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Investigation of the Persistence of Penicillin G and Dihydrostreptomycin Residues in Milk of Lactating Buffaloes (*Bubalus bubalis*) Using Ultra-High-Performance Liquid Chromatography and Tandem Mass Spectrometry

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ABSTRACT: The purpose of this research was to evaluate the persistence of penicillin G and dihydrostreptomycin in milk of lactating buffaloes following intramuscular injection of procaine penicillin G (200000 IU/mL) and dihydrostreptomycin sulfate (250 mg/mL) every 24 h for 3 days. Milk samples were collected twice daily up to the 13th milking post-treatment and analyzed by ultra-high-performance liquid chromatography coupled to tandem mass spectrometry. The analytical method has been validated according to Commission Decision 2002/657/EC. The highest concentrations of penicillin G (275 μg kg^{-1}) and dihydrostreptomycin (220.5 μg kg^{-1}) were detected in the milk of the first milkings post-treatment, and levels were below the maximum residue limit of 4 and 200 μg kg^{-1} in all treated buffaloes at milkings 12 and 2, respectively. The results of this study demonstrate that a nine-milking withdrawal time set for bovine milk was not adequate for depletion of penicillin G in lactating buffaloes.

KEYWORDS: *buffaloes, residues, milk, penicillin G, dihydrostreptomycin, HPLC−MS/MS*

■ INTRODUCTION

The water buffalo (*Bubalus bubalis*) is recognized as the world's second most important milk-producing species and plays an important role in the economy of developing countries like India, Nepal, and Pakistan (80% of the world's production of buffalo milk). In Europe, Italy is the only country where Mediterranean buffalo breeding is remarkably zootechnically important for the production of "mozzarella di bufala campana" cheese with a protected designation of origin (PDO) according to European Union legislation.[1]

Therapeutics, such as antimicrobials, are used in lactating buffaloes for the treatment of mastitis and postpartum diseases.[2] Existing legislation on veterinary medicines requires that pharmacologically active substances authorized in cattle (*Bos taurus*) be targeted to buffaloes (*B. bubalis*) with the same withdrawal periods,[3] although no data on depletion of antibiotics in buffalo milk are available in the literature. Synergistic effects of combination compounds are frequently preferred in livestock, to increase the effectiveness and to improve the treatment outcome as the combined effect of both antibiotics is better than their individual effect. In this regard, penicillin with aminoglycoside is considered a first-line antibiotic choice in dairy animals with clinical metritis and mastitis.

Penicillin G (penG) belongs to the oldest group but is still one of the most used among the classes of antimicrobials.[4] However, allergic reactions in sensitive populations have been described,[5,6] because of β-lactam group residues in milk. For this reason, molecules belonging to the β-lactam group are scarcely tolerated in the European Union among antimicrobials.[7] To adequately protect the consumer and secure dairy production, the Committee for Veterinary Medicinal Products recommends a maximum residue level (MRL) of 4 μg kg^{-1} for penG in milk of all producing animals. Dihydrostreptomycin (DHS) is an aminoglycoside antibiotic that acts directly on bacterial protein synthesis.[8] It is available in many formulations for the treatment of infections induced by Gram-negative and Gram-positive bacteria. Residues of this compound in the milk of all ruminants must be below the MRL of 200 μg kg^{-1} set by the European Commission. In veterinary medicine, DHS is commonly combined with penG to increase the single-drug potential in severe postpartum infections.[9]

If these antibiotics are not properly managed, drug residues can remain in animal tissues and carry over into milk. This condition can represent a risk for consumer safety, because of the toxicity, allergic reactions, and the increase in antimicrobial resistance.[10] The screening methods largely used to detect these compounds are microbial assays. However, confirmatory methods are based on high-performance liquid chromatography (LC) methods coupled to different detectors. The β-lactam antibiotics can be detected by LC using diode array,[11,12]

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fluorescence,[13,14] and mass spectrometry detectors,[15,16] using different sample preparation methods.

Many procedures have been evaluated for the analyses of aminoglycosides using very different approaches,[17,18] but liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) with electrospray ionization (ESI) has been chosen as the best option for the confirmatory method.[19] This class of antibiotics nevertheless has a high polarity for the presence of amino and hydroxyl groups in the structure; moreover, they do not have chromophore or fluorophore groups and try to create connections with matrix proteins.[20,21] In the literature, there have been many studies of aminoglycosides using SPE extraction, preceded by organic solvents and/or an acid deproteinization of the matrix.[22] These techniques are time-consuming and may increase the costs for daily laboratory analyses.

Detailed penG/DHS pharmacokinetic studies have been conducted with cattle,[22,23] but information concerning antibiotic depletion is lacking for buffalo species. In this paper, a very simple in-house LC–MS/MS method has been used and validated for the simultaneous determination of dihydrostreptomycin and penicillin G residues in buffalo milk, modifying the extraction procedure described by Jank et al.[24] for the detection of β-lactams in bovine milk. This method has several advantages, such as a very simple sample preparation procedure (centrifugation steps at low temperatures), a high extraction capacity, good reproducibility, and it being environmentally friendly.

MATERIALS AND METHODS

Animal Studies. Eight lactating water buffaloes with postpartum disease were selected for this study from a farm located in the Campania Region. Each animal received three intramuscular injections of 30 mL of Repen (Fatro) containing a suspension of procaine penicillin G (200000 IU/mL) and dihydrostreptomycin sulfate (250 mg/mL) at 24 h intervals. Milk samples were collected twice daily up to milking 13 post-treatment (four milkings more compared to the bovine withdrawal period) and stored at −25 °C until they were analyzed. The selected buffaloes were not treated with any antimicrobial drug during the previous 2 months. Animals were under veterinary supervision during the first 3 days of the study (every 12 h) for possible adverse reactions and then daily until the end of the trial.

Materials and Reagents. Dihydrostreptomycin (DHS), as its sulfate salt, and penicillin G (penG) used as analytical standards (AS), as its procaine salt, were purchased from Sigma-Aldrich (Bornem, Belgium). HPLC grade water, acetonitrile, and C18 bulk were from Baker Mallinckrodt (Phillisburg, NJ). Formic acid was obtained from Merck (Darmstadt, Germany).

Sample Preparation. Milk samples were thawed at room temperature prior to analyses to avoid separation of aqueous and lipidic phases. Milk samples (2 ± 0.1 g) were transferred into 50 mL centrifuge tubes. The extraction process was based on a procedure described by Jank et al.[24] using only 4 mL of acetonitrile (ACN) and 100 mg of C18 bulk for each sample. Three centrifugation steps (10 min at 4000 rpm) at a low temperature (the first two at 4 °C and the last at 2 °C) were necessary to complete the purification. The final phase was characterized by the evaporation of the supernatant in a water bath (≤45 °C) under a N_2 stream. The milk extracts (250 μL), after an additional ultracentrifugation step (10 min at 13000 rpm) in 1.5 mL tubes, were placed in HPLC vials, and 1 μL was injected into LC-ESI-QTrap-MS system.

LC–MS and LC–MS/MS Analyses. A NexeraX2 UHPLC apparatus (Shimadzu, Kyoto, Japan), coupled with an ABSciex (Foster City, CA) API6500 Q-Trap instrument, working in a positive multiple-reaction monitoring (MRM) system was selected for the LC–MS/MS analyses. A standard solution (10 μg mL^{-1} in water into the source at a flow rate of 0.01 mL/min) was diluted with acetonitrile and water (50/50) for instrument optimization. All the analyses of the MS data were performed in positive ion mode.

Samples (1 μL) were loaded on a Luna Omega Polar column (Phenomenex) (1.6 μm Polar C18 100 A, 50 mm × 2.1 mm), and compounds were separated using a linear gradient from 2 to 25% acetonitrile (eluent B) in H_2O containing 0.1% formic acid (eluent A) over 7 min. The flow rate was 0.4 mL min^{-1}, and the injection volume was 1 μL for standards and milk extract samples; the total run time was 7 min.

All the parameters, fragmentation ions of each analyte, and dwell times are listed in Table 1. All data were processed using Analyst software (ABSciex).

Table 1. Optimized Mass Spectrometry Parameters for Dihydrostreptomycin (DHS) and Penicillin G (penG)

	precursor ion m/z	product ion m/z	DP[a] (eV)	CE[b] (eV)	CXP[c] (eV)	t_R[d] (min)
DHS	584.3	263	300	40	20	0.32
		246				
penG	335.1	160	30	13	10	3.97
		176				

[a]Declustering potential. [b]Collision energy. [c]Collision cell exit potential. [d]Retention time.

Calibration. A primary stock standard solution (1 mg mL^{-1}) for DHS and penG was obtained by adding the correct amount of standard in water. Solutions were stored frozen (−20 °C) and were stable for 1 month. Eight point working solutions of 1−1000 ng mL^{-1} DHS and 0.1−500 ng mL^{-1} penG were assembled by dilution of the respective stock solutions with water. The working standard solutions were stored at −20 °C in small plastic tubes to allow the use of only one cap during each extraction day and to avoid degradation. Negative buffalo milk samples were fortified, to obtain calibration curves, with penG and DHS standard solutions to give matrix final concentrations of 1, 10, 20, 50, 100, 200, 500, and 1000 μg kg^{-1} for DHS and 0.1, 1, 2, 4, 10, 50, 100, and 500 μg kg^{-1} for penG.

Validation Procedures. The LC–MS/MS method was validated following the requirements of Commission Decision 2002/657/EC.[25] All calibration curves were obtained by comparing the standard and the known concentration of each chemical by plotting the areas of the generated curves; all the analyses were performed in triplicate. The linearity, selectivity, within-laboratory repeatability (WLr), within-laboratory reproducibility (WLR), and recovery were determined. The validation precision [accuracy and relative standard deviation (RSD)] was determined using buffalo bulk milk (compliant for the dairy industry during official controls)[26] and fortifying samples at 0.5, 1, and 2 times the MRL levels of 4 and 200 μg kg^{-1} for penG and DHS, respectively.

RESULTS AND DISCUSSION

Extraction and Cleanup. ACN had been selected as an extraction solvent for its capacity to precipitate protein.[27] The centrifugations at low temperatures (4 and 2 °C) had been used to avoid compound degradation because, under refrigerator conditions, these antibiotics have good stability for short-term storage for a maximum of 3 days.[28]

Optimization of Chromatographic Separation. Chromatographic separation was performed using an HPLC technique already confirmed for β-lactams,[29] but to improve the resolution, it was necessary to optimize the extraction procedure and change the column. The gradient composition was the same, while methanol was replaced by ACN as the organic solvent in the mobile phase to improve the resolution, reproducibility, and peak shape. The initially used chromato-

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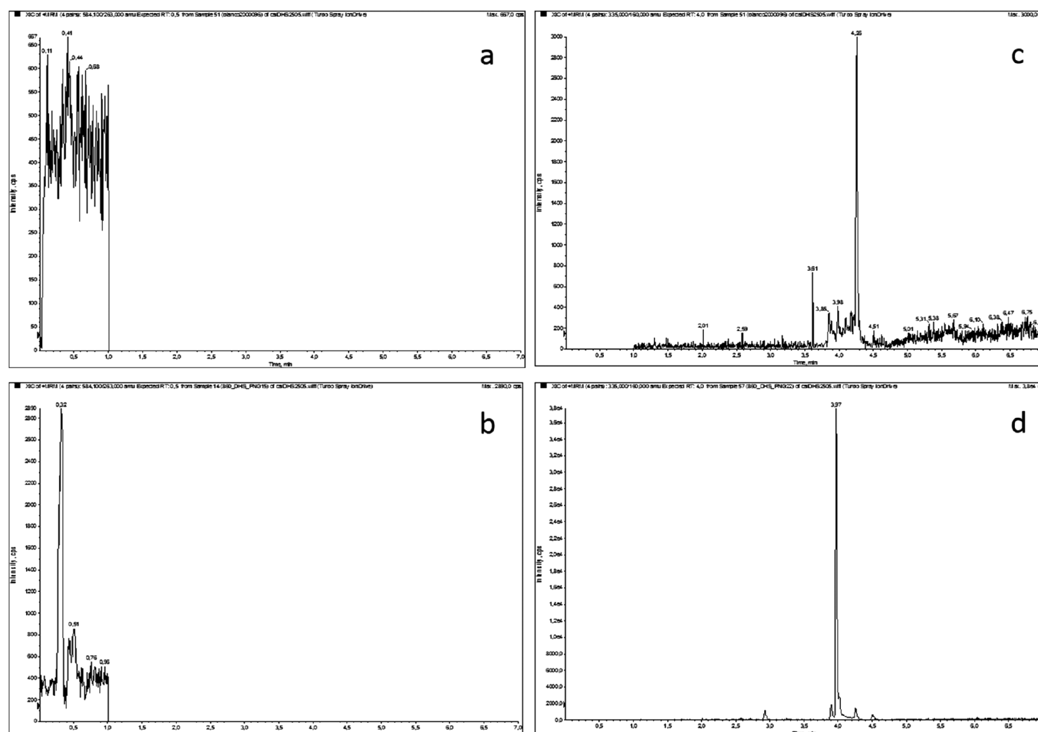

Figure 1. Extracted ion chromatograms of DHS [blank (a) and spiked sample (b)] and penG [blank (c) and spiked sample (d)].

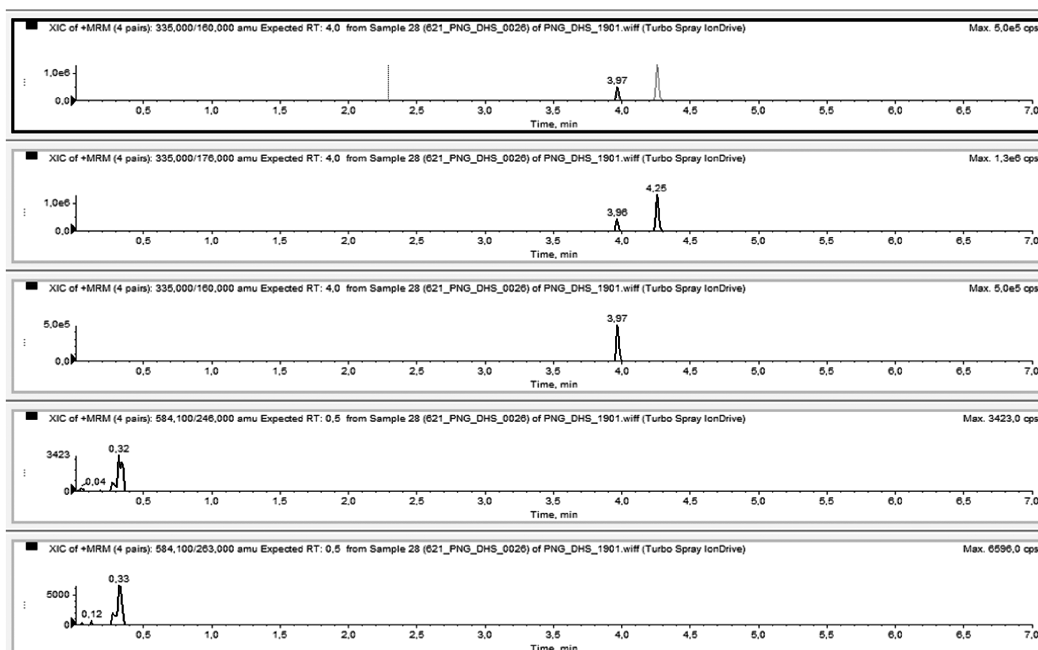

Figure 2. LC-ESI-QTrap-MS and LC-ESI-QTrap-MS/MS profiles of a milk extract in positive ion mode.

graphic column (Kinetex 2.6 μm F5 100 Å, LC Column, 100 mm × 2.1 mm) was also modified because that column has given insufficient results for DHS added to the method scope. DHS showed unsatisfactory responses (not retained), while the retention time for penG was 6.12 min. Thus, other columns were evaluated, and the Luna Omega column (1.6 μm Polar C18 100 A, 100 mm × 2.1 mm) was chosen because of the absence of stabilization time between subsequent injections and the shorter runs (<7 min).

Both antibiotics showed very good results in the optimized chromatographic column with retention times that ranged from 0.32 to 3.97 min. Extracted ion chromatograms for each compound are presented in Figure 1.

Optimization of Mass Spectrometry Parameters. Individual standard solutions (100 ng mL^{-1}) were injected with a syringe pump at a flow rate of 10 μL min^{-1}. The parent compounds and product ions were chosen for penG and DHS. Ion fragmentations were acquired using an ESI source at 300

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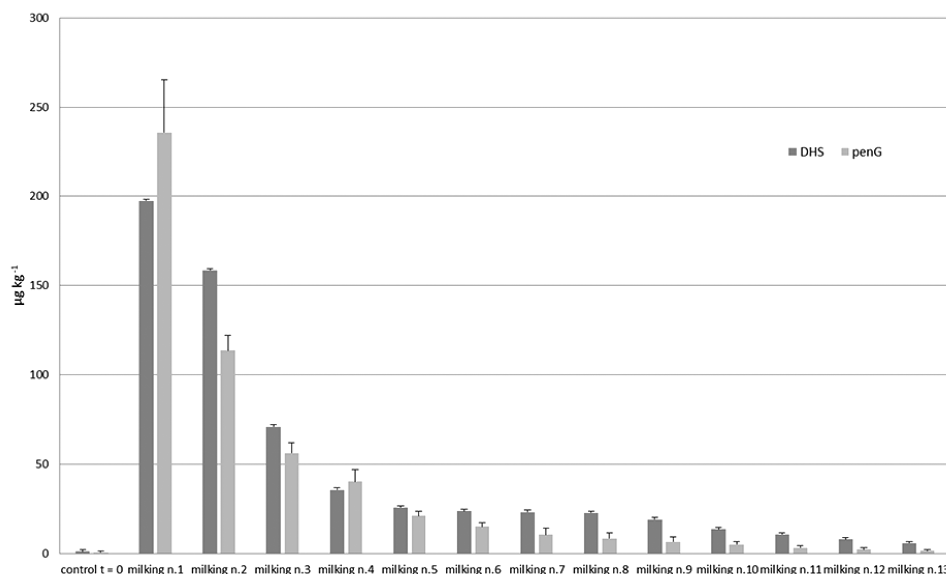

Figure 3. DHS and penG in the milk extracts of eight buffalo cows. Average values with standard deviations.

°C with an injection flow of 10 μL min^{-1}. Formic acid (0.1%) was selected to gain peak resolution and increase sensitivity.

Retention times and mass spectrometry data are listed in Table 1.

All analytes indicated a very good response with electrospray operating in positive mode. Two fragments are obtained for penG: the class-specific fragment m/z 160 and the compound-specific daughter ion that corresponds to $[M + H - 159]^+$.[30−32]

The common ions for DHS, at m/z 263 and 246, coincide with fragments not incorporating the variable R group and are the consequence of the loss of the lower and middle ring with cleavage between the glycosidic bond and the upper ring.[33]

Figure 2 shows the obtained results from the antibiotic detection by LC-ESI-QTrap-MS/MS in positive ionization mode.

The recognition of the two antibiotics was achieved via retention times and MS/MS data, investigation of mass spectral data (Mass Bank), and comparison of previous studies reported in the literature.

DHS showed a pseudomolecular ion at m/z 584.2889 that after ionization gave two principal daughter fragments at m/z 263 and 246. penG showed a pseudomolecular ion at m/z 335.1069 that after fragmentation provided two principal daughter ions at m/z 160 and 176.[34] LC-ESI-QTrap-MS analyses of milk extracts are reported in Figure 3. It always presents an intense signal, even in blank samples [t_R = 4.25 min (Figures 1 and 2)], and does not interfere with analyses of the compounds.

Method Validation. Correlation coefficients (r) from 0.992 to 0.998 were observed for all the tested compounds in the explored concentration range.

The limit of detection (LOD) and limit of quantification (LOQ) were detected using spiked samples at low concentrations and analysis of the signal-to-noise ratio. The extracted matrix curve was determined by spiking blank samples with defined standard dilutions. The concentration generating a peak whose intensity had a signal-to-noise ratio (S/N) of 3:1 was defined as the LOD instead of the LOQ (S/N) at 10:1.

The LODs for the two analytes were 0.01 ng mL^{-1} for penG and 0.1 ng mL^{-1} for DHS, whereas LOQs of 0.1 ng mL^{-1} for penG and 1 ng mL^{-1} for DHS were measured.

Three aliquots of the same sample were analyzed on the same day, and three others were examined during three consecutive days, one for each day to evaluate the reproducibility of the method. To evaluate the precision of the method, the percent relative standard deviation (RSD) was adopted.

The recovery within the same day ranged from 94 to 98%, which confirms the method has a good recovery and precision.

Results of the within-laboratory repeatability (WLr) and reproducibility (WLR) study at three fortification levels for penG (2, 4, and 8 μg kg^{-1}) and DHS (100, 200, and 400 μg kg^{-1}) are listed in Table 2. The accuracy for WLR ranged from 98 to 103%. The precision varied between 2.4 and 4.7%.

Table 2. Accuracy and Precision for Fortified Buffalo Milk under Within-Laboratory Repeatability (WLr) and Reproducibility (WLR) Conditions (analyses performed in triplicate)

	fortification (μg kg^{-1})	accuracy of WLr (%)	RSD of WLr (%)
DHS	2	99	4.7
	4	101	3.3
	8	99	2.4
penG	100	103	4.5
	200	98	2.4
	400	100	3.2

Persistence of Penicillin G and Dihydrostreptomycin in Buffalo Milk. The order of magnitude of the content of DHS and penG compounds is the same in different animals, with the higher total values shown in the first post-treatment milking.

The highest concentrations of penicillin G (275 μg kg^{-1}) and dihydrostreptomycin (220.5 μg kg^{-1}) were detected in the milk of the first milkings post-treatment, and the levels were below the maximum residue limit of 4 and 200 μg kg^{-1} (EU No 37/2010) in all treated buffaloes at milkings 12 and 2, respectively (Table 3).

In conclusion, a simple, fast, and low-cost method for simultaneous analyses of DHS and penG residues in milk of water buffalo was elaborated and validated. The sample

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Table 3. DHS and penG in the Milk Extracts of Eight Buffalo Cows (average values with standard deviations)

	DHS ± standard deviation (μg kg^{-1})	penG ± standard rdeviation (μg kg^{-1})
control, $t = 0$	1.23 ± 0.26	0.66 ± 0.65
milking 1	197.35 ± 16.64	235.88 ± 29.46
milking 2	158.64 ± 13.62	113.71 ± 8.50
milking 3	71.08 ± 10.51	56.26 ± 5.95
milking 4	35.72 ± 5.94	40.42 ± 6.72
milking 5	25.83 ± 8.86	21.18 ± 2.39
milking 6	23.82 ± 6.83	15.14 ± 2.02
milking 7	23.32 ± 6.51	10.70 ± 3.49
milking 8	22.82 ± 8.14	8.61 ± 2.91
milking 9	19.12 ± 6.24	6.70 ± 2.52
milking 10	13.71 ± 5.50	5.24 ± 1.50
milking 11	10.76 ± 6.62	3.29 ± 1.05
milking 12	7.90 ± 5.28	2.44 ± 0.86
milking 13	5.85 ± 3.71	1.51 ± 0.59

preparation method was easy, quick, and environmentally friendly when correlated with reported methods for analysis of β-lactams and aminoglycosides in food matrices, which normally requests the use of SPE and many cleanup steps. The use of a quantitative LC-ESI-QTrap-MS/MS method indicates a sensitive and selective instrument for studying antibiotic residues in milk without needing several purification steps.

Data from this study show that the presented method is a capable and reliable method for quantifying dihydrostreptomycin and penicillin G in milk and is useful in routine residue analyses for confirmatory purposes as required by Decision 2002/657/EC.

Finally, the results of this study deserve careful attention as the levels of penicillin G in buffalo milk were found to be above the MRL of 4 μg kg^{-1} at nine milkings post-treatment (withdrawal time set for bovine species) and need at least three more milkings to decrease to safety levels and to be compliant for the dairy industry.

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Notes

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REFERENCES

(1) European Commission.. *Off. J. Eur. Communities: Legis.* **2008**, *L31*, 31.

(2) Fagiolo, A.; Lai, O. Mastitis in buffalo. *Ital. J. Anim. Sci.* **2007**, *6* (sup2), 200−206.

(3) European Commission.. Commission Decision 2010/37/EC of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. *Off. J. Eur. Communities: Legis.* **2010**, *L15*, 1−69.

(4) Oates, J. A.; Wood, A. J. J.; Donowitz, G. R.; Mandell, G. L. Beta-lactam antibiotics. *N. Engl. J. Med.* **1988**, *318* (8), 490−500.

(5) Freeman, T. R. Milk quality. *J. Milk Food Technol.* **1953**, *15*, 162−166.

(6) Kindred, T. P.; Hubbert, W. T. Residue prevention strategies in the United States. *J. Am. Vet. Med. Assoc.* **1993**, *202*, 46−49.

(7) EMEA. Penicillins, summary report (3) committee for veterinary medicinal products. EMEA/Revision 1, 2008.

(8) EMEA. Dihydrostreptomycin (extrapolation to all ruminants) EMEA/CVMP/211249/2005-Final Summary Report, 2005.

(9) Whittem, T.; Hanlon, D. Dihydrostreptomycin or streptomycin in combination with penicillin G in dairy cattle therapeutics: A review and re-analysis of published data Part 1: Clinical pharmacology. *N. Z. Vet. J.* **1997**, *45*, 178−184.

(10) Whittern, T.; Hanlon, D. Dihydrostreptomycin or streptomycin in combination with penicillin G in dairy cattle therapeutics: A review and re-analysis of published data Part 2: Resistance and residues. *N. Z. Vet. J.* **1997**, *45*, 223−229.

(11) Bailon-Perez, M. I.; Garcia-Campana, A. M.; Del Olmo-Iruela, M.; Gamiz-Gracia, L.; Cruces-Blanco, C. Trace determination of 10 beta-lactam antibiotics in environmental and food samples by capillary liquid chromatography. *J. Chromatogr. A* **2009**, *1216*, 8355−8361.

(12) Cámara, M.; Gallego-Picó, A.; Garcinuño, R.; Fernández-Hernando, P.; Durand-Alegría, J.; Sánchez, P. An HPLC-DAD method for the simultaneous determination of nine β-lactam antibiotics in ewe milk. *Food Chem.* **2013**, *141*, 829−834.

(13) Benito-Peña, E.; Moreno-Bondi, M. C.; Orellana, G.; Maquieira, Á.; van Amerongen, A. Development of a Novel and Automated Fluorescent Immunoassay for the Analysis of β-Lactam Antibiotics. *J. Agric. Food Chem.* **2005**, *53*, 6635−6642.

(14) Kantiani, L.; Farré, M.; Barceló, D. Analytical methodologies for the detection of β-lactam antibiotics in milk and feed samples. *TrAC, Trends Anal. Chem.* **2009**, *28* (6), 729−744.

(15) Liu, C.; Wang, H.; Jiang, Y.; Du, Z. Rapid and simultaneous determination of amoxicillin, penicillin G, and their major metabolites in bovine milk by ultra-high-performance liquid chromatography−tandem mass spectrometry. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2011**, *879* (7), 533−540.

(16) Stead, D. A. Current methodologies for the analysis of aminoglycosides. *J. Chromatogr., Biomed. Appl.* **2000**, *747* (1−2), 69−93.

(17) McGlinchey, T. A.; Rafter, P. A.; Regan, F.; McMahon, G. P. A review of analytical methods for the determination of aminoglycoside and macrolide residues in food matrices. *Anal. Chim. Acta* **2008**, *624*, 1−15.

(18) Cherlet, M.; De Baere, S.; De Backer, P. Quantitative determination of dihydrostreptomycin in bovine tissues and milk by liquid chromatography electrospray ionization-tandem mass spectrometry. *J. Mass Spectrom.* **2007**, *42*, 647−656.

(19) Arsand, J. B.; Jank, L.; Martins, M. T.; Hoff, R. B.; Barreto, F.; Pizzolato, T. M.; Sirtori, C. Determination of aminoglycoside residues in milk and muscle based on a simple and fast extraction procedure followed by liquid chromatography coupled to tandem mass spectrometry and time of flight mass spectrometry. *Talanta* **2016**, *154*, 38−45.

(20) Moreno-González, D.; Hamed, A. M.; García-Campaña, A. M.; Gámiz-Gracia, L. Evaluation of hydrophilic interaction liquid chromatography−tandem mass spectrometry and extraction with molecularly imprinted polymers for determination of aminoglycosides in milk and milk-based functional foods. *Talanta* **2017**, *171*, 74−80.

(21) Kaufmann, A.; Butcher, P.; Maden, K. Determination of aminoglycoside residues by liquid chromatography and tandem mass

spectrometry in a variety of matrices. *Anal. Chim. Acta* **2012**, *711*, 46−53.

(22) Vilim, A.; Larocque, L.; Macintosh, A. Depletion of brilliant blue FCF, penicillin G and dihydrostreptomycin in milk from treated cows with experimentally induced mastitis. *J. Food Prot.* **1980**, *43* (5), 356−359.

(23) Dubreuil, P.; Daigneault, J.; Couture, Y.; Guay, P.; Landry, D. Penicillin concentrations in serum, milk, and urine following intramuscular and subcutaneous administration of increasing doses of procaine penicillin G in lactating dairy cows. *Can. J. Vet. Res.* **2001**, *65* (3), 173−180.

(24) Jank, L.; Martins, M. T.; Arsand, J. B.; Hoff, R. B.; Barreto, F.; Pizzolato, T. M. High-throughput method for the determination of residues of β-lactam antibiotics in bovine milk by LC-MS/MS. *Food Addit. Contam., Part A* **2015**, *32* (12), 1992−2001.

(25) Commission decision 2002/657/EC of 12 August 2002. Implementing council directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off. J. Eur. Communities: Legis.* **2000**, *L221*, 8−36.

(26) Heitzman, R. J., Ed. *Veterinary Drug Residues, Residues in Food Producing Animals and Their Products: Reference Materials and Methods*; Report Eur.15127-EN; Publications Office of the European Union, 1994.

(27) Polson, C.; Sarkar, P.; Incledon, B.; Raguvaran, V.; Grant, R. Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2003**, *785* (2), 263−275.

(28) Riediker, S.; Rytz, A.; Stadler, R. H. Cold-temperature stability of five β-lactam antibiotics in bovine milk and milk extracts prepared for liquid chromatography−electrospray ionization tandem mass spectrometry analysis. *J. Chromatogr A* **2004**, *1054* (1−2), 359−363.

(29) Jank, L.; Hoff, R. B.; Tarouco, P. C.; Barreto, F.; Pizzolato, T. M. β- lactam antibiotics residues analysis in bovine milk by LC-ESI-MS/MS: a simple and fast liquid-liquid extraction method. *Food Addit. Contam., Part A* **2012**, *29*, 497−507.

(30) Straub, R. F.; Voyksner, R. D. Determination of penicillin G, ampicillin, amoxicillin, cloxacillin and cephapirin by high-performance liquid chromatography-electrospray mass spectrometry. *J. Chromatogr. A* **1993**, *647* (1), 167−181.

(31) Tyczkowska, K. L.; Voyksner, R. D.; Straub, R. F.; Aronson, A. L. Simultaneous multiresidue analysis of beta-lactam antibiotics in bovine milk by liquid chromatography with ultraviolet detection and confirmation by electrospray mass spectrometry. *J. AOAC Int.* **1994**, *77* (5), 1122−1131.

(32) Lara, F. J.; del Olmo-Iruela, M.; Cruces-Blanco, C.; Quesada-Molina, C.; García-Campaña, A. M. Advances in the determination of β-lactam antibiotics by liquid chromatography. *TrAC, Trends Anal. Chem.* **2012**, *38*, 52−66.

(33) Goolsby, B. J.; Brodbelt, J. S. Analysis of protonated and alkali metal cationized aminoglycoside antibiotics by collision-activated dissociation and infrared multi-photon dissociation in the quadrupole ion trap. *J. Mass Spectrom.* **2000**, *35* (8), 1011−1024.

(34) Gaugain-Juhel, M.; Delépine, B.; Gautier, S.; Fourmond, M. P.; Gaudin, V.; Hurtaud-Pessel, D.; Verdon, E.; Sanders, P. Validation of a liquid chromatography-tandem mass spectrometry screening method to monitor 58 antibiotics in milk: qualitative approach. *Food Addit. Contam., Part A* **2009**, *26* (11), 1459−1471.

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