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Tissue depletion of tylosin after multiple intramuscular administration in calves and piglets

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Summary

Tylosin (TYL) is widely used to treat different bacterial infections in calves and piglets; however, there is no information available about the withdrawal period necessary for growing calves and piglets to be safe for human consumption. A high-performance liquid chromatography method with ultraviolet-visible light detection was adapted and validated for the determination of TYL in calf and piglet tissues. The procedure is based on the isolation of the TYL compound from edible calf and piglet tissues (muscle, liver, kidneys, and fat with skin) with satisfactory recovery (81.55 ± 10.49) and specificity. The residue depletion of TYL in calves and piglets was assessed after a dose of 10 mg/kg body weight/day intramuscullary for 5 consecutive days. After the treatment was discontinued, residue concentrations were detected in tissues till the 11th and 19th day in calves and piglets, respectively. The highest TYL concentrations were measured in the liver, in both species. Based on the results presented in this study, it could be assumed that a withdrawal period of 12 days for calves and 20 days for piglets before slaughter would be sufficient to ensure consumer safety.

Keywords: tylosin, residue, withdrawal period, calves, piglets

Tylosin (TYL) is a macrocyclic antibiotic that belongs to the group of 16-member-ring macrolides. It was first derived in 1960, during the fermentation process by *Streptomyces fradiae* cultures. TYL is a mixture of four compounds: the main product, tylosin A (> 80%), and the minor components: tylosin B, C, and D, which may be present in varying amounts (8, 19, 30, 99). Due to its bacteriostatic action against Gram-positive bacteria, anaerobic bacteria, Mycoplasmas, and certain Gram-negative bacteria (82), TYL has been widely used primarily to treat chronic respiratory disease (CRD) complex in chickens and infectious sinusitis in turkey caused by *Mycoplasma gallisepticum*. It is also used to treat swine and bovine respiratory diseases, calf arthritis, swine dysentery or piglets streptococcosis, and other infections caused by organisms sensitive to TYL (16, 40, 64, 81, 98).

As with other antibiotics, its use could result in residues in edible tissues, potentially harmful to consumer health, causing toxic effects in the short term or allergic reactions and the development of resistance in the long term. In humans, a variety of toxic and irritative effects have been reported from the use of TYL (such as allergic contact dermatitis (ACD), etc.) (7, 12, 18, 31, 38, 41, 42, 50, 52, 54, 71, 87-90). Such reactions are exceptionally rare, and the risk can be minimized by careful use and observing sufficiently long withdrawal periods of substances fed to livestock.

Moreover, the intense use of TYL in veterinary practice can give rise to an increasing risk for human and animal health. Therefore, the European Union (EU) has established maximum residue limits (MRLs) for edible tissues of cattle, pigs, and chickens, expressed as the marker residue, tylosin A: muscle, liver, kidney, fat, 100 µg/kg (bovine and porcine); and skin/fat, 100 µg/kg (poultry). The Committee also recommended an MRL for milk of 50 µg/kg and for eggs of 200 µg/kg, both expressed as the marker residue, tylosin A (24).

Although the pharmacokinetics and tissue depletion study of TYL have been described in a variety of animals, such as broiler chickens (2, 51, 56, 91, 95-97), goats (4, 6), dogs (3, 94), pigs (33, 39, 45, 46, 74, 75, 79, 80) cows and calves (1, 5, 10, 11, 13, 21, 22, 30, 37, 45, 49, 55, 67-69, 78, 99), in milk (17, 20, 47, 58, 61, 65), including hens and residues in eggs (9, 28, 34,

35, 43, 48, 57, 59, 77, 93) or even honey (27, 66, 85), there is limited data available on the residue of TYL in edible tissues of very young, fast-growing animals like calves and piglets when administered according to the manufacturer's recommendations for standard therapy. Therefore, it is difficult to predict the withdrawal time. Most of the EMEA reports set the withdrawal time for parenteral administration in cattle and pigs at less than 3 days.

Various instrumental methods have been introduced for the determination of TYL residues (14, 32, 35, 70, 72, 76, 83, 86, 92). However, one of the most prominent methods is high-pressure liquid chromatography (HPLC), which is highly effective in monitoring veterinary drugs, particularly for determining TYL concentrations in various biological matrices (29, 53, 74, 75).

This study describes the determination of TYL and tissue residue depletion in fast growing calves and piglets after multiple intramuscular administrations and its influence on withdrawal time. To our knowledge, this is the first time a complete residue depletion study has been conducted for TYL in edible tissues of calves and piglets.

Material and methods

Animals and experimental design. All experiments were carried out on 31 calves (Polish red and white cows) with an initial weight of 58-72 kg, at the age of 5-6 weeks and of both sexes, and on 30 piglets (Polish Landrace, PBC) with an initial weight of 11.5-15 kg, at the age of 5-7 weeks, and of both sexes. After a 7-day acclimatization period, clinical observation and examination were carried out to assess the health state of the animals. They had not received any prior antibiotic treatment. Calves were randomly divide dinto 5 experimental groups (A, n = 6; B, n = 6; C, n = 6; D, n = 6; E, n = 6), to determine the termination point for each group and the collection of tissue samples, with the last group – F, n = 1, serving as a control group. Piglets were divided in the same way into 7 experimental groups (A, n = 6; B, n = 6; C, n = 6; D, n = 4; E, n = 2; F, n = 2; G, n = 2) with the last control group, H (n = 2). The animals were housed in pens in numbers corresponding to each respective group and fed twice a day (in the morning, 1 hour after administering the medicine, and in the evening). Water was provided *ad libitum* throughout the study period. The experiments were approved by the Local Ethics Committee for Animal Experimentation in Lublin (28/2008; 32/2008) and conducted in accordance with European law (2010/63/EU).

Biotyl 50 (Biowet Drwalew sp. z o.o, Poland) for injection containing 5 g/100 ml of TYL, was administered intramuscularly (into the neck muscles) at a dose of 10 mg/kg body weight/day for 5 consecutive days. The control group received *aqua pro injectione* continuously for 5 days. The animals were slaughtered and tissue samples were taken after 6, 8, 10, 12, and 14 days in calves, and after 7, 10, 13, 15, 18, 21, and 23 days in piglets after the last drug administration. Tissue samples were extracted in the following quantities: muscles (40 g), liver (entire), kidneys (entire), and skin with fat (40 g). Samples were collected from each animal and each group. Calves and piglets from control groups were slaughtered and marker tissues were taken for TYL recovery. Tissue samples were stored at –30°C until the day of analysis using HPLC.

Chemicals and reagents. An analytical standard (tylosin base – Biowet Peshtera JSC, Bulgaria) was obtained from a pharmaceutical company (Biowet Drwalew sp. z o.o., Poland). HPLC-grade reagents (acetonitrile, methanol) and analytical-grade reagents (dichloromethane, chloroform, ethylacetate, orthophosphoric acid, hydrochloric acid, sodium hydroxide, potassium dihydrogen phosphate) were purchased from POCH chemical company (Gliwice, Poland). HPLC-grade sodium perchlorate monohydrate was obtained from Sigma-Aldrich (St. Louis, MO, USA). Water (18.2 MΩcm) was purified using the reverse osmosis method with a Milli-Q-Plus 185 system (Millipore, Molsheim, France).

Tylosin quantification. The analytical procedure was validated for all tissues collected from the calves and piglets according to the metod previously described by Prats et al., with a few modifications (73).

Liver and skin + fat: Frozen tissue samples were thawed to room temperature prior to extraction. 2.5 g of minced skin + fat was transferred into a test tube and mixed with 1 mL of 0.2 M buffer KH_2PO_4 (pH = 9.00) and 5 mL of ethylacetate, then homogenized for 1 minute. Afterward, the sample was shaken for 20 minutes and centrifuged for 20 minutes at 5000 × g. The upper ethylacetate layer was transferred into a clean tube and evaporated under a stream of nitrogen in a water bath at 50°C until approximately 1.5 mL of an oily residue remained. Next, 2 mL of metanol were added, the tube was mixed for 30 seconds on a vortex mixer, and then centrifuged for 10 minutes at 5000 × g. The metanol layer (~1.8 mL) was measured and evaporated under a stream of nitrogen in a water bath at 50°C. The dry extract was dissolved in 200 µL of a mixture of acetonitrile and water (50 : 50; v/v) and mixed. Subsequently, a 50 µL volume of the eluate was injected into the HPLC system. The same procedure was used for liver samples, but the first evaporation step was to dry the residue.

Kidney and muscle: Frozen kidney samples were thawed to room temperature prior to extraction. 2.5 g of kidney was transferred into a test tube and mixed with 1 mL of 1 M NaOH and 5 mL of acetonitrile, then homogenized for 1 minute. Afterward, the sample was shaken for 20 minutes and centrifuged for 20 minutes at 5000 × g. The acetonitrile was collected and dried under a stream of nitrogen in a water bath at 50°C. The dry extract was then redissolved in 2 mL of acetonitrile and water (8: 2) and subjected to liquid-liquid partition with 5 mL of dichloromethane for 2 minutes. After a second centrifugation for 10 minutes at 5000 × g, the organic phase was filtered into a clean tube. Another extraction of the non organic phase was carried out with 2.5 mL of dichloromethane. After shaking for 2 minutes and centrifuging for 10 minutes at 5000 × g, the upper layer was discarded, and the lower dichloromethane layer was combined with the organic phase. All the collected dichloromethane was evaporated under a stream of nitrogen in a water bath at 50°C. After dissolving the dried extracts

in 200 µL of a mixture of acetonitrile and water (50 : 50; v/v), 50 µL of sample was injected into the chromatographic column. Muscle samples were processed in the same way as kidney samples, but instead of using 1 mL of 1 M NaOH and 5 mL of acetonitrile at the beginning of the procedure, 0.5 mL of 0.2 M buffer KH_2PO_4 (pH = 9.00) and 5 mL of chloroform were used, respectively.

Preparation of standard solutions. A standard stock solution of TYL (400 µg/mL) was prepared by dissolving 10 mg in 25 mL of a mixture of acetonitrile and H_2O (50 : 50; v/v). Final working solutions (1000, 750, 500, 250, 150, 50 ng/mL) were prepared by appropriate serial dilution of the stock solution with the same mixture used for the stock solution. These solutions were then injected to obtain the calibration curve and remained stable for at least 2 months at 4°C.

HPLC instrumentation. The HPLC system consisted of an isocratic delivery pump (STAR 9002), a 50 µL manual injector Rheodyne, and a variable wavelength UV-VIS 9050 detector, all controlled by a thermostat MetaTherm (all Varian Analytical Instruments, Walnut Creek, CA, USA). The separation was performed on a reversed-phase column (XTerra RP18, 5 µm, 4.6 × 250 mm, Waters) equipped with a guard column (XTerra RP18, 5 µm, 3.9 × 20 mm, Waters). The column temperature was maintained at 35°C.

Animal tissue was homogenized using a HeidolphDiax 900 (Bochem Laborbedarf, Weilburg, Germany), and a centrifuge (2-16K Sigma) was used to separate the supernatant from the solid phase. Other instruments used included an analytical balance (Sartorius BP 61S), Vortex mixer (WL-1, Bio-mix, Warsaw, Poland), shaker (BWZ 23S, Bio-mix, Warsaw, Poland), pH meter (Φ 34 pH Meter BECKMAN, Brea, USA), water bath (MLL 547 AJL electronic, Kraków, Poland), and Milli Q Plus 185 system (Millipore-Waters).

The mobile phase consisted of acetonitrile and sodium perchlorate monohydrate (200 g/L) adjusted to pH 2.5 (40 : 60, v/v) and was injected onto the HPLC system (50 µL of this solution) isocratically at a flow rate of 1.2 mL/min. This type of mobile phase is recommended by the European Pharmacopoeia. The chromatograph was operated at 35°C, and the column effluent was monitored at a wavelength of 290 nm.

Validation of the analytical method. The quantitative HPLC method was validated for each of the calves and piglets tissues (liver, kidney, fat with skin, and muscle) in terms of linearity, intra-day and inter-day precision, stability, recovery, limits of detection (LOD) and quantification (LOQ), as well as decision limit (CCα) and detection capability (CCβ) according to the Commission Implementing Regulation (EU) draft document (15).

Linearity was determined by linear regression analysis, using calibration curves constructed using replicates (n = 3) of control samples from calves' and piglets' matrices spiked with TYL. The calibration curves were prepared at concentrations of standards of 5.50, 11.0, 55.0, 110.0, 320.0, 650.0 ng/g for calves and 5.50, 11.0, 55.0, 110.0, 268.0, 590.0 ng/g for pigs, respectively.

The intra-day and inter-day precision was calculated after analyzing six samples of matrices spiked with TYL at three different concentrations (5.50, 110.0, and 590.0 ng/g) and is expressed as percentage coefficients of variation (CV, %).

The stability of TYL was determined in two different ways in sample of purified extracts (spiked all the tissues of calf at 100 $ng \cdot g^{-1}$) stored before the HPLC analysis and in solvent (stock solutions). First, the stability of the working standard solutions prepared with the mixture acetonitrile – H_2O (50 : 50; v/v) was studied. The solutions were analyzed every week and the instrumental responses were compared with the peak areas obtained at the moment of the preparation of solutions. The acceptance criterion was a response between 95% and 105% of the initial one and no degradation phenomena observable during a storage time of 2 months at 4°C. In order to check of the purified extracts, homogenized blank tissues of calf were divided into four aliquots and each was fortified at 100 $ng \cdot g^{-1}$. One aliquot was analyzed immediately and the remaining aliquots were stored in the freezer at –20°C and analyzed after 7, 14 and 21 days. The concentration of TYL in each aliquot was calculated by considering the solution of the analyte freshly prepared at the time of analysis to be 100%. It was shown to be stable up to 21 days. Consequently, the purified extracts can be preserved in a freezer at least 3 weeks before instrumental determination.

The extraction recovery experiment was carried out by comparing the response (in an area) of high, middle, and low standards to the response of equivalent standards. Each sample of the control calf tissue was spiked at three levels: 50.0, 100.0, and 150.0 ng/g. These fortified levels were chosen depending on the MRL of the analyte (0.5, 1, and 1.5 times the MRL established for TYL).

The CC*a* and CC*b* were determined using the calibration curve procedure according to ISO 11843 (ISO 2003). The CC*a* = MRL + 1.64 × SD of blank samples and blanks fortified around the MRL and CC*b* = CC*a* + 1.64 × SD of blank samples and blanks fortified around the MRL.

Withdrawal period estimation. Exploratory withdrawal times (WT) were computed for TYL in calves' and piglets' livers using the software WT 1.4, developed by the EMA (23). The withdrawal time was established as the point when the upper one-sided tolerance limit (95%) with a 95% confidence interwal fell below the MRL. In the present study, the MRL (100 ng/g) reported for TYL in calves' and piglets' tissues by the EMA was used (25).

When the majority of data from one slaughter point were below the limit of detection (LOD), that time point was excluded. However, three time points were retained to enable meaningful regression analysis. Assumptions for linear regression analysis were assessed to validate withdrawal time estimation. Since the time points did not encompass a full day, the withdrawal period had to be rounded up to the next day (23).

Results and discussion

TYL is widely used in bovine and pig livestock husbandry, despite inadequate information about its pharmacokinetics and the lack of available data regarding tissue concentrations in these growing species after multiple administrations, such as during complete therapy. This study represents the first attempt to describe the depletion of TYL residues in edible calves and piglets tissues using a validated HPLC method.

Validation performance. Table 1 lists the results for the main parameters from the analytical method validation in all evaluated tissues. Under our experimental conditions, TYL showed a retention time of about 7 and 8 minutes in calves' and piglets' samples, respectively. The analytical method showed a linearity in the range of 5.5-650 ng/g for every matrix considered. The limit of detections (LOD = $3.3 \cdot S_{yx/a}$, where S_{yx} is the standard deviation and a is the slope) for calve and porcine tissues were calculated in the ranges 3.06-12.93 ng/g and 3.35-19.72 ng/g, respectively. The limit of quantification (LOQ = $10 \cdot S_{yx/a}$) for tissues of calves and porcine were in the range of 9.28-31.04 ng/g and 10.16-59.76 ng/g, respectively. The calculated relative standard deviation (RSD) fell within the range of 0.71% to 5.19% in calf tissues and 3.17% to 14.83% in piglets' tissues. The calculated mean recovery of TYL was dependent on the tested tissues: 93.42% and 96.77% for muscle, 82.00% and 87.19% for kidney, 68.64% and 54.81% for liver, and 82.75% and 86.85% for fat with skin in calves and piglets, respectively.

Regarding stability verification, TYL remained stable throughout the freezing process. No degradation phenomena were observed in the working solution during storage for a period of 21 days at 4°C, nor between the amount of TYL spiked and that observed in the samples stored for different periods of time.

The CC*a* and CC*b* were respectively: 103.79 ng/g and 107.58 ng/g in calves and 104.81 ng/g and 109.62 ng/g in piglets.

Residue depletion study. The concentrations of TYL measured in muscle, kidney, liver, and fat with skin from calves and piglets are summarized in Table 2 and Table 3, respectively. These results indicate the presence of TYL residues in the tissues of calves and piglets after treatment with this drug.

Residues in calves' tissues: On the sixth day of the withdrawal period following the complete treatment, the TYL residue content in the liver was more than four times higher (459.68 ± 97.64 ng/g) than the MRL of 100 ng/g, and disproportionately higher compared to the residues in the kidneys (164.79 ± 71.27 ng/g) ($P < 0.05$), where the TYL concentrations amounted to about one-third of those in the liver. This difference was also observed in the muscles (128.63 ± 40.27 ng/g) and fat with skin, where no residues of the administered drug were detected (< LOD). The trend persisted in the subsequent days of tissue collection and after the drug was withdrawn. On the 12th day post-treatment, concentrations of TYL detected in all studied tissues were below the accepted EU MRL (Tab. 2 and Fig. 1).

Tab. 1. Results of the HPLC method validation for TYL quantification in different tissues in calves and piglets

Parameter	Unit	Matrix							
		Muscle		Kidneys		Liver		Fat + Skin	
		calves	piglets	calves	piglets	calves	piglets	calves	piglets
R^2		0.9983	0.9997	0.9989	0.9998	0.9993	0.9999	0.9984	0.9993
Inter-day CV	%	2.95	9.93	1.64	7.28	3.39	14.76	3.58	10.65
Intra-day CV	%	4.17	3.17	3.11	12.43	1.75	4.95	2.80	5.56
Recovery	%	93.42	96.77	82.00	87.19	68.64	54.81	82.75	86.85
LOD	ng/g	4.60	3.35	12.93	3.50	3.32	19.72	3.06	3.91
LOQ	ng/g	13.88	10.16	31.04	10.61	10.06	59.76	9.28	11.84

Tab. 2. Mean (± SD) tylosin (TYL) residues (ng/g) in calves edible tissues after 5 days intramuscular administration in the dose 10 mg/kg bw, with 24 h intervals. Because all the values in the day 14th were below the LOD, the time points were excluded from statistical analysis of withdrawal time

Time after last dose (days)	Mean TYL calves tissue residues (ng/g)			
	Muscle	Kidney	Liver	Skin + fat
6	128.63 (± 40.27)	164.79 (± 71.27)	459.68 (± 97.64)	< LOD
8	49.33 (± 30.83)	81.10 (± 37.61)	207.47 (± 29.38)	< LOD
10	< LOD	< LOD	82.25 (± 27.61)	< LOD
12	< LOD	< LOD	< LOD	< LOD
14	< LOD	< LOD	< LOD	< LOD

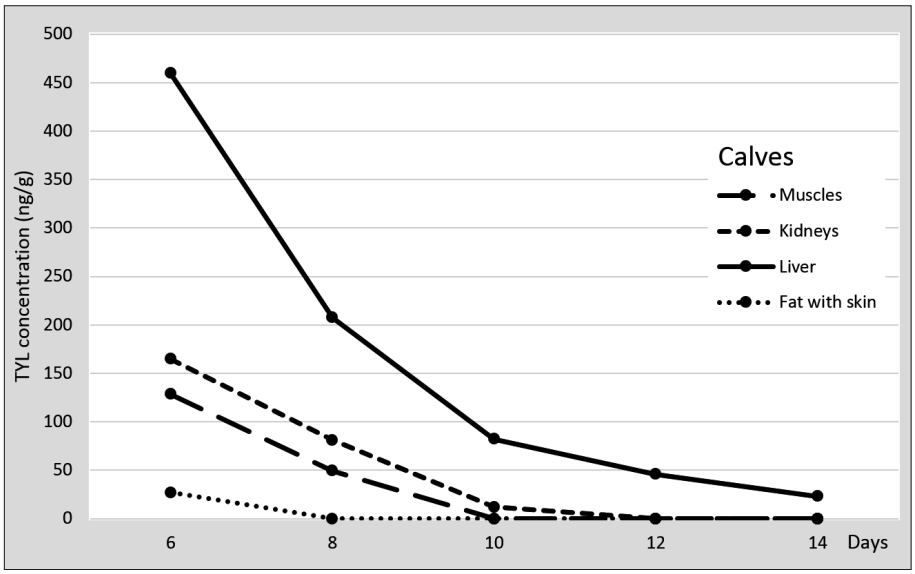

Fig. 1. Mean concentrations in muscles, kidneys, liver and fat with skin of tylosin (TYL) in calves after 5 days of intramuscular administration in the dose 10 mg/kg bw, with 24 h intervals (n = 30)

This study represents the first examination of residue depletion over a 14-day period following intramuscular administration of TYL at a dose of 10 mg/kg b.w. for 5 consecutive days, mirroring routine therapy recommended by the manufacturer. In contrast to previous reports, our findings indicate that the primary storage organ for TYL is the liver. Concentrations were significantly higher and persisted for a longer duration compared to the kidneys, as reported by Kennington et al. (44, 45) and Luperi et al. (55). This divergence could be attributed to differences in experimental conditions. Due to the scarcity of data in the literature, it is challenging to draw comparisons with studies conducted under alternate conditions. For instance, in some studies, TYL was administered at the same dosage but for only 3 consecutive days, with tissue collection for testing conducted only 4 hours after the end of drug administration (45).

It also appears inconclusive to compare the results of our study with the study conducted by Luperi et al., where TYL was administered at a dose of 10 mg/kg for 4 days, although tissues were collected from 7 to 42 days after the end of treatment (55). For instance, the values of residue concentrations in the kidneys in our study on day 5 were slightly higher (128.63 ± 40.27 ng/g) compared to those found on day 7 by Luperi et al. (73.7 ± 37.7 ng/g) (55). However, in other tissues, including the liver, TYL residues were below LOQ of the method (50 ng/g) from day 7 onwards. This concentration is even 9 times lower on day 5 and 4 times lower on day 8 than those found in our experiment (459.68 ± 97.64 ng/g) and (207.47 ± 29.38 ng/g), respectively.

Our results are partially supported by the study conducted by Thomson et al., where TYL was administered at a dose of 10 mg/kg for 5 consecutive days (84). Sections of the liver and kidneys, where similarly to the results obtained in this experiment, no TYL residues were found, were collected only on the 21st day after the end of treatment.

The experimental setup most similar to ours, which assessed TYL residues for 21 days after intramuscular administration at a dose of 10 mg/kg for 5 consecutive days, yielded comparable values of TYL residue concentrations in the kidneys on day 7 to the values obtained in our experiment on the 8th day after the end of treatment. However, there were different findings regarding residues in the liver. In the study by Moran et al. (60, 61), TYL was not detected in the liver tissues on day 3, contrary to our experiment where the liver was identified as the main deposit organ with residues nearing the MRL (100 ng/g) by the 10th day (82.25 ± 27.61 ng/g).

In another study where calves were treated with 20 mg/kg of TYL for 5 days and slaughtered on the 7th and 14th day post-administration, differences in TYL concentration in the liver were observed (73). Residues were below quantifiable levels after 7 days of administration, compared to our findings on the 6th day (459.68 ± 97.64 ng/g). Moreover, kidney concentrations were very similar to our findings and slightly exceeded the fixed MRL (100 µg/kg). Fourteen days after administration, all tissue concentrations were below the LOQ of the analytical method, similar to our study. However, it should be emphasized that the drug dose was double that used in our study.

Residues in piglets' tissues: The highest TYL concentrations were measured in the liver on the seventh day after the completion of treatment in piglets. On that day, the TYL

Tab. 3. Mean (± SD) tylosin (TYL) residues (ng/g) in piglets edible tissues after 5 days intramuscular administration in the dose 10 mg/kg bw, with 24 h intervals. Because all the values in the day 21st and 23rd in piglets tissues were below the LOD, the time points were excluded from statistical analysis of withdrawal time

Time after last dose (days)	Mean TYL piglets tissue residues (ng/g)			
	Muscle	Kidney	Liver	Skin + fat
7	61.77 (± 19.91)	188.8 (± 32.65)	410.63 (± 32.63)	< LOD
10	< LOD	63.94 (± 34.04)	241.02 (± 39.26)	< LOD
13	< LOD	< LOD	145.68 (± 23.08)	< LOD
15	< LOD	< LOD	119.96 (± 17.26)	< LOD
18	< LOD	< LOD	90.33 (± 2.34)	< LOD
21	< LOD	< LOD	< LOD	< LOD
23	< LOD	< LOD	< LOD	< LOD

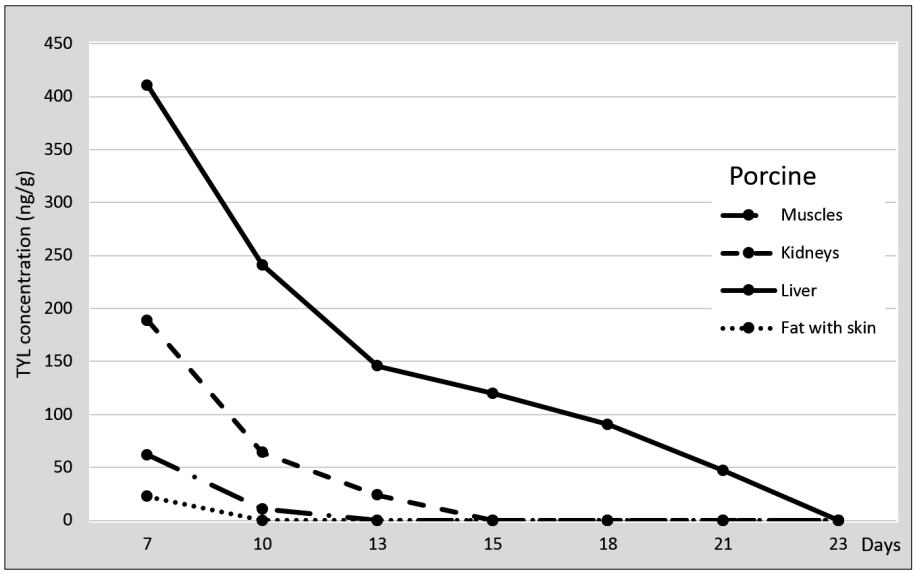

Fig. 2. Mean concentrations in muscles, kidneys, liver and fat with skin of tylosin (TYL) in piglets after 5 days of intramuscular administration in the dose 10 mg/kg bw, with 24 h intervals (n = 28)

residue content in piglets' liver was four times higher (410.63 ± 32.63 ng/g) than the MRL of 100 ng/g, while in the kidneys, it was half the TYL concentration of the liver (188.8 ± 32.65 ng/g) ($p < 0.05$). By the 18th day, TYL concentrations in all studied tissues were lower than the accepted EU MRL (Tab. 3 and Fig. 2).

In the present study, TYL residue levels in the kidney and liver differ significantly from those obtained by Prats et al. (74, 75). TYL concentrations above the MRL were found here on day 7 in the kidney, in contrast to our study where on day 7 all values were below the LOD of the method (36.46 ng/mL). Moreover, in our study, TYL residues in the liver on day 7 were even 5 times higher (410.63 ± 32.63 ng/g) than those found on day 3 in the study by Prats et al. (approximately 75 ng/g) (74, 75).

Other experiments conducted in the same experimental setup also showed differences in residue distribution, where the drug was detected in all tested tissues only on the first day after the end of intramuscular administration of the drug at a dose of 10 mg/kg b.w. for 5 consecutive days (62).

Considering the large discrepancies in the values of TYL residue concentrations in the available literature, it was decided to also refer to the results of TYL residue tests after oral administration. Nevertheless, our study did not confirm previous reports where, after using TYL for 28 consecutive days at a dose 20 times higher (200 mg/kg) than that used in our experiment, no residues were found in the tested tissues (liver and kidneys) above the LOD of the method (20 ng/g), even on the 3rd day after the end of drug administration (24). Moreover, monitoring TYL levels in swine tissue following administration of 0.5 g tylosin/L in the drinking water for 10 days does not corroborate our results. The study showed that at least 0 days withdrawal was required, as TYL residues in all tested tissues were below the detection limits, although tissues were collected from pigs sacrificed also at 2 and 5 days of withdrawal (63).

At the same time, it should be emphasized that our study does not confirm such rapid metabolism and elimination of TYL as previously reported. This may be related to changes in these processes depending on age. Previous studies were conducted mostly on adult or adolescent animals, while very young animals were used in our study. Additionally, the kidney does not seem to be an appropriate organ for routine monitoring of TYL residues in calves or piglets, although it is the recommended target tissue for routine monitoring purposes in both adult bovine and swine (24, 26). According to the study by Handy et al., where TYL was orally administered to young calves, residues were highest and persisted longest in liver tissue until the 12th day post-treatment when they were below the LOD (36). This finding is very similar to ours, although the route of administration, dose, and treatment period differed from our study. Certainly, further pharmacokinetic studies, along with the assessment of residue concentrations, should be conducted to clarify this issue.

Due to the lack of information in the open literature about TYL withdrawal time in calves and piglets, it could be speculative and may depend on periods estimated for other macrolides or other domestic species.

Withdrawal time estimation. It is crucial to consider the pre-slaughter withdrawal interval in the context of an overall risk-benefit assessment for consumers of calves' and piglets' products. As previously mentioned, the withdrawal periods for TYL were determined based on the MRL using the statistical method (95% tolerance limit and 95% confidence) outlined in EU Regulation 37/2010 guidelines (25).

Since all values on the 14th day in calf tissues and the 21st and 23rd days in piglets' tissues were below the LOD, those points were excluded from the statistical analysis of withdrawal time.

The withdrawal periods for TYL in calves were calculated only for liver tissue (11.83 days) due to meeting the basic statistical requirements of the WT1.4 program. The assumptions of linear regression analysis ensured the validation of the method. Due to the in-

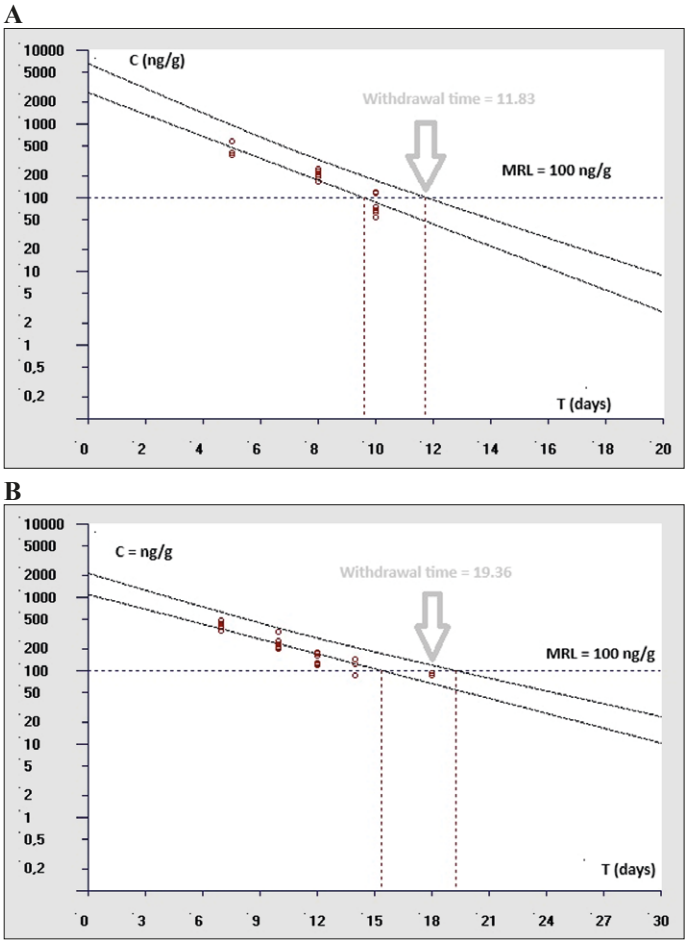

Fig. 3. Plot of withdrawal periods of tylosin (TYL) calculated for calves (A) and piglets (B) liver tissue respectively, with the one-sided 95% upper tolerance limit below the EU MRL of 100 ng/g

complete day coverage of time points, the withdrawal period needed to be rounded up to the next day. Hence, the withdrawal time of 12 days for edible tissues of calves can be considered as the definitive withdrawal time to ensure consumer safety.

The process of determining the withdrawal period for piglets' edible tissues followed a similar approach. The withdrawal periods for TYL were calculated, as in the calves study, only for liver tissue (19.36 days) due to fulfilling the basic statistical requirements of the WT1.4 program. For piglets' edible tissues, the withdrawal time was set to 20 days.

In conclusion, an analytical method for the determination of TYL in calves and piglets tissues has been developed and validated. The extraction procedure of TYL from calves and piglets tissues uses small volumes of organic solvents, making the method cheaper and safer than that published by Prats et al. (73). The chromatographic conditions adopted with LLE clean-up allow the separation of TYL from any co-extractives. The method developed in this study could be effectively utilized as a foundation for the development and validation of TYL analysis in tissues of other animal species. Liver residue depletion appears to be significant for estimating the withdrawal time of TYL in calves and piglets. The withdrawal period for the concentration of TYL in this tissue was set at 12 days for calves and 20 days for piglets, respectively, to ensure compliance with the EU MRL.

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