



# Development and validation of a UV–Vis spectrophotometric method for the quantification of affinin in ethanolic extracts of *Heliopsis longipes* and commercial herbal products

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## Abstract

Affinin, the main bioactive compound of *Heliopsis longipes* root, is an alkamide known for its multiple pharmacological effects, including anti-inflammatory, antihypertensive, and analgesic. Accurate quantification of affinin in extracts and commercial products is essential to ensure their quality and efficacy. In this study, a simple, robust, and accessible UV–Vis spectrophotometric method was developed and validated for the quantification of affinin in *H. longipes* ethanolic extracts and five marketed formulations in Mexico, and its performance was benchmarked against the gold-standard HPLC method. A linear calibration curve was established over 0.5 to 10 µg/mL, and the method was validated by evaluating linearity, accuracy, precision, specificity, and limits of detection (LOD) and quantification (LOQ). The maximum absorbance of affinin was determined at 230 nm in ethanol. Linearity was confirmed over the range of 0.5–10 µg/mL, with a correlation coefficient ( $R^2$ ) of 0.998. Precision, evaluated as intra- and inter-day reproducibility, showed relative standard deviations below 2%. Method accuracy was confirmed by standard addition experiments, with recovery values ranging from 98 to 102%. Specificity was supported by the absence of significant spectral interference in the UV spectra of the extract compared to the affinin standard, with an absorbance of  $0.6795 \pm 0.0026$ . The method also demonstrated adequate sensitivity, with LOD and LOQ of 0.298 µg/mL and 0.903 µg/mL, respectively. Affinin quantification in commercial formulations revealed variability in content, highlighting the need for rigorous quality control to ensure consistency among products. In conclusion, the validated UV–Vis method provides a rapid, cost-effective, and reliable tool for the quality control and standardization of *H. longipes* extracts and commercial products. Moreover, its use of ethanol as a bio-based solvent, low solvent consumption, and minimal waste generation align with green analytical chemistry principles, supporting more sustainable and accessible quality-control practices in the growing herbal product market.

**Keywords** UV-Vis spectrophotometry · Affinin · *Heliopsis longipes* · Herbal products · Phytochemicals

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## Introduction

The use of medicinal plants has been an integral component of therapeutic practices across diverse cultures throughout history. Today, herbal products remain widely employed as alternative or complementary treatments for a broad range of ailments. However, the growing interest in these products demands the establishment of rigorous quality control measures to ensure their efficacy, safety, and reproducibility [1]. Standardization through chemical markers is a key strategy to guarantee consistency in the chemical composition of plant extracts [2, 3].

*Heliopsis longipes* (A. Gray) Blake, commonly known as “chilcuague” or “gold root,” is an endemic species of Mexico recognized for its analgesic, anti-inflammatory, and antimicrobial properties, which are mainly attributed to the presence of alkaloids, particularly affinin [4–6]. In addition, our research group demonstrated that affinin exerts antihypertensive, antinociceptive, anti-hypothermic, and anti-inflammatory activities through mechanisms involving the activation of CB1 receptors, TRPV1 and TRPA1 channels, and the suppression of pro-inflammatory cytokines [7, 8]. Due to its biological activity and high concentration in the root of *H. longipes*, affinin has been proposed as a chemical marker for products derived from this plant. However, its quantification usually requires sophisticated analytical methods such as liquid chromatography (LC) [9, 10], which represents an economic and technical barrier for many laboratories or producers.

Chromatographic techniques, particularly HPLC or UPLC, typically employ mobile phases containing organic solvents such as methanol, acetonitrile, and dichloromethane. These solvents are often toxic, hazardous to handle, and environmentally detrimental [11, 12]. In addition, HPLC analysis typically involves labor-intensive sample preparation, costly instrumentation, and extended analysis times [13, 14], which may limit its routine use in laboratories with limited resources, highlighting the need for simpler, cheaper, and more sustainable analytical alternatives.

Beyond analytical considerations, the availability of accessible and sustainable quality control methodologies is increasingly recognized as a key enabling factor for the responsible valorization of endemic medicinal plants [1]. In regions such as Mexico, where *H. longipes* is native, reliable quality assessment tools are closely linked to the development of local agro-industrial value chains, economic resilience, and fair use of plant-based resources. Recent studies emphasize that sustainable natural product research and green analytical technologies play a pivotal role in supporting small-scale producers and local enterprises by reducing

technical and economic barriers to market participation, while promoting environmentally responsible practices [15].

Validating rapid and cost-accessible analytical methods, such as UV–Vis spectrophotometry, plays a crucial role in ensuring the quality, safety, and efficacy of herbal products. Unlike more complex and costly techniques like HPLC or UPLC, UV–Vis methods offer simplicity, affordability, and rapid execution, making them especially valuable in settings with limited analytical infrastructure [16]. UV–Vis spectrophotometric determination is widely used for quantifying phytochemicals such as polyphenols [17], alkaloids [18, 19], and alkaloids [20] due to their accessibility and suitability for routine quality control in herbal medicines. Furthermore, affinin, an alkaloid with a conjugated double-bond system, exhibits strong absorption in the ultraviolet region, making it suitable for analysis by spectrophotometry. Comparable spectroscopic behavior has been successfully exploited for structurally related alkaloids such as capsaicin, for which UV–Vis-based analytical methods have been reported [21]. In addition, previous studies have demonstrated that UV–Vis spectrophotometry can be successfully validated against HPLC for quantitative purposes, showing comparable performance for routine analysis when appropriately optimized [14, 18]. In contrast, affinin quantification has been addressed almost exclusively through chromatographic techniques, and a validated UV–Vis spectrophotometric method for its determination in plant extracts and commercial formulations has not been previously reported.

Phytopharmaceutical products derived from *H. longipes* and other medicinal plants commonly employ ethanol as a bio-based extraction solvent, since it offers a safer and more environmentally friendly alternative to conventional organic solvents [22, 23]. The use of ethanol and the reduction of hazardous reagents and waste are consistent with broader efforts toward more sustainable and circular agri-food and bio-based systems. In this context, greener analytical workflows represent practical steps toward improving sustainability across industrial value chains [24].

Strengthening the standardization and quality assurance of herbal products also supports the responsible integration of plant-based traditional medicines into sustainable health-care systems, while enhancing consumer protection. In the specific case of *H. longipes* (chilcuague), commercialization remains largely regional, limiting broader market development. Therefore, the promotion of cultivation, circular-economy approaches, and wider commercialization of this species requires simple, cost-effective, and sustainable analytical methods that enable reliable product standardization [25, 26]. Accordingly, this study aimed to develop and validate a UV–Vis spectrophotometric method for the quantification of affinin in *H. longipes* extracts and commercial

formulations, providing an accessible, reliable, and environmentally sustainable tool for routine quality control aligned with green analytical chemistry principles.

## Materials and methods

### Materials

Affinin standard was purchased from ChromaDex<sup>®</sup> Standards (Los Angeles, California, USA). Absolute ethanol was obtained from CTR Scientific (Queretaro, Mexico). HPLC-grade solvents, including ethanol, acetonitrile, acetic acid, and water, were acquired from Tedia<sup>®</sup> (Fairfield, Ohio, USA).

### Preparation of the ethanolic extract from *H. longipes* root

The ethanolic extract of *H. longipes* root (EEH) was obtained by macerating 100 g of dried and pulverized root material in absolute ethanol at a 1:10 (w/v) ratio. Roots were air-dried prior to pulverization and used as dried material for all extractions; individual moisture content was not determined. All macerations were performed under identical controlled conditions using the same batch of dried root material. Maceration was carried out at room temperature ( $25 \pm 2$  °C) for seven days. After filtration, the residual plant material was subjected to two additional maceration cycles using fresh solvent each time. All filtrates were pooled and concentrated under reduced pressure using a rotary evaporator (Büchi R-114, Büchi, Flawil, Switzerland). The extraction procedure was performed in three independent batches following the same protocol, which were subsequently pooled to obtain a single homogeneous extract for analytical validation. The extraction yield was % (w/w) relative to the initial dry root mass.

### Commercial formulations

Five liquid-based commercial topical and buccal spray formulations containing ethanolic extracts of *H. longipes* root were acquired from different Mexican manufacturers. All products were labeled as containing *H. longipes* as the active ingredient. The formulations were stored at room temperature and protected from light until analysis. Prior to dilution, samples were homogenized by gentle shaking and visually inspected, showing no visible turbidity or phase separation. Each formulation was then directly diluted in absolute ethanol without additional extraction, filtration, or centrifugation steps. After dilution, all samples were optically clear, minimizing potential light scattering or

baseline shifts during UV–Vis measurements. Appropriate dilution factors were selected to ensure absorbance values fell within the linear range of the calibration curve, preferably near its midpoint. Diluted samples were vortex-mixed to ensure homogeneity and analyzed immediately under the same conditions used for standards and extracts.

The commercial products did not provide standardized dose units or recommended administration volumes; therefore, affinin content could not be normalized to mg per dose. Results were additionally expressed as % w/w, calculated using the density of ethanol, to enable meaningful comparison among formulations without introducing dosing assumptions.

### Quantification of affinin by HPLC

Affinin content was determined using a previously validated HPLC method on a Waters<sup>®</sup> 600E system equipped with a diode array detector (DAD) and a ZORBAX<sup>®</sup> Eclipse XDB-C8 column ( $4.6 \times 150$  mm, 5  $\mu$ m). Chromatographic separation was performed under isocratic conditions using acetonitrile:1% acetic acid in water (1:1, v/v) at a flow rate of 1.0 mL/min. Analyses were carried out at ambient temperature ( $25 \pm 2$  °C). Manual injections of 20  $\mu$ L were performed, and the total run time was 9 min. Detection was carried out at 236 nm using the DAD. A calibration curve was constructed using affinin standards in the range of 15–90  $\mu$ g/mL. Ethanolic, methanolic, and dichloromethane extracts of *H. longipes* roots were analyzed in triplicate, and the results were compared with those obtained by UV–Vis spectrophotometry [10].

### Quantification of affinin by UV-Vis spectrophotometry

For the quantification of affinin, a stock solution of the standard was prepared at 100  $\mu$ g/mL in absolute ethanol. Subsequently, a spectral scan was performed in the range of 200 to 400 nm using a UV–Vis spectrophotometer (Genesys 10 S UV–Vis spectrophotometer, Thermo Fisher, Madison, WI, USA) to determine the maximum absorption wavelength ( $\lambda_{\text{max}}$ ) of affinin. Prior to measurements, the instrument was allowed to warm up for 20 min to ensure lamp stability. Spectra were recorded using matched quartz cuvettes (1 cm path length), employing absolute ethanol (99.5% purity) as the solvent blank. Baseline correction was performed by automatic subtraction of the blank spectrum recorded under identical conditions. Measurements were carried out at room temperature ( $25 \pm 2$  °C) without active temperature control. Samples were appropriately diluted to keep absorbance values within the linear working range of the instrument; linearity at higher concentrations was verified

by serial dilution, with no evidence of stray-light effects or detector saturation. Subsequently, a calibration curve was constructed within the concentration range of 0.5 to 10 µg/mL (0.5, 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 µg/mL).

Methanolic and dichloromethane extracts of *H. longipes* root were also evaluated for comparison with the results obtained by HPLC. Spectrophotometric measurements were performed in triplicate for each sample. Samples were appropriately diluted prior to HPLC or UV–Vis analysis to ensure that affinin concentrations fell within the respective linear calibration ranges of each method.

### Determination of the molar extinction coefficient ( $\epsilon$ )

The molar extinction coefficient ( $\epsilon$ ) of affinin in absolute ethanol was determined according to the Beer–Lambert law. A calibration curve was first constructed using affinin standard solutions in the concentration range of 0.5–10 µg/mL. For the calculation of  $\epsilon$ , the concentrations expressed in µg/mL were converted to molar concentration (M) using the molecular weight of affinin (221.34 g/mol). Each concentration value was converted to moles and then divided by the corresponding solution volume (1 mL) to obtain molarity.

The calibration curve was plotted as absorbance (A) versus molar concentration (M), and linear regression analysis was performed. According to the Beer–Lambert equation ( $A = \epsilon \cdot b \cdot C$ ), the slope of the regression line corresponds to the molar extinction coefficient when a path length (b) of 1 cm is used. The molar extinction coefficient was therefore obtained directly from the slope and expressed in units of  $M^{-1} \text{ cm}^{-1}$ .

### Validation of the UV–Vis method

The spectrophotometric method for the quantification of affinin was validated in accordance with the International Conference on Harmonisation (ICH) guideline Q2(R1). The validation parameters included linearity, precision (intra- and inter-day), accuracy, specificity, limit of detection (LOD), and limit of quantification (LOQ).

#### Linearity

A calibration curve was prepared using seven different concentrations of affinin standard solutions ranging from 0.5 to 10 µg/mL. Each concentration was measured in triplicate, and the absorbance was recorded at the determined maximum wavelength ( $\lambda_{\text{max}} = 230 \text{ nm}$ ). The linear relationship between absorbance and concentration was assessed using least-squares linear regression, and the coefficient of determination ( $R^2$ ), slope, and intercept were calculated. The method was considered linear when  $R^2$  was greater than

0.995. Additionally, the relative root mean square error (RRMSE) and the mean absolute percentage error (MAPE) were calculated to evaluate the magnitude of prediction errors across the calibration range.

#### Precision

Repeatability (intra-day precision) was assessed by performing five replicate ( $n = 5$ ) analyses of a middle-range concentration (4 µg affinin/mL) on the same day, under the same conditions, at three different times (12:00, 14:00, and 16:00; UTC-6). Results were expressed as the relative standard deviation (%RSD) of the measured absorbances or calculated concentrations. Intermediate precision (inter-day) was evaluated on two different days by two independent analysts using the same procedure. A %RSD of less than 2% for intra-day analysis and less than 3% for inter-day analyses was considered acceptable.

#### Accuracy (standard addition approach)

For the evaluation of method accuracy in the extract matrix, a standard addition approach was applied. An affinin standard solution (4 µg/mL) was spiked into the ethanolic extract at three concentration levels corresponding to 80%, 100%, and 120% of the previously determined affinin content. Each level was analyzed in triplicate, and method accuracy was expressed as percentage recovery, calculated using Eq. 1. Recoveries within the range of 98–102% were considered acceptable.

Equation 1:

$$\text{Accuracy (\%recovery)} = \frac{(C_s \text{ in spiked samples} - C_s \text{ in unspiked samples})}{C_{\text{StA}}} \times 100$$

where:

$C_s$  in spiked samples = concentration of affinin determined in the ethanolic extract matrix at each level (80%, 100%, and 120%) after the addition of the affinin standard.

$C_s$  in unspiked samples = concentration of affinin determined in the ethanolic extract matrix at each level (80%, 100%, and 120%) prior to spiking.

$C_{\text{StA}}$  = concentration of affinin standard added to the extract (4 µg affinin/mL).

This approach was used to assess method performance and recovery in the presence of the extract matrix.

#### Specificity

To evaluate method specificity and potential matrix effects, an extract solution was prepared by diluting the crude *H. longipes* ethanolic extract to a concentration of 2 mg extract/mL. An aliquot of this solution was

adjusted to a final volume of 2.5 mL. The affinin content of the unspiked extract was first determined by UV–Vis spectrophotometry and corresponded to 2 µg affinin/mL. Subsequently, 1.0 mL of an affinin standard solution (5 µg/mL) was added to the extract solution, and the mixture was vortex-mixed to ensure homogeneity prior to analysis.

Specificity was assessed by comparing the absorbance at the affinin  $\lambda_{\text{max}}$  (230 nm) between spiked samples and the theoretical sum of the absorbances of the unspiked extract and the affinin standard. Statistical analysis was performed using the Two One-Sided Tests (TOST) equivalence test applied to paired absorbance differences. Given the absence of chromatographic separation in UV–Vis spectrophotometry, specificity in this context is defined as the absence of analytically significant interference at the selected wavelength under the experimental conditions employed.

#### Limit of detection (LOD) and limit of quantification (LOQ)

The limits were estimated based on the standard deviation of the response ( $\sigma$ ) and the slope ( $S$ ) of the calibration curve, using the following formulas:  $\text{LOD} = 3.3 \times (\sigma/S)$  and  $\text{LOQ} = 10 \times (\sigma/S)$ . These parameters reflect the sensitivity of the method, ensuring detection and reliable quantification of affinin even at low concentrations.

#### AGREE and agreeprep analysis

The greenness of the UV-Vis and HPLC methods was assessed using the AGREE and AGREEprep metrics, two standardized tools derived from the 12 principles of Green Analytical Chemistry. AGREE was employed to evaluate the environmental performance of the overall analytical procedures, including reagent toxicity, energy consumption, waste generation, sample handling, and operator safety. In parallel, AGREEprep was used to independently assess the sustainability of the sample preparation steps, including the number of operational stages, solvent type and volume, degree of miniaturization, and potential environmental hazards. Both evaluations were performed using their respective software platforms [27, 28]. AGREE, and AGREEprep provides a 0–1 score; higher values indicate stronger overall alignment. The tool is intended for comparative, method-level benchmarking and does not replace a full life-cycle assessment. To support interpretability, we report both the overall score and the contribution of individual principles, highlighting the operational drivers of the score differences (Figures S1 and S2).

#### Statistical analysis

Data were expressed as mean  $\pm$  standard deviation (SD). Statistical comparison between UV–Vis spectrophotometric and HPLC results was performed using a paired Student's  $t$ -test at a 95% confidence level ( $p < 0.05$ ). Method agreement was further evaluated by Bland–Altman analysis, including estimation of bias and 95% limits of agreement. For specificity evaluation, equivalence between spiked samples and the theoretical sum of absorbances was assessed using the Two One-Sided Tests (TOST) equivalence test applied to paired absorbance differences at a 95% confidence level. Statistical analyses were performed using Minitab, while graphical representations were generated using OriginPro.

### Results

#### Determination of the maximum absorbance wavelength ( $\lambda_{\text{max}}$ )

The UV-Vis spectrum of affinin standard was recorded over the range of 200–400 nm to determine its wavelength of maximum absorbance ( $\lambda_{\text{max}}$ ). As shown in Fig. 1, a distinct absorption peak was observed at 230 nm, which was used for all subsequent quantitative analyses of affinin in extracts and commercial formulations.

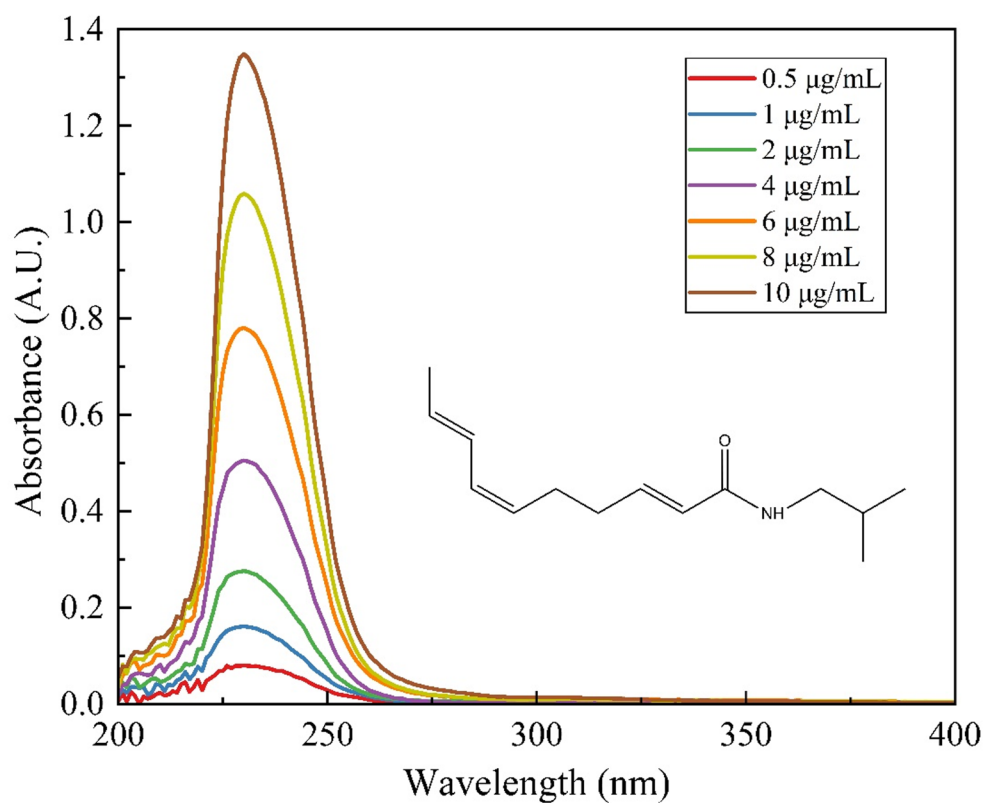
After obtaining the absorption spectrum of the affinin standard (Fig. 1), the ethanolic extract of *H. longipes* root was also analyzed under the same conditions to identify its characteristic absorption bands. The spectrum of the extract (Fig. 2) showed a single absorption band at the same wavelength, confirming the presence of affinin as the main UV-absorbing component.

#### Validation of the UV-Vis method

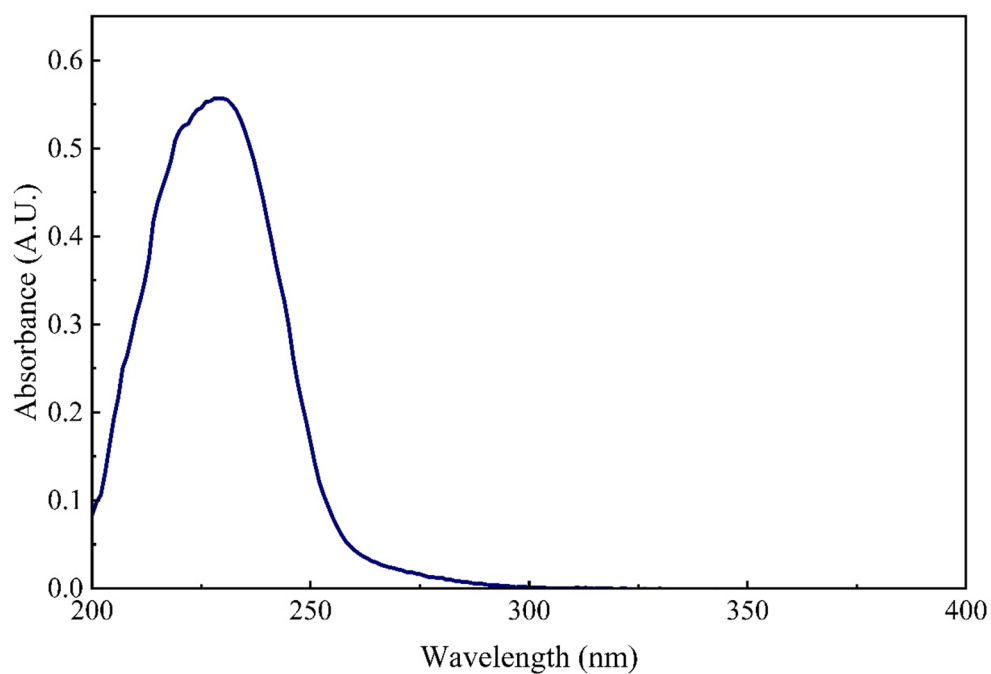
##### Linearity

The calibration curve for affinin, constructed in the concentration range of 0.5 to 10 µg/mL, showed a correlation coefficient ( $R^2$ ) of 0.998. The regression equation was determined as  $y = 0.12881x + 0.0307$ , where  $y$  represents absorbance and  $x$  the affinin concentration. As shown in Figs. 1 and 3, the absorbance increased proportionally with concentration. In addition to  $R^2$ , the calibration performance was further assessed using error-based metrics. The relative root mean square error (RRMSE) was 3.30%, and the mean absolute percentage error (MAPE) was 4.22%, indicating low prediction error and confirming the adequacy of the linear model over the studied concentration range.

**Fig. 1** UV–Vis absorption spectra of the affinin standard recorded in ethanol over the 200–400 nm range at different concentrations (0.5–10  $\mu\text{g/mL}$ ), showing a consistent maximum absorption wavelength ( $\lambda_{\text{max}}$ ) at 230 nm



**Fig. 2** UV–Vis absorption spectrum of the ethanolic extract of *H. longipes* root (4  $\mu\text{g/mL}$ ), showing an absorption maximum at 230 nm that coincides with the  $\lambda_{\text{max}}$  of the affinin standard



Following the construction of the calibration curve, the molar extinction coefficient ( $\epsilon$ ) of affinin in ethanol was determined according to the Beer–Lambert law using the slope of the linear regression. The calculated  $\epsilon$  value was  $2.851 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ , which indicates a strong ultraviolet absorption capacity consistent with the presence of a conjugated double-bond system in the affinin structure. This parameter further supports the suitability of UV–Vis spectrophotometry for the quantitative determination of affinin in *H. longipes* extracts.

### Precision

The precision of the UV–Vis method was evaluated for intra-day (repeatability) and inter-day (reproducibility) precision. Intra-day precision was assessed by measuring absorbance at multiple time points for a middle-range concentration (4  $\mu\text{g/mL}$ ), while inter-day precision was evaluated on two separate days by two different analysts. All %RSD

values were below 2%, indicating acceptable precision. The detailed results are presented in Table 1.

### Accuracy (standard addition approach)

The accuracy of the UV–Vis method was evaluated by spiking affinin into the ethanolic extract matrix, all recovery values were within the acceptable range (98–102%), confirming the accuracy of the method. The detailed results are presented in Table 2.

### Specificity

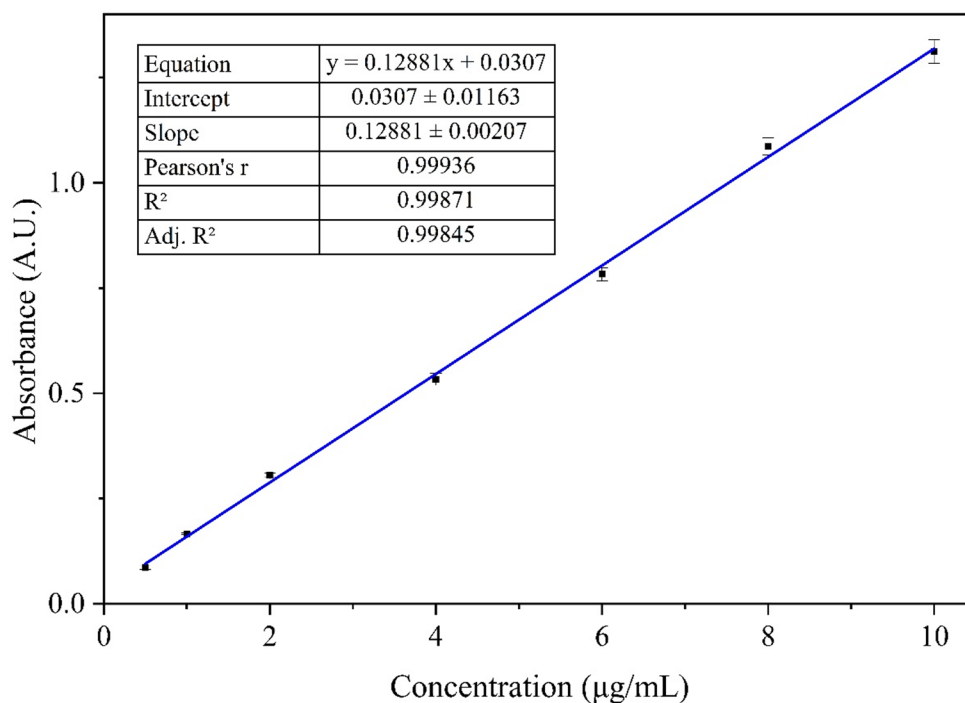
The specificity of the UV–Vis method was evaluated by comparing spiked and unspiked EEH samples with the affinin standard at the same concentration. As shown in Table 3, the absorbance difference obtained for the spiked samples showed excellent agreement with the absorbance

**Table 1** Values of intra-day (repeatability) and inter-day (reproducibility) precision for affinin quantification by UV–Vis spectrophotometry

\*The 95% confidence interval (CI) corresponds to the overall mean value

| Precision level             | Measurement      | Absorbance (mean $\pm$ SD) | Overall mean $\pm$ SD | %RSD  | CI (95%) *         |
|-----------------------------|------------------|----------------------------|-----------------------|-------|--------------------|
| Intra-day (repeatability)   | 12:00 pm         | 0.549 $\pm$ 0.008          | 0.546 $\pm$ 0.006     | 1.055 | 0.546 $\pm$ 0.0149 |
|                             | 2:00 pm          | 0.546 $\pm$ 0.006          |                       |       |                    |
|                             | 4:00 pm          | 0.542 $\pm$ 0.004          |                       |       |                    |
| Inter-day (reproducibility) | Day 1, analyst 1 | 0.552 $\pm$ 0.007          | 0.546 $\pm$ 0.009     | 1.654 | 0.546 $\pm$ 0.0224 |
|                             | Day 2, analyst 2 | 0.541 $\pm$ 0.011          |                       |       |                    |

**Fig. 3** Calibration curve of affinin standard by UV–Vis spectrophotometry



**Table 2** Accuracy of affinin quantification by UV-Vis spectrophotometry in EEH at three spiking levels

| Sample | Concentration in un-spiked samples (µg/mL) | Amount added (µg/mL) | Concentration in spiked samples (µg/mL) | Recovery (%) |
|--------|--------------------------------------------|----------------------|-----------------------------------------|--------------|
| 80%    | 3.201                                      | 4                    | 7.122                                   | 98.991       |
|        | 3.201                                      | 4                    | 7.161                                   |              |
|        | 3.193                                      | 4                    | 7.192                                   |              |
| 100%   | 4.086                                      | 4                    | 8.085                                   | 99.702       |
|        | 3.985                                      | 4                    | 7.937                                   |              |
|        | 3.993                                      | 4                    | 8.007                                   |              |
| 120%   | 4.738                                      | 4                    | 8.869                                   | 99.314       |
|        | 4.855                                      | 4                    | 8.846                                   |              |
|        | 4.707                                      | 4                    | 8.706                                   |              |

**Table 3** Specificity assessment of affinin quantification by UV-Vis spectrophotometry in EEH

| [Affinin standard added µg/mL] | Abs. (Affinin standard) | Abs. (Unspiked extract) | Abs. (Spiked extract) | Difference (D) | Overall Mean D±SD | CV (%RSD) | CI (95%) *   |
|--------------------------------|-------------------------|-------------------------|-----------------------|----------------|-------------------|-----------|--------------|
| 5                              | 0.677                   | 0.292                   | 0.972                 | 0.680          | 0.679±0.0026      | 0.389     | 0.679±0.0065 |
| 5                              | 0.675                   | 0.295                   | 0.974                 | 0.679          |                   |           |              |
| 5                              | 0.671                   | 0.300                   | 0.975                 | 0.675          |                   |           |              |
| Mean±SD                        | 0.674±0.0031            | 0.296±0.0040            | 0.974±0.0015          | 0.678±0.0026   |                   |           |              |

Abs absorbance, D Abs (Spiked extract) – Abs (Unspiked extract). \*The 95% confidence interval (CI) corresponds to the overall mean value

of the affinin standard (5 µg/mL), with an overall mean difference of  $0.679 \pm 0.0026$  and a low coefficient of variation (0.39% RSD). The narrow 95% confidence interval indicates high repeatability of the analytical response.

### Limit of detection and quantification

The sensitivity of the UV-Vis method was evaluated by calculating the limit of detection (LOD) and limit of quantification (LOQ) (Table 4). These results indicate that the method is suitable for detecting and quantifying affinin at low concentrations typically found in commercial formulations, as will be discussed below.

**Table 4** LOD and LOQ for affinin quantification by UV-Vis spectrophotometry

| Parameters from the calibration curve |                    |                                                | LOD (µg/mL) | LOQ (µg/mL) |
|---------------------------------------|--------------------|------------------------------------------------|-------------|-------------|
| Slope (S) (mean)                      | Y-intercept (mean) | SDY-intercept (standard deviation of the mean) |             |             |
| 0.129                                 | 0.031              | 0.012±0.0021                                   | 0.298       | 0.903       |

### Quantification of affinin in extracts of *H. longipes* root

Ethanollic, methanollic, and dichloromethane extracts of *H. longipes* roots were analyzed in triplicate using the validated UV–Vis method and the previously validated HPLC method for comparison. The concentrations of affinin determined by both methods are summarized in Table 5. Statistical comparison using a paired t-test showed no significant differences between UV–Vis and HPLC results for any extract ( $p > 0.05$ ). Agreement between the UV–Vis and HPLC methods was further evaluated using Bland–Altman analysis. As shown in Table 5, the differences between both methods were contained within the 95% limits of agreement. The magnitude of these limits was relatively small in relation to the concentration range analyzed, supporting a close agreement between UV–Vis and HPLC measurements.

### Quantification of affinin in commercial topical/buccal formulations

Commercial topical/buccal formulations containing ethanolic extract of *H. longipes* root were analyzed in triplicate

**Table 5** Quantification of affinin in extracts of *H. longipes* root by UV-Vis spectrophotometry and HPLC

| Extracts                | µg affinin/mg extract |               | Mean D±SD     | P value ( $p > 0.05$ ) | Bias (D) 95% | LoA*            |
|-------------------------|-----------------------|---------------|---------------|------------------------|--------------|-----------------|
|                         | HPLC                  | UV-VIS        |               |                        |              |                 |
| Ethanollic extract      | 585.48±7.163          | 576.49±8.000  | 580.98±6.354  | 0.062                  | +8.63        | (–12.42, 29.68) |
| Methanollic extract     | 245.37±0.695          | 260.47±11.789 | 252.92±10.679 | 0.147                  | –15.10       | (–37.05, 6.85)  |
| Dichloromethane extract | 354.70±3.088          | 344.45±17.589 | 349.58±7.250  | 0.285                  | +10.25       | (–8.32, 28.82)  |
| Overall                 |                       |               |               |                        | +1.26        | (–26.31, 28.83) |

\*LoA (Lower, Upper)

using the validated UV-Vis spectrophotometric method. The results, summarized in Table 6, provide an estimation of the affinin concentration in commercially available products, ensuring the applicability of the method for routine quality control.

### AGREE and agreeprep analysis

The greenness assessment showed clear differences between the two analytical procedures. In the AGREE evaluation, the UV-Vis method achieved a higher global score (0.66) than HPLC (0.53), reflecting the score difference is mainly driven by solvent selection/toxicity, renewability and waste

generation (criteria 10–11 and 7), while energy-related contributions are comparable (criterion 9). Similarly, the AGREEprep analysis indicated a markedly higher score for UV-Vis (0.72) than for HPLC (0.38), consistent with the fewer operational steps and lower use of consumables in the UV-Vis workflow (Fig. 4).

### Discussion

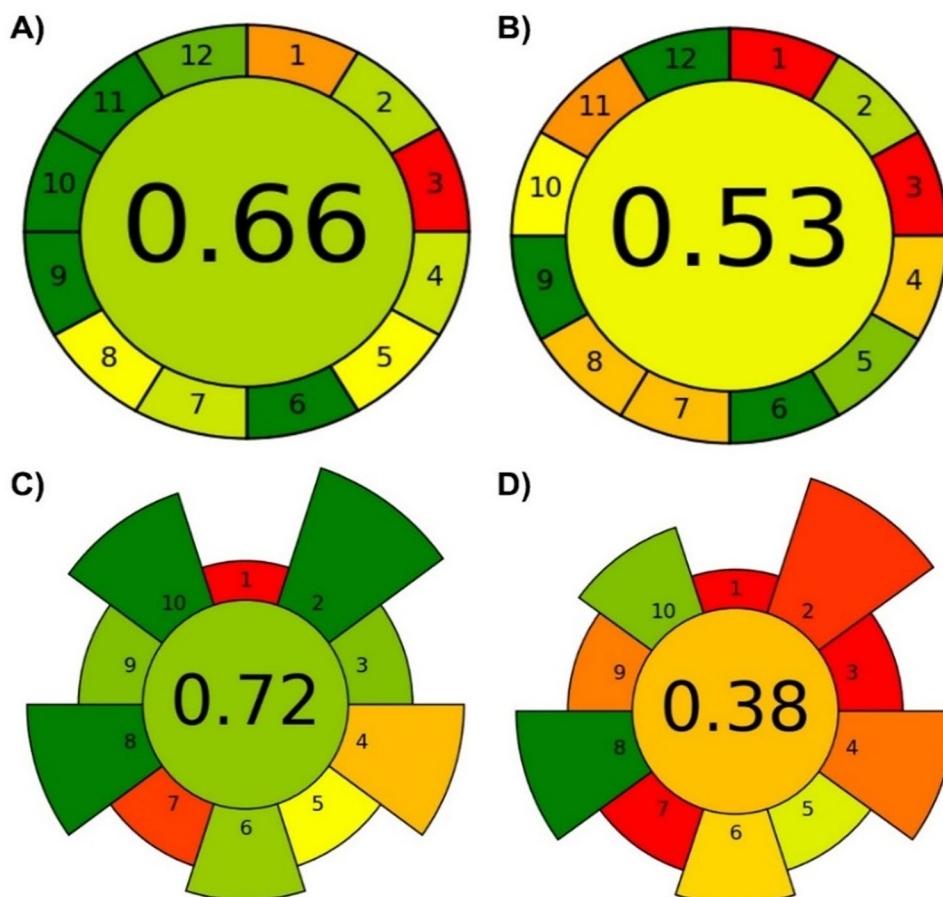
The UV-Vis spectrophotometric method developed for affinin quantification in *H. longipes* extracts showed that the selected wavelength ( $\lambda_{\text{max}}$ ) enables specific and accurate measurements under the experimental conditions. A single, well-defined absorption band was observed at 230 nm, coinciding with the  $\lambda_{\text{max}}$  of affinin (Fig. 1). This absorption maximum is consistent with experimental spectroscopic studies of spilanthal (affinin), the same alkaloid reported from different botanical sources, which describe a strong UV-Vis absorption band with a maximum excitation at 230 nm attributed to  $\pi \rightarrow \pi^*$  electronic transitions of its conjugated double-bond system [29].

The coincidence of  $\lambda_{\text{max}}$  between the pure standard and the extract is consistent with measurements performed in

**Table 6** Affinin content in commercial topical/buccal formulations

| Formulation   | Type of Formulation  | Affinin ( $\mu\text{g/mL}$ ) | % w/w               |
|---------------|----------------------|------------------------------|---------------------|
| Formulation 1 | Topical spray        | $1334.05 \pm 20.449$         | $0.169 \pm 0.003$   |
| Formulation 2 | Topical/Buccal spray | $103.15 \pm 6.457$           | $0.0131 \pm 0.001$  |
| Formulation 3 | Topical spray        | $4.09 \pm 0.709$             | $0.00052 \pm 0.000$ |
| Formulation 4 | Topical/Buccal spray | $111.67 \pm 2.361$           | $0.0141 \pm 0.000$  |
| Formulation 5 | Buccal spray         | $183.07 \pm 9.770$           | $0.0232 \pm 0.001$  |

**Fig. 4** AGREE and AGREE-Prep scores from the analytical determination of affinin **(A)** UV-Vis AGREE scores. **(B)** HPLC AGREE scores. **(C)** UV-Vis AGREEPrep scores. **(D)** HPLC AGREEPrep scores. The numbered segments (1–12) represent performance for each criterion, and the color code indicates compliance level (green = higher, yellow = intermediate, red = lower). Meaning of each numbered criterion: sample treatment, sample amount, device positioning, sample preparation stages, automation, miniaturization, derivatization, waste, analysis throughput, energy consumption, source of reagents, toxicity, and operator's safety



the same solvent (absolute ethanol), where  $\pi \rightarrow \pi^*$  transitions of conjugated alkamides are not expected to undergo significant solvatochromic or bathochromic shifts in the absence of strong specific solute–matrix interactions. In addition, the absence of relevant secondary absorption signals in the 250–400 nm region suggests a low contribution of other UV-active chromophores in this range. This behavior is consistent with the previously reported chemical composition of the ethanolic extract, which is dominated by alkamides ( $\approx 45\%$ ), mainly affinin, with minor proportions of triterpenes and sterols [30] that typically exhibit weak or no UV absorption [31–33].

The molar extinction coefficient of affinin in ethanol ( $\epsilon = 2.851 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ) reflects a strong capacity for UV absorption, characteristic of molecules containing conjugated double bonds. This magnitude is comparable to that reported for other natural compounds with extended  $\pi$ -electron systems, such as piperine ( $\epsilon \approx 3.1 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$  in ethanol), whose UV absorption is likewise governed by allowed  $\pi \rightarrow \pi^*$  transitions. This magnitude is consistent with general photophysical principles linking conjugation length to absorptivity and supports the analytical sensitivity of the proposed method [34]. Comparable UV–Vis behavior has also been reported for other alkamides and structurally related capsaicinoids, such as capsaicin, which exhibit well-defined UV absorption bands and molar absorptivities on the order of  $\epsilon \approx 3.4 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$  in organic solvent [20]. In general, the molar absorptivity of organic chromophores increases with the degree of conjugation and the presence of electron-donating substituents, as observed for flavonoids and other phenolic compounds that typically exhibit  $\epsilon$  values in the range of  $2\text{--}3 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$  [35]. Therefore, the obtained  $\epsilon$  value is consistent with the structural features of affinin and support the reliability of UV–Vis spectrophotometry for its quantification in plant extracts. From a practical analytical perspective, this relatively high molar absorptivity enables measurable absorbance signals at low analyte concentrations, enhancing method sensitivity and supporting reliable quantification within the concentration range required for routine quality control applications [36].

It should nevertheless be acknowledged that UV–Vis spectrophotometry inherently lacks chromatographic separation, therefore, absolute specificity cannot be demonstrated in the same manner as with separation-based techniques. However, under the experimental conditions employed, the predominance of affinin as the main UV-absorbing compound at 230 nm, together with the absence of relevant secondary absorption signals and the results of the standard-addition assessment, indicates that potential matrix interferences are not analytically significant. Furthermore, the identity and purity of affinin were independently confirmed by HPLC analysis of the reference standard (Figure

S5), providing orthogonal chromatographic support to the spectrophotometric specificity assessment. Consequently, the UV–Vis approach can be considered fit-for-purpose for routine affinin quantification in *H. longipes* extracts and commercial formulations.

The method exhibited good linearity within the concentration range of 0.5–10  $\mu\text{g/mL}$  ( $R^2 = 0.998$ ). In addition to the high coefficient of determination, low relative prediction errors were observed, as reflected by the RRMSE (3.30%) and MAPE (4.22%), indicating that the linear model provides reliable quantitative performance across the evaluated concentration range [18, 37]. Both intra-day and inter-day precision were below 2% RSD. Accuracy was confirmed with recovery values within the acceptable range, while the LOD and LOQ indicated sufficient sensitivity for routine analysis. Method specificity was evaluated using a standard addition approach, comparing the absorbance at the affinin  $\lambda_{\text{max}}$  (230 nm) between spiked samples and the theoretical sum of the individual absorbances. An equivalence interval of  $\pm 0.01$  absorbance units was defined based on acceptable validation limits for UV–Vis methods. The mean difference between experimental and theoretical values was 0.0037 AU, and the 95% confidence interval for this difference (0.0000–0.0046 AU) was entirely contained within the predefined equivalence limits. The TOST analysis confirmed equivalence ( $p = 0.001$ ), indicating the absence of relevant matrix interference at the selected wavelength.

Due to the absence of an affinin-free matrix, absolute accuracy against an independent reference method could not be established solely from this experiment; however, the relative accuracy of the UV–Vis method was further supported by its agreement with HPLC results obtained for the same samples. Overall, these findings confirm that the method is reliable for the quantitative determination of affinin in both extracts and commercial formulations. Furthermore, compared to more sensitive techniques such as HPLC, UV–Vis offers a simple, cost-effective, and rapid alternative, particularly suitable for laboratories with limited analytical infrastructure.

The comparison between UV–Vis and HPLC for the quantification of affinin in ethanolic, methanolic, and dichloromethane extracts showed no significant differences between the methods, confirming the validity of the UV–Vis method across solvent with different polarity. This consistency, supported by statistical comparison and method agreement analyses, demonstrates that UV–Vis provides quantitatively comparable results to HPLC for affinin determination. Consequently, UV–Vis can be considered a reliable alternative for routine quality control, maintaining concordance with validated chromatographic methods while reducing analytical complexity and cost.

Beyond its analytical performance, the validated UV–Vis spectrophotometric method developed in this study has important implications for the sustainable valorization of *H. longipes*, an endemic Mexican medicinal plant whose bioactivity and commercial relevance are strongly linked to the presence of affinin. In this species, affinin is not only the main bioactive compound responsible for analgesic [7, 38], anti-inflammatory [5, 39], and antihypertensive effects [8, 30], but also a reliable chemical marker for quality control and standardization of extracts and derived products [10]. Therefore, the availability of a simple and accessible method for its quantification represents a critical enabling factor for the responsible use and commercialization of *H. longipes*-based products.

Within the analytical framework of this study, the use of ethanol as solvent represents a rational choice consistent with phytopharmaceutical matrices, while offering favorable safety and environmental attributes for routine analytical applications. Accordingly, the selection of ethanol for UV–Vis measurements was guided by both physicochemical and practical considerations. For instance, affinin is an alkamide with a conjugated system that permits a UV absorption primarily governed by  $\pi$ – $\pi^*$  electronic transitions. Such transitions are sensitive to solvent polarity and hydrogen-bonding capacity through solvatochromic effects that may influence the position and stability of absorption bands [40].

Beyond its spectroscopic suitability, ethanol offers advantages from a sustainability and laboratory safety perspective, as it can be produced from renewable biomass and is associated with a lower hazard burden in routine laboratory settings. In contrast, acetonitrile commonly used in HPLC analyses is petrochemical-based and requires stricter risk management due to its toxicity, volatility, and complete water miscibility [41, 42].

The AGREE and AGREEprep analyses highlight the advantages of the UV–Vis method over HPLC from a green analytical chemistry perspective, providing relative, normalized indicators (0–1 scale) of the environmental performance of each workflow. In this study, these tools are used as comparative, method-level metrics to evaluate differences in solvent toxicity, renewability, waste generation, and operational simplicity within the laboratory context, and should not be interpreted as full life-cycle sustainability assessments [27]. The 0.13-point (AGREE) and 0.34-point (AGREEprep) higher scores obtained for UV–Vis indicate a lower environmental burden per determination, mainly driven by solvent selection and waste prevention. The UV–Vis workflow benefits from the use of ethanol as a potentially renewable, bio-based solvent, whereas HPLC is penalized by the use of acetonitrile. Under the reported HPLC conditions (1 mL min<sup>−1</sup> for 9 min), each run generates

approximately 9 mL of liquid waste, including ~ 4.5 mL of acetonitrile, while UV–Vis requires only 3 mL of ethanol per measurement. Consequently, replacing HPLC with UV–Vis would reduce solvent waste by ~ 6 mL per analysis, corresponding to ~ 6 L less total solvent waste and ~ 4.5 L less acetonitrile per 1,000 samples, for instance (Figures S1–S4).

Beyond these current metrics, an additional advantage of UV–Vis spectrophotometry relies on its potential for further greening through miniaturization of the measurement volume (e.g., microvolume cuvettes) and streamlined extraction procedures, which would further reduce solvent consumption and liquid waste [27]. In contrast, greener strategies for HPLC are primarily limited to reducing solvent throughput—such as shorter runs, lower flow rates, shorter or higher-efficiency columns, and avoidance of unnecessary heating—and, when feasible, switching to greener mobile phases or implementing solvent recovery systems [43]. Overall, UV–Vis spectrophotometry offers clear advantages for at-line or near-site implementation, enabling faster and more accessible routine analyses while supporting greener analytical practices through reduced solvent use, toxicity, and waste. Nevertheless, AGREE and AGREEprep assessments are restricted to laboratory-level impacts and do not capture full life-cycle sustainability considerations. For botanically derived analytes, broader aspects such as sourcing, traceability, and sustainable cultivation are also relevant. In this context, the validation of accessible analytical methods may support small-scale cultivation of *H. longipes* and contribute to local bioeconomies, although these aspects remain beyond the scope of the present study and warrant future investigation.

In regions where *H. longipes* is traditionally harvested and processed, reliance on chromatographic analyses performed in external laboratories further increases costs and limits the participation of small-scale producers, cooperatives, and local enterprises in formal value chains. By comparison, the UV–Vis method proposed here enables routine quantification of affinin using minimal sample preparation, ethanol as a bio-based solvent, and widely available instrumentation. This combination substantially reduces both environmental and economic burdens, lowering technical barriers to quality control and facilitating on-site or near-site evaluation of affinin content in raw materials, extracts, and finished formulations.

From a socio-economic perspective, reliable affinin quantification supports the transition of *H. longipes* from a predominantly wild-harvested medicinal resource toward a more structured and sustainable agro-industrial system. Moreover, by integrating green analytical chemistry principles—such as the use of ethanol, reduced solvent consumption, and simplified workflows—the proposed method aligns with broader sustainability goals in natural product research

[23]. When applied specifically to *H. longipes*, these methodological advantages support not only environmentally responsible analysis, but also the long-term conservation of the species by promoting controlled production, cultivation, and standardized commercialization pathways.

Regarding the analysis of commercial *H. longipes* formulations, it is noteworthy that none of the evaluated products declared standardized affinin content on their labels, indicating *H. longipes* only as the active ingredient. Additionally, no information regarding manufacturing dates or shelf life was available. The analysis revealed a wide variability in affinin content among the commercial formulations, ranging from 4.09 to 1334.05  $\mu\text{g/mL}$ , which constitutes one of the most relevant findings of the present study. This variability likely reflects the combined influence of multiple factors along the value chain. At the raw material level, differences in cultivation conditions, harvest timing, phenological stage, and root age are well known to affect the biosynthesis and accumulation of secondary metabolites in medicinal plants [44], and may therefore contribute to batch-to-batch heterogeneity in affinin content. The use of wild-harvested material rather than standardized cultivation practices may further amplify this intrinsic chemical variability.

Additional sources of inconsistency may arise from downstream processes, including differences in extraction efficiency, extract concentration, solvent-to-solid ratios, excipients, preparation methods, and storage conditions, as well as the intended route of administration (topical, buccal, or combined formulations). At the formulation stage, dilution practices, lack of standardized specifications, or suboptimal manufacturing controls may further reduce or alter affinin levels. This variability underscores the importance of standardized quantitative methods when affinin is used as a chemical marker for quality control, as reliable quantification is essential not only for regulatory compliance but also for ensuring product efficacy and consumer safety. Although affinin is generally considered safe at pharmacologically relevant concentrations, its reported  $\text{LD}_{50}$  of approximately 113 mg/kg in mice highlights the need for accurate determination of affinin content in extracts and commercial formulations to avoid both ineffective dosing and potential safety risks [45]. In this context, quantitative control of affinin content provides a necessary analytical basis for risk assessment and supports informed regulatory oversight.

The variability observed in the affinin content of commercial *H. longipes* formulations should be viewed not only as a quality control issue, but also as a matter of resource governance, transparency, and consumer protection. Limited access to reliable analytical verification can generate information asymmetries along the value chain, resulting in products with inconsistent or sub-therapeutic affinin levels.

This situation may disadvantage both local producers, who may be undervalued for high-quality plant material, and consumers, who may unknowingly purchase ineffective products. In this context, robust and accessible analytical methods, such as the validated UV–Vis spectrophotometric approach presented here, support transparency and equity by enabling routine verification of affinin content using widely available instrumentation. Consequently, affinin-based standardization contributes to fairer value distribution and the ethical commercialization of *H. longipes*-derived products, in line with emerging frameworks for sustainable resource governance [46, 47].

In comparison with chromatographic techniques such as HPLC, the UV–Vis spectrophotometric method shows several practical advantages, including simplicity, lower operational costs, shorter analysis time, and reduced solvent consumption. These characteristics make UV–Vis particularly suitable for routine screening and quality control applications, especially in laboratories with limited analytical infrastructure. However, unlike HPLC, UV–Vis spectrophotometry does not provide chromatographic separation and may therefore be more susceptible to matrix interferences in complex samples. In this regard, HPLC offers higher selectivity and sensitivity and remains advantageous for confirmatory analyses or regulatory purposes. Within this framework, UV–Vis and HPLC can be considered complementary rather than competing techniques, with UV–Vis serving as a rapid and accessible tool for routine analysis and HPLC providing confirmatory capability when higher analytical specificity is required.

## Conclusion

A simple, green, and cost-effective UV–Vis spectrophotometric method was developed and validated for the quantification of affinin in *H. longipes* ethanolic extracts and commercial topical and buccal formulations. The method showed satisfactory linearity, precision, accuracy, specificity, and sensitivity under the experimental conditions employed, in accordance with ICH Q2(R1) guidelines. From a sustainability perspective, the use of ethanol as a renewable, bio-based solvent, the low solvent consumption, minimal sample preparation, and reduced energy requirements contribute to a lower environmental footprint compared with chromatographic approaches. These features align the proposed method with the principles of green analytical chemistry, supporting its suitability for routine quality control while reducing analytical cost, solvent waste, and operational hazards.

Comparison with a previously validated HPLC method demonstrated good agreement between both approaches for

affinin quantification across different extracts, supporting the reliability of the UV–Vis method for routine determination of this marker compound. However, it is acknowledged that UV–Vis spectrophotometry lacks chromatographic separation and therefore cannot provide information on chemical purity or degradation products, for which chromatographic techniques remain essential.

Within these limitations, the proposed UV–Vis method represents a suitable screening and routine quantification tool for affinin in *H. longipes* extracts and commercial formulations, particularly in quality control settings where rapid analysis and reduced analytical costs are required. Thus, UV–Vis and HPLC should be regarded as complementary techniques, with the spectrophotometric approach offering a practical alternative for preliminary quantification and standardization, and chromatographic methods remaining indispensable for confirmatory and regulatory analyses.

## Future perspectives

The validated UV–Vis spectrophotometric method developed in this study offers several perspectives for future research and practical applications. It can be applied in breeding and selection programs to identify *H. longipes* populations or varieties with high affinin content, thereby supporting the development of standardized plant material. In industrial settings, the method may be used to optimize extraction processes through rapid monitoring of affinin yield under different processing conditions.

In addition, its simplicity, low cost, and reproducibility make it suitable for routine quality control in industrial and regulatory laboratories, particularly in contexts with limited analytical infrastructure. The method is also well suited for stability studies of extracts and commercial formulations, enabling the evaluation of affinin content during storage and shelf-life assessment. From a technological perspective, future work could explore partial automation of the analytical workflow and its adaptation to portable or miniaturized UV–Vis devices, which would further enhance throughput and facilitate on-site analysis. Overall, these applications extend the utility of the proposed method beyond routine analysis, supporting future standardization efforts and the sustainable use of *H. longipes*-derived products.

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**Author contributions** C.D.M.-H.: Methodology; Formal analysis; Writing—original draft preparation; C.I.-A.: Resources, Supervision; F.J.L.-V.: Methodology, Resources; Supervision; V.R.-C.: Methodology, Formal analysis; L.A.J.-O.: Investigation, Writing—original draft preparation, Supervision; D.M.-M.: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing—original draft preparation; A.R.-M.: Conceptualization, Investigation, Validation, Writing—review and editing, Resources, Supervision, Project administration, Funding acquisition. J.D.M.-M.: Validation, Writing—review and editing, Resources, Supervision. All authors have read and agreed to the published version of the manuscript.

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## Declarations

**Institutional review board statement** This study did not involve human participants or animal experimentation. In accordance with institutional requirements, the analytical protocol was reviewed and approved by the Bioethics Committee of the Faculty of Chemistry, Autonomous University of Querétaro (approval number: CBQ25/097).

**Conflict of interest** The authors declare no conflicts of interest.

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