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Determination of quinolones by fluorescent excitation emission

D. Giménez [a], D. Grasso [b], L. Sarabia [c], M.C. Ortiz [a,*]

[a] Department of Chemistry, Faculty of Sciences, University of Burgos, Plaza Misael Bañuelos s/n, 09001 Burgos, Spain
[b] Department of Chemistry and Technology, University of Genova, Via Brigata Salerno s/n, 16147 Genova, Italy
[c] Department of Mathematics and Computation, Faculty of Sciences, University of Burgos, Plaza Misael Bañuelos s/n, 09001 Burgos, Spain

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Abstract

In this work the viability of a fluorescent technique for the determination of quinolones is studied. This analytical technique allows one to analyze the effect of the increasing order of the analytical signal from a univariate calibration (zero order data) to partial least squares (PLS) calibration (first order). The comparison has been done through the figures of merit of the analytical procedure (technique and calibration) in accordance with the ISO norm and the 2002/657/EC European Decision about residuals.

Keywords: Detection capability; Maximum residual limit; Accuracy; Repeatability; Flumequine; Ciprofloxacin; Fluorescence; Partial least squares

1. Introduction

The quinolones are a family of recently synthesized antibiotics and one of the main antibacterial agents. They originate from nalidixic acid [1] (discovered in 1962) and their activity is based on the inhibition of bacterial DNA gyrase, being effective against gram-negative aerobic bacteria [2,3]. The addition of fluor and other substitutes gave rise to the fluoroquinolones [4], from the second to the fourth generation of quinolones, improving their power and spectrum for activity and expanding their coverage to anaerobic gram-positive bacteria. Currently, they are widely used in veterinary and medicine.

The concern for the sharp increase in antimicrobial resistances and its possible relation with the excessive use of medicines in animals for human consumption has led the World Health Organization to elaborate studies and strategies for action [5–7] to control the use of antibiotics. Maximal residual limits (MRLs) for the concentration of antibiotics according to the type of animal and the target tissue analyzed have been established to protect the health of consumers in the European Union (EU).

There is a lot of bibliography on the analysis of fluoroquinolones. The most commonly used techniques are liquid chromatography with fluorescence detection [8,9], mass spectrometry (MS), [10,11], or MSMS [12,13]. Other separation techniques used are capillary electrophoresis [14] and high-performance thin-layer chromatography [15]. They are usually preceded by liquid–liquid extraction and different purification techniques, habitually solid phase extraction [10,12], new liquid extractions [15] or even microdialysis [16].

A recent review [17] illustrates that, although the detection limit has been evaluated, these limits are not calculated in the same way and often the authors do not explain how they were established.

To corroborate this, a bibliographic search was done by using the ISI Web of Science [18]. A search for the fluorescence detection of quinolones (quinolone, flumequine, ciprofloxacin and enrofloxacin) showed a result of 78 recent papers (since 1999). In 44 of them the limit of detection (LOD) was calculated usually at three times the standard deviation of the signal from the blank, in accordance with previous EU norms. More than 50% of these LOD values were between 1 and 15 $\mu g\,l^{-1}$. In some cases a limit of quantification (LOQ) is also included, but calculated in different ways. Only in four of them is there a reference to the MRL. Since August 2002 a new European norm, 2002/657/EC European Decision [19], has established the requirements

* Corresponding author. Tel.: +34-947-258-800x8211; fax: +34-947-258-831.
E-mail address: mcortiz@ubu.es (M.C. Ortiz).

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to calculate new figures of merit which substitute the LOD and LOQ. Only one paper using this norm has been found [20]. The norm in addition explicitly takes into account that the variance of an analytical procedure increases as the nominal levels of analyte increase. It therefore, establishes not only the detection capability of a procedure but also the MRL which varies from some target tissues to others and also depends on the animal from which the tissue was taken.

In this work the reliability of a fluorescent technique for the determination of quinolones is studied. Of the fluoroquinolones we have chosen flumequine, enrofloxacin and ciprofloxacin (Fig. 1), which present overlapping signals. MRLs for these quinolones vary according to the target tissue and animal from 50 to 1500 $\mu g\,kg^{-1}$, their presence being forbidden in animals producing eggs for human consumption. The analysis of these substances has been carried out on synthetic samples as a previous step to its use in real samples.

The analytical technique considered allows one to analyze the effect of increasing order of the analytical signal from a univariate calibration (zero order data) to PLS calibration (first order). Multivariate techniques are necessary to determine mixtures of quinolones because of the high degree of overlapping of the analytical signals. Comparison has been made through the validation of the analytical procedure (technique and calibration) in accordance with the ISO 11843 norm [21,22] and the 2002/657/EC European Decision [19] about residuals. In this way the detection capability is established as a hypothesis test with an estimation of α and β probabilities, and these parameters should be calculated to validate the method. When a substance is banned, then the nominal value, x_0, is 0. In this case α is the probability of false positive and β the probability of false negative. When a substance has a maximum permitted limit (PL) then the nominal value, x_0, is PL; α is the probability of false noncompliance and β is the probability of false compliance. In both of them the null hypothesis is the true concentration of analyte, x_0.

The norm establishes the calculation of the parameters indicated above when the analytical technique used is univariate. This requires specificity in the signals which is not always possible. However, multivariate calibration techniques (e.g. PLS) allow quantification even though the signal is not specific. It suffices to take into account the interferents in the calibration. These multivariate techniques also allow one to assure the similarity of the sample problem with the calibration standards in order to avoid the incorrect application of the calibration function. The determination of the capability of detection with the multivariate calibration used in this work is a generalization of the univariate case. Methodologically this generalization can be classified as one of those performed for evaluating of the multivariate detection capability and that have been carried out in our working group recently [23,24], being also akin to the concept of multivariate analytical sensitivity [25].

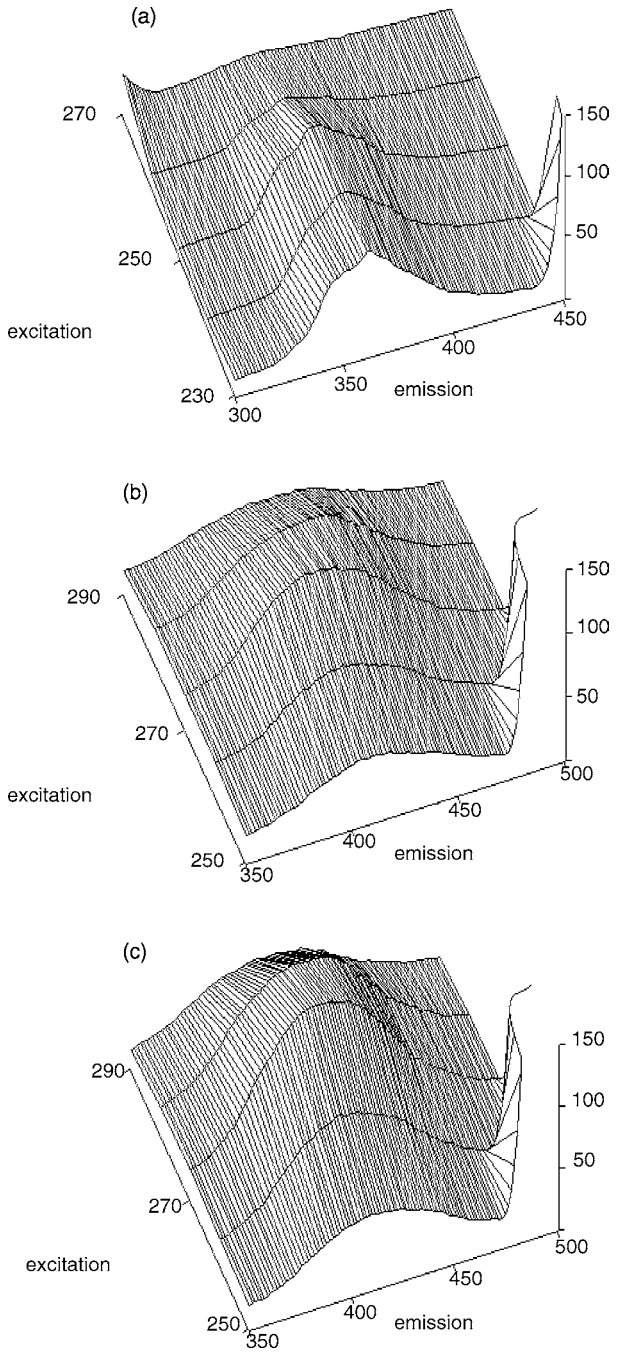

Fig. 1. Fluorescence spectra of (a) flumequine (100 $\mu g\,l^{-1}$), (b) ciprofloxacin (200 $\mu g\,l^{-1}$) and (c) ciprofloxacin (200 $\mu g\,l^{-1}$) in presence of enrofloxacin as interference (100 $\mu g\,l^{-1}$).

In addition, this work evaluates various figures of merit following the guidelines of the ISO 5725 norm [26]. The figures of merit are: accuracy (trueness and precision), repeatability as the minimum variation of the procedure and the intermediate repeatability, because the experiments have been carried out by two analysts.

In all cases nonparametric and/or robust alternatives of estimations were used as advocated in part 5 of ISO 5725 norm [27] to avoid the effect of outliers and to prevent the

underestimation of the figures of merit. The least median squares regression, LMS, has been incorporated in all the steps in which a univariate regression intervenes in order to detect possible outliers. For this task, the LMS regression has proved to be very useful in the validation of linear regressions and has been incorporated in our working group for the validation of many analytical procedures since 1993 [28].

2. Theory

2002/657/EC European Decision [19] defined the validation procedures for the confirmatory and screening methods. This includes the following new definitions to estimate the performance of measuring analytical processes when a linear univariate calibration is used.

'*Decision limit (CCα) means the limit at and above which it can be concluded with an error probability of α that a sample is non compliant*.' This value can be calculated by the calibration curve procedure according to ISO 11843.

This term, CCα, is related with the α error, which is '*the probability that the tested sample is compliant, even though a non-compliant measurement has been obtained (false non-compliance decision)*' and so evaluates the type I error (to affirm that the analyte concentration is not compliant when it is). But it has no information about the type II error (to affirm that the analyte concentration is compliant when it is not) which is established by β '*the probability that the tested sample is truly non-compliant, even though a compliant measurement has been obtained (false compliant decision)*'. CCα substitutes the definitions of the limit of decision (LOD) usually calculated as three times the standard deviation from the blank.

To deal with the type II error the norm defines the detection capability (CCβ) as '*the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β*'. It establishes two possibilities depending on whether or not there is a permitted limit: '*In the case of substances for which no permitted limit has been established, the detection capability is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of 1 − β. In the case of substances with an established permitted limit, this means that the detection capability is the concentration at which the method is able to detect permitted limit concentrations with a statistical certainty of 1 − β*,' It also defines the permitted limit as the '*maximum residue limit, maximum level or other maximum tolerance for substances established elsewhere in community legislation*.'

In this way the detection capability is established with an estimation of α and β probabilities.

The definitions included in the norm are directly applicable to the case of zero order analytical signals, but they have to be adapted for upper order signals. Generalizations of the detection capability for multivariate calibrations have been carried out recently by our research group [23,24,29].

The procedure implies the α and β probabilities estimation of the hypothesis test:

- Null hypothesis, H_0: the sample true concentration (X) is x_0, that is $X = x_0$.
- Alternative hypothesis, H_A: the sample true concentration is greater than x_0, that is $X > x_0$.
- Significance level α: the probability of rejecting H_0 when H_0 is true.
- Probability β: the probability of accepting H_0 when H_0 is false.

When a substance is banned then $x_0 = 0$, α is the probability of false positive and β the probability of false negative. When a substance has a PL then $x_0 = \mathrm{PL}$, α is the probability of false noncompliance and β is the probability of false compliance. The contrast critical value, that is, the concentration value from which the decision to reject H_0 will be taken, is denominated CCα in this directive. In both cases, the operative curve defines CCβ in function of the analyte concentration in the sample.

There are some references in the literature about multivariate figures of merit, including limits of detection. A critical review of the successive contributions to the subject can be seen in Faber et al. [30]. Most of these approaches are based on the reduction of the *N*th-order data to the scalar net analyte signal, NAS. Although different definitions of NAS have been proposed, they can be basically grouped in two classes: (i) "NAS for a component is the part of its spectrum which is orthogonal to the spectra of the other components (considered for calibration)" [31] and (ii) "The (multivariate) NAS vector is the part of the gross signal (of the mixture spectrum) that is useful for prediction" [30,32]. In the majority of the cases, the term NAS has been used according to definition (i) but in a recent paper Ferré and Faber [33] have shown that this orthogonal component is not directly related to the predicted concentration.

With definition (ii) the Euclidean norm of NAS is linearly related to the predicted concentration. As a result, the same result is obtained for CCα and CCβ using the concentration calculated with the PLS model versus the reference analyte concentration as with the norm of the NAS versus the reference analyte concentration. This is because the detection capability used in this work has the property of being unchanged by linear transformations of the estimated concentration [24]. However, the NAS based approach is limited to linear multivariant calibration methods (classical least squares, principal component regression, partial least squares, etc.) and still has some unsolved difficulties for two and higher order data.

The approach used in this paper is applicable to any type of regression, whether linear or not, although it does not use latent variables (for example a calibration based on a neuronal network). Neither is it limited by the order of the data and continues to be applicable when the data are not bilinear (trilinear, ...). Another difference with respect to the approximate limit of detection in the signal space developed

in Ref. [30] is that it is not necessary to include any bias correction because the procedure to calculate the capability of detection used in this paper does not use any estimation of the true model, i.e. the true relationship between analyte concentration and net signal. On the contrary, by using the prediction of the concentration this difficulty does not arise.

3. Experimental

3.1. Reagents

Flumequine (R&D use only) was obtained from Sigma, ciprofloxacin and enrofloxacin were kindly provided by the Burgos Health Lab. Tris(hydroxymethyl)aminomethane (quality for analysis) was obtained from Scharlau laboratories, HCl (for analysis) and methanol (HPLC grade) were supplied by Merck. Deionised water was obtained with a Millipore Milli-Q gradient A10 system.

Stock solutions of 100 mg l^{-1} flumequine were prepared monthly in 4 mM of NaOH methanolic and stored under refrigeration in amber glass bottles. Ciprofloxacin and enrofloxacin stock solutions were prepared at 1000 mg l^{-1}. Solutions in 0.02 N NaOH solution has been stored under refrigeration in the dark.

A 6.05 g l^{-1} of tris(hydroxymethyl)aminomethane solution was prepared for buffering. Working solutions were prepared daily by diluting stock solution in a methanol/buffer mixture (75:25, (v/v)) and was adjusted to pH 9.1 with 0.2 ml 1 M HCl. Measured solutions were prepared by dilution in the same methanol/buffer solution just before being measured and were deoxygenated for 5 min by ultrasonication.

3.2. Apparatus

Fluorimetric assays were carried out at room temperature on a Perkin–Elmer LS50-B Luminescence Spectrometer controlled by means of FLWinLab software. Excitation–emission data were extracted using the Biolight 3D Exporter software. Deoxygenation by ultrasonication was performed with an Elma Transsonic T460.

Spectra were collected at wavelengths from 300 to 500 nm in emission (intensities recorded every 0.5 nm) with excitation wavelengths from 230 to 300 nm (every 10 nm). Excitation and emission monochromator slit-widths were both set to 10 nm and emission was scanned at 1500 nm min^{-1}.

3.3. Calibration conditions

The experimental work was done by two different analysts (analyst 1 and analyst 2) and each one of them performed all the steps in the measurement of that data set. The process involved the daily preparation of working solutions, and from these, of the measured solutions, and then the measurement of each one of the samples for the calibration and test sets corresponding to each experiment. From the data stored in the matrix of the excitation–emission intensities, the variables used for both types of regression were selected according to Table 1.

Table 1
Excitation and emission wavelengths selected for the calibration of flumequine and ciprofloxacin

Excitation (nm)	Emission (nm)	Regression
Flumequine		
240	362	Univariate
240	300–450	PLS
Ciprofloxacin		
270	415	Univariate
270	350–500	PLS

One value of intensity was selected for univariate regression, whereas for PLS regression a vector of intensities collected at a fixed value of the excitation wavelength when the emission wavelength is increased was selected.

The univariate regressions of luminescence intensity against the sample concentration were performed by least squares (OLS) and were validated using the LMS regression as described in [28], which allows for the detection of outliers. More details regarding the theory of this regression can be seen in Ref. [34].

PLS calibrations were performed with Q-PARVUS [35] over mean centered data and the number of latent variables was determined by the maximum response explained variance in crossvalidation (leave one out). The detection capability was obtained from the OLS regression y_{calc} versus y_{true} as indicated in Ref. [23]. This regression was also validated using the LMS regression.

The errors found are shown as means of the absolute error value and as root mean standard error in calibration (RMSEC) or in prediction (RMSEP) in units of μg l^{-1}. This latter was calculated as the square root of the mean squared error, either in calibration or in prediction.

$$\mathrm{RMSEP} = \sqrt{\frac{\sum_{i=1}^{n}(c_i - \hat{c}_i)^2}{n}}$$

where c_i, $\hat{c}_i$, and n are the true concentration of i-th sample, the concentration estimated for this sample and the number of test samples respectively.

The calculation of the capability of detection as the probability of false negative (β) for a fixed probability of false positive (α) when there is no permitted limit was done with the DETARCHI program [36] (available free from the authors). For the PL case, the calculation was made using a new program known as MRL. The calculation of accuracy was obtained using classical statistics and robust statistics based on the Huber H15 estimator [37], which was calculated using a home made program called ROBUSTO. The estimations of the measures of centralization and dispersion based on mean and dispersion parameters have been accepted and advocated by ISO 5725-5 and also by the AOAC for several years now.

4. Results and discussion

4.1. Detection capability

For flumequine European norms establish different values of MRLs depending on the type of animal and for each type of tissue. These values range between 50 $\mu g\,l^{-1}$ in milk to 1500 $\mu g\,l^{-1}$ in kidney for bovine, porcine, ovine and caprine. Likewise the norms establish that flumequine should not be used in animals from which eggs are produced for human consumption. For this reason we have determined the detection capability for flumequine both when there is no established permitted limit and when there is one MLR established value.

When there is no permitted limit, the design of the calibration standards was done with 10 samples, evenly distributed in the 10–100 $\mu g\,l^{-1}$ range. To test the predictive capability four additional samples with 10, 30, 80 and 100 $\mu g\,l^{-1}$ were used. Each one of the two analysts performed the measurement of a complete data set. Thus, data set A was measured by analyst 1 and data set B by analyst 2. The results obtained from the two data sets were compared by using the two types of regression (univariate and PLS), the PLS regression being adjusted with two latent variables. Through the loadings, the first one allows for the identification of the size factor and the second one allows for a correction of the base line, justified by its proximity to the detection limit and the bad signal/noise ratio. Table 2 summarizes the results obtained for the two data sets according to the type of regression used. The error data are included (both in percentage and in terms of RMSEC or RMSEP) resulting both in adjustment and in prediction of the sample test outside the calibration, as well as the value found for the capability of detection. For the PLS regression, we include the number of latent variables with which it was fitted, and the response variances obtained with this number of latent variables, both in the elaboration of the model (explained variance) and in the validation stage.

The calibration performed using the PLS regression improves the results obtained by the univariate regression, both in modeling and in prediction at these concentration levels. The capability of detection was obtained in accordance with the European norms [19] from the regression y_{calc} versus y_{true}, using the procedure established in [23]. The power curve is obtained for a nominal value $x_0 = 0$ and a probability of false positive $\alpha = 0.05$. Fig. 2 (a) shows the power curves for a replicate for each one of the calibrations, and Table 2 shows the values for a probability of false negative $\beta = 0.05$. These values vary between 4.4 and 14.7 $\mu g\,l^{-1}$, which is the smallest value different from zero which can be detected with statistical certainty $1 - \beta$. To avoid extrapolation, taking into account that the concentration of the smallest standard measured is 10 $\mu g\,l^{-1}$, we consider that the capability of detection obtained varies between 10 μl^{-1} and 14.7 $\mu g\,l^{-1}$. Therefore, the probability of false negative is lower than 0.05 for the first of the cases.

When there is a PL, the capability of detection must be evaluated with respect to this value. In this case the PL value chosen was 400 $\mu g\,l^{-1}$, which corresponds to the MRL established for flumequine in chicken and turkey muscle. In this case the calibration samples were selected at around the value of the MRL and the working interval consisted of five different concentrations for the calibration (evenly distributed from 300 to 500 $\mu g\,l^{-1}$ with two replicates at the ends of the interval) and eight test samples (replicates at the nominal concentration of 400 $\mu g\,l^{-1}$).

Table 3 summarizes the results for the two types of regression. By increasing the working concentration, there is a considerable reduction in the errors obtained, while the results are similar for the two types of regression, univariate and PLS. In this case the PLS regression was adjusted with a single latent variable, a result which was coherent with the fact that at this working range, the analytical signal had a better signal/noise ratio. The capability of detection was obtained by selecting x_0 at the nominal value of 400 $\mu g\,l^{-1}$, and was calculated as the minimum net concentration which can be differentiated from the nominal value ($|X - x_0|$). In this case α is the probability of false noncompliance and β the probability of false compliance. The values of detection capability obtained at 400 $\mu g\,l^{-1}$ are shown in Fig. 2b. For $\alpha = \beta = 0.05$ these values ranged between 31.9 and 43.2 $\mu g\,l^{-1}$, being considerably higher than the detection limit values. It should be noted that, at these concentrations, despite the better signal/noise ratio, the method is not

Table 2
Results of calibration for two data sets of flumequine (A and B) measured by two different analysts

Type of regression	Data set	Fitting ($n = 10$)		Prediction ($n = 4$)		Detection capability ($\mu g\,l^{-1}$)	Response variance explained	
		Error[a] (%)	RMSEC ($\mu g\,l^{-1}$)	Error[a] (%)	RMSEP ($\mu g\,l^{-1}$)		In fitting (%)	In prediction (%)
Univariate	A	9.6	3.0	18.8	4.9	14.7	–	–
	B	3.0	1.4	3.8	2.4	6.9	–	–
PLS[b]	A	4.0	1.8	7.6	1.5	8.9	99.49	98.87
	B	1.9	0.9	4.5	2.5	4.4	99.83	99.78

Both sets were calibrated by using two types of regression (univariate and PLS). Each data set has 10 fitting samples and four test samples in the 10–100 $\mu g\,l^{-1}$ range. The detection capability was calculated for the case of no permitted limit ($x_0 = 0$).

[a] Mean of the absolute residuals.

[b] With two latent variables.

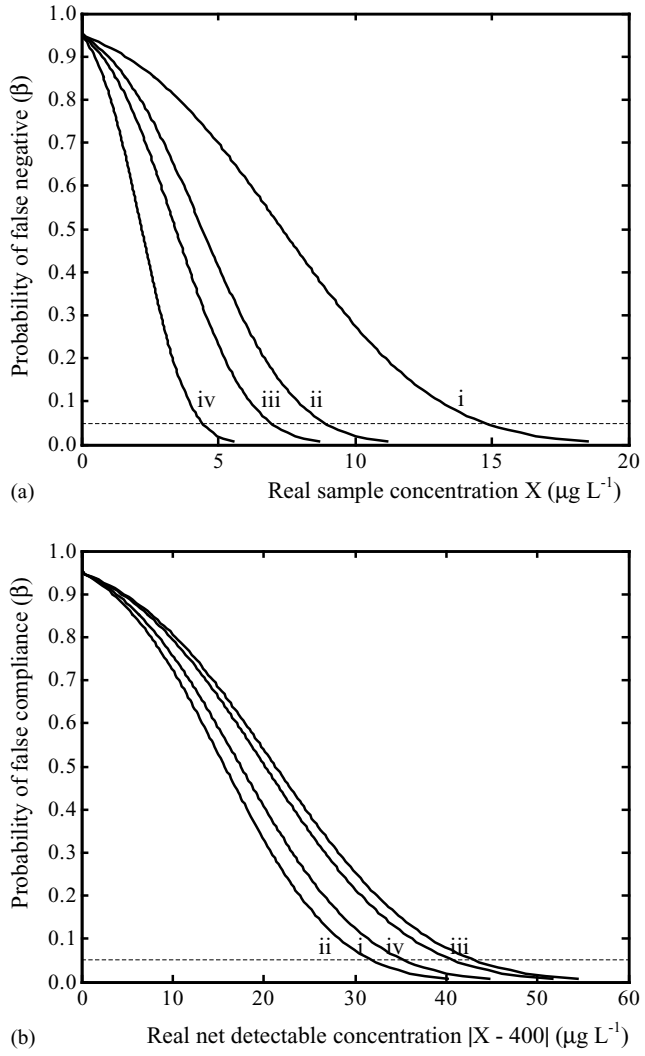

Fig. 2. Power curves for one replicate ($\alpha = 0.05$) for the true net detectable concentration (X) of flumequine when the reference concentration is $x_0 = 0$ (a) and when it is equal to $x_0 = 400\,\mu g\,l^{-1}$ (b). Results obtained for (i) data set A with univariate regression, (ii) data set A with PLS regression, (iii) data set B with univariate regression, (iv) data set B with PLS regression.

capable of distinguishing differences in concentrations of the order of $10\,\mu g\,l^{-1}$ which were detected around concentration zero.

4.2. Interferences

The possible effect of interferences on the detection capability was studied using the pair of analytes enrofloxacin and ciprofloxacin. These quinolones have established overall MRLs because enrofloxacin is metabolized in ciprofloxacin and both have a very similar fluorescence spectrum as can be seen in Fig. 1. These MRLs vary between 100 and $300\,\mu g\,l^{-1}$ and are banned in animals from which eggs are produced for human consumption. We have studied the situation in which there is no established permitted limit. Measurements were made of ciprofloxacin alone (Table 4) or in the presence of enrofloxacin as interferent (Table 5). In both cases the distribution of the calibration standards was of ten samples evenly distributed in the 20–$200\,\mu g\,l^{-1}$ range, and another four test samples with 20, 60, 160 and $200\,\mu g\,l^{-1}$. Three data sets were measured for each series, the first one (A) being measured by analyst 1 and the other two (B and C) by analyst 2. To calculate the interferent effect, a fixed quantity of $100\,\mu g\,l^{-1}$ enrofloxacin was added to each one of the samples in the second series.

Considering the results of the calibration of ciprofloxacin alone (Table 4) the calibration carried out using the PLS regression (with 2 latent variables) shows improvements as compared with the univariate regression, especially in prediction. The values of detection capability are obtained from the power curves in Fig. 3a and are situated between 14.1 and 28.6 $\mu g\,l^{-1}$ for the PLS regression and between 21.2 and 50.6 $\mu g\,l^{-1}$ when the univariate regression is used, being almost always above the concentration of the lowest standard measured. These values are poorer than those obtained for flumequine because the intensity of the fluorescence of the ciprofloxacin is much smaller. Comparing the values of luminescence intensity in the univariate calibration conditions, for a concentration of $100\,\mu g\,l^{-1}$ luminescence intensities below 55, in units of fluorescence intensity, were found for the ciprofloxacin against values above 160 of intensity for the flumequine.

Table 3
Results of two calibration sets of flumequine (A and B) measured by two different analysts

Type of regression	Data set	Fitting ($n = 7$)		Prediction ($n = 8$)		Detection capability ($\mu g\,l^{-1}$)	Response variance explained	
		Error[a] (%)	RMSEC ($\mu g\,l^{-1}$)	Error[a] (%)	RMSEP ($\mu g\,l^{-1}$)		In fitting (%)	In prediction (%)
Univariate	A	1.7	7.2	2.3	12.4	35.4	–	–
	B	1.9	8.8	2.5	13.3	43.2	–	–
PLS[b]	A	1.6	6.5	2.0	10.5	31.9	99.21	99.01
	B	2.0	8.3	2.7	14.9	40.9	98.71	98.27

Both sets were calibrated by using two types of regression (univariate and PLS). Each data set has seven fitting samples and 8 test samples in the 300–$500\,\mu g\,l^{-1}$ range. The detection capability was calculated at $400\,\mu g\,l^{-1}$.

[a] Mean of the absolute residuals.

[b] With one latent variable.

Table 4
Results of calibration for three data sets of ciprofloxacin (A, B, and C) measured by two different analysts

Type of regression	Data set	Fitting ($n = 10$)		Prediction ($n = 4$)		Detection capability ($\mu g\,l^{-1}$)	Response variance explained	
		Error[a] (%)	RMSEC ($\mu g\,l^{-1}$)	Error[a] (%)	RMSEP ($\mu g\,l^{-1}$)		In fitting (%)	In prediction (%)
Univariate	A	15.3	17.8	43.5	25.6	50.6	–	–
	B	13.6	8.2	20.0	11.9	40.0	–	–
	C	5.1	4.3	10.2	4.5	21.2	–	–
PLS[b]	A	4.4	4.9	7.7	9.8	24.1	99.06	98.69
	B	10.2	5.8	7.1	5.6	28.6	98.69	98.33
	C	3.4	2.9	5.8	2.7	14.1	99.68	99.50

The three sets were calibrated by using two types of regression (univariate and PLS). Each data set has 10 fitting samples and four test samples in the range 20–200 $\mu g\,l^{-1}$. The detection capability was calculated for the case of no permitted limit ($x_0 = 0$).
[a] Mean of the absolute residuals.
[b] With two latent variables.

The results obtained in the presence of interferents (Table 5) also give somewhat better results (particularly in prediction) with the PLS than with the univariate regression. The detection capability obtained was between 17.5 and 31.5 $\mu g\,l^{-1}$ for the univariate regression and between 12.7 and 27.3 $\mu g\,l^{-1}$ when the PLS regression was used (power curves in Fig. 3b). The latter is adjusted with two latent variables; no more were needed to model the presence of the interferent because their only effect was to shift the base line.

The fact that the presence of the interferent did not affect the detection capability can be explained as due to the conditions of standard addition which have been used, namely, with a fixed interferent level. So, the effect over the signal is a linear one and the detection capability is invariant by linear transformations of the signal [24].

The most marked difference when comparing these results with those for the ciprofloxacin alone is a worsening of the results in prediction. With the univariate calibration, the mean of absolute error (in percentage) when determining the problem samples varies between 17.6 and 57.8% and the RMSEP between 7.2 and 58.7 $\mu g\,l^{-1}$. Thus, in prediction the univariate calibration of ciprofloxacin in the presence of enrofloxacin is poorer than prediction obtained without interferent. However, in dataset A, the PLS model identifies the sample with 200 $\mu g\,l^{-1}$ as an outlier because its residual is significantly greater than that of the calibration samples (P-level > 0.99) and its leverage is 1.72 but in the calibration samples the leverage varies between 0.11 and 0.61. As a consequence the error and RMSEP are 24.6% and 15.2 $\mu g\,l^{-1}$ with the three remaining samples, instead of 33.6% and 62.0 $\mu g\,l^{-1}$ obtained with the four test samples.

The same applies to dataset B; in this case the anomalous sample was that of 20 $\mu g\,l^{-1}$ with a leverage 0.81 (for the calibration samples the leverage varies between 0.11 and 0.43) and a significant residual at probability 0.99. Without the sample the error in prediction was 11.1% and the RMSEP was 11.1 $\mu g\,l^{-1}$ instead of 40.3% and 16.0 $\mu g\,l^{-1}$ with the four samples. Therefore the possibility to detect samples different from that of calibration and as a consequence to avoid erroneous application of the model was a clear advantage of the PLS calibration.

4.3. Accuracy

EU norms [19] define accuracy as '*the closeness of agreement between a test result and an accepted reference value. It is determined by determining trueness and precision.*' It also defines trueness as '*the closeness of agreement between the average value obtained from a large series of test*

Table 5
Results of calibration for three data sets of ciprofloxacin in presence of 100 $\mu g\,l^{-1}$ of enrofloxacin (A, B, and C) measured by two different analysts

Type of regression	Data set	Fitting ($n = 10$)		Prediction ($n = 4$)		Detection capability ($\mu g\,l^{-1}$)	Response variance explained	
		Error[a] (%)	RMSEC ($\mu g\,l^{-1}$)	Error[a] (%)	RMSEP ($\mu g\,l^{-1}$)		In fitting	In prediction
Univariate	A	8.1	5.9	40.2	58.7	28.9	–	–
	B	2.5	3.6	57.8	22.5	17.5	–	–
	C	5.2	6.4	17.6	7.2	31.5	–	–
PLS[b]	A	8.3	4.7	24.6[c]	15.2[c]	23.2	99.13	98.77
	B	2.7	2.6	11.1[c]	11.1[c]	12.7	99.74	99.66
	C	6.4	5.5	4.8	3.8	27.3	98.81	98.30

Both sets were calibrated by using two types of regression (univariate and PLS). Each data set has 10 fitting samples and 4 test samples in the 20–200 $\mu g\,l^{-1}$ range. The detection capability was calculated for the case of no permitted limit ($x_0 = 0$).
[a] Mean of the absolute residuals.
[b] With two latent variables.
[c] A sample outlier was removed. Leverage (T^2) and lack of fit (Q) were significant at the 95% level.

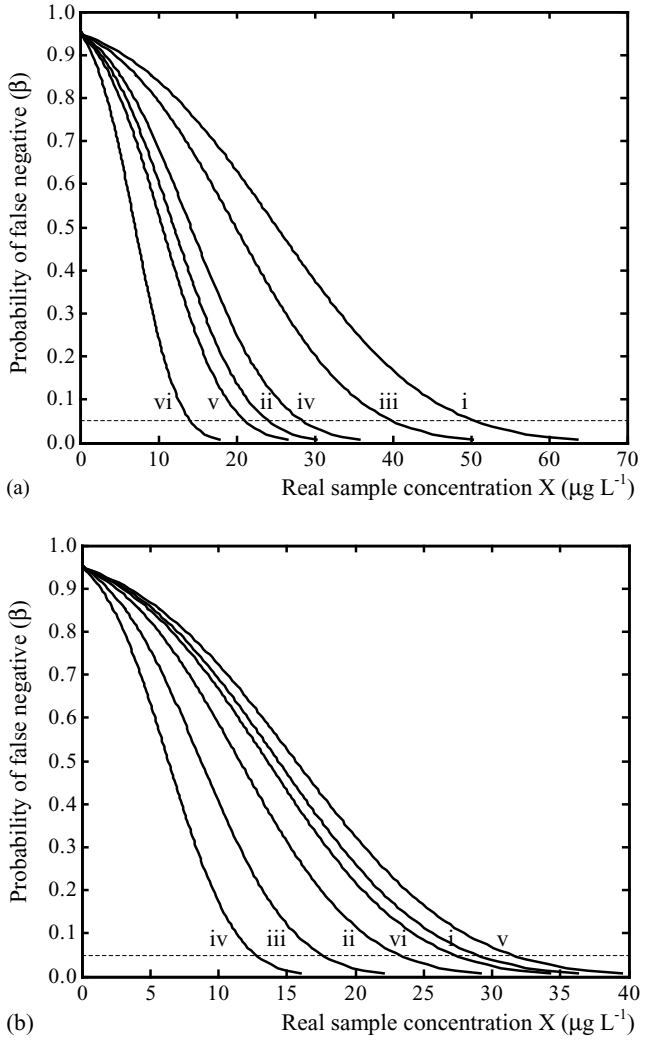

Fig. 3. Power curves for one replicate ($\alpha = 0.05$) for the detection limit for ciprofloxacin (a) and ciprofloxacin in presence of 100 μg l^{-1} of enrofloxacin as interferent (b). Results obtained for (i) data set A with univariate regression, (ii) data set A with PLS regression, (iii) data set B with univariate regression, (iv) data set B with PLS regression, (v) data set C with univariate regression, (vi) data set C with PLS regression.

results and an accepted reference value. Trueness is usually expressed as bias.' And precision as '*the closeness of agreement between independent test results obtained under stipulated (predetermined) conditions. The measure of precision usually is expressed in terms of imprecision and computed as standard deviation of the test result. Less precision is determined by a larger standard deviation*'. These definitions are similar to those found in ISO norm 5725.

From these definitions it is clear that the calculation of accuracy must be performed in terms of the trueness and precision and requires a large number of determinations of test samples. To do this, we used the calibration of flumequine in the range 300–500 μg l^{-1}, where eight test samples were measured at the nominal value of 400 μg l^{-1}. Taking into account that two different types of regression were used and the calibrations were carried out by two different analysts, we opted to obtain the accuracy in conditions of repeatability, defined as '*conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment.*'

It is essential to verify the absence of outliers, and we used the Grubb's test, proposed by ISO norm 5725. The latter tests the null hypothesis 'the value is outlier' as against the alternative hypothesis 'this is not the case'. It is basically a repeatability test to detect possible individual anomalous data. This test must be applied over the smallest data, the largest, the smallest two and over the largest two. If the statistic value is greater than its 5% critical value and less than or equal to its 1% critical value, then the item tested is called struggler. If the statistic is greater than its 1% critical value, then the item is called a statistical outlier and must be withdrawn from the calculations of the precision. In our case none of the objects were qualified as outlier.

For each of the data sets (each done by a different analyst) classical statistics were used to calculate the mean and standard deviation of the eight test samples for each one of the two regression techniques employed (Table 6). Testing of trueness was done using a hypothesis test where the null hypothesis H_0: $x_0 = 400$ μg l^{-1} (the concentration obtained is effectively the nominal concentration) with H_A: $x_0 \neq 400$ μg l^{-1} (the alternative hypothesis) was tested. This

Table 6
Comparison between classical and robust statistic parameters

	Classical statistic				Robust statistics (μg l^{-1})	
	Mean ($n = 8$) (μg l^{-1})	Standard deviation (μg l^{-1})	t calculated	P-level	H15 centralization	H15 dispersion
Data set A						
Univariate	408.9	9.2	2.75	0.028	407.6	7.1
PLS	407.7	7.6	2.88	0.024	406.3	5.2
Data set B						
Univariate	390.7	10.2	−2.57	0.037	391.9	8.9
PLS	389.5	11.2	−2.63	0.034	391.7	6.8

Data are from eight test samples at the nominal value of 400 μg l^{-1} obtained from flumequine calibration in the range from 300 to 500 μg l^{-1}. The two data sets (A and B) were measured by two different analysts and calibrated by using two types of regression (univariate and PLS).

test concludes the trueness of the method at a 99% confidence level for both analysts and both types of regression. The measurement of precision gives values of standard deviation between 7.6 and 11.2 $\mu g\,l^{-1}$, which are a measure of the repeatability of the method expressed as standard deviation.

An alternative to the elimination of anomalous data suggested in ISO standard 5725-5 is based on the use of robust estimators. We used the H15 Huber estimator [37] which limits the maximum influence of the outlier data without eliminating them and offers joint measures of centralization and dispersion. The calculation was done using the ROBUSTO program (results of robust statistic in Table 6). The almost complete coincidence between the results obtained with those of the classical statistics allows us to affirm the nonexistence of outliers. This is a simple process to apply, especially when compared with the scheme of elimination of data using the Grubb test. The intermediate repeatability could be evaluated by the pooled variance between the standard deviations obtained by the two analysts.

5. Conclusions

The capability of detection, with probabilities of errors of false positive and false negative equal to 5%, has been evaluated for flumequine in the case where there is no permitted limit (14.7 $\mu g\,l^{-1}$ with univariate regression versus 8.9 $\mu g\,l^{-1}$ with PLS regression for the data set A) and considering as permitted limit a value of maximum residue limit of 400 $\mu g\,l^{-1}$ (results of 35.4 $\mu g\,l^{-1}$ with univariate regression versus 31.9 $\mu g\,l^{-1}$ with PLS regression for data set A).

Here it is found that PLS calibration offers significant advantages over univariate calibration at low concentration ranges whereas at higher concentration levels (nominal value of 400 $\mu g\,l^{-1}$) results are very similar.

The capability of detection of ciprofloxacin alone is from 14.1 to 24.1 $\mu g\,l^{-1}$ when PLS is used versus 21.2 to 50.6 $\mu g\,l^{-1}$ when univariate calibration is carried out (for the three data sets). The presence of an interference (ciprofloxacin with enrofloxacin) has no effect on the capability of detection when PLS regression is used.

Validation of the method performed at the nominal value of 400 $\mu g\,l^{-1}$ shows that it is accurate both in trueness and precision and an alternative robust statistic was used to confirm the results.

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