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In-house validation for multi-residue analysis of tetracycline in cow milk by HPLC with UV detection

Validação intralaboratorial para análise múltipla dos resíduos de tetraciclina em leite bovino por cromatografia líquida de alta eficiência com detecção UV

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Abstract

The indiscriminate use of antibiotics in dairy cattle without complying with the waiting period results in residual contamination, whose effective control in produced milk requires validated methods to ensure analytical results. The aim of this study was to optimize and validate the HPLC-UV/VIS method at 365 nm for analyzing the tetracycline in pasteurized cow milk in accordance with the European Community (2002/657/EC). Spiked milk with analytes (oxytetracycline, tetracycline, doxycycline, and chlortetracycline) was submitted to deproteinization and cleaning by a C_{18} solid-phase column and analyzed by HPLC using a gradient system with 0.01 mol L^{-1} oxalic acid-acetonitrile-triethylamine (90:9.9:0.1) and acetonitrile on a reverse phase (C_{18}) column. Accuracy and precision were assessed by adding analytes to levels of 0.5, 1, and 1.5 times the permissible maximum limit allowed in Brazil. The method presented selectivity with a decision limit (CCα) and detection capability (CCβ) ranging from 114.2 to 143.7 and from 129.3 to 188.7 μg kg^{-1}, respectively. The recovery of tetracyclines was higher than 82.5% with a precision of 7.1%, demonstrating the efficiency in determining tetracycline residues in cow milk.

Key words: In-house validation. Tetracycline residues. High-performance liquid chromatography. Cow milk. Toxicological analysis.

Resumo

O uso indiscriminado de antibiótico em gado leiteiro, sem cumprimento do período de carência, resulta em contaminação residual, cujo controle efetivo no leite produzido requer métodos validados que garantam os resultados analíticos. O estudo visou otimizar e validar o método de CLAE-UV/VIS a 365 nm para análise de tetraciclina em leite bovino pasteurizado em conformidade com a Comunidade Europeia (2002/657/EC). O leite fortificado com analitos (oxitetraciclina, tetraciclina, doxiciclina e clortetraciclina) foi submetido à extração por desproteinização e limpeza por coluna de fase sólida C_{18} e submetido à análise por CLAE empregando sistema gradiente com 0,01 mol L-1 de ácido oxálico-acetonitrila-trietilamina (90:9,9:0,1) e acetonitrila, em coluna de fase reversa (C_{18}). A exatidão e precisão foram avaliadas adicionando o analitos em níveis de 0,5, 1 e 1,5 vezes o limite máximo permitido no Brasil. O método apresentou seletividade com limite de decisão (CCα) e capacidade de detecção (CCβ)

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variando de 114,2 a 143,7 e 129,3 a 188,7 µg kg^{-1}, respectivamente. A recuperação de tetraciclinas foi superior a 82,5% e a precisão de 7,1%, demonstrando eficiência para a determinação de resíduos de tetraciclina em leite bovino.
Palavras-chave: Validação intralaboratorial. Resíduos de tetraciclina. Cromatografia líquida de alta eficiência. Leite bovino. Análise toxicológica.

Introduction

Veterinary antimicrobials in dairy cattle are intended for treatment and prevention of diseases, as well as for use as growth promoters (CHOPRA; ROBERTS, 2001). Their indiscriminate use may result in residues in milk, causing harm to consumer health, bacterial resistance, and allergic reaction such as nausea, vomiting, and gastric irritation in hypersensitive individuals (JECFA, 1996). In addition, to the retardation or inhibition of growth of fermenting bacteria and hence economic losses to the dairy industry (COGAN, 1972).

Tetracyclines belong to the broad-spectrum class of antibiotics against gram-positive and gram-negative bacteria (CHOPRA; ROBERTS, 2001). After the discovery of the chlortetracycline produced by *Streptomyces aureofaciens* by Duggar in 1948, eight commercial tetracyclines can be found, four of which predominant in the treatment of animals for human consumption: oxytetracycline, tetracycline, doxycycline, and chlortetracycline (OKA et al., 2000).

Pontes Netto et al. (2005) performed a survey of the main drugs used in the dairy cattle in the Paraná State, Brazil from September to October 2003 and observed tetracycline as the third main group of antimicrobials, with emphasis on oxytetracycline. To verify data collected by Pontes Netto et al. (2005), Bando et al. (2009), from March 2005 to April 2006, and Zanella et al. (2010), from January 2006 to June 2007, demonstrated the occurrence of tetracycline residues as the main contaminant among the assessed antimicrobials when analyzing milk produced and marketed in the Paraná State by methods of qualitative immunoenzymatic tests. However, quantitative and confirmatory techniques are necessary to assess whether the detected levels are in conformity with health legislation. Countries establish sanitary regulations for the use of antimicrobials in livestock defining the waiting period and maximum residue limit (MRL) in the food of animal origin (RUELA et al., 2005). Brazil follows the legislation proposed by Mercosur, which was established by the *Codex Alimentarius* (CAC, 2009), i.e. MRL for tetracycline alone or in combination with other antibiotics of the same class is100 µg kg^{-1} in milk.

Immunological screening or microbial inhibition tests are commonly used in determining antimicrobial residues in milk. However, these tests are unable to identify the residues and specific chemical identification and quantification methods, such as the liquid chromatography, are necessary (AGUILERA-LUIZ et al., 2008; BOGIALLI et al., 2008). High-performance liquid chromatography (HPLC) presents a high reproducibility, accuracy, and selectivity (WANG et al., 2008). Tetracyclines absorb wavelengths in the UV range between 270 and 360 nm in the acid and neutral pH used in the detection system (OKA et al., 2000).

The European Union established in August 2002 a specific legislation (657/EC) for official analytical methods in the control of residues in products of animal origin, encompassing the performance and interpretation of their results (EC, 2002). Thus, the aim of this study was to validate a multi-residue method for determiningthe tetracycline (oxytetracycline, tetracycline, chlortetracycline, and doxycycline) in cow milk by high-performance liquid chromatography with UV-VIS detection according to the analytical criteria defined by the European Commission 2002/657/EC.

Material and Methods

Equipment and reagents

The chromatographic grade were methanol (Honeywell Burdick & Jackson, Muskegon, USA), acetonitrile (J.T. Baker, Mexico City, Mexico), and triethylamine (Vetec, Rio de Janeiro, Brazil) whereas the grade reagents were citric acid (Reagen, Rio de Janeiro, Brazil), EDTA disodium salt (Labsynth, São Paulo, Brazil), oxalic acid crystal (Dinâmica, Diadema, Brazil), anhydrous dibasic sodium phosphate (Nuclear, Brazil), and trichloroacetic acid (Reagen, Rio de Janeiro, Brazil). Ultrapure water was obtained in the Milli-Q (Millipore, Bedford, USA) system and used for preparing solutions.

Oxytetracycline, tetracycline, chlortetracycline, and doxycycline standards with a purity of 77, 95, 91.3, and 98%, respectively, were from Sigma Aldrich (St. Louis, USA).

Filters and Millex with a 0.45-µm modified PTFE membrane (Millipore, Saint Louis, USA), as well as a 3-cc, 60-mg C_{18} SPE HLB OASIS column (Waters, Wexford, Ireland) were used in the preparation of extract.

The equipment used were an Universal 320R ultra-centrifuge (HettichZentrifugen, Tuttlingen, Germany), KMC-1300V vortex mixer (Vision Scientific CO., LTD, Kyeonggi-do Korea), USC 700 ultrasonic washer (Unique, Indaiatuba, Brazil), vacuum elute (Varian, Walnut Creek, USA), Tec Vap TE-0194 concentrator (Tecnal, Piracicaba, Brazil), and CP225D analytical balance (Sartorius, Göttingen, Germany).

Standard and working solutions

A standard stock solution of 1 mg mL^{-1} was preparedfor each antibiotic by using 10 mg of standard for 10 mL of methanol, considering the degree of purity of each tetracycline standard. The stock solution was stored at 4 °C for up to 30 days where as the working solution in the concentration of 10 µg mL^{-1} was prepared daily according to the need for analysis. The McIlvaine buffer solution was prepared using 11.956 g anhydrous bibasic sodium phosphate, 3.72 g EDTA, and 13.0 g citric acid monohydrate dissolved in ultrapure water, as in Fritz and Zuo (2007). The 20% trichloroacetic acid solution was prepared by dissolving the acid in ultrapure water.

Preparation of samples

Pasteurized milk of different brands was submitted to a rapid test SNAP for tetracyclines. A total of 10 negative samples, in a total volume of 5 liters, constituted the milk pool used for validation. This pool was divided into 100-µL aliquots and stored at −20 °C for up to 3 months.

For validation tests, milk aliquots were thawed at ambient temperature and spiked at concentrations of 0.5, 1, and 1.5 times the MRL (50, 100, and 150 µg kg^{-1}) with the standard working solution of the four tetracyclines (10 µg mL^{-1}).

Extraction

Milk samplesof 5 mL were pipetted into a 50-mL polypropylene tube with 2 mL of 20% trichloroacetic acid solution and vortexed for 1 minute. Afterwards, 20-mL McIlvaine buffer solution was added and centrifuged (1842 *g* for 20 min at ambient temperature).

Cleaning

The supernatant from the previous step was applied to the extraction cartridge pre-conditioned with 3 mL of methanol and 2 mL of ultrapure water. The cartridge was washed with 2 mL of 5% methanol in water and the tetracycline eluted with 3 mL of methanol and concentrated at 35 °C. At the time of the analysis, tetracycline was reconstituted with 1 mL of methanol, homogenized, and filtered in Millex (CINQUINA et al., 2003). The blank

(negative control) was obtained under the same procedure and consisted of milk extract without the addition of antimicrobials.

Chromatographic conditions

The liquid chromatography used was a Finnigan Surveyor (Thermo Scientific, San Jose, USA) model composed of an automatic injector coupled to an UV-Vis detector (Thermo Scientific, San Jose, USA) and Intersil C_8column (150 × 4.6mm) with a 5-µm particle and 100-Å porosity (GL Sciences Inc., Tokyo, Japan). The chromatographic conditions of the mobile phase 1 was composed of 0.01 mol L^{-1} oxalic acid-acetonitrile-triethylamine (90:9.9:0.1) and the mobile phase 2 was composed of acetonitrile at ambient temperature (25 °C), at a wavelength of 365 nm, and 10-minute run under the gradient described in Table 1.

Table 1. Gradient of mobile phase used in the tetracycline analysis.

Time (min)	Mobile phase 1 (%)	Mobile phase 2 (%)	Flow (mL min^{-1})
0	85	15	1.5
0.3	70	30	1.2
1.0	70	30	1.5
5.0	85	15	1.5
10.0	85	15	1.5

Mobile phase 1: 0.01 mol L^{-1} oxalic acid-acetonitrile-triethylamine (90: 9.9:0.1); mobile phase 2: acetonitrile.

In-house validation

The assessed validation parameters were selectivity and specificity, recovery (accuracy), precision (repeatability and reproducibility), decision limit (CCα), and detection capability (CCβ), as the European Union regulation 2002/657/EC.

Analytical curves

The analytical curves assessed the linearity and sensitivity of the method, obtaining the correlation coefficients between the concentrations used to construct the curve. To assess the parameter, six curves with seven different points (blank, 50, 100, 200, 400, 800, and 1600 µg kg^{-1}) were constructed for each analyte. Each curve was constructed by adding the four tetracyclines combined in the blank extract.

Selectivity and specificity

The occurrence of interferent in the retention time of each tetracycline was assessed by performing an analysis on 20 blank samples.

Decision limit (CCα) and detection capability (CCβ)

These parameters were calculated from 20 spiked samples at MRL level (100 µg kg^{-1}). CCα is the limit at which a sample can be declared as noncompliant with a probability of error α equal to 5%. CCα was calculated according to Equation (1):

$$Icc\alpha = \mu_{MRL} + 1.64 \times \sigma_{MRL} \quad (1)$$

Wherein: μ_{MRL} is the mean and σ_{MRL} is the standard deviation of the signal amplitude of the analyte at MRL level. To calculate CCα, a calibration curve constructed from spiked blank samples (50 to 1600 µg kg^{-1}) was used for each tetracycline.

CCβ is the limit at which a substance can be detected, identified, and/or quantified with a probability of error β equal to 5%. It stands for the concentration the method is able to detect in the MRL,with a statistical certainty of 1−, and is calculated according to Equation (2):

$$Icc\beta = Icc\alpha + 1.64 \times \sigma_{MRL} \quad (2)$$

Recovery

The spiked milk at concentrations of 0.5, 1, and 1.5 times the MRL (50, 100, and 150 μg kg^{-1}) was analyzed at each concentration insix replications, being calculated as Equation (3):

$$\text{Recovery (\%)} = 100 \times \text{Measured content} / \text{Spiking level} \quad (3)$$

Precision

Precision assessed the repeatability and reproducibility of the method. For repeatability, three spiking levels (50, 100, and 150 μg kg^{-1}) were analyzed in six replications, while for reproducibility, the same analyses were performed on three different days. Repeatability and reproducibility were presented in coefficient of variation determined by Equation (4):

$$CV = \delta / \mu \quad (4)$$

Wherein:δ is the standard deviation and μ is the mean of results.

The results of reproducibility were compared with the Horwitz equation (HORWITZ et al., 1993):

$$CV = 2^{(1 - 0.5\log C)} \quad (5)$$

Wherein: C represents the concentration expressed as a power of 10 (for example, 1 μg mL^{-1} = 10^{-9}).

Results and Discussion

Aiming at a simple and rapid method for determining tetracyclines in cow milk, the method developed by Denobile and Nascimento (2004) was assessed. However, this method was ineffective in separating oxytetracycline, tetracycline, chlortetracycline, and doxycycline. This method with gradient and flow modifications (Table 1) showed selectivity and the blank presented no interferents in the regions of elution peaks of the tetracycline group (Figure 1).

The linearity assessment consisted of six analytical curves for each tetracycline analyzed in theblank extract (blank, 50, 100, 200, 400, 800 and 1600 μg kg^{-1}). Linearity occurred in a range of 50 to 1600 μg kg^{-1}, with determination coefficients of 0.9936, 0.9999, 0.9797, and 0.9988, respectively for oxytetracycline, tetracycline, chlortetracycline, and doxycycline.

Recovery determination using concentrations of 0.5, 1, and 1.5 times the MRL (50, 100, and 150 μg kg^{-1}) was based on the value stipulated by the European Union (EC, 2002). The results presented in Table 2 show that the recovery for oxytetracycline and doxycycline at a concentration of 50 μg kg^{-1} was not calculated due to the high coefficient of variation. When determining the quantification limit (QL) of the method, according to the concentration of analyte added in the blank, and diluted with a coefficient of variation lower than 20% (data not shown since this study is based on the 2002/657/EC of the European Union), we obtained a QL of 76, 49, 43, and 56 μg kg^{-1} for oxytetracycline, tetracycline, chlortetracycline, and doxycycline, respectively. These data confirm that the concentration of 50 μg kg^{-1} for oxytetracycline and doxycycline should not be used to assess the accuracy and precision of this method. In general, recoveries were satisfactory for the tetracyclines under study, with values ranging from 70.7 to 114.5%. According to the Association of Official Analytical Chemists International (AOAC, 1999), recoveries from 70 to 120% for concentrations between 10 and 100 μg kg^{-1} and recoveries from 70 to 110% for concentrations between 100 and 1000 μg kg^{-1} are acceptable for validating multi-residue methods.

Figure 1. Chromatogram of oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), and doxycycline (DC). Blank sample (Blank) and sample spiked with 100 µg kg^{-1} for each tetracycline (Standard).

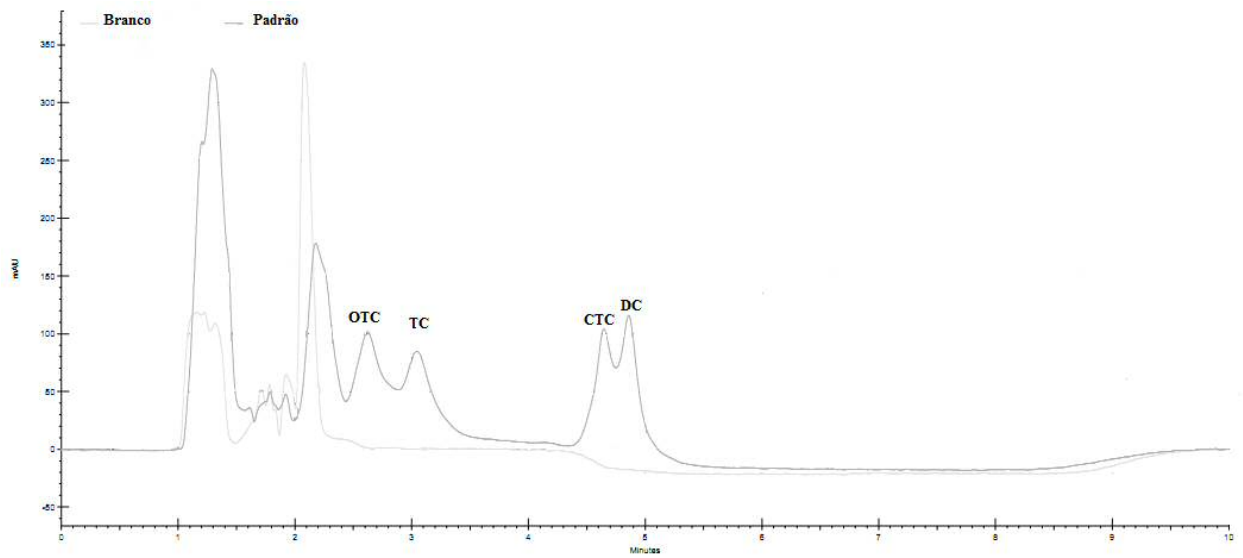

Table 2. Means of concentration, recovery, and coefficient of variation in the analysis of pasteurized cow milk spiked with tetracyclines.

Tetracyclines	**Spiking level** (µg kg^{-1})	**Concentration means ± standard deviation** (µg kg^{-1})	**Average recovery (%) (n=6)**	**Coefficient of variation (%)**
Oxytetracycline	100	98.8 ± 3.1	98.8	3.1
	150	127.9 ± 8.7	85.3	6.8
Tetracycline	50	52.2 ± 3.6	104.5	6.9
	100	114.1 ± 3.1	114.5	2.7
	150	140.4 ± 8.6	93.6	6.1
Chlortetracycline	50	37.1 ± 7.0	70.7	19.8
	100	114.1 ± 3.1	94.2	4.4
	150	123.4 ± 12.7	82.5	10.2
Doxycycline	100	90.8 ± 2.4	90.8	2.6
	150	141.9 ± 11.4	94.6	8.0

Table 2 also shows the coefficient of variation, whose average of three concentrations of the four tetracyclines demonstrated the method repeatability, i.e. 7.1%. This value is considered acceptable according to the European Union, which recommends a coefficient of variation lower than 15-20% for concentrations between 10 and 1000 µg kg^{-1}.Thus, the method presented a good accuracy when considering repeatability.

Table 3 shows the results of decision limit (CCα) and detection capability (CCβ) for oxytetracycline, tetracycline, chlortetracycline, and doxycycline. Cinquina et al. (2003) used an HPLC coupled to a diode array detector and observed CCα values of 113.2, 114.9, 121, 6, and 127 µg kg^{-1} respectively for oxytetracycline, tetracycline, chlortetracycline, and doxycycline, being close to those shown in our study (Table 3). Regarding CCβ, these authors

found values of 117.2, 120.9, 126.5, 131.3 μg kg^{-1} for oxytetracycline, tetracycline, chlortetracycline, and doxycycline, respectively. These values are considerably lower when compared to those found in our study, mainly for chlortetracycline.

Table 3. Decision limits (CCα) and detection capability (CCβ) for tetracyclines in milk.

Tetracyclines	**CCα** (μg kg^{-1})	**CCβ** (μg kg^{-1})
Oxytetracycline	114.2	129.3
Tetracycline	138.4	176.9
Chlortetracycline	143.7	188.7
Doxycycline	135.2	160.0

The reproducibility of the method is shown in Table 4 in terms of coefficient of variation. According to European Union regulations (EC, 2002), for concentrations below 100 μg kg^{-1}, the use of the Horwitz equation does not provide acceptable values since the lower the concentration is, the higher the coefficient of variation determined by this equation. Therefore, Table 4 does not present the results for concentrations of 50 μg kg^{-1}. The coefficient of variation cannot exceed 2/3 of the coefficient of variation determined by the Horwitz equation. The CV found in our study was 23% and 2/3 of this valueis 15.3%. Thus, all CVs for all determined tetracyclines are below this value, showing a good reproducibility of the method.

Table 4. Study of the method reproducibility for tetracyclines in milk.

Tetracyclines	**Spiking level** (μg kg^{-1})	**Obtained CV (%)**	**CV (%) of the Horwitz equation**	**2/3 of the CV (%) of the Horwitz equation**	**Method reproducibility (%)**
Oxytetracycline	100	6.9	23	15.3	8.0
	150	9.3			
Tetracycline	100	6.3			
	150	7.7			
Chlortetracycline	100	12.4			
	150	9.4			
Doxycycline	100	3.8			
	150	7.8			

(HORWITZ et al., 1993).

The method validated in this study for determining tetracyclines in pasteurized cow milk, according to the European Union regulation 2002/657/EC, showed to be linear, specific and selective, precise, and accurate in the range of 82.5 to 114.5%. In addition, the decision limit and detection capability were found in the ranges of 114.2 to 143.7 μg kg^{-1} and 129.3 to 188.7 μg kg^{-1}, respectively. Therefore, the assessed method presented a sufficient efficiency for monitoring tetracyclines in milk considering the requirements of the European Union.

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