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ARTICLE

Design of Experiments (DoE) Application in Two Cases of Study in Pharmaceutical Industries

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This study illustrates the practical application of Design of Experiments (DoE) in two real-life scenarios within the pharmaceutical industry. The first case involved optimizing a chromatographic method to determine multiple analytes and their degradation products. The primary variable of interest was gradient time, and the most favorable outcomes were achieved at a pH value of 2. In the second case, we conducted a shelf-life study for a veterinary product, revealing that the vial filling variable exerted a statistically significant impact (p-value < 0.05). The incorporation of DoE in both cases played an important role in ensuring the attainment of dependable and statistically validated results.

Keywords: factorial design, shelf-life, optimization, quality by design, method development

INTRODUCTION

Design of Experiments (DoE)[1] is an important tool for variables characterization, analytical methods optimization,[2] quality assurance in industry, and a variety of important applications in many industrial and scientific fields.[3,4] The use of DoE techniques in pharmaceutical area (by companies or researchers) is,[5] in several cases, an obligation to save time, economic resources, improve the speed for results analysis and acquisition, and mainly to fulfill requirements described by international regulatory agencies,[6] complying with the Quality by Design (QbD),[7,8] and Analytical QbD (AQbD) directives.[9,10]

In a review presented by Patel and Kothari in 2018, the authors presented many aspects related to the implementation of multivariate approaches to degradation study and impurities detection in pharmaceutical companies. The authors emphasized the guidelines presented by the International Council for Harmonisation (ICH).[6]

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The optimization of chromatographic methods[11-13] takes special advantage of DoE[14] and several applications are found in the scientific literature. Peng *et al.*, for instance, presented a review focused in DoE tools for chromatographic conditions optimization.[15] Another field that currently employ DoE is product shelf-life and stability studies in pharmaceutical or food applications. Table I shows some selected papers from the period of 2015 to 2023 that employed DoE to shad light in several pharmaceutical problems. Most of the studies are related to method optimization employing QbD concepts combined with DoE.[16,17]

Table I. Scientific articles related to the use of DoE, chromatographic methods optimization, and shelf-life studies

Goal	Remarks
Formulation optimization[18]	KI tablet formulation optimization using a direct compression method. The authors employed mixture design.
Formulation optimization[19]	Investigation of 3 variables in the preparation of diclofenac sodium.
Stability prediction[20]	The authors proposed phenomenological models to predict the stability of vaccines.
Method optimization[21]	Simultaneous determination of Quinabut and its impurities using HPLC method.
Method optimization[22]	The authors used a central composite design (CCD) to optimize a method for Torsemide and Eplerenone determination.
Dissolution stability[23]	Investigation of modified-release drug product stability.
Method optimization[24]	Chromatographic method optimization to study the stability of ubidecarenone.
Method development[25]	The authors optimized a liquid chromatographic method for determination of glycopyrrolate in formulations.
Increase shelf-life of liposomes[26]	Study of 5 variables (cholesterol concentration, freezing conditions, among others) contribution in physico-chemical properties.
Determination of bioactive cannabinoids[27]	The authors combined DoE and exploratory analysis with (principal component analysis) PCA for data interpretation
Quantification of two analytes: Lamivudine and Zidovudine[28]	The authors used desirability function to simultaneously optimize method conditions for both analytes.

The main goal of this study was to report two frequent pharmaceutical problems that were analyzed using DoE. The first case is related to an analytical method developing using chromatography and the second, to shelf-life (stability) veterinary product investigation. Both studies were performed at pharmaceutical company laboratories by former students from the Chemistry Professional Master Graduation Program (https://www.ppgpq.ufscar.br).

MATERIAL AND METHODS

Material

As described in the previous sections, the experimental part of this study was divided in two sets: (1) chromatographic method development for separation of three actives pharmaceutical ingredients (API) and its impurities, developed at Libbs pharmaceutical company (Embu, São Paulo State, Brazil), and (2) shelf-life study of an API veterinary product. Some details about the experimental part are described in the next sections.

Chromatographic method development (case 1)

The method development was performed using HPLC and UPLC instruments from Agilent (model 1290) and Waters (model Acquity H-Class), respectively. The instruments were equipped with diode array detector (DAD) for chromatographic purity check. The chromatographic columns were Xterra RP8 and RP18 (both Waters), Eclipse XDB C18 and Zorbax SB C18 (both Agilent). Reference analytical standards were used as described by RDC 166/2017.[29] The organic solvents employed (methanol and acetonitrile) were from Merck and a Milli-Q system (Millipore) was used for ultrapure water.

Gradient time (variable 1) and mobile phase pH (variable 2) were investigated using a central composite design (CCD)[30] with centered face and all experiments were made in two authentic replicates. These variables were tested in three different levels (-1, 0 and 1) and varied from 30 min to 50 min in the case of variable 1 and from 2.0 to 4.0 for variable 2. A total of 21 experiments were performed: a 2^2 full factorial design (4 duplicated experiments), axial points with centered face (4 duplicated experiments) and 5 experiments in the center point (variables coded as 0). Figure 1 shows the CCD configuration with all experiments performed.

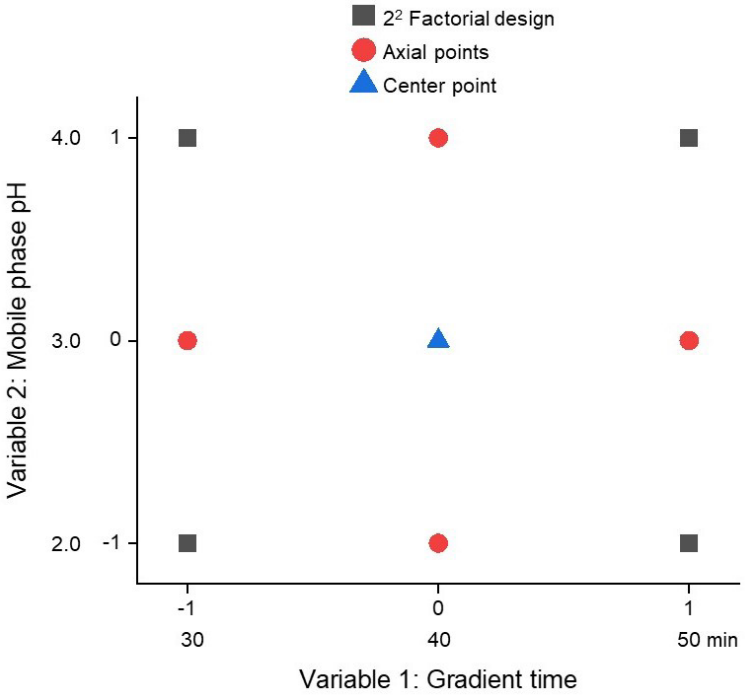

Figure 1. CCD performed in the case 1: 2^2 factorial design (black squares), axial points (red circles) and center point (blue triangle). Both normalized (between -1 and 1) and real conditions for variables 1 and 2 are presented.

Table II shows the experiments performed, and four responses were evaluated: (1) number of obtained peaks (y_1), (2) number of obtained peaks with resolution (R) higher than 1.5 (y_2), (3) global desirability (D) combining both y_1 and y_2,[31,32] and (4) resolution for a critical pair (R_{cp}).

Table II. CCD for case 1 (chromatographic method development) / The resolution values are related to the critical pair (R_{cp})

Experiment	Variables in normalized scale		Monitored responses			
	Time	pH	y_1	y_2	D	Resolution for the critical pair (R_{cp})
1	-1	-1	11	10	0.53	3.1
2	-1	-1	11	9	0.46	3.1
3	1	-1	10	9	0.33	3.0
4	1	-1	10	9	0.33	3.0
5	-1	1	10	9	0.33	2.8
6	-1	1	11	10	0.53	2.8
7	1	1	11	8	0.38	2.5
8	1	1	11	7	0.27	2.5
9	-1	0	15	7	0.46	2.9
10	-1	0	15	7	0.46	2.9
11	1	0	16	7	0.50	2.7
12	1	0	16	8	0.71	2.7
13	0	1	9	6	0.00	2.5
14	0	1	9	6	0.00	2.5
15	0	-1	11	8	0.38	2.9
16	0	-1	10	7	0.19	2.9
17	0	0	10	7	0.19	2.8
18	0	0	10	7	0.19	2.8
19	0	0	10	7	0.19	2.8
20	0	0	10	7	0.19	2.8
21	0	0	10	7	0.19	2.8

Responses y_1 and y_2 were combining after normalizing each one between 0 (not desired response, lowest resolution) and 1 (target response, highest resolution). In this case, each response (y_i) was transformed in individual desirability (d_i) as described in Equation 1.

$$d_i = \left(\frac{y_i - L}{T - L}\right)^s \qquad \text{Equation 1}$$

Where T and L are the target (highest value) and the lowest value, respectively. The index s is a weight, and in this specific case is 1.

The results for experiment 1 (see Table II, y_1 = 11 and y_2 = 10), for instance, can be normalized using the following mathematical expressions:

$$d_1 = \left(\frac{y_1 - L_1}{T_1 - L_1}\right)^S = \left(\frac{11 - 9}{16 - 9}\right)^1 = \frac{2}{7} = 0.29$$

$$d_2 = \left(\frac{y_2 - L_1}{T_2 - L_2}\right)^S = \left(\frac{10 - 6}{10 - 6}\right)^1 = \frac{4}{4} = 1$$

Both values can be combined using geometric mean, to obtain the Global desirability (D) for the first experiment:

$$D = \sqrt[n]{d_1 \times d_2 \times \cdots \times d_n} = \sqrt[2]{0.29 \times 1} = \sqrt{0.29} = 0.53$$

These calculations were repeated for all experiments and can be seen at Table II. More details will be presented in the Results and Discussion section.

All regression models obtained were calculated using Octave version 7.2.0 and the data organization and visualization were performed using Microsoft Excel®. Homemade mathematical routines[30,33] were prepared to perform analysis of variance (Anova) and statistical evaluation of the obtained regression models. In all cases for model performance evaluation, the confidence level was 95%.

Shelf-life study (case 2)

In this part of the study, HPLC was used to monitor the concentration of an API used in veterinary pharmaceutical product. The monitoring method employed a stationary phase Gemini C18 (Sigma-Aldrich), and the mobile phase was composed of methanol:acid solution (55:45% v v^{-1}) at gradient mode. The analytical signal was monitored at 330 nm.

Fifteen experiments were performed using a Doehlert design.[34] In this type of design, the variables can be verified in different number of levels. Then, three variables were tested: (1) time (days) in seven different levels (1, 21, 41, 61, 80, 100, and 120), (2) temperature (°C) in five levels (-20, -5, 10, 25 and 40), and (3) vial filling (%) in three levels (25, 62.5 and 100).

The normalized values for the time were from -0.866 (1 day) up to 0.866 (120 days). In the case of variable (temperature), the coded values varied from -1 (-20 ºC) up to 1 (40 ºC). Variable 3 (vial filling) coded values goes from -0.817 (25%) up to 0.817 (100%). As the time is a very important aspect of shelf-life study, more importance was given to this variable that was monitored in 7 levels (from 1 day up to 120 days). The monitored response was the API concentration in % m m^{-1}. The variable 3 (vial filling) was intended to understand how the air inside de vial and its interaction with time and temperature can contribute to the API stability. The level 25% means that 75% of the vial was empty. Table III shows the performed experiments and three replicates were prepared at the central point (variables coded as 0). Like the previous section, the data and regression models obtained were also handled using Microsoft Excel® and Octave, respectively. The confidence level for model evaluation was also 95%. Figure 2 shows the configuration of the 3 variables in a 3D visualization.

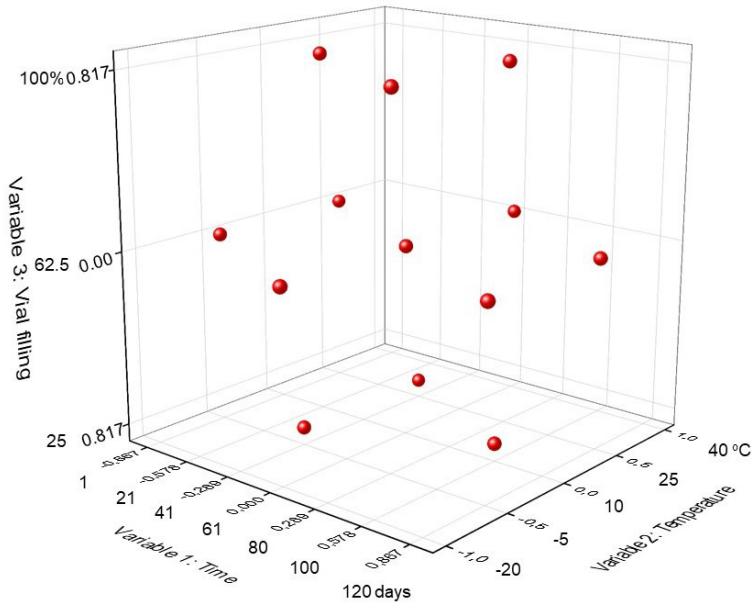

Figure 2. Doehlert design performed in the case 2. Both normalized and real conditions for the three variables (time, temperature and vial filling) are presented.

Table III. Doehlert design for case 2 (shelf-life study for a veterinary API)

Experiment	Variables in normalized scale			Monitored response
	Time	Temperature	Vial filling	[Analyte] % m m^{-1}
1	0	1	0	60.48
2	0.866	0.5	0	60.54
3	0.289	0.5	0.817	60.94
4	0	-1	0	61.01
5	-0.866	-0.5	0	60.21
6	-0.289	-0.5	-0.817	59.84
7	-0.866	0.5	0	60.29
8	-0.289	0.5	-0.817	60.06
9	0.866	-0.5	0	59.43
10	0.577	0	-0.817	60.41
11	0.289	-0.5	0.817	61.66
12	-0.577	0	0.817	61.35
13	0	0	0	61.24
14	0	0	0	60.41
15	0	0	0	60.93

RESULTS AND DISCUSSION

Chromatographic method development (case 1)

Figure 3 shows a pictorial description of a typical obtained chromatogram. The two red peaks are the critical pair that was also monitored, and the resolution (R_{cp}) between them was considered as response.

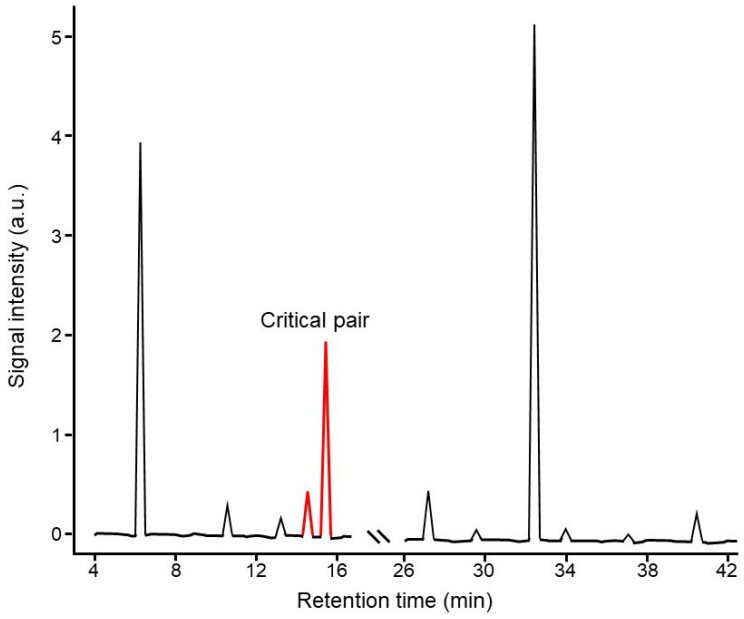

Figure 3. Pictorial description of chromatogram for case 1 (HPLC method optimization). The peaks illustrated in red are related to the critical pair.

Table II shows the four responses obtained: y_1, y_2, D (Global desirability combining y_1 and y_2), and resolution of the critical pair. The goal of this first study of case was to maximize all monitored responses. The individual models for y_1 and y_2 presented lack-of-fit and the *p*-values[35] obtained when the mean square of regression (MSR) and mean square of residue (MSr) were 0.002 and 0.003, respectively. A global model was calculated combining both y_1 and y_2 (see details in section *Chromatographic method development (case 1)*), and global desirability (D) was taken into consideration.[2] Six coefficients(b_i) were calculated using least squares[36] as describe in Equation 2.

$$b = (X^T X)^{-1} \times X^T y \qquad \text{Equation 2}$$

Where X is a matrix with n rows (number of experiments) and m columns (coefficients b that will be calculated). In the specific situation of this case of study, n = 21 (experiments, see Table II) and m = 6 (coefficients). The calculated coefficients are b_0 (constant), b_1 and b_2 (linear coefficients for the variables, time and pH), b_1^2 and b_2^2 (quadratic coefficients for the variables), and b_{12} (interaction between the two variables). The general idea behind Equation 2 is to minimize the error between the predicted response $\hat{y}$ (in this case $\hat{D}$) and the experimental value y (*D*, see Table II).

The *p*-value obtained for MSR and MSr was 0.0006 that demonstrate the both MS are statistically different. On the other hand, the proposed model presented LoF, and the *p*-value comparing MSLoF and MS of pure error (MSPE) was 0.01 (lower than 0.05, then significative). As the model presented lack-of-fit, the MSLoF was used to calculate the confidence interval of the coefficients.[30] Figure 4 shows the results obtained. As can be observed only the intercept or constant (b_0) and the quadratic coefficient for variable 1, time (b_1^2) were statistically significative.

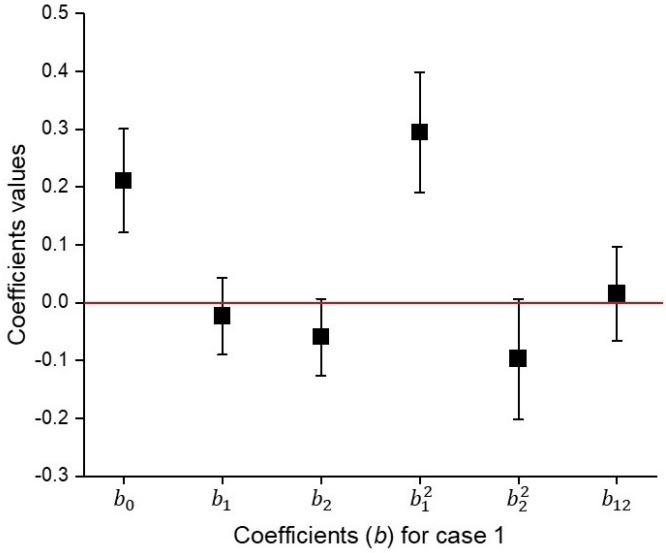

Figure 4. Coefficients (b) and its confidence interval (error bars) for the case 1 considering D as response.

From Figure 4 it is noted that some confidence intervals (C.I.) can assume the 0 value, and their correspondent coefficients are not significant. The C.I. was calculated according to Equation 3.

$$C.I. = \sqrt{MSLoF \times main\ diagonal\ of\ (X^t X)^{-1}} \times t_{(n-1.95\%)} \qquad \text{Equation 3}$$

Where $t_{(n-1.95\%)}$ is the tabulated value of *t* for *n* – 1 degree of freedom of the MSLoF.

The Anova table of the proposed model can been shown in Table IV.

Table IV. Anova table for the proposed model using D as response

Parameters	Sum of Squares (SS)	Degrees of freedom	Mean of Squares (MS)	Calculated F	*p*-value
Regression	0.480	5	0.096	$\frac{MSR}{MSr} = \frac{0.480}{0.173} = 8.34$	0.0006
Residue	0.173	15	0.012		
Total	0.653	20	0.033		
Pure error	0.069	12	0.006	$\frac{MSLoF}{MPE} = \frac{0.104}{0.069} = 6.01$	0.01
Lack-of-fit	0.104	3	0.035		

The non-significant coefficients (b_1, b_2, b_{12} and b_2^2, see Figure 4) were removed, and the Sum of Square of Pure Error was calculated again using the new replicates. The model was also calculated again, and Equation 4 shows the final proposed model (95% of confidence level) when D was monitored.

$$D = 0.17 + 0.27 v_1^2 \qquad \text{Equation 4}$$

As can be noted, only the constant (b_0 = 0.17) and the quadratic coefficient for variable 1 (b_1^2 = 0.27, time) were statistically significant (*p*-value < 0.05). Figure 5 shows the response surface obtained and it is possible to see that both short (30 min) or long (50 min) gradient times can be used. In the case of pH, any value in the range from 2.0 to 4.0 can be used to obtain both high y_1 and y_2 (high D value).

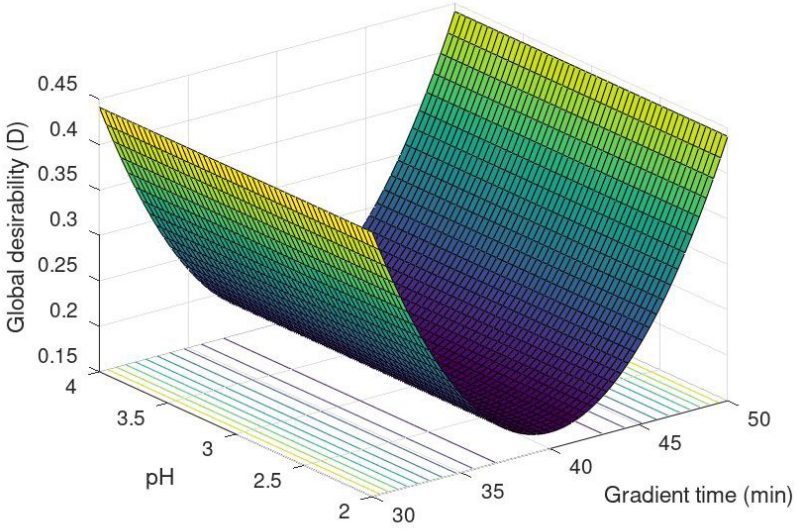

Figure 5. Surface response for case 1 with variables 1 (gradient time, min) and 2 (pH). The monitored response was the global desirability (D) considering responses y_1 and y_2 (see details in Table II).

On the other hand, the D was the only response monitored and the R_{cp} need to be also considered. The regression model for the R_{cp} can be seen at Equation 5.

$$R_{cp} = 2.76 - 0.10v_1 - 0.20v_2 + 0.078v_1^2 - 0.050v_1v_2 \qquad \text{Equation 5}$$

This regression model presented more significative coefficients and Figure 6 shows the surface response obtained. The best results can be obtained using only 30 min for variable 1 (shorter time) and pH = 2.0. Several validation experiments were performed using these instrumental conditions, and the precision values (%) for the APIs monitored varied from 99.9±0.7 up to 101.2±0.2.

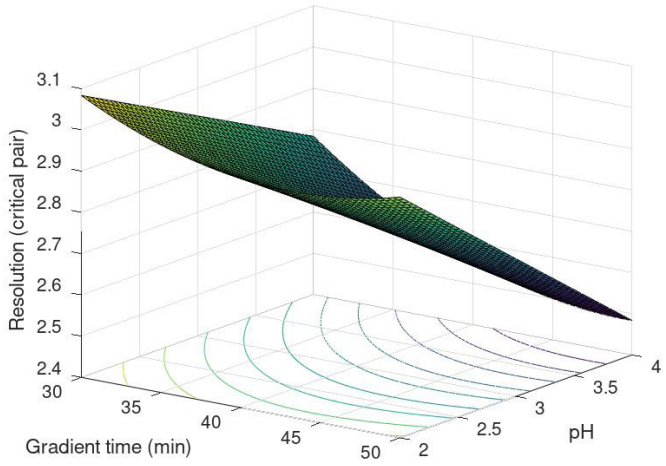

Figure 6. Surface response for case 1 with variables 1 (gradient time, min) and 2 (pH). The monitored response was the R_{cp} (see details in FIGURE 1 and Table II).

Shelf-life study (case 2)

The goal of the second case was to observe if variables related to shelf-life are affecting the concentration of the API in the final product. Figure 7 shows a pictorial description of typical chromatogram, and the concentration of the API is proportional to the signal area sum of the four analytes depicted: 1, 1a, 2a+2b and 2. Table III shows the results obtained for the Doehlert design performed, and as can be observed, the concentration of the API presented a very narrow range: from 59.43% m m^{-1} (experiment 9) up to 61.66 (experiment 11), with average, standard deviation and median of 60.59, 0.60 and 60.48, respectively. The Kurtosis and Skewness[37] of the values were -0.31 and -0.03, respectively, that reflects in a flat distribution of the data and similarity between average and median.

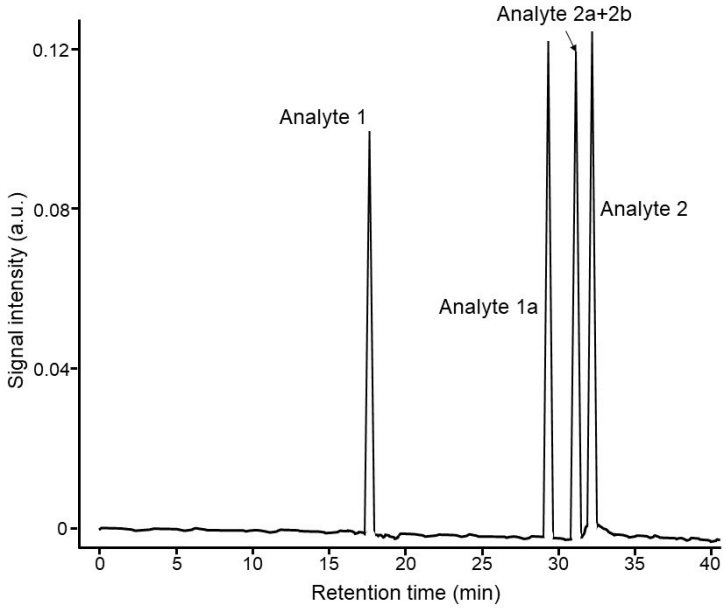

Figure 7. Pictorial description of chromatogram for case 2 (shelf-life study). The peaks identified (1, 1a, 2a+2b and 2) correspond to the veterinary API.

A regression model was calculated and evaluated to observe which variable and in which extension affect the response. But now 10 coefficients were calculated: b_0 (constant), b_1, b_2, b_3 (the linear coefficients for each variable), b_1^2, b_2^2, b_3^2 (quadratic coefficients), b_{12}, b_{13} and b_{23} (interaction coefficients). The three replicates performed at the central point (experiments 13, 14 and 15) were used to calculate the MSPE. Figure 8 shows the coefficients calculated and its interval of confidence (error bars). As can be noted only b_0 and b_3 were significant with 95% of confidence level.

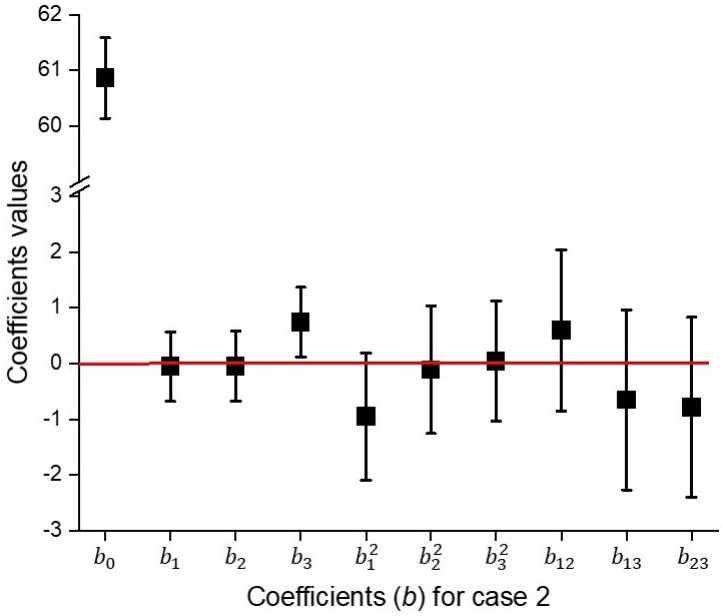

Figure 8. Coefficients (b) and its confidence interval (error bars) for the case 2 considering the concentration of the API as response.

As can be observed using Table III and Figure 8, the regression model calculated presented very poor statistical parameters due to the fact the low variation of the response. The model was recalculated excluding the non-significative coefficients and the obtained one can be seen in Equation 6.

$$[API]\%\, m\, m^{-1} = 60.86 + 0.74 v_3 \qquad \text{Equation 6}$$

Figure 9 shows the distribution of the residues when the experimental and predicted values were compared. As can be noted the residues follow a normal distribution with average, standard deviation and median of -0.00013, 0.45, and -0.047. The Kurtoses and Skewness[37] of the results were 1.87 and -0.96 that reflects in distribution in cume and low asymmetry (similarity between average and median).

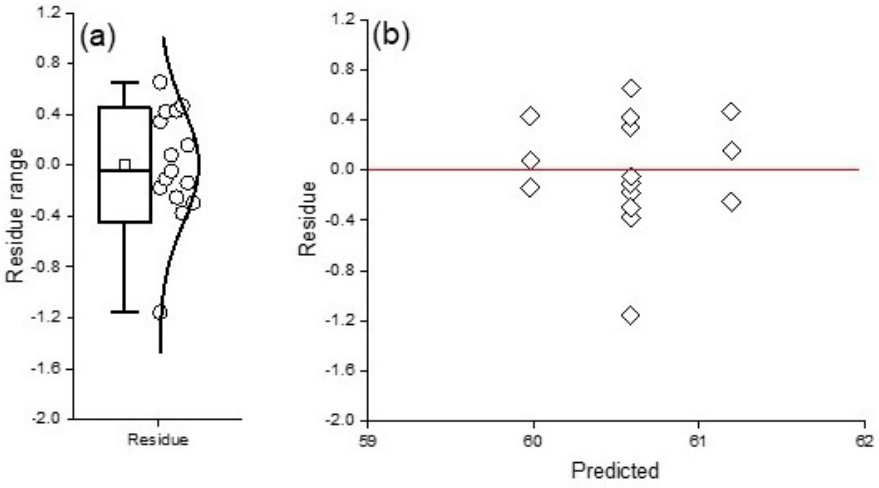

Figure 9. Statistical evaluation of the residues of the proposed model: (a) The box size is proportional to the standard deviation, the small square and the horizontal line inside the box are the average and median, respectively. The bars show the range of the values (minimum and maximum); (b) predicted $\hat{y}$) *versus* residues values for the proposed model.

The residues were evaluated in combination with the predicted values using Shapiro-Wilk[38] test, and it was observed a normal distribution

Figure 10 shows the surface response for the regression model obtained. The vial filling parameter effects and its confidence interval is positive and around 1.5±1.2% m m^{-1}. The other variables contributions were negligible (*p*-value > 0.05) in the studied range, being the API stable for a period of up to 4 months, even when submitted to temperatures from -20 up to 40 ºC. The volume of product inside the vial is a significative variable (variable 3), but its contribution for the final concentration is not critical for the purpose of the veterinary pharmaceutical product.

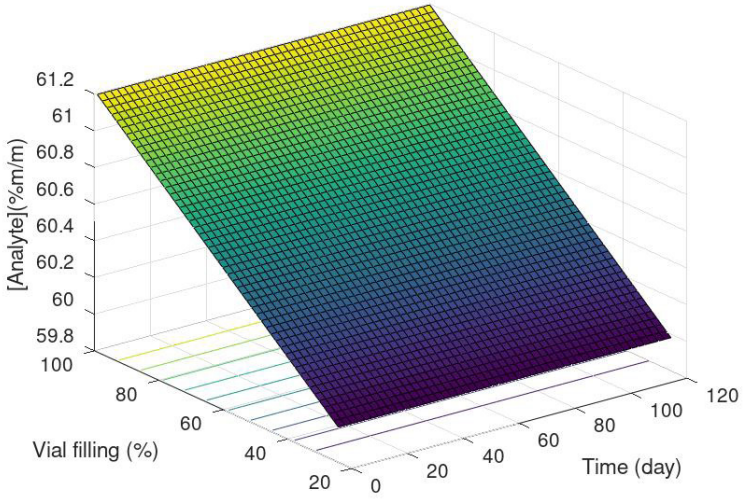

Figure 10. Surface response for case 2 with variables 1 (time, day) and 3 (Vial filling). The monitored response was the concentration of the veterinary API. The variable 2 (Temperature) was fixed in 10 ºC (normalized as 0).

CONCLUSION

Both examples presented in this study yielded reliable results that underwent statistical evaluation. Furthermore, the optimization of methods and assessment of shelf life reflected into significant economic benefits for the companies involved, underscoring the essential role of Design of Experiments (DoE) in achieving diverse goals. In the context of method optimization, retention time emerged as the most important variable, while pH played a critical role in enhancing resolution for the critical pair (see Figure 6). In the stability study (case 2), vial filling was the most critical variable. Notably, the contact between the product and air, although statistically significant, had a relatively minor effect, accounting for less than 2% of the observed variance.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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