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OPTIMIZATION OF ANALYTICAL METHOD FOR QUANTIFICATION OF 5-METHOXYCANTHIN-6-ONE BY FACTORIAL DESIGN AND PRELIMINARY STABILITY TEST

Vinicius Martins Bamberg [1], Larissa Araújo Rolim [1], Miguel Martínez [2], Cybelly Marques de Melo [3], Emanuella Chiara Valença Pereira [1], María Elena Ferreira [4], Ninfa Vera de Bilbao [4], Erwan Poupon [5], Alain Fournet [6], Gerardo Cebrián-Torrejón [*7] and Pedro José Rolim-Neto [3]

Federal University of Vale do São Francisco [1], UNIVASF, Petrolina - PE, Brasil.
Universidad Nacional de Asunción [2], Facultad de Ciencias Exactas y Naturales, Departamento de Biología, Laboratorio de Análisis de Recursos Vegetales, Área Fitoquímica, Paraguay.
Federal University of Pernambuco [3], UFPE, Recife, Brasil.
Departamento de Medicina Tropical [4], Instituto de Investigaciones em Ciencias de la Salud. Universidad Nacional de Asunción. Paraguay.
Laboratoire de Pharmacognosie et Chimie des Substances Naturelles [5], BioCIS, CNRS, Université Paris-Sud, Université Paris-Saclay, France.
Laboratoire de Pharmacognosie [6], Faculté de Pharmacie, France.
Laboratoire COVACHIM-M2E EA 3592 [7], Université des Antilles, 97157 Pointe-à-Pitre Cedex, France.

Keywords:

5-methoxycanthin-6-one, HPLC-DAD, Factorial design, Validation, Stability test

Correspondence to Author:
Gerardo Cebrián-Torrejón

Laboratoire COVACHIM-M2E EA 3592, Université des Antilles, 97157 Pointe-à-Pitre Cedex, France.

E-mail: Gerardo.cebrian-torrejon@univ-antilles.fr.

ABSTRACT: Studies of natural products emerge as an important medicinal resource. An example is the use of *Zanthoxylum chiloperone* (Rutaceae) for medicinal purposes against neglected diseases, as for example, Chagas disease and leishmaniasis. The analysis of the chemical composition of this medicinal plant was carried out by chromatrographic techniques to evaluate its pharmacological applications. The use of factorial design is currently the most common optimization technique used in the design of chromatographic experiments. We proposed the optimization and validation of the analytical methods, working with high-performance liquid chromatographic coupled with array diode array detection (HPLC-DAD), and finally, we developed the first stability test of natural 5-methoxycanthin-6-one (5-MCT). The establishment of the analytical methodology was performed using a full factorial design. The quantitative determination of 5-MCT was validated with the parameters of the factorial design: temperature of 25 ºC, methanol concentration of 70%, and flow of 0,8 mL/min. Preliminary stability tests showed that some conditions presented significant reductions of 5-MCT, and the respective equations were formulated. Ten related degradation products were characterized. The factorial design utilized in this study allowed to enlarge the spectrum of analysis of canthinones, providing more reliable data for the construction of analytical methodologies for natural products.

INTRODUCTION: Studies of natural products emerge as an important medicinal resource.

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An example is the use of *Zanthoxylum chiloperone* var. angustifolium Engl. (Rutaceae), synonym of *Zanthoxylum caribaeum* Lam., for medicinal purposes against neglected diseases, such Chagas disease and leishmaniasis [1-2].

The use of factorial design is currently the most common optimization technique applied in the design of chromatographic experiments **Fig. 1** [3-10]. For the phytochemical characterization of Z.

chiloperone, fast methods of analysis were employed, like the high-performance liquid chromatography (HPLC) [11]. A factorial design is the optimization technique most commonly used in this kind of experiment, mainly because it provides reliable statistical data, minimizing the number of experiments [12]. The full factorial design verifies which variables can influence the proposed experiment and analyze the interaction between them and the final results, always minimizing the experimental effort required [13]. We can find applications of these methods in different fields, such as the pharmaceutical industry 14, environmental chemistry and waste management 15, food engineering 16, phytomedicine 17, or analytical chemistry [18].

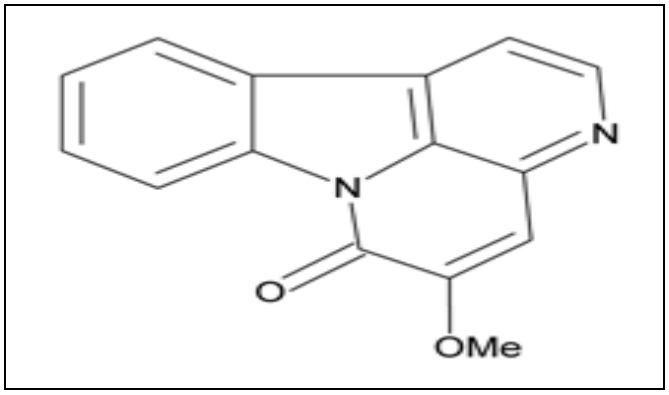

FIG. 1: STRUCTURE OF 5-METHOXYCANTHIN-6-ONE (5-MTC6)

In the present manuscript, we propose the optimization and validation of an analytical methodology by HPLC-DAD and the preliminary stability test for the bioactive alkaloid 5-methoxycanthin-6-one isolated from *Z. chiloperone*. The present data supposes an optimization of the chromatographic conditions and presents the first step to the standardization of 5-MCT identification and quantification and its implications to the quality control for future phytotherapeutic formulations.

A decoction of *Z. chiloperone* var. angustifolium stem bark is traditionally used in Paraguay for its antimalaric [19], emmenagogue, and antirheumatic properties (ethnobotanical data collected in the field). Due to its also known antiparasitic properties, we evaluated herein the bioactivity of various extracts of *Z. chiloperone* var. angustifolium against *Trypanosoma cruzi*, which is responsible for Chagas disease.

MATERIALS AND METHODS:

Instrumentation: The experiments were performed with the following equipment and materials: High-Performance Liquid Chromatography Coupled Diode Array Detection (HPLC-DAD) Shimadzu® model and Shimadzu LC Solution® software 1.0 (Japan); Analytical balance model FA 2204B; Cristófoli ultrasonic vessel model with frequency 42 KHz; Water purification system Smart model – Ultra-pure Water System (Heal Force®); PVDF filters with 0.20 µm mesh size (Chromafil®Xtra PVDF - 20/25).

Plant Material: Stem bark of *Zanthoxylum chiloperone* var. angustifolium Engl., Rutaceae, was collected by Maria Elena Ferreira, in Paraguay near Piribebuy, Department of Cordillera and identified by N. Soria (Department of Botany, National University of Asunción, Paraguay). A voucher specimen (AF 917) has been deposited at the Herbarium of Chemical Sciences Faculty, San Lorenzo, Paraguay.

Extraction and Isolation of 5-methoxycanthin-6-one: The extractive procedure was inspired by the already described methods for the isolation of these alkaloids [1, 8]. Stem bark (3 kg) were dried and grinded, sprayed, and then impregnated with a 5% aqueous solution of Na_2CO_3 followed with concentrated NH4OH and homogenized to a wet paste. It was allowed to dry and transferred to a suitable vessel; commercial dichloromethane was added to cover all the material. It was allowed to stand for 30 days with shaking twice daily. The same process was filtered and repeated 4 times, but often 15 days of rest.

The filtrates were combined and the solvent was evaporated under reduced pressure until the crude extract was obtained. The amount of crude extract obtained was 49.7 g (yield 1.66% relative to the starting vegetable material). The crude extract (49.7g) was chromatographed after preparation of a chromatographic tablet as a column head. The pellet was introduced as a seed in a liquid chromatography open column using the stationary phase silica gel 60 (0.063-0.200 mm) and a binary mixture of the hexane-ethyl acetate (Hex / EA) system as eluent. Several volumes of the Hex / EA: binary mixture (100/0, 100 mL), (95/5, 100 mL), (90/10, 100 mL), (85/15, 100 mL), (80/20, 100

mL), (75/25, 100 mL), (70/30, 100 mL), (65/35, 100 mL), (60/40, 100 mL), (55/45, 100 mL), (50/50, 100 mL), (45/55, 100 mL), (40/60, 100 mL), (35/65, 100 mL), (30/70, 100 mL), (25/75, 100 mL), (20/80, 100 mL), (15/85, 100 mL), (10/90, 100 mL). (5/95, 100 mL), (0/100, 100 mL). 205 fractions of 15 mL each were collected. The composition of the fractions collected with increasing polarity of the binary mixture used as column eluent was monitored by TLC plates using a Hex / EA mixture ratio of 8: 2 as eluent of the plates and Dragendorff reagent to identify the presence of alkaloids. Fractions from 45 to 60 were pooled and purified in acetone. The pure compound was identified as 5-methoxycanthin-6-one, which is of interest in this study. The obtained amount of methoxycanthin-6-one was 352.5 mg (yield of 0.709% relative to the initial crude extract).

Chemicals: The reagents used were HPLC grade methanol (JT. Baker®) and trifluoracetic acid (Sigma Chromasolv®- Aldrich®).

Sample Preparation: 5-methoxycanthin-6-one was weighed directly into a 10 mL or 25 mL volumetric flask to get stock solutions of 1 mg/mL. It was added amounts of methanol, 5 mL and 15 mL, respectively, to occur the solubilization of the substance, and the flask was placed in an ultrasound bath at room temperature for 280 seconds to degassing. Then, the flask was supplemented with methanol until the calibration mark. The working standard solutions were obtained before starting the experiments through appropriate dilutions of aliquots of stock solution using HPLC grade methanol. Then, the solutions were filtered through a membrane filter PVDF 0.20 µm and placed in vials of 1.5 mL with a mixture of 0.75 mL of 5-methoxycantin-6-one to be injected and analyzed by HPLC equipment.

Chromatographic Conditions: The solutions were subjected to analysis according to by the following conditions: stationary phase consisted of a C18 column (Supelco Analytical, Ascentis®, 150 mm x 4.6 mm, 5 µm); the detection was made within the range the ultraviolet; the mobile phase was done separately in which the solution A (ultrapure water + trifluoroacetic acid [99,9:0,1(v/v)] and solvent B (methanol) were sonicated and placed on the machine's reservoir; the injection volume used was 5 µL, and wavelength was 240 nm; the temperature, flow, and gradient elution were modified by the proposed method in the factorial design. The experiments were performed in a fixed sequence flow and modification of the other two variables to minimize the effects of pressure column chromatography by reducing or increasing the flow.

TABLE 1: PARAMETERS AND LEVELS FOR THE FULL FACTORIAL DESIGN 3^3

Parameter	Level		
	-1	0	+1
Temperature	25 ºC	30 ºC	35 ºC
Flow	0,6 mL	0,8 mL	1 mL
ElutionGradient	X	Y	Z

Legend: X: 60%A/40%B (30 min) → 70%A/30%B (5 min) → 60%A/40%B (5 min), Y: 60%A/40%B (30 min) → 85%A/15%B (5 min) → 60%A/40%B (5 min), Z: 60%A/40%B (30 min) → 100%A/0%B (5min) → 60%A/40%B (5 min), A: Ultrapure water + Trifluoroacetic acid (99,9:0,1(v/v)), B: Methanol

TABLE 2: MATRIX OF THE FACTORIAL DESIGN 3^3

Analysis	Flow	Temperature	Gradient
1	-1	-1	-1
2	-1	0	-1
3	-1	+1	-1
4	-1	-1	0
5	-1	0	0
6	-1	+1	0
7	-1	-1	+1
8	-1	0	+1
9	-1	+1	+1
10	0	-1	-1
11	0	0	-1
12	0	+1	-1
13	0	-1	0
14	0	0	0
15	0	+1	0
16	0	-1	+1
17	0	0	+1
18	0	+1	+1
19	+1	-1	-1
20	+1	0	-1
21	+1	+1	-1
22	+1	-1	0
23	+1	0	0
24	+1	+1	0
25	+1	-1	+1
26	+1	0	+1
27	+1	+1	+1

Factorial Design: The optimization of an analytical methodology for the determination of 5-methoxycanthin-6-one was performed using a full factorial design 3 **Table 1**.

The parameters selected and analyzed in three levels -1, 0, and +1 for this study were the temperature, flow, and gradient elution. A planning matrix was constructed considering the possible combinations of all parameters to perform a total of 27 tests **Table 2**. Factorial design 3^3 was used because three conditions were considered important for the chromatographic response and it was desired to analyze each variable in three levels in order to obtain more precise data on the influence of these variables on the efficiency of the chromatographic method.

HCRF, Pareto and 3D Surface: The results of the factorial design were obtained by equation 1 of Hierarchical Chromatographic Response Function (HCRF) that it was adapted to project by the existence of few substances [20]. HCRF was chosen as the dependent variable in place of the analyte area of interest because it allows considering other important chromatographic parameters in the choice of the best analytical method such as resolution factors, number of peaks, and chromatographic elution time in the analysis of several secondary metabolites of plants in a single chromatogram. The HCRF was expressed in **Equation 1**.

Equation 1 – Calculation for HCRF:

$$HCRF = 100\Sigma Rs + 10Rs1 + tf \text{.....Eq.1}$$

Legend: ΣRs is the sum of all the resolutions of the characteristic peaks in the chromatogram obtained; Rs1 is the resolution of peak 1; tf is the time of the last peak in the chromatogram.

Thus, the Pareto chart was done with HCRF values acquired. The obtaining of Pareto chart is a basic process to analyze the parameters and interactions that influence the experiment. In this work, the Pareto chart was obtained in the linear and quadratic effect of the variables and their interactions. In addition, they have obtained the surface response graph in 3D of the most significant variables for the experiment.

Validation: The validation of the method was based according to the International Conference on Harmonization guide [21], by Resolution RE nº. 166 of 07/24/2017 the National Health Surveillance Agency [22] and literature [23] for determining parameters of precision, accuracy, linearity, range, selectivity, robustness, limit of detection, and limit of quantization. The parameters were evaluated as follows: the specificity was evaluated by index of peak purity, resolution, retention time, area, tailing factor, and theoretical plates; the linearity was obtained by analytical calibration curve; the precision was performed intraday (repetitiveness) with one analyst and inter-day (intermediate precision) with two analysts; the recuperation test was performed with 80%, 100% and 120% of the added substance in working standard solution; the robustness was performed with 4 parameters varying for more or less of proposed method value.

Preliminary Stability Test: The preliminary stability tests were performed according to the parameters specified by the Resolution RDC Nº 45/201224 and the characteristic of the substance. The degradation conditions are shown in **Table 3**. The working standard solutions with a concentration of 250 µg/mL of the 5-methoxycanthin-6-one were placed for an exposition of the agents. Thus, these were filtered through a membrane of mesh 0.2 µm and placed in vials for the HPLC analysis. Samples were done in duplicate and assayed at times 0, 2, 4, 6, 8, 10, and 12 h.

TABLE 3: SAMPLE PREPARATION FOR STRESS TEST

Degradation condition	Agent	Exposition duration	Blank	Control
Acidic hydrolysis	HCl 0,1M (pH = 2)		HCl 0,1 M	5-MCT
Basic hydrolysis	NaOH 0,01M (pH = 10)	12 h	NaOH 0,01 M	250 µg/mL
Photodegradation	Ultraviolet light (365 nm)		MeOH	
Oxidation	H_2O_2 3%		H_2O_2 3%	

Statistical Analysis: The data were analyzed by analysis of variance (ANOVA) using Statistical software, version 8.0 (StatSoft®). The Microsoft Excel program (Microsoft Office®) and Origin Pro version 8.0 (Origin Lab Corporation®) also served to support data analysis. All experiments were performed in triplicate and expressed as mean ± standard deviation or variation coefficient with the significance level of 5%.

RESULTS AND DISCUSSION:

Factorial Design: The sample of 5-methoxy-canthin-6-one was subjected to analysis in HPLC-DAD. **Fig. 2** shows the chromatogram reported. Based to the literature and in the objectives of our project, the factorial design was made and calculated by the factor HCRF.

The HCRF was performed for all analysis, and the high results obtained were 2.547, 2.336 e 2.407 in the flow parameters 0.6, 0.8, and 1, respectively, with temperatures of 25 °C and methanol concentration of 70%.

For this analysis, the pH of the media was 2-3. Despite this, the HCRF measurement was not compromised because peak 1 (5-MCT) has become more important, and the chromatogram maintains its profile without the detection of any new peaks in the chromatogram.

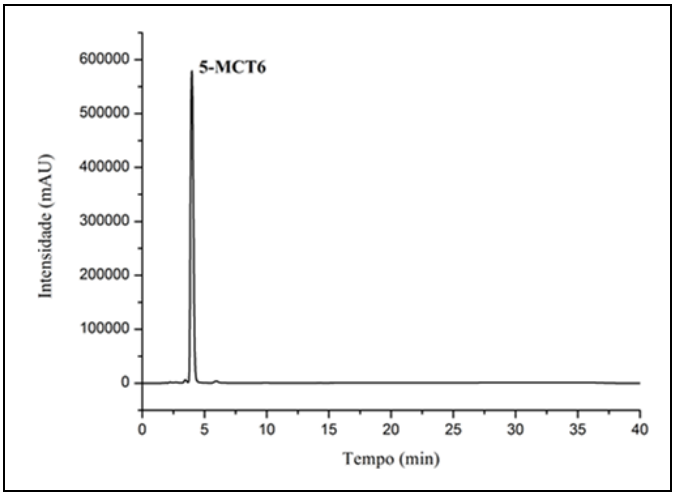

FIG. 2: HPLC-DAD PROFILE OF SAMPLE OF 5-MCT6

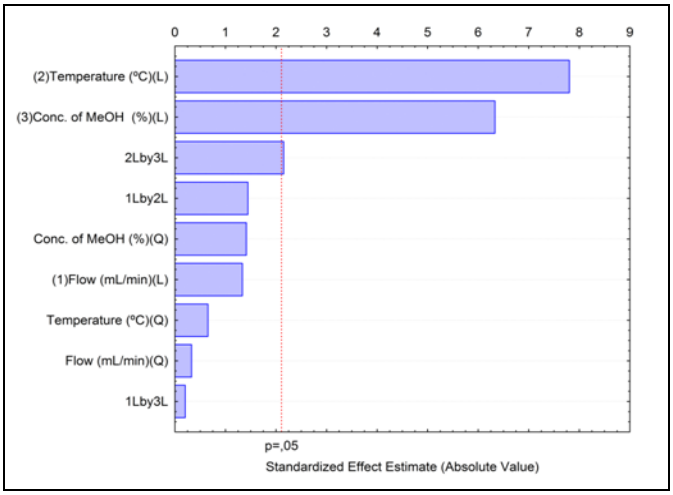

FIG. 3: PARETO GRAPH ILLUSTRATING HOW VARIABLES THAT HAS A SIGNIFICANT CHROMATOGRAPHIC RESPONSE; VARIABLE: HCRF

The values obtained to HCRF were an important step to perform the Pareto chart. The influence of factors in the experiment allowed the control and fixing of the more important variables, aiming at the improvement of chromatographic conditions.

The Pareto chart demonstrated that the most important factor was the temperature followed by methanol concentration, as shown in **Fig. 3**. These factors were more significant than flow as well as the linear interactions between all the factors. The observation of 3D surface graphic showed as well this influence, as is illustrated by **Fig. 4**.

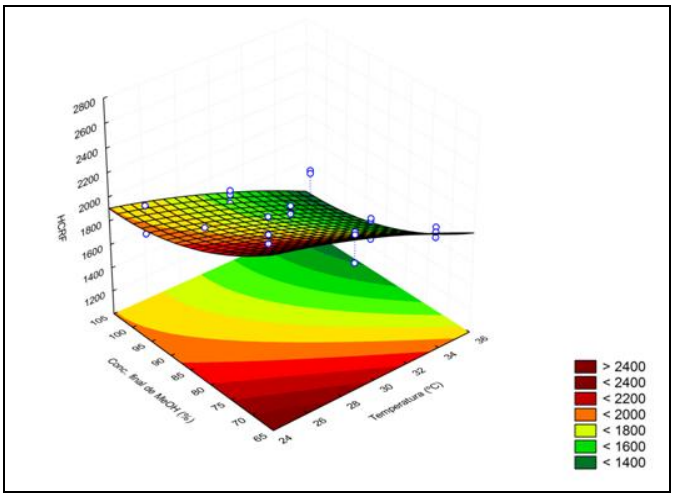

FIG. 4: SURFACE RESPONSE GRAPH REPRESENTING THE RESULTS OBTAINED IN THE 27 EXPERIMENTS CARRIED OUT IN THE 3^3 FACTORIAL DESIGNS

The graphic trend was balanced to the dark red side. This characteristic showed that the best conditions were the temperature at 25 ºC and the methanol concentration at 70%. Therefore, these parameters were fixed.

The comparison of the HCRF values allowed fixing the parameter flow in 0.8 mL/min). Thus, all the factors of the analytical methodology were settled in order to determine the best conditions, and it proceeded to validation.

Validation: Once the best chromatographic conditions were chosen for the analysis of 5-MCT, the validation of the analytical method was the next stage. The method showed to be selective for the analyte of interest, and the chromatographic peak presented optimum analytical parameters, as can be observed in **Table 4**. The method produced a linear response (r^2> .99), and from the calibration curve was obtained equation used for quantification

calculations of the 5-MCT (y = 25380, 17x - 172, 374.64). The limits of detection and quantification of this analytical method were estimated from 18.02 (µg/mL) for detection limit and 60.06 (µg/mL) for quantification limit. Precision was evaluated intraday and inter-day, and at both levels, the method showed satisfactory results, presenting a variance of 2.37% and 3.93% for intraday and inter-day precision, respectively.

The method was also found to be accurate, with recovery values ranging from 86.66% to 97.41%. The analytical method demonstrated robustness in all evaluated conditions, resisting slight variations in gradient, elution time, temperature, and flow. All values obtained in the validation of the analytical method can be observed in **Table 4**.

TABLE 4: RESULTS OF VALIDATION EXPERIMENTS

Parameter	Sub-parameter	Results (Mean)
Specificity (250 µg/ml)	Peak purity	0,9999
	Resolution	2,63
	Retention time (min)	4,55
	Area	$6{,}18 \times 10^6$
	Tailing factor	1,42
	Theoretical plates	28601,98
Linearity	Function	Y = 25380,17x - 172374,64
	R^2*	0,99
	Range	97, 10 - 385,33 (µg/mL)
Precision**	Intraday	2,37% (n=6)
	Inter-day	3,93% (n=12)
Accuracy	Recuperation 80%	86,66%
	Recuperation100%	96,01%
	Recuperation 120%	97,41%
Robustness**	Gradient 68%	4,78%
	Gradient 72%	5,05%
	Eluition time 25 min	4,72%
	Eluition time 35 min	4,70%
	Temperature 23 °C	4,96%
	Temperature 27 °C	4,87%
	Flow 0,7 mL/min	5,09%
	Flow 0,9 mL/min	4,91%
Detection limit		18,02 (µg/mL)
Quantification limit		60,06 (µg/mL)

*R2: Determination coefficient, **RSD or VC: Relative standard deviation or variation coefficient

Preliminary Stability Test: The preliminary stability tests allowed analyzing the stability of the compound exposed to several adverse conditions. Thus, chromatograms showed related substances (RS) formed in the specified adverse conditions. Overall, 10 RS were observed in the analysis of the different chromatograms. The related substances were compared with 5-methoxycanthin-6-one to observe the evolution (disappearance of 5-MCT and apparition of RS) in relation to the time. **Fig. 5** shows this rate in the case of acidic hydrolysis (yielding a salt of the alkaloid) and basic hydrolysis. As it was presented in study [25], the β-carboline alkaloids can present different structural forms in solution; these data were demonstrated by various authors [1, 7].

Complementarily, **Fig. 5** also illustrated the correlation with the oxidation and photo-degradation conditions. In recent work, [26] showed that the canthin-6-one alkaloids can be degraded in acidic, neutral, and alkaline media for electro-chemical reductive pathways.

The basic hydrolysis and oxidation conditions showed that the signals of 5-MCTare reduced significantly to approximately 25% and 89%, respectively. On the other hand, only the RS1 and RS3 in the basic experimental hydrolysis conditions were statistically significant, with 3.2% and 52.5%, respectively. The other conditions presented different RSs with lower values that are not significant.

Consequently, we can affirm that the basic medium is probably one factor of degradation of the 5-MCT. The oxidative medium can also be destabilizing the structure of 5-MCT, for example disrupting the electron delocalization between the π-π bindings that they are important to resonance, or producing the N-oxidation of the pyridine ring of the β–carboline, as was already described [7].

The reduction of 5-MCT was calculated from **Equations 2** and **3** for basic hydrolysis and oxidation conditions respectively.

Equation 2 - Equation of reducing of 5-MCT in the basic hydrolysis:

$$y = 75{,}68*e^{((-x/0{,}75))} + 24{,}31 \quad \text{.....Eq.2}$$

Legend: "e" is exponential.

Equation 3 - Equation of reducing of 5-MCT in the oxidation.

$$Y = 99{,}95 - 0{,}79x + 0{,}12x^2 - 0{,}01x^3 \quad \text{.....Eq.3}$$

The photodegradation conditions didn't reduce the quantities of the 5-MCT either increase the presence of the RSs significantly. The 5-MCT presents high stability to the acidic hydrolysis conditions, without significant changes in the quantity of 5-MCT and yielding a lower number of RSs than the rest of degraded conditions. Thus, the acidic medium is probably the best condition to preserve this substance. The determination of 5-methoxycanthin-6-one was performed by factorial design; this strategy proved to be efficient and covered a wide range of analyses.

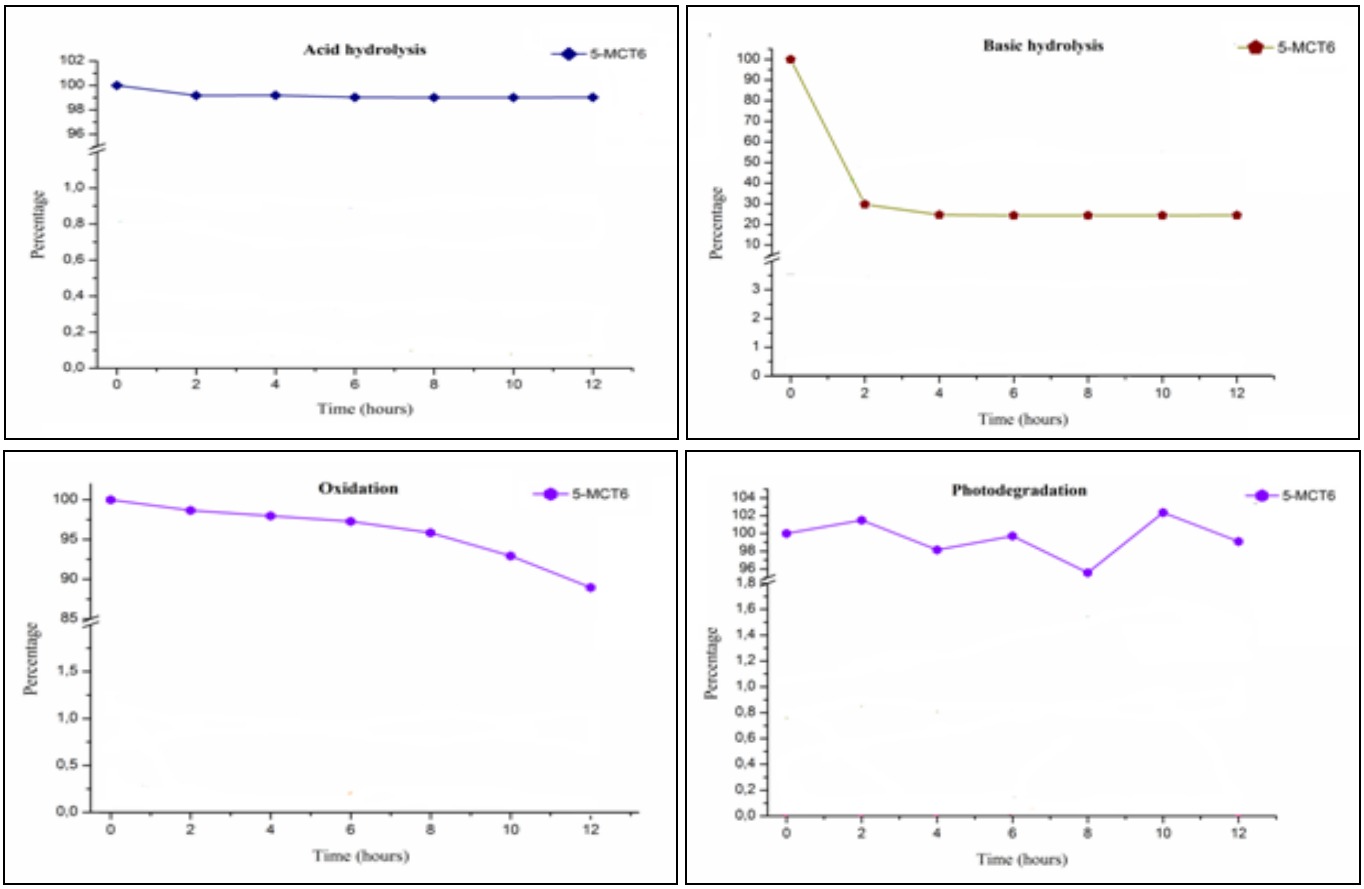

FIG. 5: RESULTS OBTAINED FROM THE STUDY OF STABILITY TO 5-MCT IN VARIOUS DEGRADATION CONDITIONS

CONCLUSION: The proposed method was obtained according to the parameters of the factorial design, selecting and validating the most favorable conditions. The preliminary stability study allowed us to consider the stability of 5-MCT against several stressful mediums. The acidic hydrolysis condition appears to be the best condition, and not photo ability was detected. In another side, the 5-MCT is not stable in basic or oxidative conditions.

Thus, the factorial design utilized allowed to enlarge the spectrum of analysis of canthinones, providing more reliable data for the construction of analytical methodologies for natural products. In the specific case of 5-MCT, these results are very important to advance in the development of a phytomedicine to the treatment of Chagas disease, were this natural product presents a very promising pharmacological properties.

Human and Animal Rights: No Animals / Humans were used for studies that are base off this research

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CONFLICTS OF INTEREST: All authors declare to have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials studied in the manuscript.

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