

# Identification and quantification of tylosin in animal feed by liquid chromatography combined with electrospray ionisation mass spectrometry

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## Abstract

**Introduction:** The authorisation of tylosin as feed additive was withdrawn for reasons of human health concerning resistance of pathogenic bacteria. An analytical method for the identification and quantification of tylosin in animal feed was developed and validated. **Material and Methods:** The samples were extracted using an acidified methanol:water mixture and solid-phase extraction was employed for the isolation of the antibiotic from diluted feed samples. Tylosin was analysed by liquid chromatography with electrospray ionisation mass spectrometric detection. The method's performance was evaluated in adherence to the Commission Decision 2002/657/EC. **Results:** The recovery of the analyte from spiked samples was determined to be in the range from 78.9% to 108.3% depending on tylosin concentrations. The CC $\alpha$  and CC $\beta$  values for tylosin in feeds were determined at 0.085 mg kg<sup>-1</sup> and 0.091 mg kg<sup>-1</sup>, respectively. The method detection limit was found to be 0.035 mg kg<sup>-1</sup> and the quantification limit 0.05 mg kg<sup>-1</sup>. The applicability of the developed method was tested by analysing real feed samples. **Conclusion:** A reliable LC-MS method was developed to identify and quantify tylosin in animal feed with a good repeatability and a high specificity and sensitivity. Because of these characteristics, the proposed method is applicable and could be deemed necessary within the field of feed control and safety.

**Keywords:** feed, antibiotics, tylosin, feed analysis, LC-MS.

## Introduction

Tylosin is a macrolide-type and bacteriostatic antibiotic produced by a strain of *Streptomyces fradiae*. It is widely and solely used in veterinary medicine and is classed as medium-spectrum because of high activity against Gram-positive bacteria, anaerobic bacteria, and mycoplasmas (20). Tylosin and its salts are used in food-producing animals and may be administered by oral or parenteral routes.

Antibiotics including tylosin were used as antibiotic growth promoters (AGPs), and their application became common agricultural practice during the late 1950s and 1960s. In the European Union (EU), tylosin was authorised as a growth promoter up to 1998 and added to the feed of farm animals in order to affect the intestinal microbial flora, thus increasing growth rates. To obtain improvements in

feed conversion and growth rate efficiency, the antibiotic was administered at subtherapeutic levels over an extended period (3, 27, 30). However, the wide application of antibiotics as feed additives for longer period could encourage the development of bacteria resistant to drugs used to treat infections (30, 31). Thus, the authorisation for tylosin (and four other antibiotics) was withdrawn in the EU in 1999 in order to protect human health (5, 8). The foundation for the ban was concerns over cross-resistances to macrolides, glycopeptides, and streptogramins used for human therapy, and those concerns led to a total ban in 2006 (7). Tylosin was withdrawn due to the cross-resistance to erythromycin and clarithromycin as therapeutic agents in human medicine. Since then, the use of tylosin is allowed only for direct applications or as medicated feed on veterinary prescription.

How animals are fed has a marked influence on food safety. Animal feedingstuffs must meet the requirements regarding contaminants and antibacterial agents. Monitoring of antibiotics in feed is important as a part of the overall control of contaminants within the food-processing chain, as elements potentially causing deleterious effects in consumer and animal health and violating good manufacturing guidelines. Feed contamination with antibiotics may cause harmful effects for both farm animals ingesting the contaminated material and consumers eating products from food-producing animals (1, 2, 15, 18). The inclusion of potentially harmful residues in edible products may pose a genuine threat to the consumer either by exposure to residue concentrations or through the transfer and development of resistant bacterial strains in humans.

Consequently, the official laboratories have to be responsible for a control strategy applying appropriate methods for a considerable volume of feed samples and identifying and quantifying a large number of analytes. Available methods of analysis have to be suitable for the effective and efficient control of the possible illegal use of banned or unwanted veterinary antibiotics to ensure feed and food safety (23, 27, 28). The methods should be implemented in an overall control strategy, which ensures the effective control of the ban throughout the EU, ranging from the fast and inexpensive screening methods to confirmatory methods, applying sophisticated instrumentation. Microbiological plate tests may be regarded as screening methods (21), whereas liquid chromatography with diode array detection (DAD) and especially liquid chromatography coupled with mass spectrometry (LC-MS) are powerful techniques for confirmatory analysis (9, 10, 16). However, it should be emphasised that various approaches are recommended in feed analysis, for instance, the confirmatory analysis of banned substances requires the use of MS detection according to Decision 2002/657/EC, but Regulation (EC) 152/2009 provides methods for analytes corresponding to this category that use UV detection (1, 4, 6). Few chemical methods for the detection of tylosin in animal feed have been developed, those that have been based on UV (14) and UV/DAD (3), ECD (13) or MS detection (27–29). Due to its multiple advantages, liquid chromatography coupled with mass spectrometry has become an essential technique in food and feed analysis laboratories.

The main purpose of the presented study was to develop a suitable and reliable LC-MS method, which could be applied to detect the illegal presence of tylosin in feed at a 1 mg kg<sup>-1</sup> level. Such a limit has been accepted according to the reported lowest level of additive still able to induce a considerable growth promoting effect (10). The secondary aim of the work was to elaborate a method appropriate to monitor the carry-over of tylosin in feed mills during the production

of medicated feed. To the authors' knowledge, this method is the first one for the determination of tylosin in animal feed developed and implemented in laboratory practice in Poland.

## Material and Methods

**Materials and reagents.** Tylosin tartrate was purchased from Sigma-Aldrich (USA). Acetonitrile and methanol (HPLC grade) were supplied by J.T. Baker (the Netherlands), *n*-hexane was obtained from Avantor Performance Materials (Poland), and formic acid was supplied by Sigma-Aldrich (USA). Water was deionised by the Millipore system (USA). Oasis HLB SPE cartridges (3 mL, 60 mg) were obtained from Waters (USA), and 0.45 mm nylon filters were from Agilent Technologies (USA).

**Preparation of matrix-based standard solutions.** The standard of tylosin was prepared in HPLC grade methanol at a concentration of 1 mg mL<sup>-1</sup> to obtain the standard stock solution, which was diluted with methanol at a concentration of 0.1 mg mL<sup>-1</sup> to prepare a set of matrix-based standards with concentrations ranging from 0.05 to 2 mg kg<sup>-1</sup>. Matrix-based standard solutions were prepared by extracting 5 g of blank feed fortified with working standards according to the procedure presented in the following section.

**Sample extraction.** All feed samples including negative and positive control samples were prepared according to the following procedure. The samples were homogenised and weighed (5 g) into a volumetric flask, 20 mL of extraction solvent (methanol/water (70/30, v/v) + 0.2% formic acid) was added and the samples were extracted for 30 min on a horizontal shaker. After the extraction, the samples were centrifuged for 10 min at 4,000×g, and then, 3 mL of the supernatants were diluted with 27 mL of water and 5 mL of *n*-hexane was added. Then, the samples were shaken for 5 min on a horizontal shaker and centrifuged for 10 min at 4,000×g at 10°C.

**SPE clean-up.** The aqueous phase of the sample was loaded onto an Oasis HLB cartridge that had been preconditioned with 3 mL of methanol and 5 mL of water. The cartridges were rinsed with 3 mL of water and then dried under vacuum to remove any excess of water. Finally, tylosin was eluted with 2 mL of 1:1 (v/v) acetonitrile:water mixture. The eluate was filtered through a nylon membrane filter (0.45 μm), and then, the solution was injected into the liquid chromatograph.

**Chromatographic analysis.** Analyses were conducted using a 1200 HPLC system from Agilent Technologies (USA) connected to a 6140 single mass spectrometer (Agilent Technologies). The mass spectrometer was equipped with an electrospray positive ionisation mode (ESI+) using a capillary voltage of 3000 V. The other optimum values of the ESI-MS parameters were drying gas temperature of

300°C, drying gas flow of 11 L/min, and nebulising gas pressure of 35 psi. HPLC separation was performed on a Zorbax Eclipse XDB-C18 column (150 × 4.6 mm, 5 µm; Agilent). The mobile phase was 0.05M formic acid in water (eluent A) and 0.05M formic acid in acetonitrile (eluent B) in the gradient elution programme. The mobile phase flow rate was 0.4 mL min<sup>-1</sup>, the injection volume was 5 µL, the column temperature was maintained at 35°C, and the run time was 15 min. The monitored precursor ion for tylosin was 916.5 *m/z*.

**Evaluation of the procedure.** Due to the lack of adequate validation guidelines for the feed area, the procedure was validated according to the Commission Decision 2002/657/EC to prove that the method is fit for purpose. The validation study was performed in terms of linearity, specificity, accuracy, precision (repeatability and within-laboratory reproducibility), as well as calculation of decision limit (CC $\alpha$ ) and detection capability (CC $\beta$ ). Representative blank feed samples (pig and poultry) were used to optimise and validate the method. A six-point matrix-matched calibration curve spiked at the levels of 0.05, 0.1, 0.25, 0.5, 1.0, and 2.0 mg kg<sup>-1</sup> was built. Linearity was evaluated by preparing a matrix-based calibration curve in the range of 0.05–2.0 mg kg<sup>-1</sup>. Specificity was determined by analysing 20 blank feed samples. Accuracy expressed as recovery, as well as repeatability, and within-laboratory reproducibility were determined by the repeated analysis (n = 6) of feed samples spiked with the analyte at three concentration levels: 0.05, 0.25, and 2.0 mg kg<sup>-1</sup>. The experiments were carried out on three consecutive days. Precision was evaluated by calculating the relative standard deviation (RSD) of the results obtained for each level of the target compound. The CC $\alpha$  and CC $\beta$  values were calculated using within-laboratory reproducibility results. Additionally, the limit of detection (LOD) and limit of quantification (LOQ) were estimated. The LOD and LOQ values were calculated on the basis of signal-to-noise ratio and were S/N = 3 for LOD and S/N = 10 for LOQ.

**Application to real samples.** The developed method was evaluated in proficiency tests for antibiotics in animal feed organised by Wageningen Food Safety Research (formerly known as RIKILT) in the Netherlands (11, 12). Our laboratory reported correct results without any false positives and negatives, and the *z*-scores were satisfactory. Moreover, the method was applied for real samples of commercial feeds. The real feed samples were obtained from the national feed control plan and from feed producers' internal quality control systems. In 2013–2017, we used our method to analyse 173 samples of feeds suspected of containing antibacterial substances and determine the presence of tylosin in them. Additionally, 64 feed samples were examined to identify tylosin as cross-contamination in non-target feed.

## Results

The presented method was developed to obtain qualitative and quantitative surveillance information about the analyte. Evaluation of the method demonstrates satisfactory validation parameters for its application in accordance with the Commission Decision 2002/657/EC. Good linearity was achieved with correlation coefficient (R<sup>2</sup>) = 0.9985. No interfering peaks were revealed in the region of interest. The recovery of tylosin for spiked samples was determined to be within the range of 78.9% to 108.3%. The validation results were repeatable and reproducible with the repeatability CV and within-laboratory reproducibility CV lower than 10% at all fortification levels. The CC $\alpha$  and CC $\beta$  values for tylosin in feeds were determined at 0.085 mg kg<sup>-1</sup> and 0.091 mg kg<sup>-1</sup>, respectively. The LOD was found to be 0.035 mg kg<sup>-1</sup> and the LOQ 0.05 mg kg<sup>-1</sup>. Validation data of the method are given in Table 1.

**Table 1.** Results of the validation of the analytical method for the determination of tylosin in feeds

Parameter	Fortification level (mg kg <sup>-1</sup> )	Results
Correlation coefficient		0.9985
Linearity (working range), mg kg <sup>-1</sup>		0.05-2.0
LOD, mg kg <sup>-1</sup>		0.035
LOQ, mg kg <sup>-1</sup>		0.05
CC $\alpha$ , mg kg <sup>-1</sup>		0.085
CC $\beta$ , mg kg <sup>-1</sup>		0.091
Recovery, % (n = 18)	0.05	88.2
	0.25	78.9
	2.0	108.3
Repeatability, CV % (n = 6)	0.05	2.4
	0.25	9.9
	2.0	3.0
Within-laboratory reproducibility, CV % (n = 18)	0.05	6.5
	0.25	6.9
	2.0	6.5
Uncertainty (k = 2, p < 95), %	0.05	21.4
	0.25	19.7
	2.0	23.8

A total of 237 real feed samples were tested. Among 173 feed samples analysed for antibacterial substances, six (3.5%) samples were found to be positive for tylosin. The analyte was identified at concentrations ranging from 0.26 mg kg<sup>-1</sup> to 64.8 mg kg<sup>-1</sup>. Cross-contamination of tylosin in non-target feed was revealed in 32 (50%) feed samples at concentrations ranging from 0.06 mg kg<sup>-1</sup> to 14.7 mg kg<sup>-1</sup>.

## Discussion

Concerns for human health related to antimicrobial resistance as well as consumer and political pressure prompted the European Union to ban the use of some antibacterial growth promoters including tylosin as feed additives in 1999. After the prohibition, improved analytical methods for the identification of prohibited medicinal additives were required. For the official control, a two-step control strategy has been implemented for the effective and efficient surveillance of the possible illegal use of antibiotics in feed. A common practice is the use of screening methods to detect antimicrobial substances; however, these often do not distinguish antibiotics well enough and give false positive non-compliant results (3). However, screening procedures are still often used because of their sensitivity, simplicity, speed, and low cost (24). Confirmatory tests should provide complete information and enable unequivocal identification and/or quantification of the target compounds, showing a low rate of false positive results.

Current feed-production practices lead inevitably to transfer, called “carry-over”, between consecutive production batches, so cross-contamination of the batch following the production of a medicated feed is practically unavoidable (1, 26). In some feed mills, different compounds including medicated feed are manufactured in the same production line, therefore traces of the first product may remain in the line and get mixed with the first batches of the next feed for non-target animals. This carry-over can lead to contamination of non-medicated feed with veterinary drugs. In this scenario, analytical methods for the effective control of non-medicated feeds play a crucial role.

The aim of the presented study was to develop and validate a highly sensitive and selective method for identifying both illegal usage of tylosin and unintentional contamination where concentrations are at a very low mg/kg level. The optimisation study was performed by identifying some factors: percentage of methanol, percentage of formic acid, addition of *n*-hexane, and dilution of the supernatant with water. Extraction solvents for tylosin were tested to find which was optimal for carrying out the analysis, and the recovery was established. Similarly to previously reported methods, methanol and water was used; however, they were utilised with our own modification (3, 13, 27). The extraction mixture with 70% methanol, 30% water, and 0.2% formic acid was maintained as the preferred extraction solvent. To improve the recovery of the compound, the addition of formic acid to the extraction solvent was necessary. Feed is recognized as one of the most difficult matrices to perform chemical analyses due to the presence of many components like plants, cereals, nutrients, and additives. Therefore, feed matrices are highly composite and very varied, which makes cleaning up

the extracts almost obligatory to obtain reliable results. The most common approach for cleanup is based on solid-phase extraction (SPE) (3, 17, 19, 27). Our method involves feed sample extraction followed by an SPE purification step. The method is actually based on two purification steps: the first with *n*-hexane in order to remove fats, and the second with an SPE column which allowed for the elimination of the major chromatographic interferences. We selected polymeric cartridges, on which we obtained good recoveries and which are used in most cases (2, 13, 17, 27). A cleanup procedure was developed for purification and concentration of feed extracts aimed at obtaining satisfactory tylosin recoveries and LOD and LOQ values. The dilution step of the extracts allowed higher sensitivity to be achieved. Chromatographic conditions were optimised to improve selectivity and sensitivity, and the addition of formic acid to the mobile phase contributed to the improvement of peak shape and ionisation efficiency.

An important advantage of the developed method is the possibility of detecting the presence of tylosin in feed not only as an unauthorised growth promoter but also as a result of cross-contamination after the production of medicated feed with tylosin. This method gave a limit of quantification of 0.05 mg kg<sup>-1</sup>, which allows the determination of tylosin at a very low level. The values of CC $\alpha$  and CC $\beta$  for tylosin obtained in our studies were 0.085 and 0.091 mg kg<sup>-1</sup>, respectively, and were much lower than the values presented by other authors. In the method presented by Van Poucke (27), the CC $\beta$  for tylosin was set at 0.31 mg kg<sup>-1</sup>, while in another method the CC $\beta$  parameter was 0.35 mg kg<sup>-1</sup> (3). The obtained repeatability and within-laboratory reproducibility were less than 10% and 7%, respectively, and were comparable to those achieved by other authors: within-laboratory reproducibility of <12% (3), and repeatability of <10% (27). The recoveries ranged from 79% to 108% and were also similar to those obtained by other authors: 68%–98% (27) and 96% (9).

The obtained results for real samples revealed that 38 (16%) of them contained tylosin. High concentrations of tylosin in pig feed could occur due to intentional non-medicinal use of the antibiotic (prophylaxis, growth promotion or arbitrary use by animal holders) with the highest determined concentration at 64.8 mg kg<sup>-1</sup>. However, the excuse for the relatively low concentrations of the antibiotic could be cross-contamination, which may appear either during manufacturing of medicated feed in a feed mill, during transport to farms, or even at the farm itself (storage, manipulation, and mixing operations). The study shows that in 50% of non-target feed samples, residues of tylosin were detected. The most frequently identified contaminating concentrations of tylosin in positive samples were in the range of 0.05–1 mg kg<sup>-1</sup>, many fewer samples contained tylosin in amounts above 1 mg kg<sup>-1</sup>, but there was also a sample containing

a much higher concentration. In most samples, the determined levels of the antibiotic were below the proposed maximum “carry-over” level for tylosin (1 mg kg<sup>-1</sup>), laid down in new Council Regulation (EU) 2019/4 (22). Generally, in the literature, there are few data regarding the problem of cross-contamination. A study conducted in the Netherlands (25) showed that a portion of flushing feed samples collected after production of medicated feed was contaminated with antibiotic residues. Overall, contaminating antimicrobials were detected in 87.1% of all samples tested. The most frequently detected antibiotic was oxytetracycline; however, tylosin was detected in seven samples with concentrations ranging between 0.6 and 6.0 mg kg<sup>-1</sup>. It is remarkable that some concentrations measured are in the same range as the banned AGP.

In conclusion, a new analytical procedure for the identification and quantification of tylosin based on LC–MS was introduced. The presented procedure provides a sensitive and selective method for the determination of tylosin at low levels in animal feed. The applicability of the method for the intended purpose was demonstrated by the satisfactory results obtained from the validation. The results show that the developed LC–MS method has satisfactory accuracy and precision and is sensitive and reliable. The method is a useful tool for identification of tylosin in routine analysis of commercial feed in combination with screening by microbiological inhibition. By means of the proposed method, the presence of tylosin can be determined in order to discover fraudulent feeds to which this substance could have been added. Moreover, low levels of tylosin in feedstuff can be also analysed in order to control cross-contamination in non-target feed in which this antibiotic could have been incorporated. For this reason, the presented method is applicable and might be considered necessary in the field of feed control and safety.

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