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Short Report

Application of a 3^3 Box-Behnken Design to Optimize the Extraction of Eleven Fluoroquinolones from Poultry Muscle and Kidney Using a QuEChERS Approach via Liquid Chromatography Tandem Mass Spectrometry: the Easy Use of Microsoft Excel® in Multivariate Analysis

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This work presents an optimization of a quick, easy, cheap, effective, rugged and safe (QuEChERS) extraction approach of eleven fluoroquinolones in poultry muscle and kidney using a 3^3 Box-Behnken factorial design. All the data treatment was performed using Microsoft Excel® 2010. The suitability of the developed method was confirmed by two proficiency tests for ciprofloxacin and enrofloxacin.

Keywords: Box-Behnken design, fluoroquinolones, QuEChERS, liquid chromatography, mass spectrometry

Introduction

The growing demands of domestic consumption and export of foodstuff have stimulated an increase in the production capacity and quality of agricultural and livestock products. Therefore, the use of drugs in animal production has been virtually an imposition.[1,2]

Fluoroquinolones (FQs) are synthetic antibiotics extensively used in veterinary medicine and also in the treatment of several human infections.[3] In order to avoid bacterial resistance, regulatory agencies have established maximum residue limits (MRLs) for FQs in animal tissues and derivatives.[4] In that sense, various studies have been conducted to develop methods of extraction and quantification of residues of these compounds in different matrices.

The development of a methodology for analyzing drug residues in foodstuff involves the critical steps of extraction and purification of target analytes, which consume most of the analysis time and are more susceptible to experimental errors.[5-8] Thus, many efforts have been devoted to develop methods for sample preparation that reduce the consumption of organic solvents, waste production and operating costs.[9-13]

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Within this context, the quick, easy, cheap, effective, rugged and safe (QuEChERS) method was developed by Anastassiades *et al.*[14] for the extraction of pesticide residues in fruits and vegetables. This method consists of a single extraction step with acetonitrile, followed by the addition of anhydrous magnesium sulfate and sodium chloride, providing the partition effect known as salting-out, and subsequent clean-up by dispersive solid phase extraction, in which anhydrous magnesium sulfate and primary secondary amine (PSA) polymer phase are stirred together with the supernatant extract.[14,15]

Since then, various modifications have been proposed to this method in order to increase its applicability,[15] such as the addition of acetic acid to the extraction phase to provide an enhanced partition; the replacement of sodium chloride by sodium acetate, forming a buffer with the former acid; and the use of other polymeric phases, combined or not with PSA, such as C18 and graphitized carbon black (GCB).[16-18]

The approach of the multivariate experimental design is a current trend in analytical chemistry, since it allows the simultaneous optimization of multiple variables in complex processes and the interaction effects between them.[19-21] The advantages in using such designs include reduction in the number of experiments, which enables a less laborious and time-consuming optimization process; and enhanced

statistical evaluations, since interaction effects between the parameters under investigation can be determined.[22]

The Box-Behnken design (BBD) is a response surface methodology (RSM) that has been widely used in various experiments involving extraction of veterinary drugs from foodstuff.[23] BBD is a second-order multivariate design based on a three-level incomplete factorial design with no axial points, which permits the assessment of the critical factors that significantly affect the analytical responses by a reduced number of experiments.[24] The number of experiments (N) necessary for the optimization of a BBD is calculated as:

$$N = 2k(k-1) + C_0 \quad (1)$$

where k is the factor number and C_0 is the replicate number at the central point.[23] Therefore, the factors are never set simultaneously at their highest or lowest levels, which avoids an optimization performed under extreme conditions. So the design points are included in a secure operating zone.[24,25]

The use of multivariate designs for the extraction of FQs is relatively scarce in recent literature. Cáceres *et al.*[26] developed a high performance liquid chromatography with electrochemical detection for the determination of danofloxacin, sarafloxacin and difloxacin in chicken tissues. Prieto *et al.*[27] optimized a microextraction by packed sorbent process for the quantification of ciprofloxacin (CIP), norfloxacin, ofloxacin and flumequine in municipal wastewater samples by liquid chromatography tandem mass spectrometry (LC-MS/MS). Rodriguez *et al.*[28] performed an optimization of a pressurized liquid extraction method for the determination of six FQs residues in infant foods by liquid chromatography with fluorescence detection.

The purpose of this work is the optimization of an extraction procedure of eleven FQs from poultry muscle and kidney using a QuEChERS approach. The factors under investigation that significantly influence the recoveries of FQs are evaluated by analysis of variance (ANOVA) and a 3^3 Box-Behnken design was used to achieve the best conditions to extract the analytes studied. The Microsoft Excel® 2010 software was chosen as a tool to perform statistical data treatment, taking into account the wide availability of this software and the easy use of its interface. The complete validation of the developed method has already been reported by the authors in a previous report.[29]

Experimental

Reagents, preparation of stock and standard solutions, LC-MS/MS instrument, chromatographic conditions, LC-MS/MS parameters and optimization of sample preparations steps were previously described by Rocha *et al.*[29]

Software

The statistical treatment was performed using Microsoft Excel® 2010 (Microsoft Corp., Redmond, WA, USA).

Statistical treatment

The Excel formulas and the matrices used in statistical treatment are available on an electronic link.[30] In this electronic worksheet it is possible to introduce the response for a 3^3 BBD and obtain the model coefficients, pure errors of the coefficients, confidence intervals for the coefficients and one-way ANOVA.

The estimators of the population parameters (b), once considered linear, were estimated by the least squares method from equation 2:[31]

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

where $\mathbf{X}$ corresponds to matrix containing the encoded factors and their interactions and $\mathbf{y}$ represents a vector containing the weighted sums of recoveries for each experiment in poultry muscle and kidney.

Since the 3^3 BBD was carried out in triplicate at the central point, the pure error for each coefficient was estimated using the variance for the replicates at the central point (s^2), according to equation 3:[31]

$$\text{p.e.} = \sqrt{\text{main diagonal of the matrix } (\mathbf{X}^t\mathbf{X})^{-1} \cdot s^2} \quad (3)$$

The confidence interval for the coefficients was estimated by equation 4:[31]

$$b_x \pm \left(\text{p.e.}_{b_x} \cdot t_{(0.05,\,2)}\right) \quad (4)$$

where b_x is the corresponding coefficient; p.e._{b_x} is the pure error of each coefficient; $t_{(0.05,\,2)}$ is the value of Student's *t*-test for two degrees of freedom at 95% confidence.

The quality of the adjustments was evaluated by ANOVA after the models be obtained. Firstly, the predicted values $\hat{y}$ were calculated from the equation 5:

$$\hat{y} = \mathbf{X} \cdot \mathbf{c} \quad (5)$$

where $\mathbf{X}$ is the matrix containing the encoded factors and their interactions and $\mathbf{c}$ is the vector of the model coefficients.

After, the values of the sum of squares due to regression (SSR), residual sum of squares (RSS), pure error sum of squares (SSEP) and lack-of-fit sum of squares (SSLF) were calculated by the matrix equations shown in Table 1.[31] The mean squares were calculated by the ratio between the sum of squares and the degrees of freedom (Table 1).

Next, F-test was performed by the ratio between the lack-of-fit mean squares and the pure error mean squares to evaluate the lack of fit of the proposed models.[31]

Table 1. Analysis of variance (ANOVA) for the evaluation of the proposed models fit by the least squares method

Sum of square			Degree of freedom
SSR	$(\hat{y} - \bar{y})^t - (\hat{y} - \bar{y})$	(5)	p – 1
RSS	$(y_{ij} - \hat{y})^t - (y_{ij} - \hat{y})$	(6)	n – p
SSEP	$(y_{ij} - \bar{y}_i)^t - (y_{ij} - \bar{y}_i)$	(7)	n – m
SSLF	$(\hat{y} - \bar{y}_i)^t - (\hat{y} - \bar{y}_i)$	(8)	m – p

SSR: sum of squares due to regression; RSS: residual sum of squares; SSEP: pure error sum of squares; SSLF: lack-of-fit sum of squares; p: number of model parameters (p = 10); n: total number of assays (n = 15); m: number of distinct assays (m = 13); $\hat{y}$: predicted value; $\bar{y}$: mean value; t: transposed matrix.

Proficiency test

In order to evaluate the accuracy of the developed method, as well as its potential applicability to other matrices and species, two proficiency tests were executed. The assays were conducted by the proficiency testing unit Centre for Veterinary Drug Residues (Canadian Food Inspection Agency, CFIA; Saskatoon Laboratory, Saskatoon, SK, Canada) in July 2013 and in January 2014. The first assay involved the analysis of four lyophilized equine kidney samples: XP1419, XP1420, XP1421 and XP1422, performed among ten laboratories. The four lyophilized veal liver samples related to the second assay were: XP 1343, XP1344, XP 1345 and XP1346, performed among twelve laboratories.

Results and Discussion

Optimization of extraction and cleanup steps by 3^3 BBD

The optimization of a QuEChERS method for extraction of FQs in poultry muscle and kidney was conducted directly by a 3^3 BBD, since the relevant variables related to this process are widely known by practical laboratorial experience and correlated references.[32,33] Fifteen experiments were then performed, using triplicate at the central point. The following factors were evaluated: water content in the extraction phase (–) acetonitrile, (0) acetonitrile:H_2O (90:10, v/v) and (+) acetonitrile:H_2O (80:20, v/v); percentage of acetic acid in the extraction phase at (–) 1, (0) 3 and (+) 5%; sorbent used at clean up stage at (–) 50 mg PSA, (0) 25 mg PSA + 25 mg C18 and (+) 50 mg C18.

In order to perform the statistical evaluation, the analytical responses were taken as the sum of the recoveries of each FQ divided by the higher recovery in the corresponding assay (weighted sum). This combined statistical evaluation was accomplished employing Microsoft Excel® 2010 software and is available on the electronic worksheet.[30]

Table 2 presents the coefficients related to the models set by the least squares method, for muscle and kidney and their pure errors and confidence intervals. For muscle samples, at the confidence level of 95%, the factors that were statistically significant in recoveries of FQs were linear term of the water content in the extraction phase and percentage of acetic acid in the extraction phase and their interaction. For kidney, the linear and quadratic terms of the percentage of acetic acid in the extraction phase, linear term of sorbent used in clean up, their interaction and the interaction between water content in the extraction phase and percentage of acetic acid in the extraction phase, were statistically significant. It is worth noting that the significance of the interactions between the evaluated factors could only be checked due to the multivariate optimization.

Using ANOVA, it was observed that the statistical models did not present evidence of lack-of-fit at 95% confidence. For the muscle samples, $F_{calculated}$ was equal to 10.45; and for the kidney samples, $F_{calculated}$ was equal to 8.72. These values are both lower than $F_{critical(0.05;\,3;\,2)} = 19.16$.

The residue graphics for poultry muscle and kidney showed a random distribution of residuals. The profile of these graphics was also used to confirm that there was a suitable fit between the models and the experimental data (electronic worksheet).[30]

The response surfaces (electronic worksheet)[30] showed that the addition of water to the extraction phase had the effect of improving analyte recovery, which can be explained by the fact that the water content present in poultry muscle samples (average 70%) was not high enough to promote good partition of analytes in the initial liquid-liquid extraction step of the QuEChERS method.[32,34] A higher acid content in the extraction phase also contributed to an increase in the recovery values, which can be explained by the formation of ion pairs between protonated structures of the FQs and acetate anions produced in the acid dissociation.[32] The formation of ion pairs likely neutralizes the charge of FQs, facilitating the migration of the compound formed to the organic phase. The evaluation

Table 2. Statistical evaluation by the least squares method for the extraction of fluoroquinolones (FQs) in poultry muscle and kidney

Parameter	Sample	Coefficient	Pure error	Confidence limit	
				–95%	95%
b0	Muscle	7.37	0.09	6.99	7.74
	Kidney	7.90	0.05	7.69	8.11
(1) Extraction phase (L)	Muscle	0.25	0.05	0.02	0.48
	Kidney	–0.43	0.03	–0.56	–0.30
(2) Acetic acid / % (L)	Muscle	0.60	0.05	0.37	0.83
	Kidney	0.65	0.03	0.52	0.78
(3) Sorbent (L)	Muscle	0.20	0.05	–0.03	0.43
	Kidney	0.42	0.03	0.30	0.55
(1) Extraction phase (Q)	Muscle	0.16	0.08	–0.18	0.50
	Kidney	–0.15	0.04	–0.34	0.04
(2) Acetic acid / % (Q)	Muscle	–0.25	0.08	–0.59	0.09
	Kidney	–0.63	0.04	–0.82	–0.44
(3) Sorbent (Q)	Muscle	0.02	0.08	–0.31	0.36
	Kidney	–0.04	0.04	–0.23	0.15
(1)*(2)	Muscle	0.40	0.08	0.07	0.73
	Kidney	–0.85	0.04	–1.03	–0.67
(1)*(3)	Muscle	0.12	0.08	–0.21	0.44
	Kidney	–0.22	0.04	–0.40	–0.03
(2)*(3)	Muscle	0.10	0.08	–0.22	0.43
	Kidney	–0.14	0.04	–0.32	0.05

L: linear term of the fitted model; Q: quadratic term of the fitted model.

of the use of the dispersive phase containing a mixture of C18 and PSA also indicated a positive effect on recoveries, which can be explained by the greater efficiency of C18 sorbent for the removal of non-polar compounds present in the matrix, whereas the PSA sorbent is suitable to remove interfering ions.[12,14,15] In that way, the extraction phase consisting of the mixture acetonitrile:H_2O (80:20, v/v) with addition of 5% acetic acid and the clean-up with the sorbent mixture of 25 mg PSA and 25 mg C18 were optimized for the extraction of FQs from poultry muscle.

In case of the extraction performed in poultry kidney, the factors percentage of acetic acid in the extraction phase and sorbent used in clean-up showed positive effects. The extraction phase composition showed a significant and negative effect, demonstrating that the addition of water led to lower recovery levels. This behavior can be explained considering the average value of water content in poultry kidney samples of 82%,[33,34] which compromised the partition of the target compounds to the organic phase, at a higher water level. Therefore, the extraction phase acetonitrile with addition of 5% acetic acid was chosen, and the same clean-up sorbent mixture was selected for the extraction of FQs from poultry kidney.

Proficiency test

Eight samples were analyzed by the developed method, in duplicates. The first proficiency test comprised the determination of CIP and enrofloxacin (ENR) in four samples (XP1343, XP1344, XP1345 and XP1346), in which one of them was a blind blank sample for the two analytes and another one was a blind blank to ENR. The second test involved the determination of ENR in four samples (XP1419, XP1420, XP1421 and XP1422), in which two of them were blind blank samples. The results presented in Table 3 showed Z-score absolute values lower than two for all analyzed samples, which meant that the results obtained by different laboratories were statistically similar. Therefore, the optimized QuEChERS method proved to be able to determine FQs in different matrices of animal origin.

Conclusions

This work presented the optimization of the extraction process of FQs from poultry muscle and kidney through a multifactorial approach using a 3^3 BBD and subsequent separation by LC-MS/MS.

Table 3. Samples analyzed by the proficiency tests performed to evaluate the method's accuracy for enrofloxacin and ciprofloxacin

Specie	Tissue	Sample	Analyte	Lab's results / (μg g^{-1})	Assigned value / (μg g^{-1})	Z-score
Veal	liver	XP1343	CIP	0.024 ± 0.002	0.021	0.65
			ENR	0.000 ± 0.003	0.000	0.00
		XP1344	CIP	0.028 ± 0.002	0.025	0.55
			ENR	0.019 ± 0.003	0.017	0.53
		XP1345	CIP	0.014 ± 0.002	0.016	–0.57
			ENR	0.034 ± 0.003	0.034	0.00
		XP1346	CIP	0.000 ± 0.002	0.000	0.00
			ENR	0.000 ± 0.003	0.000	0.00
Equine	kidney	XP1419	ENR	0.000 ± 0.003	0.000	0.00
		XP1420	ENR	0.000 ± 0.003	0.000	0.00
		XP1421	ENR	0.042 ± 0.003	0.042	0.00
		XP1422	ENR	0.016 ± 0.003	0.015	0.30

CIP: ciprofloxacin; ENR: enrofloxacin; n = 2.

All data treatment was performed using Microsoft Excel® 2010, which is widespread software available to most computer users. The worksheets employed are available on the electronic link.[30] The results in terms of analyte recovery were evaluated by ANOVA and RSM. The factors selected for the multifactorial optimization were statistically significant, as well as the interactions between them. Response surfaces were used to evaluate the influence of the investigated factors on the recoveries of the analytes. The significance of the fitted models was also confirmed, proving the suitability of the proposed method for the extraction of FQs.

The two proficiency tests showed that the QuEChERS approach in this work proved to be adequate and promising for the extraction of FQs from animal tissue matrices. The method reconciles suitable extraction efficiency, low consumption of supplies and short analysis time.

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