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A simple and high-throughput method for determination and confirmation of 14 coccidiostats in poultry muscle and eggs using liquid chromatography – quadrupole linear ion trap - tandem mass spectrometry (HPLC–QqLIT-MS/MS): Validation according to European Union 2002/657/EC

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ABSTRACT

A simple and fast quantitative and confirmatory multi-residue method was developed and validated for the determination of 14 coccidiostats residues in poultry muscle and eggs using liquid chromatography-tandem mass spectrometry (LC/MS-MS). The compounds were analyzed in a single run including lasalocid A, maduramicin, monensin, narasin, salinomycin, semduramicin, robenidine, diclazuril, toltrazuril, trimethoprim, clopidol, amprolium, diaveridine and nicarbazin (as the marker residue dinitrocarbanilide). A low-cost extraction and clean up procedure was optimized without the need of solid-phase extraction. Samples were extracted with acetonitrile followed by low-temperature clean up. Chromatographic separation was achieved using a C18 column, using water and acetonitrile, both containing 5 mmol L^{-1} of formic acid and 1 mmol L^{-1} ammonium acetate, as mobile phase. Coccidiostats were ionized in negative and positive mode and monitored simultaneously. The method was fully validated according with Commission Decision 2002/657/EC and was applied for > 100 samples from the Brazilian National Residue Control Plan (NRCP). Parameters as precision, reproducibility, trueness, CCα and CCβ were determined. Trueness values were within the range 73–115%. Precision (repeatability and intermediate precision) ranged from 0.4% to 21% and intralaboratory reproducibility ranged from 6.3% to 27%, depending on matrix.

1. Introduction

The poultry production has a significant impact on the performance of Brazilian agribusiness, making Brazil the current third largest world producer [1–3]. Among the conditions that sustain Brazil in this position, the efficient management and an adequate control of diseases should be highlighted. The intensive poultry production has considerable risks for diseases occurrence being that coccidiosis incidence is one of the most impactful in both economic and sanitary terms [4]. Coccidiosis is a highly infectious poultry disease caused by protozoa of the genus *Eimeria* and is one of the main health problems in poultry industry, impacting on production costs related to its prevention and control [5,6].

Thus, the use of coccidiostats drugs is widespread in poultry production, generally as feed additives. The intensive use of these drugs is a frequent concern for sanitary and health authorities, due to the risk of antimicrobial resistance emergence. A recent review published by Huyghebaert et al. showed an overview about absence or restricted alternatives for coccidiostats replacement on poultry production [7]. The authors comment the high risks involved and remark the correct planning needed to achieve coccidiostats replacement, avoiding severe impacts on poultry industry viability. Consequently, in absence of technological improvements, it is expected the maintenance or even the increasing of the coccidiostats use in poultry production. Some coccidiostats drugs are authorized in poultry production for treatment as well as prophylactic purposes. In recent years, the emergence of *Coccidia* resistance has been systematically reported. To overcome this challenge, combinations of different agents are used in order to reduce risks of treatment failure and increase the efficiency of the therapeutic schemes [8].

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The inadequate utilization of coccidiostats drugs in therapy or prophylaxis treatments could lead to the presence of undesirable drug residues in edible tissues. To guarantee the food safety for the consumers, maximum residues limits (MRLs) were established for several coccidiostats drugs. In some countries, the use of coccidiostats drugs as feed additives was restricted or even banned [9,10]. In Brazil, the Ministry of Agriculture, Livestock and Supply (MAPA) publish annually the list of compounds under surveillance in the National Residue Control Plan (NRCP) [11,12]. Currently, although vaccination against *Coccidia* is widely implemented [13], it is still possible to identify the systematic utilization of coccidiostats in Brazil through evaluation of NRCP samples analyzed in our laboratory, even if at concentrations below or slightly above the quantification limits for these compounds [11]. A comparison between the MRL established for some coccidiostats drugs in several countries are showed at Table 1 [14].

Currently, the coccidiostats under control in the NRCP include ionophore antibiotics (lasalocid A, maduramicin, monensin, narasin, salinomycin and semduramicin) and the chemical coccidiostats robenidine, diclazuril, toltrazuril, trimethoprim, clopidol, amprolium, diaveridine and nicarbazin (this last has dinitrocarbanilide (DNC) as marker residue). Although ionophore antibiotics were not classified by the Joint FAO/WHO/OIE Expert Meeting on Critically Important Antimicrobials as high priority group for monitoring programs, their control is important for public health protection as well as to obtain information about its usage and possible correlations with the antimicrobial resistance [15]. The coccidiostats administration is generally performed via the introduction of those drugs in the feed [16].

Due to the global use of coccidiostats agents, the development and validation of useful methods for determination of these compounds is a concern for food and analytical chemists. In recent years, the AOAC have been published several official methods for some ionophores and coccidiostats in order to harmonize analytical procedures for the determination of these analytes in feed and animal tissues [17–20].

Different methods are available for the determination of coccidiostats in food products from animal origin as high performance liquid chromatography with UV or fluorescence detection (HPLC-UV and HPLC-FD), immunoassay and liquid chromatography coupled to mass spectrometry (LC-MS/MS) [21,22]. For both Brazilian and European guidelines for the performance of analytical methods for residues determination in food, positive results must be confirmed using confirmatory methods [23,24]. The Commission Decision 2002/657/EC proposed an identification point criterion to be achieved by these confirmatory methods. For confirmatory purposes of drugs with legal limits, a minimum of three points are required, which can be easily accomplished using mass spectrometry methods with selected reaction monitoring mode (MRM). Thus, hyphenated methods, such as liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) is currently one of the best choices.

For some methods, the clean-up procedures are complex, especially for confirmatory analysis purposes [21,25]. Recent reports are limited to LC-MS/MS methodologies due to sensitivity, speed and possibility of monitoring a large number of compounds simultaneously [26–31]. Several analytical methods based on LC–MS/MS have been used to determine one or more residues of coccidiostats in different matrices [4,32,33] and were recently reviewed. by Clarke et al. [25]. Notwithstanding, just few reports deal with analysis of poultry muscle and eggs including analytes from different classes, as polyether ionophore and presenting simple sample preparation protocols, adequate for routine analysis laboratories. Clarke et al. developed a method for coccidiostats determination in bovine milk, duck muscle and non-avian species. Sample preparation was based on solvent extraction with acetonitrile and further concentration. These authors used rapid polarity switching to analyzed all compounds in a single injection for both negatively and positively charged ions. The method was validated for 20 coccidiostats [26]. Pereira et al. recently reported a method to determine residues of six ionophores in milk using QuEChERS extraction and analysis by LC-MS/MS [30].

Ion suppression is a problem when electrospray is used to ionize drugs in complex matrices using MS/MS systems [34,35]. To reduce ion suppression, sample preparation techniques as solid phase extraction (SPE) are generally applied in order to obtain clean extracts and to reduce or avoid matrix effects. Although SPE is very useful for sample clean up, this technique presents limitations related to optimization and process automation. SPE procedures are time consuming and high waste generator when compared with alternative protocols. For routine methods that deal with a high number of samples, approaches that ally sensitivity, reproducibility and high-throughput capacity with low operational costs are mandatory. In previous published reports, our research group demonstrate the feasibility to work with minimal sample preparation maintaining the analytical results confidence using liquid-liquid extraction with low temperature purification as sample clean up step [36,37].

In the present work, we describe the development and validation of a simple and fast confirmatory LC-MS/MS method for determination of the most relevant coccidiostats drugs residues used in poultry production for the matrices muscle and whole egg. The analytes included in the study comprehend all coccidiostats available in the veterinary market in Brazil. Sample preparation was easily performed without the need of SPE procedures.

Table 1
MRL adopted for some coccidiostats in chicken muscle and eggs (in μg kg^{-1}). Values are for muscle, except when indicated.

Compound	Codex Alimentarius	Brazil	USA	EU	Canada
Amprolium	–	500 10 (E)	500 4000 (E)		500
Avilamycin	200	–	–	50	–
Clopidol	–	5000 10 (E)	5000	–	5000
Decoquinate	–	–	1000	20 (E)	1000
Diaveridine	–	50 10 (E)	–	–	–
Diclazuril	500	500 10 (E)	500	500 2 (E)	500
Lasaocid A	–	20 10 (E)	1200 (S)	20 150 (E)	–
Maduramicin	–	240 10 (E)	240	30 2 (E)	–
Monensin	10	10 10 (E)	–	8 2 (E)	50
Narasin	15	15 10 (E)	480 (AF)	50 2 (E)	50
Nicarbazin	200	200 10 (E)	4000	4000 100 (E)	4000
Robenidine	–	100 10 (E)	100	200 25 (E)	100
Salinomycin	–	100 10 (E)	–	5 3 (E)	–
Semduramicin	–	50 10 (E)	130	2 (E)	–
Toltrazuril	–	500 10 (E)	–	100 Not permitted (E)	–

E=eggs; S=skin with adherent fat; AF=abdominal fat.

2. Materials and methods

2.1. Chemicals and standard solutions

Lasalocid A (LASA), maduramicin (MAD), monensin (MON), narasin (NAR), salinomycin (SAL), semduramicin (SEM), clopidol (CLOP), robenedine (ROB), amprolium (AMP), diaveridine (DIAV),