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Quantitative Analysis of Tylosin in Eggs by High Performance Liquid Chromatography with Electrospray Ionization Tandem Mass Spectrometry: Residue Depletion Kinetics after Administration via Feed and Drinking Water in Laying Hens

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Maximum residue limits (MRLs) have been established by the European Union when tylosin is used therapeutically. They are fixed at 200 μg/kg for eggs. A highly sensitive and selective quantitative liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS) method suitable for monitoring tylosin residues in eggs to determine its depletion kinetics was developed and validated. For sample pretreatment all samples were liquid−liquid extracted with citrate buffer (pH 5.0) and acetonitrile. Liquid chromatographic separation was carried out on a reversed phase C18 column employing a 0.5% formic acid/acetonitrile gradient system. The tylosin recovery in eggs at a concentration range from 1.0−400 μg/kg was >82% with relative standard deviations between 1.5 and 11.0%. In two experimental studies administrating tylosin via feed (final dosage: 1.5 g/kg) or drinking water (final dosage: 0.5 g/L), no residues above the MRL were found during and after treatment. Moreover, all samples were well below the actual MRL of 200 μg/kg. Therefore, our residue data suggest that a withholding period for eggs is not required when laying hens are treated with tylosin in recommended dosages via feed or drinking water.

KEYWORDS: Tylosin; residue; depletion; laying hen; withholding period; mass spectrometry

INTRODUCTION

Tylosin is a macrolide antibiotic frequently used in veterinary medicine with a high molecular weight of 916. The log k_{ow} value of tylosin is 1.63 (*1*), and thus the compound may be classified as a moderate hydrophilic veterinary drug. It has a macrocyclic ring structure containing 16 carbon lactones. This ring system is coupled to neutral and amino sugars, giving the molecule a basic character. The molecular structure of tylosin is shown in **Figure 1**.

In general, macrolides (e.g. tylosin, erythromycin, oleandomycin) possess antimicrobial activity against gram-positive organisms and—in rare cases—against gram-negative ones. They are nevertheless useful against mycoplasms, which have no cell wall. Macrolides inhibit protein synthesis by binding to the bacterial 50S ribosome, thus inhibiting polypeptide translation.

Salts (tartrates, phosphates, lauryl sulfates) and esters (estolate, adipate, ethyl succinate) of the macrolides are synthesized to enhance their water solubility and/or bioavailability. After hydrolytic or enzymatic cleavage of the derivatives, the free bases are absorbed, and accumulate preferably in tissue. However, the oral bioavailability of macrolides depends on individual differences and on feed consumption, and is in the range of approximately 9% to 45% (*2*).

Tylosin was previously reevaluated by the Committee for Veterinary Medicinal Products (CVMP). The committee considered a microbiological acceptable daily intake (ADI) of 0.360 mg per person (*3*). Finally, CVMP recommended the inclusion of tylosin in Annex I of Council Regulation (EEC) No. 2377/90. The maximum residue limits (MRLs) for the marker residue tylosin in all food producing species were as follows: muscle (100 μg/kg), fat (100 μg/kg), liver (100 μg/kg), kidney (100 μg/kg), milk (50 μg/kg), and eggs (200 μg/kg) (*4*). However, the maximum daily intake of tylosin based on these MRLs values will represent 80% of the ADI (*3*).

The aims of the current study were to develop and validate an appropriate quantitative analytical method suitable for monitoring tylosin residues in whole eggs and to determine its depletion kinetics after oral administration via feed and drinking water.

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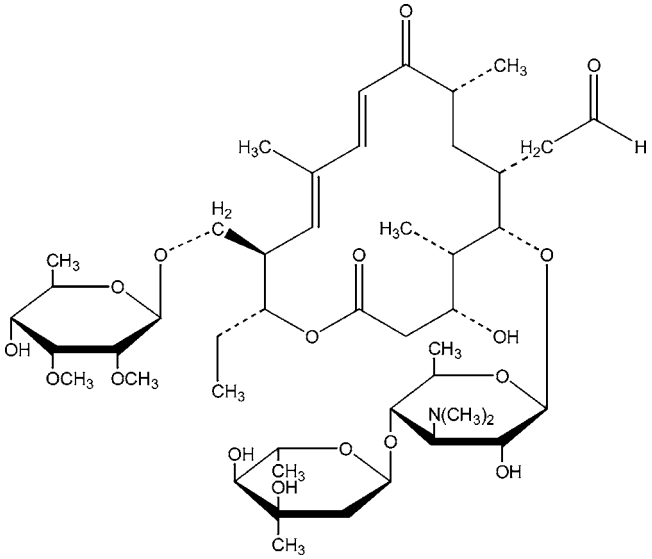

Figure 1. Molecular structure of tylosin.

MATERIALS AND METHODS

Hens and Experimental Design. Eighteen laying hens, 20 weeks old and weighing 2.0 (1.7−2.4) kg, were individually kept in cages, fed with a basal diet (Deuka all-mash L B 300/62; Düsseldorf, Germany), and given tap water ad libitum. They were divided into two groups of nine hens each.

Tylosin 25% (Lilly, ELANCO Division, Bad Homburg, Germany), which consists of 25 g tylosin phosphate per 100 g, was mixed into their basal diet to obtain a final tylosin concentration of 1.5 g/kg. Tylan soluble (Lilly, ELANCO Division, Bad Homburg, Germany), which consists of 100% tylosin tartrate, was diluted in tap water to a final tylosin concentration of 0.5 g/L. The dosages were chosen according to the recommendations of the manufacturer. The drug was administered via feed and drinking water for five consecutive days. Food and water consumption during the study was recorded individually for each hen (see **Tables 1** and **2**). Eggs were collected—whenever possible—at 1 day intervals over a period of 15 days. The whole egg was immediately separated from the shell, weighed, and stored in individual polyethylene tubes with caps (84 × 30 mm, Sarstedt, Nümbrecht, Germany) and frozen at −20 °C until they were analyzed for tylosin residues.

Chemicals and Reference Substance. Acetonitrile (J.T. Baker, Griesheim, Germany) was of HPLC grade, and ammonium acetate, citric acid, formic acid, and sodium hydroxide (all obtained from Merck, Darmstadt, Germany) were of analytical reagent grade. Water was prepared in-house by a Milli-Q-System (Millipore, Eschborn, Germany) with a resistance of >18.2 MΩ/cm. Tylosin tartrate was obtained from Sigma (Munich, Germany) and had a purity of 90.8% (confirmed by Sigma employing HPLC analysis). A stock solution of tylosin was prepared by dissolving 1 mg of the drug in 1 mL methanol. The stock solution was stored at −20 °C and was stable for at least 2 months. Working dilutions were prepared freshly on the day of use. For that purpose, prediluted methanolic solutions of 10 μg/mL, 1 μg/mL, and 0.1 μg/mL were prepared. From these solutions calibration standards from 0.00125 μg/mL to 1.25 μg/mL were prepared independantly in methanol.

Extraction of Eggs. The extraction procedure was developed under consideration of a previously published method for the determination of various tetracyclines in whole eggs (*5*). Whole egg was homogenized using an Ultra Turrax homogenizer (T25 basic, IKA Labortechnik, Staufen, Germany). One gram of the homogenate was mixed with 0.6 mL of 1 M citrate buffer (pH 5) in 25 mL plastic tubes (Beckman, Munich, Germany) for 1 min using a magnetic stirrer. Ten milliliters of acetonitrile (HPLC gradient grade, J.T.Baker, Gross-Gerau, Germany) was then added, and the whole mixture was stirred for another 10 min. The sample was centrifuged for 10 min at approximately 11000*g*. The resulting supernatant was transferred into a 250 mL round-bottom glass flask, and the residue was mixed with 0.6 mL of distilled water and homogenized. Another 10 mL of acetonitrile was added to the sample, and the extraction procedure was repeated. The supernatants were combined and rotoevaporated to dryness under vacuum at 40 °C. It is highly recommended to use 250 mL flasks because the egg extract may foam heavily during evaporation. Furthermore, a continuous observation of the evaporation process should be performed. The dried sample was reconstituted with 400 μL methanol under sonification, transferred with a glass Pasteur pipet into a 1.5 mL plastic tube (Eppendorf, Germany), and centrifuged for 1 min at 20000*g*. The supernatant was transferred into a 1.5 mL autosampler vial (Thermo Electron Cooperation, Dreieich, Germany) equipped with a 300 μL insert (Chromatography Service, Langerwehe, Germany). Prior to LC/MS/MS analysis, the samples were kept at 10 °C in the autosampler.

Liquid Chromatography/Tandem Mass Spectrometry and Quantification. The LC/MS/MS procedure was developed under consideration of a previously published method for the determination of tylosin in soil and water (*6*). The HPLC system used was a gradient system consisting of a Thermoquest P4000 pump, an AS3000 autosampler (San Jose, CA), and a Puresil C18 column (5 μm, 4.6 × 150 mm, Waters Corporation, Milford, CT) operated at 23 °C. The flow of 1 mL/min was split 1:10 before entrance into the mass spectrometer. The mobile phase consisted of 0.5% formic acid in water with 1 mM ammonium acetate (solvent A, pH 2.5) and acetonitrile (solvent B). The gradient run was 0−50% B in 9 min, then held at 50% B for 1 min. After each run, the column was rinsed for 3 min with 99% acetonitrile and reequilibrated for 8 min with solvent A. The injection volume was 8 μL. The autosampler was rinsed after each injection with 3 mL of 90% methanol/10% 10 mM oxalic acid in water.

Mass spectrometry was carried out using a LCQ ion trap with an electrospray ionization source (Finnigan Mat, San Jose, CA). A standard solution of tylosin (10 ng/μL) was infused through an integrated syringe pump at a flow rate of 10 μL/min for tuning the mass spectrometer and optimizing capillary temperature, sheath gas, and auxiliary gas flow rates. The source polarity was set positive, the spray needle voltage was 5 kV. Drying gas was nitrogen generated from pressurized air in an Ecoinert 2 ESP nitrogen generator (DWT-GmbH, Gelsenkirchen,

Table 1. Water Consumption of the Hens in Milliliters per Day During Treatment with Tylan Soluble[a]

	water consumption [mL per day]					total tylosin consumption [mg]
hen [no.]	1[b]	2[b]	3[b]	4[b]	5[b]	1–5[b]
W1	300	270	300	290	350	755
W2	300	270	220	190	200	590
W3	300	270	210	160	200	570
W4	430	370	230	200	250	740
W5	430	370	260	260	290	805
W6	430	370	600	490	380	1135
W7	300	270	210	260	220	630
W8	300	270	20	20	20	315
W9	300	270	260	240	260	665

[a] Final tylosin dosage: 0.5 g/L. [b] Day(s) of treatment.

Table 2. Feed Consumption of the Hens in Grams per Day During Treatment with Tylan 25%[a]

	food consumption [g per day]					total tylosin consumption [mg]
hen [no.]	1[b]	2[b]	3[b]	4[b]	5[b]	1–5[b]
F1[c]	15	15	15	35	35	173
F2	130	130	115	110	85	855
F3	120	130	110	95	95	825
F4	105	120	115	130	90	840
F5	145	140	130	125	120	990
F6	155	130	130	135	130	1020
F7	140	120	130	120	115	938
F8	120	130	125	120	130	938
F9	135	120	120	130	115	930

[a] Final tylosin dosage: 1.5 g/kg feed. [b] Day(s) of treatment. [c] Hen F1 was sick during treatment thus leading to a decreased food consumption.

Table 3. Recoveries ($n = 3$) at Various Concentrations and Day-to-Day Variation at the 50 μg/kg Level ($n = 7$) and the Corresponding Relative Standard Deviations (RSD) of Tylosin in Whole Egg

concn [μg/kg]	recovery [%]	RSD [%]
1	102.1 ± 3.4	3.3
5	82.0 ± 9.0	11.0
10	104.2 ± 5.4	5.2
20	88.8 ± 1.3	1.5
50[a]	92.5 ± 8.8	9.5
100	88.3 ± 7.5	8.5
200	105.1 ± 7.0	6.7
400	110.9 ± 3.9	3.5

[a] Please note that day-to-day variation was only performed at this level.

Germany). The optimized conditions were as follows: sheath gas flow was set at 100 units, the auxiliary gas was turned off, and the capillary temperature was 150 °C. The isolation width (m/z) was 2.0 and optimal collision energy in MS/MS mode was found to be 27%, causing a nearly quantitative fragmentation of the protonated molecular ion of tylosin. A calibration curve constructed for tylosin ranged from 0.01−10 ng on column with a constant injection volume of 8 μL and was linear, with $r^2 > 0.99$ for the MS/MS procedure. We used the MS2 scan mode for quantitative analysis. Quantification was obtained by comparing the peak areas of the sample with that of the external calibration curve, and all data were corrected for mean recovery.

The criteria for the confirmation of peak identity were according to Decision 2002/657/EU (*7*) for MRL substances: retention time (RSD < 2.5%) and at least two MS/MS ions specific for the standard spectrum. The maximum permitted tolerances for relative ion intensities were ±20% for a relative intensity >50%, ±25% for a relative intensity between 20% and 50%, ±30% for a relative intensity between 10% and <20%, and ±50% for a relative intensity <10%.

Method Validation. At least 20 control eggs free of tylosin residues were extracted and analyzed with the method mentioned above for checking the specifity of the method. For this purpose, white and brown eggs were bought in local supermarkets. Recovery studies were carried out with control eggs spiked with methanolic stock solutions at the 0.5, 1, 5, 10, 20, 100, 200, and 400 μg/kg level. The volume of the methanolic stock solutions used for spiking did not exceed 40 μL. The extraction procedure was performed after 10 min equilibration at room temperature. The recovery rate was calculated as an average of three experiments.

Day-to-day variation of the method was tested during the study at a concentration of 50 μg/kg by spiking control eggs. The day-to-day variation was calculated using seven independent experiments performed on seven different days.

RESULTS AND DISCUSSION

Accuracy, Precision, Quantification, and Detection Limits for LC/MS/MS. HPLC employing a simple gradient system combined with ESI/MS/MS permitted a highly sensitive and selective detection of tylosin residues in whole eggs. The mean recovery of fortified samples in the 1.0−400 μg/kg range was 97.3% with excellent linearity ($r^2 = 0.9961$) and acceptable relative standard deviations (1.5−11.0%) over a broad concentration range. Moreover, the day-to-day-variation of tylosin analysis in eggs at seven different occasions spiked at 50 μg/kg was 9.5% (see **Table 3**).

From our recovery data, a limit of quantification of 1 μg/kg was established for this method in eggs. The smallest amount that could be detected in spiked samples based on a signal-to-noise ratio greater than 6 was 0.5 μg/kg, which corresponds to approximately 10 pg of tylosin on-column. In addition, the unequivocal confirmation of tylosin residues can be obtained via MS/MS spectra, which provide at least three significant diagnostic ions (m/z 407, 754, 772; see **Figures 2B−C** and **3A**). When a standard solution is compared to an egg sample the

Table 4. Tylosin Concentrations [μg/kg] in Whole Eggs Laid by Hens Fed a Diet Containing 1.5 g/kg Tylosin or Receiving Drinking Water Containing 0.5 g/L Tylosin[a]

	tylosin [μg/kg]			
hen [no.]	day 0 (control)	day 5	day 6[b]	day 7
W1	X[c]	3.7		2.4
W2	<0.5	X		2.8
W3	<0.5	7.4		3.7
W4	X	13.9		5.8
W5	<0.5	39.0		21.3
W6	<0.5	X	3.8	6.1
			4.7	4.3
W7	<0.5	7.1	5.2	X
W8	<0.5	X	5.3	X
			5.9	X
W9	<0.5	12.7	6.0	7.8
			6.9	6.3
F1	<0.5	X	7.6	5.0
			8.1	7.8
F2	<0.5	16.6	16.4	
F3	<0.5	15.1	19.2	6.3
			19.6	
F4	<0.5	X	19.8	5.0
F5	<0.5	58.6	30.0	22.0
			37.2	
F6	X	27.0		9.8
F7	<0.5	10.1		3.6
F8	<0.5	8.1		3.2
F9	<0.5	46.8		27.9

[a] Unfortunately, eggs were not sampled individually on day 6. [b] Please note that, because eggs were not individually collected at this day, tylosin concentrations are simply listed starting from the lowest value found to the highest one. [c] X = no egg laid on this day.

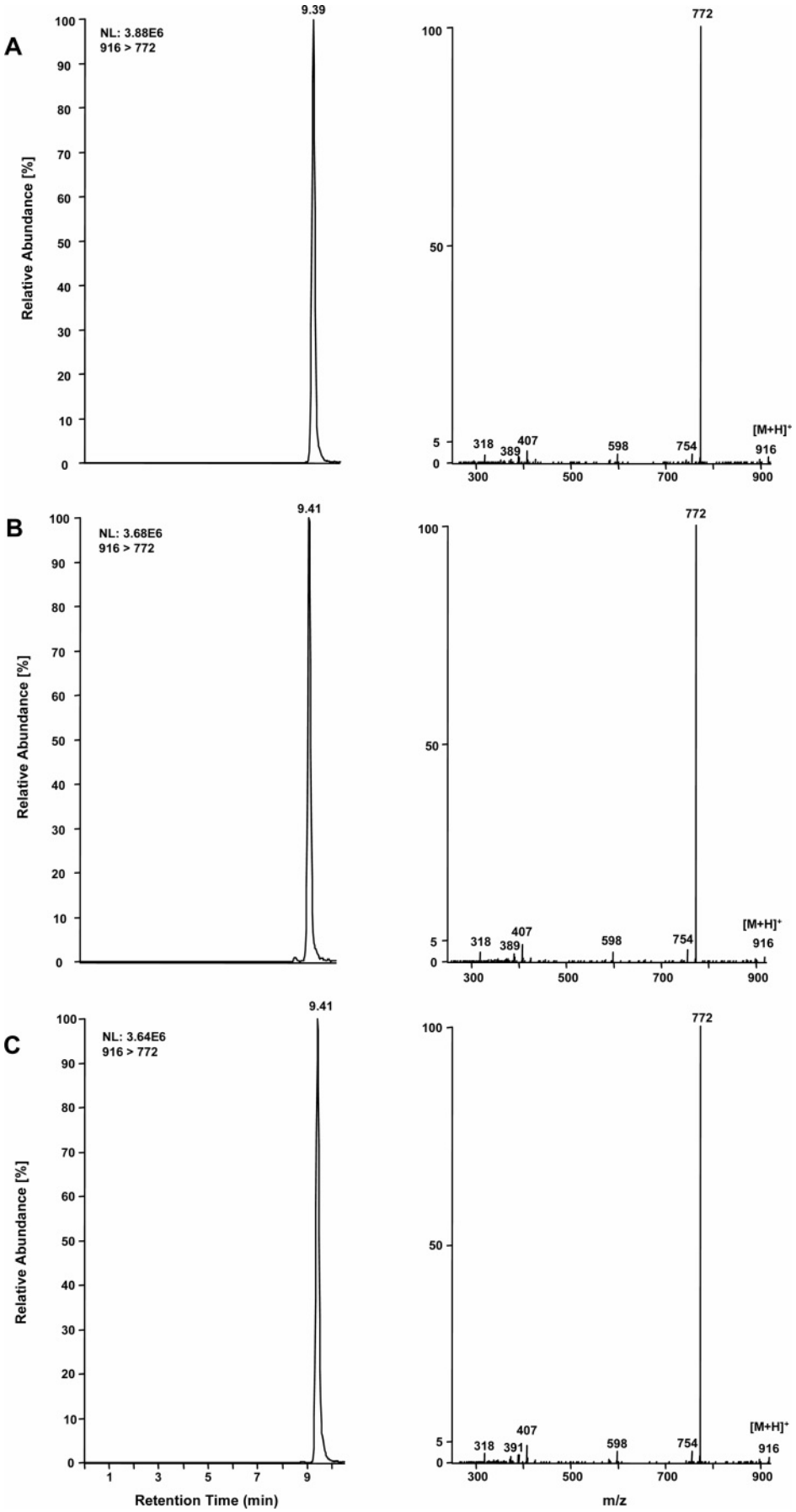

Figure 2. (**A**) Mass chromatogram for a standard sample and the corresponding MS/MS spectrum (1 ng on column). (**B**) Mass chromatogram of a control egg spiked with 50 μg/kg tylosin and the corresponding MS/MS spectrum (approximately 1 ng on column). (**C**) Mass chromatogram of an egg sample (W5/1) containing 45.1 μg/kg tylosin and the corresponding MS/MS spectrum (approximately 1 ng on column). NL: normalized level.

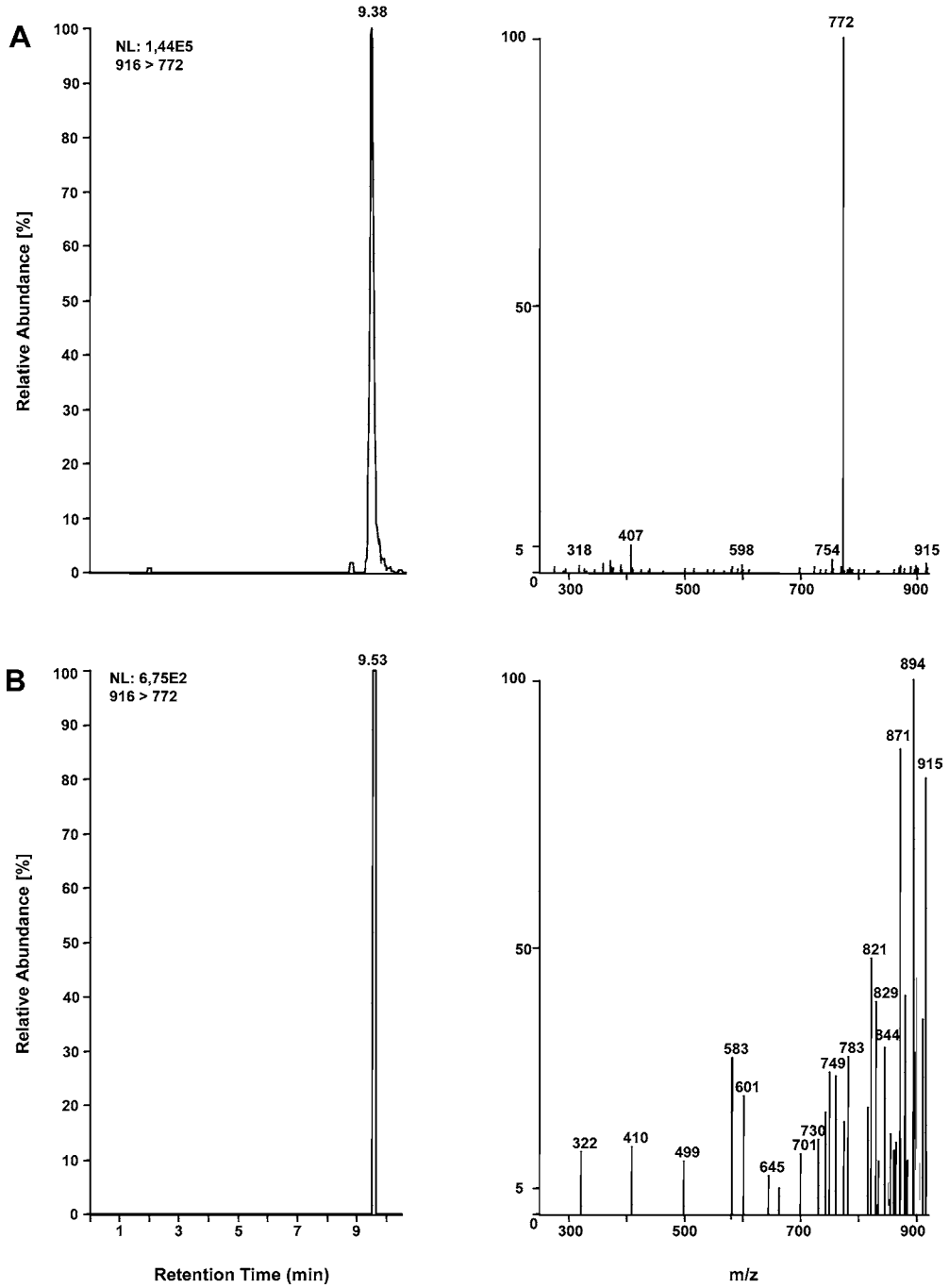

Figure 3. (**A**) Mass chromatogram of a control egg spiked at the limit of quantification with 1 μg/kg tylosin and the corresponding MS/MS spectrum (approximately 20 pg on column). (**B**) Mass chromatogram of a tylosin-free control egg and the corresponding MS/MS spectrum. No significant interferences at *m*/*z* 407, 754, or 772 were detected at the retention time of tylosin. NL: normalized level.

relative standard deviation (RSD) of the retention times is generally less than 0.5% (data not shown). Thus, the confirmation criteria for compounds listed in Annex I of Council Regulation (EEC) No. 2377/90, which include RSDs for the retention time of <2.5% and at least two diagnostic MS/MS ions when low-resolution tandem mass spectrometry is applied are fulfilled (*7*). The variations within the relative abundancies are even fulfilled by the molecular ion (see **Figure 2**), demonstrating that the simple sample pretreatment is highly efficient and results in clear chromatograms and clear MS/MS spectra. In **Figure 2A**−**C**, LC/MS/MS chromatograms and mass spectra of a standard sample (representing 1 ng of tylosin on column), a spiked egg sample, and an egg sample containing tylosin (both representing approximately 1 ng of tylosin on column) are shown. In **Figure 3A** the performance of the method at the LOQ (1 μg/kg, representing approximately 20 pg of tylosin on column) is demonstrated. Blank egg samples extracted and analyzed with the newly developed method showed nearly no endogenous interferences at the elution time of tylosin (**Figure 3B**).

Matrix effects in quantitative LC/MS/MS analysis of biological matrixes are well-known by many investigators (*8*). However, when tylosin was added to blank egg extracts, we observed neither signal suppression nor signal enhancement. Other groups found substantial matrix effects during tylosin LC/MS-analysis (*9*). One reason for these effects may be the use of 5 g of whole egg during sample cleanup in this investigation which is fivefold higher, if compared to our method. Another reason for the

Table 5. Tylosin Depletion Kinetics in Whole Eggs Laid by Four Selected Hens Fed a Diet Containing 1.5 g/kg Tylosin or Receiving Drinking Water Containing 0.5 g/L Tylosin[a]

	tylosin [μg/kg]											
hen [no.]	1[b]	2[b]	3[b]	4[b]	8[b]	9[b]	10[b]	11[b]	12[b]	13[b]	14[b]	15[b]
W4	7.6	53.4	16.9	19.3	4.4	1.2	2.2	1.2	X	X	<0.5	<0.5
W5	45.1	35.4	22.4	60.1	13.9	12.1	4.7	5.8	2.4	<1.0	<0.5	<0.5
F5	16.0	20.9	39.7	40.9	X	9.6	3.9	2.0	<0.5	<0.5	<0.5	<0.5
F9	19.9	36.8	40.0	46.0	40.8	22.2	6.0	5.8	3.3	<1.0	<0.5	<0.5

[a] Please note that the corresponding residue data for days 5–7 can be found in Table 4. [b] Day(s) of treatment. [c] X = no egg layed on this day.

absence of matrix effects in our system may be the use of a conventional 4.6 mm HPLC column and splitting the flow rate 1:10 before entering the mass spectrometer. This conventional approach seems to be robust against matrix interferences and has been successfully applied to various environmental and food matrixes in recent years (*5*, *6*, *10*−*12*).

Various multimethods based on solid-phase extraction have been developed employing LC/MS/MS for the analysis of tylosin and several other macrolides in eggs (*9*, *13*−*14*). Dubois et al. (*13*) obtained a limit of quantification of 100 μg/kg for each macrolide including tylosin, while Heller et al. (*14*) reported a method detection limit for tylosin of approximately 1 μg/kg. Wang et al. (*9*) found LC/ESI/MS/MS method detection limits of all five macrolides under investigation of <1.0 μg/kg. When compared to these methods, the LOQ reported here for tylosin is at least 2-fold lower. Furthermore, the use of an appropriate solid-phase extraction is not necessary prior to tylosin analysis when applying our method. One advantage of that may be the reduction of analysis time. Therefore, a well-trained person may be able to extract at least 24 egg samples per day with overnight LC/MS/MS analysis.

Residue Depletion Studies. The applicability of the new method was successfully demonstrated in whole eggs from two residue depletion studies. In these studies, one would have expected the highest tylosin concentrations on day 6 of the investigations following 5 days of treatment. However, the highest average tylosin concentrations were found on day 5. No tylosin residues were detected above 60.1 μg/kg on days 4, 5, 6, or 7 of the study, which is far below the current MRL of 200 μg/kg (see **Table 4**). The residue level rapidly decreased in the following days and dropped below the limit of detection on day 14 of the studies (see **Table 5**), which has been subsequently demonstrated in the eggs obtained from four selected hens showing the highest tylosin concentrations on days 5 and 7. To our best knowledge, there is currently only one other study available demonstrating residue depletion of tylosin after medication via drinking water. However, in this study the elimination kinetics of tylosin and other macrolides in eggs were determined separately for albumen and yolk with a plate diffusion assay employing *Micrococcus luteus* as test-organism. Therefore, only semiquantitative results were obtained which generally showed that drug excretion was usually over a longer time in the yolk and that 7−8 times less tylosin was transferred into the egg when compared to spiramycin (*15*). Another study has been performed mixing tylosin in feed at a concentration of 500 mg/kg (*16*). Although these authors used a 3-fold lower tylosin concentration in the diet when compared to our investigation, they found a very similar residue level for tylosin in whole eggs collected 2−14 days after the start of feeding (10−60 μg/kg).

In conclusion, we have demonstrated that LC/MS/MS, combined with rather simple but very effective sample cleanup, is an excellent method for the detection of tylosin in whole eggs. The analytical performance data and the outstanding confirmation abilities recommend the method for food monitoring even at the sub-ppb-level. The application of the newly developed method clearly demonstrated, that a withholding period for eggs is not required when laying hens are treated in recommended dosages with tylosin. Moreover, no substantial differences regarding tylosin residues in whole eggs were found between treatment via feed or drinking water.

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