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Development and Validation of a Novel TLC-Image Analysis Method for Taspine Quantification in Latex of Colombian *Croton* spp.

Abstract

This study presents the development and validation of a quantitative method based on thin-layer chromatography (TLC) coupled with a UVP gel documentation system for the determination of the alkaloid taspine in *Croton* spp. latex. Taspine, a bioactive metabolite of pharmacological interest, was extracted and purified from samples collected in Chinácota and Pamplonita (Colombia). A calibration curve was constructed by quantifying the pixel intensity of chromatographic bands analyzed with GelAnalyzer 19.1, and a polynomial model with a high coefficient of determination ($R^2 > 0.99$) was obtained. The method demonstrated excellent linearity, good reproducibility, and accuracy, confirmed by gas chromatography–mass spectrometry (GC-MS) validation. Taspine concentrations were 25.37 µg/mL in Chinácota and 10 µg/mL in Pamplonita, with statistically significant differences ($p < 0.05$), suggesting environmental and genetic variability in metabolite production. The combination of TLC with digital documentation and software integration was validated as an accurate and reproducible alternative to instrumental methods and as a viable tool for phytochemical studies, particularly in resource-limited settings. This research is the first reported application of a validated TLC-image analysis method for taspine quantification.

Palabras clave: alcaloide; cromatografía; validación de métodos.

Desarrollo y validación de un método novedoso de CCF con análisis de imagen para la cuantificación de taspina en látex de especies colombianas de *Croton*

Resumen

Este estudio presenta el desarrollo y validación de un método cuantitativo basado en cromatografía en capa fina (CCF) acoplada a un sistema de documentación UVP para la determinación del alcaloide taspina en látex de especies de *Croton*. La taspina, metabolito bioactivo de interés farmacológico, fue extraída y purificada de muestras recolectadas en Chinácota y Pamplonita (Colombia). Se construyó una curva de calibración mediante la cuantificación de la intensidad de píxeles en bandas cromatográficas analizadas con GelAnalyzer 19,1 y se obtuvo un modelo polinómico con un alto coeficiente de determinación ($R^2 > 0,99$). El método demostró excelente linealidad, buena reproducibilidad y exactitud, confirmada por validación mediante cromatografía de gases y espectrometría de masas (GC-MS). Las concentraciones de taspina fueron de 25,37 µg/mL en Chinácota y 10 µg/mL en Pamplonita, con diferencias estadísticamente significativas ($p < 0,05$), lo que sugiere variabilidad ambiental y genética en la producción del metabolito. La combinación de TLC con documentación digital y la integración de un software se validó como una alternativa precisa y reproducible frente a métodos instrumentales y como una herramienta viable para estudios fitoquímicos, particularmente en entornos con recursos limitados. Este trabajo constituye la primera aplicación reportada de un método validado de CCF con análisis de imagen para la cuantificación de taspina.

Keywords: Alkaloid; chromatography; method validation.

Desenvolvimento e validação de um método inovador de CCD com análise de imagem para quantificação de taspina no látex de espécies de *Croton* colombianas

Resumo

Este estudo apresenta o desenvolvimento e a validação de um método quantitativo baseado em cromatografia em camada delgada (CCD) acoplado a um sistema de documentação em gel UVP para a determinação do alcaloide taspina no látex de espécies de *Croton*. A taspina, um metabólito bioativo de interesse farmacológico, foi extraída e purificada de amostras coletadas em Chinácota e Pamplonita (Colômbia). Uma curva de calibração foi construída quantificando a intensidade de pixels das bandas cromatográficas analisadas com o GelAnalyzer 19,1, e um modelo polinomial com um alto coeficiente de determinação ($R^2 > 0,99$) foi obtido. O método demonstrou excelente linearidade, boa reprodutibilidade e exatidão confirmada pela validação por cromatografia gasosa-espectrometria de massas (CG-EM). As concentrações de taspina foram de 25.37 µg/mL em Chinácota e 10 µg/mL em Pamplonita, com diferenças estatisticamente significativas ($p < 0,05$), sugerindo variabilidade ambiental e genética na produção desse metabólito. A combinação de CCD com documentação digital e integração de software foi validada como uma alternativa precisa e reprodutível aos métodos instrumentais e como uma ferramenta viável para estudos fitoquímicos, particularmente em ambientes com recursos limitados. Esta pesquisa constitui a primeira aplicação relatada de um método validado de CCD com análise de imagem para a quantificação de taspina.

Palavras-chave: alcaloide; cromatografia; validação de método.

Introduction

Plant biodiversity is an inexhaustible source of chemical compounds that are a fundamental pillar in the research and development of new therapeutic agents [1, 2]. Phytochemistry, as a scientific discipline, focuses on the study, isolation, and characterization of these compounds, revealing a plethora of metabolites with potential pharmacological, agricultural, and biotechnological applications [3]. Among these, alkaloids stand out for their remarkable structural diversity, their wide distribution in the plant kingdom, and their recognized pharmacological properties: Anti-inflammatory, anticancer, and neuroprotective [4, 5].

The genus *Croton*, belonging to the *Euphorbiaceae* family, has attracted considerable scientific interest due to its exceptional richness in secondary metabolites, particularly alkaloids and diterpenes [6, 7]. This genus, with nearly 1300 species distributed globally, finds one of its main centers of diversification in the Neotropics, and specifically in South America [8]. Colombia, recognized as one of the most megadiverse nations on the planet, harbors a notable representation of this genus, with 83 reported species, primarily distributed in the Andean and Caribbean regions [9, 10]. The reddish latex produced by several of these species, ethnobotanically known as "Dragon's Blood", has been an essential component in the traditional medicine of Amazonian and Andean communities for the treatment of wounds, inflammations, and various gastrointestinal ailments [11].

From the complex phytochemical matrix of "Dragon's Blood", taspine (**Figure 1**) emerges, a benzylisoquinoline alkaloid that has been identified as one of the main compounds responsible for the potent wound-healing activity of the latex [12, 13]. Subsequent research has corroborated and expanded taspine's pharmacological profile, demonstrating its significant anti-inflammatory and antiviral properties, and, more recently, its potential as an antitumor agent by inhibiting angiogenesis and cell migration in cancer cell lines [14, 15]. This biological multifunctionality positions taspine as a promising phytopharmaceutical and a lead compound for the development of new therapies.

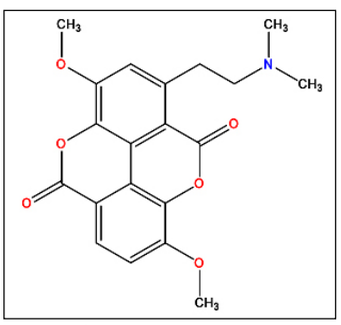

Figure 1. Chemical structure of the alkaloid taspine.

The advancement in the research and potential pharmaceutical exploitation of taspine depends on the development of analytical methods that are precise, reproducible, and economically viable for its quantification in plant extracts. Traditionally, alkaloid quantification has relied on robust instrumental techniques such as high-performance liquid chromatography (HPLC) and gas chromatography coupled with mass spectrometry (GC-MS) [16]. While these methods offer high sensitivity and specificity, their implementation is often limited by the high cost of equipment, the need for highly qualified personnel, and complex sample preparation processes, significant barriers for laboratories in resource-limited settings [17].

In this context, thin-layer chromatography (TLC), a classic planar chromatographic technique, re-emerges as a strategic alternative. Its simplicity, low cost, speed, and ability to process multiple samples in parallel make it an ideal tool for phytochemical screening and quality control [18]. The evolution of TLC towards high-performance thin-layer chromatography (HPTLC) and its coupling with densitometry and digital image analysis have overcome many of its historical limitations, allowing for quantitative data with precision and accuracy levels comparable to those of HPLC for various applications [19, 20]. The validation of TLC-densitometric methods, following the International Council for Harmonisation (ICH) guidelines, has proven to be a robust practice for the analysis of pharmaceutical formulations, ensuring the reliability of the results [21].

This study aims to settle the need for accessible analytical methods and the demand for reliable quantitative results in phytochemistry. The principal objective was to develop and validate, for the first time, a quantitative method for the determination of taspine in the latex of *Croton* species native to Colombia, using an innovative approach that combines TLC with a UVP® (Analytik Jena, Upland, CA, USA) ultraviolet gel documentation system for imaging TLC plates and the GelAnalyzer image analysis software. This work not only presents a novel and low-cost analytical tool but also highlights the importance of valuing Colombian biodiversity as a source of scientific innovation and technological development, laying the groundwork for the standardization and sustainable utilization of local phytogenetic resources.

Material and Methods

Chemicals and Reagents

Taspine standard (≥ 98% purity) was purchased from ChemCruz Biotechnology (Santa Cruz, CA, USA). Silica gel 60 G F254 plates for TLC were obtained from Merck (Darmstadt, Germany). All solvents used, including absolute ethanol, chloroform, methanol, and hydrochloric acid, were of analytical grade and also procured from Merck. Deionized water was used throughout the experiments.

Equipment

TLC plates were visualized and documented using a UVP Gel Documentation System (Analytik Jena, Upland, CA, USA) equipped with a Canon EOS Rebel T5 1105 C001 digital camera under UV illumination at 254 nm. A vortex mixer (VWR, Radnor, PA, USA) and an ultrasonic bath (Elmasonic, Singen, Germany) were used for sample preparation. A rotary evaporator (IKA RV 10 Control S1 with MPC105T vacuum pump, IKA-Werke GmbH & Co. KG, Staufen, Germany) was used for solvent evaporation. Centrifugation was performed using a microcentrifuge (Eppendorf, Hamburg, Germany). GC-MS analysis was conducted on an Agilent Technologies AT 6890 Series Plus GC system coupled with an MSD 5973 mass selective detector (Agilent Technologies, Santa Clara, CA, USA).

Chromatographic Conditions

TLC was performed on 20 × 20 cm silica gel 60 G F254 plates. The mobile phase consisted of a mixture of chloroform and methanol in a 20:1 (*v*/*v*) ratio. The development was carried out in a pre-saturated chromatographic chamber at room temperature (25 ± 5 °C) for 10 min. Iodine vapor was used for the visualization of the bands.

Preparation of Standard Solutions and Calibration Curve

A standard stock solution of taspine (100 µg/mL) was prepared by dissolving 0.5 mg of the standard compound in 5 mL of absolute ethanol. The solution was homogenized using a vortex mixer for 5 cycles of 60 s each and sonicated for 20 min. From this stock solution, a series of ten working standard solutions were prepared by serial dilution with ethanol to obtain concentrations ranging from 10 to 100 µg/mL.

For the calibration curve, 60 µL of each standard solution was carefully applied to the TLC plate. After development and visualization, the plates were digitized, and the images were analyzed using GelAnalyzer v.19.1 software. The calibration curve was constructed

by plotting the pixel intensity (measured as total integrated peak area) of the chromatographic bands against the corresponding taspine concentrations. A polynomial regression model was applied to establish the relationship.

Method Performance Evaluation

The performance of the developed TLC–image analysis method was evaluated using an analytical-grade commercial taspine reference standard. A calibration curve was constructed employing ten standard concentrations ranging from 10 to 100 µg/mL, each analyzed in triplicate. Densitometric quantification was performed by measuring the integrated peak area obtained from digital image analysis. For each concentration level, results were expressed as mean ± one standard deviation (SD).

The relationship between taspine concentration (x) and integrated peak area (y) was evaluated using polynomial regression analysis. The calibration model obtained was described by Eq. (1):

$$y = -636.76667 + 161.44231x - 7.26163x^2 + 0.18215x^3 - 0.00248x^4 + 1.71527 \times 10^{-5}x^5 - 4.71528 \times 10^{-8}x^6 \quad (1)$$

where y corresponds to integrated peak area and x to taspine concentration (µg/mL). The adjusted coefficient of determination ($R^2 = 0.99952$) was calculated to assess the goodness of fit of the model. Method reproducibility was assessed through triplicate analyses of all samples.

Plant Material Collection and Sample Preparation

Latex samples from *Croton* spp. were collected from two municipalities in Norte de Santander, Colombia: Chinácota (07°33'57.24" N, 72°34'48.295" W) and Pamplonita (07°26'40.859" N, 72°38'41.855" W). Three individual trees were sampled at each location. The collected specimens were taxonomically assigned to the genus *Croton* based on morphological characteristics and field identification criteria. Species-level identification was not established, as the principal objective of this study was the development and validation of an analytical method rather than a taxonomic assessment. Therefore, samples are referred to as *Croton* spp. throughout the manuscript. Latex was collected from morphologically comparable individuals in both locations; however, no species-level differentiation was performed within the scope of this work. The collected latex was stored in Eppendorf tubes, protected from light with aluminum foil, and kept at 4 °C until processing.

Extraction and Isolation of Taspine from Latex Samples

The collected latex (1 g) was mixed with 1.5 mL of deionized water, and the pH was adjusted to 2 with hydrochloric acid (HCl). An equal volume of chloroform ($CHCl_3$) was added, and the mixture was stirred for 12 h. The organic phase was separated, and the aqueous phase was then adjusted to pH 10 with ammonium hydroxide (NH_4OH). A fresh volume of $CHCl_3$ was added, and the mixture was stirred for another 12 h. The resulting organic phase, containing the alkaloid, was concentrated using a rotary evaporator. The precipitate was redissolved in methanol and stored at 4 °C for 12 h to facilitate the precipitation of impurities. The solution was then centrifuged at 13,000 rpm for 10 min, and the supernatant was collected and evaporated to dryness to yield the purified taspine extract. The dried extract was reconstituted in 1 mL of methanol prior to chromatographic analyses.

Quantification of Taspine in Croton spp. Samples

Sixty microliters of the purified taspine extract from each sample were applied in triplicate to the TLC plates. The plates were developed and analyzed under the same chromatographic and imaging conditions described previously. The pixel intensity of the bands corresponding to taspine in the samples was measured using GelAnalyzer v.19.1, and the concentration was determined by interpolating these values into the validated calibration curve.

Gas Chromatography Coupled with Mass Spectrometry (GC-MS) Confirmation

GC-MS analysis was performed by an external certified analytical laboratory using an Agilent 6890N gas chromatograph coupled to an Agilent 5973N mass selective detector. Separation was achieved on a DB-1MS capillary column with helium as the carrier gas. The analyzed sample corresponded to the isolated TLC band previously identified as taspine by co-chromatography with an analytical-grade reference standard. The recovered band was dissolved in methanol prior to injection.

The injection volume was 0.2 µL in split mode. The injector and detector temperatures were set at 330 and 250 °C, respectively. The oven temperature program was as follows: Initial temperature of 100 °C (held for 1 min), increased at a rate of 5 °C/min to 150 °C (held for 3 min), then increased at 5 °C/min to 250 °C (held for 5 min), and finally increased at 5 °C/min to 300 °C (held for 3 min). The total run time was 52 min.

Mass spectra were obtained under electron ionization (EI) conditions and compared with National Institute of Standards and Technology (NIST) library databases (NIST02.L, NIST5a.L, and NIST98) for compound identification. GC-MS analysis was performed exclusively for qualitative confirmation and not for quantitative determination.

Statistical Analysis

All quantitative experiments were performed in triplicate. Data were analyzed using polynomial regression for calibration curve fitting. A Student's t-test was used to compare the mean taspine concentrations between the two sampling locations. A p-value < 0.05 was considered statistically significant.

Results

Method Development and Validation

The developed TLC-image analysis method proved to be a reliable and effective tool for the quantification of taspine (**Figure 2**). The chromatographic separation yielded well-defined, compact, and clearly resolved bands for taspine, with a retention factor (Rf) value of 0.45 ± 0.02, allowing for accurate densitometric analysis. The use of a chloroform/methanol (20:1, *v*/*v*) mobile phase provided optimal separation from other compounds present in the crude latex extract.

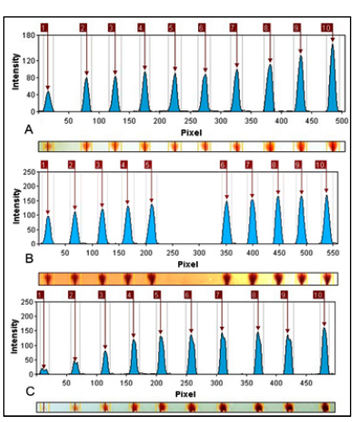

Figure 2. Intensity pixel profile obtained from thin-layer chromatography (TLC) chromatograms developed in triplicate on silica gel 60 G plates using chloroform/methanol (20:1, *v*/*v*) as the mobile phase. Panels A, B, and C correspond to independent densitometric analyses of the calibration series. Chromatographic bands numbered 1–10 correspond to taspine standard solutions ranging from 10 to 100 µg/mL, applied sequentially on the TLC plate.

Linearity and Method Performance

The calibration curve for taspine was constructed by plotting the average pixel intensity of the chromatographic bands against the corresponding concentrations (10 to 100 µg/mL) (**Figure 3**).

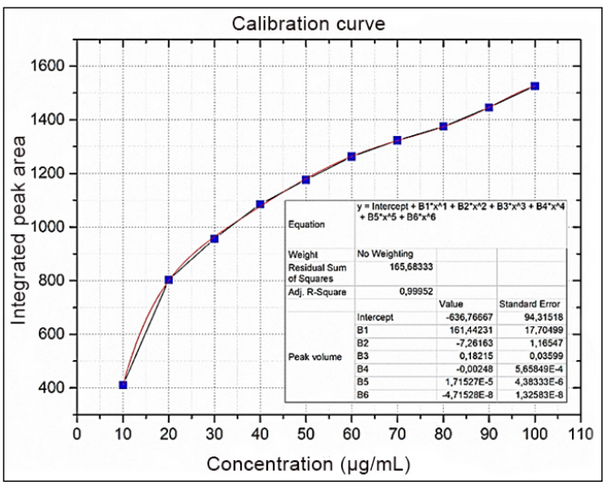

Figure 3. Calibration curve adjusted to the polynomial model.

The relationship was found to be linear over the tested concentration range. A polynomial regression model yielded a high coefficient of determination ($R^2 > 0.99$), indicating an excellent correlation between concentration and pixel intensity (**Table 1**). This demonstrates that the method is suitable for the quantitative determination of taspine in the collected *Croton* spp. latex samples, in accordance with the sensitivity reported for similar HPTLC methods for other alkaloids [19, 21].

Table 1. Mean integrated peak area for each taspine concentration (n = 3).

Concentration (µg/mL)	Integrated peak area (a.u.)*
10	410
20	803
30	956
40	1086
50	1176
60	1263
70	1323
80	1375
90	1446
100	1526

*Values correspond to the mean of triplicate analyses (A, B, and C).
a.u: Arbitrary units.

The developed method demonstrated good repeatability, as indicated by the consistent results obtained from triplicate analyses. The strong correlation coefficient ($R^2 > 0.99$) indicates the reliability of the pixel intensity measurement as a quantitative parameter for taspine determination. The identity of the quantified compound was independently confirmed by GC–MS analysis.

Quantification of Taspine in Croton spp. Samples

The validated TLC-image analysis method was applied to quantify taspine in latex samples collected from two different geographical locations: Chinácota and Pamplonita (**Figure 4**).

Figure 4. Red exudate extracted from Croton spp. of the municipality Chinácota (left) and Pamplonita (right).

The analysis revealed a significant variation in taspine concentration between the two populations. The samples from Chinácota exhibited a mean taspine concentration of 25.37 ± 1.80 µg/mL, whereas the samples from Pamplonita showed a lower mean concentration of 10.00 ± 0.90 µg/mL. The difference was found to be statistically significant ($p < 0.05$) based on the Student's *t*-test. The pixel intensity profiles for the samples are shown in **Figure 5**.

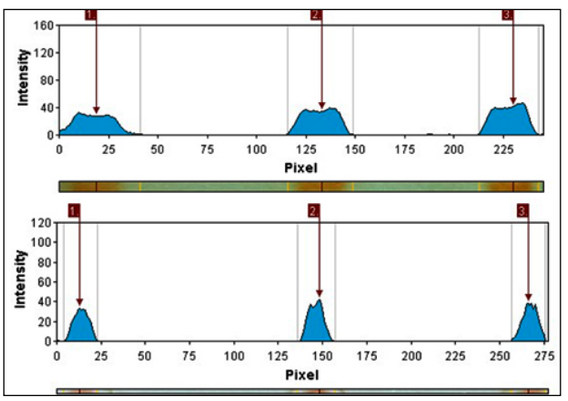

Figure 5. Intensity pixel profile of thin-layer chromatography (TLC) chromatograms obtained from latex samples collected in the municipality of Chinácota (upper panel) and Pamplonita (lower panel). The peaks correspond to the chromatographic band identified as taspine (Rf ≈ 0.45). The numbers 1, 2, and 3 indicate triplicate applications of the same sample extract on the TLC plate. The x-axis represents migration distance expressed in pixel units obtained from digital image analysis.

GC-MS Confirmation

The GC-MS chromatogram of the purified extract (**Figure 6**) showed more than one detectable signal. The peak identified as taspine was observed at a retention time (RT) of 1.34 min (**Figure 6**) and was identified based on its mass spectrum, which exhibited a characteristic signal at *m*/*z* = 348, consistent with the reference spectrum from the NIST library database. It should be emphasized that GC-MS analysis was used exclusively for qualitative confirmation of compound identity, whereas quantitative determination was performed using the validated TLC-image analysis method.

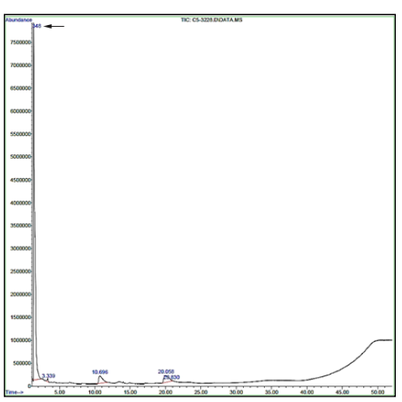

Figure 6. Gas chromatography coupled with mass spectrometry (GC-MS) chromatogram of the purified extract. The arrow indicates the peak identified as taspine (RT = 1.34 min), identified based on its mass spectral profile (m/z = 348) and comparison with the NIST library database.

Discussion

This study successfully developed and validated a novel, low-cost, and reliable quantitative method for the determination of taspine in *Croton* spp. latex using TLC coupled with digital image analysis. The results demonstrate that the method is linear and provides consistent results for taspine determination using TLC coupled with digital image analysis, which has performance characteristics suitable for phytochemical analysis, and agrees with established analytical practices [21]. This is a significant contribution, as it provides an accessible analytical alternative to the more sophisticated and expensive instrumental techniques like HPLC and GC-MS, which are often inaccessible in resource-limited settings [17].

While HPLC and GC-MS are the gold standard for phytochemical analysis, our method proves that well-validated TLC-densitometric approaches can offer comparable reliability for specific applications, a conclusion supported by recent comparative studies [17, 20, 22]. Recent advances in HPTLC methodology have demonstrated excellent performance for pharmaceutical analysis [23, 24], and the integration of digital image analysis with TLC has shown promising results for various natural product applications [25, 26]. The use of readily available software like GelAnalyzer and a standard gel documentation system further enhances the method's accessibility, democratizing the quality control of phytopharmaceutical products.

The significant difference in taspine concentration between the Chinácota (25.37 µg/mL) and Pamplonita (10.00 µg/mL) populations is a noteworthy finding. This variation can be attributed to a combination of genetic and environmental factors, including differences in soil composition, altitude, climate, and sun exposure, which are known to influence the biosynthesis and accumulation of secondary metabolites in plants [7]. Such chemodiversity within the same species from different geographical locations is a well-documented phenomenon and has important implications for the standardization of herbal medicines [6]. Our findings highlight the necessity of chemical profiling and quantitative analysis when searching for *Croton* latex for medicinal purposes to ensure consistent potency and therapeutic efficacy.

When comparing our results with previous research, it is evident that taspine concentrations can vary significantly across different regions and species. For example, Milanowski *et al*. conducted an extensive study in 22 sites across northern Peru and Ecuador and reported that taspine is the only alkaloid consistently present in the latex of *Croton lechleri*, with a mean concentration of approximately 9% of the latex by dry weight [30]. Other reviews have corroborated that taspine is a major constituent, representing between 7 and 9% of the dry weight in certain *Croton* species [6, 35].

In contrast, the concentrations quantified in the present study for Colombian samples (10.00 to 25.37 µg/mL in fresh latex extracts) appear lower in absolute numerical terms; however, direct comparison should be made cautiously due to differences in analytical methods, sample preparation procedures, and expression of results (dry weight vs. liquid extract basis). Furthermore, Froldi *et al*. reported a latex density of 1.08 g/mL and a dry residue of 27.5% for *Croton lechleri*, providing a useful reference framework for interpreting concentration values under different reporting conditions [29].

These variations highlight the influence of geographic origin, environmental conditions, plant physiology, and methodological differences on the production and quantification of secondary metabolites, reinforcing the importance of local characterization studies such as the present work.

The concentration of taspine is a critical quality parameter, given its role as a primary bioactive constituent responsible for the wound-healing and anti-inflammatory properties of "Dragon's Blood" [12, 13]. Recent studies have further elucidated the pharmacological importance of taspine, demonstrating its potential in various therapeutic applications [27, 28]. The development of this simple and rapid quantification method can be crucial for bioprospecting studies, allowing the rapid screening of different *Croton* species or populations to identify high-yield sources of taspine. Furthermore, it can be implemented for routine quality control by local producers and small-scale enterprises involved in the commercialization of "Dragon's Blood" products, ensuring they meet quality and safety standards [29, 30].

This research represents the first reported application of a validated TLC-image analysis method for taspine quantification. The approach aligns with the growing trend of developing "green" analytical methods that reduce solvent consumption and are more environmentally friendly compared to traditional HPLC methods [18, 31]. Future research should focus on expanding the geographical scope of sample collection to create a more comprehensive map of taspine variability in Colombian *Croton* species [32]. Additionally, correlating the observed chemical variations with genetic markers could provide deeper insights into the biosynthesis of this valuable alkaloid and contribute to the understanding of secondary metabolite production in medicinal plants [33, 34].

Conclusion

This study successfully achieved the primary objective of developing and validating a novel, simple, and cost-effective quantitative method for the determination of taspine in *Croton* spp. latex. The standardized approach, which combines TLC with UVP gel documentation and digital image analysis, demonstrated excellent performance characteristics. The method proved to be linear ($R^2 > 0.99$) and showed consistent results under the experimental conditions, making it a useful analytical tool for phytochemical research.

The application of this validated method revealed a statistically significant difference in taspine concentrations between samples collected in Chinácota (25.37 µg/mL) and Pamplonita (10.00 µg/mL), underscoring the influence of geographic and environmental factors on secondary metabolite production. The identity of taspine was confirmed by GC-MS, validating the specificity of the TLC-based quantification.

Compared to conventional instrumental methods, this methodology presents an accessible and affordable alternative, particularly advantageous for laboratories with limited resources. It facilitates the rapid screening of plant materials and can be effectively implemented for the quality control of herbal products derived from "Dragon's Blood". This work not only contributes a valuable and novel analytical procedure for taspine quantification but also highlights the potential of Colombia's rich biodiversity for scientific and industrial advancement. The developed method has the potential to be adapted for the quantification of other bioactive molecules in complex plant extracts, thus providing a valuable tool for future phytochemical and pharmacological investigations.

Acknowledgments

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