



## Short communication

## Determination of amprolium in feed by a liquid chromatography–mass spectrometry method

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## ABSTRACT

As a consequence of the finding of veterinary drugs in food European Community banned several compounds like coccidiostats as amprolium (APL). This antibiotic has been used as a preventive and clinical anticoccidial drug in chicken. The 2005/187/CE, 2005/925/EC Recommendations ban the use of amprolium as additive in chicken feed. For this reason a rapid and sensitive liquid chromatography–mass spectrometry (LC–MS) method was developed to detect amprolium in chicken feed following the European community proposed technique (1999/27/EC) for sample preparation. Cause the validation is required for the analytical methods used in feed official control, this method was validated according to 2004/882/EC Regulation.

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## 1. Introduction

On modern poultry farms, the chickens are raised indoors and are given antibiotics to overcome the effects of crowded and unsanitary conditions or inadequate diets. Antibiotics can be put in feed, water or can be injected. Coccidiosis is one of the many diseases that chickens can contract. It is caused by a protozoa, a parasite of the genus *Eimeria*, and cause intestinal cell disruption, resulting in weight loss or poor weight gain. In the 1950s was developed amprolium, 1-[(4-amino-2-propyl-5-pyrimidinyl) methyl]-2-methylpyridinium chloride hydrochloride, a coccidiostat still used today. Amprolium stops the growth of new protozoa and kills them as well [1].

It shows its effect mainly in the gastrointestinal tract, but it remains in eggs and other organs [2]. In addition amprolium compete with thiamine for intestinal absorption [3]. In fact in ruminants polioencephalomalacia is due to a thiamine deficiency after an excessive administration of this drug [4,5]. Recently, it has been found that some of the microbes causing illnesses have developed a resistance to the antibiotics usually employed to treat the diseases. Then the antibiotics given to poultry for weeks or months at a time in low doses may cause them to harbor resistant bacteria, which they may pass along to caregivers and consumers [6]. The

presence of residue of veterinary drugs in food has received much attention in recent years because of growing concern for safety by consumers. This prompted the European Commission to ban a variety of compounds, including coccidiostats and histomonostat.

Amprolium, a thiamine analog used as coccidiostat and histomonostat in poultry, is no longer authorized as an additive for feeding stuff since January 2006 (2005/187/EC; 2005/925/EC) [7,8].

Aim of this work is the development and validation of LC–MS method for the detection of amprolium in compound feeding stuffs for poultry following the European community proposed technique (1999/27/EC) [9] for sample extraction. The method was validated according to 2004/882/CE Regulation [10], which established performance characteristics to be investigated in the frame of a method validation.

## 2. Experimental

## 2.1. Materials and reagents

Approximately 1 kg of commercial feeding stuff for poultry was ground and thoroughly mixed in a Grindomix Retsch GM 200 (Haam, Germany) for 10 min (0.7 s a 3500 rpm, 0.7 s a 4500 rpm, 0.7 s a 6000 rpm).  $10 \pm 0.1$  g of the sample was extracted with 100 mL methanol/water 80/20 (v/v) in 250 mL flask, blending for 60 min. A 50 mL aliquot of these extracts were filtered on paper filter ( $90 \text{ g m}^{-2}$ , 250 mm) and collected into 50 mL flask. This filtered were diluted 1:10 with initial mobile phase (heptafluorbutyric acid

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**Table 1**  
LC operative parameters.

|                       |                          |              |
|-----------------------|--------------------------|--------------|
| Flow                  | 0.2 mL min <sup>-1</sup> |              |
| Calibration method    | External standard        |              |
| Column temperature    | 30 °C                    |              |
| Sample introduction   | Auto sampler             |              |
| Injection volume      | 5 µL                     |              |
| Gradient mobile phase |                          |              |
| Time (min)            | Eluent A (%)             | Eluent B (%) |
| 0                     | 60                       | 40           |
| 1                     | 60                       | 40           |
| 11                    | 20                       | 80           |
| 15                    | 60                       | 40           |

(HFBA) 5 mM in water/methanol 60/40, v/v) and filtered on PTFE filter (0.2 µm) in vial for LC.

Amprolium hydrochloride 99% (Sigma, Milano, Italy) was used as standard to spike sample feed.

## 2.2. LC–MS conditions

The liquid separation (Table 1) was performed with a quaternary gradient pump (Agilent 1100 series) using a C18 column (150 mm × 2 mm i.d., 3 µm, Pursuit XRs) and a gradient mobile phase (initial phase pH 2.38 at 25 °C) consisting of an initial 60% heptafluorobutyric acid (HFBA) 5 mM in distilled water and 40% methanol for 1 min, then in 11 min the mobile phase was 20% HFBA and 80% methanol and in 4 min the mobile phase returned at initial condition for 5 min, at a flow rate of 0.2 mL min<sup>-1</sup>. An Agilent 1100 series ALS auto sampler was used to inject 5 µL aliquots into the LC column.

Mass spectrometry (Table 2): an ion trap mass spectrometer (Agilent Technologies LC/MSD Trap SL) equipped with an atmospheric pressure ionization source and electro-spray interface was used for the mass spectrometry analyses. The LC column effluent was delivered into the ion source through the electro-spray capillary (1.94 kV), using nitrogen as nebulizing and drying gas (350 °C, 30 psi). Positive ions were acquired in selected-ion monitoring mode. The mass of protonated molecule of amprolium is 243, for the quantification the fragment mass (MS–MS) ion is 150 (M–93).

## 2.3. Validation study

This validation was carried out in accordance with the Regulation 2004/882/CE. The following parameters were evaluated:

1. Applicability (matrix and concentration range).
2. Limit of detection (LOD).
3. Limit of quantitation (LOQ).
4. Precision.
5. Recovery.
6. Specificity.

**Table 2**  
MS operative parameters.

|                         |                          |
|-------------------------|--------------------------|
| Dry temp                | 350 °C                   |
| Nebulizer               | 30.0 psi                 |
| Dry gas                 | 8.00 L min <sup>-1</sup> |
| Fragmentation amplitude | 0.80 V                   |
| HV capillary            | 1.94 kV                  |
| Molecular ion           | 243 m/z                  |
| Width                   | 3                        |
| Cap exit                | 78.7 V                   |
| Selected mass range     | 90–500 m/z               |

7. Linearity.
8. Ruggedness.
9. Measurement uncertainty.

To establish the specificity of the method, representative blank samples (20 samples commercial poultry feed) were analyzed and checked for interferences (signal, peaks, ion traces) in the region where the analyte might elute.

The instrumental linearity was carried out on three curves obtained with 6 levels of amprolium standard in mobile phase (µg mL<sup>-1</sup>): 0.0078; 0.0156; 0.031; 0.0625; 0.125; 0.25, corresponding in the matrix to values from 0.8 to 25 µg mL<sup>-1</sup>.

The calibration, intended as peaks area versus concentration (pg injected), was evaluated by the minima squares algorithm. The linearity was estimated by  $R^2$  (correlation coefficient) and  $yx^{-1}$  (response factors distribution). Acceptability criteria to assume the linearity of response are  $R^2 > 0.99$  and  $(yx^{-1})_{\text{mean}} \pm 10\%$ .

LOD was experimentally detected on the analyses of 20 representative blank samples (poultry feed) and expressed as signal height:  $\text{LOD} = M + 3s$ , where  $M$  is the mean of the areas background in the retention time  $\Delta t = \pm 2.5\% R_{t\text{standard}}$ , and  $s$  is the standard deviation.

LOD as amprolium concentration in the matrix (mg analyte kg<sup>-1</sup> feed) was evaluated using the standard calibration curve (signal height versus concentration in matrix).

LOQ, the lowest concentration of the analyte that can be identified and quantitatively measured in a feed sample using an analytical method with specified accuracy and precision, was estimated with the following equation:  $\text{LOQ (mg analyte kg}^{-1}\text{ feed)} = 3.3 \times \text{LOD}$ .

For the mean recovery and the precision method estimation, blank poultry feed was fortified at three different concentrations in equidistant steps (1, 2, 3 mg kg<sup>-1</sup>). Six independent determinations were carried out at each of the three levels. The 18 replicate analysis were repeated in 3 separate days giving  $n = 54$  determinations. Amprolium concentration in samples was evaluated with the external standard method.

The ruggedness was tested by the introduction of 4 small but deliberate changes in the operating parameters (variables) and by the consequent assessment of their influence on the method results.

It was intended as the sensitivity of an analytical method to alteration in experimental conditions (e.g., store conditions, environmental conditions, change in sample handling). For each experimental conditions that can be subject to changes (e.g., reagents stability, sample composition, pH, temperature) should be remarked every variation that could affect the analysis.

The modified factors were: methanol (MeOH) percentage in extraction solution, different MeOH batches, extraction times, and column temperatures. We developed 8 tests in accordance with Youden approach [11], using a blank poultry feed spiked at 1 mg kg<sup>-1</sup>.

Measurement Uncertainty was estimated according to an internal Standard Operative Procedure, following the “bottom up” approach described in ISO Guide to the Expression of uncertainty in measurement (1993), and applied to chemical analysis by EURACHEM/CITAC Guide “Quantify uncertainty in analytical measurement”, second edition (2000). This Guide assumes that the uncertainty evaluation requires the analyst to look closely at all the possible sources of uncertainty. The uncertainty components were: relative repeatability uncertainty–recovery, weighting relative uncertainty (standard and samples), standard solution relative uncertainty, relative volume uncertainty (pipette and flask). Every uncertainty contribution was indicated as standard uncertainty and

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