfer. Increasing the solid-liquid ratio may provide a greater surface area available for the action of ultrasound, facilitating the dispersion or extraction process, which leads to greater efficiency in the TF response.

Optimization by the desirability tool

After analyzing the ANOVA and RSM and obtaining the mathematical models, the optimization analysis was performed using the desirability tool, as shown in **Fig. 4.**

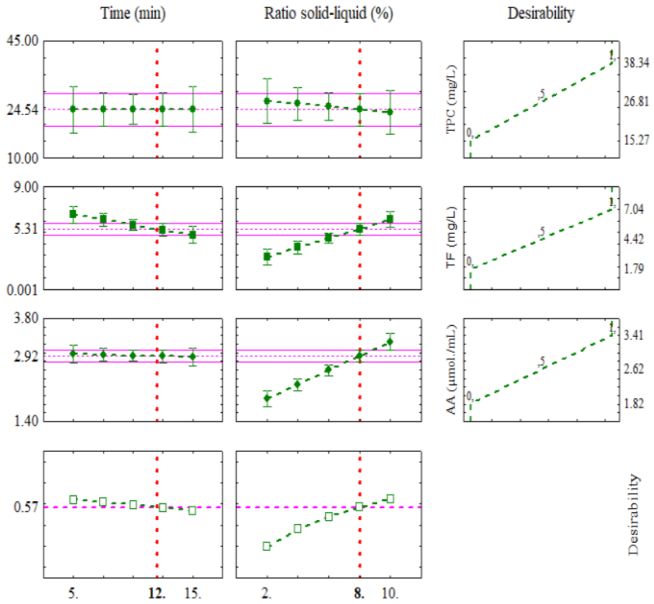


Fig. 4. Profiles for predicted values and desirability of bioactive extraction of pollen from *Scaptotrigona depilis.*

The overall desirability value for this extraction was 0.57, indicating that our experimental data fit into a satisfactory desirability range between 0.37 and 0.62 (Table 1). Thus, our mathematical models can be considered good for predicting the extraction of phenolic and flavonoid compounds from pollen samples by using pure water as a solvent, assisted by ultrasound.

The values predicted by the model as observed in Fig. 4., were 12 min for the residence time and solid-liquid ratio of 8 % (w/v), which would result in 24.54 mg GAE/L, 5.31 mg QE/L and 2.92 µmol TE/mL for TPC, TF and AA, respectively.

This condition was performed in the laboratory, where values for TPC of 30.02 mg GAE/L, TF of 5.1 mg QE/L, and AA of 3.10 µmol TE/mL were obtained. The mean relative error between the experimental values and those predicted by the model was 18.25% for TPC, 3.95% for TF, and 5.81% for AA.

The mean relative error showed good reproducibility of the optimal condition; values up to 10% are considered good; about TPC, this value exceeded what is regarded as a metric; however, we must assume that this variable was not considered significant ($p>0.1$) by the model, being only used as an indication of trend.

Oroian et al. [11] evaluated the extraction efficiency of polyphenols from *Apis mellifera* raw pollen by an ultrasonic process using solid/liquid ratios of 1, 2, and 3% and residence times of 10, 20, and 30 min in 80% (v/v) methanol. The optimal conditions were a solid-liquid ratio of 3% and a residence time of 14.30 min, leading to a maximum concentration of 366.1 mg GAE/L of TPC. These authors also varied the temperature and power parameters of the ultrasound.

Chehraghi et al. [33] studied the effect of ultrasound on the extraction of TPC from the pollen of the bee species Apis

mellifera by evaluating ultrasonication times of 15–45 min on the disintegration of the pollen wall, the amount of ethanol in the hydroalcoholic solvent of 0–100% (v/v). The highest TPC content achieved was 200 mg/L using 95% (v/v) ethanol in the solvent and an ultrasonication time of 44 min.

Although the desirability value was set as moderate, the selected condition was a feasible and efficient compromise that ensured good yields and, at the same time, avoided too short extraction times, which could compromise the reproducibility of the results, or highly elevated solid-liquid ratios, which could impair the scalability of the process.

Analysis of phenolic compounds by high-performance liquid chromatography (HPLC)

Fig. 5 shows the chromatogram of the analysis of phenolic and flavonoid compounds for the optimized pollen extract. The phenolic compounds, gallic acid and quercetin, were identified by checking the coincidence of retention times found in the analytical standards and in the samples under identical chromatographic conditions. Thus, the presence of these molecules was concluded from the similarity of the retention times because this indicates chromatographic behavior compatible with respective standards.

In our optimized extract, there may be the presence of gallic acid and quercetin, although we have the analytical standards of both substances, it should be remembered that gallic acid can exist in the protonated form or form derivatives such as esters or salts that can behave in different ways in a chromatographic analysis, the same occurs for quercetin that can also be found in the aglycone or glycosylated form; therefore, a chromatographic analysis alone is not sufficient to determine the presence of these molecules, additional studies such as mass spectrometry are necessary to ensure more accurate and reliable identification, therefore, our group only suggests that in the pollen extract obtained in the present research, there may be a majority of gallic acid and quercetin in the sample.

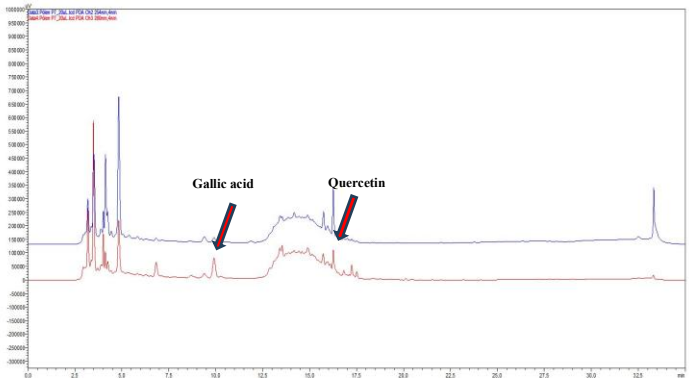


Fig. 5. Chromatogram obtained for the optimal condition of extraction of bioactive compounds of pollen from *Scaptotrigona depilis*, indicating the presence of gallic acid and quercetin.

Gallic acid (3,4,5-trihydroxybenzoic acid) is a phenol that occurs naturally in plants and is produced by hydrolysis. Numerous pharmacological characteristics of this trihydroxylated phenolic structure are being thoroughly investigated [34]. Its acidic activity allows the insertion of a wide range of substituents, producing several ester analogues with the most diverse pharmacological effects [34], it is considered a potent natural antioxidant due to its ability to scavenge reactive oxygen species, including hydrogen peroxide, superoxide anions, and hydroxyl radicals. Other studies have documented antifungal, anticancer, antibacterial, and antiulcer benefits in addition to antioxidant action [35].

Flavonoids can occur as aglycones, glycosides, or other flavonoid-containing structures such as flavolignans, but are most commonly found as glycosides. They can be divided into 13 categories, with over 5,000 compounds identified by 1990. Flavonoid subcategories include: Calcium, dihydrocalcium, aurones, flavones (apigenin, luteolin, diosmetin), flavonols (quercetin, miracetin, kaempferol), dihydroflavonol, flavanones (naringin, hesperidin), flavanol, flavandiol, anthocyanidin, isoflavonoids (genistein, daizdein), bioflavonoids, and proanthocyanins [36], [37].

Quercetin (3,5,7,3'4'-pentahydroxy flavone) is the predominant flavonoid found in the human diet. Several therapeutic benefits of flavonoids, particularly quercetin, have been investigated in recent decades, emphasizing their antioxidant and anticarcinogenic potential and their protection of the renal, cardiovascular, and hepatic system [36].

IV. CONCLUSION

The ultrasound-assisted water extraction process showed an interesting approach to extract bioactive compounds from *Scaptotrigona depilis* pollen. Although the initial findings are not definitive, water as a solvent provides good advantages, such as the absence of toxic reagents, cost reduction, and a lower environmental impact, making it a viable alternative for more sustainable processes. Furthermore, the positive relationship between total phenolic compound content and antioxidant activity reinforces the chemical potential of the natural matrix. Parallel measurements, including color analysis and hue angle determination, also provided useful clues on the composition of the extract to characterize it further.

To reach full potential in the Amazonian resource, both from an environmental conservation standpoint and to add value to this product, increased work towards optimizing conditions for extraction and further knowledge of the pollen's bioactivity profile is necessary.

SUPPLEMENTARY MATERIALS

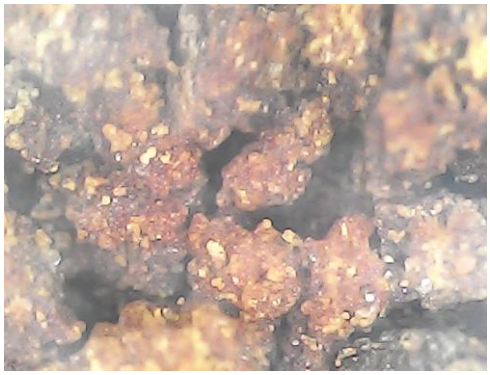


Fig. S1. Pollen sample magnified 1300 times with the aid of the MaiXpro 1600x HD USB Digital Microscope.

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