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Determination of tylosin residues in different animal tissues by high performance liquid chromatography

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Abstract

A HPLC method to determine and quantify tylosin residues from calves, pigs and poultry is reported. This procedure permitted tylosin to be separated from muscle, liver, kidney and fat after a simple extraction with chloroform or ethyl acetate under basic conditions. The analytical methodology showed a high specificity and sensitivity and an adequate precision and accuracy with a limit of quantification of 50 μg/kg. Eight calves were administered 20 mg/kg/day of tylosin for 5 days and slaughtered at 7 and 14 days post-administration. Results showed that at the 14th day tylosin levels were lower than the MRL in all target tissues.

Keywords: Tylosin

1. Introduction

Tylosin is a macrolid antibiotic produced by *Streptomyces fradiae*. It is active mostly against Gram-positive bacteria, anaerobic bacteria and mycoplasma. Tylosin is used in the treatment of dysentery and atrophic rhinitis in pigs, pneumonia, arthritis and mastitis in cattle, and mycoplasma infections in poultry [1].

To ensure human food safety, the European Union (EU) has established maximum residue limits (MRLs). When tylosin is administered to domestic species, the fixed MRLs for each target tissue (liver, kidney, muscle and fat/skin+fat) are 100 μg/kg.

Different methods have been developed to confirm the presence of tylosin in tissues. Among these studies there are microbiologic assays [2–5], thin-layer chromatography (TLC) [6–8], liquid chromatography (LC) [5–7,9–12], liquid chromatography–mass spectrometry (LC–MS) [13,15–17] or pyrolysis–gas chromatography methods [19].

High performance liquid chromatography (HPLC) by UV detection methods for determination of tylosin residues has also been reported [11,12,14,16,18]. These methods use different solid extractions, such as cation exchange or silica gel cartridge [11,12,14,17,18], but this procedure is sometimes expensive and could not be repetitive when a high number of samples need to be analysed.

Some studies used different ranges of pH to separate tylosin from the biological matrix [6–8,16], but these extractions tend to be complicated and longer.

Nevertheless, there is not much information avail-

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able about techniques that could be useful at the same time for different animal species and for a wide range of tissues.

The aim of this study was to develop a HPLC method to detect and quantify tylosin residues in all target tissues of calves, pigs and poultry with a limit of quantification (LOQ) at less than half the MRL fixed by the health authorities of the EU and also dispose of a methodology which allowed to determine the withdrawal period necessary to avoid any risk for human health after consuming tissues proceeding from animals treated with the drug.

2. Experimental

2.1. Chemicals

Tylosin tartrate was obtained from Sigma (St. Louis, MO, USA). Tylosin base was obtained from LABIANA Life Sciences, Labs. (Terrassa, Spain). Albendazole was obtained from Shering-Plough (NJ, USA). HPLC reagent grade (acetonitrile, methanol and ethyl acetate) and analytical reagent grade (chloroform, dichloromethane and orthophosphoric acid) were purchased from Riedel-de Haën (Seelze, Germany). Na_2HPO_4 and KH_2PO_4 were obtained from Merck (Mollet, Spain) and NaOH from Panreac (Montcada i Reixac, Spain). Water was double distilled.

2.2. Chromatographic system

The HPLC system consisted of an automatic injector Model 712 Wisp, a 515 pump and a 486 UV detector from Waters. Separation was achieved on a C_{18} reversed-phase column (20 mm×4.6 mm, ODS2, 5 μm, Waters, Milford, MA, USA). The column was used at room temperature.

The mobile phase was acetonitrile–H_2O 0.04 *M* Na_2HPO_4 pH 2.4 (33:66 v/v) and the flow-rate was 1.5 ml/min. The chromatogram was monitored at a wavelength of 280 nm, 0.01 AUF. Data processing was handled by a computer system (PE Nelson Turbochrom.4).

2.3. Animals

A multiple dose of 20 mg/kg of tylosin base was administered by i.m. route to eight calves every 24 h for 5 days.

Afterwards, the animals were slaughtered in groups of four at the 7th and 14th days post-administration. Samples of the different target tissues (muscle, liver, kidney and fat) were collected and frozen in 50-g capacity tubes at −80°C until processing.

2.4. Samples

Samples (tissues from calves, pigs and poultry) used for the method validation came from untreated animals that were slaughtered in order to collect samples from the different tissues. All the samples were then homogenised, stored in different containers and kept frozen at −80°C until processing.

In order to know the stability of tylosin, the different tissues were spiked with a known concentration of tylosin and stored in the same conditions as the problem samples. Samples were then defrosted and processed at different times to quantify the amount of tylosin.

2.5. Tissue sample procedure

2.5.1. Muscle, liver and kidney

Five grams of muscle were collected and 3 μg of albendazole (30 μl from a solution of 0.1 mg/ml) were added to the sample as internal standard. The aim of adding the internal standard was to correct easily the loss of tylosin that happens during the extraction and to make the procedure easier.

Samples were homogenised with 10 ml of chloroform and 1 ml of phosphate buffer (Na_2HPO_4 and KH_2PO_4, 0.07 *M*, pH 8.5). After shaking for 20 min, samples were centrifuged for 20 min at 1932 *g*. The chloroform was then collected and dried in a Speed Vac at 40°C. The dry extracts were redissolved in 4 ml of acetonitrile–water (8:2) and a liquid–liquid partition was performed with 10 ml of dichloromethane.

After shaking and centrifuging for 10 min, the organic phase was collected and another extraction was carried out with 5 ml of dichloromethane. All

the dichloromethane collected was evaporated in a rotary-evaporator. After dissolving the dried extracts in 200 μl of mobile phase, 50 μl of sample were injected in the HPLC system.

Liver and kidney samples were processed in the same way as muscle but, instead of using phosphate buffer (Na_2HPO_4 and KH_2PO_4, 0.07 *M*, pH 8.5) at the beginning of the procedure, NaOH 7 N and NaOH 50% were used, respectively.

The same extraction method was used for muscle, liver and kidney of the three different studied species (calf, pig and poultry).

2.5.2. Poultry and pig skin+fat

Five grams of poultry skin+fat were collected and 100 μl of NaOH 7 N and 10 ml of ethyl acetate were added. No internal standard was added in this tissue. After homogenisation, tubes were shaken and centrifuged at 1932 *g* for 20 min. Aqueous phase was discarded and the organic phase was evaporated until ~3 ml of an oily residue were left. Then, 4 ml of methanol were added and the tubes were shaken and centrifuged for 10 min. The methanol was measured (ml) and evaporated until dry. The dry residue was dissolved in 200 μl mobile phase and determined by HPLC.

The same procedure was used in pig skin+fat (subcutaneous fat) but, instead of 100 μl of NaOH 7 N, 2 ml of phosphate buffer (Na_2HPO_4 and KH_2PO_4, 0.07 *M*, pH 8.5) were added.

2.5.3. Calf fat

The procedure used in calf fat (subcutaneous fat) was the same as that described in pig skin+fat, but instead of 10 ml of ethyl acetate, 15 ml of ethyl acetate were used.

2.6. Recovery

2.6.1. Muscle and liver

Muscle and liver drug-free samples were spiked with 100 and 200 μg/kg from a tylosin solution of 0.01 mg/ml and 500 and 1000 μg/kg from a solution of 0.1 mg/ml of standard tylosin and were processed in triplicate as described in Section 2.5.1. After extraction, 3 μg of albendazole (internal standard) were added for analysis in order to dispose of the totality of albendazole since there is no loss due to the extraction procedure. Simultaneously, and also in triplicate, tubes were prepared with the same final volume, the same amount of tylosin and albendazole as that added to the internal samples, but without the different tissues and without submitting them to the extraction procedure described above (external samples).

Extraction recovery was determined by comparison of HPLC area ratio tylosin/albendazole of internal and external samples. Albendazole response was exactly the same in both samples and only the response of tylosin varied depending on the concentration added and if they were internal or external samples.

2.6.2. Kidney

Blank kidney samples were spiked with concentrations of tylosin of 50 and 100 μg/kg from a solution of 0.01 mg/ml and 250 and 500 μg/kg from a solution of 0.1 mg/ml following the same procedure described in Section 2.5.1.

2.6.3. Pig and poultry skin+fat and calf fat

Blank target tissues of the three species were spiked with concentrations of tylosin of 50, 100 and 200 μg/kg from a solution of 0.01 mg/ml and 400 μg/kg of a solution of 0.1 mg/ml following the same procedure described in Section 2.5.2. and 2.5.3.

2.7. Calibration

A standard series in the range 50–500 μg/kg of tylosin in drug-free muscle and kidney samples, 50–300 μg/kg in drug-free liver of the species studied and pig fat+skin samples and 50–400 μg/kg in drug-free calf fat and poultry skin+fat samples were prepared and processed as described in Section 2.5. Method linearity, precision and accuracy, quantification limit and specificity were calculated ($n=4$).

Precision of the method was expressed as inter-day variability in the tylosin concentration range for the different tissue samples. The accuracy of the method was measured by the difference between the concentrations observed and those calculated, and can be expressed as the relative error.

The limit of quantification (LOQ) was determined

by studying the inter-day accuracy and precision at a concentration of 50 ppb of tylosin. The limit of quantification represents the minimum concentration with an accuracy and precision within the established range.

2.8. Stability

In order to confirm the stability of fortified samples of the different target tissues, 250 μg/kg of tylosin were added to samples of the different matrices. Samples were then frozen at −80°C and analysed after 80 days in the muscle, after 120 days in liver samples, 110 days in skin+fat samples and after 400 days in kidney samples, evaluating the amounts of tylosin present.

3. Results and discussion

3.1. Extraction

The method described allows tylosin to be extracted, in a simple and reliable way, from all the target tissues of the different species studied with only a few changes. Because of the particular tissue properties of kidney, it was necessary to carry to extremes basic pH conditions to obtain the best recovery from this tissue.

Almost the same extraction procedure employed in the other tissue analysis was used for both fat and skin+fat, tissues with particular characteristics. Only a change in the extraction solvents was necessary because ethyl acetate and methanol were considered the most suitable ones for tylosin recovery from this tissue. Different previously reported methods used different solvents such as acetonitrile [7] or chloroform–ethyl acetate (2:1), also at basic pH [8], for tylosin extraction. In some studies, acid pH was used to make the union of the molecule to column solid-phase extraction (SPE) easier [9] or to help in the proteic structure destabilisation [5], mainly in liver and kidney tissue [6,16]. All these methods use large quantities of sample to increase the sensitivity; this therefore implies increasing the number of extraction steps, which involves a loss of precision and accuracy of the method, and the volume of solvents employed in order to achieve a good resolution and specificity of the method.

Extraction with SPE cartridges has the disadvantages that it needs a high accuracy and repeatability and a lot of attention because at some points of the extraction it is very important to not allow the column to dry and it is more expensive than liquid–liquid extraction.

In our method, there was no need to increase the size of the sample, only 5 g of the different tissues were enough to obtain a good sensitivity, recovery and specificity.

3.2. HPLC determination

Figs. 1–5 show the chromatograms from calf muscle, pig liver, poultry kidney, calf fat and poultry skin+fat blank samples and samples spiked with tylosin and albendazole. The retention times were 4.64±0.20 min and 5.27±0.02 min for tylosin and albendazole, respectively. The tylosin peak corresponded to tylosin A (marker residue of tylosin drug and its main component). The chromatogram runtime was 10 min, a time shorter than other studies described [9,11,12,16,17,19], where tylosin eluted later. This fact permitted us to analyse a relatively high number of samples in a short period of time.

Tylosin and albendazole peaks showed a good resolution and no interference with other peaks (impurities or minor components of tylosin), indicating a high specificity and sensitivity of this method.

In case there exists a suspicion of a possible interference with the internal standard, then it would be better to quantify tylosin without the use of the internal standard, but measuring the volumes of the solvents recovered from the extraction procedure.

Other studies [13,15–17] used detection systems (mass spectrometry) more sophisticated than UV detection, but sometimes not available to the majority of the analysis laboratories or with no relevant differences in sensitivity with UV detection methods [17].

Some authors used a gradient elution [10–12,17], which has a lower repeatability than the isocratic elution used in the method described in the present paper and also needs extra time to achieve column conditioning.

In this study, the same mobile phase could be used

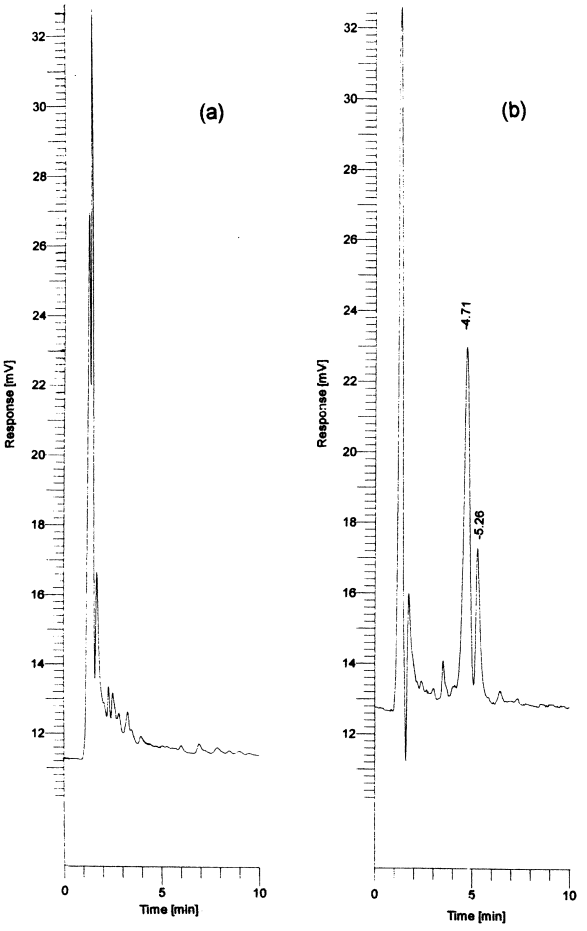

Fig. 1. Chromatograms obtained from 5 g, respectively, of a blank (a) and a calf muscle sample spiked with 500 ng of tylosin and 125 ng of albendazole (b).

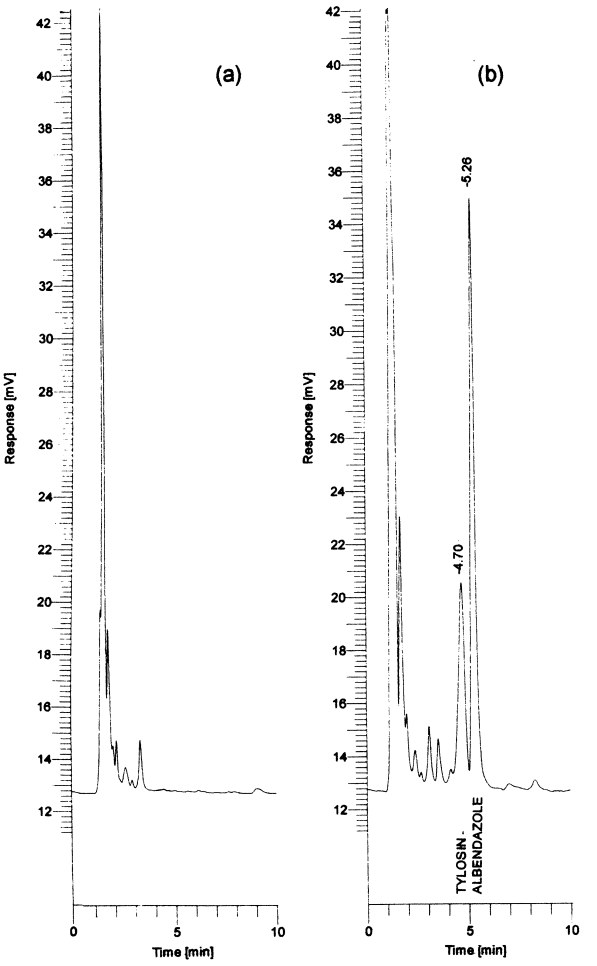

Fig. 2. Chromatograms obtained from 5 g, respectively, of a blank (a) and a pig liver sample spiked with 500 ng of tylosin and 750 ng of albendazole (b).

for the different tissue analysis, whereas other methods described needed to modify the proportion of the mobile phase depending on the type of sample analysed [5,6].

3.3. Recovery and calibration experiments

Recovery values (as quantity of tylosin recovered after submitting samples to the extraction procedure) calculated for muscle, liver, kidney, fat and fat+skin samples spiked with different concentrations are reported in Table 1.

The addition of a higher concentration provoked a saturation of the extraction system. The procedure showed saturation when concentrations were 10 times higher than the MRL.

Tylosin, a weak base with a pK_a of 7.1, needs a pH of 8.5 to be recovered in an organic solvent such as chloroform.

In muscle, liver and kidney, after evaporating chloroform, the addition of $CH_3CN–H_2O$ in a proportion (8:2) followed by a double liquid–liquid partition is a suitable way to obtain a good recovery. The recovery obtained in muscle (97.1%) at 1000 μg/kg was higher than others reported at 84.1% and 61%, respectively [11,12]. The result of the recovery

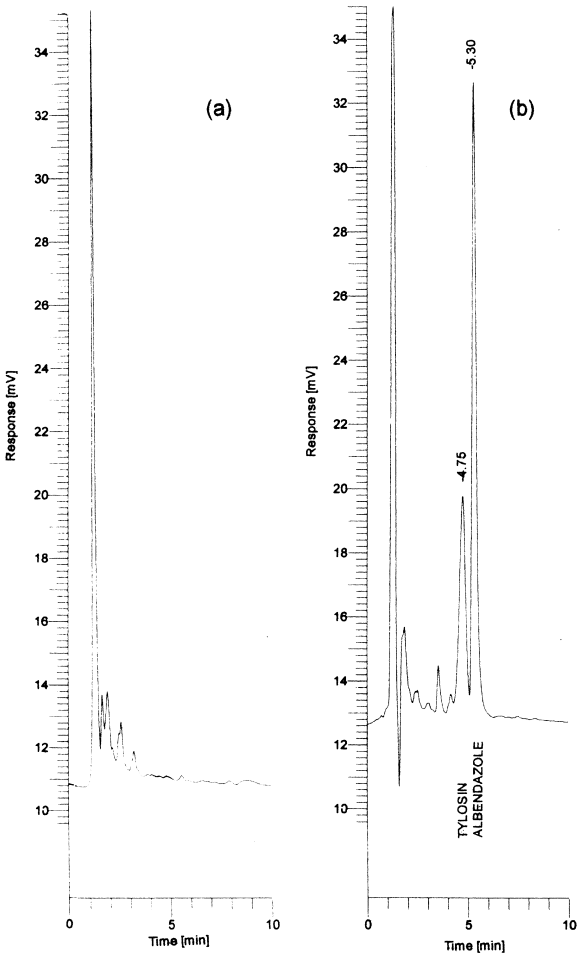

Fig. 3. Chromatograms obtained from 5 g, respectively, of a blank (a) and a poultry kidney sample spiked with 500 ng of tylosin and 750 ng of albendazole (b).

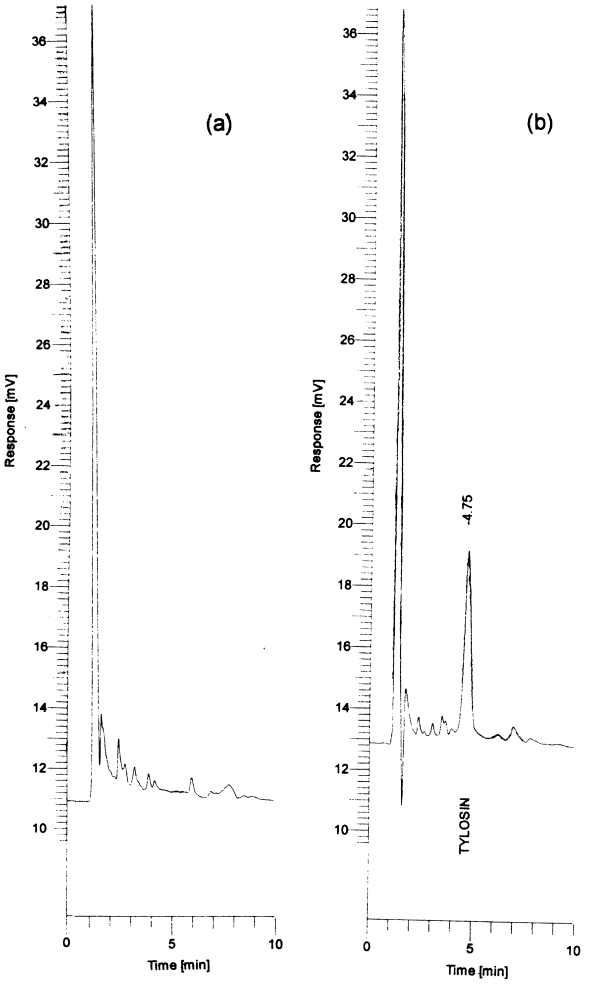

Fig. 4. Chromatograms obtained from 5 g, respectively, of a blank (a) and a calve fat sample spiked with 500 ng of tylosin (b).

of kidney (77.2%) was higher than another study but similar results of liver and muscle recoveries were obtained in both studies [17].

In the evaluation of the calibration, samples of the different target tissues were spiked with five different concentrations of tylosin and all analyses were performed in quadruplicate.

The peak area ratios (tylosin to albendazole as internal standard) showed a linear relationship with a concentration over the range 50–500 μg/kg for muscle and kidney, 50–300 μg/kg for liver and pig skin+fat and 50–400 μg/kg for poultry skin+fat.

Precision and accuracy, in most of the different spiked concentrations, were below the limit values fixed by the EC at concentrations (>1–10 μg/kg and ≥10 μg/kg in the case of accuracy and between 10 and 100 μg/kg or 100–1000 μg/kg for precision). In all the tissues analysed, precision and accuracy were below the proposed limits when the spiked concentration was at the MRLs levels [20].

Precision and accuracy of the different spiked concentrations from the tissues analysed are shown in Table 2.

In all the tissues, precision was better at the

Table 1
Tylosin recoveries observed for the different matrices studied

Conc. added (μg/kg)	Recovery (%)	Conc. added (μg/kg)	Recovery (%)	Conc. added (μg/kg)	Recovery (%)
Muscle		Liver		Kidney	
100	81	100	85	50	70
200	82	200	82	100	66
500	76	500	63	250	96
1000	97	1000	54	500	77
Calf fat		Pig fat+skin		Poultry fat+skin	
50	79	50	73	50	91
100	75	100	77	100	95
200	72	200	91	200	87
400	79	400	100	400	95

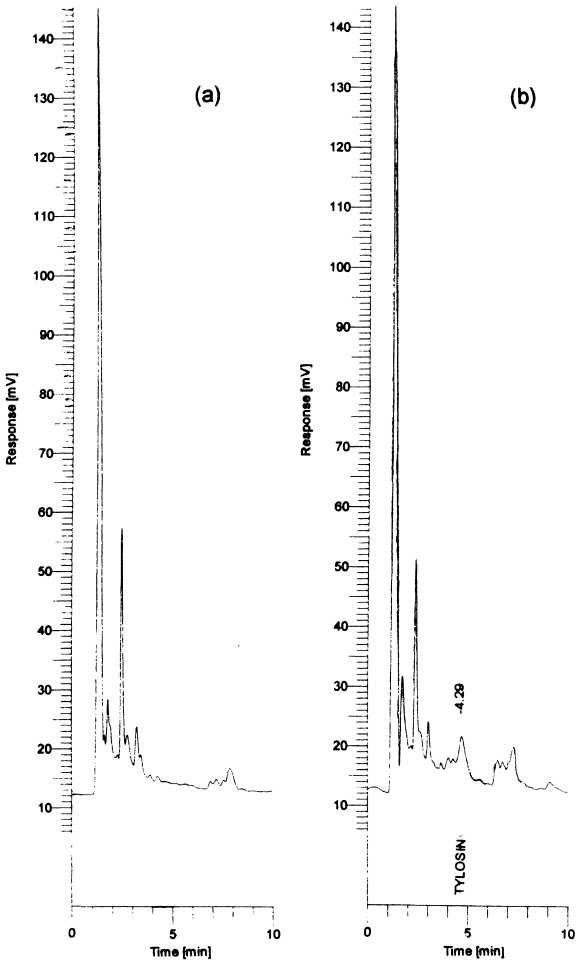

Fig. 5. Chromatograms obtained from 5 g, respectively, of a blank (a) and a poultry skin+fat sample spiked with 500 ng of tylosin (b).

highest concentration; however, a good precision was obtained in all the concentrations. The limit of quantification for tylosin was 50 μg/kg in all the tissues. The LOQ was the same as reported by other authors that use mass spectrometry [17] and less than other studies [11].

The precision and accuracy obtained between a concentration half the MRL and at least threefold the MRL, permit us to decide with certainty if tylosin concentrations of samples are or not below the MRL levels established by the legislation.

Results in all the tissues analysed showed a total stability of tylosin at the stored conditions.

3.4. Animal experiment

Results obtained from the sample analyses of the treated animals are shown in Table 3.

When the animals were treated by i.m. route with the therapeutic dose of tylosin, drug levels in muscle, liver and fat were below the quantifiable levels (<50 μg/kg) after 7 days of administration. At this time, kidney concentrations were slightly over the fixed MRL (100 μg/kg).

Fourteen days after administration, all the tissular concentrations were below the limit of quantification of the analytical method.

In conclusion, the meat proceeding from animals slaughtered at the 14th day after drug administration would not represent a risk for consumer health when the drug is administered at a dose of 20 mg/kg/day for 5 days.

Table 2
Precision and accuracy of tylosin validation method in different matrices studied

Tissue	Concentration added (μg/kg)	Concentration found (μg/kg)	Accuracy (%)	Precision (C.V.) (%)
Muscle	50	57	+14	13.1
	100	99	−1	11.5
	250	236	−5.6	11.2
	500	505	+1	2.1
Liver	50	47	−6	12
	100	103	+3	8.9
	200	198	−1	10.4
	300	298	−0.7	3.8
Kidney	50	48	−4	34
	100	108	+8	20
	250	259	+3.6	4.9
	500	491	−1.8	1.9
Calf fat	50	50	0	20.3
	100	90	−10	9.3
	200	180	−10	3.9
	400	410	+2.5	0.9
Pig fat+skin	50	42	−16	19.4
	100	105	+5	11.4
	200	199	−0.5	7.2
	300	293	−2.3	2.4
Poultry fat+skin	50	36	−28	13.5
	100	110	+10	13.4
	200	200	0	8.8
	400	395	−1.2	1.4

4. Conclusions

An easy and quite quick method was developed to quantify tylosin residues in tissues from calves, pigs and poultry with a good resolution and a high specificity and sensitivity. The same method with only few changes permitted to analyse different tissues of the different species studied, using for all of them the same mobile phase. Peak area ratios showed a linear relationship and a precision and accuracy below the limits fixed by the EC in most of the different spiked concentrations, and also good recoveries.

An experiment carried out with calves treated with tylosin administered by i.m. route for 5 days showed that in the animals slaughtered 7 days after the administration, only kidney concentrations were over the MRL and 14 days after the administration all the tissular concentrations were below the LOQ of the analytical method.

Table 3
Tissue mean concentrations from calves treated with 20 mg/kg of tylosin for 5 days and slaughtered at the 7th day post-administration

Tissue	Tylosin concentration (μg/kg)			
	Calf 1	Calf 2	Calf 3	Calf 4
Muscle	<50	<50	<50	<50
Liver	<50	<50	<50	<50
Kidney	142	108	126	116
Fat	<50	<50	<50	<50

Different tissue levels corresponding to 14th day of slaughter were below the limit of quantification (LOQ) (50 μg/kg).

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