Experimental design approach applied to the development of chitosan coated poly(isobutylcyanoacrylate) nanocapsules encapsulating copaiba oil

Francisco Humberto Xavier-Junior, Eryvaldo Sócrates Tabosa Do Egito, Andreza Rochelle Do Vale Morais, Everton Do Nascimento Alencar, Alexandre Maciuk, Christine Vauthier

EXPERIMENTAL DESIGN APPROACH APPLIED TO THE DEVELOPMENT OF CHITOSAN COATED POLY(ISOBUTYLCYANOACRYLATE) NANOCAPSULES ENCAPSULATING COPAIBA OIL

Francisco Humberto Xavier-Junior [a,b], Eryvaldo Sócrates Tabosa do Egito [a], Andreza Rochelle do Vale Morais [a,b], Everton do Nascimento Alencar [a], Alexandre Maciuk [c], Christine Vauthier [b]*

[a] Universidade Federal do Rio Grande do Norte, Centro de Ciências da Saúde, Departamento de Farmácia, Laboratório de Sistemas Dispersos (LaSiD). Av. Gal. Gustavo Cordeiro de Farias, S/N, Petrópolis, 59010-180, Natal-RN-Brazil.

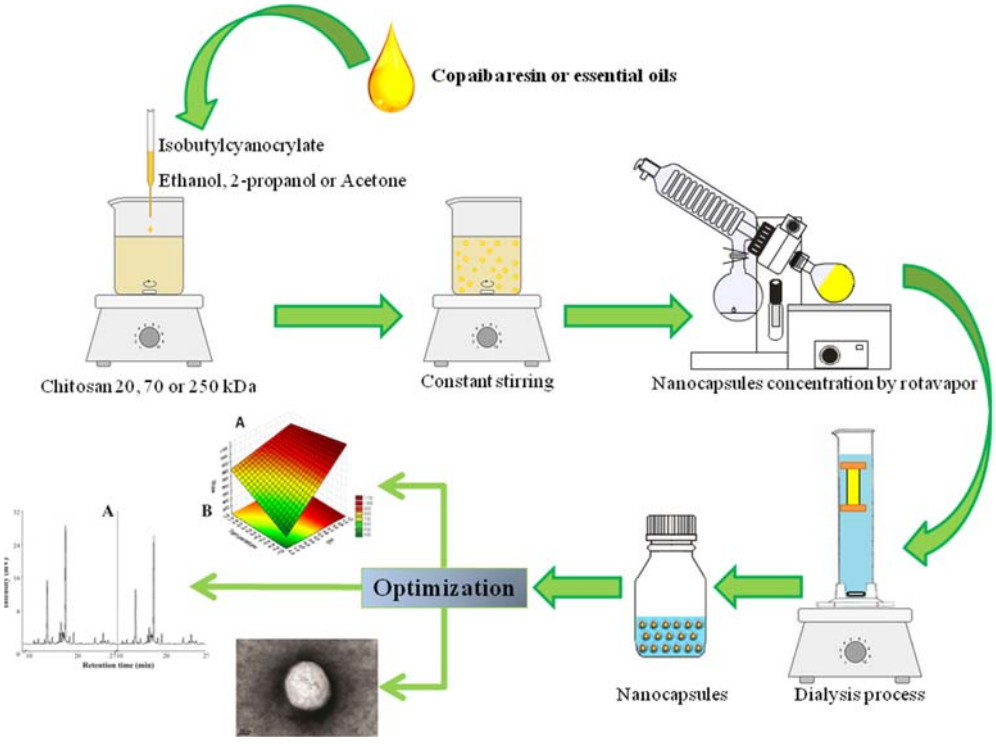
[b] Université Paris Sud, Institut Galien Paris-Sud - UMR CNRS 8612 - Faculté de Pharmacie, 92296 Chatenay-Malabry Cedex – France.

[c] Université Paris Sud, Laboratoire de Pharmacognosie - UMR CNRS 8076 BioCIS - Faculté de Pharmacie, 92296 Chatenay-Malabry Cedex – France.

Running head: copaiba oil-loaded chitosan decorated nanocapsules

Published in: Colloids Surf A Physicochem Eng Asp. 2018;536: 251-258. Available on https://doi.org/10.1016/j.colsurfa.2017.02.055

***Corresponding author**:
Christine Vauthier, Université Paris Sud, Institut Galien Paris-Sud - UMR CNRS 8612 - Faculté de Pharmacie, 5 Rue J.B. Clément, 92296 Chatenay-Malabry Cedex – France. Tel.: +33 01 46 83 56 03; fax: +33 01 46 83 53 12. E-mail address: christine.vauthier@u-psud.fr



Abstract

The aim of this work was to develop, characterize and optimize the natural copaiba oil-loaded chitosan decorated poly (isobutylcyanoacrylate) nanocapsules. These innovatively obtained natural-based systems were developed by an original method of interfacial polymerization of isobutyl cyanoacrylate using chitosan as a stabilizer for the nanocapsules. A preliminary study investigated the influence of the molecular weight of chitosan, the type of copaiba oil extract and the solvent phase. Nanocapsules could only be produced with copaiba resin oil, with size ranging from 300 to 1200 nm. Nanocapsules size and zeta potential were then optimized by two-level three-variable full-factorial experimental design. Samples showed spherical objects when analyzed by transmission electron microscopy. The copaiba oil encapsulated in the nanocapsules showed all compounds of the parent oil. Nanocapsules with positive zeta potential were obtained consistently with the expected distribution of chitosan on the nanocapsule surface. Optimal nanocapsules showed a diameter of 473 nm, a zeta potential of +34 mV and an encapsulation efficiency of the oil of 74 % including 55.5 µg of β- caryophyllene/mg of nanocapsules. The obtained nanocapsules can be suggested as oral delivery system for anticancer molecules including paclitaxel assuming a synergistic effect with anticancer active components of the oil.

Keywords: nanocapsules, copaiba oil, chitosan, poly(isobutylcyanoacrylate), experimental design approach

Highlights

- Copaiba oil-loaded chitosan decorated nanocapsules was produced
- Nanocapsules size and zeta potential were optimized by experimental design
- Chitosan was used as a stabilizer for the nanocapsules production
- pH and the temperature of polymerization influenced both the size and zeta potential
- Copaiba oil was efficiently encapsulated and showed all compounds of the parent oil

Abbreviations

$CO_{dispersed\ phase}$	the amount of copaiba oil found in the dispersed media of the nanocapsules
CO_{total}	total amount of copaiba oil used in the preparation
Adj R^2	adjusted determination coefficient
F_{model}	F-value of the model
F_{Tab}	tabulated F- value
$F_{\text{Tab residues}}$	tabulated F- value of the residues
F_{residues}	F- value of the residues
R^2	coefficient of determination
x_1	pH of the polymerization medium
x_2	temperature of polymerization
x_3	concentration of chitosan in the polymerization medium
Y_1	predicted droplet size (nm)

1 INTRODUCTION

Nanoparticles composed of mucoadhesive polymers are promising systems for oral drug delivery applications [1, 2]. Generally, nanoparticles are defined as solid colloidal particles that include both nanospheres and nanocapsules [3]. The latter are vesicular systems in which the drug is confined in a liquid/solid cavity surrounded by a polymer envelope.

Proposed to improve drug delivery, nanoparticle systems can modulate drug biodistribution and release in a controlled manner, increase intracellular uptake and improve the stability of active substances [4-6]. Many types of nanoparticles made of biodegradable polymers, including poly (isobutylcyanoacrylate), were considered to improve drug delivery by oral route. To this purpose, their surface property may be tuned to increase mucoadhesion [7, 8]. Chitosan has been a widely used polysaccharide to formulate mucoadhesive systems [9, 10]. In addition, this polysaccharide is biocompatible for oral administration. Its inherent mucoadhesive properties comes from the amino groups included in the chemical structure that can interact with sialic acid groups of mucins composing the mucus via electrostatic interactions [11]. Besides, the positive charges of chitosan are also believed to play an essential role increasing the permeability of the intestinal epithelium thanks to its capacity to disturb the calcium concentration balance near the tight junction [12]. Although widely used to improve mucoadhesion of nanospheres, this polysaccharide was not yet used to improve mucoadhesion of polymeric nanocapsules, which are interesting as drug delivery systems for lipophilic drugs.

The copaiba oil-resin (*Copaifera langsdorffii*) is an oily plant extract used in folk medicine in its *in-natura* form [13]. Phytochemical studies on oil-resin reveal that it contains a complex mixture of diterpenes and sesquiterpenes hydrocarbons [14], giving this oil many interesting therapeutic activities. For instance, these include anti-inflammatory, antitumor, anti-tetanus, antimicrobial, antileishmania activities, among others [15-17]. Although used for years in folk medicine, it is believed that pharmacological activities of this oil may be increased by developing appropriate formulations.

Therefore, the aim of the present work was to develop an original formulation of nanocapsules coated with chitosan as mucoadhesive compound and copaiba. The polymer composing the nanocapsule envelope was a critical choice. Poly (isobutylcyanoacrylate) was selected because of its capacity to formulate nanocapsule that resist well to the gastric medium and promote release in the intestinal medium [18]. Although the development of mucoadhesive copaiba oil-containing nanocapsules of poly (isobutylcyanoacrylate) have not been described before. This process was based on the use of an experimental design approach that was never applied so far while developing new formulations of oil-containing nanocapsules prepared by interfacial polymerization of isobutylcyanoacrylate. It is noteworthy that experimental design approach was not so much applied for the development of nanocapsules while such an approach could be helpful to optimize formulations, limiting the number of experiments to perform hence the amount of reagent and time [19].

2 MATERIALS AND METHODS

2. 1. Materials

Copaiba resin oil was purchased from Flores & Ervas (Piracicaba, SP, Brazil). Isobutylcyanoacrylate was provided by ORAPI engineered solutions worldwide (Vaulx-en-Velin, France). Water soluble chitosan Mw 20,000 g/mol was purchased from Amicogen (Jinju, South Gyeongsang, South Korea). Ethanol, acetone, 2-propanol, sodium hydroxide, ethyl acetate, nitric acid were provided by Fisher Scientific (Pittsburgh, PA, EUA). Diazomethane and β-caryophyllene were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Ultrapure water was obtained from a Millipore purification system (Milli-Q plus, Millipore, St Quentin en Yvelines, France). All chemicals were reagent grade and used as received.

2.2. Copaiba essential oil extraction

Copaiba essential oil was obtained from the hydrodistillation of 400 mL of copaiba resin oil using a Clevenger-type apparatus for 3 h. The extracted essential oil was dried with sodium sulphate, filtered, stored in the refrigerator and protected from light until use.

2.3. Preparation of the nanocapsules

Copaiba oil-loaded chitosan-decorated poly (isobutylcyanoacrylate) nanocapsules were elaborated by the method of interfacial polymerization [20, 21] that was adapted because of the use of chitosan. A preliminary study investigated the influence of the molecular weight of chitosan (20, 70 and 250 kDa), the type of copaiba oil (resin and essential oil) and the nature of the solvent phase (ethanol, 2-propanol and acetone) in order to identify the best substances to produce nanocapsule. Thus, 0.25 mL of copaiba oil and 0.032mL of isobutylcyanoacrylate were solubilized in 6.25 mL of solvent to produce the organic phase. This phase was slowly injected dropwise in 12.5 mL of the polymerization medium prepared with 0.6% of chitosan at pH 6 and homogenized for 10 min at 250 rpm at 25 °C (Fisher-Bioblock Scientific AM 3001K, Illkirch, France). The obtained colloidal dispersion was concentrated by rotary evaporator for 20 min at 35°C / 43mBar (BÜCHI Rotavapor R-125, Heating Bath B-491, Vacuum pump V-700, recirculating Chiller F-108, Flawil, Switzerland) to eliminate the solvent. Then, the dispersion was filtered through a 5 µm minisart NML membrane (Sartorius GmbH, Goettingen, Germany). The obtained nanocapsule dispersions were purified by dialysis (Spectra/Por Biotech membranes, cellulose ester, 100,000 g/mol molecular weight, Rancho Dominquez, CA, USA) against ultrapure water three times for 60 min and once overnight to remove non-associated chitosan. After dialysis, the nanocapsules were stored at +4°C for 24 hours before characterization.

2.4. Experimental design and nanocapsules preparation optimization process

In the present study, a 2^3 full-factorial experimental design with center points leading to 11 experimental randomized runs was used to optimize formulation and process parameters for the preparation of copaiba oil-loaded chitosan decorated- poly (isobutylcyanoacrylate)

nanocapsules. Nanocapsules were prepared as described in the section 2.3 using copaiba resin oil, chitosan 20 kDa and ethanol as solvent. For the optimization of the nanocapsules, three independent variables including, the pH of the polymerization media (x_1) (3, 6 and 9), the temperature of production (x_2) (5, 25, 45 °C) and the concentration of chitosan 20 kDa (x_3) (0.3, 0.6, 0.9 %) were selected. For each variable, a low, medium or high level value was attributed (see Table 1). The size and zeta potential of nanocapsules were chosen as the dependent output response variables. Optimization was aimed to obtain small and positively charged nanocapsules. The effects of the studied variables were graphically and statistically interpreted using the Statistic software (Version 7.0, StatSoft Inc., USA) to validate the statistical design. Response surface plots were generated to visualize the simultaneous effect of each variable on each response parameter.

Table 1: *Variables and levels chosen to define the experimental region and their corresponding coded values for nanocapsule production*

Independent Variable		Level		
i	x_i	-1	0	+1
1	pH	3	6	9
2	Temperature (°C)	5	25	45
3	Chitosan 20kDa concentration (%)	0.3	0.6	0.9
Dependent Variable (Y_i)		Desired Response		
1	Mean globule size (nm)	Minimize		
2	Zeta potential (mV)	Maximize		

2.5. Characterization of the nanocapsules.

Size measurement: Hydrodynamic mean diameter and size distribution of the nanocapsule dispersions were determined at 25°C by quasi-elastic light scattering using a Zetasizer Nano ZS90 (Malvern Instruments Ltd, Orsay, France). The scattered angle was fixed at 90°. The samples were diluted 1:100 before analysis with Milli® Q water. Each measurement was done in triplicate meaning that the average was calculated from 9 values.

Determination of the zeta potential: Zeta potential of the nanocapsules was deduced from the determination of the electrophoretic mobility by Laser Doppler Electrophoresis (Zetasizer Nano ZS90 (Malvern Instruments Ltd, Orsay, France). Nanocapsule dispersions were diluted (1:100) with NaCl at 1 mmol/L. Values are presented as mean of measurements performed on three replicate samples.

Morphology of nanocapsules: Transmission electron microscopy observation of copaiba oil-loaded chitosan-decorated poly (isobutylcyanoacrylate) nanocapsules was performed using a JEOL 1400 electron microscope (JEOL Ltd, Tokyo, Japan) equipped with a Gatan CCD digital camera (Orius SC1000) high-resolution. Samples were observed at 60kV after staining with phosphotungstic acid 2% (pH 7.4) for 30 seconds.

2.6. Analysis of the encapsulated copaiba oil

Copaiba oil composition was analyzed by PR2100 gas chromatography – Flame Ionization Detector (Alpha MOS, Toulouse, France). A fused silica capillary column (25 m × 0.32 mm i.d., 0.5 µm) coated with cross-linked 5% phenyl polysilphenylene-siloxane (SGE Analytical Science Pty Ltd, Victoria, Australia) was used. The method was previously validated for the analysis of the composition of copaiba oil (Xavier-Junior *et al.*, submitted for publication). Samples were diluted with ethyl acetate and 2.5 µL was injected in the chromatograph. The operating conditions to the samples were: oven temperature program from 90 °C (2 °C min^{-1}) to 150 °C, after isothermally heating 20 °C.min^{-1} to 300 °C, kept for 5 min at the final temperature. Split injection was 1:80, Nitrogen was the carrier gas at a pressure of 166 kPa, flow rate 1 mL.min^{-1}, temperature of injector and detector fixed at 250 °C and 300 °C, respectively. Composition of the major compounds present in the copaiba oil encapsulated in the nanocapsules was analyzed and compared with that of the oil taken prior to encapsulation.

2.7. Determination of encapsulation efficiency, encapsulation rate and concentration in the nanocapsule dispersion

For the determination of the encapsulation efficiency, the encapsulation rate and the concentration of copaiba oil in the nanocapsules, samples were prepared as explained bellow prior to their analysis by gas chromatography.

Copaiba oil-loaded chitosan-decorated poly (isobutylcyanoacrylate) nanocapsules were recovered by an ultrafiltration method. 0.5 mL of the nanocapsule dispersion was centrifuged in a Microcon centrifugal filter unit (Ultracel YM-100, regenerated cellulose, cut-off of 100 kDa, Merck Millipore**,** Billerica, MA, USA) at a speed of 10,000 rpm for 20 min (Eppendorf centrifuge 5418, Rotor FA-45-18-11, Hamburg, Germany) to remove the dispersion phase. Copaiba oil-loaded chitosan-decorated poly (isobutylcyanoacrylate) nanocapsules were separated in the different fractions. The parent nanocapsule dispersion, nanocapsules retained on the membrane and dispersion phase (i.e. the ultrafiltrate) were analyzed. These fractions were then resuspended in 1 mL of ethyl acetate, sonicated for 1 hour and filtered through a 0.22 µm Millipore filter. The amount of β-Caryophyllene was determined by gas chromatography as described in section 2.6 to evaluate the amount of copaiba oil in each fraction. The encapsulation efficiency was calculated as follows (Equation 1):

$$EE\ (\%) = \frac{(CO_{total} - CO_{dispersed\ phase})}{CO_{total}} \times 100 \qquad (1)$$

Where CO_{total} was the total amount of copaiba oil used in the preparation and $CO_{dispersed\text{-}phase}$ was the amount of copaiba oil found in the dispersed media of the nanocapsules at the end of the preparation.

The nanocapsule concentration in the dispersion was evaluated by gravimetry. 1g of the purified nanocapsule dispersion was freeze-dried and the dry residue was weighted to deduce the percentage of nanocapsules contained in 1g of the dispersion. The encapsulation rate was determined by the ratio between weights of the β-caryophyllene present in copaiba

oil loaded in the nanocapsules and the total weight of the nanocapsule analyzed by gas chromatography.

2.8. Statistical analysis

The results of these experiments were compared using analysis of variance (ANOVA), which was able to determine if the variables and the interactions between variables were significant. Regression model, t-tests and F-test with a 95% confidence level ($p<0.05$) were performed. The softwares Graph Pad Prism (Version 5.0, La Jolla, CA, USA) and Statistic software (Version 7.0, StatSoft Inc., USA) were used to perform the statistical analysis of the data.

3 RESULTS

Copaiba oil nanocapsules were obtained by designing new conditions of interfacial polymerization of poly (isobutylcyanoacrylate), performed with a bioactive natural oil, chitosan and in the absence of surface-active compounds. As a first step of the work that aimed to identify the best conditions to be used to produce copaiba oil containing nanocapsules by interfacial polymerization, several experiments were performed to select suitable materials. To this aim, preparations of nanocapsules were performed with chitosan (0.6 %) of different molecular weight (20, 70 and 250 kDa), with copaiba resin and essential oils and with different solvents including ethanol, 2-propanol and acetone at pH=6 and temperature of 25°C. The solvents were chosen based on their miscibility with water, a condition to achieve the synthesis of nanocapsules by interfacial polymerization of alkylcyanoacrylates. Particle size and zeta potential of the obtained nanocapsules are summarized in Figure 1.

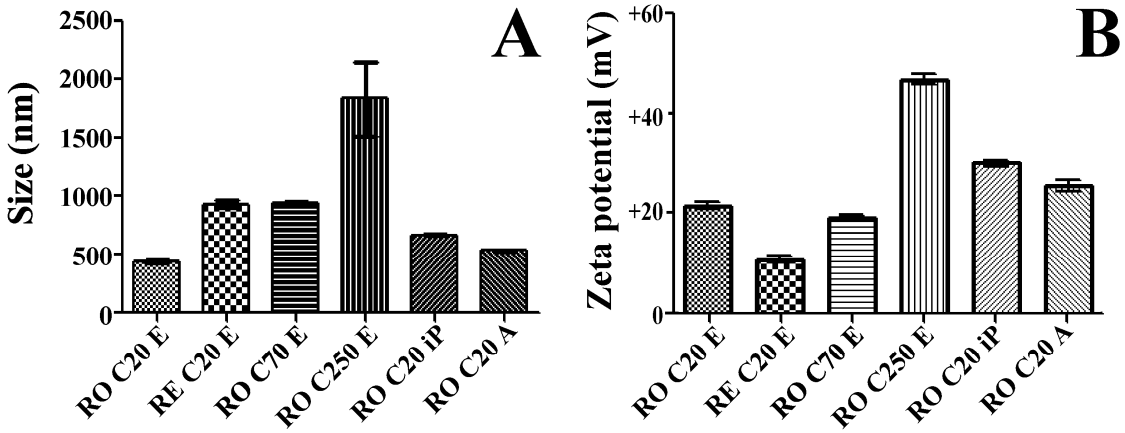


Figure 1: *Particle size (A) and zeta potential (B) of copaiba oil nanocapsules with chitosan coated in the surface at pH=6 and temperature of 25°C. Wherein RO and RE corresponds to nanocapsules produced with copaiba resin and essential oils, respectively; C20, C70 and C250 referred to the three different chitosan (0.6 %) molecular weights of 20, 70 and 250 kDa; and E, iP and A indicated the type of organic solvent used, ethanol, 2-propanol and acetone respectively, used to nanocapsule production.*

The mean diameters of the nanocapsules ranged from 440 ± 8 nm to 932 ± 28 nm when they were prepared with copaiba resin oil and copaiba essential oil, respectively. Nanocapsules obtained with chitosan 20 and 70 kDa were spherical as observed by transmission electron microscopy. The morphology of nanocapsules suggests that they were formed by an oily core surrounded by a polymer envelope. The size of copaiba resin oil-loaded nanocapsules varied with the different solvents. The average particle size were 440 ± 8, 669 ± 8 and 536 ± 7 nm, respectively (p<0.05) for nanocapsules prepared with ethanol, 2-propanol and acetone (Figure 1A).

All nanocapsules were characterized by positive values of zeta potential indicating the presence of chitosan on the nanocapsule surface. The charges of the nanocapsule envelope were statistically different whether they were prepared with copaiba resin oil or copaiba essential oil. Nanocapsules prepared with copaiba essential oil displayed the lowest zeta potential (less than +12 mV).

Based on results obtained from this first series of preparations, optimization of conditions for the preparation of chitosan-coated nanocapsules containing copaiba oil was pursue using chitosan 20kDa, ethanol and copaiba resin oil. The experimental design approach used in this work represented an easy methodology to provide important data about the natural oil-containing nanocapsules limiting the number of experiments to be performed hence the amount of reagent and time. Thus, the optimization was carried out following a 2^3 full-factorial experimental design approach with center points. Three independent variables were considered including pH of the polymerization media (x_1), the temperature of production (x_2) and the concentration of chitosan 20 kDa (x_3) (Table 1). The optimal parameters chosen were those which produced small size nanocapsules with positive surface charges.

The mean hydrodynamic diameter of the produced nanocapsules was strongly influenced by the selected variables, emphasizing their relevancy as critical parameters which influence on the preparation process. The size ranged from 200 to 1200 nm. The Pareto chart (Figure 2) gives the relative importance of the individual and interaction effects [22]. Initially, Pareto chart was created to analyses the individual standardized effects of studied variables, and posteriorly their interactions effects on the size and zeta potential, as well as it permits the identification of the statistically significant variables for the process, with a significance level (ρ-value of 0.05). The length of each bar in the chart indicated the standardized effect of the corresponding variable on the response. Negative values indicated unfavorable or antagonistic effect for the development of nanocapsules with the desired characteristics, i.e. small size and positive zeta potential, while positive coefficients showed a favorable or synergistic effect. As shown by the Pareto's chart and considering each variable separately, a decrease of the polymerization medium pH had a statistically positive effect in the decrease of the nanocapsule size. Furthermore, it was observed that the nanocapsule size decreased with an increase of the temperature while the concentration of chitosan had no significant effect on this parameter. Interactions between the different variables can be highlighted by investigating the combined influence of several variables.

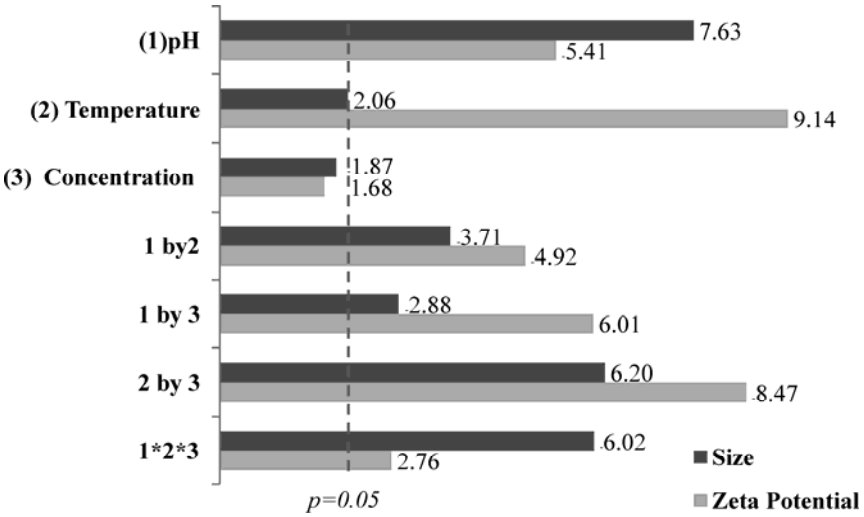


Figure 2: *The Pareto Chart of standardized effects to size and zeta potential dependent variables (p<0.05).*

The Pareto's chart highlighted a clear significant interaction between the pH of the polymerization medium and the other studied variables, i.e. pH × temperature and pH × concentration. In the acid solution at high concentration of chitosan, the increase of the temperature from 5 to 45 °C induced an increase of the nanocapsule size from 250 to 1070 nm. In contrast, in basic polymerization medium, the temperature variation did not show significant size changes (approximately 930 nm). Opposite effect on the nanocapsules size was observed between the concentration of chitosan and the pH of the medium.

The analysis of variance was used to test the significance of independent variables and their interactions. The model's goodness of fit was checked by the coefficient of determination (R^2). The R^2 values provide a measure of how much variability in the observed response values can be explained by the experimental variables and their interactions (fluctuation) [23]. The matrix of the experiments developed were considered as statistically significant with linear relationship of R^2= 0.968 which indicates that the model can explain 96.8 % of the variability in the response variable. In addition, the value of the adjusted determination coefficient (Adj R^2 = 0.942) was also very important to confirm a high significance of the model [24], which value was very close to the experimental R^2 value. Therefore, these matrix of the experiments ensured a satisfactory adjustment of the polynomial model to the experimental data [25]. By applying multiple regression analysis on the design matrix and analyzing the responses given in the experiments, the first-order polynomial equation given in Equation 2 in the coded form was established to size droplets:

$$Y_1 = 834 + 214x_1 + 58x_2 - 104x_1x_2 - 81x_1x_3 + 174x_2x_3 - 169x_1x_2x_3 \quad (2)$$

Where Y_1 was the predicted droplet size (nm), x_1, x_2 and x_3 were the coded terms for three independent test variables: the pH of the polymerization medium, the temperature at which the interfacial polymerization was carried out and the concentration of chitosan added in the polymerization medium, respectively.

According to the regression model's ANOVA, it was possible to observe that the linear model was significant (p<0.05). This was evidenced from the Fisher's F –test which provided an *F*-

value of the model (F_{model} = 23.5) much greater than the tabulated F- value (F_{Tab}= 2.4). Concerning the statistical analysis used to investigate the significance of independent variables and their interactions, results from the Fisher's F-test suggested that the computed Fisher's variance ratio at 5 % level was large enough to justify a very high degree of adequacy of the linear model and also to indicate that treatment combinations were highly significant [26].

The normal (percentage) probability plot of the residuals was an important diagnostic tool to detect and explain the systematic departures from the assumptions that errors were normally distributed and were independent of each other and that the error variances are homogeneous. In this study, a plot of normal probability of the residuals indicated almost no serious violation of the assumptions underlying the analyses ($F_{residues}$= 3.5). This value was found to be lower than the tabulated F- value ($F_{Tab\ residues}$ = 4.26) at the 5% level, indicating that the experiment exhibited predictive results ($F_{residues}$/ $F_{Tab\ residues}$ < 1). This satisfactory result confirmed the normality distribution assumptions previously made and the independence of the residuals.

Aiming the straightforward examination of the experimental variables on the responses, the three-dimensional response surfaces were drawn. Figure 3 (A) shows a three-dimensional diagram of calculated size response surface relating both pH and temperature to copaiba oil-containing nanocapsule size.

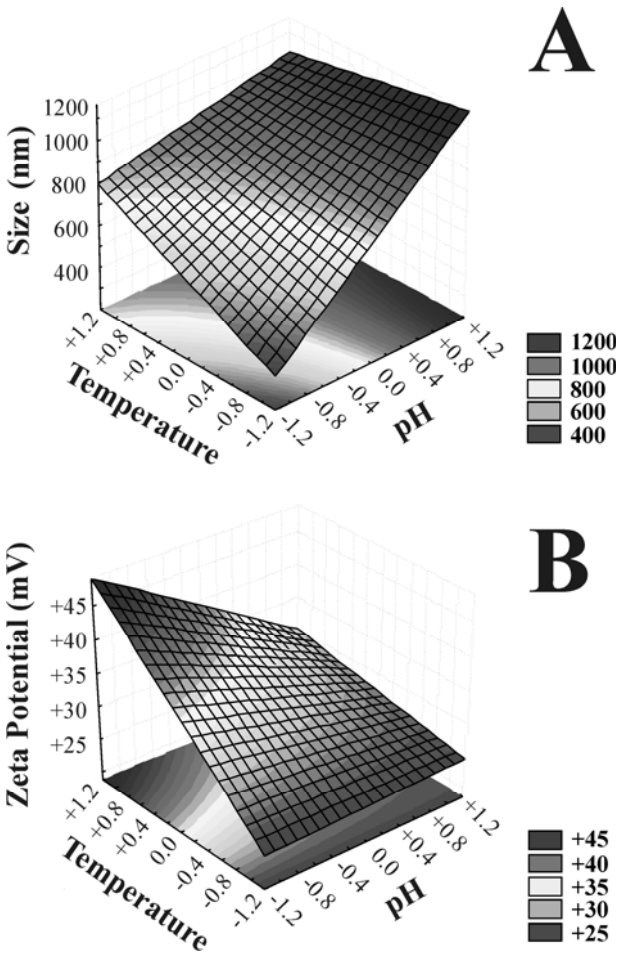


Figure 3: *3D Response surface for size droplets (A) and zeta potential (B) variable dependency with temperature and pH independent variables to production of nanocapsules.*

This diagram indicated that the size of the nanocapsules can be reduced decreasing the pH and the temperature applied during preparation. The response considering the size of the nanocapsules varied linearly with each of the variables demonstrating that there were considerable interactions between each of the independent variables and the size of the nanocapsules obtained from the different tested conditions.

Regarding the zeta potential, the copaiba oil nanocapsules showed values ranging from + 15 to + 55 mV. The Pareto's chart showed a higher increase of the zeta potential of the nanocapsules increasing the temperature and decreasing the pH of the polymerization medium comparatively to the smaller influence revealed by varying the concentration in chitosan (Figure 2). The first-order polynomial equation was established to zeta potential analyzing the responses given in the experiments (Equation 3):

$$Y_2 = 31.8 - 3.8x_1 + 6.4x_2 - 3.4x_1x_2 + 4.2x_1x_3 - 5.9x_2x_3 + 1.9x_1x_2x_3 \quad (3)$$

Where Y_2 was the predicted zeta potential (mV), x_1, x_2 and x_3 were the coded terms for three independent test variables: the pH of the polymerization medium, the temperature at which the interfacial polymerization was carried out and the concentration of chitosan added in the polymerization medium, respectively.

These experiments presented the linearity regression of R^2=0.94, therefore this model explains 94% of the variability in the response. The value of the adjusted determination coefficient (Adj R^2 = 0.92) was accepted as high significance of the model. The *F* test applied in the mathematical model shows the significant ($F_{model} > F_{tab}$) and predictive results ($F_{residues}/F_{Tab\ residues} < 1$) of these experiments. In addition, the full first-order response surface was plotted for analysis of the optimal zeta potential on the nanocapsules (Figure 3B). Nanocapsules with high positive values of the zeta potential can be obtained at low pH of the polymerization medium and performing the polymerization at high temperature. However, increasing the temperature of the polymerization increased the size of the nanocapsules that was not a desired result. To define optimal conditions, it was taken into account that the size decreased more than the zeta potential increased in the same range of variation of the temperature at which the polymerization was performed.

The result of the optimization study following a 2^3 full-factorial experimental design approach allowed to identify optimal conditions for nanocapsule preparation that have both a small particle size and positive zeta potential. The optimal characteristics of the nanocapsule preparation were pH 3, temperature 4 °C and a concentration of chitosan of 0.9 % (w/v). The samples prepared under these conditions showed a size of 473 ± 1 nm, the size distribution was unimodal with a polydispersion index of 0.20 ± 0.02 and a zeta potential of +34.8 ± 0.2 mV. Observation of the sample by transmission electron microscopy showed that the optimal nanocapsules prepared by interfacial polymerization were spherical and well individualized (Figure 4). They showed typical images of nanocapsules composed of an oily core surrounded by a polymer envelope.

The composition of copaiba oil recovered from the nanocapsules was determined by gas chromatography and compared with the oil prior to its nanoencapsulation (Figure 5). All components found in the original oil were also found in the nanocapsules. Differences in concentrations of components present in copaiba oil versus the copaiba oil extracted from the nanocapsules were in the range of precision (bellow 2%) of the gas chromatography method used to perform the analysis (Figure 5C). Results indicated that the encapsulated oil was identical in term of its composition than the initial oil. The nanoencapsulation process

fully preserved the complexity of composition of the oil. Therefore, the encapsulation efficiency of copaiba oil was high at 75.8 ± 3 %. It could be determined that the nanocapsule dispersion contained 2.5 mg of β-caryophyllene encapsulated in the nanocapsules per mL of the dispersion while the β-caryophyllene payload was 55.5 µg/mg of nanocapsules.

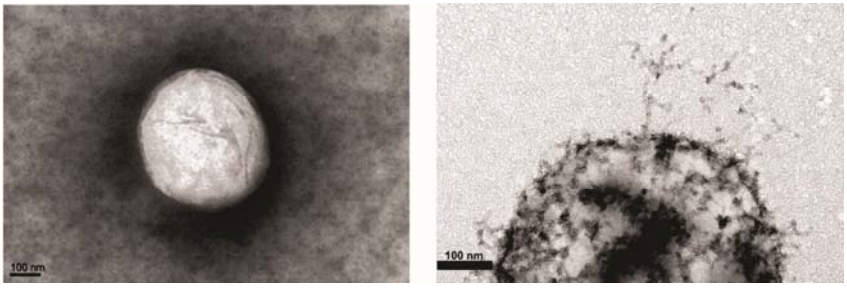


Figure 4: *Transmission electron microscopy analysis of the optimal formulation of copaiba oil-loaded chitosan- poly (isobutylcyanoacrylate) nanocapsules stained with phosphotungstic acid (2%) at 60 kV (Imagif). Scale bar of 100nm.*

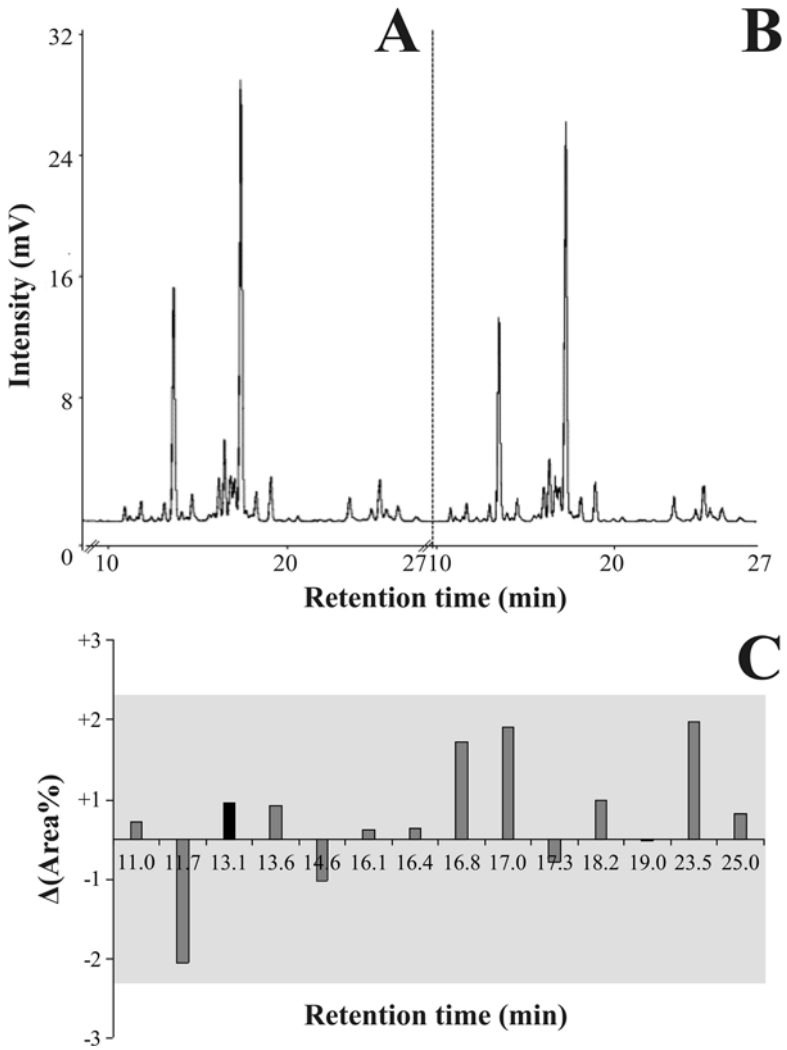


Figure 5: *Major compounds encapsulated in copaiba resin oil-nanocapsules coated with chitosan. Figures A and B represent the copaiba resin oil chromatograms before and after recovery from the nanocapsule. Figure C gives the difference in the area of the peaks between the copaiba resin oil in native form and the oil encapsulated in the nanocapsules. The gray color represents the precision of the method to determination of copaiba oil compounds*

4 DISCUSSION

This work aimed to identify optimal conditions for the encapsulation of copaiba oil in chitosan-coated nanocapsules by the method of interfacial polymerization of isobutylcyanoacrylate. Although the method of the obtaining of the nanocapsules is well known, in the present work it was applied to the encapsulation of copaiba oil, a natural complex oil mixture, that was never described to our knowledge. The second originality of the work was to design those innovatively obtained nanocapsules with positive charge on their surface. It was assumed that stable dispersion of nanocapsules could be prepared in the absence of surfactants thanks to the incorporation of chitosan in the nanocapsule envelope conferring the surface with the desired positive charges. Because chitosan include chemical groups that are able to initiate the polymerization of isobutylcyanoacrylate monomers [27], it was postulated that chitosan could be incorporated in the nanocapsule envelope thanks to a reaction with isobutylcyanoacrylate that lead to the synthesis of an amphiphilic copolymer including poly(isobutylcyanoacrylate) chains grafted on chitosan. Thus, the grafted poly(isobutylcyanoacrylate) chains could serve as anchor to attach chitosan at the nanocapsule surface.

Preparations of nanocapsules were investigated changing the type of solvents used to produce the oil-in-water emulsion based on a solvent diffusion method. The nature of the solvent is an important factor as it conditions the nucleation of emulsion droplets of the oil that formed by a mechanism of homogenous nucleation (Ouzo effect) [28]. The feasibility of the encapsulation of copaiba oil was assessed considering the resin and the essential oil fractions. Small and highly positively charged nanocapsules containing copaiba resin oil could be obtained working with ethanol as the dispersing solvent. The results are consistent with those of the literature where ethanol is widely used as a suitable solvent to produce nanocapsules by interfacial polymerization of alkylcyanoacrylate [29]. On the other hand, nanocapsules produced with the essential oil were of much larger size and showed a lower zeta potential. Copaiba resin and essential oils show different composition which volatile components were extracted from the resin to prepare the essential oil fraction. This difference in composition may explain the difference in the size of droplets that were formed by the Ouzo effect during the preparation of the emulsion in the beginning of the process and hence, affecting the nanocapsules size. Regarding the effect of the molecular weight of chitosan, at constant temperature and concentration, viscosity of polymer solutions increases with the molecular weight of the polymer [30] and the smallest nanocapsules were obtained with the shorter chitosan. They were obtained with the aqueous phase of the expected lower viscosity favoring the dispersion of the organic phase during formation of the emulsion by the solvent diffusion technique. Data obtained from these preliminary experiments provided with enough information to make the selection of the components producing positively charged copaiba oil-loaded nanocapsules.

For the optimization step, temperature, pH of the polymerization medium and concentration of chitosan were the variables analyzed with a full- factorial 2^3 experimental design

approach. In all tested conditions, the nanocapsules showed a positive zeta potential. Nanocapsules from the literature that were prepared in the absence of chitosan were characterized with a negative zeta potential [18, 31]. The positive value of the zeta potential acknowledged the presence of chitosan on the nanoparticle surface consistently with the assumption drawn for this work. Formation of nanocapsules with a small hydrodynamic diameter was promoted in conditions of preparation favoring a slow polymerization of isobutylcyanoacrylate. These included acid pH and lower temperature. In contrast, submitted to conditions in which the polymerization happened more rapidly because of the basic pH, high temperature, and low degree of protonation of chitosan, the nanocapsules size was markedly increased. These results were consistent with those of the literature suggesting that slowing down the polymerization carried out in presence of chitosan is needed to obtain well define nanoparticles [32-35].

The high encapsulation efficiency of copaiba resin oil indicated that the oil immediately diffused to the internal phase of nanocapsules, where it was entrapped by the newly polymer formed nanocapsules envelope. The maintenance of oil composition during the nanoencapsulation process was an important parameter to evaluate because the oil contains biologically active components that are believed to promote a synergistic effect when they are associated with classical anticancer drugs with a high potential for the development of new treatments against cancer. The synergistic effect may arise from the accumulation of β-caryophyllene in membranes of cancer cells that can promote membrane permeability to bioactive compounds as suggested in a recent report [36]. A simulation of the dosage of nanocapsules required to fulfill a therapeutic dose of β-caryophyllene in human adults (0.16-3.3 mg/kg for a 60kg human) was calculated from the amount of this compound found in the nanocapsules. The requested dose range of β-caryophyllene can be achieved by administering 0.18 to 3.6 g of nanocapsules. This seems reasonable to achieve in practice emphasizing the interest of the proposed nanocapsules that are easy to produce. Since the developed nanocapsules were coated with chitosan, they are expected to display mucoadhesive properties that are of interest to improve drug delivery by mucosal routes of administration, including the oral route.

5 CONCLUSION

Poly (isobutylcyanoacrylate) nanocapsules incorporating copaiba oil could be prepared by interfacial polymerization in a surfactant free polymerization medium thanks to the use of chitosan. As assumed, chitosan was associated with the nanocapsules and provided positives charges to the nanocapsules surface. An experimental design approach was used to optimize the formulation aiming to prepare nanocapsules with small diameter and highly positive zeta potential. The three variables of the process, i.e. pH, concentration of chitosan in the polymerization medium and the temperature of polymerization, were identified influencing both the size and zeta potential of the prepared nanocapsules. These variables are known to affect the polymerization of isobutylcyanoacrylate. Copaiba oil was efficiently encapsulated while the composition of the encapsulated oil was identical to that of the original oil. These nanocapsules are expected to be mucoadhesive and suitable to serve as carrier system for lipophilic anticancer drugs by the oral route with possible synergistic anticancer activity

between the oil and the drug. The overall work presented itself as a rich information source for further research aiming to develop potential mucoadhesive nanoparticles containing natural oils by performing a small amount of experiments, saving time and reagents on development.

6 ACKNOWLEDGEMENTS

Authors acknowledge financial support from CAPES COFECUB 721/11 and would like to thank Imagif Cell Biology Unit of the Gif campus (www.imagif.cnrs.fr) which is supported by the Conseil Général de l'Essonne for providing facilities for transmission electron microscopy analysis.

7 REFERENCES

[1] G. Ponchel, J. Irache, Specific and non-specific bioadhesive particulate systems for oral delivery to the gastrointestinal tract, Adv. Drug Deliv. Rev., 34 (1998) 191-219.
[2] B. Petit, K. Bouchemal, C. Vauthier, M. Djabourov, G. Ponchel, The counterbalanced effect of size and surface properties of chitosan-coated poly(isobutylcyanoacrylate) nanoparticles on mucoadhesion due to pluronic F68 addition, Pharm. Res., 29 (2012) 943-952.
[3] S.S. Guterres, M.P. Alves, A.R. Pohlmann, Polymeric nanoparticles, nanospheres and nanocapsules, for cutaneous applications, Drug Target Insights, 2 (2007) 147-157.
[4] C. Pinto Reis, R.J. Neufeld, A.J. Ribeiro, F. Veiga, Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles, Nanomedicine, 2 (2006) 8-21.
[5] E.A. Leite, A. Grabe-Guimaraes, H.N. Guimaraes, G.L. Machado-Coelho, G. Barratt, V.C. Mosqueira, Cardiotoxicity reduction induced by halofantrine entrapped in nanocapsule devices, Life Sci., 80 (2007) 1327-1334.
[6] N. Anton, J.P. Benoit, P. Saulnier, Design and production of nanoparticles formulated from nano-emulsion templates - A review, J. Control. Release, 128 (2008) 185-199.
[7] I. Bravo-Osuna, C. Vauthier, A. Farabollini, G.F. Palmieri, G. Ponchel, Mucoadhesion mechanism of chitosan and thiolated chitosan-poly(isobutyl cyanoacrylate) core-shell nanoparticles, Biomater., 28 (2007) 2233-2243.
[8] I. Bravo-Osuna, C. Vauthier, H. Chacun, G. Ponchel, Specific permeability modulation of intestinal paracellular pathway by chitosan-poly(isobutylcyanoacrylate) core-shell nanoparticles, Eur. J. Pharm. Biopharm., 69 (2008) 436-444.
[9] M. Thanou, J.C. Verhoef, H.E. Junginger, Oral drug absorption enhancement by chitosan and its derivatives, Adv. Drug Deliv. Rev., 52 (2001) 117-126.
[10] C.K. Chen, Q. Wang, C.H. Jones, Y. Yu, H. Zhang, W.C. Law, C.K. Lai, Q. Zeng, P.N. Prasad, B.A. Pfeifer, C. Cheng, Synthesis of pH-responsive chitosan nanocapsules for the controlled delivery of doxorubicin, Langmuir, 30 (2014) 4111-4119.
[11] L. Illum, I. Jabbal-Gill, M. Hinchcliffe, A.N. Fisher, S.S. Davis, Chitosan as a novel nasal delivery system for vaccines, Adv. Drug Deliv. Rev., 51 (2001) 81-96.
[12] A. Bernkop-Schnurch, A. Heinrich, A. Greimel, Development of a novel method for the preparation of submicron particles based on thiolated chitosan, Eur. J. Pharm. Biopharm., 63 (2006) 166-172.
[13] J.P. Sousa, A.P. Brancalion, A.B. Souza, I.C. Turatti, S.R. Ambrosio, N.A. Furtado, N.P. Lopes, J.K. Bastos, Validation of a gas chromatographic method to quantify sesquiterpenes in copaiba oils, J. Pharm. Biomed. Anal., 54 (2011) 653-659.

[14] E.N. Alencar, F.H. Xavier-Junior, A.R.V. Morais, T.R.F. Dantas, N. Dantas-Santos, L.M. Verissimo, V.L.G. Rehder, G.M. Chaves, A.G. Oliveira, E.S.T. Egito, Chemical characterization and antimicrobial activity evaluation of natural oil nanostructured emulsions, J. Nanosci. Nanotechnol., 15 (2015) 880-888.

[15] A.O. Santos, T. Ueda-Nakamura, B.P. Dias Filho, V.F. Veiga Junior, A.C. Pinto, C.V. Nakamura, Antimicrobial activity of Brazilian copaiba oils obtained from different species of the Copaifera genus, Mem. Inst. Oswaldo Cruz, 103 (2008) 277-281.

[16] N.M. Gomes, C.M. Rezende, S.P. Fontes, M.E. Matheus, P.D. Fernandes, Antinociceptive activity of amazonian copaiba oils, J. Ethnopharmacol., 109 (2007) 486-492.

[17] L.M. Leandro, S. Vargas F, P.C. Barbosa, J.K. Neves, J.A. Silva, V.F. Veiga-Junior, Chemistry and biological activities of terpenoids from copaiba (Copaifera spp.) oleoresins, Molecules, 17 (2012) 3866-3889.

[18] M. Aboubakar, P. Couvreur, H. Pinto-Alphandary, B. Gouritin, B. Lacour, R. Farinotti, F. Puisieux, C. Vauthier, Insulin-loaded nanocapsules for oral administration: In vitro and in vivo investigation, Drug Dev. Res., 49 (2000) 109-117.

[19] B.K. Patel, R.H. Parikh, P.S. Aboti, Development of oral sustained release rifampicin loaded chitosan nanoparticles by design of experiment, J. Drug Deliv., 2013 (2013) 370938-370948.

[20] P. Couvreur, B. Kante, M. Roland, P. Guiot, P. Bauduin, P. Speiser, Polycyanoacrylate nanocapsules as potential lysosomotropic carriers: preparation, morphological and sorptive properties, J. Pharm. Pharmacol., 31 (1979) 331-332.

[21] N.A. Fallouh, L. Roblottreupel, H. Fessi, J.P. Devissaguet, F. Puisieux, Development of a New Process for the Manufacture of Polyisobutylcyanoacrylate Nanocapsules, Int. J. Pharm., 28 (1986) 125-132.

[22] Y.G. Zu, Q. Zhao, X.H. Zhao, S.C. Zu, L. Meng, Process optimization for the preparation of oligomycin-loaded folate-conjugated chitosan nanoparticles as a tumor-targeted drug delivery system using a two-level factorial design method, Inter. J. Nanomedicine, 6 (2011) 3429-3441.

[23] W.G. Cochran, G.M. Cox, Experimental designs, 2 ed., Wiley, New York, 1957.

[24] D.C. Montgomery, Design and Analysis of Experiments, 7 ed., Wiley, New York, 2008.

[25] H.L. Liu, Y.W. Lan, Y.C. Cheng, Optimal production of sulphuric acid by Thiobacillus thiooxidans using response surface methodology, Process. Biochem. , 39 (2004) 1953-1961.

[26] R. Sen, I. Swaminathan, Response surface modeling and optimization to elucidate and analyze the effects of inoculum age and size on surfactin production, Biochem. Eng. J., 21 (2004) 141-148.

[27] C. Zandanel, C. Vauthier, Characterization of fluorescent poly(isobutylcyanoacrylate) nanoparticles obtained by copolymerization of a fluorescent probe during Redox Radical Emulsion Polymerization (RREP), Eur. J. Pharm. Biopharm., 82 (2012) 66-75.

[28] F. Ganachaud, J.L. Katz, Nanoparticles and nanocapsules created using the Ouzo effect: Spontaneous emulsification as an alternative to ultrasonic and high-shear devices, Chem. Phys. Chem., 6 (2005) 209-216.

[29] M. Gallardo, G. Couarraze, B. Denizot, L. Treupel, P. Couvreur, F. Puisieux, Study of the mechanisms of formation of nanoparticles and nanocapsules of polyisobutyl-2-cyanoacrylate, Int. J. Pharm., 100 (1993) 55-64.

[30] J. Desbrieres, Viscosity of semiflexible chitosan solutions: influence of concentration, temperature, and role of intermolecular interactions, Biomacromolecules, 3 (2002) 342-349.

[31] F. Cournarie, M. Cheron, M. Besnard, C. Vauthier, Evidence for restrictive parameters in formulation of insulin-loaded nanocapsules, Eur. J. Pharm. Biopharm., 57 (2004) 171-179.

[32] P.A. McCarron, A.D. Woolfson, S.M. Keating, Response surface methodology as a predictive tool for determining the effects of preparation conditions on the physicochemical properties of poly(isobutylcyanoacrylate) nanoparticles, Int. J. Pharm., 193 (1999) 37-47.
[33] N.R. Ravikumara, B. Madhusudhan, Chitosan nanoparticles for tamoxifen delivery and cytotoxicity to MCF-7 and Vero cells, Pure Appl. Chem., 83 (2011) 2027-2040.
[34] S.C. Yang, H.X. Ge, Y. Hu, X.Q. Jiang, C.Z. Yang, Formation of positively charged poly(butyl cyanoacrylate) nanoparticles stabilized with chitosan, Colloid Polym Sci, 278 (2000) 285-292.
[35] M. Aboubakar, F. Puisieux, P. Couvreur, M. Deyme, C. Vauthier, Study of the mechanism of insulin encapsulation in poly(isobutylcyanoacrylate) nanocapsules obtained by interfacial polymerization, J. Biomed. Mater. Res., 47 (1999) 568-576.
[36] J. Legault, A. Pichette, Potentiating effect of beta-caryophyllene on anticancer activity of alpha-humulene, isocaryophyllene and paclitaxel, J. Pharm. Pharmacol., 59 (2007) 1643-1647.