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Standardization, Validation, and Application of a Rapid and Reliable HPLC-UV Method for the Determination of Minoxidil in Hair Growth Products

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ABSTRACT

Background: Minoxidil is a medication used for hypertension. Nevertheless, a significant side effect of hypertrichosis was observed, which is now widely used in the formulation of topical medications for the treatment of alopecia. International regulations, however, prohibit its use in cosmetic products due to its antihypertensive activity and the potential for systemic absorption following application to the scalp. Despite these regulatory restrictions, several studies have reported the presence of minoxidil in cosmetic products. **Objectives**: to determine the presence of minoxidil in cosmetic products for hair growth, using a standardized and validated HPLC-UV method for different cosmetic matrices. **Methods**: the analytical method was statistically validated by parameters such as selectivity, linearity, precision, accuracy, precision, robustness, limit of detection, and limit of quantification according to International Conference on Harmonisation (ICH) guidelines. **Results:** The validated method meets the ICH requirements. Additionally, it was found that 2 of the 9 cosmetic products analyzed contained the banned analyte minoxidil, corresponding to 22.22 % of the products analyzed. **Conclusions:** It was possible to standardize and validate an HPLC-UV method for the identification of minoxidil in cosmetic matrices, which gives reliability to the results obtained. However, it is important to verify these results with other techniques, such as HPLC-MS, to support these statements.

Keywords: minoxidil, validation study, HPLC-UV, adulterated products for hair growth, cosmetovigilance.

RESUMEN

Antecedentes: El minoxidil es un medicamento utilizado para la hipertensión. Sin embargo, desde que se empezó a comercializar se ha observado un efecto secundario significativo de hipertricosis, por lo cual, se utiliza ampliamente en la formulación de medicamentos tópicos para el tratamiento de la alopecia. No obstante, las regulaciones internacionales prohíben su uso en productos cosméticos debido a su actividad antihipertensiva y al potencial de absorción sistémica después de su aplicación en el cuero cabelludo. A pesar de estas restricciones regulatorias, varios estudios han reportado la presencia de minoxidil en productos cosméticos. **Objetivos:** Determinar la presencia de minoxidil en productos cosméticos para el crecimiento del cabello, mediante un método estandarizado y validado de HPLC-UV para diferentes matrices cosméticas. **Métodos:** El método analítico fue validado estadísticamente por parámetros como selectividad, linealidad, precisión, exactitud, robustez, límite de detección y límite de cuantificación de acuerdo con las directrices de la Conferencia Internacional sobre Armonización (ICH, por sus siglas en inglés). **Resultados:** El método validado cumple con los requerimientos de la ICH. Además, se encontró que presumiblemente 2 de los 9 productos cosméticos analizados contenían el analito prohibido minoxidil, lo que corresponde al 22.22 % de los productos analizados. **Conclusiones:** Fue posible estandarizar y validar un método de HPLC-UV para la identificación de minoxidil en matrices cosméticas, lo que da fiabilidad a los resultados obtenidos. Sin embargo, es importante verificar estos resultados con otras técnicas como HPLC-MS para respaldar estas afirmaciones.

Palabras claves: minoxidil, estudio de validación, HPLC-UV, productos adulterados para el crecimiento del cabello, cosmetovigilancia.

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1. INTRODUCTION

Minoxidil was initially used as an antihypertensive medication in the 1970s due to its potent oral vasodilatory action. After its development and commercialization, adverse effects such as the growth of hair in various parts of the body began to be reported. Scientific evidence demonstrated its powerful effect in inducing hypertrichosis and hirsutism when the drug was ingested, leading to excessive growth of fine, dark, or coarse hair in localized areas or across the entire body, in both men and women. As a result, topical formulations of the medication began to be developed and marketed (1–4).

In Colombia and many countries around the world, minoxidil is approved as a medication for the treatment of alopecia, and there is sufficient evidence proven by clinical trials to corroborate its effectiveness (5,6). However, this ingredient cannot be used in cosmetic products, as Colombian regulations adhere to international lists of ingredients that can or cannot be incorporated into cosmetics (7). An example of this is the Cosmetic Ingredient Database (CosIng) of the European Commission (Cosmetic Regulation (EC) No. 1223/2009, Annex II (List of Substances Prohibited in Cosmetic Products)), which states that minoxidil and its salts should not be used in cosmetic products due to their antihypertensive activity, as the absorption of minoxidil after application to the scalp could lead to systemic effects (2,8,9). Furthermore, the Scientific Committee on Cosmetic and Non-Food Products Intended for Consumers (SCCNFP), which advises the European Commission, stated on January 21, 1998, that "minoxidil and its salts should not be used in cosmetic products due to their antihypertensive activity and because the absorption of minoxidil after application to the scalp could lead to systemic effects". The SCCNFP also provides relevant toxicity data, including significant cutaneous absorption in humans, fetotoxicity and maternal toxicity from subcutaneous injections in rats, and cardiotoxicity in long-term oral studies in rats and dogs (10).

Additionally, every cosmetic product must declare on the label or packaging its qualitative composition, which should correspond to the content and nature of the product. If the content does not match what is authorized, it will be considered an adulterated product and subject to sanctions by the competent authorities in accordance with the relevant regulations (7,11).

Despite the regulatory restrictions currently in place in Colombia and other countries, minoxidil is still being illegally used in cosmetic products for hair growth to enhance effectiveness and achieve better short-term results. Therefore, it has become of great importance to assess the use of unauthorized ingredients in cosmetics, and various research efforts have been undertaken to develop and validate methods for evaluating the presence of prohibited compounds such as minoxidil in these types of products (12–17).

Various studies have reported the presence of minoxidil and other unauthorized compounds in cosmetic products (12,14,15,17). Wu CS et al. (2013) (17) determined the presence of seven prohibited substances in cosmetic products, including minoxidil, hydrocortisone, spironolactone, estrone, canrenone, triamcinolone acetonide, and progesterone. Of the 37 products evaluated, minoxidil was found in eight of them, and concomitantly with spironolactone in one product. In a study by De Orsi D. et al. (2008) (18), six cosmetic products (two creams and four lotions) sold on internet websites were analyzed to determine the presence of minoxidil, progesterone, estrone, spironolactone, canrenone, hydrocortisone, and triamcinolone acetonide. Not only was the presence of various prohibited substances demonstrated, but the percentages of these substances were extremely high. Furthermore, in a study by Park HN et al. (2018) (12), 76 samples of products claimed to be hair growth treatments were analyzed, and the results once again showed the presence of unapproved compounds in cosmetics. It was found that around 10 % of the samples were adulterated with five compounds, including minoxidil. In these types of research, the use of different analytical techniques has been reported, including liquid chromatography coupled with tandem mass spectrometry (LC-MS) (12,17,18), UV spectrophotometry (12), capillary electrophoresis (14,16), and high-performance liquid chromatography-DAD (HPLC-DAD) (15,18).

In this study, the goal was to develop and validate a rapid, cost-effective, and reliable method with suitable selectivity for the determination of minoxidil in multi-matrix cosmetic products for hair growth. In this regard, most hair growth products available in the cosmetic market are hair tonics-cosmetic solutions enriched with extracts and other active ingredients. Although they pose an analytical challenge, they

are not as complex as heterodisperse systems such as emulsions, suspensions, and others. To achieve this, high-performance liquid chromatography with reverse-phase coupled to UV detection (HPLC-UV) was employed. The results obtained are expected to contribute to the development of cosmetovigilance activities, as well as the control and inspection of cosmetics, serving as an important strategy to prevent illegality or regulatory non-compliance by manufacturers.

2. MATERIALS AND METHODS

2.1 Reagents

Secondary standard grade certified reference material (CRM) minoxidil was obtained from Sigma-Aldrich. Raw minoxidil material was donated by Licol Laboratories (Medellín, Colombia). HPLC-grade and analytical-grade methanol were obtained from Merck Chemical Supplies (Darmstadt, Germany). Niacinamide, biotin, onion extract, rosemary extract, and Minoxidil Oxothiazolidinecarboxylate were donated by Samara Cosmetic (Medellín, Colombia). The other ingredients for placebo preparation were obtained from LyM and LyF chemicals (Medellín, Colombia). The hair growth cosmetic products were purchased from beauty stores, pharmacies, and online retailers. Two medications, topical solutions of minoxidil, products I and J, and the nine cosmetic products hair tonics (A-H and K), classified based on their ingredients as hydroalcoholic, hydroglycerin, and/or hydro-propylene glycol solutions, enriched with extracts and other active ingredients of interest for hair care, were evaluated.

2.2 Method validation

2.2.1 Placebo preparation

The placebo was developed by us based on a comprehensive review of the total ingredients of the products evaluated in this study. The ingredients were weighed in the quantities described in Table 1. First, water was weighed, and then each of the other ingredients was individually weighed and dissolved in the water. Between the addition of each ingredient, the mixture was stirred for approximately 2 minutes. Finally, it was packaged and labeled for subsequent use in method validation.

Table 1. Qualitative and quantitative formula of the placebo.

Ingredient (INCI name)	%P/P
Niacinamide	0.25
Biotin	0.25
Water	40.21
Allium cepa bulb extract	1.01
Thermo Liss[a]	5.01
Rosmarinus officinalis leaf extract	2.13
Water	51.34

Contains: aqua, acrylates/c 10-30 alkylacrylate crosspolymer, aqua (and) sodium laneth - 40 maleate/styrene sulfonate copolymer, polyquaternium-7, peg-150 distearate, poliquaternium-70 (and) dipropylene glycol, argania spinosa kernel oil, ppg - 3 miristato de eter de bencilo, peg/ppg-15/15 dimeticona, fenil trimeticona, bis etilo (isostearylimidazoline) isostearamida, hydrolyzed keratin, hydrolyzed milk protein, diazolidinilurea (and) yodopropinyl butilcarbamato, sodio metilparaben, trietanolamine, lactic acid, DMDM hidantoína, metylcloro isothiazolinone methylisothiazolinone, fragrance.

2.2.2 Chromatographic system

Analysis was performed using a Waters HPLC Refurbished, equipped with a UV detector to 280 nm (2487), an isocratic pump (515), an autosampler, and a column thermostat. The analytical column (Waters Symmetry 4.6 mm x 75 mm x 3.5 µm) was thermo-stated at 25° C. The mobile phase consisted of methanol: water with 2 % acetic acid (35:65). After preparing the mobile phase, it was filtered through 0.45 µm nylon filters and ultrasonicated for 5 minutes to degas (18).

2.2.3 Minoxidil stock solution

12.5 mg of minoxidil CMR were weighed and placed in a 50 mL flask. 40 mL of methanol was added, and the mixture was shaken in an ultrasonicator for 5 min. Then, it was made up to volume with methanol to obtain a stock solution with a concentration of 0.25 mg/mL.

2.2.4 Minoxidil analytical curve

Aliquots of 100 µL, 200 µL, 400 µL, 600 µL, and 800 µL were taken from the stock solution. Each aliquot was adjusted with a remaining volume of methanol to obtain a final volume of 1 mL, leaving final concentrations of 0.025, 0.05, 0.1, 0.15, and 0.2 mg/mL, equivalent to active percentages of 10%, 20%, 40%, 60%, 80%, and 100%.

2.2.5 Selectivity

Preparation of placebo: 250 mg of placebo was weighed into a 50 mL flask, and approximately 40 mL of methanol was added. The mixture was subjected to ultrasound for 5 minutes and then made up to volume with methanol. It was filtered through 0.45 µm nylon filters, and 20 µL of this solution was injected into the HPLC.

Preparation of the spiked sample: 12.5 mg were weighed into a 50 mL flask and dissolved in a small volume of methanol. Then, in the same flask, 250 mg of placebo were weighed, approximately 40 mL of methanol was added, and the mixture was subjected to ultrasound for 5 minutes. It was then made up to volume with methanol, filtered through 0.45 µm nylon filters, and 20 µL of this solution was injected into the HPLC (19,20).

2.2.6 Linearity and accuracy

To evaluate the linearity parameter, minoxidil solutions were prepared at five concentration levels: 100%, 75%, 50%, 25%, and 10%. To prepare the 100% concentration, 125 mg of minoxidil raw material was weighed into a 50 mL flask. Enough methanol was added to dissolve it, and 2500 mg of placebo was added to the same flask. Then, it was sonicated for 5 minutes and topped up with methanol. Subsequently, a 1 mL aliquot was taken, transferred to a 10 mL flask, and topped up with methanol. Finally, it was filtered using 0.45 µm nylon filters, and 20 µL of this solution was injected into the HPLC. This procedure was performed in triplicate for each of the concentration levels to be prepared (20,21).

2.2.7 Limits of detection and quantification

For the determination of LOD (Limit of Detection) and LOQ (Limit of Quantification), the standard deviation of the placebo (Sb) and the slope of the analytical curve was used. The Sb was obtained from the analysis of placebo samples. Three samples were prepared, injected into the HPLC, and the area corresponding to the base of the peak observed in the spiked placebo was integrated. In this way, Sb was calculated from the areas of these three samples. The detection limit was determined using the equation (20,21):

$$L = \frac{Kx\ S_b}{b} \qquad \text{equation (1)}$$

L *Limit of detection (LOD) or limit of quantification (LOQ)*

K *constant, 10 for LOQ and 3 for LOD*

S_b *Placebo standard deviation (selectivity)*

b *Analytical curve slope (linearity)*

2.2.8 Precision

Repeatability

Samples were prepared at concentrations equivalent to 50 %, 75 %, and 100 %, as de-scribed in section 2.2.6, Linearity, and accuracy (20,21).

Intermediate precision

Samples were prepared at concentrations equivalent to 100 %, as described in section 2.2.6. Linearity and Accuracy. The variation of two factors, day (two days), and researchers (three researchers), was evaluated (20,21).

2.2.9 Robustness

To evaluate the method's robustness, a Youden-Steiner factorial design was conducted, as represented in Table 2. The treatment of the samples was carried out following the description in each of the assays (columns 1-8). The percentage recovery was calculated for each assay (20,21).

Table 2. Youden-Steiner Factorial Design.

	Assay							
Factor	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
A/a	A	A	A	A	a	a	a	a
B/b	B	B	b	b	B	B	b	b
C/c	C	c	C	c	C	c	C	c
D/d	D	D	d	d	d	d	D	D
E/e	E	e	E	e	e	E	e	E
F/f	F	f	f	F	F	f	f	F
G/g	G	g	g	G	g	G	G	g

Where:

A: Sample preparation 16 mg/25*4/10 mL mg/25*4/10 mL

a: Sample preparation 32 mg/25*2/10 mL

B: Ultrasound 5 min

b: Ultrasound 2 min

C: Room temperature bath

c: Hot water bath (50°C c.a.)

D: Pre-dissolution of minoxidil in methanol

d: No pre-dissolution of minoxidil in methanol

E: Injection volume 20 uL

e: Injection volume 10 uL

F: Flow rate of MP 1.0 mL/min

f: Flow rate of MP 1.1 mL/min

G: New sample filters

g: Reused sample filters

2.2.10 Determination of minoxidil in hair growth products:

For each product, 50 mg were weighed and transferred to a 10 mL flask. 8 mL of methanol was added, and it was sonicated for 5 minutes. Then, it was topped up, filtered using 0.45 µm nylon filters, and 20 µL of this solution was injected into the HPLC. Each product was evaluated in triplicate by three different analysts.

2.2.11 Statistical analysis

Results were expressed as the means of repetition, and standard deviation and coefficients of variation were calculated. For linearity, R and R^2 were calculated, and additionally, an analysis of variance (ANOVA) and a student's t-test were performed to evaluate the slope and intercept. For accuracy, the percentage recovery was evaluated, and a Cochran's G test was conducted to assess the influence of concentration levels on variances. A reduced factorial design of Youden-Steiner was used for robustness. All calculations were performed using Microsoft Excel.

RESULTS AND DISCUSSION

Method validation

To launch any cosmetic product on the market, it is necessary to verify compliance with legal requirements, which encompass both the evaluation of product efficacy and safety, as well as active cosmetovigilance activities through the detection of prohibited substances in cosmetics. Therefore, it is essential to develop analytical methods that are not only reliable and cost-effective but also easily applicable during post-marketing surveillance and control stages. For this reason, we proceeded to carry out assays for the determination of minoxidil using UV spectrophotometry (data not shown); however, good selectivity was not achieved to differentiate minoxidil base from its derivatives. In this regard, we proceeded to develop and validate a method that would meet the minimum quality parameters according to the ICH for the determination of the analyte of interest by HPLC-UV (19–21).

With the analytical curve (Figure 1), the range of concentrations with linear behavior was determined. In addition to this, the slope and intercept of the straight-line equation were used to calculate the concentrations or recovery percentages of minoxidil from the obtained areas. This allowed the identification and quantification of minoxidil in the evaluated products.

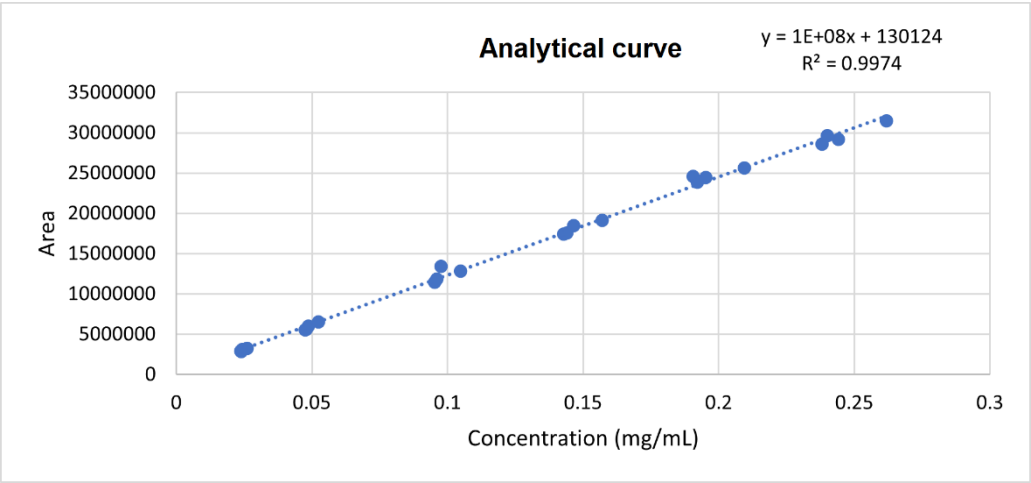

Figure 1. Analytical curve of minoxidil CMR

Selectivity

The placebo formulation was prepared considering the different ingredients used in the evaluated products, aiming to create a matrix similar to hair growth cosmetic products. This was done to determine potential interferences in the quantification of the analyte. Figure 2 shows that the placebo does not interfere with the detection of the analyte of interest, as no significant peaks are observed in the retention time (tR) between 3.8 and 4.0 that could interfere with the detection and quantification of minoxidil, in comparison with the height and area ratio observed in the spiked placebo (Figure 3). Therefore, it can be concluded that the placebo does not interfere with the measurements, indicating the selectivity of the method.

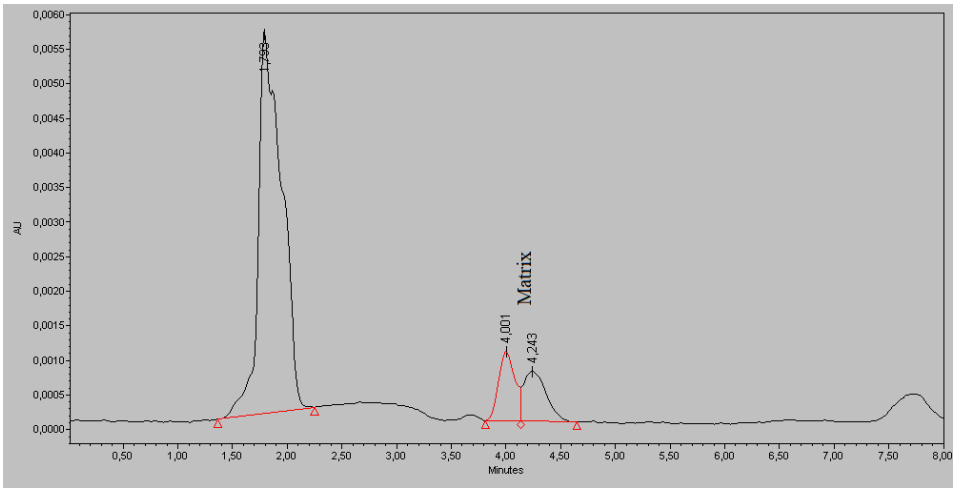

Figure 2. Chromatogram of the placebo sample – Selectivity test

It was determined that the placebo spiked with the analyte at a concentration of 0.25 mg/mL did not present interferences in the detection and quantification of minoxidil, allowing the real recovery of the analyte (98.56 %), with a retention time (RT) for minoxidil of 3.803 according to the standard, showing that despite being within the matrix, there was no matrix effect that significantly increased or decreased the recovery of the analyte (Figure 3). In this sense, Park HN et al. (2018) (12) demonstrated the selectivity of their analytical method similarly by comparing the matrix before and after adding the analyte of interest to identify potential interferences present in the sample. The results showed that there were no interference peaks at the target retention time (RT).

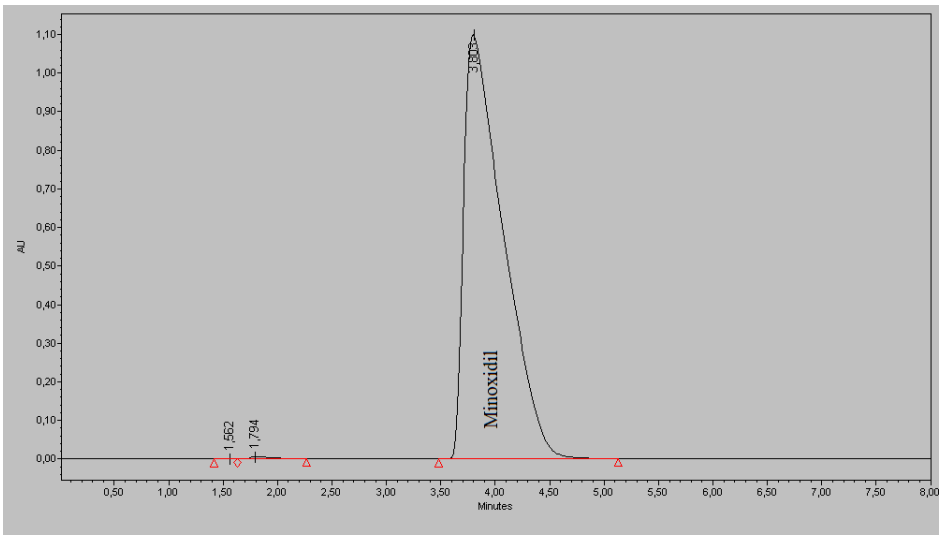

Figure 3. Chromatogram of placebo spiked with Minoxidil – Selectivity test

Linearity

Linear regression allows evaluating the fit to the proposed linear model through the correlation coefficient (R) indicated at the top of Table 3. In this case, considering products with a matrix composed of a large number of different ingredients, the obtained R value is adequate, indicating the data's non-variability and their accommodation to the defined acceptance criteria. Using least squares, the equation of the straight-line y = 135270081.5x – 737205.57 was obtained, which proportionally relates the instrumental response 'y' (area) to the analyte concentration 'x', as observed in Figure 4.

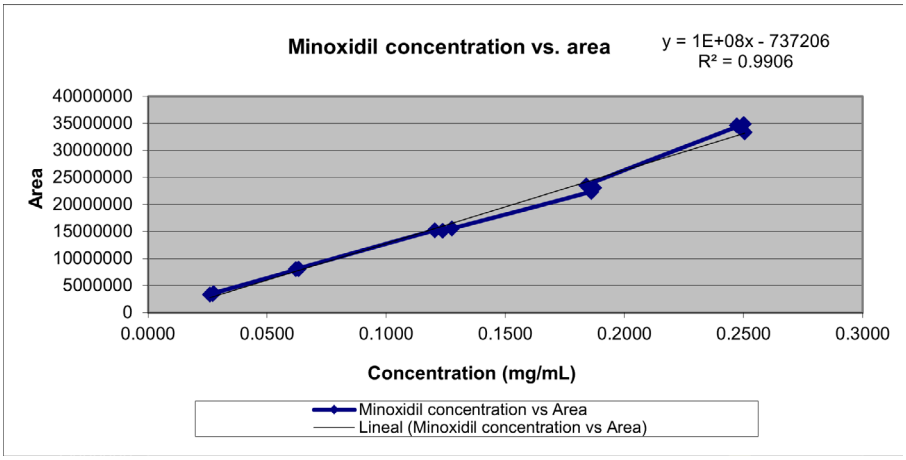

Figure 4. Linearity test: Minoxidil concentration vs. area

Another key statistical measure derived from linear regression is the coefficient of determination (R^2), which reflects the goodness of fit of a model to the variable being explained. The obtained value of R^2 demonstrates that there is a direct relationship between the analyte concentration and the instrumental response obtained.

Additionally, an analysis of variance (ANOVA) was conducted to statistically demonstrate the existence of a non-zero slope and the linear relationship of the results obtained. This was confirmed, as observed in Table 3, where the calculated F1 is greater than the tabulated F, thus rejecting the null hypothesis representing a slope equal to zero. On the other hand, the calculated F2 is less than the tabulated F, confirming the null hypothesis, which, in this case, represents the existence of a linear relationship in the data.

The t-test was also employed to demonstrate that the method responds to changes in concentration, meaning that the slope is different from zero (calculated t > tabulated t for the slope) and that the confidence interval of the slope does not include zero. Additionally, it was observed that the method is proportional, as the confidence interval of the intercept includes zero and the calculated t is less than the tabulated t for the intercept. It is concluded that the method is linear within the evaluated concentration range.

Table 3. Statistical Analysis of Linearity Test

Linear Regression	Correlation coefficient - R	0.9953
	Acceptance criterion - R	≥ 0.995
	Coefficient of determination - R^2	0.9906
	Acceptance criterion - R^2	≥ 0.990
Analysis of Variance (ANOVA)	F1	1377.15
	F2	-3.19
	Tabulated F	2.51
Student's t-test	**Analysis of the slope**	
	Alpha α	0.05
	t calculated	37.11
	t tabulated	2.16
	Intercept test	
	Alpha α	0.05
	t calculated	-1.32
	t tabulated	2.16

Accuracy

To evaluate accuracy, a criterion of acceptance was established for the recovery percentage of 95-105 %. Table 4 shows the mean of the results

obtained for the 5 levels evaluated, standard deviations, coefficients of variation, and variances for each of the measurements, meeting the established parameters and showing better recoveries (between 100.3 and 104.3%) than those reported in the literature. In this regard, Park HN et al. (2018) (12) developed and validated a method for the determination of 12 hair growth compounds in adulterated products by UHPLC-MS/MS, and in the assessment of the accuracy parameter, they observed recoveries between 81-117 % for minoxidil. These values were defined as acceptable for method validation. Comparing our method with the one mentioned in the present study, it can be said that ours has greater reliability.

However, it is important to emphasize that although the recovery reported by Park NH et al. shows greater variability than those reported in this manuscript, the analysis was performed using UHPLC-MS/MS, a much more sensitive technique both for detecting the analyte of interest and for assessing the risk of interference due to the matrix effect in the formulation under analysis. Therefore, although this technique exhibits greater variability, it has the advantage of achieving higher analytical sensitivity. Additionally, in the present study, the Cochran's G test was applied to evaluate the variances at the five concentration levels, concluding that the concentration factor does not influence the variability of the results; therefore, the method is accurate.

Table 4. Evaluated levels and recovery percentage for accuracy.

Level (%)	10	25	50	75	100
Mean % recovery	101.67	103.33	100.25	100.88	104.30
Standard deviation (SD)	1.24	1.13	1.95	3.14	0.33
Coefficient of variation (%CV)	1.22	1.02	1.94	3.11	0.31
Variance S^2	1.53	1.28	3.80	9.86	0.11

Precision - Repeatability.

The repeatability parameter was evaluated at 50, 75, and 100 %, and a global coefficient of variation equal to or less than 3% was established as the acceptance criterion. The obtained global coefficient of variation (2.6 %) for this parameter falls within the established range, as observed in Table 5. Therefore, it is concluded that the percentages found in the repetitions of the samples analyzed did not exhibit significant variability.

Table 5. Levels evaluated for repeatability.

Level (%)	50	75	100
Mean % recovery	100.25	100.88	104.30
Standard deviation (SD)	1.95	3.14	0.33
Coefficient of variation (%CV)	1.94	3.11	0.31
Global statistics	Mean	101.81	
	SD	2.65	
	%CV	2.60	

Intermediate precision.

In the current study, intermediate precision was assessed considering two variables: the analyst and the day. The acceptance criterion for the evaluation of intermediate precision was defined as a global coefficient of variation less than or equal to twice the coefficient of variation for repeatability, that is, 5.2 %, and the coefficient of variation for each subgroup less than or equal to 3%. In Table 6, it is observed that analysts 1 and 3 obtained a similarly low coefficient of variation between them; however, analyst 2 had higher inter-day variations. Despite this, the average global coefficient of variation is below the established acceptance criterion. Therefore, the variability that occurs when conducting the assay on different days and with different analysts is low, which does not significantly impact the results. Based on the results obtained in this parameter, it is concluded that the method is precise.

In the previously mentioned study for the validation of a method for the determination of non-permitted compounds using UHPLC-MS/MS, precision was measured considering inter-day parameters, evaluating on three different days, and intra-day parameters that were determined from repeated measurements on a single day. The results obtained show that the intra-day precision was below 4%, and for the inter-day case, it was at 11%. Both values were considered suitable to determine that the method is precise; however, both results are higher than those obtained in our study (12). Moreover, Patterson SC et al. (2005) (16) found in a method they developed for determining Minoxidil, CV% values similar to those we found (Intra-day: 0.0114-0.0133 and inter-day: 0.0243-0.0329).

Table 6. Summary of Intermediate Precision Results.

Level (%)	Analyst 1		Analyst 2		Analyst 3	
100	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
Mean	107.1	102.3	105.2	103.7	104.1	106.1
Standard deviation (SD)	0.1	1.6	3.1	1.4	0.8	1.8
Coefficient of variation (%CV)	0.1	1.5	3.0	1.3	0.7	1.7
	Mean		104.76			
Global statistics	SD		2.18			
	%CV (%)		2.08			

Detection and quantification limits

According to equation 1, a detection limit of 8.03 × 10^-6 mg/mL (0.00803 ppm) and a quantification limit of 0.000027 mg/mL (0.027 ppm) were obtained. Therefore, the method allows for the detection and quantification of small amounts of the analyte under study.

Robustness

The objective of the robustness test was to determine which processes or steps of the method are critical. It may affect the recovery of the analyte, and thus the accuracy of the measurement. For this parameter, a reduced Youden-Steiner factorial design was used, evaluating 7 variables in 8 assays, and the % recovery was determined as the response variable (Table 7). Furthermore, as an acceptance criterion, it was defined that the influence of a factor is relevant and considered significant if, when comparing the value of the effect with the expression $s\sqrt{2}$, where s is the standard deviation of repeatability, the differences exceed in absolute value the result of this expression.

With this evaluation, it was observed that the relevant factors in the method application were reducing the time in ultrasound (Factor B), pre-dissolving minoxidil in methanol before weighing or adding the placebo (Factor D), injection volume (Factor E), and flow change (Factor F) (Figure 5). For this reason, especially these parameters must be kept unchanged as they significantly influence the results obtained, while other factors such as modification of the aliquot (Factor A), control of temperature in ultrasound (Factor C), and the use of new or used filters (Factor G), do not significantly influence. In this sense, for the method to be reliable, the significant factors must be rigorously followed when performing quantification (Table 7 and Figure 5).

Table 7. Recovery (%) results for each robustness test.

	Assay							
Factor	1	2	3	4	5	6	7	8
A/a	A	A	A	A	a	a	a	a
B/b	B	B	b	b	B	B	b	b
C/c	C	c	C	c	C	c	C	c
D/d	D	D	d	d	d	d	D	D
E/e	E	e	E	e	e	E	e	E
F/f	F	f	f	F	F	f	f	F
G/g	G	g	g	G	g	G	G	g
Recovery (%) (%) esultado	114.4	96.4	101.9	110.7	111.3	108.2	92.2	112.8

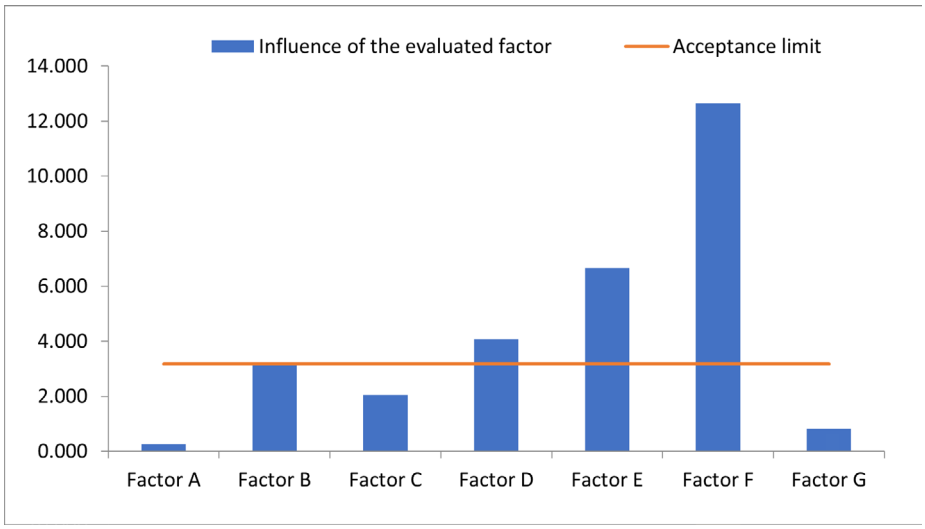

Figure 5. Graph showing the influence of the evaluated factors on the recovery.

Determination of minoxidil in hair growth products

Eleven products were selected from the market: two medications declared to contain 5 % Minoxidil and nine cosmetic hair growth products with different matrices. All products were purchased from websites, beauty stores, hair salons, and barber shops. As selection criteria, products suspected to contain Minoxidil and cosmetic hair growth products that declare Minoxidil were considered, even if the ingredient list did not specify whether it is a derivative of Minoxidil or corresponds to the base Minoxidil.

According to the method validation, it was found that the base minoxidil had an approximate retention time (RT) of 3.8, while minoxidil oxothiazolidinecarboxylate had an approximate RT of 4.3. In addition to this peak, another peak of lower intensity was presented at an RT of 2.5-3.0. This latter peak helped differentiate whether the products contained base minoxidil or minoxidil oxothiazolidinecarboxylate (Figures 6 and 7).

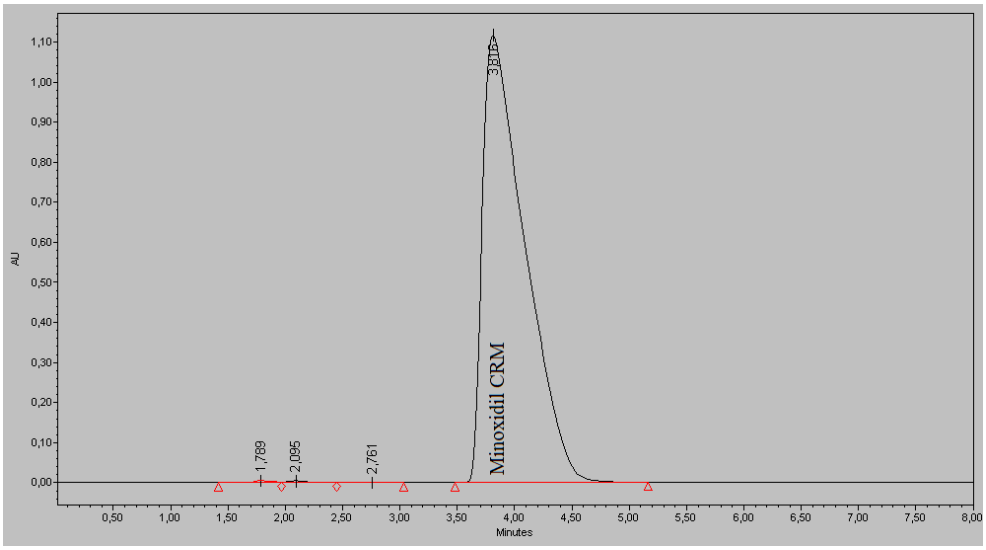

Figure 6. Chromatogram of certified reference material Minoxidil

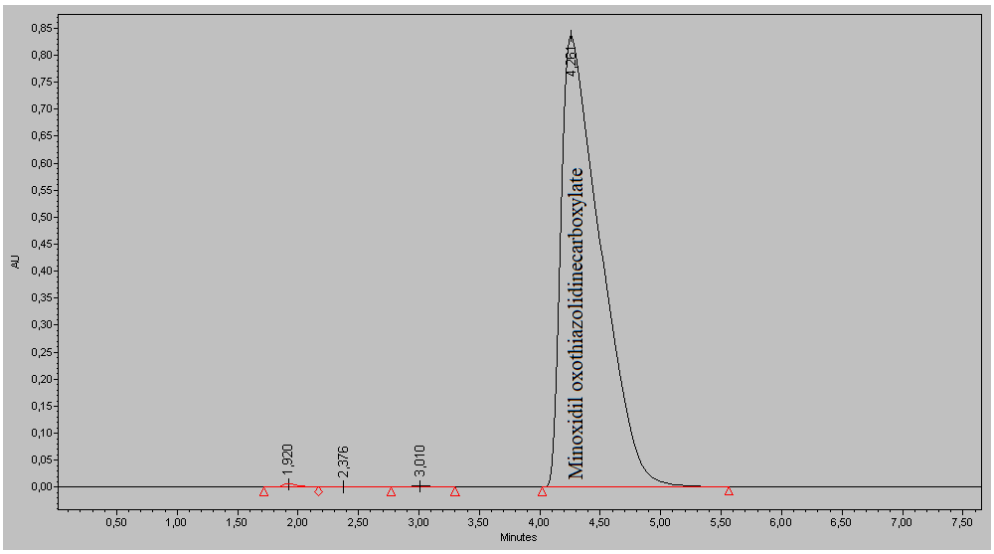

Figure 7. Chromatogram of raw material Minoxidil oxothiazolidinecarboxylate.

Table 8 shows the results of the determination of Minoxidil and its derivative Minoxidil oxothiazolidinecarboxylate. It was identified that products A, E, F, G, and K do not contain Minoxidil, which corresponds to what is declared on their respective labels. Products C and H (Figure 8) declare on their label to contain Minoxidil oxothiazolidinecarboxylate. Through identification and quantification, it was corroborated that this compound was present and not the base Minoxidil (Table 8). However, product D did not present the characteristic peak at tR 2.5-3.0 indicative of the derivative Minoxidil oxothiazolidinecarboxylate, therefore, it is presumed that this cosmetic product contains base Minoxidil (RT 3.6) (Figure 9). This adverse analytical result is compounded by the fact that this product showed appearance problems, presenting a large amount of precipitated white crystals, which were separated by filtration, washed, and dried in an oven at 60°C. Subsequently, a solution was prepared with these crystals and injected using the same chromatographic system, presenting a retention time corresponding to base Minoxidil. These findings suggest that this product does not comply with international standards for cosmetic ingredients, as discussed, this substance is prohibited for use in cosmetics (5–8).

Table 8. Results of the determination of minoxidil in the evaluated products.

Product	Label information	Results
A	Does not claim minoxidil	Does not contain minoxidil
B	Claim minoxidil	Minoxidil base is presumed (7.39±0.07%)
C	Claim derivative	Minoxidil oxothiazolidinecarboxylate (0.02±0.002%)
D	Claim derivative 5%	Minoxidil base is presumed (2.04±0.68%)
E	Does not declare minoxidil	Does not contain minoxidil
F	Does not declare minoxidil	Does not contain minoxidil
G	Does not declare minoxidil	Does not contain minoxidil
H	Claim derivative	Minoxidil oxothiazolidinecarboxylate (0,15±0,23%)
I	Claim minoxidil 5%	Minoxidil base (1.10±0.02%)
J	Claim minoxidil 5%	Minoxidil base (5.29±0.10%)
K	Does not claim minoxidil	Does not contain minoxidil

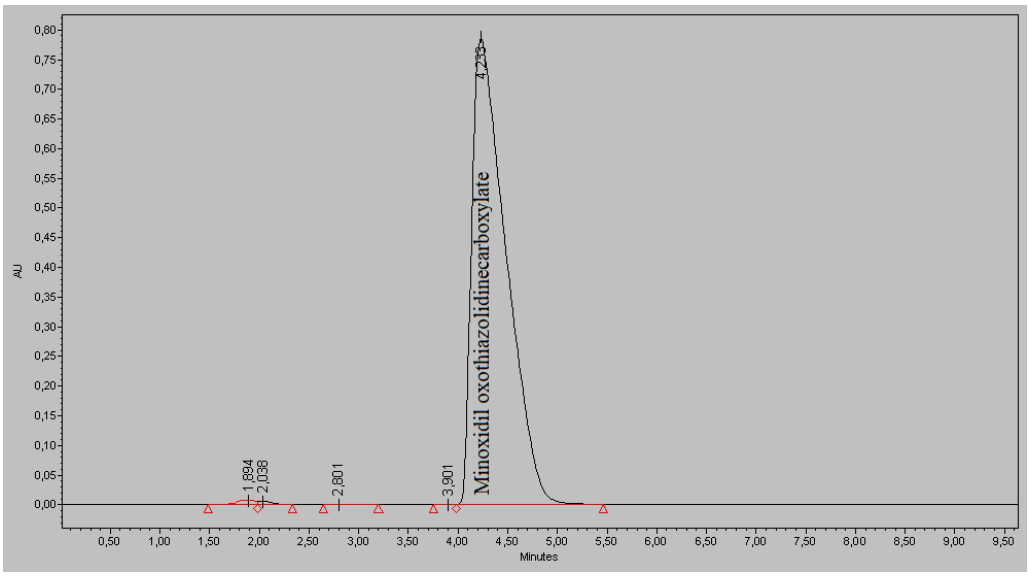

Figure 8. Product H chromatogram

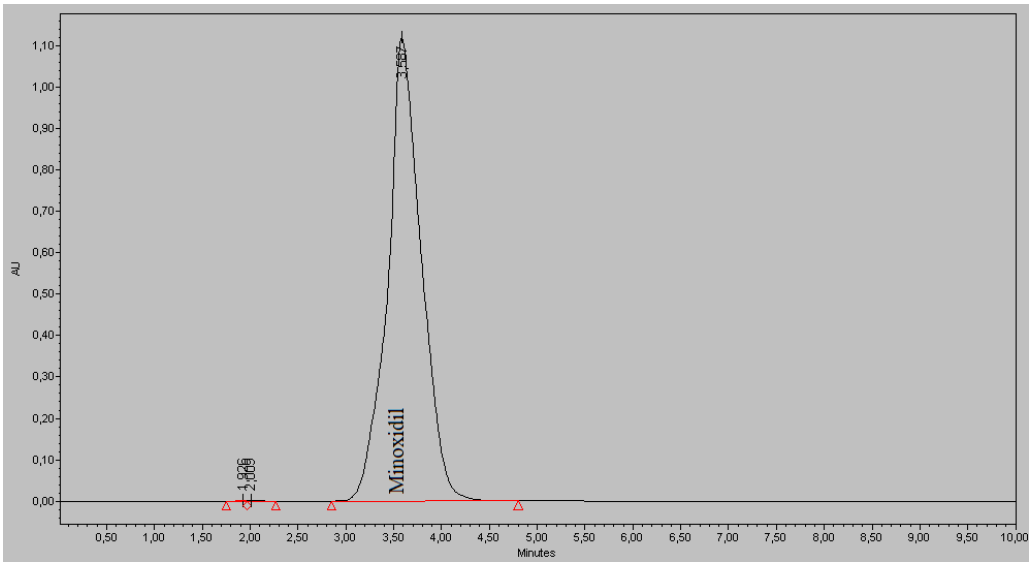

Figure 9. Product D chromatogram

On the other hand, product B declares to contain minoxidil; however, its ingredient list does not specify whether it is minoxidil oxothiazolidinecarboxylate or another approved derivative. Upon verification on the website of INVIMA (Instituto Nacional de Vigilancia de Medicamentos y Alimentos de Colombia), its Mandatory Health Notification (NSO), the search yielded no results, which raises suspicions of possible regulatory non-compliance. Additionally, the analysis of this product showed the specific peak for base Minoxidil, indicating that this product likely contains the prohibited form of minoxidil in cosmetics.

Two medications, topical solutions of minoxidil, products I and J, were also analyzed. These were evaluated as reference products for the identification of minoxidil. Indeed, it was confirmed that these products, being medications rather than cosmetics, contain declared base minoxidil at a concentration of 5 %. Drug product I, which is illegally imported into the country as it lacks a sanitary registration, had a found percentage of Minoxidil of 1.10±0.02%. In contrast, medication J, a domestically manufactured product with sanitary registration, had a found percentage of 5.29±0.10 %. According to the individual USP monograph, these

medications should have between 90 – 110% of the labeled quantity, meaning between 4.5 – 5.5% of Minoxidil per formulation. Therefore, product I, illegally imported into the country, does not meet the quality characteristics for the assay according to the USP 18.

In the results obtained in this research, it was found that 2 out of the 9 cosmetic products analyzed contained the prohibited analyte base minoxidil, i.e., 22.22 % of the analyzed products, which is consistent with what has been reported in other studies (11,13,16,17)

In this regard, De Orsi et al. (2008) determined the presence of seven prohibited substances in cosmetic products, including the substances evaluated: minoxidil, hydrocortisone, spironolactone, estrone, canrenone, triamcinolone acetonide, and progesterone, found that of the six assessed products, minoxidil was present in four of them, and concomitantly with spironolactone in two products (18). Not only did these products demonstrate the presence of different prohibited substances, but also the percentages of these substances were extremely high. Another study conducted in 2018 analyzed 76 samples of products claimed to be hair growth treatments. Once again, the results demonstrated the presence of unapproved compounds in cosmetics. It was found that around 10 % of the samples were adulterated with five prohibited ingredients, including minoxidil (12).

Finally, with this work we hope to contribute to the inspection, surveillance, and post-market control activities of cosmetic products, with the aim of reducing regulatory non-compliance due to the use of prohibited ingredients, leading to reducing the trend of the number of fraudulent products in the international markets. In this regard, compared to LC-MS methods, although they offer greater specificity and sensitivity, they are clearly not economical, easy to implement, or rapid (12). On the other hand, the method described by the USP is designed for topical drug solutions, not for complex matrices such as cosmetics. Additionally, the USP method employs ion-pair chromatography (IPC) with docusate sodium as the reagent, which is challenging to standardize, more expensive, and contaminates chromatographic columns (19). For all these reasons, the method we propose is a simpler, faster, more cost-effective, and reliable alternative for determining Minoxidil in cosmetic products.

CONCLUSIONS

This work allowed for the development, standardization, and validation of an HPLC-UV analysis methodology that is much simpler, faster, and more economical compared to other methods reported for the identification and quantification of minoxidil in cosmetic products and medications. The results obtained in this research coincide with what has been reported in the literature regarding the use of prohibited substances in cosmetic products. Additionally, it is worth noting that it was rigorously determined whether the presence of minoxidil corresponded to the base molecule or a derivative such as minoxidil oxothiazolidinecarboxylate. This distinction is not present in other related articles that have evaluated the presence of prohibited substances like minoxidil, which could lead to erroneous results. However, it is suggested to verify these results with other, more selective techniques such as HPLC-DAD or LC-MS, which can support these claims. It is hoped that the results obtained will contribute to the generation of cosmetovigilance activities, cosmetic control, and inspection, as an important strategy to prevent illegality or regulatory non-compliance by manufacturers.

CONFLICT OF INTEREST

The authors declare no conflict of interest. The paper was published as a preprint on the Prerpints.org platform DOI: 10.20944/preprints202501.2080.v1

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AUTHOR CONTRIBUTIONS:

Conceptualization, J.C.M.-G.; methodology, J.C.M.-G, M.L-L, D.M.T-L and T. R-A; validation, M.L-L, D.M.T-L and T. R-A.; formal analysis, J.C.M.-G; investigation, J.C.M.-G, M.L-L, D.M.T-L and T. R-A; resources J.C.M.-G; data curation, J.C.M.-G and M.L-L; writing—original draft preparation, M.L-L, D.M.T-L and T. R-A; writing—review and editing, J.C.M.-G; supervision, J.C.M.-G; project administration, J.C.M.-G; funding acquisition, J.C.M.-G. All authors have read and agreed to the published version of the manuscript

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