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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

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ABSTRACT

Antibiotics are widely used in veterinary medicine mainly for treatment and prevention of diseases. The aminoglycosides are one of the antibiotics classes that have been extensively employed in animal husbandry for the treatment of bacterial infections, but also as growth promotion. The European Union has issued strict Maximum Residue Levels (MRLs) for aminoglycosides in several animal origin products including bovine milk, bovine, swine and poultry muscle. This paper describes a fast and simple analytical method for the determination of ten aminoglycosides (spectinomycin, tobramycin, gentamicin, kanamycin, hygromycin, apramycin, streptomycin, dihydrostreptomycin, amikacin and neomycin) in bovine milk and bovine, swine and poultry muscle. For sample preparation, an extraction method was developed using trichloroacetic acid and clean up with low temperature precipitation and C18 bulk. Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) was used to carry out quantitative analysis and liquid chromatography-quadrupole-time of flight-mass spectrometry (LC-QTOF-MS) was used to screening purposes. Both methods were validated according to the European Union Commission Directive 2002/657/EC. Good performance characteristics were obtained for recovery, precision, calibration curve, specificity, decision limits (CCα) and detection capabilities (CCβ) in all matrices evaluated. The detection limit (LOD) and quantification limit (LOQ) were ranging from 5 to 100 ng g^{-1} and 12.5 to 250 ng g^{-1}, respectively. Good linearity (*r*)-above 0.99-was achieved in concentrations ranging from 0.0 to 2.0 × MRL. Recoveries ranged from 36.8% to 98.0% and the coefficient of variation from 0.9 to 20.2%, noting that all curves have been made into their own matrices in order to minimize the matrix effects. The CCβ values obtained in qualitative method were between 25 and 250 ng g^{-1}. The proposed method showed to be simple, easy, and adequate for high-throughput analysis of a large number of samples per day at low cost.

1. Introduction

Monitoring of animal origin food for veterinary drug residues is mandatory in many countries to ensure food security and compliance goog practices of veterinary medicine [1]. Currently, one of the major concerns in food safety is the determination of the risk associated with the presence of contaminants that may be present in products of animal origin. Matrices such as muscle and milk are often chosen as target samples, because high concentrations of veterinary drugs can be found in those tissues after systemic drug administration [2,3]. Among the various drugs used in veterinary medicine, antimicrobials are used not just for their therapeutic and/or prophylactic activity as well as their growth-promoting properties [4]. When good veterinary practices are not observed, this can result in the presence of drug residues in animal tissues above the acceptable limits. Thus, monitoring and assuring the compliance for drug residue levels in food-producing animals is a major concern.

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Aminoglycosides are a class of antibiotics that have been extensively used in both human and veterinary medicine. Aminoglycosides act directly on bacterial protein synthesis. In veterinary medicine, they are used to treat mastitis in dairy animals, leptospirosis in cattle, sheep, goats and pigs, and also in the treatment of various gram negative bacterial infections [5]. At therapeutic doses, the observed serum aminoglycoside concentrations are close to toxic doses. Cellular toxicity is a common characteristic of aminoglycosides (except for spectinomycin) due to their absorption into the intracellular medium. The most important toxic effects are ototoxicity, nephrotoxicity, and neuromuscular blockade [6].

The European Commission has set Maximum Residue Levels (MRLs) for some aminoglycosides in milk and muscle (Table I, Supplementary content) [7]. If the levels of these substances in a product are above the MRL, the product is rejected. It makes the product unsuitable for use in industry and human consumption, since there is no treatment that can inactivate these substances. The presence of aminoglycosides in food may represent a risk to consumer health, due to possible allergenicity and/or tissue toxicity, and exposure to them may lead to an increase in antimicrobial resistance. In Brazil, the monitoring program for antibacterial residues in food is performed by the laboratory network of the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) through the National Residue Control Plan (NRCP) [8,9]. Nowadays, in Brazil, aminoglycoside residue levels are monitored only in honey and kidney. For muscle, probably the most consumed edible tissue, there are no MRL established yet.

According to McGlinchey and Kaufmann, quantitation of aminoglycosides represents a considerable analytical challenge, both during sample preparation and analytical instrumental analysis, because these compounds are highly polar due to various amino and hydroxyl groups in their structure, as well as they lack chromophore or fluorophore groups and tend to establish strong links with matrix proteins. For these reasons, the presence of this class of antibiotics is less frequently assessed than other veterinary drugs [10,11].

Some purification methods, such as solid phase extraction (SPE) [10–14], *on-line* SPE [15,16] and matrix solid phase dispersion (MSPD) [11,17] have been employed to aminoglycoside extraction. Generally, extraction methods published for aminoglycosides mainly used SPE extraction, preceded by organic solvent extraction and/or an acid deproteinization of the matrix [10,12,15,18]. For instance, both Kaufmann, Zhu and Gremilogianni developed extraction methods with trichloroacetic acid followed by clean up using SPE in different matrices of animal origin [10,12,13]. However, it is important to consider that in most studies involving residues in food from animal origin, the SPE technique is considered time consuming and can be represent a high cost for routine purposes, especially for developing countries laboratories.

Multiresidue screening methods allow qualitative analysis of a large number of different pharmacological classes of drugs. Several multiresidue methods that include one or more aminoglycosides were published in recent years using different sample preparation approaches [4,19,20].

The present work is focused on the development of a screening method using liquid chromatography-quadrupole-time of flight-mass spectrometry (LC-QTOF-MS) together with a quantitative and confirmatory method using liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS), both able to analyze ten aminoglycosides in bovine milk and bovine, swine and poultry muscle samples. The sample preparation procedure is simple and environmental friendly, making this method suitable and feasible for routine analysis.

2. Experimental

2.1. Chemicals and reagents

Streptomycin (STR), dihydrostreptomycin (DHSTR), kanamycin (KNM), hygromycin B (HGR), gentamicin (GNT), neomycin (NEO) and amikacin (AMC) standards of $>90\%$ certified purity (except HGR, of 84,3%) were obtained from Dr. Ehrenstorfer (Augnburg, Germany). Spectinomycin (SPC) and apramycin (APR) were obtained from Fluka at $>95\%$ certified purity, and tobramycin (TBR) was purchased from Sigma-Aldrich Logistik (Schnelldorf, Germany), at 98% certified purity. Stock standard solutions were prepared in deionized ultra-pure water at concentrations of 1000 $\mu g\ mL^{-1}$.

For milk analysis, an intermediate solution (10 mL) was prepared using 100 μL of GNT stock solution, 150 μL of KNM stock solution, 200 μL of SPC, STR and DHSTR stock solutions, and 500 μL of APR, NEO, AMC, TBR and HGR stock solutions. This solution was prepared with deionized ultra-pure water. A working solution was prepared by diluting the intermediate solution five times with ultra-pure water. As required, milk samples were spiked by the addition of 50 μL of this working solution to a 1.0 mL milk sample resulting in concentrations equivalent to 100 $ng\ g^{-1}$ of GNT, 150 $ng\ g^{-1}$ of KNM, 200 $ng\ g^{-1}$ of SPC, STR and DHSTR, and 500 $ng\ g^{-1}$ of APR, NEO, AMC, TBR and HGR.

For muscle sample analysis, a working solution was prepared using 50 μL of GNT stock solution, 100 μL of KNM stock solution, 300 μL of SPC stock solution, 500 μL of STR, DHSTR, NEO, AMC, TBR and HGR stock solutions, and 1000 μL of APR stock solution. The volume was brought up to 10 mL with deionized ultra-pure water. The addition of 100 μL of working solution to 10 g of muscle is equivalent to 50 $ng\ g^{-1}$ of GNT, 100 $ng\ g^{-1}$ of KNM, 300 $ng\ g^{-1}$ of SPC, 500 $ng\ g^{-1}$ of STR, DHSTR, NEO, AMC, TBR and HGR, and 1000 $ng\ g^{-1}$ of APR.

Acetonitrile (ACN; HPLC grade) was purchased from Merck (Darmstadt, Germany), deionized ultra-pure water ($<18.2\ M\Omega$ cm resistivity) was obtained from a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, US), trichloroacetic acid (TCA) and ethylenediamine tetracetic acid (EDTA) were obtained from Vetec (Rio de Janeiro, Brazil). Nonafluoropentanoic acid (NFPA) of $>97\%$ certified purity was purchased from Sigma-Aldrich and Chromabond® Sorbent C18 ec, was obtained from Macherey-Nagel (Düren, Germany).

A 150 mM EDTA solution was prepared by dissolving 5.58 g of EDTA in 100 mL of deionized ultra-pure water. The deproteinization solution (TCA 15%) was prepared by dissolving 15 g of TCA with a water: ACN (1:1) solution to obtain 100 mL.

Mobile phase component A was prepared by adding 160 μL of NFPA to 100 mL of deionized ultra-pure water, and mobile phase component B was prepared by adding 160 μL of NFPA to 100 mL of ACN.

2.2. Sample preparation

A 2^4 factorial design with center point was used to optimize the variables with effects under the extraction process. The variables selected to be studied were: (1) sample volume, (2) extraction solution volume, (3) TCA concentration in the extraction solution and (4) freezing time. Experimental conditions employed in optimization of the extraction process are shown in Table II (Supplementary content). The peak area was used as the response factor and the data were processed using the spreadsheet previously described by Teófilo and Ferreira [21].

For milk analysis, raw milk samples were thawed, homogenized and a 1.0 mL aliquot was transferred to a 50 mL polypropylene centrifuge tube. An aliquot of 50 μL of 150 mM EDTA