DETERMINATION OF COCCIDIOSTATS IN EGG, MUSCLE AND FEED BY LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY

Servicio Navarro de Salud Osasunbidea

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INTRODUCTION:

The intensive farming of livestock uses a variety of drug products, either for therapeutic purposes, zootechnical or growth promoters. Most of these products can enter the food chain by not respecting the periods of suppression in those animals that have been previously treated.

The sensitivity and specificity of the HPLC-MS/MS technique makes it very suitable for analysis in complex matrices of many organic compounds that may appear in foods as a result of deliberate additions or as contaminants. Coccidiostats comprise a chemically heterogeneous group whose only common point is their activity against *coccidia* (eukaryotic cellular organisms). The anticoccidial are in the group B (Veterinary drugs and contaminants established in RD 1749/1998). If they are classified by their origin there are two groups, the polyether ionophoric anticoccidial, such as salinomycin, monensin, narasin and maduramicin, and the organic synthetic anticoccidial like diclazuril, sulfaquinoxaline, nicarbazin and robenidine.

The sulfaquinoxaline is a broad spectrum antimicrobial (sulfonamide) that also works against coccidia and therefore has been considered anticoccidial.

Ideally, these drugs should not have adverse effects in growth, feed intake and conversion, and should not leave residues in meat. However it has been found that cross-contamination of feed can cause the appearance of animal origin residues in food.

There was no legislation for these compounds until the coming into effect in February 2009 of the Commission Regulation (EC) No. 124/2009 that sets the maximum levels for the presence of coccidiostats or histomonostats in food resulting from the unavoidable carry-over of these substances in non-target feed.

MATERIALS AND METHOD:

The method is validated and is currently accredited by ENAC for analysis of Nicarbazin, Robenidine, Diclazuril and Sulfaquinoxaline in egg and muscle matrices of sheep, pigs, goats, bovine, rabbits and poultry (quail and chicken). Values of the limit of decision (CCα) and the detection capability (CCβ) were calculated as established by Commission Decision 2002/657. It is also possible to analyze other compounds such as narasin, salinomycin, lasalocid and monensin and extended to matrices as feed.

The method used for the determination is HPLC-MS/MS after extraction with acetonitrile and concentration by nitrogen stream. Quantification was carried out by the system of internal standard (*methyl diclazuril)*.

SAMPLE PREPARATION:

- Take a portion of sample previously homogenized and dehydrated it with anhydrous sodium sulfate.
- Add the appropriate volume of the internal standard solution (methyl diclazuril).
- Extracted it with acetonitrile and shake horizontaly.
- Centrifuge and evaporate to dryness an aliquot of the supernatant in a thermostatic bath with nitrogen stream.
- Reconstitute the sample in the glass tube with a mixture of acetonitrile/water and transfer it into the injection vials.
- There will be made an added to real matrix calibration curve in each work series.

CONDITIONS OF MASS SPECTROMETRY:

Method is optimized for highest sensitivity possible, choosing the transitions of each compound and assessing the collision energy and cone voltage suitable.

It uses both ES^+ and ES^- to obtain characteristic ions.

Function 1: Monitoring ES^+ 0-14 minutes MRM of 4 channels

Compound	Precursor ion < Product ion	Dwell	Cone Voltage (V)	Collision Energy (eV)
Sulfaquinoxaline	301<118	0.1	45	35
Sulfaquinoxaline	301<156	0.1	45	20
Robenidine	334<155	0.1	35	25
Robenidine	334<138	0.1	35	25

Function 2: Monitoring ES^- 14-20 minutes MRM of 6 channels

Compound	Precursor ion < Product ion	Dwell	Cone Voltage (V)	Collision Energy (eV)
Nicarbazin*	301.1<107.1	0.1	35	30
Nicarbazin*	301.1<137.1	0.1	35	30
Diclazuril	336<407	0.1	32	20
Diclazuril	334<405	0.1	32	20
Methyldiclazuril (IS)	419<321	0.1	30	25
Methyldiclazuril (IS)	421<323	0.1	50	25

* The scan for nicarbazin is shown, as an example, in Figure 1 and Figure 2.

Function 3: Monitoring ES^+ 20-32 minutes MRM of 10 channels

Compound	Precursor ion < Product ion	Dwell	Cone Voltage (V)	Collision Energy (eV)
Lasalocid	613.6<359.1	0.1	55	35
Lasalocid	613.6<377.1	0.1	55	35
Monensin	693.4<461.5	0.1	80	48
Monensin	693.4<479.5	0.1	80	50
Salinomycin	773.5<265.2	0.1	80	48
Salinomycin	773.5<431.2	0.1	80	48
Narasin	787.7<431.5	0.1	80	50
Narasin	787.7<531.6	0.1	80	43
Maduramicin	939.5<719.3	0.1	55	70
Maduramicin	939.5<877.4	0.1	55	40

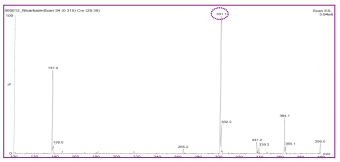

Fig. 1: Nicarbazin scan to determine the **Precursor ion** from the first quadrupole in ES-

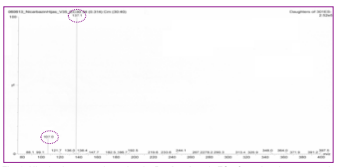

Fig. 2: Nicarbazin scan to determine the **Product ions** in ES- after select the precursor ion.

CHROMATOGRAPHIC METHOD:

Equipment used was a high pressure liquid chromatograph, injector integrated, Waters Alliance HT 2795, coupled with a mass detector Quatro Micro API triple quadrupole (HPLC-MS/MS).

The method uses a Waters Symmetry column (2.1 mm i.d. x 150 mm) C18 with a particle size of 5 µm.

The elution of the different components is achieved by gradient:

-Oven Temp.: 40 ºC
-Samples Temp.: 20 ºC
-Flow: 0.3 mL/min
-Mobile phase: A Channel: Acetonitrile 0,1% Formic acid
-B Channel: D.I. Water 0,1 % Formic acid
-Gradient:
- Minute 0: (5% A : 95% B)
- Minute 3: (5% A : 95% B)
- Minute 20: (95% A: 5% B)
- Minute 30: (95% A: 5% B)
- Minute 30,1: (5% A : 95% B)
- Minute 32: (5% A : 95% B)

-Total analysis time: 32 minutes

CHARACTERISTICS OF ANALYTICAL METHOD FOR ACCREDITED COMPOUNDS:

The Commission Decision (2002/657/EC) establishes the performance of analytical methods and the interpretation of results.

The decision limit (CCα) is defined like *the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant.*

EGG:

Analyte (µg/Kg)	ccα	LQ	LM	Range	R^2	% Recov.
Sulfaquinoxaline	0.5	1.0	---	1 - 50	> 0.998	99.5
Nicarbazin	109.3	20	100	20 - 200	> 0.998	101.4
Diclazuril	2.3	0.4	2	0.4 - 20	> 0.998	100.6
Robenidine	28.4	1	25	1 - 50	> 0.998	99.5

LQ: Limit of Quantification **LM**: Max. Limit

MUSCLE:

Analyte (µg/Kg)	ccα	LQ	LM	Range	R^2	% Recov.
Sulfaquinoxaline	112.5	5.0	100	5 – 150	> 0.998	101.2
Nicarbazin	29.3	10.0	25 (except chicken)	10 – 50	> 0.998	100.3
Diclazuril	5.6	0.5	5 (except chicken, rabbit, bovine and pigs)	0.5 – 10	> 0.998	101.0
	0.5	0.5	Absence (turkey, pigs and ruminants)			
Robenidine	5.6	0.5	5 (except chicken and rabbit)	0.5 – 10	> 0.998	100.2
	0.5	0.5	Absence (rabbit)			

RESULTS:

A review of the samples analyzed during the period 2006-2010 has been made, considering the different matrices in which you can find these residues. The total number of samples was 1133, and in 285 of them were detected the presence of anticoccidial.

The samples with coccidiostats, were distributed in 20 % of the total number of egg samples analyzed, 25 % of muscle samples from different matrices (poultry, rabbits, bovine, sheep and goats) and 49 % of feed samples.

In both, egg and feed, the most commonly compound found was nicarbazin, concentrations ranging from 0,1 to 40 µg/kg and between 1 and 108 µg/kg respectively. In muscle was diclazuril and their concentrations were between 0,1 and 23,4 µg/kg.

Figures 3, 4 and 5 show the number of positive samples according to each compound.

About the different muscle matrices analyzed should be noted that in 35 % of rabbit muscle samples and in 29 % of chicken muscle has been found presence of some anticoccidial over 5 years. As shown in Figure 6.

EGG:

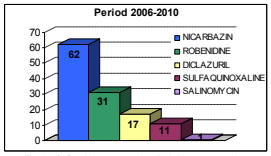

Fig. 3: Nº of positive **egg** samples divided by compound.

FEED

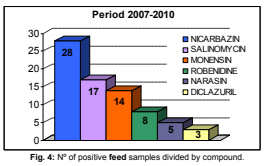

Fig. 4: Nº of positive **feed** samples divided by compound.

MUSCLE:

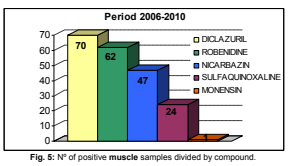

Fig. 5: Nº of positive **muscle** samples divided by compound.

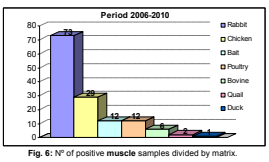

Fig. 6: Nº of positive **muscle** samples divided by matrix.

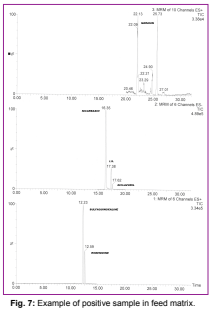

Fig. 7: Example of positive sample in feed matrix.

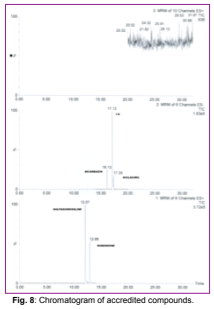

Fig. 8: Chromatogram of accredited compounds.

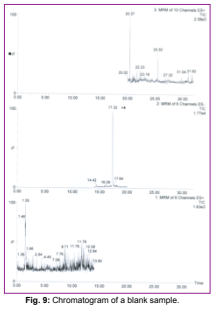

Fig. 9: Chromatogram of a blank sample.

DISCUSSION:

The positive results do not mean that the sample breaks the legal limits, it just points out that they are above the limit of quantification of the method.

The legislation that established the coccidiostats content in food has evolved from total absence, to the actual state, in which a tolerable maximum has been establish, depending on compound and matrix. These limits have been modified several times and it is likely that the regulations in this regard will continue to update based on the results observed.

Analysis of anticoccidial is important for the correct control of suppression periods of these products before human consumption and for check cross-contamination in the feed used.

The method by HPLC-MS/MS is sensitive and selective for the simultaneous determination of these compounds. It is a confirmatory method validated according to EU criteria of the Commission Decision 2002/657/EC.

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