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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 veterinarian 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.

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 methylpiridinium 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 ± 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 g m^{-2}, 250 mm) and collected into 50 mL flask. This filtered were diluted 1:10 with initial mobile phase (heptafluorbutirric acid

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Table 1
LC operative parameters.

Flow		0.2 mL min^{-1}
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 hydrocloride 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^{-1}. 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.
7. Linearity.
8. Ruggedness.
9. Measurement uncertainty.

Table 2
MS operative parameters.

Dry temp	350 °C
Nebulizer	30.0 psi
Dry gas	8.00 L min^{-1}
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*

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 carry out on three curves obtained with 6 levels of amprolium standard in mobile phase (μg mL^{-1}): 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^{-1}.

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 $(yx^{-1})_{mean} \pm 10\%$.

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

LOD as amprolium concentration in the matrix (mg analyte kg^{-1} 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: LOQ (mg analyte kg^{-1} feed) = 3.3 × 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^{-1}). Six independent determinations were carried out at each of the three levels. The 18 replicate analysis where 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^{-1}.

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

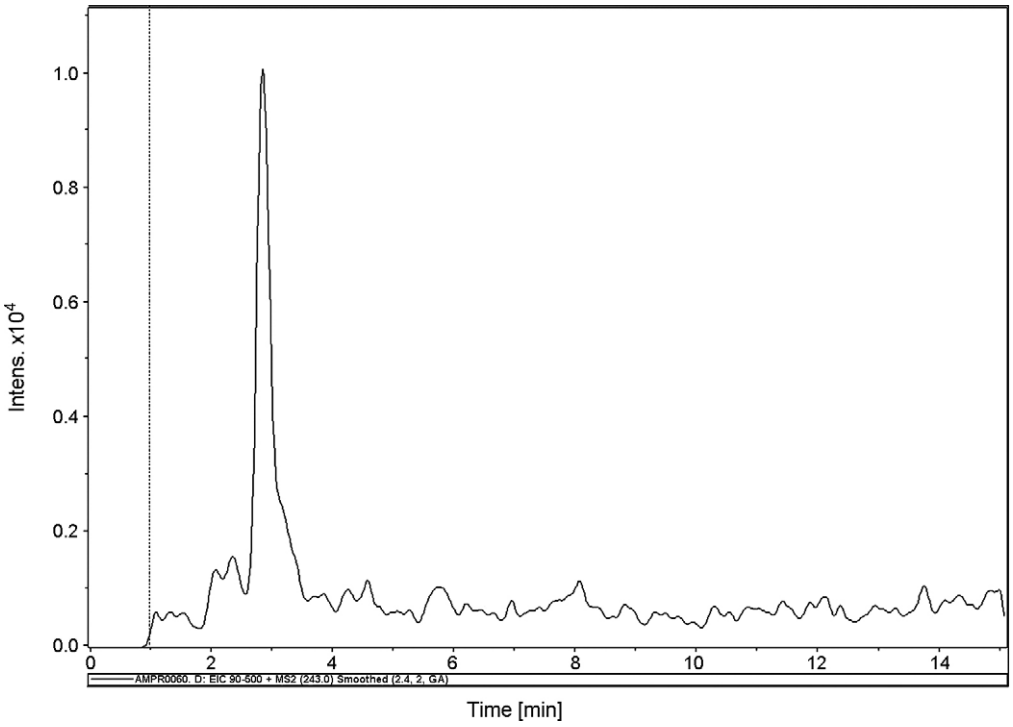

Fig. 1. Negative poultry feed chromatogram.

stated as standard deviation. The compound relative uncertainty (u_c), derived from the square root of the total variance (considering all contributors). The expanded relative uncertainty (U) was estimated multiplying $u_c \times k$ (coverage factor), rounded up 2 for 95% confidence level.

3. Results and discussion

In Fig. 1 a blank poultry feed chromatogram is shown, while in Fig. 2 a spiked poultry feed chromatogram (1 mg kg^{-1}, the LOQ) and in Fig. 3 an amprolium mass spectrum in feed are shown. Specificity

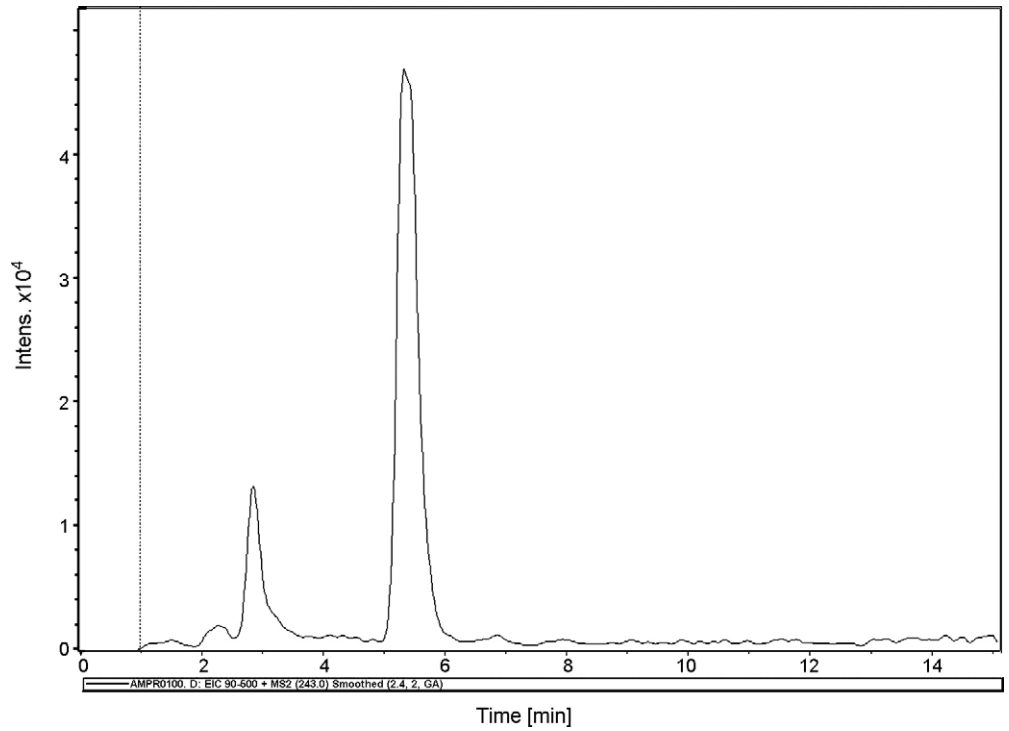

Fig. 2. Spiked poultry feed chromatogram (1 mg kg^{-1}).

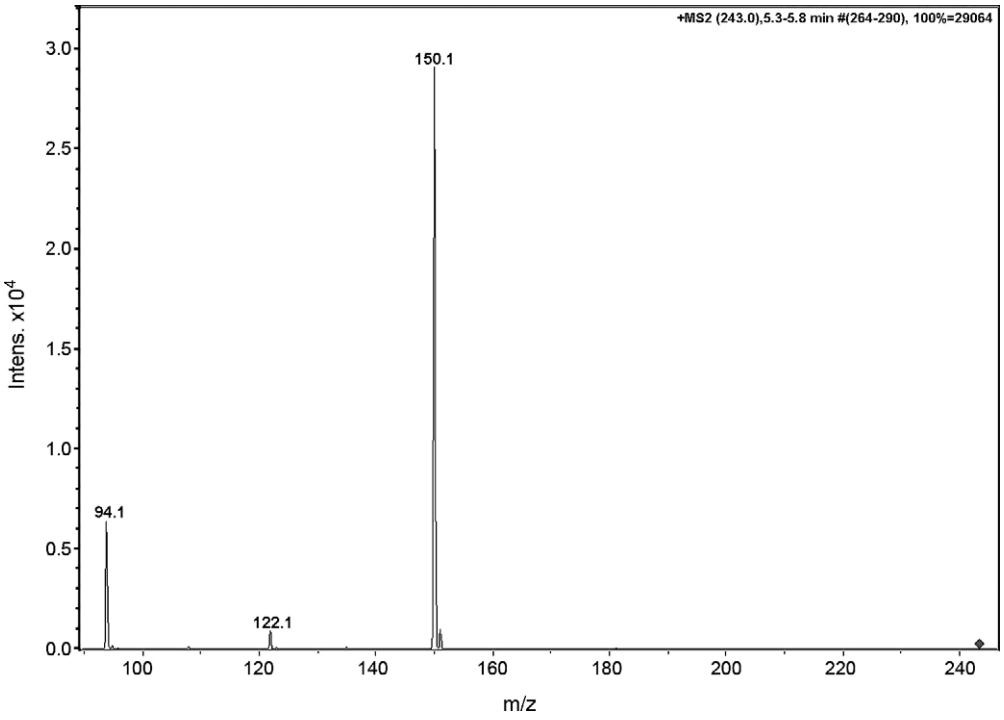

Fig. 3. Amprolium mass spectrum in feed.

trials showed that matrix components and possible additives as vitamins, microelements and other molecules in feeding stuffs do not interfere with amprolium detection and quantification. Table 3 shows regression analyses data. The calibration curve was linear in the range of 0.0078–0.25 μg mL^{-1}. All the parameters showed a linear correlation between standard concentration and peak area in the considered interval and the correlation coefficient was $R^2 > 0.99$ and $yx^{-1} \pm 10\%$. The regression equation was $y = 2E+07x - 3281.3$ where y = amprolium peak area and x = amount of amprolium injection. In Table 3 LOD and LOQ values are displayed. For our aim a LOQ of 1 mg kg^{-1} was chosen, as first spiking level for accuracy trials. Table 3 reports recovery and precision results. After verifying the normality of data distribution (Shapiro Wilk test), for each level the mean recovery and R.S.D.% were evaluated. As we did not observe remarkable differences between the levels, a total mean recovery of 96.8% was estimated. It is acceptable according to European Community criteria. The development of the chromatographic parameters aimed at maximizing sensitivity and minimizing runtime. The short retention times lead to sharp peaks and high sensitivity. In Table 4 uncertainty components and relative expanded uncertainty percentage are shown (12.2%).

Table 3
Validation parameters.

Linearity	$R^2 > 0.990$
LOD (mg kg^{-1})	0.061
LOQ (mg kg^{-1}) measured	0.202
LOQ (mg kg^{-1})	1
Field of measurement (mg kg^{-1})	1–25
Average recovery	96.8%
Average recovery (first level)	89.1%
Average recovery (second level)	98.1%
Average recovery (third level)	103.1%
Precision (1 mg kg^{-1})—R.S.D.%	13.59%
Precision (2 mg kg^{-1})—R.S.D.%	7.49%
Precision (3 mg kg^{-1})—R.S.D.%	12.22%
Uncertainty of measurement	12.2%

Table 4
Uncertainty components and relative expanded uncertainty.

Components	Contribution	Square contribution
Relative repeatability uncertainty–recovery	0.03440	1.1836E−03
Relative volume uncertainty (pipette)	0.04243	1.8000E−03
Relative volume uncertainty (flask)	0.00115	1.3333E−06
Weighting relative uncertainty (analytical scale)	0.00325	1.0580E−05
Weighting relative uncertainty (technique scale)	0.00099	9.800E−07
Relative volume uncertainty (cylinder)	0.00289	8.3333E−06
Standard (powder) relative uncertainty	0.02887	8.3333E−04
Square contribution sum		0.003838
Relative compound uncertainty		0.06195
Coverage factor k		1.96
Relative expanded uncertainty		0.122
Relative expanded uncertainty (%)		12.2

Other methods to detect amprolium in feeding stuffs were issued [6,12], however, our technique offers a number of significant advantages compared to these ones, as a lower limit of quantitation than the method proposed by Hormazabal et al. (2002). Moreover, distinctly from Tan et al. protocol (1996), there is no solid-phase extraction clean-up and this definitely increases the speed of the method for higher throughput analysis.

Besides, LC–MS technique provides a better alternative then HPLC method, cause it has higher sensitivity, selectivity and quantitative capability and represent a first choice method of identification and confirmation of amprolium in feed.

4. Conclusion

The aim of this work was to propose a simple validated method for the detection of amprolium in feed. In fact validation is required for every analytical method employed in feeding stuffs official control. Regarding the method applicability, validation results shown that the method is specific, accurate and suitable in the concen-

tration range of 1–25 mg kg^{-1}. The absence of intermediate steps of purification allows an excellent analyte recovery without an increase of the background and, consequently, of LOQ value. In fact, according to laboratory requirements, LOQ could be lowered, as in repeatability trials the amprolium signal area was enhanced and the background was really low. During the method validation process, we evaluate the measurement uncertainty of the method while, in routine controls; the sample is usually analyzed twice. Therefore, the uncertainty, which should be associated to the quantitative result, ought to be calculated assuming $n = 2$ in repeatability uncertainty–recovery contribution, instead of 12 (which is the number of tests performed during the validation process). Thus, we would obtain a higher expanded uncertainty with a value of 25% instead of 12,2%. Moreover a control chart (Shewart chart) was elaborated using validation data. It can be routinely used to check the maintenance of repeatability and accuracy conditions.

In fact in every analytical trial a control sample (fortified with 1 mg kg^{-1}) was analyzed. The trial is adequate if the recovery percentage comply with the control cart (± 3 standard deviation).

The developed method is advantageous for its simplicity, quickness and for the affordable costs of the analysis. It is consistent with the requirements of the ISO 17025 and it is suitable for quantitative official analysis for either screening and confirmatory of amprolium in feed. We can assume to develop a multiresidual method to detect at the same time, in poultry feed, amprolium and other banned coccidiostats like nicarbazine, methylchlorpindole and dimetridazole.

Acknowledgements

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