Evaluation of separation alternatives of clavulanic acid produced in *Streptomyces clavuligerus* cultures

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Dedication

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Abstract

Clavulanic acid (CA) is a soft β-lactam antibiotic with a strong inhibitory effect on β-lactamase enzymes, which confers resistance to bacteria against several known broad-spectrum antibiotics. CA is produced by the filamentous Gram-positive bacterium *Streptomyces clavuligerus* (*S. clavuligerus*) as a secondary metabolite. The production of CA at industrial scale is traditionally carried out in agitated tank bioreactors, where the mode of operation has proven to be a determining factor for obtaining high product yields.

The selection of CA separation processes is based on the physicochemical properties of the product and fermentation broth. Separation is generally carried out by solid-liquid separation operations, liquid-liquid extraction, and precipitation in the form of clavulanate. Although the metabolic restrictions to reach high CA titers are the first difficulty in the CA production process, downstream processing is compromised by the degradation of CA molecule in aqueous solution presumably by following a hydrolysis reaction mechanism catalyzed by both, acid and alkaline media.

In this research work, the extraction of CA from the fermentation broth of *S. clavuligerus* cultures was carried out at laboratory scale. For this, two separation operations were selected: liquid-liquid extraction and adsorption. For the case of liquid-liquid extraction, two experimental designs were developed aimed at finding the best conditions of the process; for the first design, temperature and pH of the

broth were selected as the controlled variables, and CA extraction yield as the response variable. With the best conditions found, a second experimental design was developed; the control variables were the ratio between the aqueous phase/extraction agent (ethyl acetate), and the CA load in the fermentation broth. For the case of adsorption, the ion exchange resin IRA 400 was selected. For this process the same experimental design, used in the previous strategy, was carried out, for comparison purposes. The optimal contact time between phases (20 min) and the ratio of the pretreatment solvent (ether) for the purification process (ratio of 0.6) were also studied experimentally. Samples (300 μL) from the aqueous phase were taken for analytical purposes. The obtained samples were derivatized with imidazole for 30 min and subsequently filtered at 0.20 μm. Finally, the samples were placed into vials for subsequent HPLC analysis. CA loss was minimized at 10 °C and pH 2.0. The adsorption was favored by increasing the adsorbent to liquid ratio. Thus, the highest separation was attained in the range of 40-45% solid/liquid ratio, adsorbing a mean value of 47.7% of the CA present in the broth. Ethyl acetate was selected as the best alternative for extraction considering its low price and good performance. Further, high solvent to aqueous ratio (>2.0) allowed to extract up to 80% of the CA in the fermentation broth. The use of low volumes of organic solvent (ratios 0.2-0.6) led to 40% less CA extracted from the broth. Both liquid-liquid extraction and adsorption seem to be appropriate separation techniques for CA.

Keywords: *Streptomyces clavuligerus*; Clavulanic acid; separation processes; liquid-liquid extraction; adsorption; bioprocess.

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Symbols

Symbol	Term	Unit SI
R_a^2	Adjusted coefficient of determination	
S_{aq}	Aqueous phase	
$C_{CA-\mathrm{a}}$	Concentrations of CA after extraction	$\frac{\mathrm{mg}}{L}$
mg/mL	Concentration	
$C_{CA-\mathrm{b}}$	Concentrations of CA before extraction	$\frac{\mathrm{mg}}{L}$
g/L	Concentration	
$x_1\ and\ x_2$	Controlled variables	M
D	Distribution ratio	
L/h	Flow	
C_0	Initial concentration of clavulanic acid	$\frac{\mathrm{mg}}{L}$
Ph	Measure of acidity	
Mm	Micrometre	
S_{org}	Organic phase	
K_D	Partition coefficient	
$\hat{y}$	Percentage of CA extraction	%
%	Percentage	%
A	Predefined significance	
Rpm	Revolution per minute	rpm
℃	Temperature in Degree Celsius	℃
T	Temperature	K and ℃
T	Time	Min
V_0	Volume of the initial aqueous phase	L
w/v	Weight/volumen	

Subindexes

Subindex	Term
Aq	Aqueous
CA-a	Concentrations of CA after extraction
CA-b	Concentrations of CA before extraction
C	Culture broth
O	Initial
Org	Organic
D	Partition
T	Total

Superindexes

Superindex	Term
O	Degree
N	Exponent, power

Abbreviations

Abbreviation	Term
ATCC	American Type Culture Collection
ANOVA	Analysis of variance
ARG	Arginine
CCRD	Central composite rotatable design
CA	Clavulanic acid
GSPA	Culture medium
GAP	GTPase activating protein
HPLC	High performance liquid chromatography
ISP	Isolate soy protein

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Introduction

Infectious diseases remain the second leading cause of death in the world. The massive use of commercial antibiotics in health, agriculture and livestock activities is an important factor in the development of bacterial resistance that decimates the effectiveness of conventional antibiotic treatments (De Lima *et al*., 2012). For lactam-type antibiotics, their effectiveness has been significantly reduced by the action of bacterial β-lactamases, which catalyze the hydrolysis of the β-lactam ring producing an acid compound lacking antibacterial activity (Barcelona *et al*., 2008).

In recent years, there has been an increase in resistant bacterial infections (Casellas, 2011; Pérez *et al*., 2011); antibiotic β-lactam combinations with β-lactamase inhibitors remain the only means to combat such infections (Barcelona *et al*., 2008; Drawz & Bonomo, 2010). There are some variants of antibiotic formulations with β-lactamase inhibitors: amoxicillin-clavulanate, ticarcillin-clavulanate, ampicillin-sulbactam, piperacillin tazobactam, cefoperazone-sulbactam, avibactam, relebactam and vaborbactam (Drawz & Bonomo, 2010; Werth, 2022; Zhang *et al*., 2024).

Amoxicillin-CA medications are widely used in the treatment of respiratory infections, which rank as the fifth leading cause of death in the country (Rios *et al*., 2011); Additionally, they are also employed in the management of enteric infections (Saudagar *et al.*, 2008). Amoxicillin-CA (Augmentin®) is the fourth most sold formulation in the antibiotic market (Business Insights Ltd., 2011). CA is a soft β-lactam antibiotic with a strong inhibitory effect on β-lactamase enzymes, which confers resistance to bacteria against several known broad-spectrum antibiotics.

The demand for formulations with CA is sustained by epidemiological and demographic factors, which constitute one of the main revenues in the antibiotic industry (Business Insights Ltd., 2011) considering that antibiotic drugs with CA are between 350% and 450% more expensive than those that do not contain it.

CA is produced by the filamentous Gram-positive bacterium *S. clavuligerus;* it is a secondary metabolite derived from arginine and glycerol metabolism (Gómez-Ríos *et al*., 2019; Ramirez-Malule, 2018; De Araujo *et al*., 2018). The production of CA at industrial scale is traditionally carried out in agitated tank bioreactors, where the mode of operation has proven to be a determining factor for obtaining high product yields (Saudagar *et al*., 2008).

The performance of the CA production process must be evaluated not only by considering the cultivation process and strain improvement strategies, but also regarding the recovery process from the culture medium. The selection of separation processes is based on the physicochemical properties of the product and fermentation broth. In the case of CA, separation is generally carried out by solid-liquid separation operations, liquid-liquid extraction, and precipitation in the form of clavulanate (Yepes *et al*., 2020; Ser *et al*., 2016). Although the metabolic restrictions to reach high CA titers are the first difficulty in the CA production process, downstream processing is compromised by the degradation of the CA molecule in aqueous solution, presumably by following a hydrolytic reaction mechanism catalyzed by both, acid and alkaline media (Bersanetti *et al*., 2005; Carvalho *et al*., 2009; Gómez-Ríos *et al*., 2019; Marques *et al*., 2009). Degradation of CA is considered because of susceptibility of its molecular structure to nucleophilic attacks at specific points; it operates as a hydrolytic mechanism in two steps: equilibrium reaction, in which CA produces an active intermediate followed by irreversible reaction of this intermediate, with an additional CA molecule to form the degradation product (Gómez-Ríos *et al.,* 2019). As a consequence, CA increases its own degradation rate when increasing its concentration in the

fermentation broth. This decomposition factor makes an efficient recovery necessary to ensure the productivity and feasibility of the process.

In addition to its instability, recovery of CA from fermentation broths is limited by the mass transfer phenomena, which might cause a product loss as high as 70% in techniques such as adsorption, liquid-liquid extraction, and two-step precipitation stages (López *et al*., 2021; Gómez-Ríos *et al*., 2019; de Mancilha *et al*., 2014; Brites *et al*., 2012; Carvalho *et al*., 2009). Therefore, CA production is costly due to low product yields in both, fermentative production and subsequent separation and precipitation stages for obtaining a stable active compound.

Some studies related to CA degradation and recovery from fermentation broths have been developed in our research group. Yepes *et al*. (Yepes *et al*., 2020) explored the use of ionic exchange resins (Amberlite IRA 400) in the recovery of CA from the fermentation broth. Gomez-Ríos *et al*. (Gomez-Ríos *et al*., 2019) determined the degradation kinetics of CA at different temperatures in fermentation broths of *S. clavuligerus* cultivated in chemically defined media, revealing a possible two-steps hydrolytic mechanism involving irreversible and equilibrium reactions, and showing a direct dependence of degradation rate with concentration.

In this work, the extraction and separation of CA from the fermentation broth of *S. clavuligerus* cultures at laboratory scale were studied. Two strategies were implemented from two separation procedures; the first strategy consisted in the development of a liquid-liquid extraction process; for this, two response surface experimental designs were developed to find the best operating conditions. The first was a central composite rotational design (CCRD) where the temperature and pH of the broth were taken as controlled variables; the CA extraction yield was selected as the response variable. With the best conditions found, a new experimental design was developed, in which the control variables were the liquid ratio of the aqueous phase-extraction agent (ethyl acetate) and the CA load in the fermentation broth.

The second strategy consisted in articulating two separation techniques: solid-liquid adsorption with liquid-liquid extraction. For the first approach the ion exchange resin IRA 400 was used. The optimal contact time between phases and the solvent ratio of pretreatment for the purification process were also studied. The pretreatment process was carried out with the objective of eliminating impurities such as amino acids and proteins, which can affect the yield of CA recovery.

1. Problem statement, Hypothesis and Objectives

1.1 Problem statement

Despite its clinical importance and implications for public health, CA has limited availability in the market, due to its high cost, which derives from low yields, and the complexity of the production process (Business Insights Ltd., 2011; Saudagar *et al*., 2008); Lopes & Badino, 2015; Saudagar *et al.,* 2008).

CA yield is also compromised by its degradation in aqueous solutions. The CA released to the fermentation broth typically exhibits a fast decline in concentration, during the first 5 h, followed by a slower but stable reaction rate in the subsequent hours (Gómez-Ríos *et al*., 2019). This reaction rate is dependent on several factors like pH, medium composition, and temperature.

Further, the high costs associated to the separation/purification operations reveal the need for developing economic alternatives for CA recovery. Therefore, it is necessary to implement strategies that reduce the rate of degradation, thus promoting its accumulation and subsequent recovery. In this research, two distinct operations are poised for implementation: firstly, the adsorption of CA through the utilization of the IRA 400 ion exchange resin within the recovery process, and secondly, the application of a liquid-liquid extraction system.

1.2 Hypothesis

The outcomes obtained from the implementation of these separation techniques would serve as the foundation for formulating a separation strategy at the laboratory scale, enabling the achievement of consistent and stable recovery yields of CA from the fermentation broth.

1.3 General objective

To evaluate the performance of different recovery alternatives for separation of CA (or clavulanate salt) from supernatants of *S. clavuligerus* cultivations.

1.4 Specific objectives

- To select and standardize the recovery process of CA in shake flasks that allows obtaining acceptable and stable levels of CA.

- To evaluate the performance of the selected separation strategies in terms of product yield and associated costs.

- To determine optimal operation conditions for the separation strategy that allows to obtain acceptable and stable levels of CA considering the technical and economic feasibility of implementation.

2. Theoretical framework

2.1 CA as secondary metabolites produced by *S. clavuligerus.*

CA is a lactam-antibiotic independently discovered by Brown and Napier in 1976 and 1981, respectively, as a secondary metabolite produced by *S. clavuligerus* (Li *et al*., 2006; Saudagar *et al*., 2008; Forte *et al*., 2011). The commercial interest for CA is given by its ability to inhibit the activity of β-lactamase enzymes, being commonly used in antibiotic formulations for antibiotic resistant-bacterial infections (Saudagar *et al*., 2008; Costa *et al*., 2015; Ser *et al*., 2016).

The *S. clavuligerus* microorganism is a gram-positive filamentous bacterium belonging to the subclass of the actinomycetes. *S. clavuligerus* was first isolated in 1971 in a strain screening program for producers of lactam compounds resistant to lactamases (Baptista-Neto *et al*., 2000; Martínez-burgo *et al*., 2014; Saudagar *et al*., 2008). Initially, *S. clavuligerus* was selected as a strain that produces the antibiotic Cefamycin C; however, years later, CA, a metabolite of great interest, was discovered, see Figure 1 for its chemical structure (Saudagar *et al*., 2008; Marquez *et al*., 2009; Costa *et al.,* 2012).

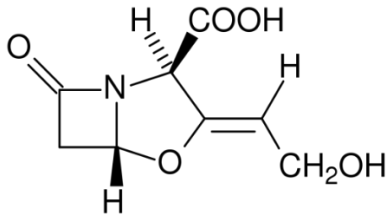

Figure 1. Chemical structure of the CA molecule (Saudagar *et al*., 2008).

2.2 The commercial interest of CA as an inhibitor of β-lactamase enzymes

CA has become a pharmaceutical product of high clinical and commercial value, available in combinations with β-lactam antibiotics such as amoxicillin, tetracycline and as a generic product (Jiang, 2016). The demand of formulations with CA is supported by epidemiological and demographic factors; these formulations constitute one of the main incomes in the antibiotic industry (Business Insights Ltd., 2011; Yepes *et al*., 2020), considering that they are between 350% and 450% more expensive than those that do not contain it. Formulations with CA are widely used in the treatment of respiratory infections, e.g., pharyngitis, bronchitis, and pneumonia, enteric infections and some sexually transmitted diseases (Business Insights Ltd., 2011).

2.3 CA biosynthesis

Previous studies have elucidated most of the anabolic and catabolic pathways involved in CA biosynthesis (Arulanantham *et al*., 2006). The precursors in the biosynthesis of CA come from glycolysis and the urea cycle, having, as an initial stage, the condensation reaction between the amino acid ARG and the glycolytic intermediate GAP to produce N2-(2-carboxyethyl) arginine. Through different reactions N2-(2-carboxyethyl) arginine is transformed into clavaminic acid, which in turn is the precursor of CA and other compounds known as clavams 5S (Arulanantham *et al*., 2006; Ramirez *et al*., 2016; Sánchez, 2013). The structural

similarities between CA and clavams reflect shared elements of a common biosynthetic pathway (Tahlan *et al*., 2007). The steps shared in the biosynthetic pathways are called “early stages”, which lead to the last intermediate, clavaminic acid, while the specific stages, used to produce clavams or CA are called “late stages” (Jensen, 2012). The known reactions of the clavam pathway are summarized in Figure 2.

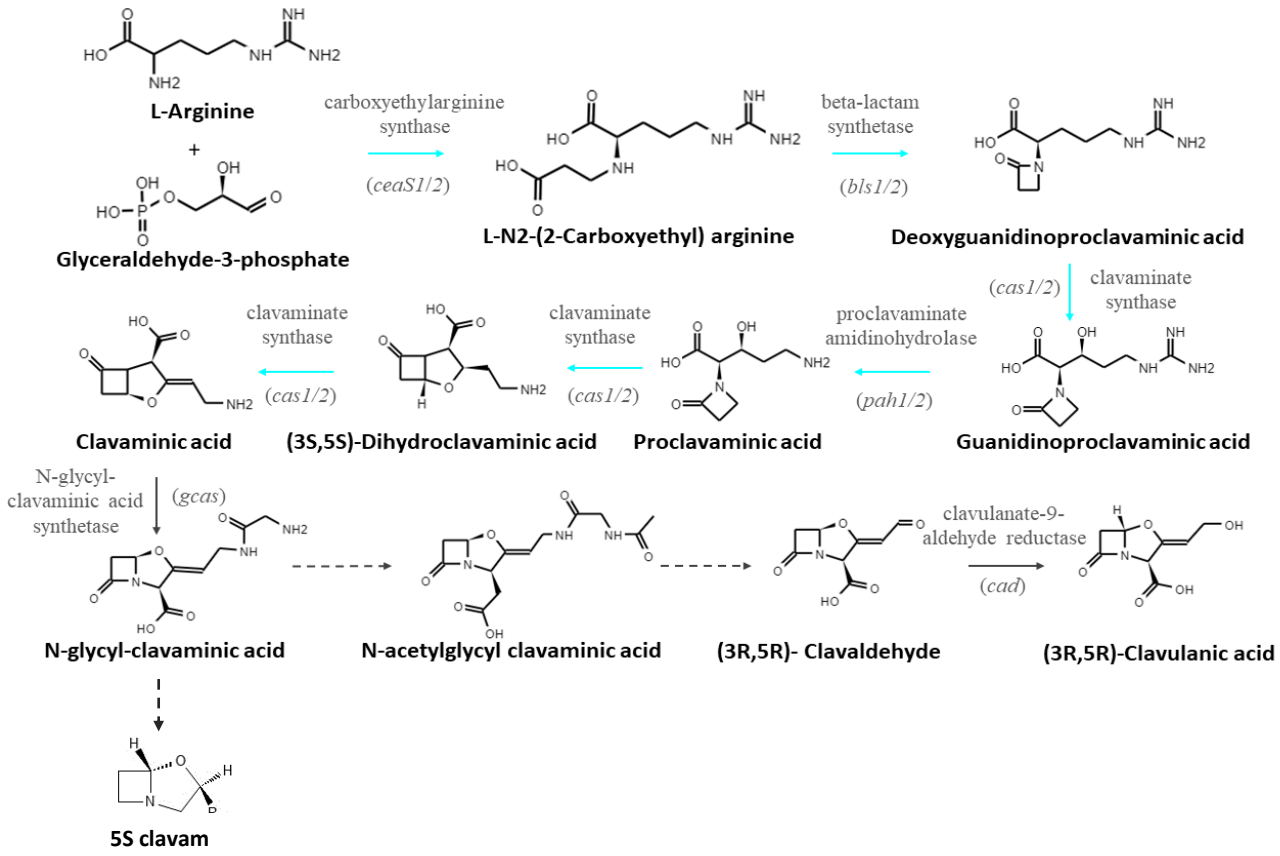

Figure 2. Summary of the CA biosynthetic pathway in *S. clavuligerus* (Arulanantham *et al*., 2006; Sanchez, 2012; Gómez *et al*., 2021).

However, given the low productivity of wild type strain, the metabolic, genetic, nutritional, environmental, and/or operational factors, related to CA yields, are still a matter of study (Ser *et al*., 2016). The production of CA at industrial scale is traditionally carried out in agitated tank bioreactors, where the mode of operation has proven to be a determining factor for obtaining high product yields (Business

Insights Ltd., 2011; Saudagar *et al*., 2008). The productivities of these operations typically oscillate around 1.5 g/L (Bushell *et al*., 2006; Rosa *et al*., 2005; Ser *et al*., 2016; Teodoro *et al*., 2010). In previous studies, some metabolic, nutritional, environmental, operating and recovery factors have been identified that impact CA production. This has allowed exploring production alternatives in batch, fed batch and continuous processes, seeking to obtain better yields (Bellão *et al*., 2013; Bushell *et al*., 2006; Costa *et al*., 2015, 2012; Ives & Bushell, 1997; Neto *et al*., 2005; Rosa *et al*., 2005; Sanchez *et al*., 2012; Saudagar & Singhal, 2007; Ser *et al*., 2016; Teodoro *et al*., 2010). The performance of the CA production process is not only a matter of study about the cultivation process and improvement of the producing strain, but also with regard to the process of recovering of the product from the culture medium.

Depending on the culture medium composition and fermentation operating conditions, it has been possible to obtain yields around 1700 mg/L of CA using the wild-type strain. *S. clavuligerus* preferably uses glycerol as carbon source; the inclusion of glycerol enhances the production of CA as compared to other carbohydrates (e.g., starch), as glycerol provides a higher energy content on a weight-by-weight basis (Ser *et al*., 2016). Furthermore, culture media formulations include a nitrogen source to provide adequate amino acid concentrations. The choice of soybean flour or isolate soy protein in complex media, significantly improves CA production. Pinilla *et al*. (Pinilla *et al*., 2018), reported an increased CA production for media containing soy protein isolate (698 mg/L) rather than soybean flour (338 mg/L) (Ser *et al*., 2016). Works published by Gouveia *et al*. (Gouveia *et al*., 1999) using soybean flour or soy protein isolate as substrates at different levels, showed that CA levels up to 920 mg/L could be achieved using a fermentation matrix based on soy protein isolate (27 g/L), glycerol (20 g/L) and peptone (10 g/L), and CA values of 472 mg/L, using soybean flour (80 g/L). Moreover, Ortiz *et al*. (Ortiz *et al*., 2007), achieved an increase in 360 mg/L of CA by replacing soy protein isolate (10.9 g/L) with soybean flour (20 g/L).

However, the influence of the primary nitrogen source (amino acids), as well as salt composition and other metabolites present in the culture media, can negatively affect CA biosynthesis. For instance, the presence of glutamate in GSPG medium negatively influence CA production. In the biosynthetic pathway in *S. clavuligerus*, glutamate can be converted into 2-oxoglutarate, which enters the citric acid cycle (Da Silva *et al*., 2018). The conversion is catalyzed by a glutamate dehydrogenase and releases ammonium, which inhibits CA biosynthesis, hence explaining the negative influence of glutamate on CA production (Da Silva *et al*., 2018).

CA is separated from the fermentation medium in several steps. These steps may involve a variety of standard techniques such as filtration and centrifugation for cell separation, followed by extraction and/or adsorption techniques for subsequent antibiotic purification. One of these methods is direct extraction using an organic solvent, whereby CA is transferred to the organic phase and subsequently purified. Chromatographic adsorption techniques can also be used with ionic or nonionic adsorbers (Mayer *et al*., 1996; Mayer *et al*., 1997; Barboza *et al*., 2002; Barboza *et al*., 2003; Gullo *et al*., 2006). These methods are the best choice for low molecular weight compounds present in diluted solutions, as it is the case of antibiotics produced by fermentation (Hirata *et al*., 2009).

2.4 Separation of CA from fermentation broths

The selection of separation processes is based on the physicochemical properties of the product and fermentation broth. In the case of CA, separation is generally carried out by solid-liquid separation operations, liquid-liquid extraction, and precipitation in the form of clavulanate (Yepes *et al*., 2020; Ser *et al*., 2016). The liquid-liquid extraction operation, also known as solvent extraction, is the separation of the components of a solution by contact with an immiscible liquid that preferentially dissolves one of the constituents of the original solution, giving rise to the appearance of two layers of immiscible liquids of different densities (Pereira *et al*., 2012). In a simple liquid-liquid extraction the solute partitions itself between two

immiscible phases. One phase usually is an aqueous solvent, and the other phase is an organic solvent. Because the phases are immiscible, they form two layers, with the denser phase on the bottom. The solute initially is present in one of the two phases; after the extraction, it is present in both phases (Pereira *et al*., 2012; Marquez *et al*., 2012; Carvalho *et al*., 2011).

Considering the merit of its low cost and effectiveness, solvent extraction is by far the most widely used separation technique for antibiotics (Soto *et al*., 2005). Several patents describe the extraction of CA onto organic solvents (Cole *et al*., 1978; Cardoso, 1998; Capuder, 2000; Cook & Nicola, 2001; Ruddick, 1996; Simon 2001). Solvent extraction from cold clarified culture medium adjusted to acid pH, and methods utilizing the anionic nature of CA at neutral pH such as the use of anion exchange resins, have been found to be particularly useful for isolation of CA (Saudagar *et al*., 2008). A further useful method is to form an ester of CA, purify the ester and regenerate the acid or its corresponding potassium salt. This salt is produced by reacting CA with potassium 2-ethylhexanoate in the solvent rich phase; the resulting clarified broth is acidified to reach pH values between 2 and 3. Suitable acids used to lower the pH include hydrochloric, sulphuric, nitric, or phosphoric acids (Carvalho *et al*., 2011). The CA is extracted using organic solvents such as ethyl acetate, n-butanol, methyl isobutyl ketone, n-butyl acetate, and others, and the salt is formed. Moreover, due to the very low concentration of CA in the fermentation broth, in which it is produced, the separation stage of the overall antibiotic production process accounts for a large share of the total manufacturing cost (Bersanetti *et al*., 2005; Brites *et al*., 2012; Mancilha *et al*., 2014).

Further, Cook *et al*. (Cook *et al*., 1981) and Cook and Wilkins (Cook & Wilkins., 1995) used a purification process in which a solution of impure CA in an organic solvent is exposed to t-butylamine to form the t-butylamine salt of CA, which is then isolated, thereby separating the CA from impurities remaining in the organic solvent. The salt is then converted back to CA or into a derivative of CA such as an alkali metal salt or an ester. Zhang and McKnight (Zhang *et al*., 2003) also reported a

similar process for the preparation of a pharmaceutically acceptable metal salt of CA. They extracted CA from aqueous fermentation broths into an organic solvent, converting it into an intermediate tertiary butylamine clavulanate at a pH below 6.0; the amine salt is finally converted into potassium clavulanate. Other known purification processes for CA involve the use of other organic amines such as diethylamine, tri-(lower alkyl) amines, dimethylaniline and N,N'-diisopropylethylenediamine to form salts and/or other derivatives thereof with CA. Cardoso (Cardoso *et al*., 1998) and Summer *et al*. (Summer *et al*., 1999) reported a process for the isolation of a pharmaceutically acceptable alkali metal salt of CA from the fermentation broth. The steps included filtration of the fermented broth, extraction of the CA in a water immiscible or partly water immiscible solvent at pH 1.2-2.0, and precipitation of the alkali metal salt.

Ethyl acetate, as the organic phase solvent, was chosen in the work of Brites *et al*. (Brites *et al*., 2012) as the best solvent to be used for the extraction of CA, present in the fermentation broth; the study considered the use of individual solvents, for which an extraction yield of 35.6% was obtained at an initial CA concentration of 161.9 mg/L. Mancilha *et al*. (Mancilha *et al*., 2014) conducted extraction experiments with ethyl acetate in order to obtain a better understanding of the influences of the variables involved in the extraction process using a single solvent. This information was used to further select the appropriate variables for the tests performed with mixtures of two solvents.

The use of ethyl acetate-rich mixtures with different combinations between solvents also resulted in extraction systems with different characteristics. For example, the system in which a mixture between methyl isobutyl ketone and ethyl acetate was used, allowed a higher extraction of CA from the fermentation broth (from 44.7% to 50.0%) compared to ethyl acetate alone (36.5%) (Brites *et al*., 2012). Therefore, ethyl acetate is used, in comparison with other organic solvents, as the solvent to work with, because of its physicochemical properties *i.e.*, solubility, polarity and

dielectric constant which allow for a good recovery performance of CA from the aqueous phase.

The extraction efficiency, that is the percentage of solute that moves from one phase to the other, is determined by the equilibrium constant for the solute's partitioning between the phases, and any other side reactions that involve the solute (Mancilha *et al*., 2014). A solute's partitioning between two phases is described by a partition coefficient, K_D. If we extract a solute from an aqueous phase into an organic phase:

$$S_{aq} \rightleftharpoons S_{org}$$

Then, the partition coefficient is

$$K_D = \frac{[S_{org}]}{[S_{aq}]} \qquad \text{[Equation 1]}$$

A large value for K_D indicates that extraction of solute into the organic phase is favorable. To evaluate an extraction's efficiency, we must consider the solute's total concentration in each phase (Mancilha *et al*., 2014), which we define as a distribution ratio, D.

$$D = \frac{[S_{org}]_{total}}{[S_{aq}]_{total}} \qquad \text{[Equation 2]}$$

The partition coefficient, and the distribution ratio, are identical if the solute has only one chemical form in each phase; however, if the solute exists in more than one chemical form in either phase, then, K_D and D usually have different values (Mancilha *et al*., 2014).

Solid-liquid separation operation

The separation of CA has been widely studied. Barboza *et al*. (Barboza *et al*., 2003) studied the influence of temperature on the adsorption and desorption of CA at laboratory scale, finding that the removal of CA is very favorable at thermal conditions between 5 and 10 °C, since the process of binding CA to the resin is exothermic. The study was carried out with the ion exchange resin Amberlite IRA 400 at four different temperatures. The authors showed that the desorption process is facilitated by high temperatures, since it increases the state of agitation of the molecules. Mayer *et al*. (Mayer *et al*., 1997) have studied CA adsorption using non-ionic and ion-exchange resins (XAD-4 and IRA 400) and found that, when the temperature was increased from 4 to 21 °C for both resins, a decrease occurred in the values of the effective diffusion coefficient. Chang *et al*. (Chang *et al*., 2006) carried out a study of lysozyme immobilization on NaY zeolites for temperatures ranging from 4 to 37 °C and found a decrease in the maximum adsorption capacity (q_m), and an increase in the dissociation constant (K_D).

Forte *et al*. studied the adsorption of CA at 10, 15 and 20 °C, respectively (Forte *et al*., 2011). The obtained equilibrium data was evaluated using a linear model, as well as by the non-linear models of Langmuir and Freundlich. The partition coefficient of the linear model (K) and the constant of the Freundlich model (K_F) were evaluated at different temperatures taking standard errors into account. The data arising from such adsorption studies were best described by the Langmuir model. However, although the parameters derived from this model failed to exhibit a well-defined trend, an increase in temperature did not favor the adsorption process. Bersanetti *et al*. (Bersanetti *et al*., 2005) demonstrated, through the fitting of CA disappearance data using the Arrhenius equation obtained at temperatures between 10 and 30 °C, that at high temperatures (30 °C) the half-life of CA was only 12.6 h, while at 10 °C it extends up to 48 h, suggesting that production systems should include strict temperature control to maintain product stability. Typically,

fermentations for CA biosynthesis are carried out at temperatures of 28 °C (Neto *et al*., 2005; Bellao *et al*., 2013; Gouveia *et al*., 2001).

Almeida *et al*. (Almeida *et al*., 2003) proposed a simultaneous adsorption/desorption system of CA using sequential stirred tanks, where the first tank operates at 10 °C with 2% NaCl and the second at 30 °C. It is important to mention that the use of sequential tanks and packing columns allows the recycling of the fermentation supernatants thus increasing the CA adsorption percentages. Hirata *et al*. (Hirata *et al*., 2013) studied the use of CA precipitation as the final step in the process of purification of CA from fermentation broths as an alternative to conventional methods traditionally employed. In the study, the authors used a stable intermediate (t-octylamine) between the conversion of CA and its salt form (potassium clavulanate), thereby enabling the resulting intermediate (amine salt of CA) to improve the purification process and to maintain the stability of the resulting potassium clavulanate. For the first reaction, five temperatures (6.60 to 23.40 °C), concentrations of CA in organic solvent (6.60 to 23.40 mg/mL) and t-octylamine inflow rates (0.33 to 1.17 drop/min) were selected, based on a central composite rotatable design (CCRD). For the second reaction, five temperatures (11.60 to 28.40 °C), concentrations of CA amine salt in organic solvent (8.20 to 41.80 mg/mL) and concentrations of potassium 2-ethylhexanoate (0.2 to 1.2 molar) were also selected using CCRD. From these results, precipitation conditions were selected and applied to the purification of CA from the fermentation broth, obtaining a yield of 72.37%.

Various studies have been developed in order to investigate the use of alternative adsorbents, organic as well as inorganic (Sanghi & Bhattacharya, 2002; Allen and Koumanova, 2005; Crini, 2006). Alternative adsorbents such as active carbon, hydrotalcites, zeolites and carbon molecular sieves, have been frequently proposed for the separation, purification and immobilization of biomolecules such as sugars, amino acids, antibiotics or enzymes (Forte *et al*., 2011; Chang *et al*. 2006; Hibino 2004; Boon *et al*. 2000). Mayer *et al*. (Mayer *et al*., 1996) studied diffusivity of CA in

different porous systems (Amberlite IRA 400, activated carbon and two ion-pair adsorption systems based on Amberlite XAD4 and activated carbon as matrixes and water-soluble quaternary ammonium salts as ion pairing substances) using batch adsorption experiments. The results described in their work enabled comparison between the different sorption systems tested for the purification of CA from supernatants. Forte *et al*. (Forte *et al*., 2012), studied Layered double hydroxides (LDHs), which are lamellar mixed hydroxides containing positively charged structural layers capable of anion-exchange. These layers consist essentially of divalent and trivalent cations and hydroxyl anions, while the interlayer domain of LDHs is mainly constituted of water molecules and anions (Goh *et al*., 2008).

Previous studies have used amberlite IRA 400 in the form of chloride, for the recovery of CA. This resin is composed of a basic polar group incorporated into a styrene-divinylbenzene synthetic copolymer matrix, with a particle size between 600 and 750 μm and a range of operation of pH between 1 and 14 (Ferrero, 2010). The amberlite IRA 400 can be used for the adsorption of negatively charged molecules, such as carbonates, silicates, sulfates, nitrates, phosphates, among many other molecules or atoms that contain excess of at least one electron in its structure (Khan *et al*., 2008). Therefore, the amberlite IRA 400 is one of the best options for adsorbing the CA fermentation product.

From the capacity of the resin to adsorb CA, the effect of variables such as the resin-supernatant ratio, the concentration of CA in the culture broth, the pretreatment of both the resin and the supernatant are significant in the recovery of CA in the broth. Adsorption takes place through a reversible reaction process that occurs until equilibrium is reached, where the rate of adsorption of CA is equal to the rate of desorption (Yepes *et al*., 2020). The mechanism of CA on the resin can be represented as:

$$Resin^{+}Cl^{-} + CA^{-} \leftrightarrow Resin^{+}CA^{-} + Cl^{-}$$

The purpose of establishing an adsorption kinetics study is to evaluate the adsorption capacity of the resin and to study the incidence of some process variables such as the change in the concentration of the supernatant and its incidence on CA degradation.

2.5 The difficulties of the CA separation process

CA or its salts may be extracted directly from the culture medium in various ways, but normally the cells of *S. clavuligerus* are first removed from the culture medium by filtration or centrifugation before such extraction procedures are commenced. Although the metabolic restrictions to reach high CA titers are the first difficulty in the CA production process, downstream processing is compromised by the degradation of the CA molecule in aqueous solution, presumably by following a hydrolysis reaction mechanism catalyzed by both, acid and alkaline media (Gómez-Ríos *et al*., 2019).

In addition to instability, recovery of CA from fermentation broths is limited by the mass transfer phenomena, which might cause a product loss as high as 70% in the adsorption, liquid-liquid extraction, and two-step precipitation stages (López-Agudelo *et al*., 2021; Carvalho *et al*., 2009; Gómez-Ríos *et al*., 2019). Therefore, CA production is costly due to low product yields in both, fermentative production and subsequent separation and precipitation stages for obtaining a stable active compound. Thus, it is necessary to implement strategies aimed to improve the CA recovery considering the degradation rate and the further efficiency of the process.

The difficulties in the purification process of CA arise from its high instability in aqueous solution, which limits its final yield (Bersanetti *et al*., 2005; Gómez *et al*., 2019). To obtain better CA yields in the separation processes, these usually include reactions that generate stable intermediates such as esters or amines derived from

CA, to then precipitate the clavulanate of sodium or potassium (Hirata *et al*., 2009). Although the formation of esters can generate good yields of the respective CA salt, the range of conditions in which this method can be applied is very narrow, which limits its industrial applicability (Hirata *et al*., 2009). The conversion to amines offers good yields and its implementation is less complex; however, for this procedure only amines, that are not toxic or hygroscopic, can be used (Hirata *et al*., 2013). Prior to the precipitation process, the alternative of using extractive fermentation as continuous extraction method of CA by adsorption with zeolites and ion exchange resins, has been explored (Lopes & Badino., 2015); after desorption, CA could be conventionally precipitated as clavulanate of potassium.

3. Materials and methods

In this chapter the physiology of the microorganism, the operating conditions for cell suspension cultures and media composition, are described. The quantification of CA in the process and the pretreatment of the resin to be used are detailed. The methodology used included the implementation of two separation strategies (liquid-liquid extraction and adsorption) for the recovery of CA from the fermentation broth. For this purpose, the analytical techniques and experimental designs, treated at laboratory scale, are described, identifying the best operating conditions (temperature, pH, contact time, solid-liquid ratio, and concentration of the fermentation broth), in order to evaluate their effect on product recovery.

3.1 Microorganism, operating conditions, and fermentation broth

The commercial strain *S. clavuligerus* ATCC 27064 was used throughout this study. Mycelium cultures were stored as 20% (v/v) glycerol stocks at -80 ºC. *S. clavuligerus* ATCC 27064 was cultivated in baffled shake flasks in glycerol-sucrose-proline-aspartate (GSPA) and isolate soy protein (ISP) media as described by Pinilla *et al*. (Pinilla *et al*., 2018). The biomass was separated by centrifugation for 10 min at 4 °C and 5000 rpm. The supernatant containing CA was separated and the biomass discarded. For the separation and purification assays, concentration of CA in supernatants was adjusted, accordingly, by adding potassium clavulanate or

by dilution. Central composite experimental designs were followed with CA removal as the response variable.

3.1.1 Preparation of the seed medium

The seed medium proposed by Roubos *et al*. (Roubos *et al*., 2002), Table 1, was inoculated with 1 mL of *S. clavuligerus* spores (for 24 h, at 28 °C and 220 rpm). Afterwards, 5 mL of seed medium were transferred to 45 mL of pre-culture medium; this medium was incubated under the same operating conditions.

Table 1. Composition of the seed medium proposed (Roubos *et al*., 2002).

Reagent	Composition (g/L)
Glycerol	15.00
Soy Peptone	15.00
Sodium chloride	3.00
Calcium carbonate	1.00

Subsequently, the pH was adjusted to 6.80 adding citric acid on a plate with magnetic stirring (CORNING PC-4200). From the developed seed medium, two 50 mL media were taken out in 250 mL Erlenmeyer flasks, respectively, as a backup for possible contamination. Finally, the prepared seed medium was autoclaved for 20 min at 15 bar and 120 °C.

3.1.2 Preparation of GSPA and ISP fermentation broths

Table 2 shows the composition of the defined medium (GSPA). The composition of ISP (Isolated Soy Protein) is presented in Table 3.

Table 2. Composition of the defined production medium GSPA.

Medium production	Composition (g/L)
Glycerol	15.00
Sucrose	20.00
NaCl	5.00

K_2HPO_4	2.00
MOPS	21.00
$CaCl_2$	0.40
$MnCl_2*4H_2O$ *	0.10
$FeSO_4*7H_2O$	0.10
$ZnCl_2$	0.05
$MgSO_4*7H_2O$	1.00
Proline	2.50
L-Aspartic Acid **	1.50

* Trace elements which are mixed in volume and prepared in solution.

** L-Aspartic acid is sterilized separately, and then added to the medium, to avoid undesirable side compounds (Millar reaction).

Table 3. Composition of the production medium ISP (Isolated Soy Protein).

Components	Composition (g/L)
Soy protein	10.00
Yeast extract	1.00
Malt extract	10.00
Glycerol	15.00
K_2HPO_4	2.50
MOPS	21.00
$MnCl_2*4H_2O$ *	0.001
$FeSO_4*7H_2O$	0.001
$ZnCl_2$	0.001
$MgSO_4*7H_2O$	0.75

* Traces, which are mixed in volume and prepared in solution.

For CA production, either ISP or GSPA culture medium was inoculated with pre-culture medium at 10% (v/v). All *S. clavuligerus* cultures were performed in 250-baffled Erlenmeyer flasks containing 50 mL of medium. Cultures for CA production were incubated for 144 h, at 220 rpm and 28 ºC. All experiments were performed in triplicate. These fermentation media were prepared with the purpose of observing how the compound matrices affected the CA separation process. Media such as ISP produce interferences caused by the presence of amino acids and proteins,

which do not allow CA molecules to efficiently bind to the active sites of the ion exchange resin or solvents to be used as extraction agents. Conversely, GSPA defined medium, provides a low concentration of amino acids, that could have a favorable impact on CA separation (Da Silva *et al*., 2018).

3.1.3 Cell harvesting and pretreatment of the spent culture medium

The spent medium from, the fermentations with *S. clavuligerus,* was pretreated to eliminate biomass and protein residues. The culture medium was transfer to 50 mL falcon™ tubes and centrifuged (Sigma Laborzentrifugen® model 2-16PK). Centrifugation was carried out at 4 °C and 5000 rpm during 10 min. After centrifugation, the supernatant was filtered (0.20 µm Sartorius®) and analyzed; the remaining biomass was discarded.

3.2 Liquid-liquid extraction of CA

3.2.1 Experimental design

The extraction of CA from the fermentation broth of *S. clavuligerus* cultures was carried out at laboratory scale; for this, two experimental designs were developed to find the best process conditions. For the first experimental design, the central composite rotatable experimental design with two central points, was considered. Two factors (temperature and pH) were evaluated, at two levels each, for a total of 10 treatments without considering triplicates, Figure 3. The process variables were pH (2.0-7.0) and temperature (10-15 °C) (see Table 4).

Table 4. Matrix of the experimental design used to investigate the influence of temperature and pH on the recovery of CA (%).

Treatment	pH	Temperature (℃)
1	4.50	12.50
2	8.00	12.50
3	2.00	10.00
4	2.00	15.00
5	7.00	10.00
6	4.50	16.00
7	0.97	12.50
8	7.00	15.00
9	4.50	12.50
10	4.50	9.00

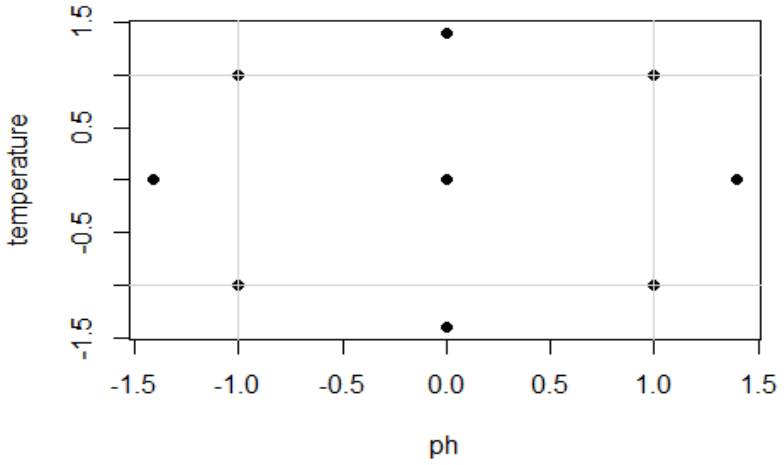

Figure 3. Rotational composite central experimental design with central points used to investigate the influence of the independent variables, temperature, and pH, on CA recovery.

A new central composite rotatable experimental design, with three central points, was considered, with the objective of establishing the most appropriate values for the different factors, that maximize the recovery of CA. The experimental factors were CA concentration in the fermentation broth, and the aqueous/organic phase ratio. Following are the levels that were implemented for the CA concentration (100 mg/L-900 mg/L) and the aqueous/organic phase ratio (1:1-1:3), Table 5.

Table 5. Matrix of the experimental design used to investigate the influence of concentration and aqueous/organic phase ratio on the yield of CA (%).

Treatment	Concentration (mg/L)	Aqueous/organic phase ratio
1	100	1:3
2	500	1:3.40
3	500	1:2
4	500	1:2
5	900	1:1
6	500	1:0.60
7	100	1:1
8	65.70	1:2
9	900	1:3
10	1066	1:2
11	500	1:2

The central composite rotatable experimental design was considered in order to evaluate the recovery of CA under extreme conditions, Table 6. Following are the levels, for the different variables, that were implemented: CA concentration (500 mg/L-1000 mg/L) and the aqueous/organic phase ratio (1:0.2-1:1).

Table 6. Matrix of the experimental design used to investigate the influence of concentration and aqueous/organic phase ratio on the recovery of CA (%).

Treatment	CA Concentration (mg/L)	Aqueous/organic phase ratio
1	750	1:0.60
2	1000	1:1
3	1000	1:0.20
4	750	1:0.034
5	500	1:1
6	750	1:0.60
7	1103.50	1:0.60
8	396.50	1:0.60
9	500	1:0.20
10	750	1:1.17

3.2.2 CA separation by liquid-liquid extraction

The following CA sources were used: Potassium clavulanate obtained from Clavulin® (875 mg of amoxicillin and 125 mg of clavulanate potassium); this source was used to ensure the initial concentration of CA was equally set in all experiments, and to ensure there was no interference of other substances, generally present in the fermentation broth. CA is produced by cell suspension cultures of *S. clavuligerus* ATCC 27064. The culture media used were those specified in the previous section. At the end of the culture, the broth was centrifuged, filtered on a 0.20 μm filter to remove residual cells, and then, extraction was performed with the organic solvent. Four organic solvents were used, two of them having an ester functional group (ethyl acetate and butyl acetate); the ethers had polyetal100 and PolyDiox. The selection was made considering factors such as selectivity and the ability to recover CA.

To use the CA present in the commercial drug, it was necessary to perform a liquid-liquid extraction for subsequent use. The drug was pulverized, then diluted into 40 mL of milli-Q water at 10 °C for 1 h, and subsequently filtered through a 0.20 μm filter paper (Sartorius®). With this procedure, 99% of the CA present in the drug was recovered, with a minimum of substances that could cause interference (Mancilha *et al*., 2014; Hirata *et al*., 2013).

Two (2) mL of the filtrate containing CA, from the commercial drug, were taken and diluted with 8 mL of each fermentation broth (20% CA of the total volume to be prepared), which was acidified with 3M sulfuric acid until reaching the pH value stipulated in the proposed experimental design. A 300 μL sample was taken, before starting the extraction, as the initial concentration; solvent (10 mL) was added as organic phase (ethyl acetate), to work with a 1:1 ratio. The aqueous and organic phases were left in contact for 20 min, at 240 rpm and at a temperature determined according to the experimental design. Next, the mixture was left to stand in the separatory funnel for the complete the separation of phases by decantation.

The response surface experimental designs, with defined and complex medium variables, were set as follows. CA load: 100 mg/L-900 mg/L; aqueous to organic phases ratio: 1:1-1:3. To set the level of the initial CA concentration, according to the experimental design, a certain amount of the CA solution was taken and diluted with spent medium, either defined or complex. Once the 10 mL solution was prepared, pH and temperature were adjusted to the best conditions found in the experimental design. Subsequently, the solution was transferred to an ice bath and left for 2 min at a temperature defined by the experimental design. Solvent was added as organic phase (ethyl acetate), in a way that the aqueous/organic phase ratio determined in the experimental design, was functional. The phases were left in contact for 20 min, at 240 rpm and at the previously determined temperature. The mixture was left on a separating funnel for 10 min, so that the phases separate from each other. During the development of the experimental designs, samples of the aqueous phase were collected before and after of the separation process for subsequent analysis. The obtained samples were imidazole-derivatized for 30 min and subsequently filtered at 0.20 μm. Finally, the samples were placed into vials for subsequent HPLC analysis (see Figure 4).

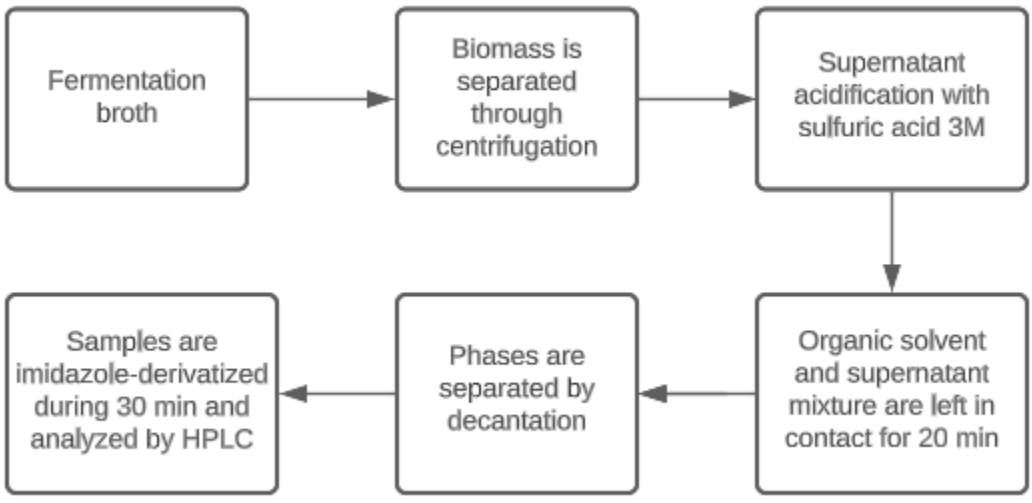

Figure 4. Schematic representation of procedures performed for the implementation of liquid-liquid extraction of CA from fermentation broths.

3.3 CA separation by adsorption methods

3.3.1 Experimental design

For the purpose of comparison between the two separations techniques, experiments, using the ion exchange resin IRA 400 for the adsorption process, were set based on a central rotational composite experimental design (with two central points) was considered (Table 7).

Table 7. Matrix of the experimental design (CCRD) used to investigate the influence of temperature and pH on the adsorption process of CA*

Treatment	pH	Temperature (℃)
1	4.50	12.50
2	8.00	12.50
3	2.00	10.00
4	2.00	15.00
5	7.00	10.00
6	4.50	16.00
7	0.97	12.50
8	7.00	15.00
9	4.50	12.50
10	4.50	9.00

* The ion exchange resin IRA 400 was used. The response variable was the percentage CA recovered (%CA).

Using the best experimental conditions, found in the design used to study the effect of temperature and pH on CA recovery, a new experimental design was set and carried out; for this, the CA load in the fermentation broth, and the ratio between the supernatant and the adsorption solid, were selected as factors. For each factor, two levels and three replicates at the center, were used, rendering a total of 11 treatments (Table 8).

Table 8. Experimental design (CCRD) used to investigate the influence of concentration and solid/liquid ratio on the CA adsorption process *.

Treatment	Concentration (mg/L)	Solid/liquid ratio (%)
1	100	40
2	500	45
3	500	25
4	500	25
5	900	10
6	500	5
7	100	10
8	65.70	25
9	900	40
10	1066	25
11	500	25

* The ion exchange resin IRA 400 was used. The response variable was the percentage of CA recovered (%CA).

3.3.2 Pretreatment of the Amberlite® IRA 400 resin

50 g of Amberlite® IRA 400 chloride form (Sigma Aldrich, Saint Louis, MO) was used; resin was pretreated by washing it with NaOH 10% (w/v) for 10 min at 1:1 solid/liquid ratio. It was then centrifuged at 5000 rpm, 4 °C for 3 min, followed by three washing steps with distilled water, for 5 min each. The pretreated resin was filtered and then regenerated using NaCl 10% (w/v) for 10 min (Almeida *et al*., 2003; Barboza *et al*., 2003; Costa *et al*., 2015; Yepes *et al*., 2020).

3.3.3 Preparation of the fermentation broth

The culture medium from the fermentations with *S. clavuligerus* was pretreated prior to any contact with the ion exchange resin, to eliminate biomass and protein residues. The process conditions were described in section 3.1.3.

3.3.4 Adsorption process

An appropriate quantity of the prepared (commercial) CA solution was used and diluted with culture medium (defined or complex). Once the 10 mL solution was prepared, pH was adjusted (sulfuric acid 3M) as specified in the proposed experimental design. Subsequently, the solution was transfer to a bath ice. A sample of 300 μL was taken before starting the adsorption as initial concentration. The required amount of pre-treated resin was added to the 10 mL solution as defined in the experimental design. The resin in the solution was left in contact at 220 rpm and 10 °C for 50 min. At the end, a sample of the supernatant was taken to evaluate CA content. All experiments were performed in triplicate. A negative control was considered, inoculating various treatments of the experimental design, under the same conditions, but without addition of the solid resin.

3.3.5 CA desorption process

The desorption of the CA retained by the ion exchange resin was carried out by filtering the resin from the adsorption process and then mixing it with a NaCl solution 10% (w/v). The levels for this variable were determined by a pre-experimental treatment. The solid/liquid ratio (45%) was selected from literature data (Yepes *et al*., 2020; Costa & Colli, 2015; Barboza *et al*., 2003). The resulting mixture was incubated at 22 °C and 240 rpm for 135 min. For quantification purposes, samples (300 μL) were taken every 15 min. Subsequently, they were derivatized and analyzed by HPLC (Figure 5). A negative control was carried out using resin that had not been in contact with the supernatant. All experiments were performed in triplicate.

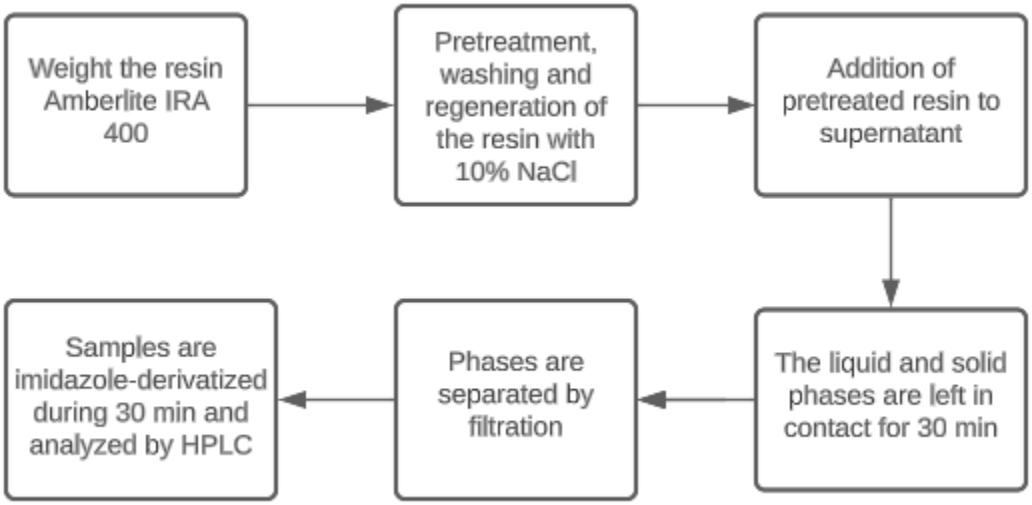

Figure 5. Schematic representation of the procedures performed for the implementation of CA adsorption using ion exchange resin IRA 400.

Finally, an experimental design was carried out (Table 9), to identify the best ratio between the solid/liquid ratio and the NaCl concentration in water, for the desorption process.

Table 9. Experimental design to evaluate the best ratio between the solid/liquid ratio and the NaCl concentration in water in the CA desorption process.

Treatment	Solid/liquid ratio	% NaCl
1	60	10
2	60	10
3	60	17.10
4	40	15
5	100	5
6	60	2.90
7	100	15
8	40	5
9	36	10
10	143	10

3.3.6 Calculation of extraction parameters

To evaluate the yield of CA extracted, the percentage of extraction (CA,%) and the extracted mass (m_{CA}, mg) were evaluated for the proposed separation alternatives. Based on these results, important decisions can be made related to the best conditions or parameters to work with according to the selected controlled variables; the final aim is to increase the yield of CA recovery from the fermentation broth.

The assessed parameters were the percentage of extraction (CA, %), and the mass of CA extracted from the fermentation broth. The percentage of CA present in the solution was calculated according to the following equation:

$$CA\ (\%) = \frac{C_{SOL}}{C_0} * 100 \qquad \text{[Equation 3]}$$

where $CA\ (\%)$ represents the percentage of CA recovered, C_{SOL} and C_0 are the CA concentration (mg/L) after the extraction process and the initial CA concentration, respectively.

The extracted CA yield (η_{CA}) can be calculated by

$$\eta_{CA} = \frac{C * V}{C_0 * V_0} * 100 \qquad \text{[Equation 4]}$$

Where C_0 is the initial concentration of CA in the aqueous phase, V_0 is the volume of the initial aqueous phase, C is the final concentration and V is the final volume of the solution containing CA after extraction.

The mass of CA extracted from the fermentation broth m_{CA} (mg) was determined from a mass balance:

$$m_{CA}(mg) = (C_{CA-b} - C_{CA-a}) * V_c \qquad \text{[Equation 5]}$$

Where, C_{CA-b} and C_{CA-a} (mg/L) are the concentrations of CA before and after extraction, respectively; and V_c (L) is the volume of the culture broth.

3.4 Analytical techniques used for CA quantification

Samples (300 μL) were placed into vials for quantification by HPLC Agilent 1200 (Agilent Technologies, Waldbrom, Germany) equipped with a Diode Array Detector (Agilent Technologies, Palo Alto, CA, USA) at 312 nm, using a reverse-phase ZORBAX Eclipse XDB-C_{18} (4.6 × 150 mm, 18 μm Agilent Technologies, Palo Alto, CA, USA) column; 94% (v/v) KH_2PO_4 (50 mM, pH 3,2) and a 6% (v/v) methanol solution were used as mobile phase at 0.7 mL/min. CA was imidazole-derivatized at a ratio of 1:3; the reaction was kept at 28 °C for 15 min (Gómez *et al*., 2019). Next, the imidazole-clavulanate complex reaction of the samples was stopped by freezing (-20 °C, 15 min), see Figure 6.

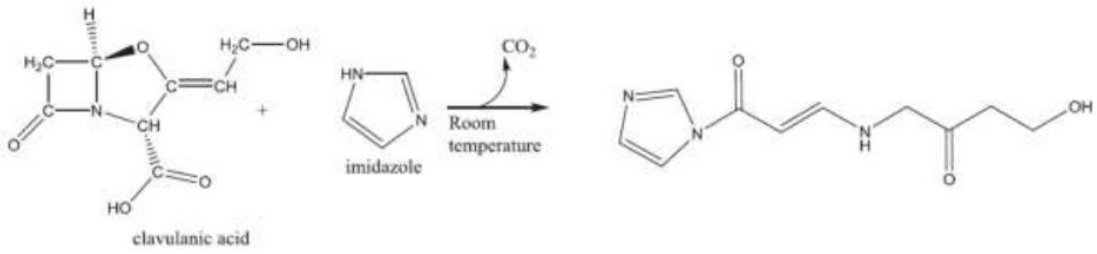

Figure 6. Derivatization reaction of CA with Imidazole (Ramírez *et al*., 2016).

Standard solutions were prepared with a stock solution of CA obained from the commercial product Clavulin® (amoxicillin 875 mg + clavulanic acid 125 mg).

3.5 Response Surface Methodology

A central composite rotational experimental design (CCRD) was considered, with the objective of establishing the values which optimize the recovery of CA. The following is the detailed master sheet of the developed design:

3.5.1 Response Variable

CA extraction yield. The usual operating values oscillate between 40% and 80%. The measuring instrument to determine the concentrations of CA was a liquid chromatography equipment (HPLC). The response variable was calculated using the yield equation, presented in the theoretical framework.

3.5.2 Control Variable

A factorial design (2^2) was performed. Factors used as the controlled variables were defined as:

Factor 1: Concentration. Level (-1): 500 mg/L, Level (1): 1000 mg/L.
Factor 2: Aqueous/organic phase ratio. Level (-1): 1:0.20, Level (1): 1:1

Therefore, for each factor we used two levels, which gave us four treatments. There were 2 replicates of the central point and 4 axial points, for a total of 10 experiments. As a predicted effect of the control variables on the response variable, the aqueous/organic phase ratio factor was expected to have the greater effect on CA extraction yield.

3.5.3 Parameters that remained constant

- Temperature of the extraction medium. This was controlled with an ice bath and thermometer.

- Volume of the fermentation broth (45 mL). The volume of the fermentation broth was measured with a measuring cylinder, which is a precision instrument.
- Extraction vessel (Erlenmeyer of 50 mL). The use of the same glassware, where the extraction was performed, was guaranteed.
- Chromatographic analysis equipment - HPLC.
- Person who is responsible for the measurement.
- Place where the extraction of the product is carried out (measurements made in the same laboratory). The sudden change in environmental conditions is reduced to a certain degree.
- Stirring plate. The calibration points of the plates are different, and therefore if the plate is changed, the shaking force will not be the same.
- Digital balance. The calibration points of the balances are different, and therefore influence the weight of the reagents in the case of adsorption.

3.5.4 Noise factor

- Environmental variables.
- Calibration of measurement instruments (HPLC column, stopwatch, stirring plate and analytical balance).

One of the strategies employed was a completely randomized design. The experimental runs were carried out in random order, so that the possible environmental and temporal effects were distributed equally, between treatments.

4. Results and discussions

This chapter details the results obtained from the implementation of the proposed CA separation strategies, as well as their interpretation and statistical analysis.

4.1 Liquid-liquid extraction of CA

The separation of a compound by extraction is based on the selective transfer of the compound of interest from a liquid mixture into a liquid phase (usually an organic solvent) (Harris, 2015). Liquid extraction is based on the differences in solubility of the components of a mixture in a suitable solvent. It is an important separation method in research and chemical analysis. As a commercial process, it is frequently used in the chemical and mining industries and in the downstream recovery of fermentation products (antibiotics, amino acids, steroids) (Berk, 2018).

4.1.1 Effect of pH and temperature on CA extraction

CA centration in spent broths was set using commercially distribuited CA-based drugs, e.g., clavulin®. This was necessary so that the variables used in the experimental design could be evaluated correctly, ensuring that the initial amount of CA used was the same in all of the considered experiments. Further, the dilution of the drug was performed using the spent fermentation broths, with the aim of mimicking the interferences and/or perturbations of other substances e.g., aminoacids and proteins, on the separation process.

Table 10 shows the percentages of CA recovery for a single extraction, obtained from the central composite rotatable experimental design (CCRD). The values varied from 16.01% (pH: 8.0 and T: 12.5 °C) to 49.09% (pH: 2.0 and T: 10 °C). It was found that the acidic medium favored the extraction process of CA in aqueous media. CA presented greater stability at acidic pH values, since less acid catalytic activity is presented in the degradation reaction. Results from experimental treatments 2, 5 and 8, which worked at alkaline conditions, showed low percentage of CA recovery in the process. Some authors have reported about CA instability under alkaline environmental conditions (Costa *et al*., 2015; Mayer *et al*., 1996). According to the findings of Bersanetti *et al*. (Bersanetti *et al*., 2005), the stability of CA is highest at pH 6.2, but it becomes highly unstable when the pH exceeds 8.0.

Table 10. Results from the central composite rotatable design (CCRD) for assessing CA liquid-liquid extraction. Study variables: pH and T. Response variable: CA yield.

Treatment	pH	Temperature (℃)	CA (%)
1	4.50	12.50	39.57 ± 0.34
2	8.00	12.50	16.01 ± 0.11
3	2.00	10.00	49.09 ± 0.23
4	2.00	15.00	48.33 ± 0.02
5	7.00	10.00	19.55 ± 0.12
6	4.50	16.00	42.17 ± 0.01
7	0.97	12.50	48.18 ± 0.21
8	7.00	15.00	23.36 ± 0.10
9	4.50	12.50	33.63 ± 0.31
10	4.50	9.00	20.26 ± 0.02

As observed in Table 10, the best experimental conditions for CA liquid-liquid extraction were pH 2.0 and 10 °C. A statistical analysis was also performed, which is detailed in Appendix A-1. Likewise, the effectiveness of ethyl acetate as a solvent in the organic phase was evidenced with respect to the other solvents used (ethyl acetate (49.09%), butyl acetate (46.70%) and the poor extraction levels of the ethers polyetal100 and PolyDiox).

The results obtained showed a higher selectivity of ethyl acetate towards CA. This behavior might be caused by the low solubility of this solvent in the aqueous phase during the process. Additionally, ethyl acetate has a lower boiling point than the other solvents used, which favors the precipitation of CA in later stages of the process, at the time of recovery in its clavulanate salt form. Non-polar or poorly soluble molecules, such as ethyl acetate, do not interact at all with the aqueous phase, allowing in the process that, instead of dissolving, they form separate layers when placed in an aqueous medium (Raven *et al*., 2014; Reece *et al*., 2011).

Temperature and pH played an important role in the extraction process since pH has a direct effect on CA hydrolysis (Soares *et al*., 2012). The degradation of CA by alkaline hydrolysis (Mayer *et al*., 1997) could contribute to losses in the process; this might be result of the irreversible interactions that occur, rendering, as an effect, the degradation of CA. Operating conditions, recomended in the literature, include pH values between 1.0-3.0, and temperatures around 10-15 °C. This is because at low pH, beta-lactam antibiotics, including CA, usually have a protonated carboxylate group causing its low solubility in water and facilitating the organic-solvent extraction (Mayer *et al*., 1996; Hirata *et al*., 2009; Brites *et al*., 2012; Cook *et al*., 1987). CA is a weak acid with pKa equal to 2.5 (Mourão *et al*., 2006); only the undissociated acid can be extracted by an organic solvent. At higher pH values the carboxylate group is deprotonated and charged, rendering the CA water soluble (clavulanate form) (Hirata *et al*., 2009).

The results obtained showed a better percentage of CA recovery at low temperatures, so it was possible to observed that the CA degradation rate increased significantly with increasing temperature. Table 11 shows the percentages of CA extraction after reprocessing.

Table 11. Results of the central composite rotatable design (CCRD) for assessing CA with double liquid–liquid extraction. Study variables: pH and T. Response variable: CA yield.

Treatment	pH	Temperature (℃)	CA (%)
1	4.50	12.50	39.88 ± 0.45
2	8.00	12.50	18.85 ± 0.52
3	2.00	10.00	57.14 ± 0.19
4	2.00	15.00	53.47 ± 0.01
5	7.00	10.00	21.74 ± 0.37
6	4.50	16.00	46.15 ± 0.09
7	0.97	12.50	56.22 ± 0.14
8	7.00	15.00	29.22 ± 1.29
9	4.50	12.50	39.32 ± 0.43
10	4.50	9.00	28.16 ± 1.51

A second extraction with fresh solvent allowed to increase the percentage of CA recovery from the fermentation broth, thus increasing its yield. The values varied from 18.85% (pH: 8.00 and T: 12.50 °C) to 57.14% (pH: 2.0 and T: 10 °C). These results showed a 10% increase in the recovery of CA with respect to the process with a single liquid-liquid extraction. It is also evident that the values of the temperature and pH variables coincide with those found in the first experimental design. However, the higher the volume of solvent used, the more diluted the CA solution would become, which increases the cost of the process at the moment of concentrating the organic phase for the subsequent precipitation processes.

The treatments carried out in the previous experimental designs, allowed analyzing that the temperature of 10 °C was the most adequate to increase the recovery yield of the CA, likewise, from the statistical analysis shown in Annex A-1, it is evident that a temperature close to 10 °C maximizes CA recovery. It has been observed in previous analyses that lower temperatures can promote a lower degradation rates of CA, thus increasing its stability. For example, Bersanetti *et al*. (Bersanetti *et al*., 2005) investigated the degradation of CA at temperatures of 10, 20, 25, 30 and 40 °C, and pH values of 6.2 and 7.0. The results showed irreversible first-order kinetics, where a relationship between the degradation rate constant and temperature was

established. It was found that the highest stability of CA was observed under slightly acidic conditions and at low temperatures (10 °C). However, it was found that at higher temperatures the partitioning of the system is favored, supporting the previous results obtained in Table 11. In this table, it is observed that, for treatments 6 (16 °C), 9 (12.5 °C) and 10 (9 °C), which were carried out under the same pH conditions (4.5), the recovered CA was 46.15%, 39.32% and 28.16%, respectively.

The partition coefficient (K) of a substance, refers to the ratio between the concentrations of that substance in the two phases of a mixture formed by two immiscible solvents in equilibrium. This coefficient measures the differential solubility of the substance in the two solvents. Forte *et al*. (Forte *et al*., 2011) investigated the partition coefficient of the linear model (K) and the constant of the Freundlich model (K_F) were evaluated at different temperatures, and reported an increase in the values of K as a consequence of the increase in temperature. The degradation reaction of CA is considered to be predominant as it degrades at high temperatures (above 35 °C) (Mancilha *et al*., 2014). The fitted regression model, described in Appendix A-1, predicted the maximum CA extraction to be 59.31%, using a pH of 0.96 and a temperature close to 9.0 °C. However, from the experimental point of view, it is not feasible to work with such high acidity values (pH close to zero). Therefore, with the experimental conditions selected in this research work, an extraction percentage of 57.14% was achieved, close to that predicted by the model using a pH of 2.0 and a temperature of 10 °C.

As can be observed, the system must be cooled to carry out the extraction process, which requires energy expenditure over time. By being able to decrease the extraction time, the energy expenditure would be lower, which turns into lower operating cost.

4.1.2 Optimum contact time between liquid phases

To have an approximation of the time required for reaching the equilibrium during the extraction process, samples, taken at regular intervals, were analyzed up to 70 min, (Figure 7).

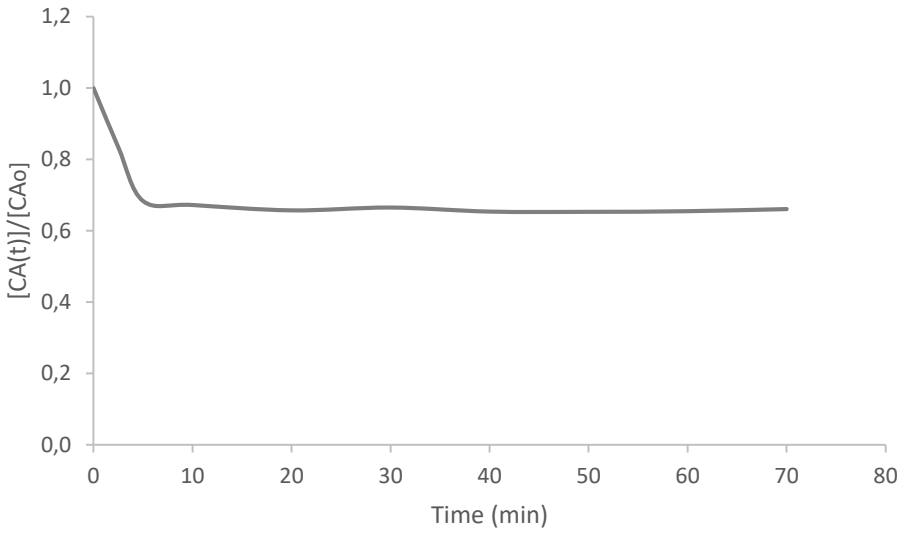

Figure 7. Analysis of the required time for reaching the equilibrium during the extraction process. Time courses of CA concentration (CA(t)). CA initial concentration (CAo).

As observed in Figure 7, after 10 min of contact between phases, the CA concentration reached the equilibrium point; undoubly, a short time favors CA yield, since time is a determining factor due to the high instability of the CA molecule and, therefore, its degradation in the medium. Therefore, it was decided to perform the experimental treatments every 20 min, thus ensuring time is sufficient to reach equilibrium during the extraction process. Moreover, at large scale, a reduction in time is equivalent to a lower energy expenditure, which would reduce operating costs.

4.1.3 Using a Non-polar solvent as a pretreatment strategy for increasing CA extraction

The CA extraction protocol was tested using the real matrix, *i.e*., fermentation broths. The complex and defined media were used to analyze how the complex mixture of nutrients/metabolites could affect CA extraction. A pretreatment strategy with a non-polar organic solvent (petroleum ether) was implemented, prior to the liquid extraction, and compared with the single extraction operation, without any previous treatment.

Table 12 shows the percentage of CA recovered, when a fermentation medium pretreatment strategy was implemented using petroleum ether as non-polar organic solvent. The level of study for the variable non-polar organic solvent/fermentation medium ratio (1:0.2-1:1) was set considering the quantity and price of the solvent. The smaller the amount, the lower the potential contamination generated, and therefore its cost.

Table 12. Percentage of CA recovered when a pretreatment strategy is applied to the fermentation medium.

Aqueous/organic phase ratio *	CA recovered (%)
1:1	60.53 ± 0.54
1:0.80	51.48 ± 0.25
1:0.60	49.65 ± 1.89
1:0.40	49.36 ± 0.92
1:0.20	48.52 ± 2.03
Control	43.06 ± 0.35

* Ratio of the amount of non-polar organic solvent to the amount of fermentation medium used in the pretreatment strategy.

Although the use of a 1:1 ratio showed a favorable increase in the recovery of CA, it was not a favorable condition due to the amount of non-polar organic solvent that was employed in the process. However, it was observed that by decreasing the

purification solvent to 20%, the yield of the purification process increased by 10%. Figure 8 presents the results of the percentage of CA obtained by implementing a pretreatment with petroleum ether as a non-polar organic solvent. It was observed that when the process considered a double extraction, using the pretreatment with the non-polar organic solvent, 60.37% recovery of CA was obtained. Mancilha *et al*. (Mancilha *et al*., 2014) reported up to 35% CA recovery, also using ethyl acetate as solvent for extraction, thus indicating the effectiveness of the strategies implemented in the process.

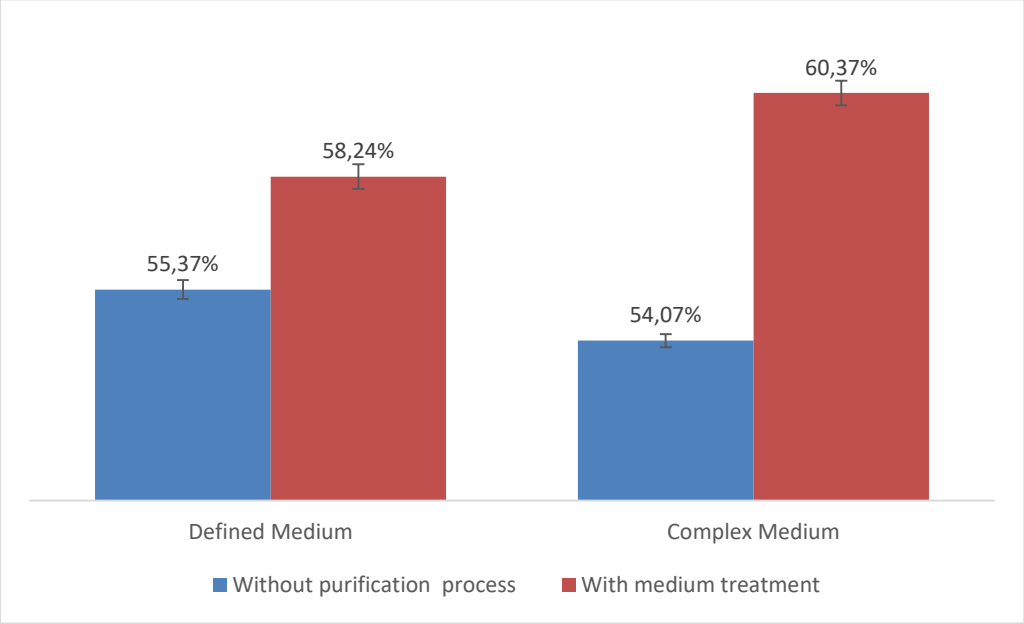

Figure 8. Results of percentage of CA obtained when a double extraction is performed, using a pretreatment with petroleum ether as a non-polar organic solvent.

Prior to the pretreatment with the non-polar solvent, recovery percentages were 55.37% for the defined medium and 54.07% for the complex medium. Once the fermentation broth was exposed to the pretreatment, a recovery of 60.37% was attained for the complex medium, which contained less impurities, and therefore, higher selectivity for CA.

Non-polar solvents, such as many hydrocarbons (e.g. benzene, hexane, ethane) or organic solvents such as ether, do not exhibit miscibility with the aqueous phase (fermentation broths) due to differences in the intermolecular forces and polarities of the substances involved. As a result, non-polar molecules tend to group together, giving rise to separate phases in the presence of water, which leads to the absence of miscibility between non-polar solvents and aqueous media. However, some impurities, such as proteins and amino acids, are soluble in the organic phase, which enables significant transfer of these impurities to the petroleum ether. This transfer reduces interference when recovering the compound of interest (CA) in the fermentation broth, resulting in an increased yield in the extraction process.

4.1.4 Effect of CA concentration on its recovery from the culture medium.

Using the process conditions (temperature and pH) obtained previously, a new experimental design was carried out having, as factors to be studied. These factors were considered because they are directly related to the cost of the process and the size of the equipment (See Table 13). The Pareto graph was used in the statistical analysis presented in Appendices A-1 and A-2. In this graph, it can be seen that the pH and liquid ratio variables have the greatest influence on the response variable and on the bioprocess economy.

Table 13. Results of CA recovery according to the central composite rotatable design (CCRD), by varying the ratio between the aqueous and organic phase.

Treatment	Concentration (mg/L)	Aqueous/organic phase ratio	CA recovered (%)
1	100	1:3	77.18 ± 0.87
2	500	1:3.40	84.08 ± 0.05
3	500	1:2	76.25 ± 1.80
4	500	1:2	75.93 ± 0.78
5	900	1:1	55.47 ± 2.58
6	500	1:0.60	44.69 ± 1.41
7	100	1:1	52.83 ± 0.02

8	65.70	1:2	81.56 ± 2.09
9	900	1:3	80.64 ± 2.19
10	1066	1:2	77.68 ± 1.71
11	500	1:2	72.80 ± 0.38

Although high solvent ratios (> 2) allowed to extract up to 80% of the CA present in the cultivation broth, high solvent volumes could increase considerably the cost of the operation at industrial scale, as more energy would need to be implemented to remove the solvent and hence, to concentrate the recovered CA. Therefore, a new experimental design at different conditions, was proposed for exploring low volumes of the extraction solvent.

The use of low volumes of organic solvent (ratios 0.20-0.60) led to 40% less CA extracted from the broth (see Table 14), with respect to that achieved when using high volumes of organic solvent. It should be mentioned that before the extraction a pretreatment of the medium was performed using petroleum ether as a non-polar organic solvent, which allowed for the partial removal of proteins and amino acids that would interfere in the process.

Table 14. Calculated values of mass and concentration of CA at low volumes of ethyl acetate.

Treatment	Concentration (mg/L)	Aqueous/organic phase ratio	CA (mg/L)	CA Mass (mg)	CA (%)	Cost ($)
1	750	1:0.60	236.88	1.18	29.84 ± 0.45	1,446
2	1000	1:1	318.91	1.59	41.46 ± 0.52	1,050
3	1000	1:0.20	235.55	1.18	19.56 ± 0.19	1,278
4	750	1:0.034	231.74	1.16	25.05 ± 0.01	1,031
5	500	1:1	213.67	1.07	44.67 ± 0.37	1,779
6	750	1:0.60	201.44	1.01	29.81 ± 1.18	1,447
7	1103.50	1:0.60	304.61	1.52	23.89 ± 0.09	1,330
8	396.50	1:0.60	166.40	0.83	42.03 ± 0.14	1,780
9	500	1:0.20	83.85	0.42	24.23 ± 1.29	1,747
10	750	1:1.20	257.70	1.30	45.44 ± 0.42	1,344

Interestingly, at the very low solvent ratio, the formation of micelles in an unstable emulsion of ethyl acetate in water at ratios below 0.1 (treatment 4) favored a high CA extraction, leading to yields comparable to those observed when using the 0.6 ratio. The results (Table 14) suggest an unfavorable effect on the recovery percentage as the concentration of CA in the cultivation broth increases when maintaining the same aqueous/organic phase ratio, which is in agreement with the fact that higher solvent ratio would increase the extraction yield, but with a consequent high dilution. A statistical analysis was also performed, which is detailed in Appendix A-2.

Extraction at an aqueous to the organic ratio of 1:0.6, as a feasible condition, led to recoveries of 42.03%, 29.81%, and 23.89% at 396.5, 750, and 1103.50 mg/L of CA initial concentrations, respectively. The observed effect of CA concentration in decreasing the final recovery in the organic phase can be explained by the critical micellar concentration, which is the minimum point where micelles start to form in a liquid solution. In aqueous-organic extraction systems, the micelles formation is favored by low temperatures and low concentrations as shown by Martino *et al.* (Martino, 2020) for extraction of amino-acids from aqueous solutions.

In relation to the costs associated with the selected separation strategy, it can be observed that treatments 2 (1000 mg/L, Liquid ratio 1:1) and 4 (750 mg/L, Liquid ratio 1:0.034) showed the lowest costs per unit mass of CA recovered ($1,050 and $1,031 pesos respectively). This can be attributed to two main reasons. First, a low liquid ratio leads to the use of reduced amounts of recovery solvent, thus reducing costs. However, it is important to note that in treatment 2, the ratio is 1:1, but with a high working concentration. Secondly, although treatment 2 requires a higher amount of solvent, the costs are offset by the high mass recovery of CA in the process.

Therefore, a stepwise contact operation could improve the efficiency of the process with low volumes of the solvent for extraction; it can be carried out in batches or

continuously. It is possible since the process driving force would be maintained. The residual fermentation medium can be repeatedly extracted with more ester solvent to further reduce the CA content, or a countercurrent cascade of stages can be rearranged. Another possibility is to use some type of continuous contact countercurrent apparatus, where no discrete stages are present. This type of operation requires fewer stages for a given amount of solvent, or less solvent for a fixed number of stages than crosscurrent methods, in which different amounts of solvent can be used in the various stages. The use of reflux, as in distillation, can further improve the final separation yield of the process.

4.2 Adsorption of CA

Adsorption processes is a surface phenomenon that is characterized by the concentration of a chemical species (adsorbate) from a solution onto or near the surfaces or pores of a solid (adsorbent) (Forte *et al*., 2016). This technique has received special attention in bioprocesses since biomolecules may be selectively adsorbed within a range of solid materials, allowing exploitation of these properties in extraction steps of the target molecule (Brito *et al*., 2008). Adsorption processes strongly depend on the nature and surface area of the adsorbent, nature of adsorbate, temperature, contact time of adsorbent and adsorbate, nature of medium and the pH of the adsorption medium (Forte *et al*., 2016). For the case of CA extraction, temperature and pH have been confirmed as the variables that have major incidence in the extraction yield.

4.2.1 Effect of pH and temperature on CA adsorption

Table 15 shows the percentages of CA adsorption using the ion exchange resin IRA 400. The values varied from 23.92% (pH: 7 and T: 10 °C) to 40.04% (pH: 4.50 and T: 12.50 °C) when the defined medium was used, and from 14.46% (pH: 7.0 and T: 10.0 °C) to 41.67% (pH: 4.50 and T: 12.50 °C) when using the complex medium. As it can be seen, the acidic medium and low temperatures favored the adsorption

process of CA. A statistical analysis was also performed, which is detailed in Appendix A-3.

Table 15. Effect of temperature and pH on the adsorption of CA, using the ion exchange resin IRA 400.

Treatment	pH	Temperature (℃)	CA recovered - defined medium (%)	CA recovered - complex medium (%)
1	4.50	12.50	39.60 ± 0.34	41.67 ± 0.41
2	8.00	12.50	25.58 ± 0.13	30.66 ± 0.35
3	2.00	10.00	30.97 ± 0.09	31.29 ± 0.52
4	2.00	15.00	37.41 ± 0.10	32.65 ± 0.34
5	7.00	10.00	23.92 ± 0.02	14.46 ± 0.28
6	4.50	16.00	35.97 ± 0.23	34.50 ± 0.30
7	0.97	12.50	27.59 ± 0.17	32.23 ± 0.17
8	7.00	15.00	27.96 ± 0.12	25.62 ± 0.65
9	4.50	12.50	40.04 ± 0.38	32.55 ± 0.10
10	4.50	9.00	34.53 ± 0.16	32.75 ± 0.39

Temperature and pH played an important role in the hydrolysis of CA in the fermentation medium, according to Soares *et al*. (Soares *et al*., 2012) the lowest hydrolysis constant occurs when temperature and pH are at their lowest levels. The degradation of CA by alkaline hydrolysis could contribute to losses; this occurs due to irreversible interactions with the resin matrix (Mayer *et al*., 1997), causing degradation of CA in the ion exchange phase and, as a consequence, a low result in the percentage recovery in the process. Therefore, it is strategic to work at slightly acidic pH and low temperatures, in order to lessen degradation and therefore, obtain higher CA recovery yields. The results obtained in the defined fermentation medium were higher than those from the complex medium; this might be the result of interferences caused by amino acids and proteins present in the ISP medium, which do not allow the CA molecules to effectively bind to the active sites of the ion exchange resin.

It can be further observed (Table 15) that small variations in temperature and pH led to a significant increase in the adsorption capacity of the resin; these outcomes agree with the work of Mayer *et al*. (Mayer *et al*., 1997). The authors studied the adsorption of CA using non-ionic and ion exchange resins (XAD-4 and IRA 400) and observed that, as the temperature increased from 4 °C to 21 °C for both resins, there was a decrease in the values of the effective diffusion coefficient. This phenomenon might be caused by an increase in the frequency of molecular collisions between the CA ionic groups and the charged sites of the resin, as argued by Barboza *et al*. (Barboza *et al*., 2002).

Another possible explanation is due to the interaction between CA ionic molecules and the resin, since this favors adsorption with decreasing temperature, as observed by Yepes *et al*. (Yepes *et al*., 2020), who studied, in a range of thermal condition (isotherms from 10 °C to 30 °C), the effect of temperature on the maximum adsorption capacity of the resin for CA recovery (q_m), as well as the affinity between the adsorbent and CA from the fit of data to the Langmuir model. In his work, Yepes *et al*. (Yepes *et al*., 2020) found that low values of k indicate a negative effect of temperature on CA adsorption, since k constitutes a measure of the affinity of the resin for the CA; therefore, the higher temperature the lower the affinity. Likewise, he noticed that the values obtained for q_m (0.64 mg_{CA}/g_{resin}) indicate the favorability of adsorption with decreasing temperature (10 °C, exothermic process). Barboza *et al.* (Barboza *et al*., 2002), determined the adsorption isotherms and investigated the influence of the temperature on the adsorption kinetics and desorption of CA in ion-exchange resins and reported an increase in the values of K as a consequence of the increase in temperature. According to these authors, this phenomenon can be justified by the increase in frequency of molecular collisions between the CA ion groups and the loaded sites of the resin, resulting in a more pronounced desorption. The methodology presented by the authors allowed estimating the adsorption enthalpy, showing a $\Delta H°$ value of -29.15 kJ/mol. These values portray an exothermic process in the formation of the CA complex on the sites of the resin.

Working at low temperature benefits the process since a higher temperature would generate an instability of the molecule, and further favors degradation rate (Gómez *et al*., 2019); as a result, a possible decrease in the percentage of CA recovery, will make the process technically unfeasible for an industrial implementation. Several authors such as Costa *et al.,* 2015; Mayer *et al.,* 1996 and Barboza *et al.,* 2003 have reported the operational advantages of using Amberlite Ira 400 resin as one of the most effective adsorbents in terms of capacity and speed of CA adsorption, compared to other materials such as zeolites, which require more time to reach equilibrium. It is important to note that temperature does not affect the time required to reach dynamic equilibrium in the resin. This time depends on the concentrations of CA in the fermentation broth and its adsorption on the specific sites of the resin, as well as the maximum adsorption capacity (q_m). Although each adsorbate has a maximum adsorption capacity, where the active sites are selectively saturated in the presence of ions, it has been observed in the literature that small variations in temperature can lead to a significant increase in adsorption capacity. For example, Costa and Badino (2015), found that the maximum adsorption capacity of CA (q_m) was considerably higher than the value reported by Barboza *et al.* (2003). The authors explain this difference by positing that the pH and temperature conditions employed in the two studies could be different. They suggest that competition between CA and other anions present in the fermentation broth for binding sites on the ionic resin could explain the higher value of CA q_m obtained.

Table 16 shows the percentages of adsorption of CA when a new experimental design was carried out. The factors for this new design were the load of CA in the fermentation broth, and the solid/liquid phases ratio. It was observed that treatments 1 and 2 were the ones that obtained the best recovery of CA, which is consistent, since they presented the highest number of solids adsorbed, when loads of 100 and 500 mg/L were used in the process. A statistical analysis was also performed, which is detailed in Appendix A-4.

Table 16. Effect of the concentration and solid/liquid ratio on adsorption percentages of CA using the ion exchange resin IRA 400.

Treatment	Concentration (mg/L)	Solid/liquid ratio (%)	CA recovered (%)
1	100	40	51.50 ± 0.15
2	500	45	52.48 ± 0.09
3	500	25	44.01 ± 0.62
4	500	25	44.79 ± 0.18
5	900	10	23.80 ± 0.33
6	500	5	11.91 ± 0.08
7	100	10	32.14 ± 0.29
8	65.70	25	39.21 ± 0.08
9	900	40	45.66 ± 0.17
10	1066	25	30.52 ± 0.33
11	500	25	44.29 ± 0.10

The adsorption was favored by increasing the adsorbent to liquid ratio. Thus, the highest separation was attained in the range of 40-45% solid/liquid ratio, adsorbing a mean of 52.48% of CA present in the broth. The response surface plot, which can be seen in Appendix A-4, shows a maximum value in the experimental region. According to the fitted regression model, a maximum CA extraction of 52.89% was predicted, very close to the value obtained in the experimental work, using a solid/liquid ratio of 43.80% and a concentration of 315.28 mg/L. Although the concentration differs, it is the solid/liquid ratio that has the greatest influence on the design. The CA concentration in the liquid does not significantly influence the mass of CA adsorbed since the available sites in the resin for the adsorption are the main factor associated with the separation. The resin is highly selective for CA, showing no significant difference between the adsorption capacity on defined and complex medium after pretreatment with the non-polar solvent, i.e., complex medium composition does not reduce the mass transfer driving force for CA adsorption.

To determine the optimum contact time between phases, samples taken at regular intervals were analyzed up to 70 min, to evaluate the adsorption capacity of the

resin and the time in which the CA reached equilibrium. To develop the procedure, conditions corresponding to 10 °C, pH 4.50, solid/liquid ratio 45% and a loading of 100 mg/L of CA in the complex fermentation broth were used. Table 17 shows the results.

Table 17. Result of the determination of the optimal time of contact in the CA adsorption process.

Time (min)	Supernatant concentration (mg/L)	CA adsorbed (mg/L)
0	100.10	0.00 ± 0.00
5	63.90	36.20 ± 0.01
10	53.80	46.30 ± 0.18
15	53.00	47.10 ± 0.37
20	51.60	48.50 ± 0.35
30	46.80	53.30 ± 0.39
40	44.80	55.30 ± 0.05
50	38.80	61.30 ± 0.06
60	38.90	61.20 ± 0.05
70	38.90	61.20 ± 0.08

It can be observed that after 50 min of contact between the solid and liquid phases, the concentration of CA reached its dynamic equilibrium. Performing the experiment at 1 h and 30 min or at 50 min will lead to similar results.

Time is a determining factor in the process due to the instability of the CA molecule. The temperature at which the experiments were carried out (10 °C), favored the CA recovery process, since the low temperature conditions allowed to decrease the degradation rate of the molecule in the fermentation media, generating a larger stability over time. Table 17 and Figure 9 show the dynamics of the adsorption process while approaching to the dynamic equilibrium.

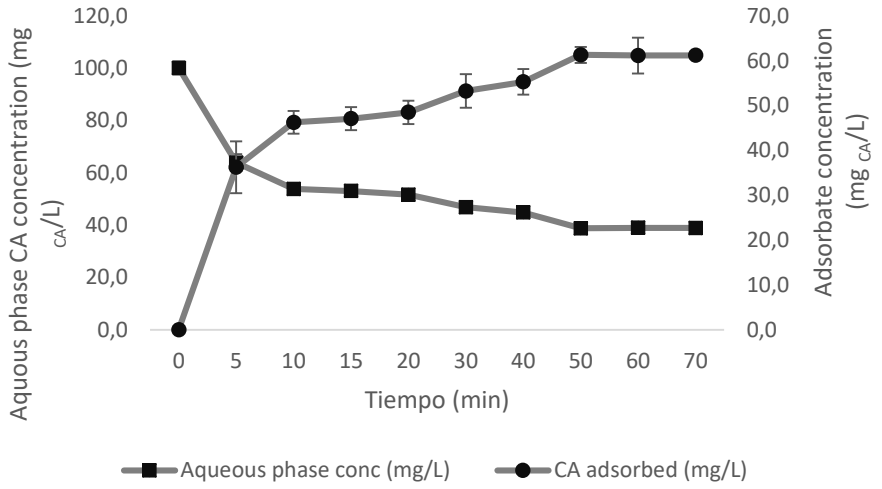

Figure 9. Determination of the optimum contact time in the CA adsorption process.

As observed in Figure 9, 46.3% of CA was recovered during the first 10 min of the process, indicating the rapid saturation of all sites available for CA adsorption. The process generates a great ionic interaction force or binding force between the resin and the CA, allowing, therefore, that the maximum adsorption capacity (q_m) is limited according to the operational conditions worked in the system, hence only 61.3% of the CA is recovered from the fermentation broth after 50 min. Barboza *et al.* (Barboza *et al*., 2002) determined the diffusivity of CA within resin pores. Additionally, they obtained adsorption isotherms and conducted kinetic studies to verify the effects of mass transfer on overall kinetics. The results found by the authors indicated complete CA adsorption within the initial 2 min. As the initial concentration increased, the total adsorbed CA decreased due to resin saturation. This suggests the potential utilization of this process in systems with brief contact times with CA, thereby mitigating pH-induced degradation effects. The analysis of the results presented leads to the conclusion that when evaluating the process, it is essential to consider both mass transfer and the adsorption step as limiting factors. In particular, the studies of Barboza *et al*. (Barboza *et al*., 2002) highlighted that when the external resistance to mass transfer is reduced, a greater amount of CA is dispersed through the resin pores. Yepes *et al.* (Yepes *et al.*, 2020) presented the CA adsorption kinetics as a function of CA_{Ce}, as well as the fit of the experimental

data to the Langmuir model. From the data presented in the results, at the 10 °C, solid/liquid ratio 40% and pH 6.0 condition, the author (Yepes *et al.*, 2020) evidenced the best capacity for CA adsorption (0.641 mg_{CA}/g_{resin}), with a maximum recovery at equilibrium corresponding to 55%. According to the authors, the value of q_m does not remain constant across different temperatures, showing a favorable relationship with decreasing temperature. This is because the sites available for CA binding undergo modifications in response to thermal changes. On the other hand, the parameter k is influenced by temperature, since an increase in temperature causes greater agitation of ionic molecules such as CA, which need to bind to the charged sites on the resin.

Also, there is a suspicion that compounds such as phosphate, sulfate, amino acids, small peptides, clavamas and cefamycin C might have attached to the resin. In addition, the authors (Yepes *et al.*, 2020) report the interference of amino acids such as tyrosine, histidine, proline and lysine in the adsorption processes. This interference limits the optimal recovery of CA, implying that the presence of these compounds could negatively affect the recovery efficiency in the process.

Degradation and stability of CA in fermentation broths

The degradation and stability of CA in fermentation media has been widely discussed (Gómez-Ríos *et al*., 2019; de Mancilha *et al*., 2014; Brites *et al*., 2012). This condition represents one of the great difficulties when implementing strategies to increase its production and subsequent recovery, due to its disappearance from the fermentation matrix, once it is obtained. Variations in environmental conditions e.g., pH and temperature, in addition to the presence of contaminating compounds, impede the recovery of the product in high percentages. In order to evaluate the effect of metabolic products and culture medium components, such as salts and nitrogen sources, on the stability of the product, the degradation associated with CA in the complex fermentation broth was performed under the best conditions of the adsorption process. Table 18 shows the corresponding results.

Table 18. Results of the degradation of CA in complex fermentation broth.

Time (min)	CA concentration in the complex fermentation broth (mg/L)
0	79.52 ± 0.00
5	78.78 ± 0.73
10	78.81 ± 0.70
15	78.38 ± 1.13
20	78.46 ± 1.05
30	78.74 ± 0.07
40	78.23 ± 1.29
50	77.55 ± 1.00
60	79.52 ± 0.02
70	78.78 ± 0.01

Under the thermal conditions of 10 °C and pH at 4.5, the degradation process of CA was not so evident. It was observed that even after 70 min, the concentration of CA remained stable; the degradation of CA in the complex fermentation broth was low, see Table 18. CA degradation is caused by several factors, including the temperature conditions, concentration, pH, and the presence of various components in the fermentation medium e.g., ammonium (Gómez *et al.,* 2019). The effect of ammonium on the CA degradation rate might be the result of the synthesis of an enzyme with degradative activity on CA (Roubos *et al*., 2002), and an increase in the thermal condition (Barboza *et al*., 2003).

Consequently, the findings of this study do not disagree with previous research, such as the study by Gómez *et al*. (Gómez *et al*., 2019) in terms of the time evaluated (70 min). In that study, the authors also observed negligible losses for all temperatures studied over a period of 1 h. This is good for the process, since after a certain time there is evidence of considerable losses of CA due to degradation, as was evidenced by Gómez *et al.,* (Gómez *et al*., 2019) who presented the study of CA degradation kinetics at low temperatures (−80, −20, 4, and 25 °C) and pH 6.8 in chemically defined fermentation broths. According to their results, the

degradation proceeded at the highest rate during the first 5-6 h. Product loss was between 8% and 12% during this time when operating at temperatures of 4 °C and 25 °C, respectively. The results reported by the authors indicate that the decomposition rate of CA tends to decrease significantly as the initial concentration of CA is reduced, and this has also been confirmed in the study in question.

These results support the idea that a lower initial concentration of CA may contribute to a higher stability and lower decomposition rate of the compound. It is important to consider these findings when designing CA production, separation and storage processes to ensure maximum preservation of the compound over time. Additionally, a shorter process time at low temperature will allow a significant reduction in the degradation rate of CA, resulting in a more efficient and cost-effective process.

The pH has been reported as another influential factor on CA degradation (Bersanetti *et al*., 2005; Mayer *et al*., 1996). Also, in Appendix A-3, it is evident from the Pareto figure that the most significant factor in the process was pH. An analysis of the response surface plot shows that treatments with basic pH values did not favor CA recovery. The supernatants employed for the degradation assays initially had a pH of 4.5. Following the adsorption process, the pH of the medium increased to 6.3. CA presents a higher stability at pH values around 6.2 (Costa *et al*., 2015), since less acid catalytic activity is presented on the degradation reaction; thus, CA decomposes more easily in solutions of basic character as explained in the previous session 4.2.1 (Bersanetti *et al*., 2005; Costa *et al*., 2015; Mayer *et al*., 1996).

The pH of the supernatant increased slightly after being exposed to the Amberlite IRA 400 resin. This allowed us to analyze that the resin promoted a basic character to the medium. The increase in pH of the supernatant after contact with Amberlite IRA 400 resin may be due to the interaction between the functional groups of the resin and the ions present in the solution. Amberlite IRA 400 resin is an ion exchange resin that contains charged functional groups, such as amino or carboxyl

groups, in its structure (Costa & Badino, 2015; Saudagar *et al.,* 2008). When the solution with an initial pH of 4.5 comes in contact with the resin, the functional groups of the resin may interact with the ions present in the solution. In this case, it is possible for the resin functional groups to act as bases, capturing hydrogen ions (H^+) from the solution and releasing hydroxyl ions (OH^-) in their place. This interaction can lead to an increase in the concentration of hydroxyl ions in the solution, which in turn would raise the pH of the medium, making it more basic (Costa & Badino, 2015; Mamani, 2018).

It is important to note that this phenomenon is characteristic of ion exchange resins and can vary depending on the functional groups present in the resin and the chemical composition of the solution (Saudagar *et al.,* 2008; Bersanetti *et al.,* 2005; Barboza *et al.,* 2003; Mayer *et al.,* 1997). However, it did not contribute to the degradation of the molecule, since the pH value in the process remained constant at 6.3, the value where the best recovery yields were obtained. It is important to highlight that a pH above 4.5 for adsorption process with IRA 400 resin would cause an increase in the supernatant final pH (basic), which would not favor the stability of the molecule, causing a high degradation rate, due to the mechanism of alkaline hydrolysis already mentioned.

On the other hand, it was evidenced that working with pH below 4.5 did not allow obtaining high values of CA recovery, which is interesting to analyze, since the basic contribution in the medium of the solid phase is limited and was not sufficient for the final pH to be in the range of greater favorability of the CA recovery conditions. This allowed us to analyze that the conditions found are acceptable for the CA recovery process in the fermentation broth. Also, the adsorption process of CA is affected by the interference of other substances present in the fermentation broth, such as the amino acids lysine, histidine, proline and tyrosine. These amino acids demonstrate significant affinity towards the charged sites of the resin. Therefore, it is necessary to implement preliminary impurity removal processes. These processes include

centrifugation, filtration and, in some cases, the application of aqueous two-phase phase systems (ATPS) (da Silva *et al*., 2012).

Similarly, removal of negatively charged ions, such as salts present in fermentation media, is essential. These ions have considerable affinity for the resin and can directly interfere with the CA recovery process (da Silva *et al.,* 2012; A. Mayer *et al.,* 1996), decreasing the driving force in the process. The implementation of broth acidification has been identified viable as a step prior to the adsorption process. It has been observed that precipitation of these impurities occurs in pH ranges below 2.0, making it an effective alternative to mitigate their presence and improve the efficiency of the adsorption process.

4.2.2 Desorption process of CA

Desorption can be defined as a process where a previously adsorbed substance is released from a surface (Thrower *et al*., 2018). Typically, desorption occurs when a given molecule gains enough energy to overcome the bounding energy that had previously been keeping it attached to the surface. Desorption is the exact opposite of adsorption. The desorption rate depends on the density of the chemical species, the strength of bonding to the surface, and the surface temperature. The spatial distribution of emitted species sometimes depends on surface atom arrangements or on the orientation of surface species (Matsushima, 2018).

The desorption of the CA retained by the ion exchange resin was carried out by elution. This elution involved the addition of a 10% (w/v) of NaCl solution at 22 °C and 240 rpm for a duration of 135 min. These operational parameters were established through pre-experimental procedures and existing bibliographies were used as references (Yepes *et al.,* 2020; Costa & Colli, 2015; Barboza *et al*., 2003). Subsequently, CA concentrations taken over time were analyzed in the chromatography equipment HPLC, see results in Table 19.

Table 19. Result of the desorption of CA from *S. clavuligerus* cultures in complex fermentation broth.

Time (min)	CA concentration (mg/L)
0	0.00 ± 0.00
15	27.76 ± 0.53
30	30.32 ± 0.28
45	31.86 ± 0.13
60	32.81 ± 0.36
75	32.88 ± 0.48
90	32.47 ± 0.13
105	30.23 ± 0.36
120	29.33 ± 0.20
135	28.66 ± 0.12

Table 19 shows the desorption of CA overtime. As observed, the larger part adsorbate was released during the first 15 min of elution, reaching its maximum concentration at 75 min. These observation match data already reported by Barboza *et al*. (Barboza *et al*., 2003). It is important to point out the larger time required for desorption compared to that of adsorption. One possible reason could be due to the strong ionic interaction between resin and CA. This interaction overcomes the adhesive strength between the resin and the chloride ion (Mayer *et al.,* 1996). The best thermal condition for adsorption (10 °C) differs from the desorption temperature (22 °C), which, according to Barboza *et al*. (Barboza *et al*., 2003) is related to the adsorption enthalpy, which showed a $\Delta H°$ value of -29.15 kJ/mol. These values portray an exothermic process in the formation of the CA complex on sites of the resin, favored at low temperatures; in turn, the process of CA release is endothermic, and requires higher temperatures (22 °C) for the dissociation of the resin-CA complex (Yepes *et al*., 2020).

Costa *et al*. (Costa *et al.,* 2015) highlighted the importance of incubating the mixture of resin-CA/NaCl solution for a minimum of 90 min to achieve the maximum possible release of the adsorbate. In their research, they were able to desorb approximately 70% of CA from the resin using NaCl at a concentration of 10% (w/v). Desorption

kinetics depend on various factors, including resin type, CA concentration, NaCl concentration, and temperature. Further investigations and optimization experiments can help to refine the desorption process and to identify the optimal conditions for maximizing CA release from the resin.

Yepes *et al*. (Yepes *et al*., 2020) found that most of the CA is released during the first 60 min of incubation, and after 120 min the maximum concentration of CA is detected in the aqueous solution reaching 65%. Accordingly, a decrease in the time required to reach equilibrium in the process is beneficial, since CA degradation of CA is minimized. Barboza *et al*., (Barboza *et al.,* 2003), carried out five experiments for the elution study; these were executed at three different temperatures (10, 25 and 30 °C). The elution results were associated to temperature by the Arrhenius equation, showing that the desorption process is facilitated at higher temperatures, which increases the agitation state of the molecules, allowing the ionic CA molecules to be more easily displaced from the sites where they are located.

This behavior can also be analyzed from the standpoint of the state of agitation of the ionic molecules (CA^- and Cl^-) that bind to the loaded sites of the resin. At higher temperatures the shock frequency is greater, which favors desorption (Barboza *et al*., 2003). Likewise, it can be evidenced that the desorption process is endothermic, so at high temperatures the process is favored. The maximum desorption percentage reached under the best experimental conditions was 53.64%. Consequently, the results of this study differ from previous investigations such as those of Costa *et al.* and Yepes *et al* (Yepes *et al.,* 2020; Costa *et al.,* 2015). who have shown that the time required to reach the maximum concentration of CA in the desorption process is longer than that recorded in this work. This discrepancy could have unfavorable implications for this stage, due to the temperatures employed (above 20 °C), as these promote the rate of CA degradation.

As observed, there were some losses between the adsorption and desorption process, due to the fact that the adsorption agent (ion exchange resin IRA 400)

presented some limitations both in the fermentation broth and in the saline solution used at the moment of CA desorption. Among the limitations presented by the resin we can mention the ionic interference of other substances in the adsorption and desorption stages; the presence of amino acids such as lysine, histidine, proline and tyrosine, which present a good affinity for the charged sites of the resin, limits a high recovery of CA (da Silva *et al*., 2012). The presence of high concentrations of negatively charged ions (Cl^-) in salt solutions can lead to ionic competition with the resin exchange sites. This can decrease desorption efficiency by interfering with the release of adsorbed CA (da Silva *et al.,* 2012; A. Mayer *et al.,* 1996). Likewise, the high concentration of ions (Cl^-) in the salt solution could accelerate the saturation of the active sites of Amberlite IRA 400, limiting its ability to completely desorb the adsorbed CA. For this, a new experimental design was made, Table 20, where, for the desorption process, a better relation between the solid/liquid ratio and the concentration of NaCl in water, was found.

It is important to mention that NaCl, as salt, presents a high ionic strength and is able to generate a high concentration of ions in solution (Chen *et al.,* 2018). This high ionic strength environment favors the competitive substitution of CA molecules adsorbed on the resin. The presence of Na^+ ions in solution effectively competes with CA for binding sites on the adsorbent, resulting in the release of CA molecules and their passage to the liquid phase (Chen *et al.,* 2018; Gupta *et al.,* 2012). The addition of NaCl helps to create an environment in which electrostatic interactions between CA and the adsorbent are weakened, thus facilitating the release of CA molecules (Nishad *et al.,* 2017).

In addition to NaCl, there are other ionic solutions that can be used to desorb CA from the resin, such as sodium hydroxide or potassium chloride (Mayer *et al*., 1996). The choice of desorbent solution will depend on several factors, such as the type of resin used, the nature of the process and the desired conditions for desorption. It is essential to take into account the specific requirements and limitations of the

process, such as the stability of the CA under alkaline conditions, which precludes the use of hydroxides (Chen *et al.,* 2018; Scott *et al.,* 2003). It is also necessary to consider the compatibility of the desorbent solution with downstream processes and the general purity requirements of the recovered CA. Performing prior experimental evaluations with different desorbent solutions can help to determine the most suitable option to achieve maximum yield in CA recovery.

To carry out the experimental design to improve the recovery of CA in the desorption process with the ion exchange resin, two factors (percentage of NaCl in water and the solid/aqueous phase ratio) were evaluated, each with two levels, for a total of 10 experiments without replicates, see table 20.

Table 20. Evaluation of the NaCl concentration in water and the solid/aqueous phase ratio in the desorption process of CA.

Treatment	Solid/liquid ratio	% NaCl	% CA recovery
1	60	10	50.72 ± 0.31
2	60	10	42.94 ± 0.20
3	60	17.10	35.06 ± 0.37
4	40	15	34.27 ± 0.29
5	100	5	29.70 ± 0.12
6	60	2.90	23.88 ± 0.46
7	100	15	24.87 ± 0.33
8	40	5	27.54 ± 0.22
9	36	10	29.26 ± 0.05
10	143	10	40.09 ± 0.18

As observed (Table 20), the concentration of salt in the solvent used coincides with that shown by the experimental design; the best treatment found was 1, which uses a solid/liquid ratio of 60% and a concentration of NaCl 10%. The performance of the desorption of CA can be improved by reducing the liquid phase in the process, which will increase the solid/liquid ratio. However, it must be considered that a larger amount of adsorbent used at industrial scale process will increase the operating

cost and the amount of waste generated. After analyzing the results found in the experimental designs, it was decided to continue working with a solid/liquid ratio of 45%, since the recovery of CA does not differ that much from that of the solid/liquid ratio at 60%.

4.3 Study of the complete strategy of the adsorption + extraction process of CA

Once the adsorption and desorption processes were carried out, the entire strategy (proposed) was performed under the best conditions found in the individual processes. For this, two CA solutions (100 mg/L and the other at 500 mg/L) were prepared. The complete process considered adsorption + desorption + liquid-liquid extraction. Table 21 shows the amount of CA recovered from the fermentation broth when using the combined adsorption and extraction strategies.

Table 21. Results of the complete process (adsorption-desorption-extraction) for obtaining CA when using a concentration of 100 mg/L and 500 mg/L.

Stage	% CA recovered (100 mg/L)	% CA recovered (500 mg/L)
Adsorption	40.76 ± 0.16	48.88 ± 0.36
Desorption	85.89 ± 0.50	88.46 ± 0.52
Extraction	55.49 ± 0.08	58.60 ± 0.19

The yields obtained in the adsorption-desorption process for both loads were between 40.76-85.89% (load of 100 mg/L) and 48.88-88.46% (load of 500 mg/L), respectively. This strategy was not entirely satisfactory for the recovery of CA, since the yields obtained were very low, *i.e*., around 25% for the whole process. This is due to the high instability of CA allied to its high solubility in water, which would subsequently hinder the formation of its solid and dry forms (salts) required for its use in medicinal preparations.

This shows a considerable loss of CA with respect to the direct extraction from the medium using organic solvents. According to the results, found in each stage, it was expected that this strategy would not be workable, since the interferences caused by impurities bound to the active sites of the resin would generate a loss in the adsorption capacity. However, the strategy would be considered interesting if an 80% recovery in the adsorption stage would be reached. This eventual condition would allow recovering the concentrated CA for the subsequent extraction and precipitation stage, thus making the process more efficient, reducing the volumes of ethyl acetate and purification solvent to be used; this clearly would generate a reduction in the cost and contamination of the process.

5. Conclusions and recommendations

5.1 Conclusions

It was observed that the effect of most significant factors on the response variable were pH and the solid/liquid ratio, since these show a significant increase or decrease in CA recovery. Ethyl acetate was selected as the best alternative for extraction considering its low price and good performance. The use of low volumes of organic solvent (ratios 0.2-0.6) led to 40% less CA mass extracted from the broth. However, the CA concentration on the organic phase was up to 3-fold higher in comparison with those extracted at high solvent rates.

CA adsorption was enhanced by increasing the adsorbent-to-liquid ratio. Consequently, the most effective separation occurred within the 40%–45% solid/liquid ratio range, leading to the adsorption of an average of 47.70% of CA present in the broth. Despite this, the overall adsorption process fell short of being entirely satisfactory for CA recovery, as the yields obtained were relatively low, indicating a significant CA loss (approximately 25%) compared to liquid–liquid extraction using ethyl acetate (which achieved a 60.37% recovery for the complex medium). Based on the results, a potential contributing factor to this outcome could be the interferences resulting from impurities adhering to the active sites of the resin, leading to a reduction in adsorption capacity. The extraction strategy potentially allows up to 80% of product recovery. Meeting this condition would

enable the retrieval of concentrated CA for subsequent precipitation and purification stages, thereby enhancing overall process efficiency and resulting in a clear reduction in both cost and environmental impact.

The separation strategy in which the adsorption and desorption stages were used as previous stages, did not allow obtaining a good result in the recovery yield of CA, due to the fact that considerable losses were presented in the process. This strategy would be promising if a recovery by stages were carried out. A recovery strategy by stages in the adsorption process would allow purifying the solution and reducing the degradation rate of the CA, thus increasing the extraction percentage in the subsequent stages with the ester solvent, such as ethyl acetate.

It was determined that to improve performance it was necessary to increase the solid/liquid ratio during the desorption process from 45% to 60%. This increase would be achieved by reducing the amount of liquid NaCl solution to a concentration of 10%. Regarding the response surface methodology, it was observed that at a concentration of approximately 396.50 mg/L and a ratio of 1:1.20, the best extraction yield of CA would be obtained in the process.

5.2 Recommendations

A possible alternative to be implemented for increasing the recovery yield would be a mechanism of recirculation or stepwise adsorption for the medium in which the CA is found. Although it was not possible to develop this proposal in this work due to time constraints and research interest, it would be interesting to explore this process in future works. Additionally, it is attractive because this process can avoid the impurities that the medium contains, such as amino acids or heavy chain proteins. This would allow greater efficiency in the subsequent stages of extraction with the organic solvent to be used. This process can minimize product degradation by reducing the contact time required in batch operations with continuous CA

extraction. In addition, the continuous process reduces costs with adsorbent because less resin is needed, which is beneficial in the case of an industrial-scale application of the process.

Another option would be to implement the addition of the organic solvent directly to the production medium, thus combining fermentation + separation of the CA product. Also, experiments were the solvent and the resin used are changed, making sure that the solvent selected is from the ester family and the ion exchange resin, are also recommended.

A. Appendix A: Response Surface Methodology

A-1. Response surface methodology is a set of techniques used to model and analyze problems in which a variable of interest is influenced by at least two quantitative factors (Gutiérrez & Salazar, 2008). Among the strategies developed to achieve the highest recovery of CA from the fermentation broth, it was found that due to optimization in process time, raw material to be used and reduction of environmental pollution in a possible industrial scale process, liquid extraction was presented as one of the best alternatives to implement.

A statistical and graphic analysis of the process was performed in the R software, to evaluate the effect of the variable's temperature and pH on the CA extraction yield. As mentioned during the written paper, the design to be used was the response surface methodology.

In relation to the experimental design in Table 11, where the temperature and pH factors are evaluated, we proceed to show the results obtained from the statistical analysis. In this, a significant interaction between the controlled variables is observed, Table 22.

Table 22. Evaluation of the estimated effects and interactions for CA recovery using temperature and pH factors in the liquid extraction process.

Effect	Estimate	Standard error
Average	39.60	0.28
A: pH	-28.12	0.28
B: Temperature	7.31	0.28
AA	-0.54	0.37
AB	5.57	0.39
BB	-0.92	0.37

To visualize the estimates in decreasing order of importance, the Pareto chart is used, Figure 10. In this graph, we can see that the pH variable has the greatest effect on the response variable.

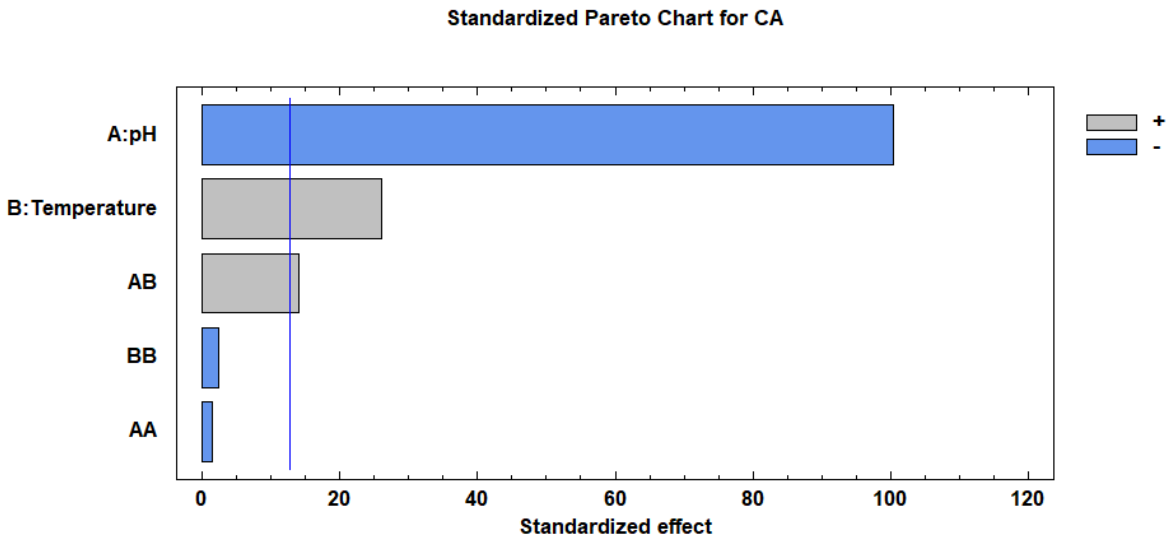

Figure 10. Normalized Pareto plot for CA recovery using temperature and pH factors in the liquid extraction process.

From the analysis of variance, it was observed that both main effects were significant, given that their p-value was less than 0.05. Furthermore, in the ANOVA results, Table 23, it was noted that the highest significant p-value corresponded to the interaction between the controlled variables, with a value of 0.0451. This value is below the predefined significance level, indicating that the interaction is

statistically significant at a 95% confidence level, thus suggesting that the interactions should be considered.

Table 23. Analysis of variance for CA recovery using temperature and pH factors in the liquid extraction process.

Source	Sum of Squares	Mean Square	F-Value	P-Value
Model	1721.05	344.21	16.59	0.0088
A: pH	1582.01	1582.01	76,24	0.0063
B: Temperature	106.96	106.96	5.15	0.0244
AB	31.08	31.08	1.50	0.0451
Lack of fit	83.85	16.77	176.12	0.0723
Pure error	0.16	0.16		
Total (error)	1804.05			

The value of R_a^2 adjusted for degrees of freedom was 0.954; which means that the developed model explains 95.4% of the variability in the percentage of CA extraction with respect to pH and temperature. This suggests that the model has a good fit to the data and can be used as a tool to estimate the behavior of the percentage of CA extraction. The equation representing the fitted model is as follows:

$$\hat{y} = \ 71.132 - 11.200x_1 - 0.544x_2 + 0.446x_1x_2 \qquad [\text{Equation } 6]$$

Where, $\hat{y}$ represents the percentage of CA extraction. While x_1 and x_2 represent the controlled variables, i.e., pH and temperature, respectively. A considerable linear effect was observed between pH and temperature, since the quadratic factors were not significant. In the response surface plot, Figure 11 it was observed that there is a maximum value in the experimental region.

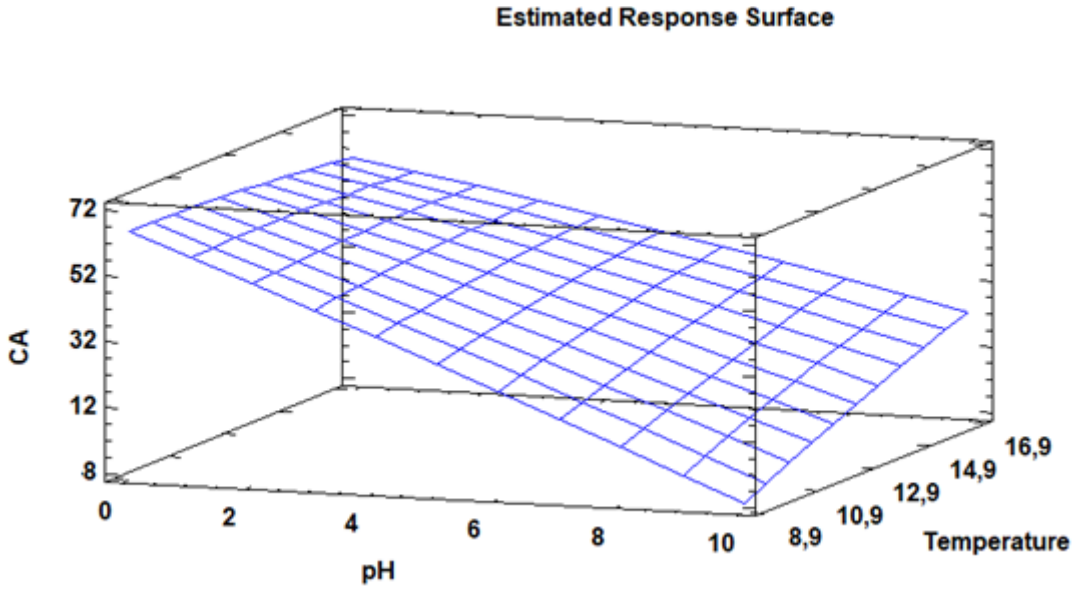

Figure 11. Estimated Response Surface for CA recovery using temperature and pH factors in the liquid extraction process

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The fitted regression model predicted that the maximum CA extraction would be 59.31%, using a pH of 0.96 and a temperature of 8.96 °C. Therefore, we can conclude that the experimental values or conditions selected in the research work (which achieved an extraction percentage of 57.14% with a pH of 2.0 and a temperature of 10 °C) were not similar to the values predicted by the model. However, the difference in values of the acid medium is not far from each other, and being the most significant variable, it allowed the recovery percentages to be very near.

As can be seen in the response surface figure 11 above, the optimum is at one extreme. This suggests the need to explore other values around this point to possibly find higher values of the response variable. Although the importance of the statistical decisions is recognized at this point, it is important to make it clear that from the beginning the decisions were made solely based on the experimental values.

A-2. In relation to the experimental design in Table 14, where the CA concentration in the fermentation broth and the aqueous/organic phase ratio factors are evaluated, we proceed to show the results obtained from the statistical analysis. Table 24 shows each of the estimated effects and interactions of the variables on the percentage of CA recovery. Also shown is the standard error of each of the effects, which measures their sampling error.

Table 24. Evaluation of estimated effects and interactions for CA recovery using concentration and liquid ratio factors in the liquid extraction process.

Effect	Estimate	Standard error
Average	29.82	0.015
A: Concentration	-8.38	0.019
B: RLL	17.79	0.019
AA	2.33	0.020
AB	0.73	0.021
BB	4.61	0.019

The p-value is a statistical measure that helps determine the significance of an observed result in a hypothesis test. It is a probability value that quantifies the strength of evidence against the null hypothesis. In hypothesis testing, the null hypothesis is the assumption being tested, and the alternative hypothesis is the alternative assumption (Greenland *et al.,* 2016). The effect whose p-value is less than the predefined significance (0.05) is declared statistically significant.

From the analysis of variance, it was observed that all the effects were influential, given that their p-value were less than $\alpha = 0.05$. It could be seen that both main effects were significant (see Figure 12). Within the effects by double interactions, the most significant was contributed by that of concentration.

In the ANOVA results, Table 25, it was observed that the highest p-value is given by the double interaction between the controlled variables, with a value of 0.0185, lower than the predefined significance value, which indicates that it is statistically significant and, therefore, all interactions should be taken into account.

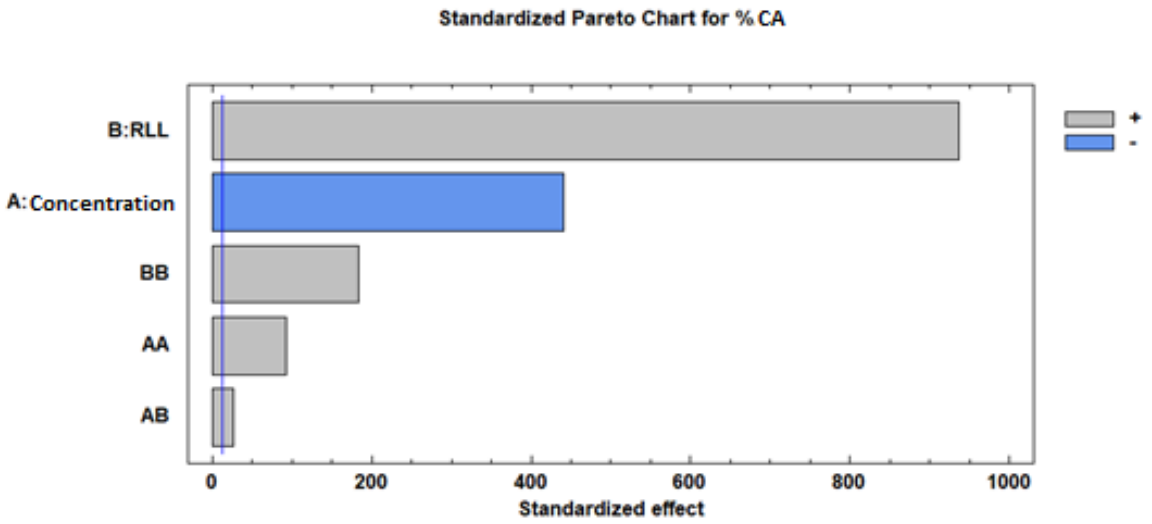

Figure 12. Normalized Pareto plot for CA recovery using concentration and liquid ratio factors in the liquid extraction process.

From the analysis of variance, it was observed that the five effects were significant, given that their p-values were less than 0.05. Furthermore, in the ANOVA results, it was noted that the highest significant p-value corresponded to the interaction between the controlled variables, with a value of 0.0185. It can be seen, Figure 12 and Table 25, that the most significant controlled variable was the liquid ratio between the aqueous and organic phase.

Table 25. Analysis of variance for CA recovery using concentration and liquid ratio factors in the liquid extraction process.

Source	Sum of Squares	Mean Square	F-Value	P-Value
Model	798.790	159.760	9.46	0.0246
A: Concentration	140.564	140.564	8.32	0.0011
B: RLL	633.247	633.247	37.50	0.0005
AA	6.171	6.171	0.37	0.0054
AB	0.533	0.533	0.03	0.0185
BB	24.274	24.274	1.44	0.0027
Lack of fit	67.549	22.517	50036.32	0.0532
Pure error	0.0004	0.0004		
Total (error)	866.342			

The liquid-liquid ratio between the aqueous/organic phase presented a strongly linear effect, and although its quadratic factor was significant, it constituted the largest effect of the model, Table 25, whereas, concentration presented a more pronounced curvature effect; in summary, the overall behavior of the effects was not linear, given that, the quadratic factors were significant.

The value of R_a^2 adjusted by degrees of freedom was 0.922; thus, the model obtained explains 92.203% of the variability of the CA extraction percentage with respect to concentration and liquid ratio. This suggests that the model fits the experimental data taken. The fitted model is represented by the following equation:

$$\hat{y} = 46.339 - 0.047x_1 + 2.222x_2 + 0.000019x_1^2 + 14.402x_2^2 + 0.0037x_1x_2 \qquad \text{[Equation 7]}$$

Where, $\hat{y}$ represents the percentage of CA extraction. x_1 and x_2 represent the controlled variables; concentration and liquid ratio between aqueous/organic phase, respectively. It should be noted that for the above equation, the variables take their real values.

In the response surface plot, Figure 13, it was observed that there is a maximum value in the experimental region. The aqueous/organic phase ratio presented a strong linear effect, and although its quadratic factor was significant, it constituted the largest effect of the model, while, the concentration presented a slight curvature effect. In summary, the overall behavior of the effects was not linear, given that, the quadratic factors were significant.

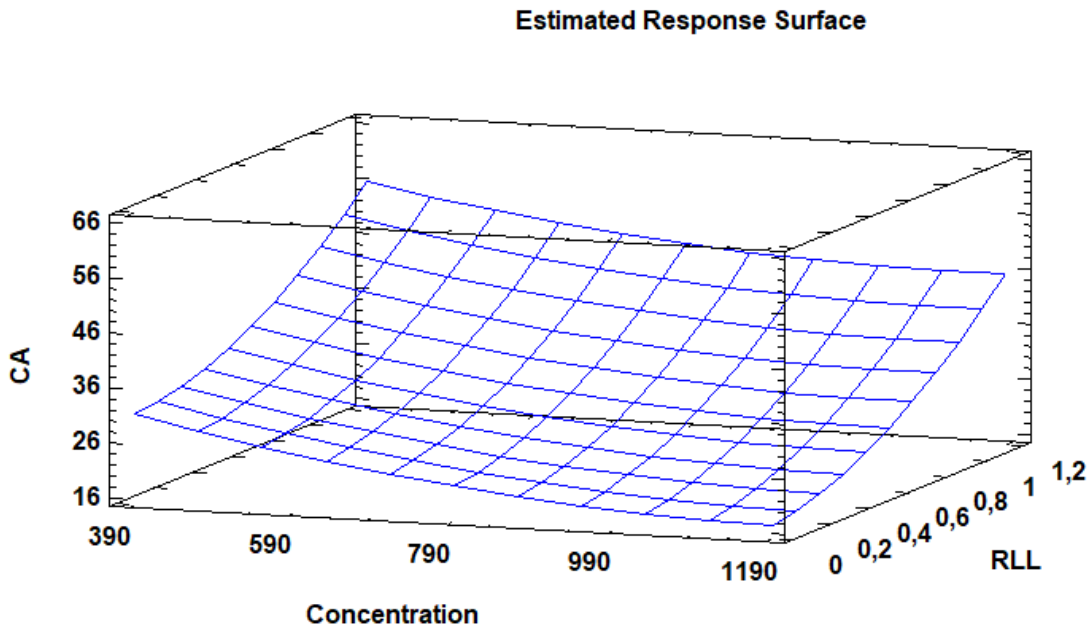

Figure 13. Response surface plot for CA recovery using concentration and liquid ratio factors in the liquid extraction process.

An analysis of the response surface, Figure 13, indicated that the region of the highest yield of CA is associated with the high liquid ratios between the aqueous/organic phases studied here. The fitted model of regression predicted the maximum CA extraction (54.538%) by using a liquid ratio of 1:1.20 and a concentration of 396.45 mg/L. The assumption of normality, constant variance and independence was verified (Table 26) because the data shown in the analytical tests obtained p-values greater than the predefined significance (α=0.05), thus proving that the distribution of the residuals of the fitted model tends to a normal distribution (Gutiérrez & Salazar, 2008).

Table 26. Analytical testing of assumptions of normality, constant variance, and independence for CA recovery using concentration and liquid ratio factors in the liquid extraction process.

Assumption verified	Test performed	P – Value
Normality	Shapiro Wilks	0.9992

Constant variance	Breusch-Pagan test	0.1437
Independence	Durbin-watson test	0.5676

A-3. In relation to the experimental design in Table 15, where the temperature and pH variables for the adsorption process are evaluated, Figure 14, we proceed to show the results obtained from the statistical analysis. Each of the estimated effects and the interactions of the temperature and pH variables for the adsorption process are shown below. In this graph, Figure 14, it can be seen that the most significant factor in the process was pH as described in the work from an experimental analysis.

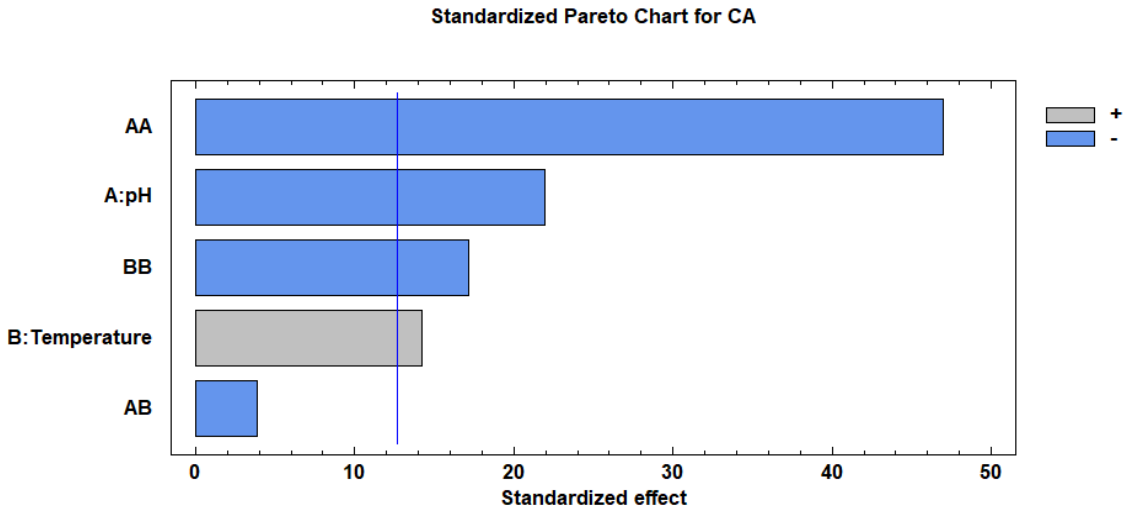

Figure 14. Normalized Pareto plot for CA recovery using temperature and pH factors in the adsorption process.

After analyzing the variance, it was observed that only four effects were significant, with a p-value of less than 0.05. Both main effects were significant, as were the quadratic effects. The interaction between the controlled variables yielded a value of 0.1615, so this interaction is not significant and was therefore not taken into account, see Table 27.

Table 27. Analysis of variance for CA recovery using temperature and pH factors in the adsorption process.

Source	Sum of Squares	Mean Square	F-Value	P-Value
Model	282.11	56.42	6.68	0.0448
A: pH	46.77	46.77	5.54	0.0289
B: Temperature	19.58	19.58	2.32	0.0447
AA	213.29	213.29	25.26	0.0136
BB	28.53	28.53	3.38	0.0370
Lack of fit	35.12	8.78	115.98	0.0775
Pure error	0.097	0.097		
Total (error)	315.89			

The R_a^2 value obtained was 0.888, indicating that the model explains 88.85% of the variability in the percentage of CA extraction as a function of temperature and pH. This indicates that the model fits the experimental data taken. A strong curvature effect was observed for pH, with a significant quadratic component. The fitted model equation is as follows:

$$\hat{y} = -48.235 + 8.869x_1 + 10.618x_2 - 1.093x_1^2 - 0.399x_2^2 \quad [\text{Equation } 8]$$

Where, $\hat{y}$ represents the percentage of CA extraction. While x_1 and x_2 represent the controlled variables, i.e., pH and temperature, respectively. In the response surface plot, Figure 15, it is observed that there is a maximum value in the experimental region.

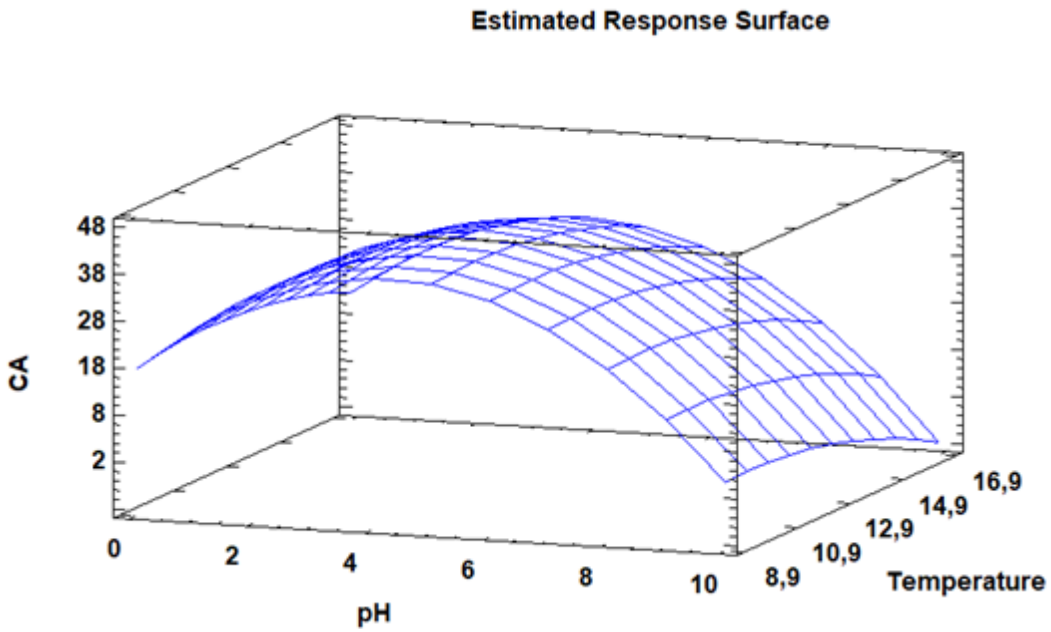

Figure 15. Estimated response surface for CA recovery using temperature and pH factors in the adsorption process.

From the response surface plot, it is observed that there is a maximum value in the experimental region. The fitted regression model predicted a maximum CA extraction of 40.28%, with a temperature of 13.28 °C and a pH of 4.06. This predicted value of recovery does not differ much from that found experimentally, where a percentage of 40.04% of CA present in the medium was reached, with a temperature of 12.5 °C and a pH of 4.5.

A-4. In relation to the experimental design in Table 16, each of the estimated effects and the interactions of the variables CA concentration in the fermentation broth and solid/liquid ratio on the percentage recovery of CA are shown below. The Figure 16 shows that the most significant factor in the process was the solid/liquid ratio.

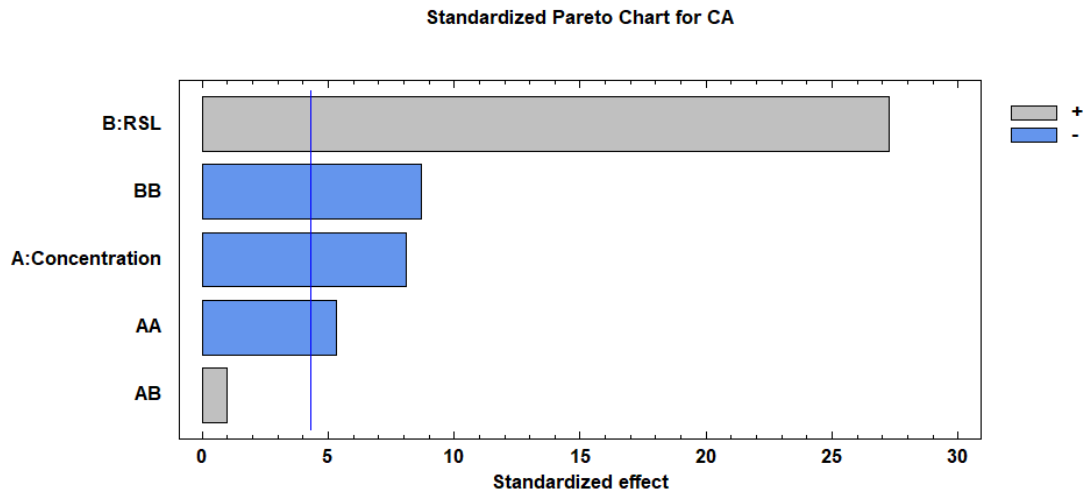

Figure 16. Normalized Pareto plot for CA recovery using concentration and solid/liquid ratio factors in the adsorption process.

After analyzing the variance, it was observed that all the effects, except for the relationship between the controlled variables, were significant, with a p-value of less than 0.05. Both main effects were found to be significant. Among the double interaction effects, Table 28, the most significant was that of the solid/liquid ratio. In the ANOVA results, it was noted that the highest p-value corresponded to the double interaction between the controlled variables, with a value of 0.4315, so this interaction is not significant. Moreover, this is also observed in the figure, since this interaction is below the threshold.

Table 28. Analysis of variance for CA recovery using concentration and solid/liquid ratio factors in the adsorption process.

Source	Sum of Squares	Mean Square	F-Value	P-Value
Model	1426.92	285.38	14.56	0.0113
A:Concentation	87.58	87.58	4.47	0.0375
B:RSL	1215.11	1215.11	61.99	0.0101
AA	58.63	58.63	2.99	0.0458
BB	110.49	110.49	5.64	0.0334
Lack of fit	79.66	19.92	85.58	0.0912
Pure error	0.31	0.31		
Total (error)	1505.32			

The R_a^2 value obtained was 0.947, indicating that the model explains 94.70% of the variability in the percentage of CA extraction as a function of concentration and solid/liquid ratio. A strong curvature effect was observed for the solid/liquid ratio, with a significant quadratic component. On the other hand, concentration exhibited a more pronounced curvature, suggesting an overall nonlinear behavior due to the significance of the quadratic terms. The fitted model equation is as follows:

$$\hat{y} = 8.743 + 0.014x_1 + 1.914x_2 - 0.000022x_1^2 - 0.021x_2^2 \qquad \text{[Equation 9]}$$

From the response surface plot, Figure 17, it can be seen that there is a maximum value in the experimental region. According to the fitted regression model, a maximum CA extraction of 52.89% was predicted, using a solid/liquid ratio of 43.80% and a concentration of 315.28 mg/L. However, in the experiment, a recovery of 52.48% of the CA present in the medium was achieved, with a solid/liquid ratio close to 45% and a concentration of 500 mg/L.

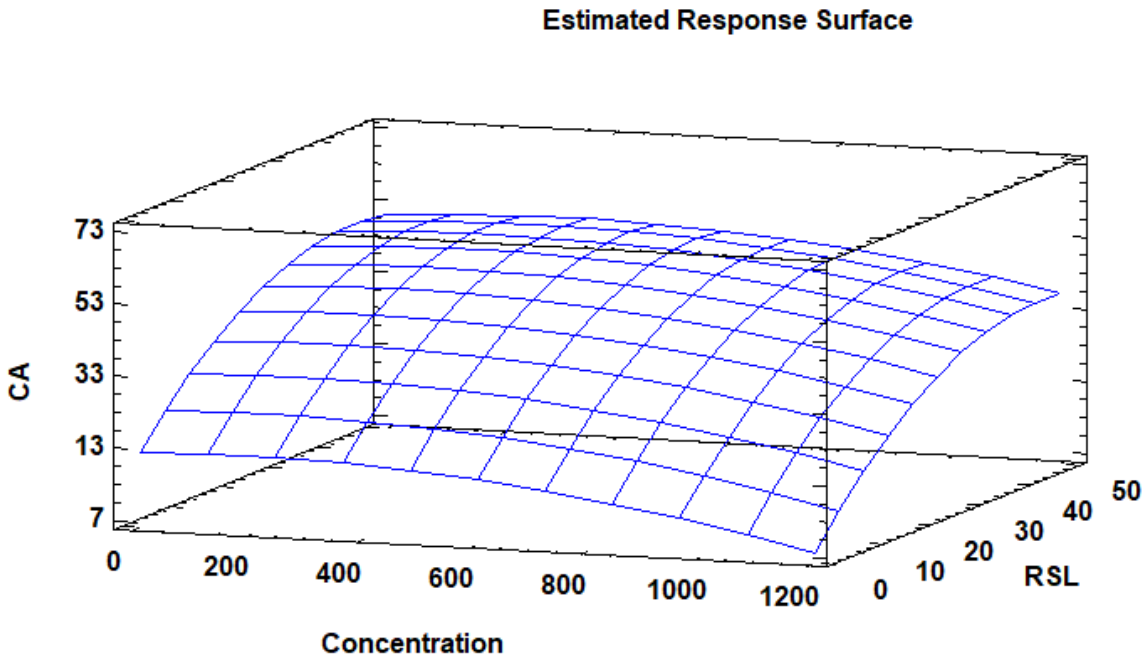

Figure 17. Estimated response surface for CA recovery using concentration and solid/liquid ratio factors in the adsorption process.

It is evident that the values obtained for the concentration variable from the model do not closely resemble the experimental values. However, it is interesting to note that the CA recovery values do not present a considerable difference. This is because the liquid/solid ratio variable was similar in both cases. Since this variable has the most impact on the process, no significant difference is observed in the results.

The model developed in the R software represented a useful tool to analyze and study the CA separation process, under the study conditions. The advantages of the simulation are evidenced in the possibility of predicting the behavior of the system, by changing one or several operating parameters without intervention in the real system. This reduces the cost of operation, the risk of accidents and increases the safety and productivity of the process, since the simulated experimental design is carried out in less time, does not require replications, does not pollute the environment (waste generation), does not require physical laboratory space, thus increasing the safety and integrity of the experimenter and, finally, does not require reagent consumption.

B. Appendix B: Structure of the code generated in the statistical software R

```
# Lectura de datos
datos <- Datos
attach(datos)

# Análisis descriptivo

par(mfrow=c(1,2))
boxplot(%CA ~ Concentration,data=datos)
boxplot(%CA ~ RLL,data=datos)

require(graphics)
par(mfrow=c(1,1))
plot.design(%CA ~ factor(Concentration)+factor(RLL),data=datos)

require(gplots)
par(mfrow=c(1,2))
plotmeans(%CA ~ Concentration, data=datos)
plotmeans(%CA ~ RLL, data=datos)

# Prueba de Normalidad par la variable respuesta
require(car)
par(mfrow=c(1,3))
qqPlot(%CA, xlab="Cuantiles teóricos", ylab="Cuantiles muestrales", main="Gráfico
cuantil-cuantil")
hist(%CA, xlab="%CA", ylab="Frecuencia", main="Histograma")
boxplot(%CA, xlab="%CA",  main="Gráfico de cajas")

# Prueba estadística
require(nortest)
shapiro.test(%CA)
ad.test(%CA)

# Metodología de superficie respuesta
library(rsm)
```

```
# Gráfico del diseño
par(mfrow=c(1,1))
plot(VarCod[ , c(1:2)], pch = 16, main = "Diseño CC")
abline(h = c(1, -1), col = "lightgrey")
abline(v = c(1, -1), col = "lightgrey")

# Modelo lineal de primer orden sin curvatura
#datos.fo2=rsm(%CA ~ FO(Concentration,RLL), data=VarCod)

# Modelo polinómico de segundo orden
datos.fo2=rsm(%CA ~
FO(Concentration,RLL)+TWI(Concentration,RLL)+PQ(Concentration,RLL), data=VarCod)

# Resultado del ajuste
summary(datos.fo2)

#Datos en la dirección óptima
#paso <- seq(0,14,0.63)
#steepest(datos.fo2, descent = FALSE, dist =paso)

# Gráfico de Superficie
par(mfrow = c(1,1))
persp(datos.fo2, as.list(c( ~Concentration*RLL)), atpos= 1, at =
list(Concentration=0,RLL=0))

# Gráfico de contornos
par(mfrow = c(1,1))
contour(datos.fo2, as.list(c( ~Concentration*RLL)), image= FALSE, at =
list(Concentration=0,RLL=0), atpos= 1)

# Análisis de residuales
residuales <- rstandard(datos.fo2)
summary(residuales)

# Prueba de Normalidad

# Procedimiento gráfico
require(car)
par(mfrow=c(1,3))
qqPlot(residuales, xlab="Cuantiles teóricos", ylab="Cuantiles muestrales", main="Gráfico
cuantil-cuantil")
hist(residuales, xlab="Residuales", ylab="Frecuencia", main="Histograma")
boxplot(residuales, xlab="Residuales",  main="Gráfico de cajas")

# Prueba estadística
require(nortest)
shapiro.test(residuales)
ad.test(residuales)
```

```
# Prueba de varianza constante

# Procedimiento gráfico
valores_ajustados<-(fitted(datos.fo2))

par(mfrow=c(2,2))
plot(valores_ajustados, residuales, xlab="Valores ajustados", ylab="Residuales")
abline(h=0, col = "gray60")
plot(as.numeric(Tiempo), residuales, xlab="Tiempo", ylab="Residuales")
abline(h=0, col = "gray60")
plot(as.numeric(Temperatura), residuales, xlab="Temperatura", ylab="Residuales")
abline(h=0, col = "gray60")

#Prueba estadística
require(lmtest)
bptest(datos.fo2)

# Prueba de independencia

#Procedimiento gráfico
par(mfrow=c(1,3))
plot(residuales, pch=16, ylab="Residuales", xlab="Orden", main="Gráfico de Orden vs
Residuales", ylim=c(-3,3))
abline(h=c(-3,0,3))
acf(residuales,ylim=c(-3,3), main="Gráfico de ACF")
pacf(residuales,ylim=c(-3,3), main="Gráfico de PACF", ylab="PACF")

#Prueba estadística
require(lmtest)
dwtest(datos.fo2,alternative="two.sided")
```

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