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Application News

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LCMS-8050

An efficient method for simultaneous determination of 15 coccidiostats in animal feed at carry-over levels by UHPLC-MS/MS using fast polarity switching

Konrad Pietruk, Małgorzata Olejnik, Teresa Szprengier-Juszkiewicz
Department of Pharmacology and Toxicology
National Veterinary Research Institute, Puławy, Poland

1. Introduction

Coccidiosis is a major problem in poultry but it also concerns other species like rabbits, pigs, and sheep. The use of coccidiostats is considered to be the best way to maintain animal health and welfare. There are 11 coccidiostats authorized as feed additives: decoquinate, diclazuril, halofuginone, lasalocid, maduramicin, monensin, narasin, nicarbazin, robenidine, salinomycin and semduramicin. Other coccidiostats such as: amprolium, clopidol, ethopabate and toltrazuril are not allowed to use as feed additives.
The usage of one production line to produce different types of feed for different species and categories of animals is a 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 pose risk for human health due to residues of coccidiostats in food of animal origin.
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. The established limits are in a wide range from 0.01 mg/kg for diclazuril to 3.75 mg/kg for lasalocid, monensin and nicarbazin.
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 at carry-over levels

2. Materials and Methods

The sample was left for one hour to improve standards' dispersal in feed. An aliquot of 12.5 mL of 1% ammonium solution in methanol (prepared by diluting 1 mL of 25% ammonium solution in 100 mL methanol) was added to each analysed sample. Then the sample was transferred to the ultrasonic bath for 15 min. After that, 12.5 mL of 2% acetic acid in methanol was added. Sample was shaken (30 min, 200 cycles/min) and centrifuged (4500 rpm, 20 °C, 15 min). Finally 1 mL of supernatant was transferred into vial for instrumental analysis.

2-1. LC-MS/MS analysis

Extracts were analyzed on a Nexera X2 (Shimadzu, Japan) UHPLC system coupled to a triple quadruple mass spectrometer LCMS-8050 (Shimadzu, Japan). Analysis was carried out in selected reaction monitoring (MRM) mode acquiring 2 transitions for each compound. A single transition was monitored for each internal standard.

Table 1 – LC conditions

Analytical column	Agilent Poroshell 120 EC-C18 2.1 x 100 mm, 2.7 µm
Mobile phase	A = 0.01 M ammonium formate pH 4.0 B = acetonitrile: methanol: mobile phase A, 60:35:5 (v:v:v)
Gradient	10%B (0.0 1.0 min), 95%B (8.0 - 15.0 min), 10%B (16-24 min), Stop (24.0 min)
Column temperature	55 °C
Injection volume	2 µL
Flow rate	0.25 mL/min

Table 2 – MS/MS conditions

Ionization mode	Heated ESI (+/-)
Temperatures	HESI: 300 ºC Desolvation line: 250 ºC Heat block: 400 ºC
Gas flows	Nebulizing gas (N_2): 3 L/min Heating gas (Air): 10 L/min Drying gas (N_2): 10 L/min
CID gas pressure	270 kPa (Ar)
Dwell time	10 to 100 ms depending on the number of concomitant transitions to ensure a maximum loop time of 500 ms (including pause time and polarity switching)

Table 3 – MRM transitions

Name	ESI mode	Ret. Time (min)	MRM Quan	MRM Qual
Amprolium	postive	4.5	242.3 > 150.2	242.3 > 122.2
Clopidol	positive	5.0	192.0 >101.2	192.0 > 87.1
Decoquinate	positive	11.0	418.2> 372.2	418.2 >204.1
Decoquinate d5 (ISTD)	positive	11.1	423.3 > 337.7	N/A
Diclazuril	negative	9.3	406.9 > 336.0	404.8 > 334.0
Dinitrocarbanilide (for Nicarbazin detection)	negative	8.9	309.1 > 138.2	309.1 > 108.2
Dinitrocarbanilide d8 (ISTD)	negative	9.0	309.1* > 141.2	
Ethopabate	positive	7.1	238.0 > 206.2	238.0 > 136.2
Halofuginone	positive	6.7	416.1 > 138.1	416.1 > 100.1
Halofuginone 13C6 (ISTD)	positive	6.8	422.0 > 138.0	N/A
Lasalocid	positive	10.9	613.3 > 577.3	613.3 > 377.2
Maduramicin	positive	13.6	934.5 > 647.4	934.5 > 629.4
Methyldiclazuril (ISTD)	negative	9.4		
Monensin	positive	12.5	693.3 > 675.4	693.3 > 479.3
Narasin	positive	14.7	787.4 > 531.3	787.4 > 431.3
Robenidine	positive	9.1	334.1 > 155.1	334.1 > 138.1
Robenidine d8 (ISTD)	positive	9.2	342.0 > 159.2	N/A
Salinomycin	positive	13.6	773.4 > 531.3	733.4 > 431.1
Semduramicin	positive	11.8	895.4 > 851.5	895.4 > 833.7
Toltrazuril	negative	9.6	424.4 > 424.4	N/A

*^{13}C isotopic ion

3. Results and discussion

3-1. Method development

The MS/MS fragmentation conditions were investigated and collision energies were optimized for each individual compound to give sufficient sensitivity. For the detection of polyether coccidiostats, sodium adducts were chosen due to more reproducible results. The chemical coccidiostats presented pseudo-molecular ions. For dinitrocarbanilide, ^{13}C isotopic ion was chosen in order to avoid detector saturation. Because diclazuril gave only one fragmentation ion, a transition of isotope ion containing ^{37}Cl was monitored for confirmation. Despite sufficient sensitivity of toltrazuril MS signal, the method can be used only for screening due to an inefficient fragmentation and lack of confirmatory SRM transition.

The Poroshell C18 column combined with gradient profile of ammonium formate and mixture of acetonitrile, methanol and ammonium formate allowed to obtain good peak shapes, sufficient separation of analytes and time efficient method.

Two manners of the extraction were checked during the method development. Two stage extraction consisted of: 1% ammonium solution in methanol and 2% of acetic acid in methanol applied one by one. The second extraction procedure, was performed with the mixture of methanol, acetonitrile and McIvaine buffer (37.5:37.5:25, v:v:v). Two stage extraction protocol gave better recovery rates and more reproducible results and was chosen in the final version of the method.

3-2. Method validation

General concept of validation experiment has been based on the performance criteria described in Regulation (EC) 882/2004. Additionally, decision limit (CCα) and detection capability (CCβ) were calculated according to Commission Decision 2002/657 as a well-recognized factor for evaluating overall analytical method performance. To prove the robustness of the method without the necessity to perform full validation, the design of a validation study assessed the matrix influence and method applicability using a relatively low number of tested samples. The validation experiment was carried out using 5 different animal feed types (for cattle broiler, chickens, turkeys, laying hens and pigs). Validation results shown in Table 4 confirm that the method fits for the purpose of the determination of coccidiostats at maximum levels introduced by EU regulations. It should be emphasized that matrix-fortified calibration curves prepared from a single feed can be used for the quantification of wide range of feed types.

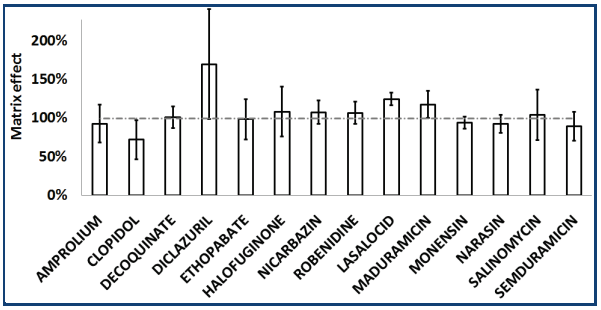
Table 4 – Validation results (limit of detection, LOD; limit of quantification, LOQ; decision limit, CCα; detection capability, CCβ; intra-laboratory reproducibility expressed as CV) obtained for fifteen coccidiostats

Name	LOD LOQ [mg/kg]	Validation levels [mg/kg]	Reproducibility CV [%]	CCα CCβ [mg/kg]
Amprolium	0.002 0.004	0.10 0.20 0.60	21 14 9	0.25 0.29
Clopidol	0.005 0.009	0.05 0.10 0.30	23 19 25	0.13 0.16
Decoquinate	0.003 0.021	0.20 0.40 1.20	9 18 9	0.51 0.62
Diclazuril	0.003 0.005	0.005 0.01 0.03	25 17 25	0.013 0.016
Nicarbazin	0.14 0.22	0.625 1.25 3.75	8 5 5	1.35 1.46
Ethopabate	0.006 0.01	0.10 0.20 0.60	20 15 26	0.25 0.30
Halofuginone	0.009 0.015	0.015 0.03 0.09	23 18 21	0.039 0.048
Lasalocid	0.063 0.10	0.625 1.25 3.75	12 17 9	1.60 1.95
Maduramicin	0.007 0.011	0.025 0.05 0.15	25 13 9	0.061 0.071
Monensin	0.074 0.12	0.625 1.25 3.75	9 6 5	1.37 1.50
Narasin	0.085 0.13	0.35 0.7 2.10	16 18 4	0.91 1.11
Robenidine	0.008 0.014	0.35 0.70 2.10	10 7 8	0.78 0.86
Salinomycin	0.073 0.13	0.35 0.70 2.10	14 11 14	0.83 0.95
Semduramycin	0.004 0.007	0.125 0.25 0.75	26 12 13	0.30 0.35
Toltrazuril	0.014 0.020	0.10 0.20 0.60	21 20 20	0.27 0.33

3-3 Evaluation of the matrix effect

An experiment was performed to assess the extent of a matrix effect. It was calculated as the percentage of a signal (analyte peak area divided by peak area of an internal standard, if applicable or simply analyte peak area) of the sample spiked after extraction to the analogue signal of a reference standard. The results of the experiment with twenty different feed samples are shown in the Figure 1.

Figure 1. Matrix effect (mean ±SD) – results after the correction with ISTD



It is commonly known that one of the ways to minimize matrix effect is simply to dilute a sample. To make that possible, sensitive instrumentation has to be applied to compensate huge sample dilution. In the case of the presented method, that approach seems to be the reason for a relatively low matrix effect despite high complexity of a feed matrix.

4. Conclusions

- A sensitive, robust and time efficient method for the determination of 15 coccidiostats in feed at carry-over levels has been developed and validated
- The method provides reliable quantification at maximum levels established by European Union in a wide range of animal feeds
- Low matrix effect was achieved by huge sample dilution

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