Article

Rapid Determination of Clavulanic Acid in Submerged Cultures of *Streptomyces clavuligerus* as an Alternative Method for Bioprocess Monitoring and Control

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Abstract

Clavulanic acid (CA) is a β-lactamase inhibitor widely used in combination with β-lactam antibiotics to overcome bacterial resistance, and it is produced by *Streptomyces clavuligerus* through submerged fermentation. To ensure its industrial-scale production, it is essential to develop analytical methods that are rapid, reliable, and suitable for process monitoring. In this work, a UV–VIS spectrophotometric method was developed and validated for the quantification of CA in simulated fermentation matrices, employing ethyl acetate as the solvent for liquid–liquid extraction. The method demonstrated linearity in the range of 100–600 ppm ($R^2 > 0.98$), with coefficients of variation below 3% and recovery rates ranging from 90% to 110%. Clavulanic acid remained stable for up to 72 h under refrigeration, and complete transfer to the organic phase was achieved within 40 min. No significant interferences were detected, and the method showed adequate sensitivity (LOD: 60 ppm; LOQ: 100 ppm). These results confirm that the proposed method is a fast, cost-effective, and reproducible alternative to chromatographic techniques such as HPLC, representing a practical tool for routine bioprocess monitoring and quality control in the industrial production of clavulanic acid.

Keywords: clavulanic acid; *Streptomyces clavuligerus*; UV–VIS spectrophotometry; ethyl acetate extraction

1. Introduction

Throughout the past decades, the widespread and improper use of antibiotics has led to a concerning increase in bacterial resistance, which is currently considered one of the main challenges in modern medicine. Among the most common resistance mechanisms is the production of β-lactamases, enzymes capable of hydrolyzing the β-lactam ring present in antibiotics such as penicillin, rendering them ineffective and compromising conventional treatments. In response to this issue, scientific research and the pharmaceutical industry have focused their efforts on developing β-lactamase inhibitors that restore the activity of β-lactam antibiotics.[1] Among them, clavulanic acid (CA) has become one of the most effective and widely used. This compound belongs to the group known as clavam metabolites, which are naturally produced by the filamentous bacterium *Streptomyces clavuligerus* (S. clavuligerus). Its characteristic structure, composed of a β-lactam ring fused to an oxazolidine and with a 3R,5R stereochemical configuration, enables it to bind irreversibly to a serine residue in the active site of the β-lactamase,

thereby inactivating the enzyme. Owing to this property, clavulanic acid is combined with antibiotics such as amoxicillin to restore their effectiveness against resistant bacteria.[2]

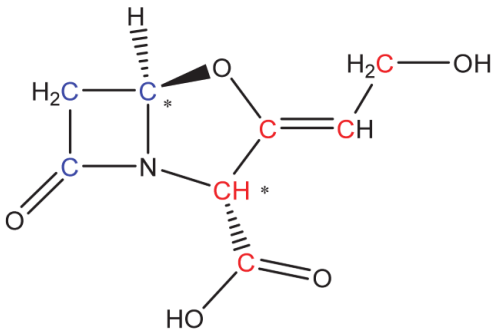

Figure 1. Chemical structure of clavulanic acid [3]

The industrial production of clavulanic acid is carried out through submerged fermentation processes using *S. clavuligerus* strains that utilize glycerol as the main carbon source[4]. However, the compound's instability in aqueous solutions and its susceptibility to degradation during handling make real-time monitoring difficult and reduce the efficiency of the production process [3,5]. In this context, liquid–liquid extraction has been established as an efficient strategy for separating clavulanic acid from fermentation broths. Several studies have shown that the use of water-immiscible organic solvents, such as ethyl acetate, enables effective recovery of the compound. According to Agudelo-Arenas et al. (2024)[4], single and double extraction with ethyl acetate demonstrated higher efficiency in recovering carboxylic compounds, due to the high affinity of clavulanic acid for this solvent. Moreover, the optimal extraction conditions were determined at a pH of 2.0 and a temperature of 10 °C, where the carboxylate group of clavulanic acid is protonated, decreasing its solubility in water and promoting its transfer to the organic phase. Compared to other solvents or mixtures, ethyl acetate increases the extraction yield by up to 30%, making it an ideal option for the recovery of clavulanic acid in fermentative systems.

Traditionally, the quantification of clavulanic acid has been carried out using high-performance liquid chromatography (HPLC), which is considered the reference method due to its high sensitivity and specificity [6,7]. However, its high operational cost, long analysis times, and the need for specialized personnel limit its routine application in industrial settings. Other developed methods, such as mass spectrometry [8,9]or liquid chromatography coupled with mass spectrometry (LC-MS/MS) [10,11], offer high accuracy but share the same limitations in terms of cost, complexity, and processing time.

In response to this, UV-VIS spectrophotometry emerges as a viable alternative due to its simplicity, speed, and low cost. This technique allows for the quantification of compounds based on their absorbance according to the Beer–Lambert law, and it is particularly useful in complex matrices when its selectivity, linearity, precision, and accuracy are properly validated [12](pp. 336-340). Pioneering studies, such as that of Bird et al. (1982)[13], demonstrated the possibility of determining clavulanic acid using a spectrophotometric method with good linear correlation, paving the way for its application in monitoring biotechnological processes.

In this context, the present study develops and validates a UV-VIS spectrophotometric method as a fast, cost-effective, and reliable alternative for quantifying clavulanic acid in simulated fermentation matrices, using ethyl acetate as the

extraction solvent. Based on the Beer–Lambert law, this approach aims to overcome the limitations of traditional chromatographic methods, whose high costs and long analysis times restrict their application in routine bioprocess monitoring[6,9,11]. It is proposed that UV-VIS spectrophotometry enables the acquisition of linear, precise, and reproducible results within a concentration range of 100 to 600 ppm, allowing its implementation in the analytical monitoring of *S. clavuligerus* cultures. The results demonstrate that this methodology provides a viable and efficient alternative for quantifying clavulanic acid, contributing to the development of more accessible and sustainable procedures for quality control in the pharmaceutical and biotechnological industries.

2. Materials and Methods

2.1 Reagents and solvents

The equipment used was a JASCO V-730 UV-VIS spectrophotometer, equipped with two 10 mm path length quartz cells, which were used for the measurement of the sample and the blank, respectively. For the quantification of clavulanic acid, potassium clavulanate was used as the sample. The components of the culture medium commonly employed for the growth of *Streptomyces clavuligerus* although in this case the biological stage was not carried out consisted, per liter of medium, of distilled water and the following compounds: glycerol (9.3 g/L), $(NH_4)_2SO_4$ (1.26 g/L), K_2HPO_4 (0.8 g/L), $MgSO_4 \cdot 7H_2O$ (0.72 g/L), $FeSO_4 \cdot 7H_2O$ (0.18 g/L), and trace elements solutions contained: H_2SO_4 (20.4 g/L), monosodium citrate·$1H_2O$ (50 g/L), $ZnSO_4 \cdot 7H_2O$ (16.75 g/L), $CuSO_4 \cdot 5H_2O$ (2.5 g/L), $MnCl_2 \cdot 4H_2O$ (1.5 g/L), H_3BO_3 (2 g/L), and $Na_2MoO_4 \cdot 2H_2O$ (2 g/L), all of analytical grade. The pH was adjusted to 6.8 through the addition of NaOH and HCl solutions at 4 M. Aeration was supplied at 0.6 VVM, while the system operated at a controlled temperature of 28 °C [5].

The dissolution of the drug in the culture medium was carried out using magnetic stirring plates to ensure complete homogenization. Subsequently, the liquid–liquid extraction of clavulanic acid was performed in 15 mL falcon tubes by adding ethyl acetate with a purity of 99.91% as the organic solvent. Phase separation was facilitated by centrifugation at 6000 rpm for the established time, using a Hettich EBA 200 centrifuge.

2.2 UV conditions

In order to determine the wavelength corresponding to the maximum absorption of clavulanic acid, a spectral scan from 200 to 600 nm was performed using the UV-VIS spectrophotometer. For this purpose, a standard solution of clavulanic acid with a concentration of 600 ppm from the calibration curve was selected, using ethyl acetate as the blank. The obtained spectrum showed a maximum absorption at a wavelength of 290 nm, a value that was selected and used in all subsequent analyses.

2.3 Preparation of solutions

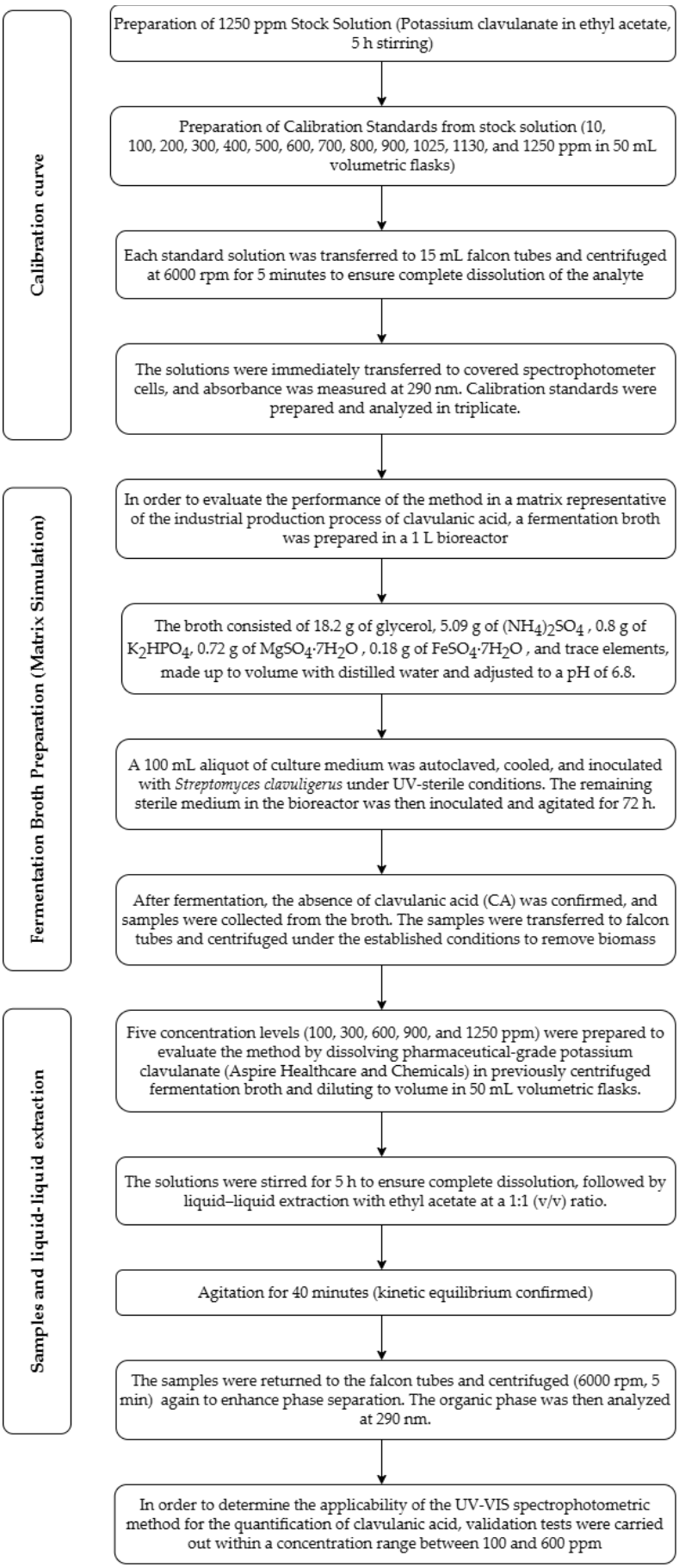

Figure 2. Flow diagram preparation of solutions

The extraction was carried out at a 1:1 ratio between the sample (fermentation broth) and ethyl acetate. Accordingly, 7 mL of each sample was transferred to 14 mL falcon tubes, followed by the addition of 7 mL of ethyl acetate. This stage allowed the separation of clavulanic acid from any other compounds present. Subsequently, the samples were centrifuged at the same previously established speed and time. Each resulting sample was then transferred to 25 mL volumetric flasks and kept under stirring for 40 minutes, simultaneously across all concentrations. This time was established based on kinetic studies conducted to evaluate the transfer of clavulanic acid into ethyl acetate. In these experiments, two concentrations (312.5 and 625 ppm) of clavulanic acid were prepared in 50 mL volumetric flasks using fermentation broth free of biomass as the solvent. These concentrations were selected because they are representative of the working range of the method, covering an intermediate and a high level, in order to confirm that the transfer time is consistent within the operating domain. The same procedure previously described was applied, following the established times and extraction with ethyl acetate, recording absorbance readings every 20 minutes until completing 2 hours. The results showed that, after 40 minutes, the clavulanic acid had been completely transferred to the organic phase; therefore, this extraction time was applied to all samples.

For the precision, accuracy, stability, sensitivity, and selectivity assays, the corresponding samples were prepared in 25 mL volumetric flasks following the same experimental procedure previously described. This procedure was kept constant to ensure reproducibility and comparability among the different validation assays. In addition, RStudio version from Posit 2024.12.1+563 was used to perform the statistical tests that could demonstrate that the analytical method employed is reliable, reproducible, and suitable for quantifying CA in fermentation broths

2.4 Validation procedure

2.4.1.Linearity and Range

Linearity is defined as the ability of the procedure to generate a response that is proportional to the concentration of the analyte within a given interval; the range corresponds to the set of concentrations over which such proportionality is demonstrated, with analytically evaluated and adequate levels of precision, accuracy, and linearity. [14]. For the evaluation of these parameters, a standard solution of CA was prepared and subsequently diluted in ethyl acetate; these dilutions were used to construct a thirteen-level calibration curve, obtained in triplicate through three independent preparations. The responses were recorded at the established wavelength. For the evaluation in a real matrix, *Streptomyces clavuligerus* broths were prepared and fortified at five concentration levels in mg/L (ppm). The samples were subjected to liquid–liquid extraction with ethyl acetate and measured in the same manner as in the calibration curve, also in triplicate. Linearity was evaluated through linear regression of absorbance versus concentration, with estimation of the slope, intercept, and correlation coefficient (R^2). The comparison among replicates was performed using an analysis of variance (ANOVA) of the model with the Conc*replicate interaction, to determine whether the slopes differed among replicates and whether there was any difference in intercepts. The validity of using linear regression was supported by verifying the following assumptions: Shapiro–Wilk for normality, Bartlett for homoscedasticity, Durbin–Watson for independence, and a t-test to confirm a zero mean of the residuals. Finally, to evaluate the influence of the matrix, a second model was fitted with the medium factor (standard vs. broth) and the Conc*medium interaction, from which the corresponding ANOVA was obtained. The working range was established as the interval of concentrations in which linearity was

demonstrated and compliance was achieved in subsequent analyses of precision and accuracy.

The comparison between replicates and evaluate the matrix effect were conducted using a linear model that accounts the effects of concentration, the effect of the comparison factor, and the concentration*factor interaction. The model is formulated as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 (X_1 X_2) + \varepsilon \quad (1)$$

Where Y is the measured absorbance, β_0 is the model intercept, β_1 is the overall slope, X_1 is the nominal concentration of CA (mg/L), X_2 is the comparison factor (replicate or average), β_2 is the replicate or average factor, β_3 is the concentration*factor interaction and ε is the random error.

2.4.2. Precision (Intraday/Interday)

Precision is understood as the degree of agreement among repeated measurements under defined conditions; repeatability evaluates intraday variation, while intermediate precision estimates the variation between series performed on different days under the same analytical protocol [11,14]. Precision was evaluated by determining the concentration of CA in analytical solutions, and measurements were carried out at a nominal level of 300 ppm, which is a representative value within the previously established range. On each working day, five independent samples were prepared, and each was measured in triplicate using the UV-VIS spectrophotometer, for a total of 15 readings per day. The procedure was repeated on three different days, keeping all measurement conditions constant. Using the daily average readings, the mean, standard deviation, and coefficient of variation were calculated, from which intraday repeatability was estimated. For intermediate precision, the means among days were compared using a one-way ANOVA with "day" as the factor, and the homogeneity of variances among days was verified using Bartlett's and Levene's tests.

2.4.3. Accuracy

Accuracy is understood as the closeness between the measured values and the true value of the analyte (AC), expressed as the percentage of recovery [14,15]. It was evaluated using three different concentrations (150, 300, and 450 ppm), prepared on three different days. Each sample was measured in triplicate under identical preparation and UV-VIS reading conditions. The percentage of recovery was calculated as a measure of accuracy, and a two-factor ANOVA (day and concentration) with interaction was applied to the recovery percentage (%R) in order to detect dependence on concentration, possible systematic differences between days, and potential changes in the effect of concentration.

2.4.4. Stability

The stability of clavulanic acid (CA) in the solvent was evaluated to verify whether the samples could be stored under refrigeration without significant loss of the analyte. For this purpose, sample 2 from the first day of the precision study was selected, stored under refrigeration (2–8 °C), and measured in triplicate at 24, 48, and 72 hours, reproducing before each reading the treatment established in the centrifuge. The response was expressed as the calculated concentration and as the percentage of recovery (%Rec) relative to 300 mg/L. In order to evaluate stability throughout storage, an ANOVA with the factor "day" was applied to the %Rec to test the equality of means among the different times. In addition, for each day, the mean, standard deviation, and %CV were estimated

to compare temporal variability with short-term precision as an operational reference for stability [14]. Finally, Bartlett's and Levene's tests were applied to verify the consistency of dispersion among days.

2.4.5. Selectivity

Selectivity is understood as the unequivocal ability to measure the analyte of interest in the presence of other substances that may interfere with the measurement [14–16]. It was evaluated by comparing the response at the working wavelength between matrices without analyte (placebos) and matrices containing the analyte, obtained from the precision study. Six placebo samples were prepared on three different days, and each was measured in triplicate under identical conditions. The absence of interference was verified using a two-sample t-test (responses with analyte vs. placebos) and a one-sample t-test applied to the placebos (mean vs. 0) with a 95% confidence interval, in order to confirm that the blank signal was indistinguishable from zero.

2.4.6. Sensitivity

The sensitivity of the method was defined as the slope of the calibration curve that relates the instrumental response to the concentration of clavulanic acid, obtained during the linearity assessment with the standard. This slope was adopted as the sensitivity parameter [14,15,17].To visually estimate the limits of detection (LOD) and quantification (LOQ), understood as the minimum detectable amount and the minimum reliably quantifiable amount, a series of solutions covering the lower concentration limit and the operational range was prepared. From the data obtained, a graph was generated to determine the LOD and LOQ values [14,15].

3. Results

3.1. Method

During the validation of the UV-VIS spectrophotometric method, a systematic bias of overestimation was identified in the measured concentrations of clavulanic acid. This behavior was observed in both standard solutions and real matrices and is attributed to the combined effect of the instrumental response of the method (method capability) and the nominal concentration declared by the standard (pharmaceutical-grade) used in the analyses. Despite this bias, it was determined that the method is reliable within a working range of 100 to 600 ppm in real matrices, where the overestimation remains constant and controlled, thus ensuring the reproducibility of the results [14,15,18]. Outside this range, the method exhibits certain limitations, such as the inability to detect low concentrations (<100 ppm) and a considerable overestimation at high concentrations (>600 ppm). Based on the aforementioned observations, the results of the different validation tests (precision, accuracy, stability, etc.) are interpreted considering the presence of this bias, prioritizing the evaluation of the method's reproducibility, consistency, and applicability within the validated range

3.2. Linearity and Range

The calibration curves obtained in the three replicates showed a proportional relationship between absorbance and analyte concentration, with highly consistent slopes and correlation coefficients (Figure 3). The analysis of variance revealed no significant differences between the slopes or the intercepts, confirming the reproducibility of the linear fit among replicates. The overall linear regression model showed a good fit (Figure

4) and met the assumptions of normality, homoscedasticity, independence, and zero mean of residuals, supporting the validity of the regression approach (Table 1). At the lower end, the deviation observed at 10 mg/L indicated that this point does not belong to the linear interval (Figure 4).

In the real matrix (broth), concentrations ranging from 100 to 600 mg/L remained within the prediction bands of the standard, while higher concentrations exhibited systematic deviations (Figure 5). The analysis, including the medium factor confirmed a matrix effect reflected in an increase in the slope for the broth, although no differences were observed in the intercept (Table 1). Based on this evidence, the results confirm the linearity of the method up to 600 mg/L and establish this interval as the operational range, supported by the concordance between graphical and statistical evidence.

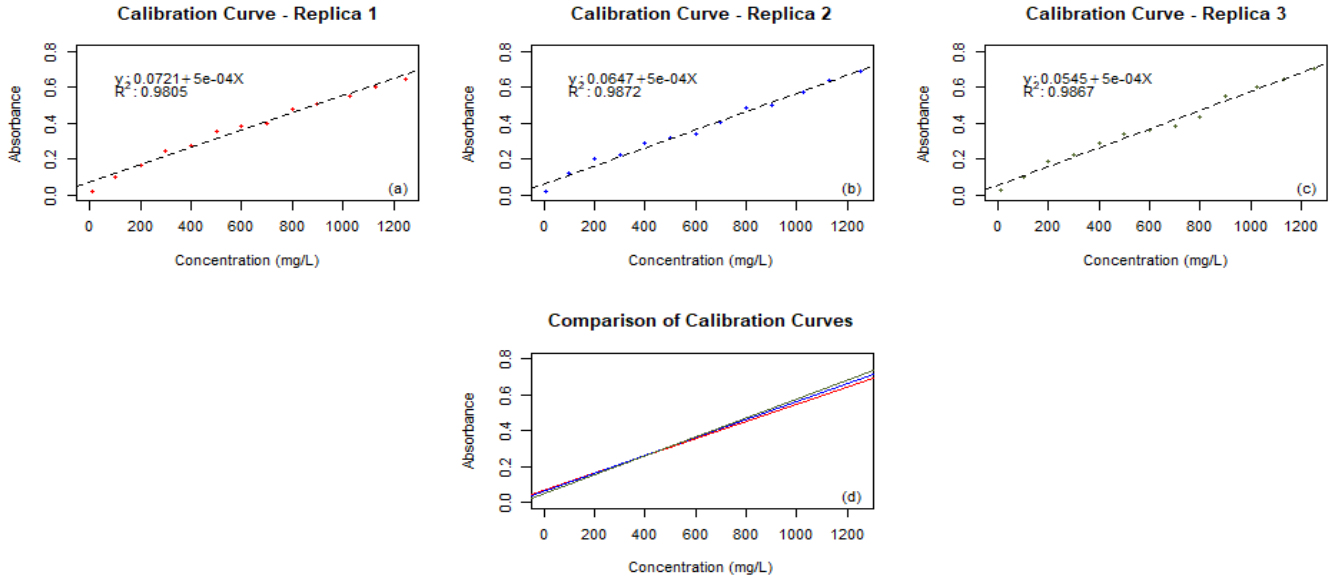

Figure 3. Calibration curve by replicate and combined integration. a) Abs vs. Conc scatter plot and linear fit for replicate 1. b) Same for replicate 2. c) Same for replicate 3. d) Superposition of the fits for visual comparison of slopes.

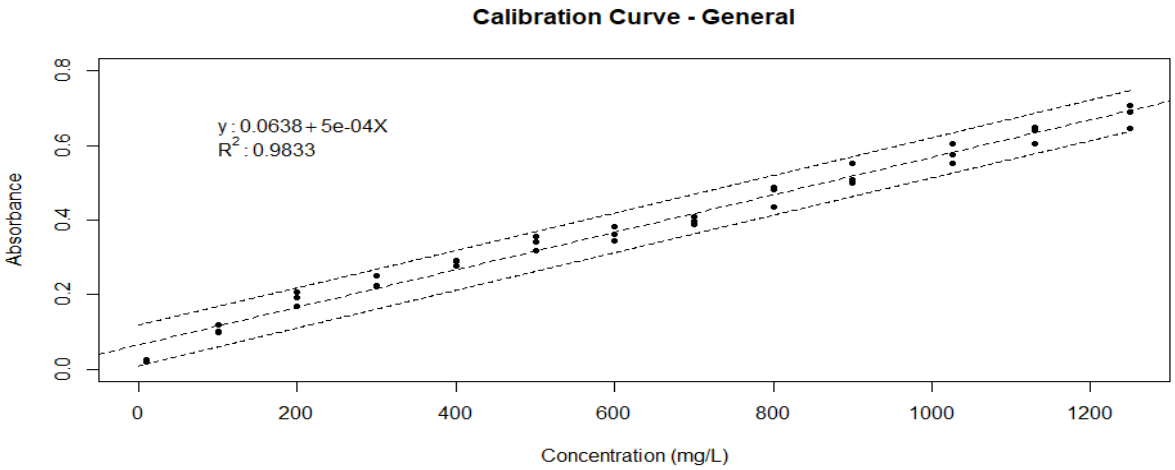

Figure 4. General calibration curve of the standard with 95% prediction bands for individual observations in the 10–1250 ppm range.

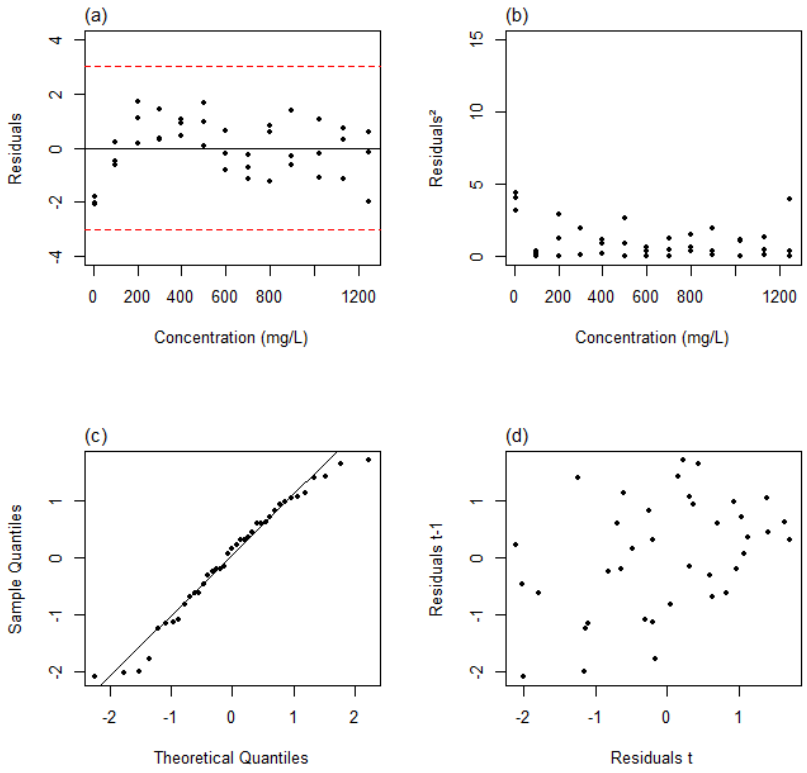

Figure 5. Validation of the assumptions of the linear regression model. (a) Zero mean; (b) Homoscedasticity; (c) Normality; (d) Independence.

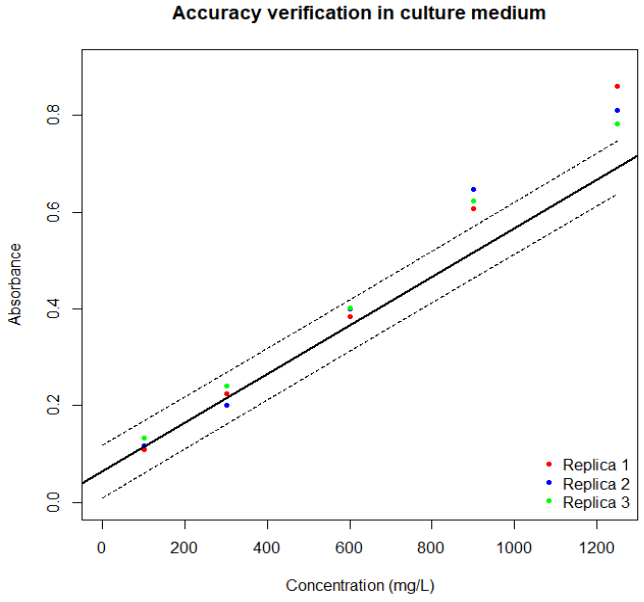

Figure 6. Verification in the real matrix versus the standard with fortified points between 100–1250 ppm, superimposed on the standard line and its 95% prediction bands; the higher levels lie above the upper limit, thus defining the working range.

Table 1. Comprehensive linearity assessment: calibration parameters, verification of assumptions, ANOVA and matrix impact.

Linearity			
General equation	**Range (mg/L)**	**LOD (mg/L)**	**LOQ (mg/L)**
y=0.06375x+0.0005028	100-600	60	100
Assumption testing			
Shapiro-Wilk	**Bartlett**	**Durbin-Watson**	**Zero mean (t-test)**
p=0.262	p=0.635	p=0.176	p=0.952
Analysis of variance (ANOVA) with replicates			
Difference in slope		**Difference in intercept**	
p=0.0955		p=0.352	
Effect of the matrix (broth) on sensitivity			
Interaction Concentration x medium interaction	**Standard slope**	**Broth slope**	**Difference in intercept**
$p=1.41\times10^{-7}$	5.029×10^{-4}	6.226×10^{-4}	p=0.178

3.3. Precision

The values of %CV 1.12, 1.21, and 1.52 were obtained for days 1, 2, and 3, respectively, demonstrating low variability and high intra-day repeatability. The analysis of variance indicated no significant differences between days and consistent inter-day precision. The homogeneity of variances across days was confirmed using Bartlett's test—appropriate under normality—and Levene's test centered on the median, which is more robust to deviations from normality; the lack of significance in both tests supports comparable variances between days. Overall, the results meet the acceptance criteria and confirm the precision of the method under the evaluated conditions (see Figure 7 for the graphical representation of the data dispersion).

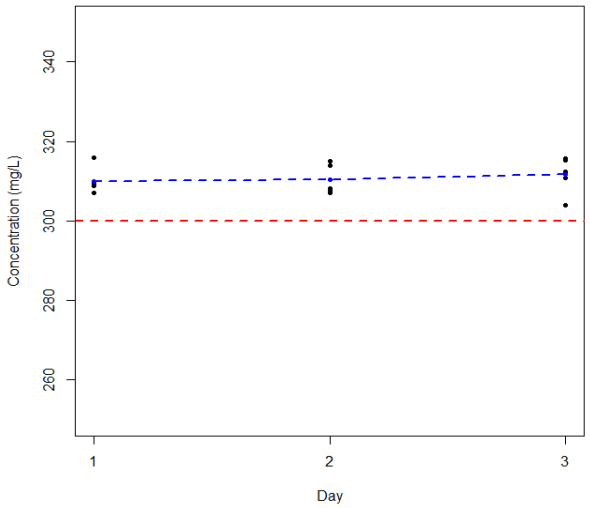

Figure 7. Distribution of determinations per day (n = 15) at 300 ppm; dashed lines: nominal level (red) and daily mean (blue). The similarity between days and the proximity of the means illustrate low intra-day variability and consistent inter-day performance.

Table 2. Method precision at 300 mg/L evaluated over three independent days.

Day	Mean ± SD (mg/L)	%CV
1	309.98 ± 3.48	1.12
2	310.43 ± 3.77	1.21
3	311.63 ± 4.72	1.52
Global tests		
ANOVA (Day factor)	Bartlett	Levene
p= 0.8016	p=0.8307	p=0.7958

3.4 Accuracy

The recoveries fell within the acceptance range of 90–110% for the three concentrations tested (Figure 8). The %recovery values remained between 102–109% with %CV ≤ 7% for all three test samples. The analysis of variance showed no effect of day on %recovery and no day * Concentration interaction, confirming interday reproducibility and consistent behavior across the experimental sessions (Figure 9). Conversely, a concentration effect was detected, reflected in a moderate increase in the mean value as the concentration increased, with a trend toward the upper limit of the acceptance range. Overall, the results support the accuracy of the method, showing low to moderate variability depending on concentration and no dependence on the day of evaluation.

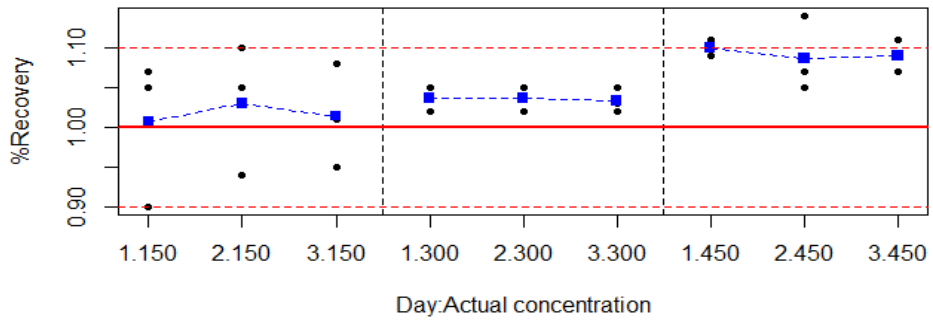

Figure 8. Recovery by concentration and day. Individual observations and mean values per concentration are shown, with a red 100% reference line and red dotted acceptance limits.

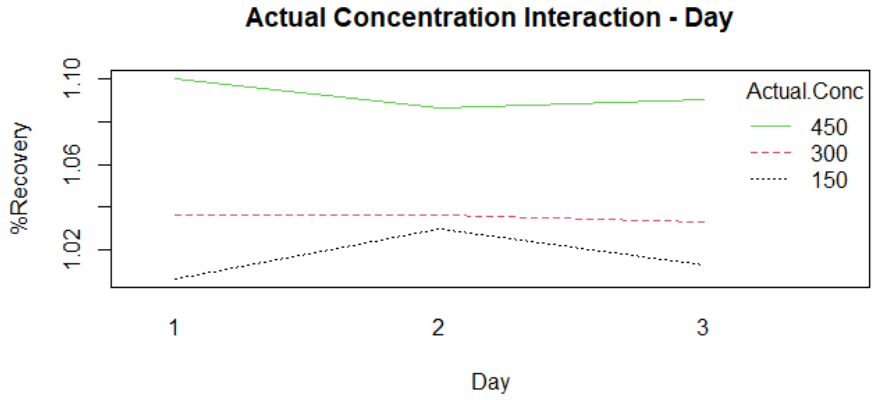

Figure 9. Recovery: concentration × day interaction; stable curves across days and a moderate trend with concentration.

Table 3. Accuracy by concentration level (50, 100, and 150% ppm) and ANOVA global contrasts.

Concentration (mg/L)	Mean ± SD (mg/L)	%CV
150	1.02 ± 0.07	6.86
300	1.04 ± 0.01	9.62x10-5
450	1.09 ± 0.03	2.75
CONTRASTS WITH ANOVA		
Day	Concentration	Interaction
p= 0.9729	p=0.0141	p=0.9806

3.5. Stability

After 72 hours of storage under refrigeration (2–8 °C), the results demonstrated the stability of AC in ethyl acetate (Figure 10). The ANOVA performed on the % recovery showed no significant differences among days (Table 4), and the mean values remained close to the nominal concentration of 300 mg/L, with recoveries around 102–103% and low variability (Table 4). Likewise, the variability of the measurements was similar across the three days, suggesting no appreciable degradation of the analyte during storage.

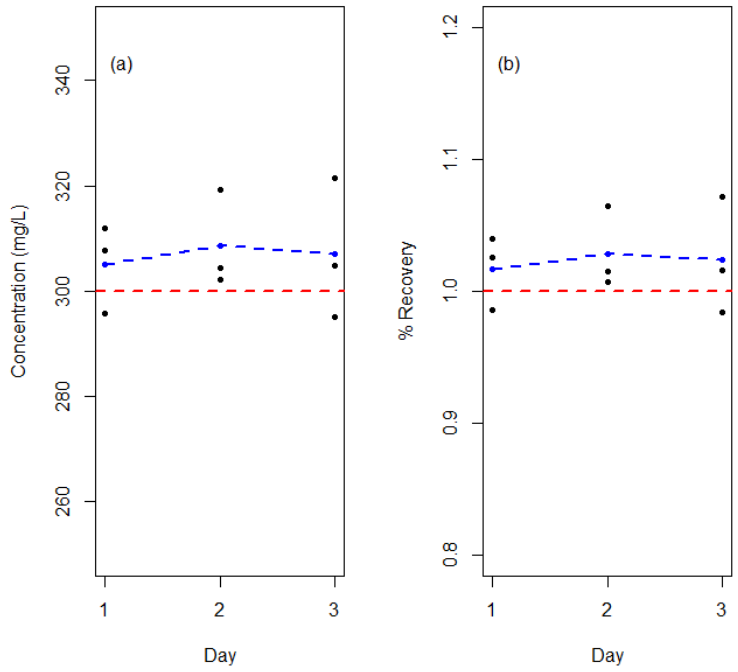

Figure 10. Stability under refrigeration over 72 h. a) Calculated concentration per day; b) % recovery per day.

Table 4. Evaluation of AC stability in ethyl acetate. Daily statistical summary of concentration, recovery, and overall tests for comparison between days.

Day	Conc mean (mg/L)	%Mean ± SD	%CV
1	305.15	102.0 ± 0.03	2.94
2	308.61	103.0 ± 0.03	2.91
3	307.15	102.0 ± 0.04	3.92
Global tests			
	ANOVA (day)	Bartlett	Levene
	p= 0.9226	p=0.8169	p=0.8367

3.6. Selectivity

In Figure 11, panel (a) shows the placebo samples close to the baseline, at approximately -77 mg/L, and the samples containing the analyte around 310 mg/L, with no overlap observed across the three days. Panel (b) reinforces this separation, with the "blank" and "Yes" boxplots displaying clearly distinct means and narrow dispersion. This graphical evidence was statistically supported by the Welch t-test between placebo and analyte-containing samples, indicating a marked difference between means, while the one-sample t-test on the placebo samples confirmed that their mean did not differ from zero. In summary, no positive signal attributable to the matrix or interferences at the working wavelength was detected, thus confirming that the method is selective.

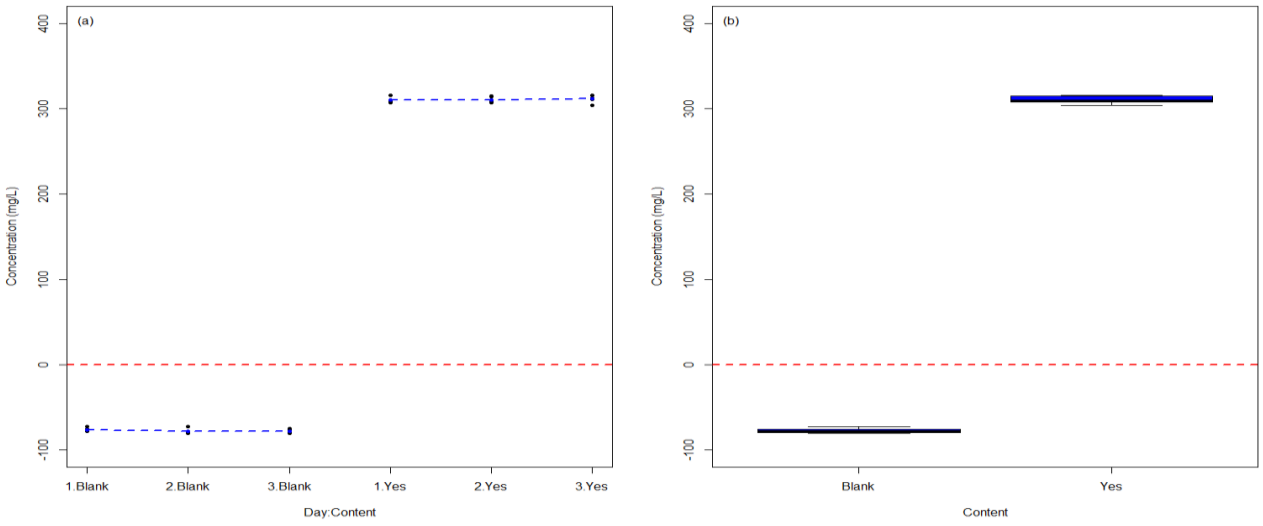

Figure 11. Selectivity evaluation. (a) Placebo samples close to the baseline and analyte-containing samples around 310 mg/L, with no overlap observed across days. (b) Boxplots confirming the separation between "blank" and "yes" samples.

Table 5. Statistical analysis of selectivity: comparison between analyte-containing samples and placebos.

	Test	
Value	**t-test of two samples (Welch´s t)**	**t of a sample (placebos)**
t	-343.01	-137.22
p	$<2.2\times10^{-16}$	$<2.2\times10^{-16}$
	Placebos	
Mean ± SD (mg/L)		**n**
-77.5 ± 2.4		**18**

3.7. Sensitivity

The slope obtained from the standard calibration curve reflects the method's ability to detect small variations in clavulanic acid (CA) concentration. The calculated slope (Table 1) indicates that the method exhibits high sensitivity for detecting CA concentrations within the evaluated range. Based on the calibration curve and the measurements at the lower limit of the concentration range (Figure 12), the LOD was established at 60 mg/L, as measurements at this concentration begin to differ significantly from the blank (placebo) readings. The mean and standard deviation of the blank are reported in Table 5, and and

its mean is shown as a red dotted line in Figure 12; thus the LOD and LOQ fall outside the normal dispersion of the blank, which reinforcing the separation between the blank and the detectable and quantifiable levels. Similarly, the LOQ was defined at 100 mg/L, since at this concentration the method begins to provide reliable quantification of the analyte, showing a stable relationship between the actual and calculated concentrations, closely following the calibration line.

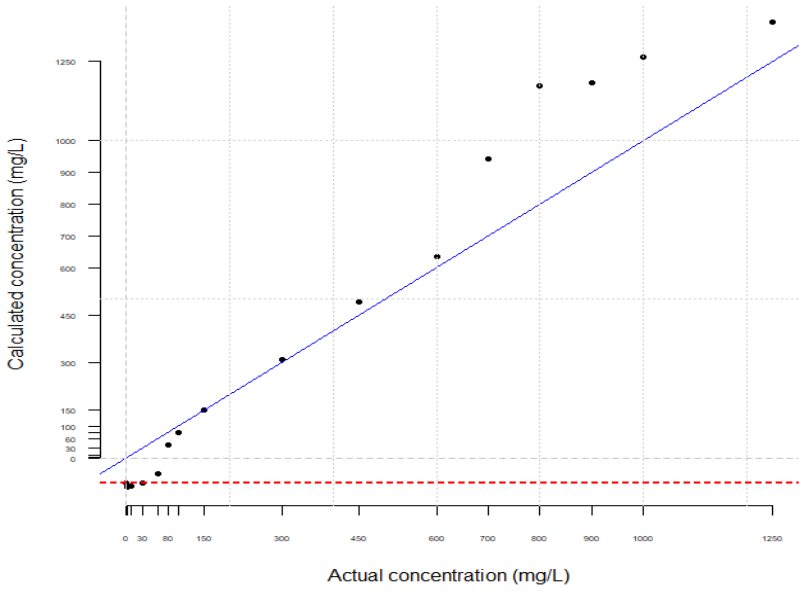

Figure 12. Relationship between actual and calculated concentration, with indicators of the LOD and LOQ based on observed values.

3.8. Kinetic

The kinetic study demonstrated that clavulanic acid reaches equilibrium in its transfer to the organic phase in approximately 40 minutes. After this time, the responses remained constant at both evaluated levels (312.5 and 625 mg/L) (Figure 13). Therefore, it is confirmed that 40 minutes of agitation, as an operational condition prior to extraction, ensures the complete migration of clavulanic acid (CA) from the real matrix (broth) into the ethyl acetate phase.

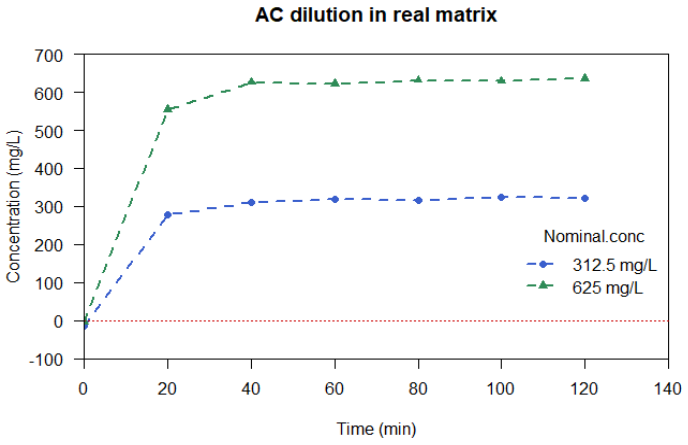

Figure 13. Time required for CA extraction in *Streptomyces* broths.

4. Discussion

UV-VIS spectrophotometry, coupled with liquid–liquid extraction using ethyl acetate, can be reliably employed to quantify clavulanic acid (CA) in real culture matrices when frequent bioprocess monitoring is required at low cost and with short response times. This positioning aligns with practices reported for in-process control, where operational speed and simplicity are prioritized over the higher structural specificity offered by chromatographic techniques such as HPLC or LC-MS.[14,15,19]. Within this framework, the results obtained show linear proportionality, low dispersion under repetition, closeness to the true value in the matrix, and sufficient temporal stability to ensure that the observed differences reflect real sample changes rather than degradation or instrumental variation. Furthermore, the method features well-defined detection and quantification limits established through visual evaluation, which clearly delineate the scope of the technique's applicability.

The response was linear between 100–600 mg/L with an adequate fit (Table 1), defining a working range in which the described procedure provides suitable analytical performance within the validated interval and maintains accuracy and precision consistent with the intended analytical use (Figure 6). Intraday and interday precision remained low throughout the range, with %CV values below 3%, a practical criterion accepted for chemical/spectrophotometric methods; this supports both the repeatability and intermediate precision of the procedure (Table 2) [19]. Likewise, recoveries in the real matrix ranged between 90–110%, showing moderate variation and no day effect (Table 3), in accordance with ICH recommendations for assessing accuracy at multiple levels (50, 100, and 150%) within the working range [14,15,19]. The combination of linearity, precision, and accuracy supports that the method reliably reports the AC content within the proposed range.

Practical selectivity was achieved through the transfer of AC to an organic phase, minimizing the contribution of co-extracts that absorb at 290 nm. The suitability of ethyl acetate is supported by the recovery of AC from broths, where its physicochemical properties correlate with extraction efficiency and interference reduction, and where phase ratios and solvent composition predictably influence yield and purity. [4,10,13].This behavior is consistent with previous reports indicating that solvents of intermediate polarity and low water miscibility, such as ethyl acetate, exhibit favorable partitioning of CA from complex matrices [20]. In complex matrices such as culture broth, where the direct UV signal can be affected by components that absorb in nearby ranges, this sample design transfers AC to a phase in which its free fraction dominates the absorbance at 290 nm and reduces the spectral background. For this reason, even without derivatization, the AC signal is predominant in the analyzed organic phase, with placebos remaining close to the baseline under the established conditions. The partition physicochemistry (distribution coefficient, effect of near-neutral pH, chemical form of clavulanate, temperature, and phase ratio) makes ethyl acetate a suitable vehicle to functionally isolate AC and, consequently, to provide the spectrophotometer with a simpler scenario in which the Beer–Lambert law holds with good approximation. The observed behavior is consistent with the theoretical framework that links solvent selectivity to its hydrophobicity and its ability to transfer β-lactam compounds into the organic phase [3,4,20]. In the same line, there is also a UV alternative that relies on derivatization with imidazole and measurement around 312 nm, which has been described as providing greater spectral selectivity. In this work, however, derivatization was intentionally avoided to preserve simplicity and short analysis times, achieving the required selectivity solely through liquid–liquid extraction [6,7]. Therefore, the selection of 290 nm for the measurement in the organic phase is consistent with the spectral profiles of AC, and if

necessary, it could be further refined through simple spectral adjustments without adding any complexity to the analytical design. [21]

Within this framework, the limits of detection and quantification (Table 1) were established through visual evaluation at the lower end of the response range (Figure 12), an alternative approach endorsed by the guidelines when the signal-to-noise ratio is not robustly applicable to the technique or instrument used [14,15]; the placement of these limits is consistent with the adopted range and clarifies from which level the signal becomes usable, without shifting the primary analytical weight that, in a quantification assay, relies on linearity, accuracy, and precision [14,15].

Regarding pre-analytical handling, the organic extracts of clavulanic acid remained stable for 72 hours under refrigeration, with no significant differences between readings (Table 4). This allows for the refrigeration of sample batches and the organization of runs by lots without loss of response, thus promoting operational reproducibility in future studies [14,19]. In the aqueous phase, the stability of CA depends on pH, ionic strength, and temperature; in fact, appreciable degradation of CA has been reported even at 4 °C within the first 36–54 h in fermentation supernatants. This difference reinforces the convenience of working under cold conditions and transferring the analyte early into the organic phase to minimize pre-analytical degradation; the experimental design combining liquid–liquid extraction with refrigeration stabilizes the response over time and minimizes pre-analytical degradation [5,22–25]. Evaluations in different real matrices, such as milk, have been demonstrated using HPLC–MS/MS, showing that the stability of clavulanic acid is strongly matrix-dependent and influenced by thermal conditioning, which aligns with the refrigeration of samples [26].

The kinetic evaluation showed that the response stabilized and remained constant after approximately 40 minutes. During the initial minutes, a concentration gradient develops between both phases, driving mass transfer; agitation increases the interfacial area and reduces diffusional resistance, causing the concentration of clavulanic acid (CA) in the organic phase to rise over time until the driving force is exhausted (Figure 13). Therefore, setting 40 minutes of agitation as an operational condition prior to measurement ensures complete transfer of CA to the organic phase and reduces variability in sample preparation. This behavior is consistent with the equilibrium times typically observed in liquid–liquid extraction, which depend on pH, phase ratio, and temperature [4,10].

The UV-Vis method enables high sampling frequency, simple preparation, and refrigerated storage of extracts without analyte loss, under operating conditions that stabilize the analytical phase (Figure 13). In practice, this means that the experimental design employed is highly efficient for processing multiple samples per day, with short and uniform preparation steps, avoiding lengthy chromatographic separations or derivatization. In process laboratories, these characteristics translate into short response times and more efficient resource utilization compared to complex techniques, particularly when the goal is to monitor the culture and adjust conditions in real time [3,15]. Compared to validated RP-HPLC methods, which offer higher specificity through separation and substantially lower quantification limits, the proposed UV-VIS workflow stands out as a rapid and cost-effective alternative when extreme sensitivity is not a primary requirement and the goal is to obtain timely operational decisions [11].

The scope of the method under the working conditions covered the range of 100–600 mg/L in the real matrix using liquid–liquid extraction with ethyl acetate and readings at a wavelength of 290 nm. The main limitations are the inherent spectral selectivity of UV-

Vis in highly complex matrices and the dependence on transfer time; nevertheless, within this range, the method yields reliable and reproducible results. To consolidate and expand its use in future evaluations, it is recommended to extend the robustness by assessing the effects of pH, percentage of organic components in the extraction, temperature, and time; to perform calibration in multi-batch matrices to strengthen longitudinal accuracy; to carry out a direct comparison with a validated HPLC method in the same matrix; and finally, to explore simple spectral treatments (baseline correction and derivatives) without compromising the simplicity of the workflow [10,13,15,18].

5. Conclusions

The quantification of clavulanic acid by UV–VIS spectroscopy, coupled with liquid–liquid extraction using ethyl acetate, is established as a simple, rapid, and reproducible alternative for routine monitoring in real culture matrices. The results obtained in this study support its analytical suitability within the defined working domain and demonstrate its usefulness for informing process decisions that require short response times and controlled costs. The standardized transfer step prior to reading stabilizes sample preparation and reduces variation before analysis, while the stability observed in refrigerated extracts provides logistical flexibility to work in batches when convenient, without being a mandatory condition of the procedure. Overall, the method offers a solid balance between performance, simplicity, and operational feasibility, making it suitable for bioprocess control applications within the framework established by this study.

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