Development and Validation of an LC–APCI-MS–MS Analytical Method for the Determination of Streptomycin and Dihydrostreptomycin Residues in Milk

Renata Cabrera de Oliveira[1,*], Jonas Augusto Rizzato Paschoal[1], Marcela Sismotto[1], Flávia Pereira da Silva Airoldi[2], and **Felix Guillermo Reyes Reyes[1]**

[1]School of Food Engineering, Department of Food Science, University of Campinas, P.O. Box 6121, 13084-971 Campinas, SP, Brazil; [2]Institute of Chemistry, Department of Organic Chemistry, University of Campinas , P.O. Box 6154, 13084-971 Campinas, SP, Brazil

Abstract

An analytical method for the quantification and identity confirmation of streptomycin and dihydrostreptomycin residues in pasteurized milk using liquid chromatography (LC)–atmospheric pressure chemical ionization (APCI)-tandem mass spectrometry (MS–MS) was developed and validated. Method validation was performed according to the recommendations of the international agencies European Community and IUPAC, and the following parameters were evaluated: analytical curve, linearity, sensitivity, precision (intra- and inter-day repeatability), accuracy, and the limit of detection (LOD) and limit of quantification (LOQ). Simple sample preparation was followed by the LC–APCI-MS–MS analysis. The method presented adequate linearity with correlation coefficients above 0.99 for both analytes in the dynamic range of 50–400 µg/kg, and average accuracies between 84–110%. The LOD and LOQ were, respectively, 25 µg/kg and 50 µg/kg for both analytes. Method selectivity was verified by the absence of interfering peaks in the retention regions of the analytes and the internal standard when a blank sample was tested. The results qualified the method for the quantification and confirmation of the analytes in milk at concentrations inferior to the established maximum residue limits (200 µg/kg).

Introduction

Streptomycin is an aminoglycoside produced by some *Streptomyces griseus* strains, and dihydrostreptomycin is the product of its catalytic hydrogenation. Aminoglycosides are protein synthesis inhibitors and, in spite of their toxicity, are widely used in veterinary medicine for the treatment of aerobic gram-negative bacterial infections such as clinical and sub-clinical mastitis in cattle. It is normally used in association with penicillin and tetracycline. All aminoglycoside antibiotics present the same toxicity spectrum (1), and the most evident adverse effects are nefrotoxicity and ototoxicity (2).

The incorrect usage of antimicrobial agents in cattle or the nonobservation of the withdrawal time after treatment may result in the presence of these substance residues in the milk, which increases the risk to consumer health due to their toxicity and possible allergic reactions in sensitive people. In addition, such residues promote bacterial resistance, as previously reported in the literature (3–5) and cause negative effects in the dairy industry, totally or partially inhibiting the cultures employed in cheese and yogurt production (6,7).

The use of streptomycin is allowed in the European Union (EU), and the maximum residue limit (MLR) for streptomycin together with dihydrostreptomycin in milk is 200 µg/kg (8). The same MRL was adopted in the MERCOSUL Resolution GMC nº 54/2000 (9).

In 1986, the Brazilian Ministry of Agriculture (MAPA) established the National Plan for the Control of Residues in Edible Products of Animal Origin (10,11). Furthermore, in 2003 the Brazilian Agency for Sanitary Surveillance (ANVISA) created the National Plan for the Analysis of Veterinary Drug Residues in Food Exposed to Consumption, which describes the control of veterinary drug residues in edible products of animal origin for human consumption (12). Aminoglycosides are highlighted in these programs, both streptomycin and dihydrostreptomycin being indicated as substances to be evaluated in milk.

The control of antimicrobial agent residues in edible products of animal origin requires the development of selective analytical methods that present adequate detectability. Liquid chromatography (LC) attached to tandem mass spectrometry (MS–MS) is recognized as a very selective technique and can be employed to analyze aminoglycosides in food products. It is possible to quantify and confirm the identity of these substances at very low concentrations. LC–MS–MS methods for the determination of streptomycin and dihydrostreptomycin with limits of quantification (LOQ) lower than 20 µg/kg have been reported (13–15). Nevertheless, the sample preparation is normally time-consuming, involving deproteinization and solid-phase extraction steps. In addition, the majority of the analytical methods involving LC–MS–MS employ triple quadrupole (QQQ) analyzers. Nevertheless, the ability of time-of-flight (Q-ToF) analyzers to provide high quality results due to their exact measurement of the analyte mass, make them instruments of great interest to take part in confirmatory analysis methods with legislative perspectives, such as those required by the EU (16)

* Author to whom correspondence should be sent: email renata29269@hotmail.com.

The aim of the present work was to develop and validate an analytical method with a simple and fast sample preparation step and to identify and quantify streptomycin and dihydrostreptomycin residues in pasteurized milk at concentration levels below the MRL (17,18), thus being adequate for adoption in veterinary drug residue monitoring programs.

Material and Methods

Samples

Pasteurized milk samples were acquired from local markets in the city of Campinas, SP, Brazil. These samples were separated into aliquots and stored at 5 ± 2°C before being analyzed.

Solvents and reagents

Trichloroacetic acid (TCA) was purchased from Acros Organics (Morris Plains, NJ) and heptafluorobutyric acid (HFBA) from Sigma-Aldrich (Munich, Germany), both of analytical-grade. HPLC-grade acetonitrile (ACN) was obtained from J.T. Baker (Phillipsburg, NJ). The Milli-Q Plus System (Millipore, Billerica, MA) was used to purify the distilled water.

Polyvinylidene difluoride (PVDF) hydrophilic and polytetrafluorethylene (PTFE) membranes (Millipore), both with 0.22-µm pore sizes, were used to filter the aqueous and organic mobile phase solutions, respectively. PVDF hydrophilic syringe filters, 33-mm diameter, 0.22-µm pore size, were used to filter the sample extracts before injection.

Table I. Elution Gradient for the Chromatographic Analysis of STP and DHSTP

Time (min)	Solvent A (%)	Solvent B (%)
0	75	25
3	75	25
10	64	36
15	75	25

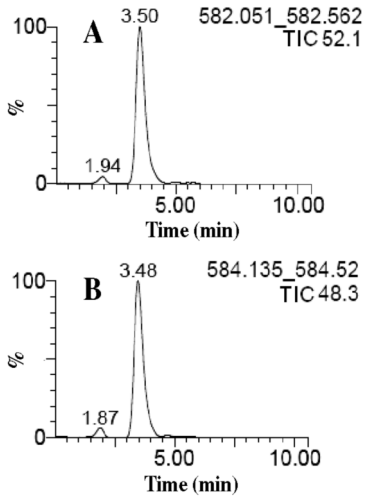

Figure 1. STP 400 µg/kg, (A); and DHSTP 400 µg/kg, (B); in standard solution extracted mass chromatograms. ESI ionization: Note the total ion count (TIC).

Standard solutions

Analytical-grade streptomycin sulfate (STP) (99.9%), dihydrostreptomycin sesquisulfate (DHSTP) (98.0%), and tobramycin (TOB) (99.9%) were obtained from Sigma-Aldrich.

Stock solutions of 1000 mg/kg were separately prepared by dilution of STP or DHSTP in solution of water–methanol (1:1, v/v) and stored in tightly closed amber vessels at 5ºC for a maximum period of six months (19).

Working solutions containing 12.5, 25.0, 50.0, 75.0, 100.0, 125.0, 150.0, and 200.0 mg/kg of STP and DHSTP were prepared weekly by dilution of the stock solutions in solution of water–methanol (1:1, v/v) and stored in tightly closed amber vessels at 5ºC. These solutions were used to construct calibration curves for both analytes by spiking blank milk samples at five concentration levels (50.0, 100.0, 200.0, 300.0, and 400.0 µg/kg). The internal standard (TOB) was also spiked in the blank milk samples at a concentration of 2.5 mg/kg. This high concentration was chosen because the ionization conditions established for analytes are not ideal for the internal standard. The analytical curves were constructed on the same day as the spiked samples were prepared.

Instruments

A Waters Alliance 2695 HPLC system (Waters, Milford, MA), composed of quaternary pumping and automatic injection systems, was employed for the chromatographic separation. The HPLC system was interfaced with electrospray (ESI) or atmospheric pressure chemical (APCI) ionization sources, followed by the hybrid Q-ToF Micro (Micromass, Hertfordshire,UK) mass spectrometer, composed of a quadrupole time of flight (Q-ToF) analyzers with a hexapole collision cell between them. The data were acquired using the Masslynx software (Micromass).

A model T14 ultrasound bath (Thornton, São Paulo, Brazil) was used for the degasification of the mobile phase solutions. A model BL 2105 analytical balance (Sartorius, Goettingen, Germany) was used to weigh the reagents and standards. A model 204-NR centrifuge (Fanem, São Paulo, Brazil) and a model MA120 rotary evaporator (Marconi, Brazil) were employed for sample preparation.

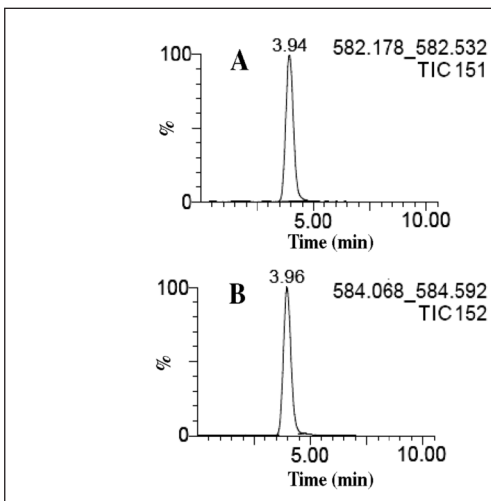

Figure 2. STP 400 µg/kg, (A); and DHSTP 400 µg/kg, (B); in standard solution extracted mass chromatograms. APCI ionization: Note the total ion count (TIC).

Sample preparation

Two milliliters aliquots of the blank or spiked milk samples were placed in 12 mL centrifuge tubes, and 0.4 mL of TCA solution (10% v/v) was added. The samples were homogenized in a vortex mixer for 3 min, and then centrifuged at 3600 g for 10 min. The supernatants were filtered using 0.22 µm pore size syringe filters and injected into the HPLC system.

For optimization of the sample preparation procedure, several concentrations of aqueous and methanolic TCA solutions were tested. Deproteinization with acetonitrile instead of TCA was also tried, and additional C_{18} solid phase extraction and partitioning with some organic solvent cleaning steps were also evaluated. None of these improved the extraction efficiency, which was expressed as the percentage of the expected concentration (obtained with external calibration curves, in which the analytes were diluted in water–methanol, 1:1 v/v), after analysis of the spiked samples using the method developed.

LC–MS–MS (QToF) Conditions

For the chromatographic separation, a C_{18} XTerra MS (150 × 2.1 mm, 5 µm, Waters, Milford, MA) reversed-phase column was used at 30°C. Aqueous 10 $mmol^{-1}$ HFBA (solvent A) and 10 $mmol^{-1}$ HFBA in acetonitrile (solvent B) constituted the mobile phase using the gradient shown in Table I at a constant flow rate of 200 µL/min.

Both positive-mode ESI and APCI ionization sources were evaluated. An optimized APCI signal was achieved under the following tuning conditions: 0.5 µA of corona current, sample cone at 50 V, extraction cone at 2 V, source temperature of 150°C, probe and dessolvation temperatures of 650°C and 150°C, respectively, ion and collision energies of 2 V and 7 V, respectively, and cone and dessolvation gas flows of 0 and 100 l/h, respectively. For ESI, the best signal conditions were: probe voltage of 2700 V, sample cone at 65 V, extraction cone at 0 V, source and dessolvation temperatures of 100°C and 250°C, respectively, ion and collision energies of 1 V and 20 V, respectively, and cone and dessolvation gas flows of 50 and 500 L/h, respectively. For both ionization sources, detection was performed monitoring the protonated $(M-H)^+$ molecules of the analytes (STP and DHSTP) and the sodium adduct $(M-Na)^+$ of the internal standard (TOB) by MS–MS in the acquisition mode, which presented the respective *m/z* ratios: 582.3, 584.3, and 490.1.

The quantitative analysis was performed using the analytical curves of the spiked samples with the analyte and internal standard concentrations mentioned previously. Confirmation was achieved under the same APCI ionization conditions with the collision energy at 28 V for STP, which was the best tuning for the simultaneous detection of the fragment ions with *m/z* ratios of 263.1 and 407.2 (besides the protonated molecule), and 25 V for DHSTP, which was the best tuning for the simultaneous detection of the fragment ions with *m/z* ratios of 263.1 and 409.2 (besides the protonated molecule).

Table II. Matrix effects for STP and DHSTP Obtained Using ESI and APCI Ionization for Spiked Milk Samples

Ionization source	Matrix effect (%)	
	STP	DHSTP
ESI	69	52
APCI	25	21

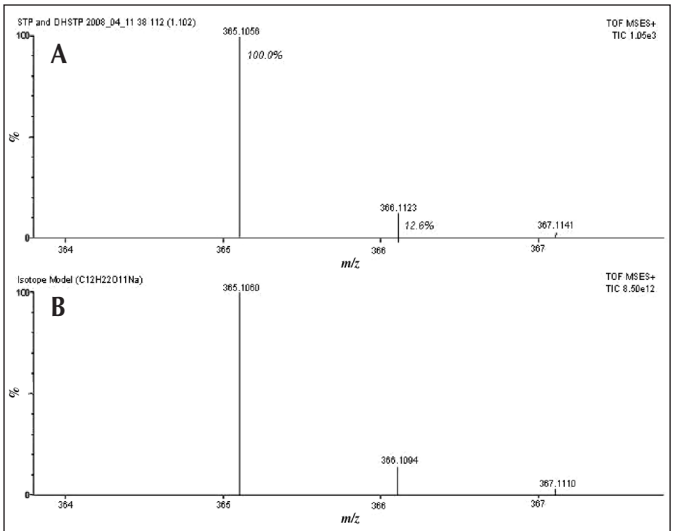

Figure 3. Isotopic profile of the suppressing interfering ion (*m/z* = 365), (A); obtained from the LC–ESI-MS–MS analysis of a blank milk sample after the extraction procedure and the isotope model of the lactose sodium adduct ion, simulated by Masslynx, (B).

Method validation

Method validation was performed according to major international regulatory agencies, such as the European Community (16) and the International Union of Pure and Applied Chemistry (IUPAC) (20). The selectivity, analytical curve, linearity, sensitivity, precision (intra- and inter-day repeatability), accuracy, matrix effect, and the LOD and LOQ of the parameters were eval-

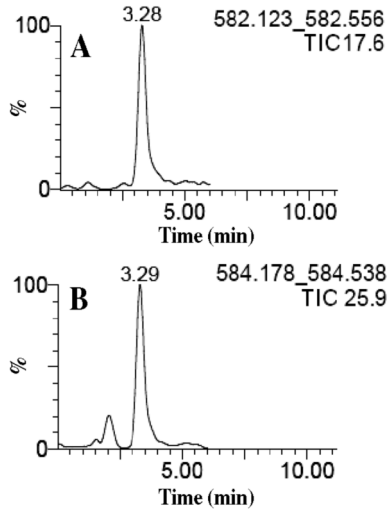

Figure 4. Spiked milk sample chromatograms–STP 400 µg/kg (A); and DHSTP 400 µg/kg (B). ESI ionization

uated using spiked samples at several concentration levels.

For both analytes, spiked samples at five concentration levels in a range that included the MRL were used to construct the analytical curves and determine the linearity and sensitivity. The data was analyzed using the quantification software Quanlynx (Micromass).

Method selectivity was evaluated by verifying the absence of interfering substances that could possibly compromise the identification or quantification of the analytes or the internal standard in five blank samples.

The precision was determined in two steps: (i) intra-day repeatability: from the variation in results of four replicates analyzed on the same day by the same analyst and using the same instrument; and (ii) inter-day repeatability: from the variation in results of three analyses performed on three different days (n = 3) by the same analyst and using the same instrument. For this purpose, blank samples were spiked with both analytes at 50, 100, 200, and 300 µg/kg (equivalent to 0.25, 0.5, 1.0, and 1.5 times the MRL, respectively).

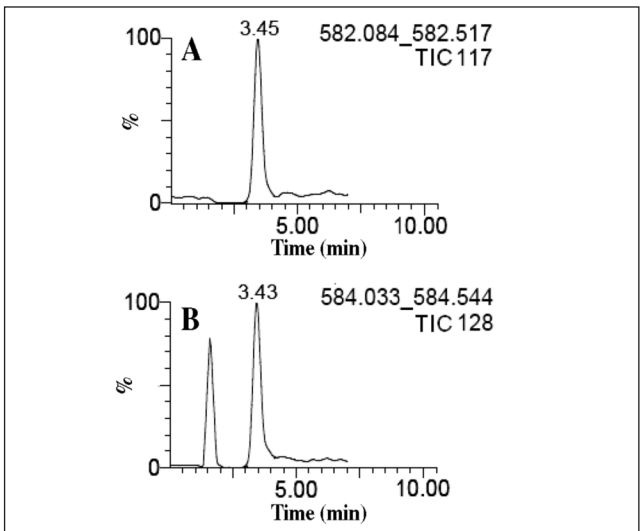

Figure 5. Spiked milk sample chromatograms–STP 400 µg/kg (A); and DHSTP 400 µg/kg (B). APCI ionization

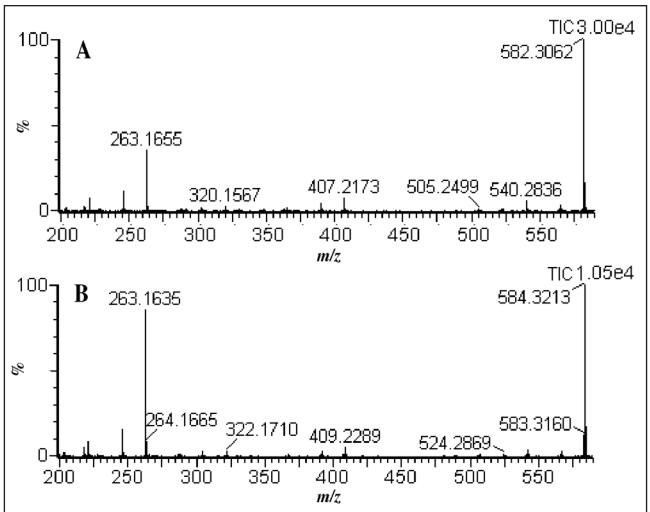

Figure 6. STP (A) and DHSTP (B) fragmentation spectra obtained using LC–APCI-MS–MS Q-ToF.

Accuracy was evaluated by recovery tests, analyzing samples spiked with the same concentration levels used in the precision tests. The results were expressed as the percentage of the expected concentrations (the amounts initially added) of the analytes.

Blank pasteurized milk samples were submitted to the established sample preparation procedure to evaluate the matrix effect. After the extraction step, the supernatant was evaporated to dryness at 45°C, and resuspended in 1.0 mL of a solution containing the analytes (200 µg/kg) and the internal standard (2.5 mg/kg) in water–methanol (1:1, v/v) solution. The results were obtained by comparison with those obtained by internal calibration, with analytical standards in pure solvent, and expressed as the percentage difference from the expected concentrations.

The LOD and LOQ were obtained by analyzing samples spiked at decreasing concentration levels and evaluating the precision and accuracy of the results. Each analyte LOD was expressed as the min-

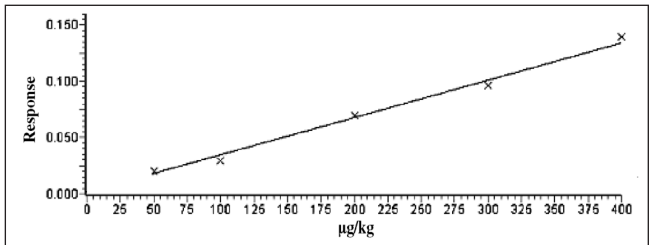

Figure 7. Analytical curve for STP. Dynamic range: 50–400 µg/kg. Correlation coefficient (r) = 0.99.

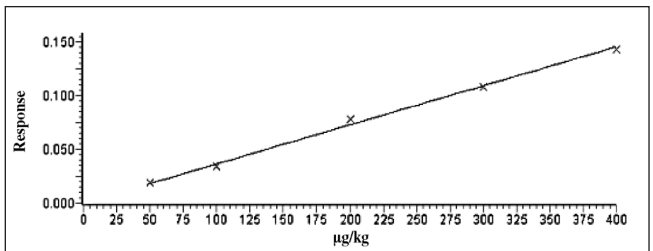

Figure 8. Analytical curve for DHSTP. Dynamic range: 50–400 µg/kg. Correlation coefficient (r) = 0.99.

Table III. Accuracy and Precision Results Obtained in the Intra- and Inter-day Assays

STP and DHSTP Concentrations in milk	Accuracy (Recovery %) STP	Accuracy (Recovery %) DHSTP	Precision (RSD %) STP	Precision (RSD %) DHSTP
Intra-day repeatability				
50 µg/kg (n = 4)	109.8	107.6	15.6	12.9
100 µg/kg (n = 4)	95.3	90.0	9.1	11.5
200 µg/kg (n = 4)	100.3	103.7	3.7	10.6
300 µg/kg (n = 4)	90.0	84.2	10.5	14.3
Inter-day repeatability (3 days)				
50 µg/kg (n = 3)	105.9	108.6	19.2	19.8
100 µg/kg (n = 3)	110.0	103.0	14.3	14.5
200 µg/kg (n = 3)	100.8	102.5	7.3	4.1
300 µg/kg (n = 3)	108.3	105.6	11.0	8.9

imum detectable concentration in the spiked sample. LOQs were expressed as the minimum concentrations that could be quantified in the spiked samples with adequate precision and accuracy.

Results and Discussion

Sample preparation was carried out by acid precipitation of the milk proteins in which the analytes were extracted with adequate efficiency (71–104% for STP, 78–107% for DHSTP). No complementary SPE or partition cleaning steps being needed. The analyte and internal standard recoveries were zero for extraction by deproteinization with acetonitrile.

The APCI source presented signal intensities for the analytes in the mass spectra approximately three times higher than the ESI source, consequently promoting better sensitivity in the chromatograms (Figures 1 and 2) and was therefore chosen as the ionization source for the method.

The tests with the ESI source showed sharp ionic suppression of the analyte signal by the matrix. This effect was evaluated and quantified as 69% for STP and 52% for DHSTP (Table II). It was observed that the suppression effect was due to an ion with a *m/z* ratio of 365, which appeared at the same retention time as the analytes. The collision energy was increased in order to evaluate the fragmentation pattern of this substance; however, even after a significant increment in the collision energy (from 7 to 35 V) no fragment ion was observed, which is a characteristic behavior of sodium adduct ions. Considering that lactose is the main sugar in the chemical composition of milk (2–8% of the milk by weight) and that its molar mass with the addition of the sodium molar mass is exactly 365 g mol^{-1}, the hypothesis of the suppression being caused by lactose was investigated. Indeed, the isotopic profile of the interfering ion coincided with the isotopic profile of lactose $(M\text{-}Na)^+$ as simulated by the Masslynx software (Figure 3) with a 1.1 ppm error in mass accuracy. It showed ~12% of ions with an *m/z* ratio of 366, which is an indication of 12 carbons in its molecular composition (equivalent to two hexoses). All these factors corroborated the theory that the suppression really was caused by lactose.

The identification of the interfering ion was necessary to establish new alternatives for the extraction procedure that could possibly separate it from the analytes. When the APCI source was tested, the matrix effect showed an important improvement (Table II): indication of less ionic suppression of the analyte signals using this ionization source (Figures 4 and 5).

Figure 6 shows the fragmentation spectra for STP and DHSTP obtained using APCI ionization under the ideal fragmentation conditions. Besides the protonated analyte (*m/z* ratio = 582.3), fragment ions with *m/z* ratios 263.1 and 407.2 were used to confirm the STP identity. For the confirmation of DHSTP, the protonated analyte and two fragment ions were observed with *m/z* ratios of 584.3, 263.1, and 409.2 respectively.

According to European Community regulation (2002), confirmatory methods for organic residues in food matrices must provide molecular structural information, and MS detection is recognizably capable of that. MS techniques showing less resolution, such as the triple quadrupole one (QQQ), may satisfy the confirmation criteria based on established systems of identification points, which are related to the masses of the fragment ions

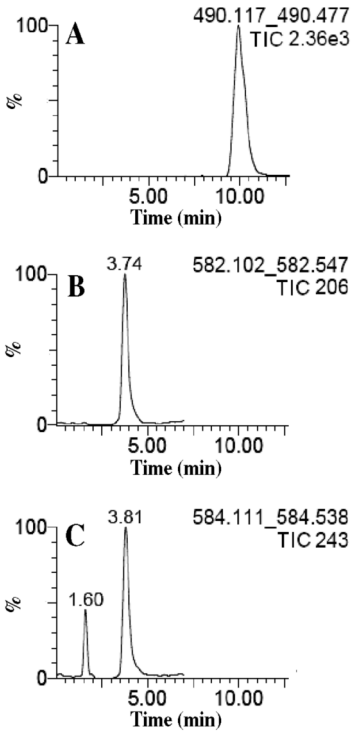

Figure 9. Extracted mass chromatograms of milk samples spiked with TOB 2.5 mg/kg, STP 200 µg/kg and DHSTP 200 µ/kg: *m/z* ratios of 490.1 (A); 582.3, (B); and 584.3 (C); respectively.

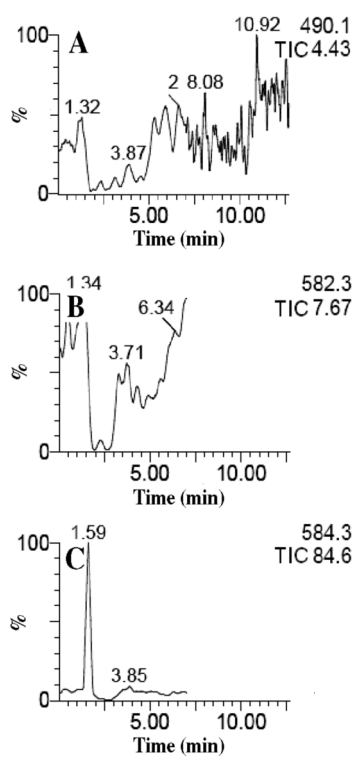

Figure 10. Extracted mass chromatograms of blank samples: *m/z* ratios of 490.1 (A); 582.3 (B); and 584.3 (C).

from the analytes (21,22). For these techniques, a minimum of three ions is necessary for the confirmation of permitted substances and four fragment ions for the banished compounds (16). The use of QToF systems may reach the minimum points for identification by the sum of just two ions: the (de)protonated or the molecular ion and one fragment ion (23).

The quantitative analysis presented adequate linearity with correlation coefficients (*r*) higher than 0.99 for both analytes in the dynamic range of 50–400 µg/kg. Calibration curves (Figures 7 and 8) were constructed in the matrix to guarantee reliability of the results.

The results for accuracy and precision (Table III) were in accordance with the EU decision nº 2002/657 (16). The average results for recovery were between 84–110%. Precision was demonstrated from the relative standard deviations in the intra- and inter- day repeatability tests, which were lower than 15% for concentrations above 100 µg/kg, and 20% for concentrations below this value.

The LOD and LOQ obtained 25 µg/kg and 50 µg/kg, respectively, were way below the MRL defined for both STP and DHSTP in milk (200 µg/kg).

Method selectivity was verified by the absence of interfering peaks in the blank sample extracted. Mass chromatograms in the areas around the retention times of the analytes are shown in Figures 9 and 10.

Conclusions

The sample preparation procedure developed was simple, which is crucial for a faster analysis that is less susceptible to analyte losses and results in adequate extraction efficiency (71–104% for STP, 78–107% for DHSTP).

The method linearity was adequate with correlation coefficients above 0.99 for both analytes in the linear range from 50–400 µg/kg. The precision was verified from the values obtained for the RSDs in the intra- and inter-day repeatability assays, which were lower than 15% for concentrations above 100 µg/kg and lower than 20% for concentrations below this level. These results are in accordance with the EU recommendations (2002). The LOD and LOQ were 25 µg/kg and 50 µg/kg, respectively. The method presented accuracy values in the range from 84–110%.

The method allowed for the monitoring of the protonated molecules and two fragment ions for each analyte, which also achieves the purpose of confirming the identity of the analytes according to EU (2002) recommendations.

The validation results showed that the method developed was adequate for the quantification and confirmation of streptomycin and dihydrostreptomycin residues in pasteurized milk at concentrations lower than the established MRL for these substances (200 µg/kg).

Acknowledgements

The authors gratefully acknowledge the financial support from CNPq, CAPES, FAPESP, and Professor Hilary Castle de Menezes for language assistance.

References

1. H.F. Chambers. Antimicrobianos: Os Aminoglicosídeos. In: A. G. Gilman, J.G. Hardman, L.E. Limbird. As Bases Farmacológicas da Terapêutica 10ed. McGraw-Hill Rio de Janeiro, Brasil, 2001, pp. 913–928.
2. FAO/WHO – Residues of Some Veterinary Drugs in Animals and Foods, Monographs prepared by the 52nd Meeting of the Joint FAO/WHO Expert Committee on Food Additives. Rome: JECFA, 1999, p. 133.
3. M.R. Baccaro, A.M. Moreno, A. Corrêa, A.J.P. Ferreira, and F.F. Calderaro. Resistência antimicrobiana de amostras de Escherichia coli isoladas de fezes de leitões com diarréia. *Arq Inst. Biol.* **69(2):** 15–18 (2002).
4. A.R. Ribeiro, A. Kellermann, L.R. Santos, A.P. Fittél, and V.P. Nascimento. Resistência antimicrobiana em Salmonella enterica subsp. Entérica Sorovar Hadar isoladas de carcaças de frango. *Arq. Inst. Biol.* **73(3):** 357–360 (2006).
5. A.E. Stella, E.C. Rigobelo, A.C. Oliveira, R.P. Maluta, J.M. Marin, and F.A. Ávila. Ocorrência e sensibilidade microbiana de linhagens de Escherichia coli enteropatogênicas isoladas de propriedades leiteiras na região de Ribeirão Preto-SP, Brasil. *Vet. Zootec.* **15:** 66–74 (2008).
6. J.M. Mitchell, M.W. Griffiths, S.A. Mcewen, W.B. Mcnab, and A. Yee. Antimicrobial drug residues in milk and meat; causes, concerns, prevalence, regulations, tests, and test performance. *J. Food Prot.* **61(6):** 42–745 (1998)
7. M.A.V.P. Brito. Resíduos de antimicrobianos no leite. Circular Técnica Embrapa Gado de Leite, n. 60, 2000. 20 p.
8. Comunidade Européia - Comission Regulation (EEC) 2377/ 90. *Off. J. Eur. Commun.* L224, n.1, 1990.
9. MERCOSUL – Mercado Comum do Cone Sul. Metodologias analíticas, ingestão diária admissível e limites máximos de resíduos para medicamentos veterinários em alimentos de origem animal. Resolução 54/2000, (s.n.l.), 2000.
10. BRASIL. Ministério da Agricultura. Portaria Nº 51, de 06 de fevereiro de 1986. http://extranet.agricultura.gov.br/sislegis-consulta/constularLegislacao.do?operacao=visualizar& id=2631. (Accessed September 1, 2008).
11. BRASIL. Ministério da Agricultura. Portaria Nº 527, de 15 de agosto de 1995. http://extranet.agricultura.gov.br/sislegis-consulta/consultarLegislacao.do?operacao=visualizar& id=16313. (Accessed September 1, 2008).
12. ANVISA. Agência Nacional de Vigilância Sanitária. Programa Nacional de Análise de Resíduos de Medi-camentos Veterinários em Alimentos Expostos ao Consumo—PAMVet, Brasília, Novembro de 2003. Available at http://www.anvisa.gov.br/alimentos/pamvet/pamvet.pdf. (Date Accessed September 1, 2008).
13. M. Van Bruijnsvoort, S.J.M. Ottik, M.J. Klaas, and E. Boer. Determination of streptomycin and dihydrostreptomycin in milk and honey by liquid chromatography with tandem mass spectrometry. *J. Chromatogr. A* **1058:** 137–142 (2004).
14. S. Bogialli, R. Curini, A. DI Corcia, A. Laganà, M. Mele, and M. Nazzari. Simple confirmatory assay for analyzing residues of aminoglycoside antibiotics in bovine milk: hot water extraction followed by liquid chromatography–tandem mass spectrometry. *J. Chromatogr. A* **1067:** 93–100 (2005).
15. D.N. Heller, J.O. Peggins, C.B. Nochetto, M.L. Smith, O.A. Chiesa, and K. Moulton. LC/MS/MS measurement of gentamicin in bovine plasma, urine, milk, and biopsy samples taken from kidneys of standing animals. *J. Chromatogr. B* **821:** 22–30 (2005).
16. Comunidade Européia - Decisão da Comissão 657/2002. *J. Ofic. Comun. Eur.* L221/8, 2002.
17. BRASIL. Ministério da Agricultura. Instrução Normativa Nº 42, de 20 de dezembro de 1999. Anexo IV. Available at http://extranet.agricultura.gov.br/sislegis-consulta/consultarLegislacao.do?operacao=visualizar&id=16716. (Accessed September 1, 2008).
18. Codex Alimentarius. Residuos de medicamentos veterinarios en los alimentos. 2ed., v. 3, Roma: Codex Alimentarius, 1993.
19. M. Horie, H. Saito, T. Natori, J. Nagata, and H. Nakazawa. Determination of streptomycin and dihydrostreptomycin in honey by liquid chromatography – electrospray mass spectrometry. *J. Liq. Chromatogr. Relat Tech.* **27(5):** 863–874 (2004).
20. M. Thompson, L.R. Stephen, and R. Wood. Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report). *Pure Appl. Chem.* **74:** 835 (2002).
21. A.A. Bergwerff and P. Scherpenisse. Determination of residues of malachite green in aquatic animals. *J. Chromatog. B* **788:** 351–359 (2003).
22. K. Halme, E. Lindfords, and K. Peltonen. Determination of malachite green residues in rainbow trout muscle with liquid chromatography and liquid chromatography coupled with tandem mass spectrometry. *Food Addit. Contam.* **21:** 641–648 (2004).
23. M.D. Hernando, M. Mezcua, J.M. Suarez-Barcena, and A.R. Fernández-Alba. Liquid chromatography with time-of-flight mass spectrometry for simultaneous determination of chemotherapeutant residues in salmon. *Anal. Chim. Acta* **562:** 176–184 (2006).

Manuscript received November 14, 2008;
Revision received April 17, 2009.