



## Validation of a multi-residue liquid chromatography–tandem mass spectrometry confirmatory method for 10 anticoccidials in eggs according to Commission Decision 2002/657/EC

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### ABSTRACT

A liquid chromatography–tandem mass spectrometric (LC–MS/MS) method for the simultaneous detection and confirmation of halofuginone, robenidine, diclazuril, nicarbazin, monensin, narasin, lasalocid, salinomycin, maduramicin and semduramicin in whole egg has been developed and validated. The anticoccidial residues were extracted by acetonitrile, evaporated and dissolved in a sodium acetate/acetonitrile mixture. Then, the samples were injected on a C8 column in a gradient mode. Diclazuril-bis, DNC-d8 and nigericin were used as internal standards. The results of the full validation in accordance with the guidelines of the Commission Decision no 2002/657/EC are presented. This rapid and sensitive method was found suitable to confirm the anticoccidials at 1 and at 75  $\mu\text{g kg}^{-1}$  for the MRL compound lasalocid.

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### 1. Introduction

Anticoccidial drugs are widely used as feed additives, particularly in poultry farming, for the prevention and treatment of the coccidiosis. This important disease is caused by protozoa of the genus *Eimeria*, which spend most of their life in the intestinal tract of the host animal. The protozoa multiply very fast excreted in the form of sporulated oocysts and the infection starts with their ingestion by the animals from the contaminated environment. Due to their facility to multiply, economic losses can be important, therefore prevention by the use of anticoccidials is necessary. The main anticoccidial compounds used in France as feed additives are divided into two classes, the well known naturally produced polyether ionophores, as monensin, narasin, lasalocid, salinomycin, maduramicin and semduramicin, and the other chemically produced anticoccidials, as halofuginone, robenidine, diclazuril and nicarbazin. Currently, anticoccidial drugs are licensed for use as feed additives for poultry in Europe Union under Regulation No 1831/2003/EC [1], but none of them is authorised for laying hens. For example, monensin sodium as the commercial additive form Elancoban®, is authorised for broiler, turkey and chicken reared for laying.

But in spite of the regulation, residues of anticoccidials are frequently found in eggs. The reasons of the occurrence of these residues in eggs are diverse, as accidental cross-contamination in feed preparation or improper use on farm [2,3]. In Europe Union, surveys or monitoring plans reported this occurrence, in particular for the compound nicarbazin found in its specific marker residue form [4], for robenidine, or for ionophore polyethers like lasalocid or salinomycin [5]. Generally, these anticoccidials have been found safe and effective in target animals given the recommended use levels. However, toxic syndromes can result from overdosage and misuse. For these reasons, anticoccidials are included in the national monitoring programmes for residues of veterinary drugs.

To detect the residues in eggs, reliable and sensitive methods are necessary. To determine one or more anticoccidial drugs in biological matrices, several analytical methods have been described. These methods are based on high performance liquid chromatography with UV [6–11] or fluorescence detection [12,13]. But to detect the substances in low concentrations and to confirm the presence of the analytes, liquid chromatography linked to mass spectrometric detection has been developed recently [14–27].

To our knowledge, only one published LC–MS/MS method [22] deals with 9 of the 10 above-mentioned anticoccidial drugs. The objective of this study was to develop and to validate a rapid and sensitive method for the screening and confirmation of residues of the anticoccidial drugs by LC–MS/MS in order to transfer it to routine laboratories for implementing French control plans in eggs.

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The validation was performed according to the guidelines of Commission Decision No. 2002/657/EC [28]. A limit of quantification of  $1 \mu\text{g kg}^{-1}$  was set except for lasalocid for which an MRL is established at  $150 \mu\text{g kg}^{-1}$ .

## 2. Experimental

### 2.1. Chemical and reagents

Acetonitrile, methanol, dimethylsulfoxide, ethanol and acetone were of HPLC or analytical grade and purchased from Fisher Scientific (Leicestershire, UK). Sodium acetate and formic acid 98–100% were purchased from Merck (Darmstadt, Germany). Demineralised water was HPLC grade generated by a Milli-Q (Millipore, Molsheim, France) purification system. Filters for filtration of the biological extract were from Millipore (Millex GV,  $0.45 \mu\text{m}$ ).

The standards halofuginone lactate and lasalocid A sodium were purchased from Cluzeau (Ste Foy-La-Grande, France) in a  $100 \mu\text{g ml}^{-1}$  acetonitrile solution; robenidine hydrochloride, nicarbazin (marker residue: DNC), salinomycin, monensin sodium, narasin and nigericin sodium were purchased from Sigma-Aldrich Co. (St Quentin Fallavier, France); DNC-d8 from Witega (Berlin, France); diclazuril-bis from Janssen (Issy-les-Moulineaux, France); semduramicin sodium from Phibro Animal Health (Ridgefield Park, USA); maduramicin ammonium from Alpharma (Berchem, Belgium).

Individual stock standard solutions at  $1 \text{ mg ml}^{-1}$  were prepared in methanol for the polyether ionophores, in ethanol for robenidine, in dimethylsulfoxide for DNC and for DNC-d8. The stock standard solutions of diclazuril and diclazuril-bis were specifically prepared at  $0.5 \text{ mg ml}^{-1}$  in acetone. The standard solutions of halofuginone and lasalocid provided by Cluzeau at  $100 \mu\text{g ml}^{-1}$  were used directly for further dilutions.

These stock standard solutions were stored at  $-20^\circ\text{C}$  for halofuginone and for the polyether ionophores, at  $+4^\circ\text{C}$  for diclazuril and diclazuril-bis, and at room temperature for DNC and DNC-d8. Intermediate working solutions were prepared in acetonitrile in order to finally give three working calibration standard solutions, four working validation standard solutions, and an internal standard solution (named 3IS), containing DNC-d8 and diclazuril-bis at  $0.2 \mu\text{g ml}^{-1}$  and nigericin at  $0.5 \mu\text{g ml}^{-1}$ .

### 2.2. Instrumentation

Chromatography was performed on a Thermo Electron Surveyor instrument (San Jose, USA, USA) fitted with a Zorbax Eclipse XDB-C8 column ( $150 \text{ mm} \times 3 \text{ mm}$ ,  $5 \mu\text{m}$ ) from Agilent (Massy, France) and protected with a C8 guard column from Phenomenex (Ashaffenburg, Germany) containing the same material. The MS equipment consisted of a Thermo Electron TSQ Quantum Ultra mass spectrometer (San Jose, CA, USA) controlled by the Xcalibur software operating in both negative and positive ion electrospray mode. The first 6.1 min were in the  $\text{ESI}^+$  mode for halofuginone and robenidine, then switched to  $\text{ESI}^-$  mode for the next 1.4 min, and switched again to  $\text{ESI}^+$  for the polyether ionophores until the end of the run.

### 2.3. Sample preparation

#### 2.3.1. For screening and confirmation of all anticoccidials (except confirmation of lasalocid)

The fresh eggs were broken and mixed. Then 2 g of homogenized egg mixture were weighed into a centrifuge tube. The internal standards (3IS) were added at a concentration of  $20 \mu\text{g ml}^{-1}$  for diclazuril-bis and DNC-d8 (IS of diclazuril and DNC, respectively), and at a concentration of  $50 \mu\text{g ml}^{-1}$  for nigericin (IS of the polyether ionophores). The sample was vortex-mixed and allowed

to stand for 10 min in a dark place. Eight millilitre of acetonitrile were added and the sample was again vortex-mixed to homogenize the material with the solvent. The sample was further placed on a mechanical rotary shaker for 10 min at 100 rpm and then centrifuged for 5 min at 14000 g refrigerated at  $4^\circ\text{C}$ . A 5 ml aliquot of the supernatant was transferred to 12 ml disposable plastic tubes and evaporated to dryness under nitrogen at  $50^\circ\text{C}$ . The sample was redissolved in  $500 \mu\text{l}$  of a 20 mM sodium acetate/acetonitrile mixture (50/50; v/v). The concentrated extract was vortex-mixed briefly, filtered through a  $0.45 \mu\text{m}$  syringe filter, and transferred to an HPLC autosampler vial before injecting  $20 \mu\text{l}$  of the extract into the LC-MS/MS instrument.

#### 2.3.2. For confirmation of lasalocid

A similar sample preparation was used, except that a more important dilution was applied for the preparation of samples containing lasalocid because of signal saturation appearing occasionally on our MS detection system. This saturation is due to the higher concentrations of lasalocid adapted to the spiking solutions to work around the MRL level. The differences with the above mentioned sample preparation consist of evaporating a 1 ml aliquot instead of 5 ml and to redissolve the sample in  $1000 \mu\text{l}$  of a 20 mM sodium acetate/acetonitrile mixture (50/50; v/v) instead of  $500 \mu\text{l}$ .

### 2.4. LC-MS/MS analysis

#### 2.4.1. Chromatography

A gradient was applied with ultra-pure water (A), methanol (B) and acetonitrile (C), each solvent contained 0.1% formic acid. The gradient conditions were as follows: 20% B is apply during the entire gradient; from 0 to 4 min ramp from 0 to 80% of C; hold for 6 min; then ramp over 0.5 min to 80% of A; and hold for 5 min to re-equilibrate the system. The flow rate was  $0.60 \text{ ml min}^{-1}$ , and no split was necessary.

#### 2.4.2. Mass spectrometry

Solutions of each compound ( $10 \mu\text{g ml}^{-1}$  in acetonitrile) were infused at a rate of  $10 \mu\text{l min}^{-1}$  into the detector in flow injection analysis with initial mobile phase conditions (80%A, 20%B) at  $0.6 \text{ ml min}^{-1}$ . The positioning of the source was adjusted; cone voltage and collision energy were tuned to optimize the transition of the precursor to the most abundant product ions monitored in the MRM mode. Dissociation of the precursor ions with argon were induced, which were determined as the molecular ions  $[\text{M} + \text{H}]^+$  for halofuginone and robenidine (1st segment),  $[\text{M} - \text{H}]^-$  for DNC, diclazuril and the internal standards DNC-d8 and diclazuril-bis (2nd segment), and the adduct ions  $[\text{M} + \text{Na}]^+$  for polyether ionophores including the internal standard nigericin (3rd segment). The following MS-MS parameters were used: sample tube or desolvation temperature,  $350^\circ\text{C}$ ; capillary voltage, 4500 V; sheat gas pressure (air), 60 (arbitrary unit); auxiliary gas pressure (air), 10 (arbitrary unit); collision gas pressure, 10 (arbitrary unit); CID source voltage, 5 eV. The tube lens offset and collision energy parameters associated with the precursor and the products ions are given in Table 1.

### 2.5. Method validation according to Commission Decision No. 2002/657/EC

According to Commission Decision No. 2002/657/EC, matrix-matched calibration standards (here named SC) and matrix-matched validation samples (here named SV) were selected in order to estimate the required qualitative and quantitative parameters. The levels to fortify the samples SC and SV were chosen following the MRL for lasalocid and therefore in accordance with the guidelines for the validation of a substance for which a MRL has been established. For the nine other anticoccidials, levels were evaluated

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