

Determination of Erythromycin, Spiramycin, Tilmicosin and Tylosin in Animal Feedingstuffs by Liquid Chromatography-Tandem Mass Spectrometry

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Makale Kodu (Article Code): KVFD-2013-10365

Summary

Detection of macrolides residues in animal feedingstuffs to ensure food safety is very important issue. For this purpose, a liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed and validated for the determination of residues of 4 macrolides (erythromycin, spiramycin, tilmicosin and tylosin) in animal feedingstuffs. Roxithromycin (ROX), a macrolide not used in veterinary medicine, was used as internal standard. The mass spectral acquisition was done in the positive-ion mode applying multiple reaction monitoring with the following ions (mass-to-charge ratio, m/z): m/z 837.4→679.2; m/z 734.4→576.2; m/z 422.4→174.1; m/z 869.1→696.1 and m/z 916.4→772.4 for roxithromycin (ROX), erythromycin (ERY), spiramycin (SPI), tilmicosin (TIL), and tylosin (TYL), respectively. Good repeatability, reproducibility and recovery values was obtained. Average recoveries ranged from 98.9% (TIL) to 101% (ERY) and an overall mean of 99.9%. Method limits of detection (LODs) of ERY, SPI, TIL and TYL 5.1, 6.6, 6.7 and 7.5 $\mu\text{g kg}^{-1}$ were achieved respectively. Method limits of quantification (LOQs) were 8.5, 11.1, 11.2 and 12.4 $\mu\text{g kg}^{-1}$ for the same drugs, respectively. Satisfactory decision limits (CC α) and detection capabilities (CC β) were also attained. The method is simple, rapid, sensitive and suitable for the simultaneous determination of macrolide antibiotics in animal feedingstuffs.

Keywords: Macrolides, Feedingstuffs, Validation, LC-MS/MS

Hayvan Yemlerinde Eritromisin, Spiramisin, Tilmikosin ve Tilosinin LC-MS/MS İle Tespiti

Özet

Makrolid grubu antibiyotiklerin hayvan yemlerinde tespit edilmesi gıda güvenliğinin sağlanması açısından çok önemli bir konudur. Bu amaçla, hayvan yemlerinde kullanılan 4 adet makrolid (eritromisin, spiramisin, tilmikosin ve tilosin)'in tespiti için sıvı kromatografi-tandem kütle spektrometrisi (LC-MS/MS) metodu valide edilerek kullanılmıştır. Roksitromisin (ROX), veteriner hekimlik alanında kullanılmayan bir makroliddir ve bu yüzden çalışmada iç standart olarak kullanılmıştır. Kütle spektrasi uygulaması pozitif-iyon modunda ve çoklu reaksiyon izleme modunda gerçekleştirilerek, (kütle/şarj oranı, m/z) roksitromisin (ROX) için m/z 837.4→679.2; eritromisin (ERY) için m/z 734.4→576.2; spiramisin (SPI) için m/z 422.4→174.1; tilmikosin (TIL) için m/z 869.1→696.1 ve tilosin (TYL) için m/z 916.4→772.4 taranmıştır. İyi bir tekrarlanabilirlik, üretilebilirlik ve geri kazanım değerleri elde edildi. Hesaplanan ortalama geri kazanım değerleri %98.9 (TIL)'den %101'e (ERY) kadar değişmekle beraber, toplam ortalama %99.9 olarak gerçekleşti. Metodun tespit limitleri (LOD) ERY, SPI, TIL ve TYL için sırasıyla, 5.1, 6.6, 6.7 ve 7.5 $\mu\text{g kg}^{-1}$ olarak hesaplandı. Metodun sayı verilebilir limitleri aynı sırayla 8.5, 11.1, 11.2 and 12.4 $\mu\text{g kg}^{-1}$ olarak gerçekleşti. Validasyon sonucunda belirtilen bu dört makrolid için memnun edici karar limitleri (CC α) ve karar verebilme kapasitelerine (CC β) de ulaşılabildi. Validasyon sonuçlarına bakıldığında basit, hızlı, duyarlı ve uygun olan bu metot yukarıda belirtilen dört adet makrolid grubu antibiyotiğin hayvan yemlerinde aranmasında kullanılabilir.

Anahtar sözcükler: Makrolid, Hayvan Yemi, Validasyon, LC-MS/MS

INTRODUCTION

Macrolides are characterised by a macrocyclic lactone ring containing 12 to 16 atoms with sugars linked via

glycosidic bonds ^[1]. They active against gram positive and some gram-negative bacteria (*Pasteurella* and *Haemophilus*),



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mycoplasmas, and members of the chlamidia group [2,3]. The macrolides reversibly bind to the 50S ribosomal unit and inhibit bacterial protein biosynthesis [4].

They are widely used in veterinary medicine and in the husbandry of livestock to prevent and treat respiratory diseases, necrotic enteritis and to promote growth in food-producing animals when incorporated at subtherapeutic level into feedingstuffs or drinking water. Antibiotics in animal feedingstuff, in general, are used regularly to induce growth promotion and feed conversion ratio and to increase weight gain than to combat specific diseases [3,5]. Overuse and incorrect use of these antibiotics in animal feedingstuffs can cause harmful residue problems in the human food chain [5,6].

Development of resistant strains of bacteria (*Campylobacter* spp.) has been the major concern regarding these drugs use in food-producing animals, as pointed out by the World Health Organization (WHO) [7]. Consequently, the use of macrolides such as for their growth promotion properties is banned in the EU and in Turkey [8-10]. In this respect, sensitive and suitable analysis and control of banned antibacterial growth promoters in animal feedingstuffs plays a key role to ensure the safety of food for consumers.

A number of methods for the detection of macrolides in various of animal tissues have been proposed [1,3,6,11,12]. The aim of this study was to determine the feasibility, sensitivity, rapidity, simplicity and suitability of LC-MS/MS for the direct detection and quantitation of erythromycin (ERY), spiramycin (SPI), tilmicosin (TIL), and tylosin (TYL) in animal feedingstuffs. The following analytical parameters of the method were validated according to the guidelines laid down by Commission Decision 2002/657 [13] using an in-house validation linearity of the standard response in matrix extracts, specificity, recovery, repeatability, decision limit (CC α), detection capability (CC β) and accuracy.

MATERIAL and METHODS

Chemicals and Reagents

All chemicals (extraction reagents and mobile phase A and B) of analytical grade were from ZIVAK® Technologies (Kocaeli, TURKEY). Ultra pure water was provided by a Milli-Q system (Millipore, Bedford, MA, USA). Roxithromycin (ROX), erythromycin (ERY), spiramycin (SPI), tilmicosin (TIL), and tylosin (TYL) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Standard Solutions

All the individual and composite stock standard solutions (10 $\mu\text{g mL}^{-1}$) were prepared by dissolution in methanol. Working standard solutions were made by diluting stock solution with methanol. Stock standard solutions for the

internal standards were also prepared in a similar way. The stock standard solutions were stored at -80°C in Eppendorf micro tubes in the dark, until use. Warmed up to room temperature before use. The working standard solutions (1 $\mu\text{g mL}^{-1}$ for all standards) were stored at $+4^{\circ}\text{C}$ in the dark.

Animal Feedingstuff Samples Used for the Validation

Animal feedingstuffs (pelleted cattle feeds) samples were collected from markets and farms. The absence of the target antibiotics was checked by LC-MS/MS analysis. Blank animal feedingstuffs were used for method development experiments. Since they proved to be blank samples in previous analysis they were used for all validation experiments.

In-house Validation of the Analytical Method

The method was validated according to the criteria laid down in the European Commission Decision 2002/657/EC for confirmatory purposes in LC technique using ROX as internal standard [3,13]. The validation included the determination of selectivity/specificity, linearity, recovery, repeatability, reproductibility, the decision limit (CC α), detection capability (CC β), the limits of detection (LOD) and quantification (LOQ).

Method linearity was verified by matrix calibration curves constructed at the five concentration levels plus zero (blank). The procedure was validated at the following spiking levels: 62.5, 125, 250 and 500 $\mu\text{g kg}^{-1}$ for ERY and SPI; 15.625, 31.25, 62.50 and 125 $\mu\text{g kg}^{-1}$ for TIL; 31.25, 62.50, 125 and 250 $\mu\text{g kg}^{-1}$ for TYL. Matrix effects were evaluated with matrix-matched calibration curves (both including zero). All samples were also fortified with an amount of internal standard (100 μL of a 10 $\mu\text{g mL}^{-1}$ working solution of ROX). The calibration curves were constructed analyte/internal standard peak area ratio versus concentration of analyte.

Recoveries were assessed for all studied concentration levels, using six replicates for each spiking level. Repeatability and within-laboratory reproducibility were also estimated for all concentrations ($n=6$, each spiking level), with the whole procedure being repeated on two other days by the same technicians (repeatability) and on more two other different days by different technicians (within-laboratory reproducibility).

The method limits of detection (LODs) and limits of quantification (LOQs) were determined using the samples fortified at the lower validation level in within-laboratory reproducibility experiments. LODs and LOQs were calculated considering a signal-to-noise ratio equal to 3 and 10, respectively, for the first confirmation transition (the second most intense transition) by S-to-N using peak-to-peak script available in Tandem Gold Workstation® Version 6.9.1 software.

The decision limits (CC α) and the detection capabilities (CC β) were calculated by overall calibration curve procedure from the same within-laboratory reproducibility data, applying weighted regression analysis.

Sample Extraction and Clean-up

Firstly, animal feedingstuffs (pelleted cattle feeds) were blended and homogenized thoroughly with an ultra turrax homogenizer (T25 basic, IKA Labortechnik, Staufen, Germany). A total of 5 g of homogenised sample was weighed into 50 mL centrifuge tubes. 100 μ L of a 10 μ g mL⁻¹ working solution of ROX (I.S.) was added. Moreover, the extraction and clean-up procedure were continued and performed according to the manufacturer's instructions [14]. Multi Speed Vortex (Biosan, MSV-3500), centrifuge (Sigma, 3K15) and nitrogen evaporator (Pierce, Rockford, IL, USA) were used for shaking, centrifugation and evaporating to dryness, respectively. At the end of the extraction procedure, the eluted solution was sonicated for 5 min using ultrasonic bath and then filtered through a 0.45 μ m membrane filter. A 20 μ L aliquot of the filtrate was injected into the LC-MS/MS system.

Instrumentation

LC-MS-MS analysis was acquired with Tandem Gold Triple Qudrople MS-MS system (ZIVAK, Kocaeli, TURKEY). Chromotography was performed on a 150 x 2 mm, 5 μ m C₁₈ column (ZV-1015-02C1). The flow rate was 0.2 mL min⁻¹, with a linear gradient at the following conditions: 0 to 6 min with 80% A, 6 to 10 min with 0% A, 10:01 to 14 with 80% A. The column temperature was maintained at 23°C and the injection volume was 20 μ L.

Nitrogen was used for the gas nebuliser. The ions were monitored by Multiple Reaction Monitoring (MRM). The source block temperature was set at 300°C and the electrospray capillary voltage was 5000 V. The MS/MS conditions are presented in Table 1 and the mass transitions of the analytes for the macrolides are given in Table 2. The mass spectrometer was operated in the positive electrospray ionisation (ESI+) mode.

RESULTS

The limits of detection (LOD=3.3xSD/m) and quantification (LOQ=10xSD/m) were determined by analysing the animal feedingstuff samples spiked with standard solutions of the macrolides. The LODs were 5.1, 6.6, 6.7, 7.5 μ g kg⁻¹, the LOQs were 8.5, 11.1, 11.2 and 12.5 μ g kg⁻¹ ERY, SPI, TIL and TYL, respectively. Response linearity was evaluated by calibration curves. The calibration curves were linear. The R² values for the system results were all >0.995 for the linear regression equations in the concentration ranges tested. Data of the calibration curves (equation and regression coefficient) performed with the standards were respectively: erythromycin: y=0.0051x-0.0547, R² 0.9983;

Table 1. MS/MS conditions

Tablo 1. MS/MS şartları

Ionisation Mode	ESI+
API nebulising gas pressure	55 psi
Drying gas temperature	300°C
Drying gas pressure	34 psi
Scan time	0.5 sec
SIM width	1.0 amu
Needle	+5000V
Shield	+600V
Capillary	30V
Detector	+1800V
CID Gas Pressure	2.00 m Torr
Spray Chamber Temperature	50°C
Mass peak width in amu	1.5
Ouad 1	1.5
Ouad 3	1.5

Table 2. Transition reactions monitored by LC-MS/MS

Tablo 2. LC-MS/MS ile taranan geçiş reaksiyonları

Compound	Transition Reactions (m/z)	Collision Energy (eV)	Dwell (ms)
ROX	837.4 → 679.2	20	0.1
ERY	734.4 → 576.2	20	0.1
SPI	422.4 → 174.1	26	0.1
TIL	869.1 → 696.1	40	0.1
TYL	916.4 → 772.4	28	0.1

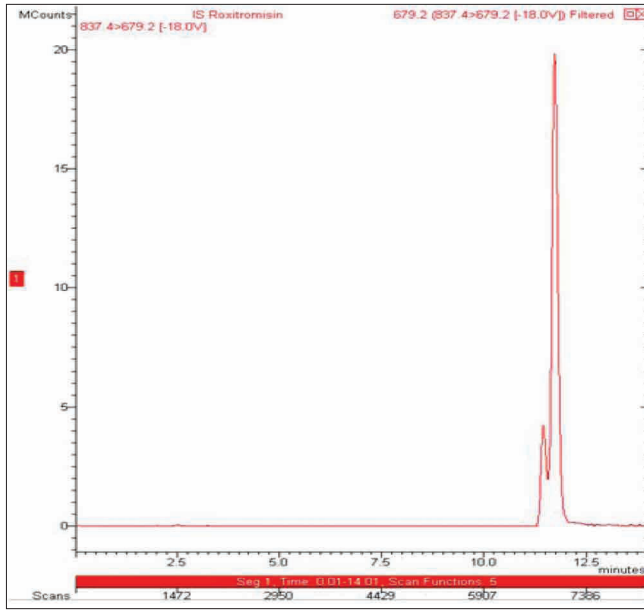
spiramycin: y=0.0047x-0.0671, R² 0.9994; tilmicosin: y=0.0151x-0.0193, R² 0.9982 and tylosin: y=0.0062x-0.0622, R² 0.9951. Measurement uncertainties (U(CO)) 0.94, 0.31, 0.82 and 0.31 were achieved respectively. The values for the decision limit (CC α) and the detection capability (CC β) were calculated using the calibration curve procedure described in Commission Decision 2002/657/EC (Table 3).

Representative chromatograms of a feedingstuff sample spiked with macrolides at concentrations equivalent to the following spiking levels: 250 μ g kg⁻¹ for ERY and SPI; 62.5 μ g kg⁻¹ for TIL; 125 μ g kg⁻¹ for TYL, and to 200 μ g kg⁻¹ for ROX (Fig. 1).

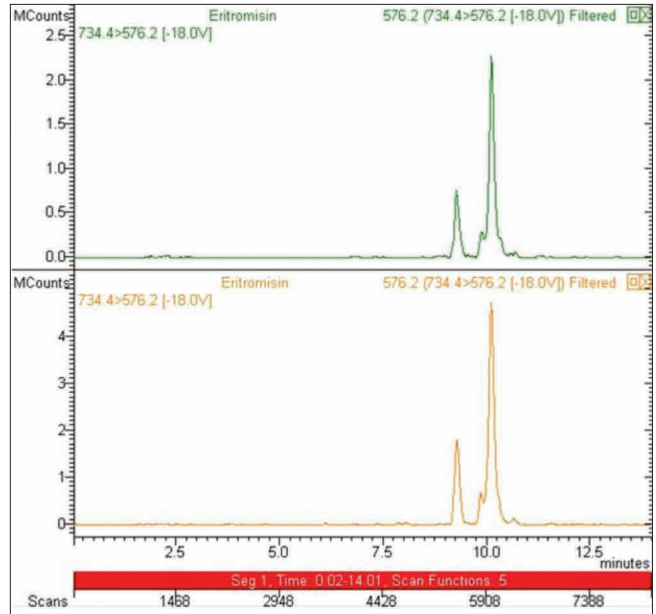
The precision (repeatability), recovery and accuracy of the method were obtained by analysing six replicates for each of the 0.5 and 1 MRL of tested fortification levels (125 and 250 μ g kg⁻¹ for ERY and SPI; 62.5 and 125 μ g kg⁻¹ for TYL; 31.25 and 62.5 μ g kg⁻¹ for TIL) on each of 3 days for the animal feedingstuff samples under investigation (Fig. 2, Fig. 3, Fig. 4 and Fig. 5). Precision was determined by calculating the relative standard deviation (RSD, %) for the replicated measurements and the accuracy (relative

Table 3. Data summary showing the LC-MS/MS method performance results in animal feedingstuffs**Tablo 3.** Hayvan yemlerinde LC-MS/MS metodu performans sonuçlarını gösteren veri özeti

Compound	CC α ($\mu\text{g kg}^{-1}$)	CC β ($\mu\text{g kg}^{-1}$)	LOD ($\mu\text{g kg}^{-1}$)	LOQ ($\mu\text{g kg}^{-1}$)	Measurement Uncertainty U(C0)	R ²
ERY	255.6	261.2	5.1	8.5	0.94	0.9983
SPI	256.1	262.3	6.6	11.1	0.31	0.9994
TIL	67	71.4	6.7	11.2	0.82	0.9982
TYL	130.6	136.2	7.5	12.5	0.31	0.9951

**Fig 1.** LC-MS/MS chromatogram of a spiked animal feedingstuff sample with roxithromycin (I.S.) ($200 \mu\text{g kg}^{-1}$)

Şekil 1. Roksitromisin (I.S.) ile yükleme yapılmış hayvan yeminin LC-MS/MS kromotogramı ($200 \mu\text{g kg}^{-1}$)

**Fig 2.** LC-MS/MS chromatogram of a spiked animal feedingstuff sample with erythromycin (125 and $250 \mu\text{g kg}^{-1}$)

Şekil 2. Eritromisin ile yükleme yapılmış hayvan yeminin LC-MS/MS kromotogramı (125 ve $250 \mu\text{g kg}^{-1}$)

Table 4. Data summary showing the recovery mean results (%) in animal feedingstuffs**Tablo 4.** Hayvan yemlerinde ortalama geri kazanım (%) sonuçlarını gösteren veri özeti

Compound	Spiked level ($\mu\text{g kg}^{-1}$)	Recovery (%)	RSD (%)
ERY	125	101	1.6
	250	100.5	1.6
SPI	125	99.9	2.2
	250	100.9	1.5
TIL	31.25	99.6	5.1
	62.5	98.9	2.5
TYL	62.5	99.8	1.6
	125	99.5	1.7

error, RE%) was calculated by the agreement between the measured and the nominal concentrations for the fortified samples. The average recoveries of the four drugs from the feedingstuffs spiked at concentrations equivalent to the 0.5 and 1 MRL levels were between 98.9% and 101% with the relative standard deviations (RSD) were between 1.5% and 5.1% (Table 4).

Selectivity/specificity of the method was demonstrated by analysing of 20 different blank samples in order to investigate possible interferences eluting on ERY, SPI, TIL and TYL retention time. No interference was observed around the macrolides retention times in the matrices with the monitored MS reactions (data not shown). Our results indicate that the method has adequate precision.

DISCUSSION

The goal of this study was to develop a specific, sensitive and reliable LC-MS/MS method for the identify and quantify macrolides (ERY, SPI, TIL and TYL) in animal feedingstuffs. ROX, a macrolide structurally related to the analyte under investigation and not used in veterinary medicine, was used as internal standard [3].

Lower LOD values for these drugs in animal feedingstuffs were also observed by De la Huebra et al. [15]. The CC α ($130.6 \mu\text{g kg}^{-1}$) and CC β ($136.2 \mu\text{g kg}^{-1}$) values for tylosin obtained in animal feedingsuffs were better than those reported by Van Poucke et al. [16]. These calculated CC α and CC β values were also below the minimum

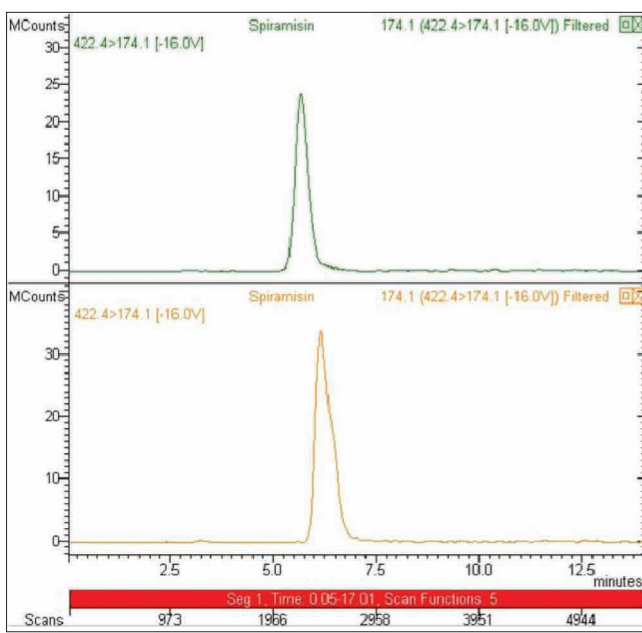


Fig 3. LC-MS/MS chromatogram of a spiked animal feedingstuff sample with spiramycin (125 and 250 $\mu\text{g kg}^{-1}$)

Şekil 3. Spiramisin ile yükleme yapılmış hayvan yeminin LC-MS/MS kromotogramı (125 ve 250 $\mu\text{g kg}^{-1}$)

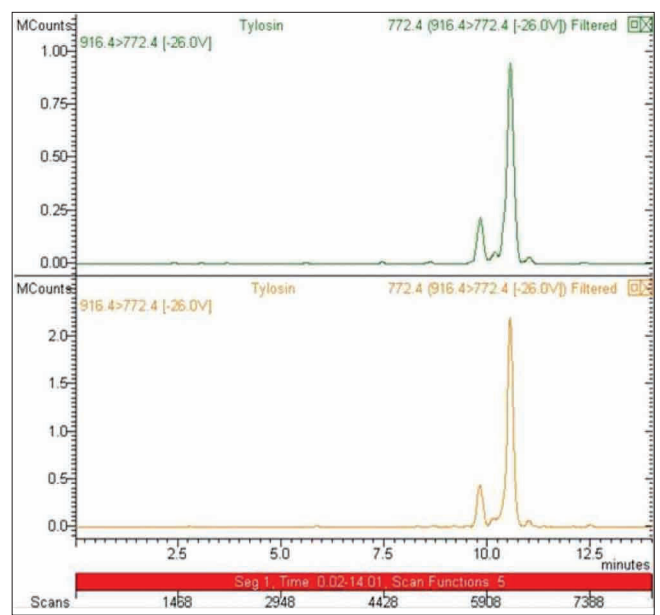


Fig 5. LC-MS/MS chromatogram of a spiked animal feedingstuff sample with tylosin (62.5 and 125 $\mu\text{g kg}^{-1}$)

Şekil 5. Tilosin ile yükleme yapılmış hayvan yeminin LC-MS/MS kromotogramı (62.5 ve 125 $\mu\text{g kg}^{-1}$)

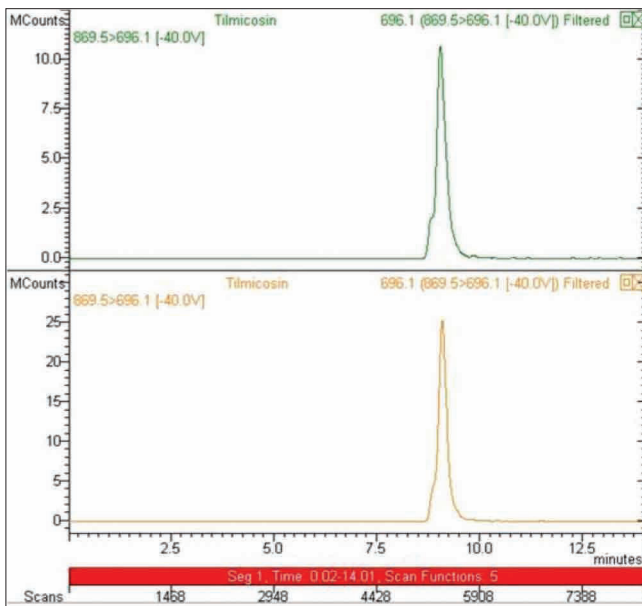


Fig 4. LC-MS/MS chromatogram of a spiked animal feedingstuff sample with tilmicosin (31.25 and 62.5 $\mu\text{g kg}^{-1}$)

Şekil 4. Tilmicosin ile yükleme yapılmış hayvan yeminin LC-MS/MS kromotogramı (31.25 ve 62.5 $\mu\text{g kg}^{-1}$)

required performance limit (MRPL) established by the EU, which is 1 mg kg^{-1} (ppm). Thus, these values appeared very satisfying. Precision values were all below 15.7% (RSD) and the overall accuracy $\leq 3.7\%$ (2.7-3.7) were acceptable. These values could be considered satisfactory, on account of the complexity of the biological matrices. Higher-than-average recoveries for ERY, SPI, TIL and TYL [15]; for ERY and TYL [17] have also been reported

for animal feedingstuffs. However, lower-than-average recoveries for TIL in pig feed samples were also observed by Kaklamanos et al. [18].

The linearities were good for all analytes in the whole range of tested concentrations, as proved by the correlation coefficients greater than 0.995 for all curves. However, the linearity of tylosin (0.9951) obtained in this study were lower than that (0.9995) reported by Civitareale et al. [19]. In addition, different types of blank feedingstuffs such as pig finisher, broiler breeder and poultry feeds [16] and macrolides such as rosamycin, clarithromycin, and lincomycin could have been used for the validation procedure [18].

In recent years, residues in animal originated foods arising from the use of zootechnical feed additives including macrolides. Furthermore, there has been increasing regulators, clinicians and the general public concern over the possible links between drug residues in edible tissues (muscle, liver and kidney) and milk the perception of widely use of antimicrobial compounds in animal feedingstuffs and the increasing number of resistant pathogenic bacteria and the spread of resistance genes from food-producing animals to humans as a result of veterinary and zoo-technical use in food-producing animals [8,20-22]. Therefore, analysis and detection of macrolides residues in animal feedingstuffs by accurate methods to maintain an efficient food safety management system across the human food chain is very important issue.

As a final result, the proposed method was successfully used for detection and quantification of these drugs in animal feedingstuffs. The method is simple, rapid, sensitive

and suitable for the simultaneous determination of ERY, SPI, TIL and TYL drugs in animal feedingstuffs.

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