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Usefulness of D-optimal designs and multicriteria optimization in laborious analytical procedures Application to the extraction of quinolones from eggs

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Abstract

An analytical method has been developed to extract ciprofloxacin and enrofloxacin from eggs. The aim of this work is to determine the experimental conditions of extraction providing high recoveries with small standard deviations. An experimental design based on the D-optimality criterion and replicated three times was built to evaluate the effect of five factors related to the extraction which is the most inaccurate stage of the procedure. This non-classical design is needed because there are several practical constraints: (i) the extraction procedure is time-consuming, quinolones are not stable and the design must be performed in a single working session. (ii) The tube capacity of the centrifuge is 6, so the number of experiments will be 6 or a multiple of 6. In the optimal experimental conditions, the extraction is performed once with 5 ml of methanol. Then, fatty acids are removed with a mixture of hexane/ether. Analytes are finally separated and detected by HPLC-fluorescence without the additional step of purification by solid-phase extraction (SPE). Under these conditions, the mean recovery is 64% and 70% and the standard deviation 5% and 4% for ciprofloxacin and enrofloxacin, respectively. The capability of decision, CCα, is 3.1 and 2.8 $\mu g\,kg^{-1}$ of ciprofloxacin and enrofloxacin, respectively. The capability of detection, CCβ, is 7.8 and 7.0 $\mu g\,kg^{-1}$ of ciprofloxacin and enrofloxacin, respectively. In both cases the probabilities of false positive, α, and of false negative, β, were fixed at 0.05.

Keywords: D-optimal design; Ciprofloxacin; Enrofloxacin; Residue analysis; Exchange algorithm; HPLC; Fluorescence; European Decision 2002/657/EC

1. Introduction

Enrofloxacin and its metabolite ciprofloxacin are two biologically active antibiotics from the second generation of the fluoroquinolones. Quinolone antibiotics are increasingly being used for veterinarian treatment because of their antibacterial activity [1] and effectiveness against infections. However, quinolone residues in food might cause allergic reactions and microbial resistance. That is why the European Union has regulated the use of antibiotics in food-producing animals through Regulation no. 2377/90 amended by successive modifications. Specifically for poultry, their use has been forbidden in animals from which eggs are produced for human consumption. Residues are proved [2] to be transmitted and accumulated in eggs about 1 week after the poultry treatment.

The aim of this paper is to develop an extraction procedure of ciprofloxacin and enrofloxacin from eggs that guarantees high recoveries with small standard deviations. Thus, other figures of merit such as the accuracy and the detection limit among others, will improve [3]. Enrofloxacin and ciprofloxacin have already been analysed in poultry eggs [4–7]. A wide review of the methods for analysing quinolones in other biological matrices has been made by Hernández-Arteseros in ref. [8]. Most of the methods consist of a first stage for quinolone extraction carried out with water-immiscible organic solvents such as dichloromethane

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[9], water-miscible organic solvents such as acetonitrile [10], hydro-organic mixtures containing acetonitrile and ammonia [4,5], or acetonitrile–water mixtures containing acetic acid [7]. In other references, methanol has been used as extracting solvent acidified with trichloroacetic acid [11] or aqueous solutions buffered [12]. After extraction and clean-up treatment, quinolones are analysed by high-performance liquid chromatography (HPLC) with fluorescence detection [4,5,7], liquid chromatography with mass spectrometry detection (LC–MS) [6,10,12], HPLC with ultraviolet detection [2,11], or by electrophoresis [13]. Other detection methods have been reported in ref. [14].

In this paper, we will focus on the extraction procedure because it is the step which introduces most of the variability in the analytical method. Five factors will be examined: type and volume of extracting agent, number of extractions and of washes and purification by solid-phase extraction (SPE).

The methodology based on the design of experiments (DOE) is a useful tool that might be employed for finding the best experimental conditions. Depending on the problem, several kinds of designs can be applied: factorial designs [15,16] are appropriate to evaluate principal effects as well as interactions between factors; fractional designs [17,18] solely deal with principal effects; central composite [19,20] and Doehlert [21] designs together with those designs based on the simplex method [19] are employed for optimizing the experimental conditions when dealing with continuous factors. In some situations it is not possible to apply these classical experimental designs due to constraints either on the experimental domain (cost of certain reagents, safety or incompatibility in the experimental conditions, etc.) or on the number of experiments (time-consuming analysis, cost, material, etc.). These limitations oblige the analyst to reduce the experimentation by selecting those experiments that, complying with the constraints enforced, keep the maximum quality of the design, the reliability of the estimations and therefore of the conclusions derived from it. In this work, two practical restrictions were found: (i) the stages of sample pre-treatment, extraction of the analytes and clean-up of the extracts are time-consuming. The number of experiments must be reduced so that the experimental plan can be performed on the same day (the full factorial design would need $2^5 = 32$ experiments). (ii) The tube capacity of the centrifuge is 6 so the experimentation should be done in series of six experiments for the correct performance of the centrifuge.

The six experiments to be performed were selected according to the D-optimality criterion [22–24]. D-optimal designs have the property that the estimations derived from the mathematical model postulated are the most precise ones. Thus, the experimental conditions maximizing the accuracy can be obtained from the analysis of the coefficients of the model. Designs based on the D-optimality criterion have already been used in those cases in which either any combination of values in the experimental variables is not possible (pH and solvent strength of a solution [25]), or the number of experiments is limited [26]. The aim of this work is to establish, by means of a D-optimal design, which factors influence the extraction of the two quinolones from eggs, to subsequently select the best extraction conditions from the point of view of the accuracy [27]. Specifically, we will explore those conditions which ensure high recoveries (trueness, response 1) with small standard deviations (precision, response 2). From a methodological point of view, the problem is general and this paper is an example of the practical interest of the made-to-measure experimental designs in the chemical analysis.

2. Methodology

2.1. Selection of the factors, of the experimental domain and of the responses

Five factors related to the extraction and clean-up procedure will be analysed in this paper. All five factors together with their variation levels, nominal level (+), extreme level (−) and the codification used in the paper are listed in Table 1. The election of the extracting solvent (X_1 in Table 1) is fundamental for precipitating the proteins from eggs, extracting both antibiotics from the sample and dissolving them. According to the review done by Hernández-Arteseros et al. [8], acetonitrile (−, in Table 1) and methanol (+) are two solvents commonly used. Other factor to be analysed is the volume of extracting solvent (X_5, in Table 1). It will be proved whether 3 ml (−) are enough for complete extraction or the procedure would require 5 ml (+). The extraction may be performed once (−) or twice (+) to assure quantitative extraction (X_2, in Table 1).

Once the analytes are in solution, it is necessary to include one (−) or two (+) clean-up steps to remove fat (X_3, in Table 1) from the extract. Finally, the extracts may be purified by SPE (−) or not (+) (X_4, in Table 1).

The effect of all five factors on the mean recovery (trueness, response 1) and on the standard deviation (precision, response 2) of the extraction will be determined.

2.2. Mathematical model postulated: D-optimal design and exchange algorithm

The selection of the mathematical model which represents the phenomenon studied is the second step of the experimental design methodology. In this paper a first-order linear

Table 1
Experimental factors together with the nominal (+) and extreme (−) levels selected for the D-optimal design

Associated variable	Factor (units)	Level	
		−	+
X_1	Extracting agent	Acetonitrile	Methanol
X_2	Times extracted	1	2
X_3	Times washed	1	2
X_4	Cartridge	Yes	No
X_5	Volume of solvent (ml)	3	5

Table 2
D-optimal design, mean recovery (%, response 1) and standard deviation (%, response 2) from the three replicates for ciprofloxacin and enrofloxacin

Experiment	Coded variables					Responses			
	X_1	X_2	X_3	X_4	X_5	Ciprofloxacin (%)		Enrofloxacin (%)	
						Recovery	SD	Recovery	SD
4	–	+	+	–	–	46	7	43	3
5[a]	–	–	–	+	–	50	2	52	3
3[a]	+	+	–	+	–	57	6	60	6
6	+	–	–	–	+	87	7	62	10
2	–	+	–	+	+	46	7	59	5
1	+	–	+	+	+	66	4	69	4

[a] An outlier has been removed for the estimation of the mean recovery and the standard deviation of ciprofloxacin and enrofloxacin.

model was postulated:

$$y = \mathbf{X}\beta + \varepsilon \quad (1)$$

where **X** is the model matrix or effect matrix with dimensions $N \times p$ (N is the number of experiments and p is the number of coefficients of the model). y is the vector of the experimental responses, β is the vector of the coefficients and ε is the vector of the experimental errors. In this paper, no interaction between two or more factors is expected and only the principal effects will be evaluated. When the model is adjusted to the experimental data, not only the experimental error but also an error in the selection of the model is transmitted to the coefficients and through them to the analysis of the significance of the factors. Consequently, the proper selection of the model according to the a priori knowledge is important to get satisfactory results.

The estimation of the coefficients of model (1), b_i, allows one to know the effect of a factor on the response and is obtained by least squares:

$$b = (\mathbf{X}^t\mathbf{X})^{-1}\mathbf{X}^t y \quad (2)$$

where $(\mathbf{X}^t\mathbf{X})$ is called the information matrix and $(\mathbf{X}^t\mathbf{X})^{-1}$ is the dispersion matrix.

The joint confidence region [22,23,28] for the estimated coefficients, b_i, is represented by hyperellipsoids and calculated according to Eq. (3).

$$(\beta - b)^t(\mathbf{X}^t\mathbf{X})(\beta - b) \leq ps^2 F_\alpha(p, \gamma) \quad (3)$$

where $F_{\alpha(p,\gamma)}$ is the critical F value with (p,γ) degrees of freedom at the significance level, α. It can be deduced from Eqs. (2) and (3) that the estimation of the coefficients along with the volume, the shape and the orientation of the confidence hyperellipsoid (precision) depend on the information matrix $(\mathbf{X}^t\mathbf{X})$ and therefore on the dispersion matrix $(\mathbf{X}^t\mathbf{X})^{-1}$. The smaller the determinant of the dispersion matrix the more precise the estimates of the model (Eq. (3)) and the more reliable the conclusions drawn from the analysis of the coefficients. This means that the quality of the coefficients (Eq. (3)) depends on the model matrix, **X**. By proper selection of **X**, step previous to the experimentation, the determinant of the dispersion matrix $|(\mathbf{X}^t\mathbf{X})^{-1}|$, and consequently, the variance of the coefficients can be minimized. This property is very interesting when the significance of the coefficients and hence the influence of the factors on a given response is analysed.

However, the determinant of the information matrix increases when an experiment is added to the design, ξ. The matrix of moments $M(\xi)$, Eq. (4), is defined to compare designs with different number of experiments, n.

$$M(\xi_n) = \frac{\mathbf{X}^t\mathbf{X}}{n} \quad (4)$$

A given design, ξ_1, is said to have greater D-efficiency than a design ξ_2, if $|M(\xi_1)| > |M(\xi_2)|$. This criterion, known as D-optimality criterion, has been applied in this paper for the selection of the experiments because it minimizes the determinant of the dispersion matrix. Hence, the coefficients will be the most precise possible.

The election of the six experiments from the full factorial design ($2^5 = 32$ experiments) is done through an exchange algorithm because it would be tedious to evaluate the determinant of all possible six-experiment combinations with the 32 experiments ($C_{32,6} = 906{,}192$ combinations). This method [23] is iterative such that the determinant of the matrix of moments will be maximized. Theory and comparative analysis of several algorithms for building D-optimal designs can be found in chapter 7 of ref. [29] and literature there cited. The D-optimal design has been built through the exchange algorithm in Nemrodw [30] and is shown in coded variables in Table 2.

3. Experimental

3.1. Chemicals and reagents

Acetonitrile, methanol, diethyl ether, hexane, phosphoric acid (85%), ammonium (25%), sodium hydroxide and potassium hydroxide were obtained from Merck (Darmstadt, Germany). Sodium chloride, potassium phosphate and di-sodium hydrogenphosphate anhydrous were purchased from Panreac (Barcelona).

Ciprofloxacin hydrochloride (94%) was obtained from the European Pharmacopoeia (Strasbourg) and enrofloxacin (Baytril, 10%) from Bayer (Leverkusen).

Deionised water was obtained by the Milli-Q Gradient A10 water purification system of Millipore (Bedford, MA, USA).

The potassium phosphate buffer (0.05 M, pH 7.4) was prepared for sample pre-treatment by diluting potassium phosphate in deionised water and adjusting the pH at 7.4 with 10 M sodium hydroxide. Di-sodium phosphate buffer (0.02 M, pH 3) was arranged for the mobile phase by dissolving di-sodium hydrogenphosphate anhydrous in water and adjusting the pH at 3 with phosphoric acid.

3.2. Standard solutions

Stock solutions (1 g l^{-1}) of ciprofloxacin and enrofloxacin were prepared in 0.02 M sodium hydroxide. A diluted solution containing both analytes at 10 mg l^{-1} was prepared by diluting the stock solutions (1 g l^{-1}) with 0.05 M phosphate buffer (pH 7.4). All solutions were stored at 4 °C in amber bottles for a maximum period of 2 months.

The calibration curves used to quantify both fluoroquinolones in eggs were built with seven standards prepared daily in 0.05 M phosphate buffer (pH 7.4) at concentrations ranging from 0 to 1275 μg l^{-1} of ciprofloxacin (in regular steps of 225 μg l^{-1}) and from 0 to 1500 μg l^{-1} of enrofloxacin (at fixed intervals of 187.4 μg l^{-1}).

3.3. Pre-treatment and clean-up procedure of egg samples

Two grams of egg (white and yolk) are homogenized and fortified at 850 and 1000 μg kg^{-1} of ciprofloxacin and enrofloxacin, respectively in egg. According to the experimental design shown in Table 2, the corresponding volume (X_5, 3 or 5 ml) of the extracting agent (X_1, acetonitrile or methanol) and 25 μl of ammonia are added. Samples are stirred in an ultrasonic bath for 10 min and then centrifuged at 20,000 rpm for 10 min in a High Speed Refrigerated Centrifuge 4239R from ALC (Milan, Italy). The supernatants are decanted and filtered. The extraction (X_2) can be performed once (−) or twice (+). Then, 3 ml of hexane, 3 ml of diethyl ether and 250 ml of 1 M sodium chloride are added to the combined extracts (X_3, −). The extracts are mixed for 10 min and centrifuged at 3000 rpm for 10 min. The upper layer is discarded and the elimination of fatty acids (X_3) can be done once (−) or twice (+) by adding 3 ml of hexane, mixing for 10 min and centrifuging at 3000 rpm for 10 min. If no SPE extraction is performed (X_4, +), the combined extracts of the lower layer are evaporated to dryness under a stream of nitrogen at 36 °C and then dissolved with 1 ml of 0.05 M phosphate buffer (pH 7.4) stirred and centrifuged at 3000 rpm for 10 min.

Taking into account the experimental design in Table 2, if SPE is performed (X_4, −), the combined extracts of the lower layer are loaded and passed across Discovery DSC-18 columns (3 ml, 500 mg) from Supelco (Bellefonte, PA, USA) previously activated with 3 ml of methanol and 3 ml of water at a pressure of 5 mmHg. The cartridges are washed with 3 ml of water and dried for 5 min. Quinolones are eluted from the column with 5 ml of methanol/ammonium hydroxide (75/25, v/v). The extracts are evaporated to dryness under a stream of nitrogen at 36 °C, reconstituted in 1 ml of 0.05 M phosphate buffer (pH 7.4) and disposed into amber autosampler vials for chromatographic analysis.

3.4. Instrumental analysis

The analysis was performed in a liquid chromatograph (Waldbronn, Germany) 1100 Series HPLC from Agilent including a G1313A autosampler, a G1322A vacuum degasser, a G1311A quaternary pump and a G1321A fluorescence detector.

Forty microliters were injected into the system and eluted at an isocratic flow rate of 1 ml min^{-1}. The chromatographic separation of the compounds was achieved with a Simmetry C18 (5 μm) column from Waters (Milford, MA, USA) with dimensions 4.6 mm × 250 mm. The mobile phase is a mixture of acetonitrile/phosphate buffer (0.02 M, pH 3) at 10/90 (v/v). The mobile phase was passed through 0.45 μm filters and degassed in an ultrasonic bath.

Analytes were detected and quantified according to the Decision 2002/657/EC [31]: selection of the excitation and emission wavelengths such that the selectivity in the chromatograms is obtained. In this work the excitation wavelength (λ_{exc}) was fixed at 267 nm and the emission wavelength (λ_{emi}) at 447 nm. A diode array detector in the ultraviolet-visible was also placed in series to help the spectral confirmation of the residues.

4. Results and discussion

4.1. Validation of the D-optimal design

The exchange algorithm is iterative so the solution may depend on the starting point and converge to a local maximum. That is why the method has been repeated several times. Since the determinant of the moment matrix $|M(\xi)|$, is constant, the solution is a stable global maximum.

The design proposed in Table 2 is not a classical experimental design but it has been planned to resolve a specific problem. Consequently, its validation is basic and it must be done before the experimentation to guarantee the reliability of the results obtained. Firstly, the absence of correlation between the coefficients was checked. If this were so, attributing significant influences to one or other factor could be false. In the case of the design in Table 2, six out of the ten correlations from the six coefficients of the model are zero, three have an absolute value of 0.30 and the other is 0.25. The design is therefore valid.

The quality of the estimations also needs to be verified. The variance of the coefficients is:

$$\mathrm{Var}(b_i) = c_{ii}s^2 \quad (5)$$

where c_{ii} is the element corresponding to the diagonal of the dispersion matrix $(\mathbf{X}^t\mathbf{X})^{-1}$. The first factor of Eq. (5), c_{ii}, depends on the design and the second, s^2, on the experimental variability. The coefficients c_{ii} depend on the size of the experimental domain. That is why c_{ii} are standardised to obtain the so-called variance inflation factors, VIFs. VIF is an index greater or equal to 1 and must be less than 4 for the design to give sufficiently precise estimations. In the case of the design in Table 2, the VIFs are 1.20 for b_1, b_2 and b_4 and 1.07 for b_3 and b_5 which indicates that the design is valid.

The variance of the response predicted by the model at a point, u, within the experimental domain, var($y(u)$), is estimated as follows:

$$\mathrm{Var}(\hat{y}(u)) = x(u)^t(\mathbf{X}^t\mathbf{X})^{-1}x(u)s^2 = d(u)s^2 \quad (6)$$

$d(u)$ is called the variance function and should be as small as possible, and never greater than 1, to have small standard deviation in the predicted response, $\hat{y}_u$. Within the experimental domain there will be a point at which the variance function will be maximum and it is designated as d_{max}. The maximum variance function, d_{max}, of the design shown in Table 2 is 1 that is, the variance of the prediction at that point u, is the variance of the experimental error, s^2. Therefore, the solution found with the model proposed is acceptable to predict the response, either the recovery or the standard deviation.

Another parameter to be examined is the G-efficiency (Eq. (7)) because it takes into account not only the variance of the response but also the number of coefficients, p, and of experiments, n.

$$G_{eff} = \left(\frac{p}{d_{max}n}\right) \quad (7)$$

The G-efficiency of the design used in this work is 100%. All these parameters prove the suitability of the proposed design to solve this particular problem.

4.2. Performance of the extraction and calibration curves

Not before has the design been validated, the experimentation is performed. With the aim of optimizing not only the recovery (maximize) but also the standard deviation (minimize) each experiment in Table 2 was done three times, that is three aliquots of homogenized egg were independently enriched and analysed. The mean recovery (response 1) and the standard deviation (response 2) are shown in Table 2. The preferred conditions will be those that provide high recoveries with small standard deviations for both compounds.

The concentration recovered from eggs was determined by building a calibration curve according to Section 3.2. The calibration curves (area of the chromatograms versus concentration of the analyte) are: $y = 1.04x + 4.19$ for ciprofloxacin and $y = 0.79x - 6.44$ for enrofloxacin. The residual standard deviation of the regression is 25.48 and 24.12 au for ciprofloxacin and enrofloxacin, respectively. The determination coefficient of both regressions is 0.997.

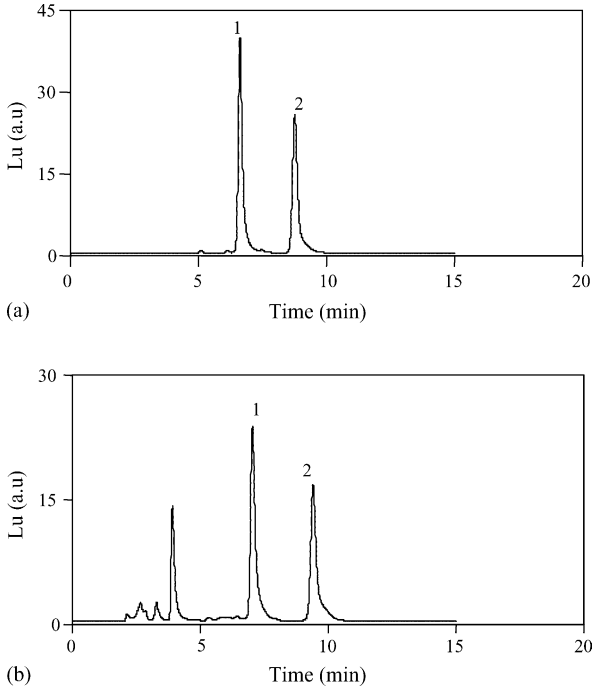

Fig. 1. Characteristic chromatograms of (a) a standard containing 425 μg l^{-1} of ciprofloxacin (1) and 500 μg l^{-1} of enrofloxacin (2). (b) An egg sample spiked with 850 μg kg^{-1} of ciprofloxacin (1) and 1000 μg kg^{-1} of enrofloxacin (2). Analytes have been extracted according to the conditions shown in experiment number 1 of Table 2.

The chromatogram of a standard containing 425 μg l^{-1} of ciprofloxacin and 500 μg l^{-1} of enrofloxacin is displayed in Fig. 1a. The retention times are 6.6 and 8.8 min for ciprofloxacin and enrofloxacin, respectively. Fig. 1b shows the chromatogram of an egg sample enriched with 850 μg kg^{-1} of ciprofloxacin and 1000 μg kg^{-1} of ciprofloxacin and subjected to the extraction conditions indicated in experiment 1 of Table 2. It can be observed that the matrix interferences elute at small retention time (less than 5 min) and therefore they do not interfere for the quinolone analysis, including quantification and confirmation of the analytes.

4.3. Analysis of the models built for the recovery

Experimental data (recovery) of Table 2 were fitted to the model in Eq. (1) by least squares [23,30]. The analysis of the standardized residuals shows that there are two outliers for both compounds: one replicate of experiment 3 and another of experiment 5. The standardized residuals (SR) of those experiments are −23 and −16 for ciprofloxacin and −24 and −18 for enrofloxacin. Both outliers have smaller recoveries than expected for both compounds, around 20%, so it may be due to an error in the sample processing. Outliers were

Table 3
Estimated effects and *p*-values of the hypothesis test for checking the significance of the coefficients of the model built for the recovery (level at which the analyst would work to maximize the recovery)

Coefficient	Ciprofloxacin			Enrofloxacin		
	Estimation	*p*-value	Level	Estimation	*p*-value	Level
b_1	8.733	<0.001*	+	3.567	0.063	+
b_2	−4.983	0.015*	−	−2.067	0.258	Indifferent
b_3	−3.383	0.050*	−	−0.633	0.690	Indifferent
b_4	−6.950	0.001*	−	4.133	0.022*	+
b_5	3.067	0.088	+	3.067	0.089	+

* The factor is significant at a significance level of 0.05.

removed from the data set and the model was then performed with the 16 experiments.

As there are three replicates of each experiment the significance of the model and of the coefficients can be tested. The hypothesis test [32] for the model significance is: null hypothesis "the regression cannot explain the experimental variation", alternative hypothesis "the regression does explain the experimental variation". The *p*-value of the test is less than 0.001 in both models so the null hypothesis will be rejected setting the significance level at 0.05. Models, consequently, explain the experimental variability found in the data referred to the recovery of both analytes.

The coefficients of the model are listed in Table 3. To determine if a coefficient is significant and consequently, if the corresponding factor affects the extraction procedure, the hypothesis test for the significance of the coefficients was applied [32]: null hypothesis, "the coefficient is zero", alternative hypothesis, "the coefficient is different from zero". Since the significance level was established at 0.05, those coefficients whose *p*-value is smaller than 0.05 (shown with an asterisk (*) in Table 3) will be considered statistically different from zero and therefore affect the recovery of the procedure. Influential factors for the ciprofloxacin extraction are: extracting solvent (X_1), number of extractions (X_2), times washed for removing fat (X_3) and SPE (X_4). For the enrofloxacin model only the factor SPE (X_4) is statistically significant. However, the significance should be carefully considered because there are some factors in the limit of the significance. For example, the *p*-value of the coefficient b_3 is 0.05 for ciprofloxacin and that of b_5 is 0.09 (setting the significance level at 0.10 both factors would be influential).

Both compounds behave similarly to changes in the factors because the sign of the coefficients is equal in all cases except in that of the factor X_4 (presence or absence of the SPE cartridge). On the other hand, the magnitude of the coefficients of ciprofloxacin is greater despite the fact that the mean recovery from the 16 experiments is comparable in both cases: 59.4% and 58.4% for ciprofloxacin and enrofloxacin, respectively. Larger variability has been found between the 16 experiments of ciprofloxacin (minimum 39% and maximum 94%) than between the experiments of enrofloxacin (minimum 41% and maximum 73%) but not between the three replicates of the same experiment (see the standard deviation in Table 2). From these results, it can be concluded that the variations of the factors have a greater effect on the recovery of ciprofloxacin than on the recovery of enrofloxacin.

The effect can be positive (effects with positive coefficients have greater recoveries at the nominal level) or negative (effects with negative coefficients have smaller recoveries at the nominal level). The following conclusions can be drawn from the study of the sign of the coefficients: the coefficient of the factor kind of solvent, b_1, is positive which indicates that greater recoveries will be obtained at the nominal level (+), that is with methanol (see codification in Table 1). The volume of extracting agent is not significant at a significance level of 0.05 but it is at 0.10 and its coefficient is positive so the extraction should be performed with 5 ml (+) of solvent. The coefficient corresponding to the number of extractions, b_2, is negative and consequently greater recoveries will be obtained with one step for protein precipitation. This conclusion is important because the extraction is complete with 5 ml of methanol and it is not necessary to extract twice and introduce additional phases which reduce the recovery because of the lost of sample in the pre-treatment. Then fatty acids can be removed (X_3) in a single step (−) with hexane/ether.

The coefficient of the factor SPE (b_4) is significant in both cases but it has contrary sign in each compound. b_4 is negative (SPE) for ciprofloxacin and positive (no SPE) for enrofloxacin. The different behaviour of both substances to this factor might be related to the fact that ciprofloxacin is the metabolite of enrofloxacin. As the analytes elute through the cartridge enrofloxacin might transform into ciprofloxacin so the ciprofloxacin recovery increases whereas the enrofloxacin recovery decreases. According to this hypothesis, either the conditions for quinolone clean-up should be changed or the SPE step should be removed. Since matrix interferences do not affect the specificity of the chromatograms, the second option is preferred. However, the effect of this factor on the standard deviation should also be taken into account and is examined in Section 4.4.

4.4. Analysis of the models built for the standard deviation

In the residue analysis, large recoveries are as important as small standard deviations. That is why the effect of all five factors on the standard deviation of the method has also been evaluated. The standard deviation from all three replicates

Table 4
Estimated coefficients of the model built for the standard deviation (level at which one should work to minimize the standard deviation)

Coefficient	Ciprofloxacin		Enrofloxacin	
	Estimation	Level	Estimation	Level
b_1	0.3	Indifferent	1.2	Indifferent
b_2	1.5	−	0.2	Indifferent
b_3	−0.3	Indifferent	−1.5	+
b_4	−1.2	+	−1.5	+
b_5	0.9	Indifferent	0.9	Indifferent

per experiment is shown in Table 2. The coefficients of the model (1) have been estimated by least squares [23,30] and are listed in Table 4. As there are not replicates of the standard deviation, neither the model significance nor the significance of the coefficients can been assessed.

Some approaches such as the Lenth's method along with the Bayesian analysis [23] of the coefficients have been applied because they do not need replicates to evaluate the significance of the coefficients.

The Lenth's method estimates a value of the standard deviation, s_b, from which the active effects will be identified. To calculate s_b, the median of the coefficients in absolute value (Table 4) is multiplied by 1.5. The coefficients which are higher than a critical value set at $2.5s_b$, are considered significant and are removed from the list. The procedure is repeated until there are not significant effects. The critical values estimated in this way are 3.5 for ciprofloxacin and 4.6 for enrofloxacin. Since no coefficient is greater than the corresponding critical value it can be concluded that variations in the effects do not affect the standard deviation of the extraction.

The bayesian analysis [23] of the coefficients consists of the computation of the a posteriori probability that the effects are significant and is shown in Fig. 2a for ciprofloxacin and in Fig. 2b for enrofloxacin. It can be observed that the maximum a posteriori probability that a factor is significant for the model of ciprofloxacin is 66% (number of extractions, X_2) and is obtained independently from the a priori probability. For enrofloxacin the factor which provides the greatest a posteriori probability (51%) is X_3 (times washed). As the probability of none factor is greater than 95%, no coefficient is significant for the standard deviation and consequently, the precision of the extraction is not affected by changes in the factors. Similar results have been found in the extraction of sulfonamides from kidney [33] by means of a Plackett–Burman design. The standard deviation of the extraction procedure, that is the precision, is less affected by the experimental factors than the recovery (trueness).

Although no coefficient is significant, it can be observed that those factors with the largest coefficients in absolute value for ciprofloxacin are the number of extractions (b_2, Table 4) and the solid-phase extraction (b_4). Their effect will be subsequently discussed. The sign of b_2 is positive and therefore one should work at the extreme level (−, one extraction) to obtain small standard deviations. This result is

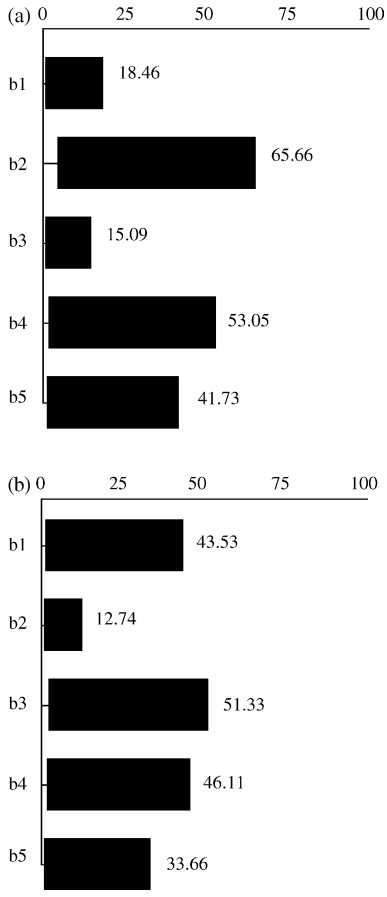

Fig. 2. Bayesian analysis of the coefficients of the model built for the standard deviation of: (a) ciprofloxacin and (b) enrofloxacin.

in agreement with that proposed to maximize the recovery (Section 4.3) and is reasonable because the greater the number of steps, the worse the precision (more variability) and the recovery (analyte lost). The coefficient corresponding to the solid-phase extraction (b_4) is negative, that is one should work at the nominal level (+, no SPE) in order to obtain acceptable precision. As concluded in Section 4.3, this factor behaves in a different way for the recovery of ciprofloxacin (with SPE) and of enrofloxacin (no SPE). With the results obtained in this section, the analysis will be performed without the step of SPE. As matrix interferences elute at small retention times and have no effect on the detection of both substances, the solid-phase extraction can be eliminated from the procedure. Besides the problem of the conversion of enrofloxacin to ciprofloxacin is minimized. An additional advantage is that the cost and the analysis time is reduced because some previous steps such as column activation, analyte elution, etc. are avoided.

4.5. *Working experimental conditions and their validation*

Taking into account the results to increase the recovery (Section 4.3) and reduce the variability (Section 4.4) of

the pre-treatment and clean-up stages, the final working conditions will be as follows. The precipitation of the proteins and the extraction of the antibiotics from eggs will be carried out once (−, X_2) with 5 ml (+, X_5) of methanol (+, X_1). Once in solution, quinolones will be separated from fatty acids in a step (−, X_3) with a hexane/ether mixture. Analytes might be detected without the need to include an additional purification step by solid-phase extraction (+, X_4). Under these conditions, the recovery predicted by the models is it has been assayed that the mean recovery is 63.78% for ciprofloxacin and 69.84% for enrofloxacin. The standard deviation from three replicates is 5% for ciprofloxacin and 4% for enrofloxacin.

The selected experiment (+ − − + +), according to the codification used in Table 1, is equal to experiment number 6 of the design (+ − − − +) except in the fourth factor (SPE). The recovery obtained in the experiment 6 (Table 2) is 87% for ciprofloxacin and 62% for enrofloxacin. The recovery of ciprofloxacin in experiment 6 (87%) is much greater than that of the experiment proposed in this paper (64%). As has already been concluded in Section 4.3, the recovery of ciprofloxacin increases with the SPE stage because of the enrofloxacin conversion. However, the chosen experiment is preferred because the recovery of the two substances is more similar (64% and 70%, range 6%) than in experiment 6 (87% and 62%, range 25%). Besides, the standard deviation is smaller in the experiment proposed in this paper, 5% and 4%, than in the experiment 6, 7% and 10%, for ciprofloxacin and enrofloxacin, respectively.

As the administration of ciprofloxacin and enrofloxacin to poultry is forbidden (Regulation no. 1181/2002 amending Regulation no. 2377/90), the evaluation of the capability of decision, CCα, and of the capability of detection, CCβ, is fundamental. Both figures of merit have been estimated with the program DETARCHI [34] according to the standard ISO 11843 [35] which evaluates the probability of false positive, α, and of false negative, β. This way to estimate the capability of decision and the capability of detection has also been accepted by the European Decision 2002/657/EC [31] which names both terms CCα and CCβ, respectively.

Fixing α and β at 0.05, CCα is 3.1 and 2.8 $\mu g\,kg^{-1}$ of ciprofloxacin and enrofloxacin, respectively. CCβ is 7.8 and 7.0 $\mu g\,kg^{-1}$ of ciprofloxacin and enrofloxacin, respectively.

5. Conclusions

The D-optimal design applied in this work is adequate to evaluate which experimental factors influence the extraction of two quinolones taking into account the practical restrictions of limited number of experiments and the tube capacity of the centrifuge. The analysis of the significance of the coefficients and their sign has been useful for finding those experimental conditions which jointly assure large recoveries (trueness, response 1) and small standard deviations (precision, response 2), that is, good accuracy. The experimental conditions selected in this work prove that the extraction of the antibiotics from the biological sample can be done once and that it is not necessary to add the solid-phase extraction step to remove matrix interferences. Besides, fat elimination can also be performed in a single stage which allows one to reduce not only the analysis time and consequently the cost but also the use of organic solvents.

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