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Identification and quantification method of spiramycin and tylosin in feedingstuffs with HPLC–UV/DAD at 1 ppm level

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Abstract

The use of the two macrolides antibiotics Spiramycin (S) and Tylosin (T) as growth promoters in animal feeding has been recently withdrawn in the European Union due to a concern about the outbreaks of farmacoresistance fenomena as a possible hazard for humans. For feed additives monitoring purposes, an analytical method has been developed for their extraction, purification and identification in different animal feedingstuffs (pelleted beef, pig, poultry feeds and calves milk replacer) at a minimum performance required limit (MRPL) of 1 $\mu g\,g^{-1}$ (ppm). Such limit has been established according to the lowest dosage of additives still able to elicit an appreciable growth promoting effect.

Blank feeds were spiked at two concentration levels, 1.0 and 2.5 ppm in six replicates. After methanolic extraction, samples were cleaned up on SPE CN columns and extracts analysed in HPLC–UV/DAD, using a gradient elution. Detection limits, calculated as the tree time mean noise of 20 blank feeds, were 176 and 118 $ng\,g^{-1}$ for S and T, respectively. Results show good repeatability (CV% not exceeding the value of 15) and mean recovery in the range of 99–74% and 81–53% for S and T, respectively, at 1 ppm. When the standards were injected up to 250 ng the chromatographic method can resolve the components of analytes (Spiramycin I, II and III; Tylosin A and B) but can not resolve the components on real feed samples at the spiked levels considered. For this reason the identification and quantification of analytes on matrix were carried out considering the main compound of the drugs (Spiramycin I and Tylosin A). As a verification, the overlapping of UV spectra in the range 220–350 nm between analytical standards and the compounds in the matrix were considered.

Keywords: Growth promoters; Animal feed additives; Tylosin; Spiramycin; Illegal use; HPLC

1. Introduction

Spiramycin and tylosin, two macrolides antibacteric drugs, were authorised in the recent past, as feeds additives (5 ppm) according to Directive 70/524/EC in order to modulate gut microbial flora, thus enhancing the growth rates performances in calves, cattle, pigs and poultry. The application of these antibiotics in feeding stuffs are regulated from Council Directive 70/524/EEC [1].

Recently in December 1998, health ministers of European Union countries voted to ban the remaining human-use or related antibiotics still administered to promote animal growth, so that they application will be restricted only to veterinary therapeutic use only [2].

The request for the withdrawal of the authorisation was based on the scientific evidence that the systematic administration of such compounds at sub-therapeuthic doses can lead to the development of antibiotic-resistant bacteria that can be transferred to people, making it more difficult to treat certain infections [3–5]. Spiramycin and tylosin are related to erythromycin and can show cross-resistance although they are not used in human medicine.

Bacteria that are present in farm animal and that have developed resistance to antibiotics can transfer these resistant genes to human pathogens. A particular gene may be resistant to more than one antibiotic. The consequences of multi-gene plasmid transfers are extremely worryng, as JETACAR points out: "... if an antibiotic from an antibiotic family that is not used in human medicine is used in animal production, it may still affect the levels of bacteria that are resistant to important human antibiotics".

In order to perform an efficient official controls of the possible illegal use of these drugs in feedingstuffs, reliable

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Table 1
Composition of feeds (%)

	Poultry	Pig	Cattle	Lactoreplacer for calves
Water	13.5	12.6	11.3	3.9
Ash	6.8	6.0	8.0	6.4
Protein	16.3	16.2	16.6	22.4
Fat	2.3	3.3	3.0	21.0
Fiber	5.7	4.9	8.0	0
Additives	ND	ND	ND	ND

ND: not detected (coccidiostats and antibiotics).

methods of analysis need to be available. Furthermore, these methods should be able to detect the drugs at contents five times lower than lowest contents for which they were formerly autorised [6]. The aim of this study was to develop a suitable HPLC–UV/DAD method, which could be used to detect, and to possibly, to identify the illegal presence of spiramycin and tylosin in different feedingstuffs at 1 ppm level. Such a limit has been chosen according to the reported lowest dosage of additive still able to elicit an appreciable growth promoting effect [7,8].

2. Experimental

2.1. Samples

Proposed blank reference samples of pig, poultry, beef and calves feeds were supplied by IRMM (Geel–Belgium). Their composition are reported in Table 1. Other samples were collected on the market and also kindly provided by the DGCCRF (Rennes, France). The sum of samples from IRMM and DGCCRF were 20, within the frame of the EU granted SIMBAG FEED project. These were previously tested for the absence of antibiotics by an analytical microbiological test. The microbiological diffusion assay was performed according the criteria described by AOAC International [9]. These samples (50 g) were thoroughly minced and test samples (5 g) were formed and stored at +4 °C.

Also fortified references samples at 5 ppm level for both analytes were supplied by IRMM. Their composition are the same of blank reference samples.

2.2. Reagents and solvents

Only reagents of recognised analytical grade and distilled water must be used; for HPLC analysis use only reagents HPLC grade.

Products used in their commercially available form include:

Acetonitrile (Carlo Erba, Milan, Italy); diethylamine (Sigma, Steinheim, Germany); *n*-hexane (Carlo Erba); methanol (Carlo Erba); orthophosphoric acid (85%) (Merck, Darmstadt, Germany); hydrochloric acid (Carlo Erba); sodium acetate (Carlo Erba); potassium dihydrogen phosphate (Merck); di-potassium hydrogen phosphate (BDH, Broom, UK); di-sodium hydrogen orthophosphate dihydrate (Merck).

2.3. Solutions

2.3.1. Acetate buffer (pH 5.5; 0.15 M)

Weight 12.30 g of sodium acetate in a 1000 ml graduated cylinder. Add 900 ml water HPLC grade until complete dissolution. Adjust the pH to 5.5 by addition of HCl 1N solution. Bring the volume to 1000 ml final with water.

2.3.2. Phosphate buffer (pH 8.0; 0.06 M)

Weight 9.08 of potassium dihydrogen phosphate in a 1000 ml graduated cylinder. Add water HPLC grade to the mark and to dissolve (A solution).

Weight 11.88 of di-sodium hydrogen orthophosphate dehydrate in a 1000 ml graduated cylinder. Add water HPLC grade to the mark and to dissolve (B solution). The buffer at pH 8.0 is prepared mixing 5.5 ml of A and 94.5 ml of B solutions to reach the final volume of 100 ml.

2.3.3. Solvent A (mobile phase for HPLC)

Weight 8.71 g of di-potassium hydrogen phosphate are in a 1000 ml graduated cylinder. Add 900 ml water HPLC grade until complete dissolution. Adjust the pH to 2.5 by addition of 85% orthophosphoric acid. Bring the volume to 1000 ml final with water.

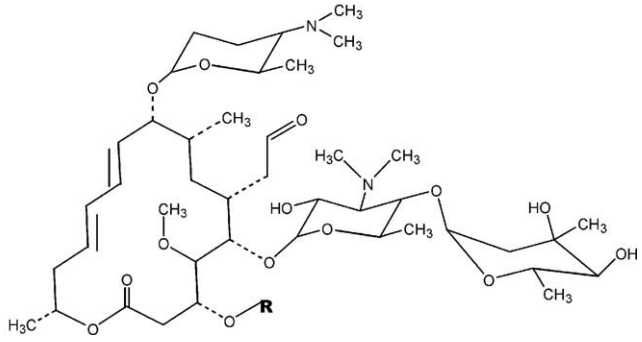
The solvent A on the mobile phase for HPLC was prepared mixing the solution above described with ACN (80:20 (v/v)).

2.4. Analytical standards

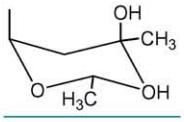
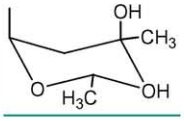
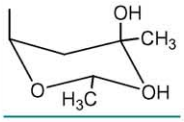
Spiramycin (S) and Tylosin tartrate (T) were purchased from Sigma Italia (Milan, Italy); yours chemical structure were following reported.

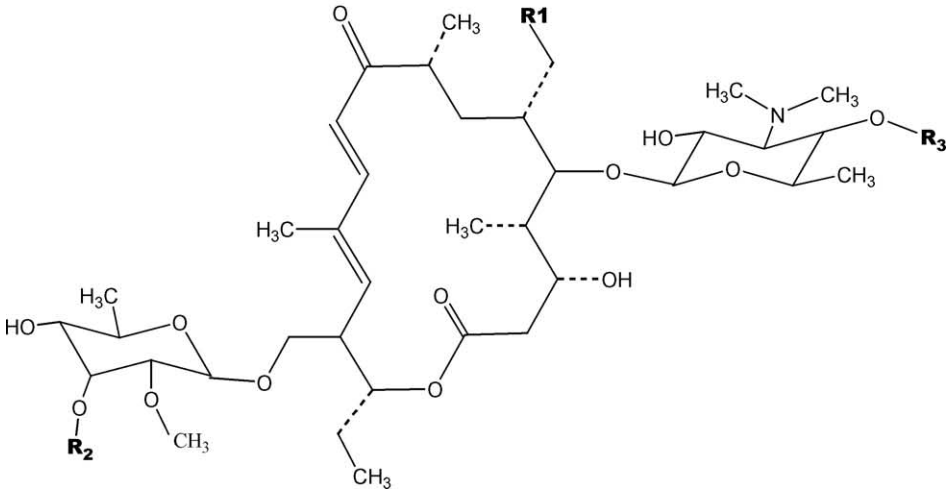
Chemical structure of macrolide antibiotic Spiramycin

Compound	R
Spiramycin I	- H
Spiramycin II	- $COCH_3$
Spiramycin III	- $COCH_2CH_3$



Chemical structure of the macrolide antibiotic Tylosin

Compound	R1	R2	R3
Tylosin (A)	- CHO	- CH3	
Desmycosin (B)	- CHO	- CH3	- H
Macrocin (C)	- CHO	- H	
Relomycin (D)	-CH2OH	- CH3	



2.5. Apparatus

Usual laboratory apparatus and, in particular, the following: Centrifuge (Heraeus); pH meter (Crison); Rotary Evaporator (Buchii); Vortex (Heidolph); Moulinette mincer; nitrogen evaporation station (Stepbio); SPE vacuum manifold (Supelco); analytical balance (Mettler); macroanalytical balance (Mettler); plastic insulin syringes (1 ml); stoppered centrifuge glass tube (40 ml); oven (Heraeus); Thermoshake (Gerhardt); HPLC–UV/DAD equipped with a model 126 pump and a model 168 photodiode-array detector (System Gold Nouveaux-Beckmann); column: RP-C18 250 × 4 (Merck).

2.6. Cartridge and matrix for the solid phase extraction

SPE column cyano-propyl non-endcapped SPE 500 mg 3 ml (Mallincrodt, J.T. Baker).

Aluminium oxide 90 active, acidic (activity I) (Merck).

2.7. Preparation of standard solutions

2.7.1. Stock solutions

25.000 mg ± 0.001 of S and 28.25 ± 0.001 mg of T were accurately weighted, transferred to a 25 ml volumetric flask and dissolved in methanol, to reach the final concentration of 1 mg ml^{-1}. In the dark such a solution is stable for 1 month at +4 °C.

2.7.2. Working solutions

Two hundred and 100 μl of the stock solutions is transferred in a 10 ml volumetric flasks and brought to the final volume with methanol to reach the nominal concentration of 20.00 and 10.00 μg ml^{-1}. The last solution is then serially diluted 1:1 in methanol to reach the final concentrations of 5.000, 2.500, 1.250, and 0.625 μg ml^{-1}. The working solution should be prepared at the time of use.

2.7.3. Spiking solution

0.250 and 0.625 ml of the 20.00 μg ml^{-1} working solutions are used to spike negative feeds (5 g) at 1 and 2.5 ppm levels.

2.8. Preparation of the standard curve

Methanolic working solutions are used to build up the calibration curve on the following concentrations: 1000, 500, 250, 125 and 62.5 ng injected into the HPLC.

2.9. Spiking of samples

Blank feeds sub-samples (5 g) were fortified with 0.250 and 0.625 ml of the 20.00 μg ml^{-1} working solutions S and T to attain the final concentration of 1 and 2.5 ppm. The spiked samples were left to stand for 1 h at room temperature in the dark.

2.10. Procedure of extraction and purification

2.10.1. Extraction

The IRMM feeds were supply thoroughly minced, so they were directly used for extraction step. The ICRFF samples that were on pelleted form were grinded with a Moulinette mixer and sieved (1 mm) before analysis.

Homogeneous feed aliquot (5 g), were extracted twice with methanol (10 ml + 10 ml) (30 min of shaker for single extraction). Supernatants were recovered by centrifugation (4000 × *g*, 15 min +15 °C) and pooled in a 50 ml round flask (final volume around 15 ml), then dried in a rotary evaporator (+35 °C). The flask was rinsed adding 200 μl methanol, gently hand-shaken, then 4.8 ml acetate buffer (pH 5.5; 0.15 M) is added and again shaken. This fraction was collected in a 50 ml centrifuge glass tube. The lipids were eliminated with hexane. Thus was add to a flask with 5 ml *n*-hexane, gently hand-shaken and the organic phase transferred into the same glass tube where the acetate buffer fraction had been recovered. The flask is rinsed again with 1 ml acetate buffer and the aqueous phase is transferred into the same glass tube. Then, vortex it to 30 s to achieve a final liquid–liquid partition. After centrifugation at 2000 × *g*, 5 min +15 °C, the organic phase was discharged. The aqueous phase (final volume 6 ml) was processed for the cleanup.

2.10.2. Clean-up

The clean-up was executed in two steps: first step involves the use of SPE cyano-propyl cartridge; in the second step an alumina column is prepared at the time of clean-up.

The first step clean-up was performed loading (flow-rate gravity) the extract (6 ml) onto SPE cyano-propyl cartridge previously conditioned with 3 ml methanol and 5 ml water (flow rate gravity). The column should not be allowed to dry. The SPE columns were then washed with 3 ml of deionized water (flow-rate 1 ml min^{-1}); 3 ml methanol (flow-rate 1 ml min^{-1}). The column is then dried by air flushing. The elution was carried out with 5 ml of methanol (1% diethylamina) (flow-rate gravity). Each elute fraction was collected, dried under nitrogen stream +35 °C in a 10 ml conical vial and resuspended in 1 ml of solution phosphate buffer (pH 8.00; 0.6 M)/methanol (1:1 (v/v)). This was loaded onto an alumina column prepared in the following way: alumina was activated in an oven (+500 °C, 12 h). After activation, preserve the alumina, not exposing it to moisture. Next, 1 ml plastic insulin syringes were plugged at the bottom with glass wool, filled with 0.5 ml of activated alumina and again plugged at the top again with glass wool. The columns were placed on a support to maintain them in a vertical position. A (1 ml) sample was loaded onto the alumina column (flow-rate gravity). First, 0.1 ml was discharged. The resulting 0.5 ml was recovered in a conic vial and 50 μl was injected into HPLC.

2.11. Determination of S and T by HPLC/DAD

The HPLC instrument was run under the following experimental conditions: mobile phase, solvent A: di-potassium hydrogen phosphate (pH 2.5; 0.05 M)/ACN (80:20 (v/v)), solvent B: ACN 100%; flow rate: 0.7 ml min^{-1}; the gradient performed for the analysis is indicated in the following scheme:

Time (min)	B%	Duration (min)
0.01	0	
0.5	40	12.00
12.50	40	2.00
14.50	0	1.00
20.00	End of run	

Injection volume: 50 μl; wavelengths: Spiramycin 232 nm, Tylosin 280 nm. UV spectra recorded in the range 220–350 nm and acquired in the purity mode.

2.12. Limits of detection and quantification/ validation study

The validation study was performed according to Commission Decision 2002/657/EC for confirmatory purposes in LC technique [10]. The following criteria was considered for spectra identification: the same maxima are present and the difference between the two spectra is at no point observed greater than 10% of the absorbance of the calibration standard, or of the fortified reference samples, with reference to the data spectrum kept in our computer library. To evaluate the power of discrimination between the compounds and possible interferences were analysed representative blank samples ($n = 20$). Verified the absence interferences at the retention times of the analytes on blank feeds, the recovery test was carried out by analysing the blank feeds that were fortified by addition of a quantity of spiramycin and tylosin at 1 and 2.5 time the minimum required performance limit. Six aliquots of each kind of blank material ($n = 4$) were selected; the total samples used for recovery test were $n = 24$). The recovery (%) was calculated as ratio between the really measured standard concentration in the spiked sample and the theoretical value of standard concentration at the fortification level * 100. The above mentioned concentrations were obtained by plotting the interest peak areas versus an calibration curve of standards. The calibration curves were derived with the standards, on five points in the range 5.33–0.33 mg kg^{-1}.

To evaluate the specificity of chromatographic method, a mixture of closely related substances was injected onto HPLC: erythromycin, oleandomycin, troleandomycin and tilmycosin (1250 ng injected).

The decision limits as CC_α (the smallest content of the analyte in matrix that may be confirmed with 95% probability) were calculated as result of three time the signal-to-noise ratio on 20 blank samples in correspondence to the retention time of each analyte. The detection capability as CC_β (smallest content of the analyte from which sample is truly violative with a confidence limit of 95%) was calculated as sum of the CC_α value plus 1.64-fold the standard deviation of the within-laboratory reproducibility at the lowest concentration level considered (1 μg g^{-1}).

The practical limit of quantification was estimated as the concentration equal to twice the detection capability of the method.

3. Results and discussion

The chromatographic analysis of standards, injected onto HPLC up 250 ng, can distinguish their main fractions components: components I, II and III for spiramycin; components A and B for tylosin). When we analysed the feed samples all evaluations were carried out considering only the main compound of analytical standards: spiramicyn I and tylosin A.

Data of the calibration curves (equation and regression coefficient) performed with the standards were respectively: spiramycin: $y = 1161.7x - 3291.2$, r^2 0.9993; tylosin: $y = 1538x - 260.11$, r^2 0.9995.

Matrices background evaluated from the 20 blank feeds as area recorded at the RT of the analytes are reported in Fig. 1. The minimum performance required limit (MRPL) was considered at level of 1 ppm, the decision limits as CC_α ($\alpha = 5\%$) (the smallest content of the analyte in matrix that may be confirmed with 95% probability) were calculated as result of three time the signal-to noise ratio on 20 blank samples in respect to the retention time of each analyte.

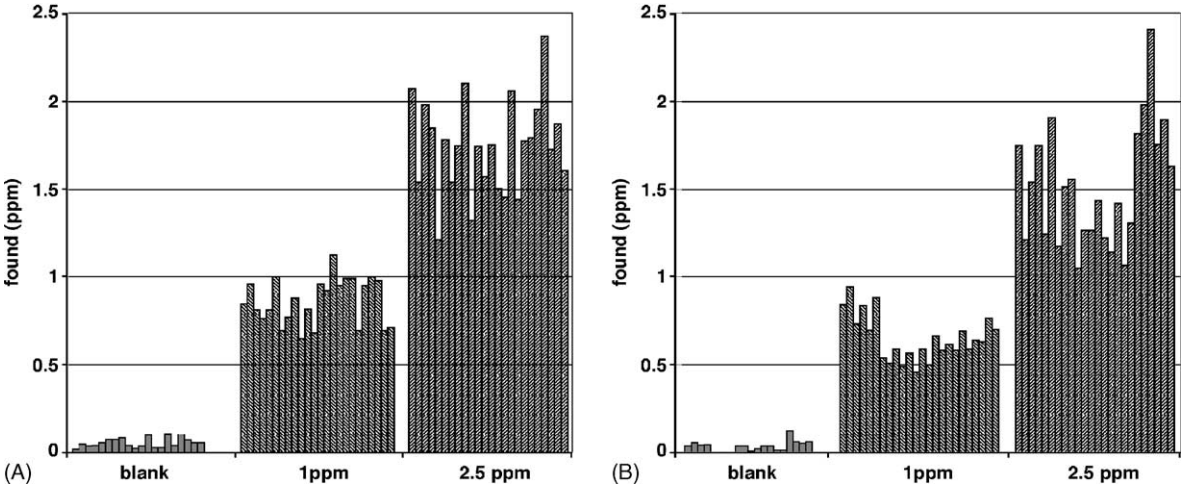


Fig. 1. Comparation between distribution of matrices background evaluated from 20 blank feeds and the amounts (μg g^{-1}) of analytes recovered in 1 and 2.5 spiked feeds: (A) spiramycin; (B) tylosin; blank feeds (▭) series $N = 20$ from left to right: cattle feed ($n = 4$); calves feed ($n = 4$); pig feed ($n = 4$); poultry feed ($n = 4$) cattle feeds ($n = 4$) from DGCCRF spiked 1 (▨) and 2.5 (▨) ppm feeds series ($N = 24$) from left to right: cattle feed ($n = 6$); calves feed ($n = 6$); pig feed ($n = 6$); poultry feed ($n = 6$).

Table 2
Recovery mean (rec %), standard deviation (S.D.) and variation coefficient (cv%) of spiramycin and tylosin on different feedingstuffs at two spiked levels 1 and 2.5 ppm

Feed	*N*	Spiramycin (ppm)						Tylosin (ppm)					
		1			2.5			1			2.5		
		rec%	S.D.	cv%	rec%	S.D.	cv%	rec%	S.D.	cv%	rec%	S.D.	cv%
Cattle	6	86.80	9.50	10.95	89.22	12.09	13.55	81.74	9.04	11.06	79.78	11.36	14.24
Milk replaced	6	74.88	8.85	11.82	84.30	12.22	14.50	53.17	4.96	9.34	64.60	9.52	14.73
Pig	6	99.26	7.24	7.30	83.22	12.12	14.56	58.91	5.32	9.03	62.61	7.42	11.84
Poultry	6	83.46	12.91	15.47	94.40	13.41	14.20	67.30	6.10	9.06	62.00	7.56	12.20

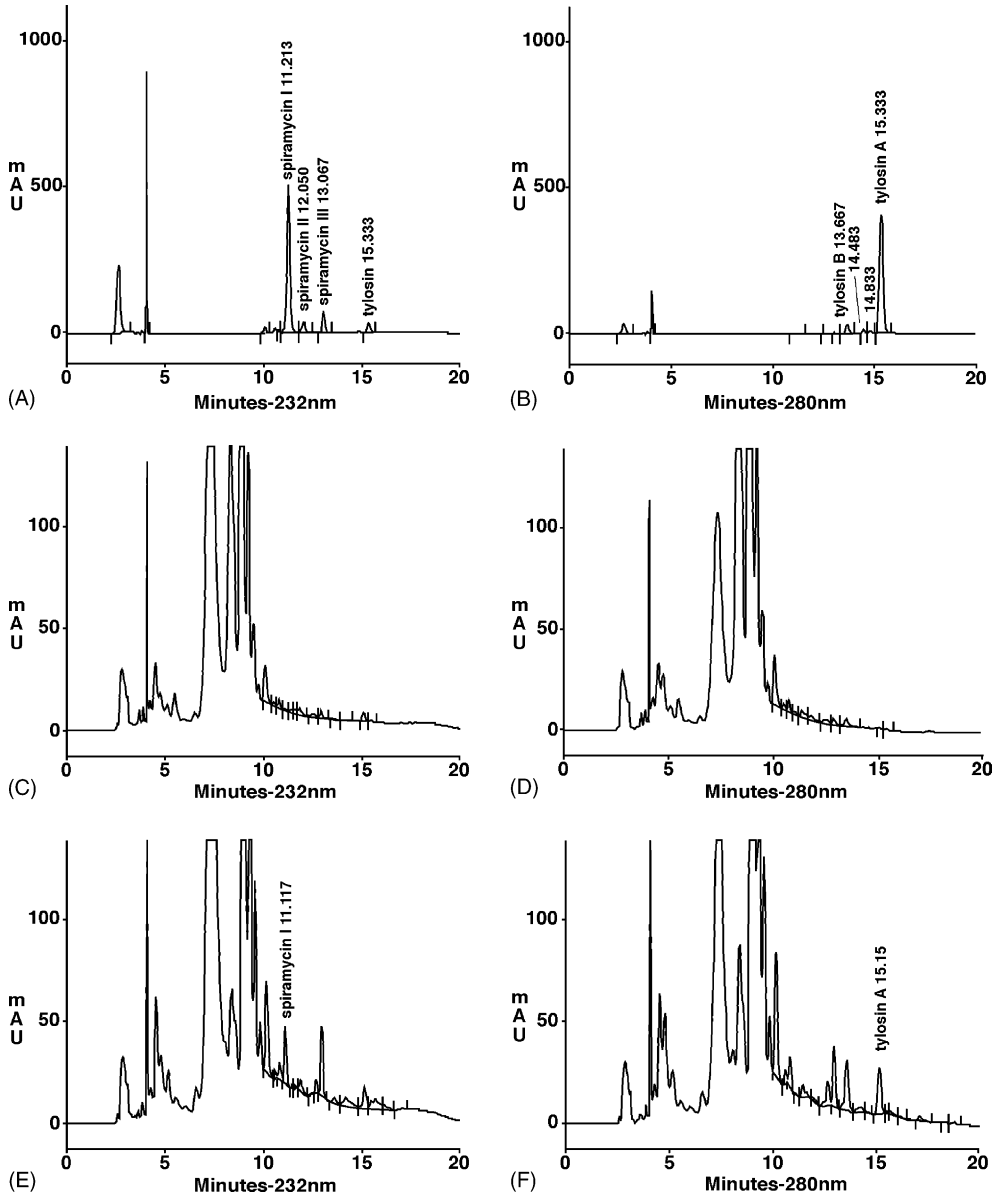


Fig. 2. Chromatograms of (A) spiramycin standards 2.5 μg injected; (B) tylosin standard 2.5 μg injected; (C and D) blank cattle feeds; (E) 1 ppm spiked with spiramycin and (F) tylosin cattle feeds.

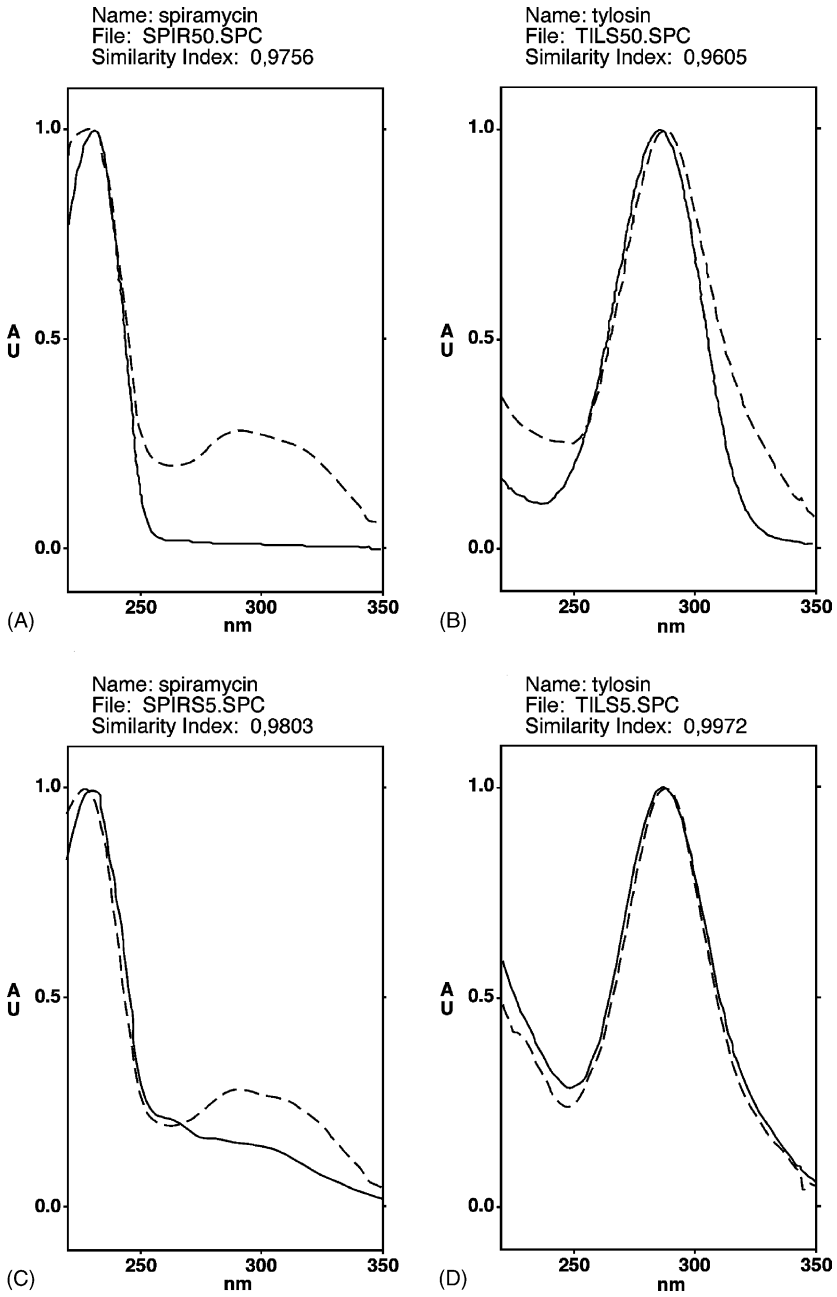


Fig. 3. Similarity index between spectrum data of spiramycin (A) and tylosin (B) spiked at 1 ppm level in swine feed and their standard solutions; Similarity index between spectrum data of spiramycin (C) and tylosin (D) spiked at 1 ppm and the reference material fortified at 5 ppm.

These were 176 and 118 ng g^{-1} for S and T, respectively. The detection capability as CC_β ($\beta = 5\%$) was 395 and 352 ng g^{-1}, respectively for S and T. In the same feeds, spiked at 1 and 2.5 ppm, the mean values of percentage recovery (Rec %), standard deviations (S.D.) and variation coefficents (CV%) for any matrix (replicates, $n = 6$) are reported on Table 2. The chromatograms of standards, blank and spiked cattle feed at 1 ppm level are reported in Fig. 2(A, B, C, D, E, F).

In Fig. 3 are shown the comparisons of spectral data in the swine test samples at 1 ppm level spiked and the standards solution (A, B) or the fortified reference material at 5 ppm level.

The specificity of chromatographic analysis for other macrolide drugs was evaluated. Displayed in Fig. 4(A, B and C) are the chromatograms of a four macrolides mixture (spiramycin, tilmycosin and tylosin) at three different wavelengths.

The results of chromatographic method show a good specificity, in fact erythromycin, oleandomycin and troleandomycin were not found while and tilmicosin showed different retention times of macrolides of interest.

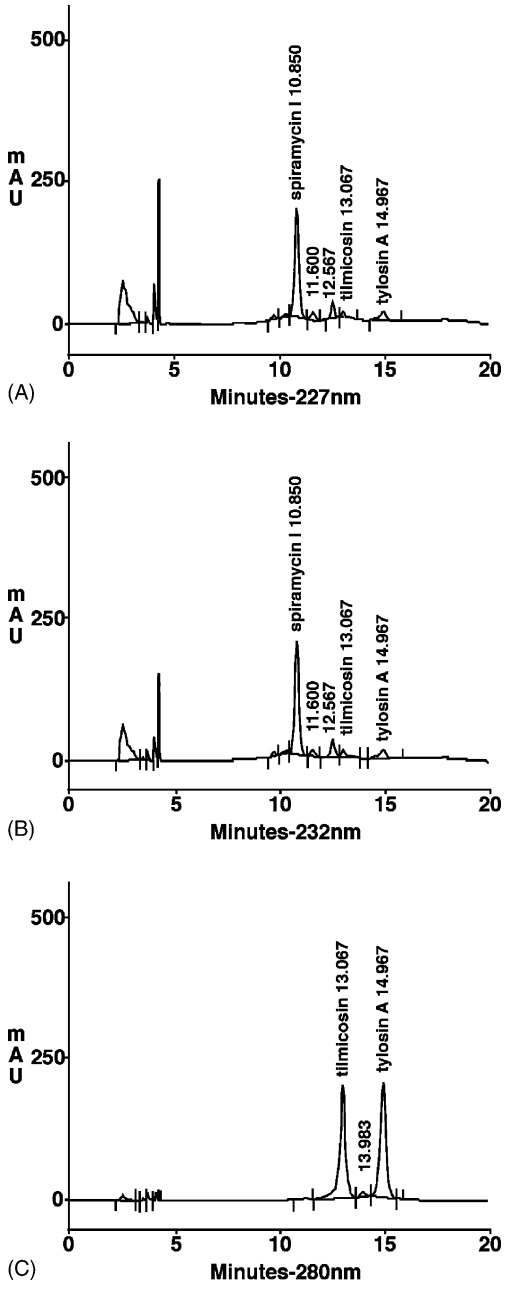


Fig. 4. Specificity of chromatographic analysis for separation of a mixture of three macrolides at three wavelengths (1250 ng injected).

4. Conclusion

After the prohibition in the use of macrolides, superior analytical methods for identification of these were required in feedingstuffs. The microbiological tests can be used for screening procedures, however these often do not discriminate antibiotics well enough and produce false positive non-compliant results. On the other hand, the confirmatory methods LC/MS(MS) are difficult to perform. The HPLC methods offers a good selectivity and specificity together with the ease of applicability. Furthermore, by the comparison of the spectral data (DAD) in the test sample to that of the calibration solution, HPLC method can be used as a simpler preliminary confirmatory mean method of verification than LC/MS(MS) able to use on official controls.

The HPLC/DAD method so far described can distinguish the molecules of the same drug family without erythromycin and oleandomycin causing very low extinction coefficient and so this can be considered a multi-drug method.

The methanolic extraction has is the double effect of to solubility the analytes and to remove the protein could interfere with the polar interaction of drugs with the functional group of CN/SPE column. Additionally, the proteins having an UV absorbency at 280 nm, could be interfering with analysis of tylosin that are also a maximum at the same wavelengths. The acidic alumina column, was effective to reduce background around the retention time of S.

The double steps of purification of the extract can permit a better clean up to be obtain and to eliminate the interference of blank matrices. This purification procedure allows the identification of the analytes at 1 ppm level.

We have investigated the applicability of method using four different animal feeds. The comparison of results on the different matrix showed that the method can be performed for the identification of S and T at MRPL level which eventually presents itself in feedingstuffs having different composition. The recovery is good throughout the different feed compositions. The removal of lipids with hexane is major factor when the lipid percentage in feed is very high (milk replacers 21%) (Table 1).

The spectrum analysis of peaks eluted in the test samples at the same retention time of the standards allow the unequivocal identification of molecules and to confirm the presence of drugs by evaluation of similarity index.

For legal purposes, the results of analysis can be basically expressed as presence/absence of spiramycin and/or tylosin above/below the MPRL in the feed. In making a decision if a sample is positive or negative the following conditions can be considered:

A sample is suspected positive, when:

(1) With respect to the RT of the Spiramycin I and Tylosin A standards there is a peak whose area is within an matrix calibration curve realised with an analogous blank feeds spiked at appropriate concentration levels of standards.
(2) The blank sample chromatogram does not show any relevant interference in respect of the standards RT (whose area does not exceed the apparent concentration of 0.150 ppm in feed both for S and T; this value corresponding at CC_α ($\alpha = 1\%$).

A sample is non-compliant when:

(1) The above mentioned criteria are fulfilled.

(2) The UV spectra of the target peak (eluted in respect of the RT of the standards) does not differ more than 10% from the UV spectra acquired from target peak of the spiked feed, or from that of the standard injected at comparable concentration.

A sample is truly negative (S and T content below the MRPL) when:

(1) The sample chromatogram does not show any relevant interference in respect of the Standards RT (whose area corresponds to an apparent concentration in the feed not exceeding 0.395 ppm for S and 0.352 ppm for T, respectively.
(2) The recovery on the spiked sample, calculated according to the external standard method, was considered by the following criteria, plotting nominal concentrations against recorded area of the points considered in the standard curve, is >55% for T and >75% for S.
(3) The blank feed chromatogram shows interfering peaks at the RT of S and T whose area corresponds to an apparent concentration in feed not exceeding 0.150 ppm both for T and S.

Acknowledgements

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