Matrix effect on the analysis of amphenicols in fish by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

L R Guidi[1], P A S Tette[1], W P Evangelista[1], C Fernandes[2] and M B A Glória[1]
[1]LBqA - Laboratório de Bioquímica de Alimentos - Faculdade de Farmácia, UFMG, Av. Antônio Carlos, 6627, 31270-901, Belo Horizonte, MG, BRAZIL
[2]Laboratório de Controle de Qualidade de Medicamentos - Faculdade de Farmácia, UFMG, Av. Antônio Carlos, 6627, 31270-901, Belo Horizonte, MG, BRAZIL
E-mail: leticiaguidi@yahoo.com.br

Abstract. Matrix effect is an important parameter to be investigated during the development and validation of a method for the quantitative determination of contaminants in food. The objective of this study was to evaluate the matrix effect, through statistical tests, in the quantification of amphenicols in fish by HPLC-MS/MS. The study was performed by comparing the standard curves prepared in solvent solutions and in a fish sample previously known to be free of amphenicols. Since matrix effect was observed for the three analytes, calibration curves for quantification of chloramphenicol, thiamphenicol and florfenicol should be constructed using the matrix.

1. Introdution

Chloramphenicol and its analogues thiamphenicol and florfenicol are antibiotics with broad spectrum activity against bacteria. However, the clinical use of chloramphenicol was banned in many countries because of their severe side effects in humans such as aplastic anemia. Thiamphenicol and florfenicol appear to be viable substitutes for chloramphenicol in veterinary medicine. In order to ensure consumers' safety, it is necessary to develop sensitive and reliable methods for routine monitoring of these compounds.

Several chemical analyzes have been performed routinely in laboratories for the determination of a wide variety of substances. Frequently, the determination of the exact amount of the substance to be analyzed is necessary, and, in such cases, a quantitative analysis is performed. In these analysis calibration curves, which relate analyte concentration to the response in the detection system, are needed. This ratio corresponds to a performance parameter in the validation of an analytical method called linearity [1, 2].

It is possible to confirm linearity and to determine the range by the construction of analytical curves relating analyte concentration and analyte peak area obtained in the detection system. The range is defined based on the Maximum Residue Limit (MRL) or the Minimum Required Performance Limits (MRPL), choosing equidistant concentration levels, above and below these limits.

The experiments described for evaluation of linearity often involve preparation of calibration curves in solvents or in the matrix, mostly with five to six concentration levels, with a minimum of two to seven replicates per level [3].

A method proposed by [4] for evaluating the linearity consists on the following steps: (a) define the range of interest, where the midpoint of the range should correspond to the expected concentration in the sample; (b) prepare calibration solutions in solvent or in matrix (in case the matrix effect is

significant), at least, six equally spaced concentration levels in three independent replicates (c) measure the response of the calibration solutions in a random order.

The sample matrix may contain components that interfere with the performance of the measurement by the detector selected, without causing a visible signal in the selectivity test. The interferences can increase or decrease the signal, and the magnitude of the effect can also depend on the concentration. Different samples, extracts and matrix concentrations may exhibit different levels of matrix effect. Then, a matrix which represents all these different levels of matrix effects should be selected during calibration curve construction [5]. When there is no significant matrix effect, the standard curve can be directly prepared in solvent [6]. If an effect is detected, the analytical curves should be constructed on the matrix. Several tests and their corresponding statistics can be used to study this effect.

The aim of this study was to evaluate the matrix effect, by statistical tests, on the quantification of amphenicols in fish by LC-MS/MS.

2. Material and methods

2.1. Material

Samples of *Tilapia* were collected in the consumer market of Itabirito, MG, Brazil.

Reagents were from analytical grade, except LC-MS/MS solvents, which were chromatographic grade. The ultrapure water used was obtained from Milli-Q Plus system (Millipore Corp., Milford, MA, USA). The internal standard used was deuterated chloramphenicol (CAP-d5). Standard solutions of thiamphenicol (1000 ppb), florfenicol (1000 ppb), chloramphenicol (10 ppb) and deuterated chloramphenicol (100 ppb) were prepared in methanol and stored at -20 ºC.

2.2. Methods

2.2.1. Determination of the linearity

To determine the linearity tests were carried out with standard solutions in solvents and in the matrix using peak area [4]. The standard curves of amphenicols in solvent (water:methanol - 80:20 v/v) were built in the following concentration levels: chloramphenicol - 0.30, 0.90, 1.50, 2.10, 2.70 and 3.30 ng/mL; thiamphenicol - 30.0, 60.0, 90.0, 120.0, 150.0 and 180.0 ng/mL; florfenicol - 50.0, 100.0, 150.0, 200.0; 250.0 and 300.0 ng/mL. A graph was constructed relating analyte peak area/internal standard peak area versus the analyte concentration. Regression analysis was performed, providing the linear equation and the correlation coefficient for each analyte.

For each concentration level, three independent replicates were prepared and analyzed randomly to evaluate the behavior of the variances along the analytical curve. After construction of the standard curve, the regression parameters (intersection and slope) were calculated by the Method of Ordinary Minimum Squares, following the method proposed by [4].

2.2.2. Determination of the matrix effect

The study of matrix effect was achieved by comparing the analytical standard curves prepared in solvent and in the fish matrix. The average response of the analytes from the two calibration curves (in the same concentration range), obtained in the same experiment, were compared using the statistical tests F (Snedecor) of variance homogeneity and *t test* (Student) of averages comparison. To accept that the matrix effect is not significant, there should be no matrix effect at any concentration of the calibration curve [3].

3. Results

3.1. Determination of the linearity

The calibration curves of chloramphenicol, thiamphenicol and florfenicol in the solvent and in the matrix were constructed and evaluated for linearity. The curves were obtained from the relationship between the analyte concentration and the analyte peak areas/internal standard peak area. Outliers were treated and all assumptions regarding the Method of Ordinary Minimum Squares (normality, homoscedasticity and independence) were confirmed for the curve constructed in the matrix. The significance of the regression and the non-significant deviations from linearity confirmed the linear model and indicated the possibility of comparing the inclinations and the intersections of both curves by *t test* to evaluate the matrix effect (Table 1).

Table 1. Evaluation of model assumptions and linearity for the standard curves of chloramphenicol, thiamphenicol and florfenicol in fish.

	Values / Curve in					
	CAP		TAP		FF	
Statistics	Solvent	Matrix	Solvent	Matrix	Solvent	Matrix
Number of observations (n)	17	16	15	16	18	16
Normality						
R	0.992	0.997	0.974	0.991	0.984	0.967
P	p > 0.10	p > 0.10	p > 0.10	p > 0.10	p > 0.10	p > 0.10
Homoscedasticity						
tL	-1.176	-1.756	-1.827	-0.679	0.924	0.784
P	0.258	0.101	0.091	0.508	0.369	0.446
Independence						
D	2.278	1.525	2.018	2.337	1.766	1.635
P	p > 0.10	p > 0.10	p > 0.10	p > 0.10	p > 0.10	p > 0.10
Regression						
F	9591.08	6419.97	562.90	979.37	1178.03	878.57
P	1.81×10^{-22}	4.84×10^{-20}	4.36×10^{-12}	2.33×10^{-14}	2.05×10^{-16}	4.93×10^{-14}
Linearity deviation						
F	1.591	1.501	1.077	2.106	4.660	25.282
P	0.245	0.274	0.423	0.155	0.168	0.327

n = number of observations, R = Ryan-Joiner correlation coefficient, p = significance, tL = t-statistics of Levene, d = statistics of Durbin-Watson, F = variance ratio.

3.2. Determination of the matrix effect

The calibration curves for the three amphenicols in solvent and in the fish matrix are shown in Figure 1.

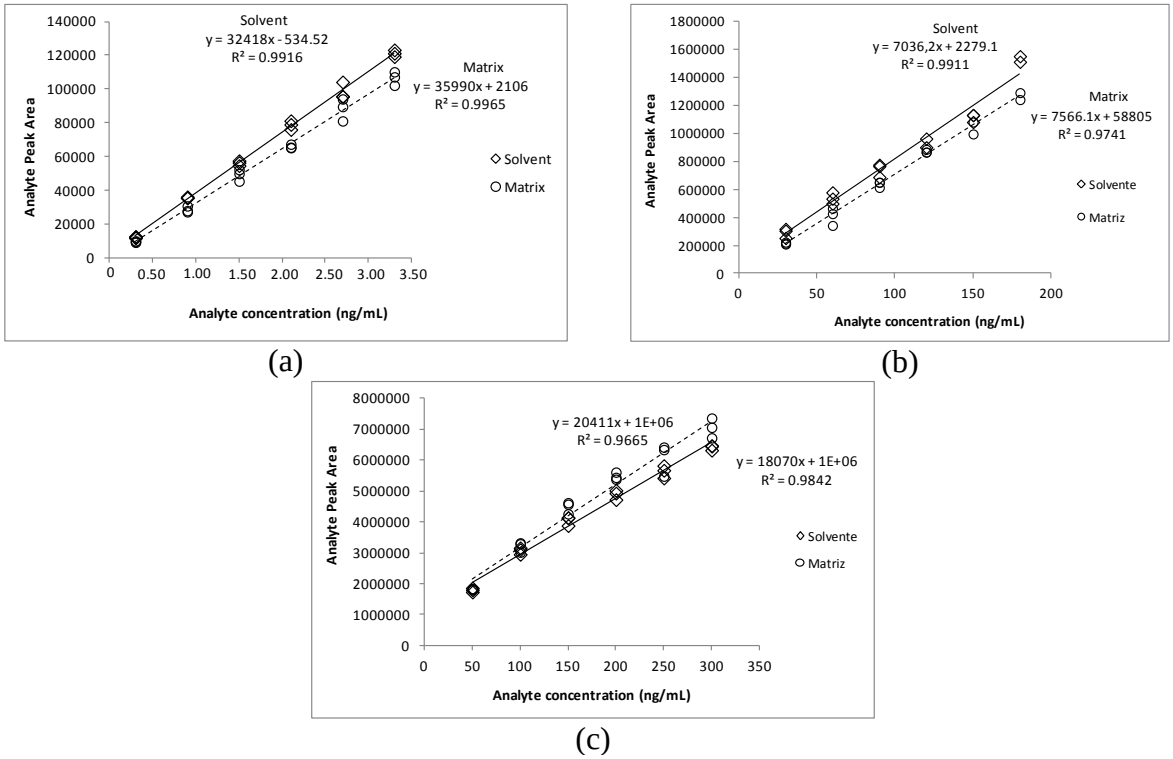

Figure 1. Calibration curves for: (a) chloramphenicol, (b) thiamphenicol (c) florfenicol in solvent and in the fish matrix in the ranges of 0.30 to 3.30 ng/mL, 30.0 to 180.0 ng/mL and 50.0 to 300.0 ng/mL, respectively.

Table 2 shows the comparisons of slopes and intersections of the calibration curves of chloramphenicol, thiamphenicol and florfenicol in solvent and in the matrix, respectively. By the t-test (Student), significant difference at 5% of probability between the slopes of the curves in solvent and in matrix was observed, indicating matrix effect for fish on the responses of chloramphenicol and florfenicol. For thiamphenicol there was significant difference at 5% probability in the slopes and intersections of the curves in solvent and in the fish matrix, indicating that there was matrix effect.

Table 2. Comparison of slopes and intersections of the chloramphenicol (0.30 to 3.30 ng/mL), thiamphenicol (30.0 to 180.0 ng/mL) and florfenicol (50.0 to 300.0 ng/mL) calibration curves in the matrix of fish and in the solvent.

	Results		
Statistics	CAP	TAP	FF
Comparison between intersections			
Ta	1.502	2.232	1.948
P	0.144	**0.034**	0.061
Comparison between slopes			
Tb	2.652	3.354	5.338
P	**p<0.05**	**p<0.05**	**p<0.05**

ta = t-statistic for contrasts between intersections, tb = t-statistic for contrasts between slopes, p = significance. Values in bold and underlined are significant at 5% probability.

4. Conclusion

Based on the results, matrix effect was observed for the three studied analytes chloramphenicol, thiamphenicol and florfenicol. Therefore, the calibration curves must be constructed using the matrix.

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References

[1] Eurachem 1998 The fitness for purpose of analytical methods. A laboratory guide to method validation and related topics 61

[2] BRASIL. Ministério da Agricultura Pecuária e Abastecimento (Mapa). Inst. Norm. nº 24. Guia para validação de métodos analíticos e controle de qualidade interna das análises de monitoramento do Plano Nacional de Resíduos e Contaminantes - PNCRC Animal. D.O.U. 2009

[3] Souza S V C 2007 Procedure for house validation of test methods: design and applicability in food analysis. Belo Horizonte – Faculty of Pharmacy UFMG (Thesis, DSc in Food Science) 296

[4] Souza S V C and Junqueira R G A 2005 A procedure to assess linearity by ordinary least squares method. *Anal. Chim. Acta* **552** 25-35

[5] Bruce P, Minkkinen P and Riekkola M L 1998 Practical method validation: validation sufficient for an analysis method *Mikrochim. Acta* **128** 93-106

[6] Thompsom M E, Ellison S L R and Wood R 2002 Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report). *Pure and Appl. Chem.* **74** 835–855