Revista de Ciências Farmacêuticas Básica e Aplicada
Journal of Basic and Applied Pharmaceutical Sciences

ISSN 2179-443X

RESEARCH ARTICLE

Development and validation of a UHPLC-ESI-MS/MS method for the quantification of artepillin C in Brazilian green propolis

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Abstract

Objectives: The aim of this study was the development and validation of a fast method to quantify artepillin C in green propolis using ultra high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UHPLC-ESI-MS/MS). **Methods:** High purity (97.8%) artepillin C was isolated from green propolis using chromatography techniques. Quantification was performed using a C_{18} (2.1 x 100 mm; 1.7 µm) column, gradient of water and methanol (with 0.01% formic acid) as mobile phase, at a flow rate of 0.4 mL/min and 45 ºC in temperature. A mass spectrometer operated in selected reaction monitoring mode to monitor the deprotonated molecular ion of artepillin C (*m/z* 299) > fragment ion (*m/z* 200.12). Several parameters such as specificity, linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy, and robustness were determined. **Results:** The method was linear in the 50 – 400 µg/mL range (r^2 = 0.9906), showing LOD = 10.79 µg/mL and LOQ = 32.70 µg/mL with satisfactory intra-day and inter-day precision with relative standard deviation (RSD %) of 1.9% and 3.4%, respectively. The accuracy showed recovery of 93-104%, the method was robust and artepillin C was quantified in green propolis at 6.51%. **Conclusions:** The proposed method showed advantages in comparison with other methods, such as short analysis time and high selectivity for artepillin C.

Keywords: Artepillin C. Brazilian Green Propolis. Liquid Chromatography. Mass Spectrometry. Quality Control.

How to cite

Riani LR, Silva LM, Silva OOZ, Junqueira LR, Nascimento JWL, Da Silva Filho AA. Development and validation of a UHPLC-ESI-MS/MS method for the quantification of artepillin C in Brazilian green propolis. Rev Ciênc Farm Básica Apl. 2021;42:e727. https://doi.org/10.4322/2179-443X.0727

INTRODUCTION

Propolis is a natural resinous material collected by bees (*Apis mellifera* L.) from certain plants and used by them mainly to protect their hives[1]. The southeastern Brazilian propolis, called as green propolis, has been used in Brazilian traditional medicine as an antimicrobial, anti-inflammatory, and to prevent diseases, such as gastrointestinal disorders[2]. Green

Financial support: LRR and LMS – received scholarship from CAPES – Coordenação de Aperfeiçoamento de Nível Superior.
Conflicts of interest: LRR, LMS, OOZS, LRJ, JWLN and AASF declare no conflict of interest.
The study was carried out at Federal University of Juiz de Fora-UFJF, Juiz de Fora, Minas Gerais, Brazil
Received on March 08, 2021. Accepted on April 14, 2021.

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propolis is produced mainly in the state of Minas Gerais, and is widely consumed worldwide not only as traditional medicine but also as a functional food[3]. The increasing use of green propolis requires relevant approaches for the quantitative determination of the active components, such as artepillin C, coumaric acid, ferulic acid, kaempferol, and kaempferide[4].

Additionally, it is known that the composition of propolis may differ according to some factors, such as season of the year and the genetic characteristics of bees[5,6]. According to the literature, the main botanical source of Brazilian green propolis is *Baccharis dracunculifolia* DC. (Asteraceae)[7,8]. Many biological activities have been reported for green propolis, such as antimicrobial[9], antimutagenic[10], gastroprotective[11] and antioxidant[12]. Also, the biological properties of Brazilian green propolis are largely due to the high levels of prenylated *p*- coumaric acids, mainly artepillin C (3,5-diprenyl-*p*-coumaric acid; Figure 1)[9].

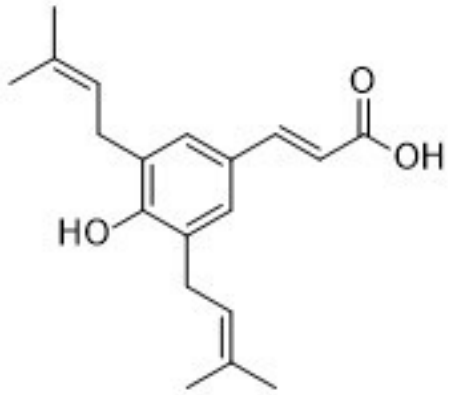

Figure 1. Chemical structure of artepillin C.

Artepillin C is the main chemical marker used for quality control of green propolis[13,14] and has demonstrated many important biological activities, including antioxidant[15], antimicrobial[16], anti-inflammatory[17] and anticancer[18, 19].

Despite artepillin C being the chemical marker of green propolis, few reliable analytical procedures to establish its content have been described, and most of them use high-performance liquid chromatography coupled with a photodiode array detector (HPLC-PDA)[13,14,20,21]. Ultra high-performance liquid chromatography coupled with mass spectrometry (UHPLC-MS) has also been used to identify artepillin C in green propolis samples[22,23].

In this regard, the legislation from the Brazilian Ministry of Agriculture in Brazil[24] designates propolis as an opotherapy drug, determining the need mainly for quantification assays of specific markers for its quality control. In this context, this work was undertaken to develop and validate a fast, sensitive, and specific method to quantify artepillin C in green propolis using efficient ultra high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UHPLC-ESI-MS/MS).

MATERIAL AND METHODS

Chemicals and reagents

Reference standard artepillin C (98.0%) was acquired from Wako Pure Chemical Industries (Osaka, Japan). Ultra-pure distilled water, with resistivity greater than 18 MΩ, was generated in-house from a Milli-Q system (Millipore Corp., Billerica, MA, USA). The green propolis sample was produced and collected from *Apis mellifera* hives and donated by Bee Propolis Brasil Ltda (Bambuí, Minas Gerais, Brazil). All other chemicals were of analytical, MS, or HPLC grade.

Isolation, identification, and purity of artepillin C

Green propolis (100 g) was maintained in a freezer at -20 ºC for 24 h and powdered in a blender. The resulting powder was submitted to maceration using methanol, followed by filtration at room temperature. The produced extract was concentrated under vacuum to furnish 30 g of a crude extract, which was chromatographed in a vacuum-liquid chromatography (VLC) system using silica gel (70-230 mesh) and chloroform/methanol mixtures as eluent, furnishing fifteen fractions (I-XV). After, fractions III and IV were subsequently joined (1.7 g) and submitted to semi-preparative reverse-phase HPLC for final purification. In this case an HPLC binary pump system (Waters 1525) equipped with a DAD detector (Waters 2998), and autosampler (Waters 2707) were used. The semi-preparative column used was a SunFire C_{18} column (10 mm x 250 mm; 5 µm particle size; Waters) with a SunFire C_{18} precolumn (10 mm x 20 mm; 5 µm particle size; Waters). Methanol/H_2O (80:20 *v/v*) was used as mobile phase at a flow rate of 5 mL/min, furnishing 100 mg of compound. The chemical structure of the isolated compound was established as artepillin C by ^{1}H- (500 MHz) and ^{13}C- (125 MHz) Nuclear Magnetic Resonance (NMR; Bruker Avance III 500 MHz spectrometer) data analysis in comparison with literature data[4,25].

The purity of the isolated artepillin C was subsequently determined in comparison with standard artepillin C (Wako Pure Chemical Industries; 98.0%) using a validated method in HPLC-PDA as previously reported[14].

Chromatographic conditions

Ultra high-performance liquid chromatography (UHPLC) was performed with a H-Class Acquity UPLC system (Waters), using an Acquity BEH C_{18} column (2.1 mm x 100 mm, 1.7 µm particle size, Waters) coupled with a Acquity BEH C_{18} guard column (2.1 mm x 5 mm, 1.7 µm particle size, Waters) under elution at a flow rate of 0.4 mL/min and column heater maintained at 45 °C. The elution system was performed with a gradient using solvents A (water) and B (methanol), both containing 0.01% formic acid. The gradient started with 40% of solvent B and increased within 8 min linearly to 100% of solvent B, which was maintained for 2 min. Starting conditions were restored immediately and maintained for 4 min, allowing the system to re-equilibrate, taking 1 µL of the aliquot and 14 min for each injection.

Mass spectrometric conditions

The UHPLC system was coupled to a Xevo G2-S quadrupole time-of-flight (Q-TOF) mass spectrometer (Waters) equipped with an electrospray (ESI) ion source, which operated in negative ionization mode. Nitrogen was used as nebulizing gas and argon was used as collision gas. The MS/MS parameters were optimized by constant infusion of artepillin C. Quantification was performed using selected reaction monitoring mode to monitor the deprotonated molecular ion (*m/z* 299) > fragment ion (*m/z* 200.12). The optimized parameters were as follows: collision energy 25 eV; capillary voltage 3 kV; cone voltage 40 V; source temperature 150 °C; desolvation temperature 500 °C; cone gas flow 50 L/h; desolvation gas flow 1000 L/h. The response acquisition was performed using MassLynx software (Version 4.1) and the quantitative analysis was performed by QuanLynx software (Version 4.1).

Preparation of standard and sample solutions

A stock standard solution was prepared dissolving the isolated artepillin C in methanol to generate a concentration of 2000 µg/mL. Additionally, stock solutions of green propolis (10 mg/mL) were prepared extracting green propolis (1.0 g) with 100 mL of methanol by ultrasonication (Sanders Medical) for 1 h. After extraction, the mixture was filtered through white-band filter paper (= 12.5 cm; JP40).

Method validation

Method validation was based on the international guidelines of the FDA[26] and ICH Q2(R1)[27], as well as to the Brazilian ANVISA (RDC n° 166/2017)[28] and INMETRO (DOQ-CGCRE-008)[29] guidelines. For this, the following validation characteristics were evaluated: specificity, linearity, range, limits of detection and quantification, precision, accuracy, and robustness.

Specificity

The specificity of the method was assessed for any possible interference in the retention time of the artepillin C under the established conditions by loading blank samples, in which methanol was used to dilute the samples and standard, and mobile phase. The analysis was performed comparing the standard, sample and blank samples, in triplicate.

Linearity, range, limit of detection (LOD) and limit of quantitation (LOQ)

Linearity was assessed by analyzing the calibration curve with the concentrations of 50, 100, 200, 300 and 400 µg/mL by three replicates. The calibration curve was analyzed statistically by defining the linear regression, correlation coefficient, determination coefficient, residues, and homoscedasticity. Range was determined from the linearity. Limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation and the slope of the calibration curve according to ICH Q2(R1)[27] (LOD = 3.3xσ/S and LOQ = 10xσ/S, where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve).

Repeatability and Intermediate Precision

The intra-day precision (repeatability) and inter-day precision (intermediate) were expressed in terms of relative standard deviation (RSD %). Six replicate samples were prepared from stock solutions of propolis samples diluted to a concentration 4 mg/mL on two different days.

Accuracy

The accuracy of method was determined in terms of percentage recovery. Recoveries of the artepillin C were determined by spiking propolis samples (from stock solutions of propolis samples diluted to a concentration 1 mg/mL) with artepillin C at three concentration levels (100, 200 and 300 µg/mL in triplicate). The accuracy was calculated by the experimental recovery value/nominal value x 100.

Robustness

The robustness parameter was evaluated by intentional modifications in the chromatographic conditions in the proposed methodology. Artepillin C was quantified in the propolis sample (4 mg/mL) and some variables were intentionally modified, such as temperature column (40 and 50 °C) and flow rate (0.3 and 0.5 mL/min), comparing with the conditions of the developed method (temperature column 45 °C and flow rate 0.4 mL/min).

Statistical analysis

Results were presented as mean ± standard deviation and the RSD % was defined as acceptable when <5%.

RESULTS AND DISCUSSION

After isolation, artepillin C was chemically identified by ^{1}H- (500 MHz) and ^{13}C- (125 MHz) NMR data analysis in comparison with the literature[4,25]. The purity of the isolated artepillin C

was estimated as more than 95% based on ^{1}H-NMR data and determined as 97.8% based on a validated HPLC-PDA analysis. The isolated artepillin C was subsequently used for method development and validation.

During method development, several parameters were evaluated to achieve better performance in sensitivity and precision. For development of MS conditions, artepillin C was directly introduced into the MS detector using ESI ionization and some parameters such as cone voltage, desolvation gas flow, and source temperature, were investigated to obtain its molecular ion ([M-H]$^-$ *m/z* 299). In addition, ionization of artepillin C was tested in positive and negative ionization modes, in which better sensitivity with higher response was achieved in negative electrospray ionization. Artepillin C was then submitted to different collision energies (0, 15, 25, and 30 eV) (Figure 2) to select the optimum collision energy, since this may affect the ion abundance and the type of ions generated in the mass spectrum[30]. Collision energy of 25 eV was subsequently selected as the most adequate for fragmentation by observing the maximum response obtained for the fragment ion peak. The mass spectrum showed the [M- H]$^-$ *m/z* 299 deprotonated molecular ion and fragments of *m/z* 255, *m/z* 200 and *m/z* 145 (Figure 2C), which agree with previous reported data[12,31].

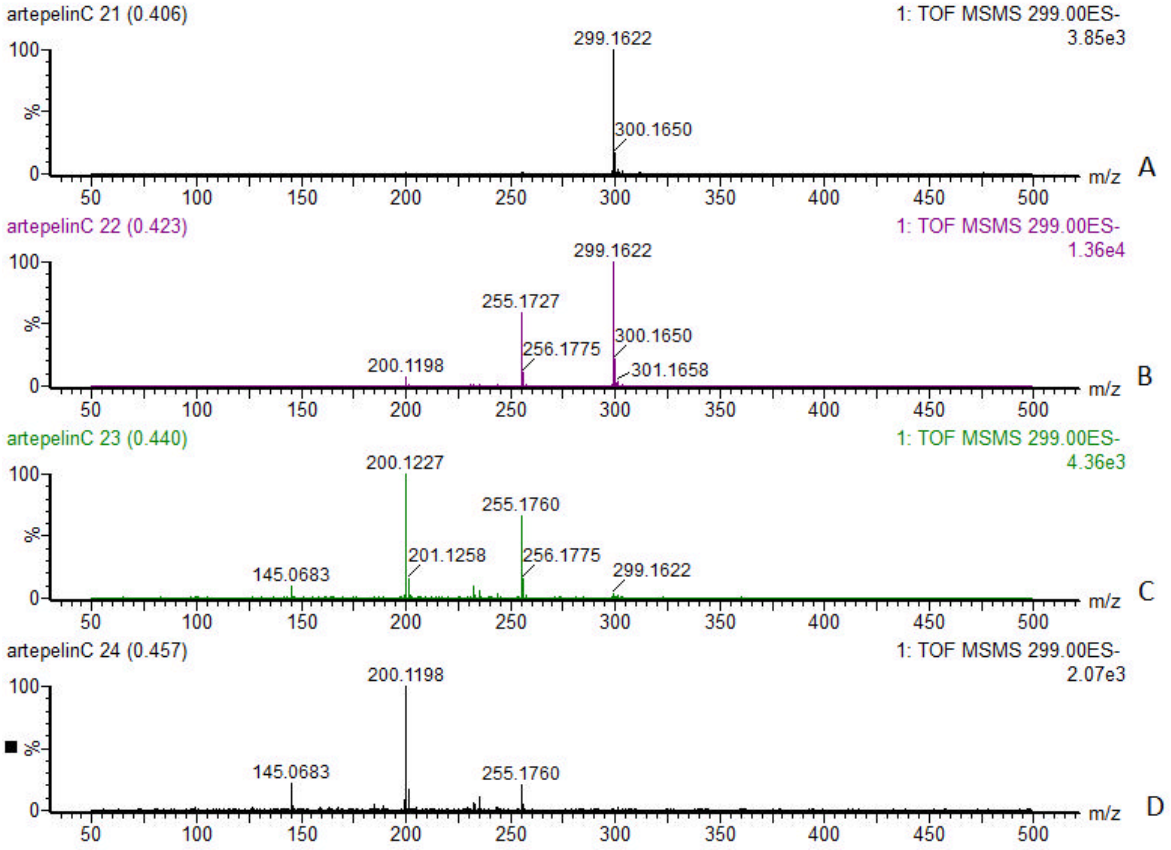

Figure 2. Mass spectra of artepillin C in different collision energies. (A) 0 eV; (B) 15 eV; (C) 25 eV; (D) 30 eV. Data represent mass fragmentation of artepillin C. 25 eV collision energy was selected as the most adequate for fragmentation, because it forms the most important mass fragments *m/z* 255, *m/z* 200 and *m/z* 145.

When the molecular ion [M-H]$^-$ *m/z* 299 was selected to acquire the green propolis chromatogram, three peaks were shown (Figure 3), referring to artepillin C and two other compounds (dihydrokaempferide and kaempferide), which possess similar molecular ions and are also present in green propolis samples[12,31]. Subsequently the base peak ion of *m/z* 200.12 (the most intense

fragment) was used as quantification ion of artepillin C (retention time 6.44 min). It is important to highlight that the deprotonated molecular ion (*m/z* 299) was selected for acquisition of the chromatogram, and the base peak fragment ion (*m/z* 200.12) to quantify artepillin C, obtaining a selective chromatogram (Figure 4), which represents a great advantage of this UHPLC-MS method over HPLC-PDA.

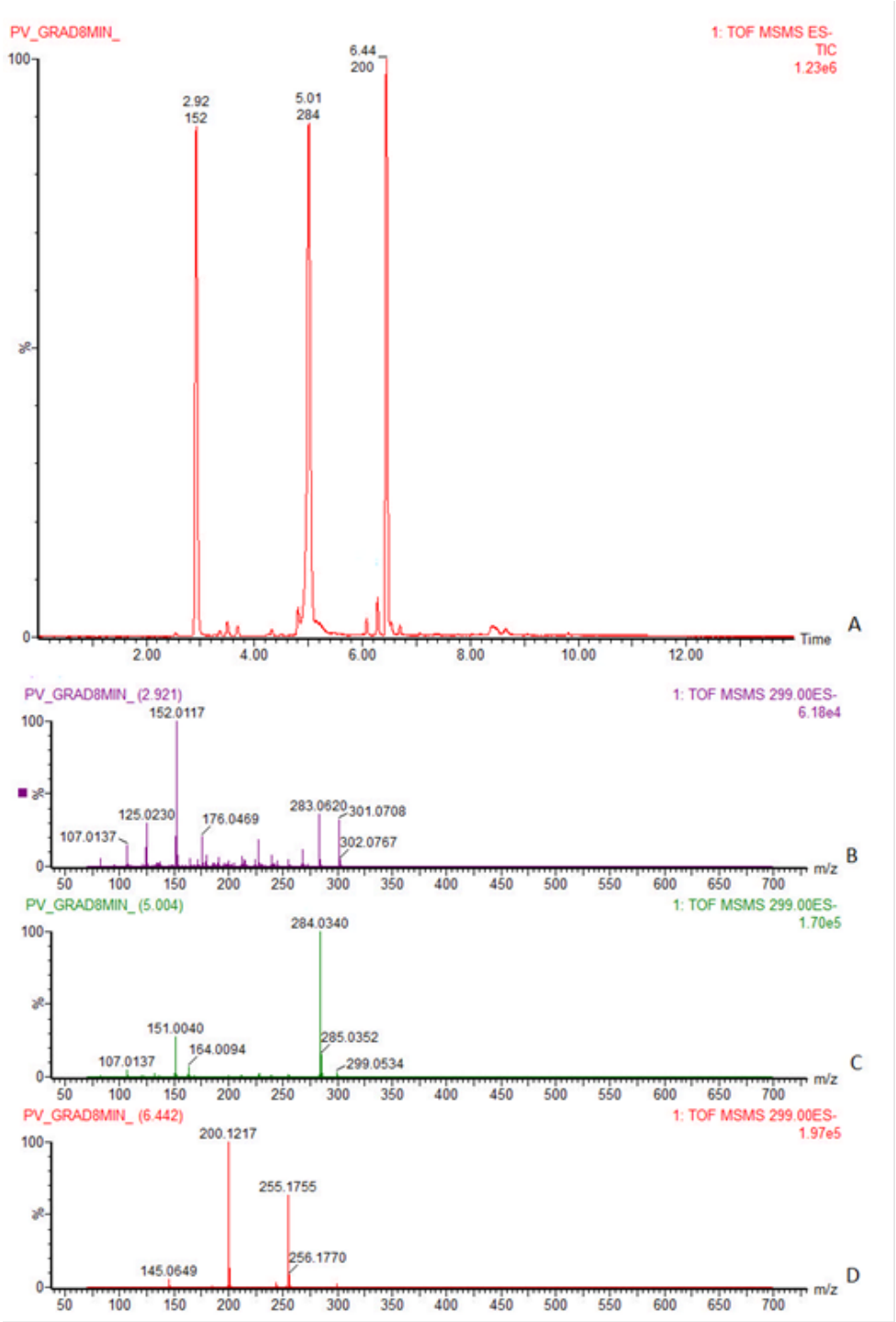

Figure 3. Chromatogram of green propolis in UHPLC-MS (with *m/z* 299 selection) and mass spectrum of the possible compounds. (A) Chromatogram; (B) Mass spectrum of peak at retention time = 2.92 min; compound (dihydrokaempferide); (C) Mass spectrum of peak at retention time = 5.00 min; compound (kaempferide); (D) Mass spectrum of peak at retention time = 6.44 min; compound (artepillin C).

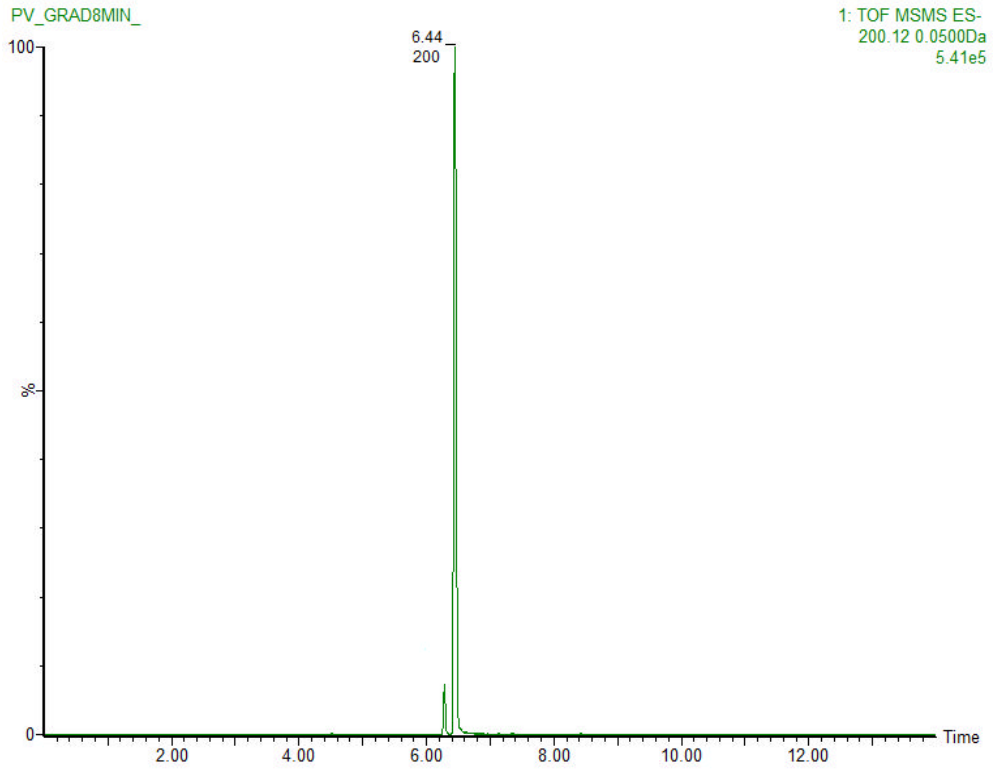

Figure 4. Chromatogram of green propolis in UHPLC-MS (with m/z 299 > m/z 200.12 selection). The chromatogram shows the selectivity of the method for artepillin C when the deprotonated molecular ion (m/z 299) and also base peak fragment ion (m/z 200.12) are selected.

The chromatographic conditions were subsequently optimized to achieve the satisfactory separation of artepillin C with good resolution, appropriate retention time, sharp peak shape, high signal response, and shorter running time. The best elution condition was observed by adding formic acid as modifier and using the linear gradient as previously described. The total running time optimized for the chromatographic conditions was 14 min, which is shorter than the total running times found in previous HPLC-PDA reported methods[13,14,20,21]. Additionally, methanol was chosen as the organic modifier in the mobile phase and a high proportion of water was used during the first minutes, since this is an important strategy for the analysis of complex matrices, such as green propolis[32]. The first 8 min were composed of a linear gradient of 40-100% methanol in water (both containing 0.01% formic acid). The next 2 min (8-10 min) maintained 100% of methanol, because at the end of the gradient, a high percentage of organic solvent provides satisfactory column cleaning, minimizing the carry-over effects[33]. The system was then re-equilibrated for 4 min with the starting conditions.

Considering the validation method, in the specificity assay, no interference was detected around the retention time of artepillin C under the established conditions by loading blank samples (Figure 5). To verify the linearity, a calibration curve of artepillin C (Figure 6) was constructed by peak areas versus known concentrations of the standard solution, showing good linearity in the range 50-400 µg/mL (Table 1). The homoscedasticity was verified by Cochran's test (same numbers of replicates into the same level), obtaining values equal to C_{Cal} = 0.648 < C_{Tab} = 0.684 and this demonstrated the homogeneity of the values found. Additionally, as presented in Figure 6 (B), in the residue analysis the data obtained follow a normal distribution.

Table 1. Results of validation method of quantification of artepillin C in green propolis by UHPLC-ESI-MS/MS

Parameter	Result	
Linearity		
Equation	y = 166.956 x + 14468.8	
r^2	0.9905	
Linear range (µg/mL)	50-400	
LOD (µg/mL)	10.79	
LOQ (µg/mL)	32.70	
Intra-day precision (n=6)		
Mean concentration (µg/mL)	252.96	
Mean concentration (%)	6.32	
RSD (%)	1.9	
Inter-day precision (n=6)		
Mean concentration (µg/mL)	260.31	
Mean concentration (%)	6.51	
RSD (%)	3.4	
Accuracy		
Amount of artepillin C added (µg/mL):		
100	103.95	
200	102.49	
300	92.57	
Robustness	% artepillin C	RSD (%)[a]
Used condition	6.80	-
Temperature column 40 °C	6.86	0.65
Temperature column 50 °C	6.93	1.35
Flow rate 0.3 mL/min	6.97	1.75
Flow rate 0.5 mL/min	6.78	0.17

LOD: limit of detection; LOQ: limit of quantitation; RSD: relative standard deviation; [a]Compared with normal conditions.

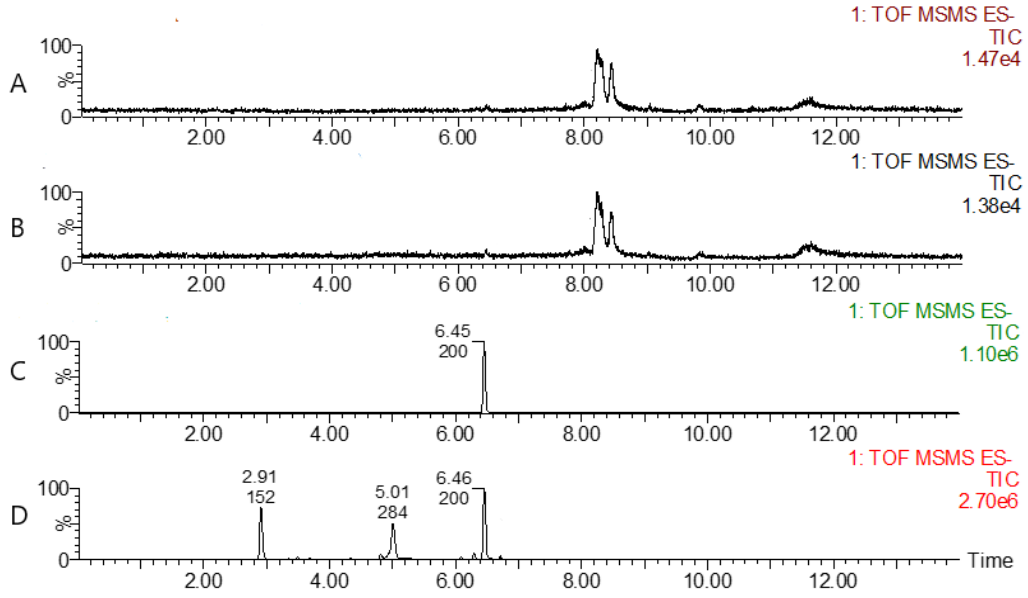

Figure 5. Chromatograms of specificity. (A) Blank - Mobile phase; (B) Blank - Methanol; (C) Standard of artepillin C; (D) Green propolis sample.

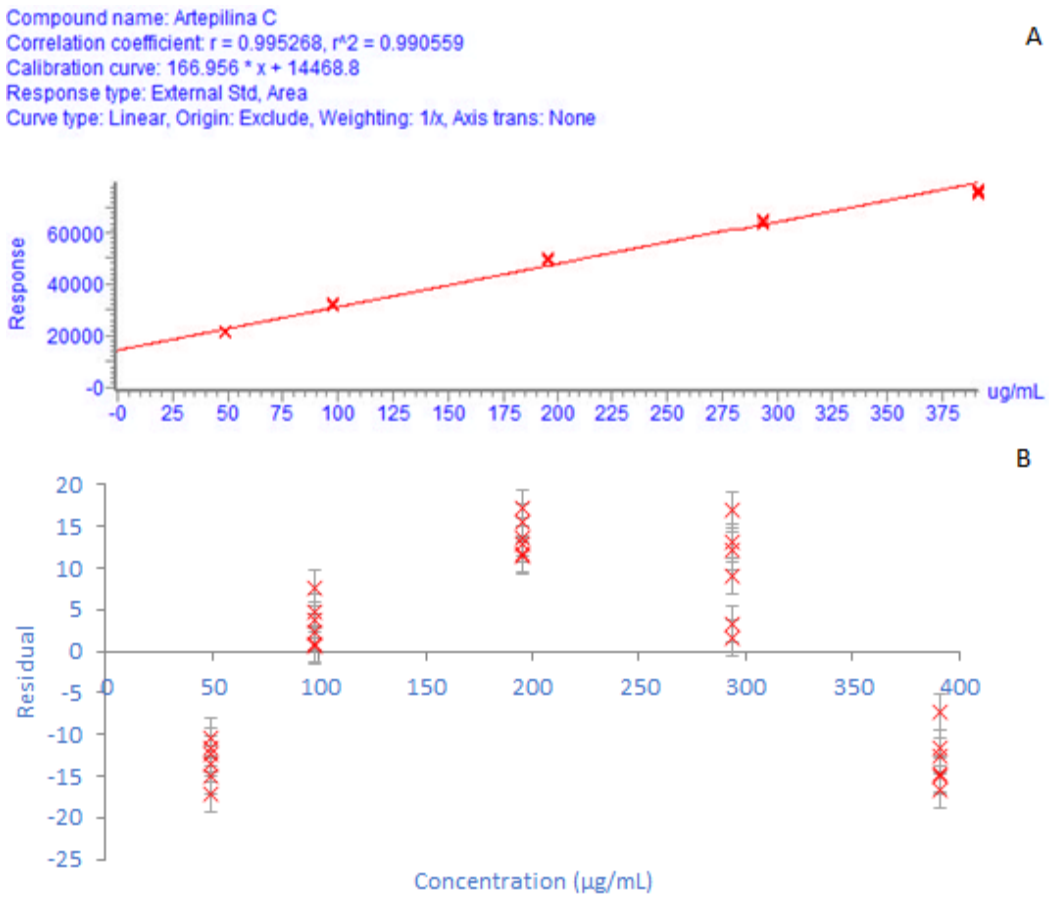

Figure 6. Calibration curve of artepillin C on linearity. The representative equation of linear regression was y = 166.956 x + 14468.8, the correlation coefficient (r) was 0.995268 and the determination coefficient (r^2) was 0.990559. (A) linear regression; (B) residue analysis.

LOD and LOQ were calculated based on the standard deviation and the slope of the calibration curve according to ICH Q2(R1)[27], being LOD = 10.79 µg/mL and LOQ = 32.70 µg/mL. In addition, the results of precision showed RSD intra-day = 1.9% and RSD inter-day = 3.4% and the content of artepillin C was quantified at 6.51% in green propolis. Our results are in accordance with previous quantification by HPLC-PDA[13], which showed amounts of artepillin C ranging from 5.68% to 10.20% in propolis samples from the state of Minas Gerais. The obtained RSD data indicated that satisfactory precision was achieved. More importantly, the present UHPLC-MS method showed smaller RSD than others, which used HPLC-PDA for artepillin C quantification. Sousa et al.[20] evaluated the intra-day precision, obtaining 3.51% RSD, while Nobushi et al.[14] showed RSD ranging from 1.28 to 5.60% for intra-day and 1.95 to 6.75% for inter-day precision.

In addition, the proposed UHPLC-MS method demonstrated to be suitable and reliable for artepillin C quantification, because recoveries obtained in the accuracy were 93-104% (Table 1). This is a range recovery considered as satisfactory for an accuracy method[20,21,34]. The method proved to be robust when subjected to small variations of the analyzed parameters (Table 1). The quantification of artepillin C in the green propolis sample, using modifications in temperature column (40 and 50 °C) and flow rate (0.3 and 0.5 mL/min), showed variations smaller than 2.0% compared to the conditions of the developed method (temperature column 45 °C and flow rate 0.4 mL/min) (Table 1). In addition, it is important to point out that the robustness was not evaluated in other quantification studies with artepillin C in green propolis.

The present UHPLC-ESI-MS/MS method showed some advantages over HPLC methods previously described[13,14,20,21]. Previous published UPLC-MS methods[13,14,20,21] used larger injection volumes, run times, and flow rates, with a longer total analysis time, since they quantified several compounds besides artepillin C[23]. In contrast, the analysis time in the present study showed artepillin C at 6.44 min, while in previous HPLC-PDA methods reported the retention times of artepillin C ranged from 15 to 54 min[13,14,20,21]. In addition, the savings in analysis time and quantities of organic solvents used, makes our present method more advantageous.

Additionally, when we are dealing with complex matrices, such as Brazilian propolis, a major challenge is to develop a selective method for a specific chemical marker. The present UHPLC study using the mass spectrometry detector, allowed high sensitivity and specificity, with the selection of specific masses related to artepillin C, which may eliminate the detection of several interfering substances present in green propolis. Another advantage is the use of a mass spectrometry detector with a Q-TOF analyzer, which possess high resolution, and is recommended not only for compound identification, but also for successful quantification of compounds[35,36]. After evaluation of all parameters, the developed UHPLC-ESI-MS/MS method was considered validated and was demonstrated to be suitable and reliable for quantification of artepillin C in green propolis samples.

CONCLUSION

The present study developed and validated a new method for quantification of artepillin C in green propolis using UHPLC-ESI-MS/MS. The method proved to be specific, linear, precise, accurate, and robust, showing many advantages in comparison to other previous HPLC methods, such as high selectivity and significant decrease in analysis time.

ACKNOWLEDGMENTS

The authors are grateful to Humberto de M. Brandão (Embrapa Gado de Leite, Juiz de Fora) for donation of standard and Carolina Miranda Gasparetto (CENTRALBIO, UFJF) for technical assistance with UHPLC-MS, as well as to "Bee Propolis Brazil Ltda" (Bambuí, MG, Brazil) for donation of Brazilian green propolis sample. We are also grateful to Minas Gerais Research Foundation (FAPEMIG, Grant numbers #APQ 02015/14; PPM-00296-16) and the National Council for Scientific and Technological Development (CNPq, Grant number # 487221/2012- 5) for financial support, as well as to CAPES, CNPq and PIBIC/CNPq/UFJF and for fellowships.

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Author contributions

LRR: Conceptualization, Methodology, Acquisition, Analysis and interpretation of data, Validation, Original draft preparation; LMS - acquisition, analysis and interpretation of data for the work; OOZS - acquisition, analysis and interpretation of data for the work; LRJ - acquisition, analysis and interpretation of data for the work; JWLN – Original draft preparation, Validation, Review & Editing; AASF – Conceptualization, Analysis and interpretation of data, original draft, supervision.