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DETERMINATION OF ELEVEN COCCIDIOSTATS IN MUSCLE USING LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

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Introduction

Coccidiostats are feed additives widely used for the prevention of coccidiosis, a severe poultry infection caused by genus *Eimeria*. Recently the European Commission has set permitted levels (PLs) for eleven coccidiostats in various foods from non target animals [1]. The regulated drugs are six ionophore antibiotics (lasalocid, maduramycin, monensin, narasin, salinomycin, semduramycin) and five chemical coccidiostats (decoquinate, diclazuril, halofuginone, nicarbazin and robenidine). The availability of analytical methods suitable for the simultaneous determination of these molecules is a very important target for laboratories involved in official controls. Hence the aim of this work was the development, optimization and validation of an LC-MS/MS confirmatory method for the determination of residues of the eleven coccidiostats in animal muscle.

LC-MS / MS Method

- Instrument: FINNIGAN TSQ QUANTUM ULTRA
- Column: Synergi Fusion (150 x 2.0 mm; 4 µm) - Phenomenex
- Guard column: Fusion RP (4 x 2.0 mm; 3.5 µm) - Phenomenex
- Flow-rate: 0.25 mL/min
- Column temperature: 40°C; Samples temperature: 16° C
- Mobile phases: acetonitrile with formic acid 0.1% and 20 µM sodium acetate [A] - Aqueous formic acid 0.1% [B]
- Gradient: 0-2 min 15% [A]; 2-3 min linear increase to 25% [A]; 3-15.5 min linear increase to 95% [A]; 15.5-33 min 95% [A]; 33-35 min linear decrease to 15% [A]; with final hold of 15% [A] for 5 min.
- Ionization mode: ESI positive and negative mode
- Capillary voltage: 4.2 kV (ESI positive) and 3.0 kV (ESI negative)
- Ionization gas (nitrogen) pressure: 40 units (Sheath); 25 units (Auxiliary); 5 units (Ion sweep)
- Capillary temperature: 320°C
- Collision gas (argon) pressure: 1.5 mtorr
- Acquisition mode: Selected Reaction Monitoring, SRM (Table 1)

Table 1 - MS / MS conditions

Analyte	ESI mode	Precursor ion (m/z)	Product ions (m/z)
Nicarbazin (DNC)	negative	301	107, 137[a]
Robenidine	positive	334	138, 155[a], 178
Diclazuril	negative	405 (407)	299 (301), 334[a] (336[a])
Halofuginone	positive	416	121, 138, 398[a]
Decoquinate	positive	418	204, 232, 372[a]
Lasalocid	positive	613	377[a], 577, 595
Monensin	positive	693	461[a], 479, 501
Salinomycin	positive	773	413, 431[a], 531
Narasin	positive	787	413, 431[a], 531
Semduramicin	positive	895	833[a], 851
Maduramicin	positive	939	719, 859, 877[a]

[a] The most abundant product ion

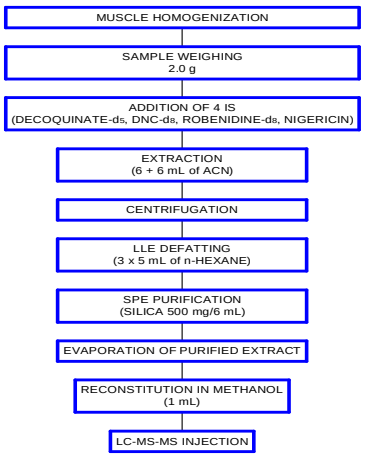

Figure 1. Flow diagram of sample preparation

Validation study

The validation was carried out on poultry muscle in accordance with the criteria described in CD 2002/657/EC [2] introducing an alternative experimental plan [3]. For the estimation of method trueness, precision, decision limit (CCα) and detection capability (CCβ) six progressive spiking levels were tested: 1.0, 3.2, 10, 32, 100 and 320 µg/kg. Four replicates (n=4) were carried out for each concentration level and repeated on three separate days (p=3) varying time, operator and calibration status of LC-MS/MS equipment. Specificity, linearity and ruggedness were also evaluated.

Table 2. Decision limits and detection capabilities for the eleven coccidiostats in muscle of non target species

Analyte	PL (µg/kg)	CCα (µg/kg)	CCβ (µg/kg)
Nicarbazin (DNC)	25	27	30
Robenidine	5	5.7	6.6
Diclazuril	5	5.7	6.5
Halofuginone	3	3.7	4.6
Decoquinate	20	24	28
Lasalocid	5	5.7	6.6
Monensin	2	2.2	2.4
Salinomycin	2	2.2	2.4
Narasin	5	5.5	6.0
Semduramicin	2	2.3	2.7
Maduramicin	2	2.4	2.8

Results

Starting from the our previously published method for coccidiostats in eggs [3], several experiments were carried out to optimize the protocol also in muscle. In fact the eggs sample preparation was not suitable for muscle since very low recoveries were observed for Halofuginone. This drawback was solved introducing an additional SPE column elution with a basic mixture containing ethanol, water and ammonia (90/10/0.5, v/v/v). The quantification was accomplished using the internal standardization method, except for Halofuginone, Monensin, Semduramicin and Maduramicin for which the external calibration was more suitable. It is important to underline that the lack of correspondent labelled internal standards for all coccidiostats must force the analyst to test the better quantification strategy. The method precision was calculated using Analysis of Variance (ANOVA). In this way at each validation level the repeatability and the within lab reproducibility precision was estimated. Since in the investigated range the Coefficients of Variation (CV) were not influenced by the analyte concentration, pooled CVs were calculated and used to establish the CCα and CCβ in Table 2. In the overall validation range the CVs evaluated in repeatability and within laboratory reproducibility conditions were lower than 10 and 15%, respectively. The real recoveries (extraction yields) ranged from 60 to 114%, except for the Halofuginone at 1 µg/kg (45%).

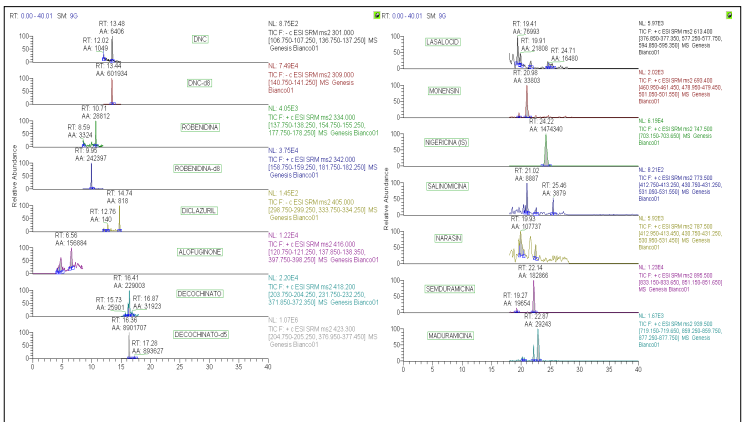

Figure 2. Blank poultry muscle sample

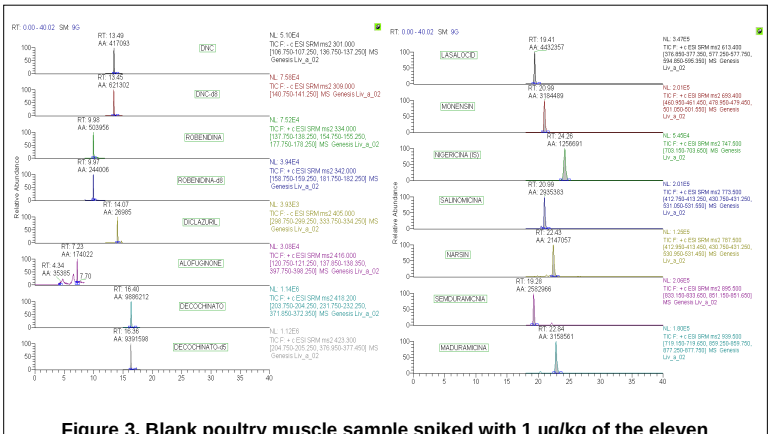

Figure 3. Blank poultry muscle sample spiked with 1 µg/kg of the eleven coccidiostats

Conclusions

It was concluded that the developed method is fit for the purpose to be used in the frame of official control of the presence of the coccidiostats. Since the PLs in Table 2 are related to non target species, recently several EU Regulations are published setting the maximum residue limits (MRLs) also in target species. Thanks to this alternative experimental plan a wide concentration range was investigated (1-320 µg/kg). Therefore this validation strategy provides extensive knowledge of the method and greater flexibility, permitting the re-evaluation of fundamental performance characteristics, especially CCα, also when a legislative limit was changed or set.

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References

[1] Commission Regulation (EC) No 124/2009, *Off. J. Eur. Commun.*, L40 (2009) 7-11;

[2] Commission Decision 2002/657/EC, *Off. J. Eur. Comm.*, L221 (2002) 8-36;

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