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Tylosin Detection in Animal Feed by Liquid Chromatography−Tandem Mass Spectrometry with Enzymatic Hydrolysis of the Tylosin Urea Adduct

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When the use of tylosin as a feed additive was forbidden by Council Regulation 2821/98, the necessity of a chemical confirmation method for the monitoring of the ban was created. Recently a method was developed for the detection of tylosin in animal feed by means of LC-MS/MS. During the validation high deviating values for the decision limit, detection capability, and repeatability for tylosin in cattle feed were observed, and the presence of urea and the formation of a tylosin urea adduct (TUA) were suggested as possible explanations. In this study two hydrolysis approaches for the TUA adduct were compared, namely, a chemical hydrolysis and an enzymatic hydrolysis with urease. The latter yielded a more complete hydrolysis of urea and was used for further validation. The recovery increased by ~15−25% depending on the amount of urea present in the feed (0.5−2%). The decision limit and detection capability were hardly influenced by the enzymatic hydrolysis.

KEYWORDS: LC-MS/MS; tylosin; tylosin urea adduct; animal feed

INTRODUCTION

Tylosin is a macrolide-type antibiotic produced by *Streptomyces fradiae* (*1*). It is extensively and exclusively used in veterinary medicine and is classified as medium-spectrum because it has a high activity against Gram-positive bacteria and mycoplasma but only a limited activity against Gram-negative bacteria. It is being used therapeutically for the treatment of dysentery in pigs, pneumonia and mastitis in cattle, and mycoplasma infections in poultry (*2*). The antibiotic is a complex mixture of several compounds (see **Figure 1**) of which tylosin A is the major compound (*1*). It normally accounts for >85% of the active ingredients in commercial preparations.

Up to 1998 tylosin was added to the feed of poultry, swine, and cattle as a growth promoter. This was done at subtherapeutic levels over an extended period of time to obtain improvements in feed conversion and growth rate efficiency (*1*, *3*). The licensing, authorization, and inclusion rate of this feed additive were regulated by Council Directive 70/524/EEC (*4*). To control if this label declared feed additive (and others) was present within specified tolerances, Council Directive 95/53/EC (*5*) prescribed the use of Community methods of analysis that were all microbiologically based methods.

Because of the risk that residues of tylosin in edible tissue could lead to the development of resistant strains of bacteria in humans (*1*), the European Union decided to ban the use of tylosin together with three other antibiotics (zinc bacitracin, spiramycin, and virginiamycin) as a growth promoter (*6*). As a consequence, these Community methods of analysis were no longer useful because they lack the specificity required to identify an unknown compound. A few chemical methods for the detection of tylosin in animal feed have been developed (*7*,

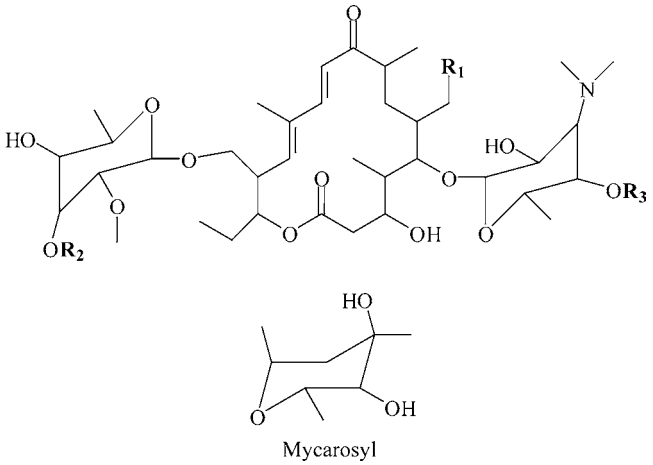

Figure 1. Structure of tylosin: TYLOSIN (tylosin A): R_1 = CHO, R_2 = CH_3, R_3 = mycarosyl; DESMYCOSIN (tylosin B): R_1 = CHO, R_2 = CH_3, R_3 = H; MACROCIN (tylosin C): R_1 = CHO, R_2 = H, R_3 = mycarosyl; RELOMYCIN (tylosin D): R_1 = CH_2OH, R_2 = CH_3, R_3 = mycarosyl.

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8)—one based on UV detection and the other on particle concentration fluorescence immunoassay. Since September 2002, however, Commission Decision 2002/657/EC (*9*) came into force. This directed that the confirmation of this banned additive should be done by either mass spectrometry (MS), infrared detection (IR), UV−vis diode array detection (DAD), fluorescence detection, electron capture detection (ECD), or two-dimensional (2D) thin-layer chromatography. The use of UV−vis detection is allowed only when two different chromatographic systems are used.

Due to the multiple advantages of liquid chromatography−tandem mass spectrometry we decided to focus on this technique. The simultaneous ban of three other antibiotics (*6*) resulted in the development a multianalyte method for the detection of the banned antibacterial growth promoters in animal feed (*10*).

During the validation of the already published method, a problem for tylosin detection in cattle feed was observed. Compared to other feed types, high values for the decision limit, detection capability, and repeatability for tylosin in cattle feed were encountered. The presence of urea in cattle feed as a nonprotein nitrogen source and the formation of a tylosin urea adduct (TUA), as described by Houglum et al. (*7*), resulting in a lower extraction yield (*10*) were suggested as possible explanations. This paper describes the research undertaken to prove this thesis and in particular the procedure of how to hydrolyze the TUA complex.

MATERIALS AND METHODS

Reagents and Chemicals. Methanol, acetonitrile, glycerol (BDH Laboratory Supplies), formic acid, phosphoric acid, sodium acetate, acetic acid, Titriplex III (disodium EDTA) (Merck), and disodium hydrogen phosphate and ammonia (UCB) were all supplied by VWR (Leuven, Belgium). Potassium dihydrogen phosphate (Ferak) was purchased from Fiers (Kortrijk, Belgium). Tylosin tartrate (containing ~90% tylosin) and urease (type IX from jack beans; 20000 units) were from Sigma-Aldrich (Bornem, Belgium). Double-deionized water (Milli-Q; Millipore Corp.) of 18.2 MΩ cm^{-1} resistivity was used throughout.

Urea-Containing Feed Samples. To see the correlation between the amount of urea present in the feed and the effects of the hydrolysis, urea-containing feeds were prepared in the laboratory. These were used to test the different approaches for hydrolyzing the TUA complex and for the validation. Urea was added to the feed at two [0.5 and 2% (w/w)] levels. Because of the European ban of tylosin, we were not able to purchase tylosin-containing feed, and thus spiking was the only possible solution. The feed samples were spiked with tylosin at a fifth of the previous allowed minimum inclusion rate (5 mg/kg), resulting in a minimum required performance level (MRPL) of 1 mg/kg.

Urease Solutions. The stock solution of urease (1 mg/mL) was prepared in a phosphate buffer (0.02 M, pH 7.6) containing 1 mM EDTA and 50% (v/v) glycerol. This stock solution was stored at 4 °C for a maximum of 1 month. The working solution of 0.2 mg/mL (±20 units/mL) was prepared freshly by diluting with water.

Apparatus and Materials. OASIS HLB columns (6 mL; 200 mg) were purchased from Waters (Brussels, Belgium). The OASIS column was conditioned by passing 5 mL of methanol followed by 5 mL of water through it.

The analyses were performed on a Waters Alliance 2695 HPLC system (Milford, MA) with a Kromasil C_{18} column (3.2 × 150 mm; 5 μm) (Alltech, Belgium) coupled to a Quattro Micro mass spectrometer (Micromass, Manchester, U.K.).

High-purity nitrogen was used as the drying and electrospray ionization (ESI) nebulizing gas. Argon was used as the collision gas for collision-induced dissociation.

Sample Cleanup. The standard sample cleanup procedure that is further adapted is described in detail by Van Poucke et al. (*10*). In

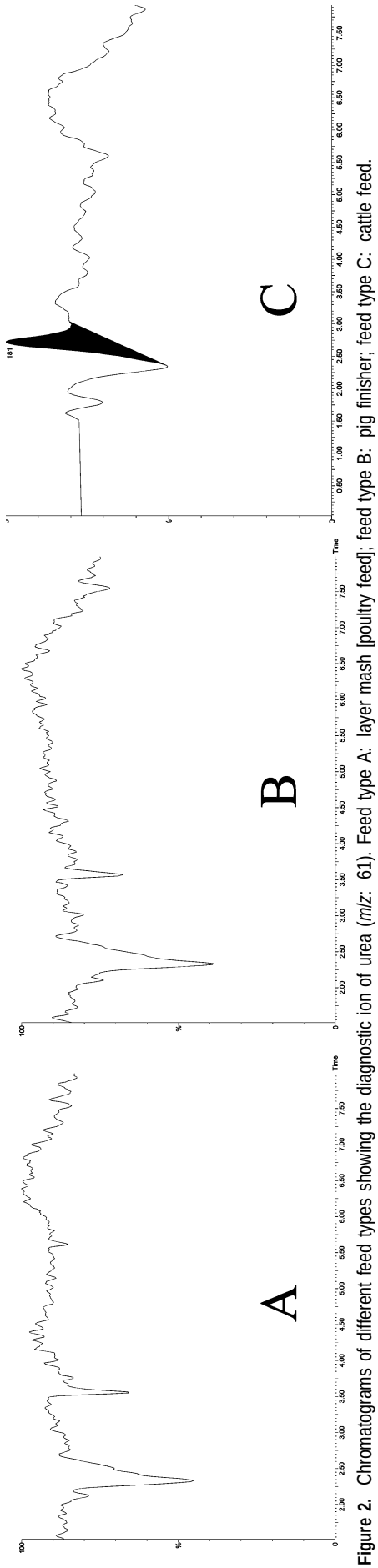

Figure 2. Chromatograms of different feed types showing the diagnostic ion of urea (*m/z*: 61). Feed type A: layer mash [poultry feed]; feed type B: pig finisher; feed type C: cattle feed.

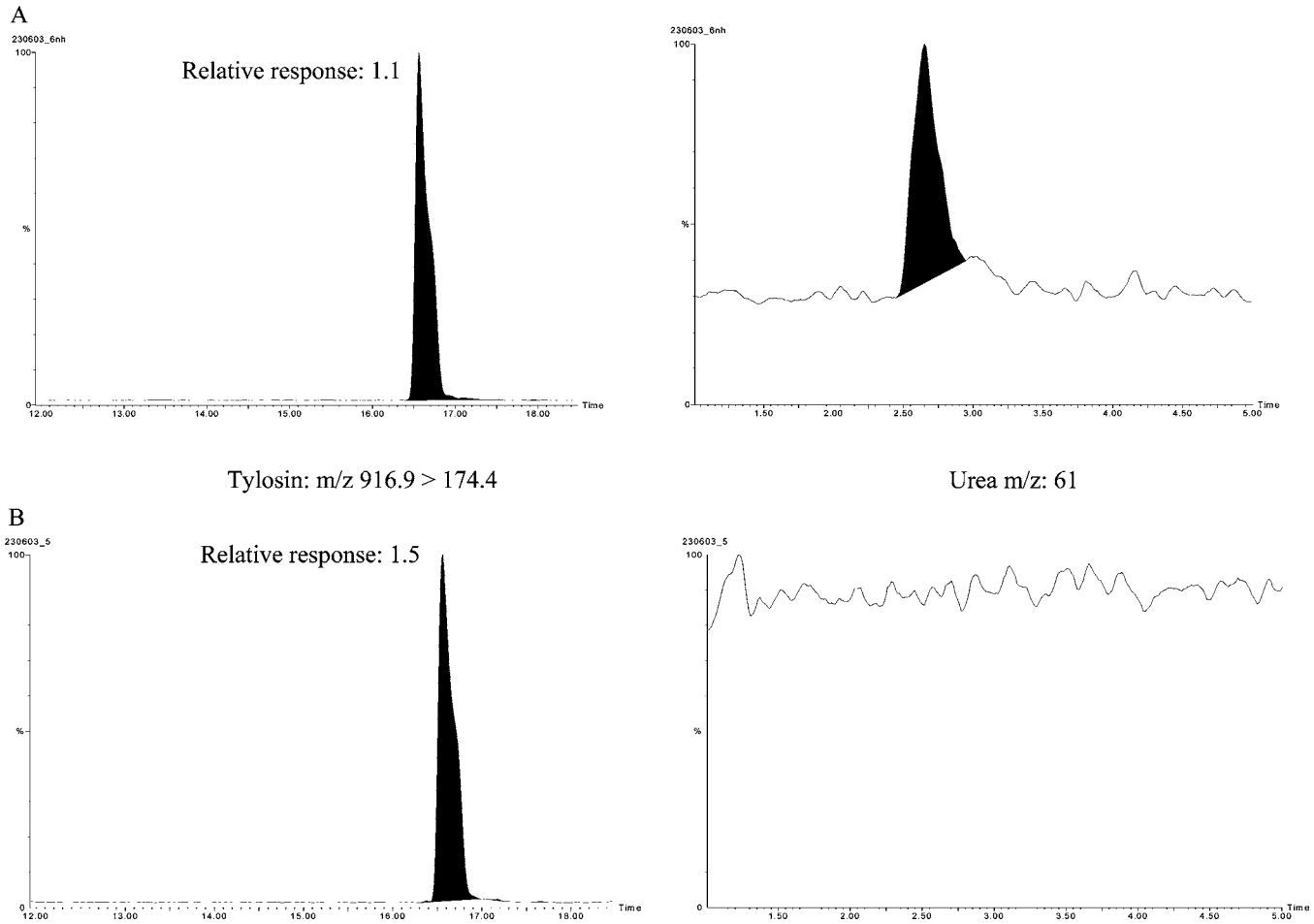

Figure 3. Chromatograms of the diagnostic ions of urea (*m*/*z*: 61) and tylosin (*m*/*z*: 916.9 > 174.4). A: without enzymatic hydrolysis; B: with enzymatic hydrolysis.

summary, the samples are extracted with a 70% methanolic (v/v) solution (containing 2% formic acid) and are cleaned up by solid-phase extraction on OASIS HLB cartridge columns.

The method used for the chemical hydrolysis was a combination of the above-mentioned method and the chemical hydrolysis described by Houglum et al. (*7*). Two and a half grams of feed was extracted with 10 mL of methanol/water (70:30, v/v) and vigorously shaken. After centrifugation, the pH of 3 mL of the supernatant was adapted by first adding 15 mL of a phosphate buffer (0.1 M, pH 8) and next phosphoric acid until the pH was ∼5.5. This solution was then heated for 1 h at 95−99 °C. After cooling, this solution was diluted to 30 mL with water and the standard OASIS cleanup procedure (*10*) was performed.

To test the enzymatic hydrolysis, the extraction of 2.5 g of feed was done with 10 mL of a 70% (v/v) methanolic solution containing no formic acid. After 0.5 h of shaking and centrifugation, 3 mL of the supernatant was diluted with 26 mL of water. After adaptation of the pH to 7.5 with a 20% ammonia solution, 1 mL of the urease working solution (0.2 mg/mL) was added. This mixture was then kept for 4 h at 26 °C. To stop the reaction, 60 μL of formic acid was added and next the normal OASIS HLB cleanup was done.

Liquid Chromatographic−Tandem Mass Spectrometric Conditions (LC-MS/MS). The gradient used to achieve chromatographic separation, the mass spectrometric settings, and the transitions monitored have been published by Van Poucke et al. (*10*).

RESULTS AND DISCUSSION

Our first objective was to evidence the presence of urea in the cattle feed and the absence in the other feed types. This was done by performing the standard cleanup procedure on different feed types and monitoring the parent ion of urea (*m*/*z* 61) with a cone voltage of 25 V. Urea eluted at ∼2.75 min. Different feed types were analyzed. No urea peak was observed with the poultry feed (layer mash) or with the pig feed (pig finisher), but a peak was visible in the cattle feed (**Figure 2**). The presumed presence of urea and the formation of the TUA complex that causes the high deviating values observed were supported by these findings.

The first approach for the hydrolysis of the TUA complex was based on the method of Houglum et al. (*7*). Urea is chemically hydrolyzed by heating the extract at pH 5.5. To test the method, a blank calf feed was mixed with 0.5% urea (w/w) and was spiked with tylosin at 1 mg/kg. Six samples followed the normal cleanup procedure, and six samples were submitted to the adapted procedure with chemical hydrolysis. After hydrolysis, an increase of ∼10% of tylosin peak area was observed, indicating the hydrolysis of the TUA complex, but the urea peak was reduced to not more than 85% of the original peak, indicating a partial hydrolysis of the urea and thus of the TUA complex.

The addition of formic acid to the extraction solvent for the experiments with the enzymatic hydrolysis was omitted for the following two reasons. First, the addition of formic acid to the original extraction solvent was necessary only to improve the recovery of one of the other compounds (zinc bacitracin) in the multianalyte method. Second, the optimal pH for the urease activity is 7.5. In contrast with the chemical hydrolysis, the 3 mL aliquot of the extract was diluted before hydrolysis to reduce the methanol content and so to avoid denaturation of the enzyme. To optimize reaction time, peak areas of tylosin and urea for two groups (each group consisting of six samples) were

Table 1. Increase (+)/Decrease (–) in Tylosin and Urea Peak Areas (Percent) after Enzymatic Hydrolysis [±Standard Deviation (Percent)]

	1 h	2 h	3 h	4 h	overnight
tylosin	+0.55 [±6.8]	–1.83 [±6.7]	–3.31 [±6.2]	+22.04 [±3.3]	+13.15 [±3.8]
signif[a]	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p < 0.001$	$p < 0.01$
urea	–100.00	–98.72	–97.98	–100.00	–100.00

[a] Significance tested at 95% level ($\alpha = 0.05$).

Table 2. Summary of Obtained Validation Parameters for Urea-Containing Feed (0.5 and 2%) Spiked with 1 mg/kg Tylosin with or without Enzymatic Hydrolysis

		decision limit (mg kg^{-1})[a]	detection capability (mg kg^{-1})	recovery ± repeatability (%)
0.5% urea	no hydrolysis	0.19	0.29	92.5 ± 7.5
	hydrolysis	0.17	0.31	107.2 ± 10.2
2% urea	no hydrolysis	0.34	0.35	77.7 ± 5.1
	hydrolysis	0.29	0.41	105.5 ± 15.5

compared. One group followed the standard cleanup procedure (however, without formic acid in the extraction solvent); the second group of samples was subjected to the enzymatic hydrolysis. The mean values of six measurements in both groups were tested for significant differences at the 95% level with an independent sample *t* test. The feed used for this experiment was mixed with 0.5% (w/w) urea and spiked with 1 mg/kg tylosin. The results are summarized in **Table 1**.

Whereas the urea has already completely disappeared after 1 h, the highest significant increase of the tylosin peak was found after 4 h. This indicates that urease first hydrolyzes the free urea before the urea of the TUA complex. The hydrolysis time was fixed at 4 h for further experiments. **Figure 3** shows chromatograms of tylosin and urea in feed, with and without enzymatic hydrolysis.

Finally this enzymatic procedure was validated in accordance with Commission Decision 2002/657/EC (*9*) using a limited number (12−24) of samples. In this procedure we determined the decision limit (CCα), detection capability (CCβ), and the accuracy, expressed in terms of recovery and repeatability [coefficient of variation (CV)] for two groups of feed, each group containing either 0.5 or 2% urea. For each group a number of samples were hydrolyzed, and an equal amount of samples followed the standard cleanup procedure. The results obtained are summarized in **Table 2**.

After hydrolysis, an increase in tylosin recovery can been observed. Moreover, the more urea is added, the lower the extraction yield of tylosin. This supports the presumption of the formation of a tylosin urea adduct. The decision limit and detection capability seem not to be influenced by the enzymatic hydrolysis. However, the high deviating values for the decision limit (0.4 mg kg^{-1}), detection capability (0.8 mg kg^{-1}), and repeatability (≥24%) seen for tylosin in cattle feed during previous validation work were not reproduced. The possible explanation given for these values, namely, the presence of urea, was not supported by these experiments and must thus be caused by something else. However, we can conclude that the approach of the enzymatic hydrolysis with urease yields an increase of tylosin recovery.

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