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Determination of fifteen coccidiostats in feed at carry-over levels using liquid chromatography–mass spectrometry

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ABSTRACT

A multi-residue method has been developed and validated for the simultaneous determination of authorized (decoquinate, diclazuril, halofuginone, lasalocid, maduramicin, monensin, narasin, nicarbazin, robenidine, salinomycin and semduramicin) and non-authorized (amprolium, clopidol, ethopabate and toltrazuril) coccidiostats in animal feed. Feed samples were extracted with basic followed by acidified solution in methanol and, after centrifugation, were injected directly into LC–MS/MS system. Detection was performed in selected reaction monitoring mode with both positive and negative electrospray ionization. The time efficient validation experiment has verified the robustness of a method in different types of feed and on two separate LC–MS/MS instruments. The comparison of different quantification methods demonstrated that, against expectations, the standard addition did not prove better in comparison with matrix-matched calibration curve. Although the sample preparation was very easy, the observed matrix effects were not significant for the most part but they could explain the problems with the quantification of some coccidiostats.

1. Introduction

Coccidiosis is a parasitic disease of the gastrointestinal tract caused by single-celled parasites belonging to the genus *Eimeria*. Coccidiosis is a major problem in poultry, but it also concerns other species like rabbits, pigs, and sheep. In acute form, coccidiosis causes high mortality rates, its subacute form leads to the distortion in weight gain, eggs production and food assimilation [1]. Nowadays animal health management cannot exclude existence of coccidian infections in animal farms. Thus, usage of anticoccidial agents (coccidiostats) is considered to be the best way to maintain animal health and welfare.

Coccidiostats and histomononostats are the only pharmacologically active compounds which can be used as feed additives according to European Union (EU) recommendation. There are 11 coccidiostats authorized as feed additives: decoquinate (DEQ), diclazuril (DIC), halofuginone (HAL), lasalocid (LAS), maduramicin (MAD), monensin (MON), narasin (NAR), nicarbazin (NIC), robenidine (ROB), salinomycin (SAL) and semduramicin (SEM). Other coccidiostats mentioned in above regulation such as: amprolium (AMP), clopidol (CLO), ethopabate (ETH) and toltrazuril (TOL) are not authorized as feed additives [2].

The usage of one production line to produce different types of feed for different species and categories of animals is a very common practice in feed production. This situation can lead to the carry-over of coccidiostats from target to non-target feeds, which could harm sensitive species (turkey, horse) and also may be a risk for human health due to residues of coccidiostats in food of animal origin [3,4].

To protect animal health and minimize risk to consumers, EU has established maximum levels (ML) of unavoidable carry-over for all authorized coccidiostats in non-target feed [5]. The established limits are in a wide range of 0.01 mg/kg for DIC to 3.75 mg/kg for LAS, MON and NIC, as shown in Table 1.

Although the use of pharmacologically active feed additives other than mentioned eleven coccidiostats has been banned for a long time, there is still a potential risk of their administration to promote growth or control coccidiosis when other anticoccidials fail. According to European Union law, the growth promotion with pharmacologically active agents is forbidden and this rule is strictly implemented. Therefore, the control of contamination of feeds with such agents should be performed at concentrations as low as reasonably achievable (ALARA approach).

As a result of EU regulations regarding cross-contamination of feeds with coccidiostats, there was an urgent need to develop reliable analytical method for the determination of coccidiostats in this matrix. Many multi-residue methods for the determination of coccidiostats in animal tissues and products of animal origin

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Table 1
Maximum levels (MLs) of coccidiostats in non-target feeds due to unavoidable carry-over in feeds. The carry-over of 1% applies to feeds for sensitive species, continuous food-producing animals and target species for the period before the slaughter, 3% carry over applies to feeds for all other non-target species and categories of animals [5].

Coccidiostat	1% carry-over level for non-target feeds (mg/kg)	3% carry-over level for non-target feeds (mg/kg)
Decoquinate	0.40	1.20
Diclazuril	0.01	0.03
Halofuginone	0.03	0.09
Lasalocid	1.25	3.75
Maduramicin	0.05	0.15
Monensin	1.25	3.75
Narasin	0.70	2.10
Nicarbazin	1.25	3.75
Robenidine	0.70	2.10
Salinomycin	0.70	2.10
Semduramicin	0.25	0.75

(liver, eggs, muscle or milk) have been published in the literature [6,7]. There are significantly fewer methods for animal feed. To the authors' knowledge, only three methods are suitable for the determination of all eleven authorized coccidiostats at carry-over levels in animal feeds [8–10].

One of the main difficulties regarding developing of reliable method for the determination of coccidiostats in animal feed is the complexity of matrix. In the method presented by Delahaut et al. [8] a very simple and time-efficient extraction protocol was used, unluckily tests using this protocol, performed in our laboratory, did not provide satisfying results, probably because the differences in available instrumentation. Method developed by Cronly et al. [9] encountered significant problems with matrix effect which was overcome by injecting extracts twice in two different dilutions. Finally, method by Moretti et al. [10] described complicated method and time-consuming validation process which required two separate protocols to cover all coccidiostats at their maximum levels, thus can be impractical in the routine work.

None of the published methods is applicable to the simultaneous determination of coccidiostats authorized and non-authorized by EU legislation in a single analytical run. Some methods focus only on one or two coccidiostats: amprolium [11], clopidol and nicarbazin [12] or are designed to cover more substances (not only coccidiostats) non-authorized as a feed additives [13].

The aim of this study was to develop an easy and labour-effective method for the simultaneous determination of authorized and non-authorized coccidiostats in animal feed. An additional objective was to show the applicability of the method for the determination of coccidiostats in animal feed to a different mass spectrometer and discuss difficulties that might occur during the inter-laboratory validation of such a method in the future.

2. Experimental

2.1. Reagents

Acetonitrile, dimethyl sulfoxide and methanol, LC–MS grade were supplied from J.T. Baker (Germany), ammonium solution p.p.a (25%) and calcium chloride, p.p.a were purchased from POCh (Poland), acetic acid and ammonium format, both HPLC grade were purchased from Merck (Germany), formic acid, HPLC grade was supplied from Sigma–Aldrich (Germany), ultrapure water was taken from Milli-Q purification system (Millipore, France).

2.2. Standards substances

Amprolium, decoquinate, diclazuril, ethopabate, halofuginone hydrobromide, maduramicin ammonium, monensin sodium, narasin, nicarbazin, nigericin, robenidine hydrochloride, salinomycin sodium, toltrazuril, decoquinate-d5, robenidine hydrochloride-d8, were purchased from Sigma–Aldrich (Germany). Dinitrocarbanilide-d8 was obtained from Witega (Germany), lasalocid sodium was purchased from Dr. Ehrenstorfer Laboratories (Germany). Clopidol, halofuginone hydrobromide $^{13}C_6$, semduramicin sodium and methyldiclazuril were kindly donated by European Union Reference Laboratory in Berlin.

2.3. Standard solutions

Stock standard solutions (1000 μg/mL) were prepared by weighing of 10.00 mg of amprolium, ethopabate, clopidol, lasalocid, maduramicin, monensin, narasin, salinomycin and semduramicin and dissolving in 10 mL of acetonitrile. Stock standard solution (1000 μg/mL) of decoquinate was prepared by weighting of 10.00 mg of substance in 10 mL of 1% solution of calcium chloride in methanol. Stock standard solution (1000 mg/mL) of halofuginone was prepared by weighting of 10 mg of halofuginone in 10 mL 50% aqueous solution of acetonitrile. Stock standard solution of diclazuril, nicarbazin and robenidine were prepared by weighting of 10.00 mg of each analyte and dissolving in 10 mL of dimethyl sulfoxide (DMSO). Dilutions in acetonitrile were then prepared from stock solutions to obtain suitable range of working solutions. All solutions were stored in 2–10 °C. Working standard solution mixture was prepared in acetonitrile by mixing suitable amounts of working solutions and diluting it in the way to receive carry-over levels in feed.

2.4. Equipment

Instrumental analysis was performed on two separate LC–MS/MS systems. The first system (MS1) was Shimadzu Nexera X2 UHPLC (Shimadzu, Japan) coupled with 8050 triple quadruple detector (Shimadzu, Japan), controlled by LabSolution 5.60 SP2 software. The second one (MS2) consisted of Agilent 1100 HPLC (Agilent, USA) coupled with API 4000 triple quadruple detector (ABSciex, USA), controlled by Analyst 1.6 software.

2.5. Liquid chromatography

Separation was performed using Agilent Poroshell 120 EC-C18, 2.1 mm × 100 mm, 2.7 μm column and gradient mode with 0.01 M ammonium formate pH 4.0 (mobile phase A) and mixture of acetonitrile, methanol and mobile phase A in a composition of 60:35:5 (v:v:v) (mobile phase B). The starting conditions were 90% mobile phase A at a flow rate of 0.25 mL/min. For MS1, the linear gradient was applied with 90% A for 1 min, increase of organic phase to 95% phase B in 8 min, which was maintained until 15 min and re-equilibration with initial conditions from 16 to 24 min. For MS2 the applied gradient was as follows: 0–1 min 90% A; 1.5–15 min 0% A; 16–24 min 90% A. Oven temperature was set to 55 °C, injection volume was 2 μL.

2.6. Mass spectrometry

The analysis was performed using positive and negative electrospray ionization mode (ESI). The parameters of MS1 instrument were as follows: nebulizing gas flow – 3 L/min, heating gas and drying gas flows – 10 L/min both, interface temperature – 300 °C, temperature of desolvation line – 250 °C, heat block temperature – 400 °C, capillary voltage – −3 kV and 4 kV for negative