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Determination of diclazuril, toltrazuril and its two metabolites in poultry tissues and eggs by gel permeation chromatography–liquid chromatography–tandem mass spectrometry

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ABSTRACT

A new procedure has been described for the extraction of diclazuril (DIZ), toltrazuril (TOZ) and its two main metabolites toltrazuril sulphoxide (TZSO) and toltrazuril sulphone (TZS) from poultry tissues and eggs, using gel permeation chromatography (GPC). The analytes and the deuterated internal standard were extracted from the samples with ethyl acetate. The analytes were measured by LC coupled to an electrospray ionization tandem mass spectrometer operating in the negative ion mode. Excellent linear dynamic range was observed from 1 to 500 μg/L with the correlation coefficients (R^2) better than 0.99 for all analytes. The method LOQ of the four analytes in real samples was 1.2 μg/kg for DIZ and TOZ, and 1.8 μg/kg for TZSO and TZS. These values are far lower than the maximum residue limits (MRLs) established by several control authorities. The developed method was accurate with overall recoveries in four matrices.

1. Introduction

Diclazuril (DIZ) and toltrazuril (TOZ) are two kinds of triazine coccidiostats, which are wildly used to prevent and combat coccidiosis, a contagious amoebic disease affecting livestock, particularly poultry that is associated with warm and humid conditions. TOZ has a high toxicity profile, which turns this drug into a candidate for high-priority risk assessment lists, and almost exclusively metabolized to two metabolites, toltrazuril sulphoxide (TZSO) and toltrazuril sulphone (TZS), which is marker residue of TOZ [1]. The chemical structures of the four compounds are shown in Fig. 1.

Owing to the widespread use of these drugs in farms, there is a risk that the triazine residues will be present in animal products intended for human consumption. The presence of their residues in animal food products may have side effects to consumers. European Union (EU) has set the following maximum residue limit (MRL) of TOZ for poultry: 100 μg/kg for muscle, 200 μg/kg for fat/skin, 400 μg/kg for kidney, 600 μg/kg for liver and not for use in animals producing eggs for human consumption [2], the Commission Regulation (EU) (No 37/2010) [3] has set pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin, and Codex Alimentarius Commission (CAC) has set the following MRLs of DIZ for poultry: 500 μg/kg for muscle, 1000 μg/kg for kidney and 1500 μg/kg for liver [4].

Several methods for the determination of DIZ or TOZ in feed, biological fluids, animal tissues and eggs have been reported [5–13]. A LC method for the simultaneous determination of the residues of DIZ and TOZ in chicken tissues was developed using matrix solid-phase dispersion (MSPD) for clean-up, and the LOQs of DIZ and TOZ were 10 and 12 μg/kg for muscle, respectively, and 15 μg/kg for liver and kidney [5]. A sensitive validated quantitative high-pressure liquid chromatography method for toltrazuril sulfone in bovine biological fluids was developed [6]. Liquid chromatography tandem mass spectrometry method (LC–MS/MS) has proven to be a promising technique for trace level residual analysis with its high selectivity, specificity and sensitivity for DIZ [7–12]. Croubels et al. [7] described a LC–MS/MS for the detection of DIZ in animal plasma with a LOQ of 1 μg/L and a LC–MS/MS for the detection of DIZ in poultry meat and feed with CCα and CCβ values of 0.5 and 0.6 μg/kg was developed by Mortier et al. [8]. There are also some multi-residue LC–MS/MS methods to detect coccidiostats including DIZ and TOZ in chicken tissues and eggs [9–13]. However, there has been no report of simultaneous analysis of DIZ, TOZ, and its two metabolites (TZSO and TZS) in poultry tissues and eggs. The matrix interference resulting from large-molecule substances such as proteins and lipophilic compounds in the animal tissues and in eggs may be a serious hindrance for the accurate quantification of the

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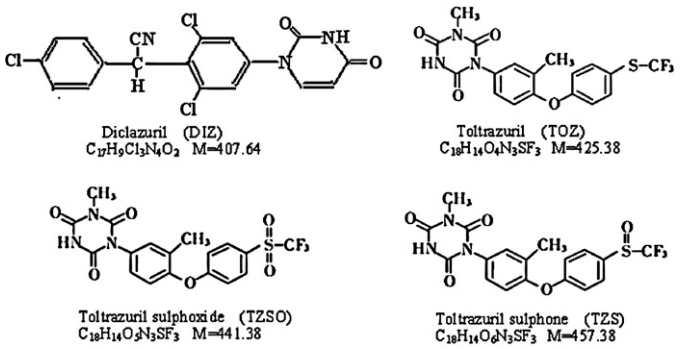

Fig. 1. Chemical structures of the four compounds.

lipophilic triazine coccidiostats. Gel permeation chromatography (GPC) clean-up has been an essential sample preparation technique of lipophilic compounds, and has been used to clean co-extractives molecular interferences in other methods such as fatty and pigment, based on the great difference in molecular size between them and the target compounds [14].

In this study, the combination of the GPC sample preparation coupled with LC–MS/MS analysis gave an extremely sensitive and robust method for the detection of DIZ, TOZ and its two metabolites in a different range of matrices. The proposed method was validated by evaluating recovery, selectivity, linearity and accuracy, and has been applied for analysis of official control samples.

2. Experimental

2.1. Chemicals

The HPLC-grade methanol solvent was obtained from Merk (Darmstadt, Germany). The formic acid (purity = 88%), ethyl acetate, acetonitrile and cyclohexane, were obtained as chromatographic grade from Yongda (Tianjin, China). The water was purified and deionized by a water purification system (Human power III plus, Korea). The solvents for LC were filtered by 0.45 membranes (Whatman, UK) and degassed in an ultrasonic bath.

2.2. Standards

The analytes DIZ and TOZ were obtained as a gift from Dr. Ehrenstorfer GmbH (Augsburg, Germany), whereas the TZSO, TZS and the internal deuterated isotopologue standard TOZ-D3 were obtained from WITEGA (Berlin, Germany).

Stock standard solutions (0.1 mg/mL) of DIZ, TOZ, TZSO, TZS and TOZ-D_3 were prepared in methanol and stored at −18 °C in the dark. Working standard mixture solutions were prepared by mixing desired volume of individual stock standard solutions and serially diluting to different levels with methanol. These solutions were stored at 4 °C.

2.3. Instrumentation

The AccuPrep MPS GPC clean-up system used was equipped with J2 M3300 Autosampler containing a 24 vials (10 mL) tray (Columbia, Missouri 65202, USA). Express glass column (300 mm × 20 mm i.d.) packed with 22 g 200–400 mesh Bio-Breads S-X3 resin and DT0001 fixed wavelength detector at 254 nm was used for GPC.

The sample analysis was performed with an LC–MS/MS system composed of a TSQ Quantum triple stage quadrupole mass spectrometer (Thermo Electron Corp., San Jose, CA, USA) equipped with an electrospray ionization (ESI) source operated in negative ion mode. The LC system consisted of a Surveyor LC pump with an online degasser and a Surveyor auto sampler. The LC separation was performed on an Inertsil ODS-3 analytical column (150 mm × 2.1 mm, 4.6 μm) (GL Sciences Inc. Japan) at ambient temperature.

The LC mobile phase was composed of acetonitrile and 0.1% acetic acid aqueous solution (55:45, v/v) and the flow rate was 200 μL/min. The TSQ Quantum mass spectrometer was calibrated with a solution of polytyrosine-1,3,6 as described by the manufacturer. The ESI source parameters were optimized based on preliminary results at LC flow of 200 μL/min. The optimized source parameters were as follows: sheath gas pressure, 20 (arbitrary units); auxiliary gas flow, 30 (arbitrary units); spray voltage, 3500 V; capillary temperature, 320 °C; and source collision-induced decomposition (CID), 10 V. For quantification, the mass spectrometer was set to the data acquisition mode of multiple-reaction monitoring (MRM). The acquisition parameters common to all analytes were: scan width (m/z) 0.01, scan time 0.2 s, peak width (FWHM) 0.7 for both Q1 and Q3, and collision gas pressure 1.5 mTorr. Time schedule and mass spectrometer parameters for acquisition of mass spectra for each analytes were showed in Table 1. Data acquisition and analysis were accomplished with LCquan software v.2.5 (Thermo Electron Corp., San Jose, CA, USA).

2.4. Sample preparation

2.4.1. Extraction

The samples of 5 g were weighed into a 50-mL centrifuge tube. A 25 μL volume of 10 μg/mL IS solution was added to all samples. This was followed by the addition of 25 mL ethyl acetate and 5 g anhydrous sodium sulfate and by blending with a homogenizer at a speed of 12 000 rpm for 1 min. The extracts were centrifuged at 4000 rpm for 10 min and 10 mL of the extracts were pipetted and condensed to dryness, under nitrogen in a water bath at 45 °C. The obtained residues were dissolved in 10 mL ethyl acetate-cyclohexane (50:50, v/v).

2.4.2. Clean-up

The extracts of sample were injected into the GPC system. The elution was carried out with a mixture of ethyl acetate–cyclohexane (50:50, v/v) at a flow rate of 5 mL/min. The eluent obtained after 8.0–12 min was collected in 100-mL heart-shape flasks. The collected solutions were evaporated to dryness at 45 °C with a rotary vacuum evaporator. The obtained dry residues were dissolved in 1.0 mL methanol–water (80:20, v/v), and the final solution was passed through 0.45 μm membrane filter, and waited for LC–MS/MS analysis.

3. Results and discussion

3.1. The extraction of target analytes

Four organic solvents namely dichloromethane, ethyl acetate, acetone and acetonitrile were investigated in this study and were used to extract the liver samples. The results are shown in Table 2. We have found that acetonitrile, dichloromethane and ethyl acetate were the most efficient for extraction of the analytes, and the results obtained with acetone were relatively lower. For acetonitrile, the concentration step is more time-consuming because the evaporation of acetonitrile was tedious due to its high boiling point. Whereas, when dichloromethane was used as extractant, an environmentally unfriendly reagent, there were some solid residues floating in the suspension after centrifugation. We have opted to use ethyl acetate as an extractant, since the recoveries were relatively higher with that solvent and thus we were able to overcome

Table 1
Time schedule and mass spectrometer parameters for acquisition of mass spectra for each analytes.

Analyte	Retention time (min)	Segments (min)	Monitored ions (*m*/*z*)	Collision energy (eV)
TZSO	4.73	3–5	440/371*	18
TZS	7.81	5–9	456/456*	0
DIZ	11.02	9–12	404/334*	19
TOZ	13.53	12–15	424/424*	0
TOZ-D3	13.61	12–15	427/427*	0

*Quantitative ion.

Table 2
Absolute recoveries of analytes using different organic solvents as extractant.

Analytes	Extraction recoveries (%)			
	Acetonitrile	Ethyl acetate	Dichloromethane	Acetone
TZSO	88.9	93.1	91.1	86.4
TZS	88.5	91.5	89.2	78.9
DIZ	89.7	91.8	90.8	64.5
TOZ	90.4	89.7	90.4	75.8

the shortcomings of using acetonitrile and dichloromethane. So this method adopted ethyl acetate as the extractant.

3.2. Clean-up efficiency of GPC

GPC has been used to clean co-extractives molecular interferences such as pigment, fat and protein, based on the great difference in molecular size. The poultry tissues and eggs were very complex matrix in which the high molecular compounds were main matrix interference. The GPC clean-up can decrease the presence of interferents in the final extract and also avoid matrix effects resulted from co-eluting residual matrix components affecting the ionization efficiency of target analytes.

We investigated separation efficiency of GPC for liver samples. 5 mL of mixed standard dilution (10 μg/mL for each analyte) and the extracts of poultry liver were injected, respectively, into the GPC system. The elution was carried out with ethyl acetate–cyclohexane (50:50, v/v) at a flow rate of 5 mL/min. The chromatogram is shown in Fig. 2. The interferents were almost eluted within 8.0 min, the standards began to be outflowed from the GPC column at the 8.0 min and the target analytes were completely eluted in 8.0–13 min.

3.3. LC–MS and LC–MS/MS analysis

In this work, MS and MS/MS behaviors of 4 analytes were investigated in ESI(−) mode. Full-scan ESI(−)-MS mass spectra of the analytes showed only the deprotonated molecule $[M-H]^-$, allowing confirmation of the molecular mass. Under ESI(−)-MS/MS conditions, characteristic fragment ions were observed in production mass spectra for TZSO and DIZ, but for TOZ and TZS there were no characteristic ions (seeing Fig. 3). So for TZSO and DIZ quantitative analysis applied characteristic ion transitions (TZSO: 440/371, DIZ: 404/334) in MS/MS mode, and for TOZ and TZS quantitative analysis applied molecule ion in MS/MS mode with no collision energy, the detail parameters are showed in Table 2.

In the test regarding optimization of equipment conditions, we found if all analytes were scanned in one scan segments using different five scan events, the responses of all analytes were lower than in separated scan segments. It was supposed to be caused by the contradiction between DIL and TZSO needing dissociation energy and TZS and TOZ needing no energy. So completely separation is necessary to set similar scan events in separately segments.

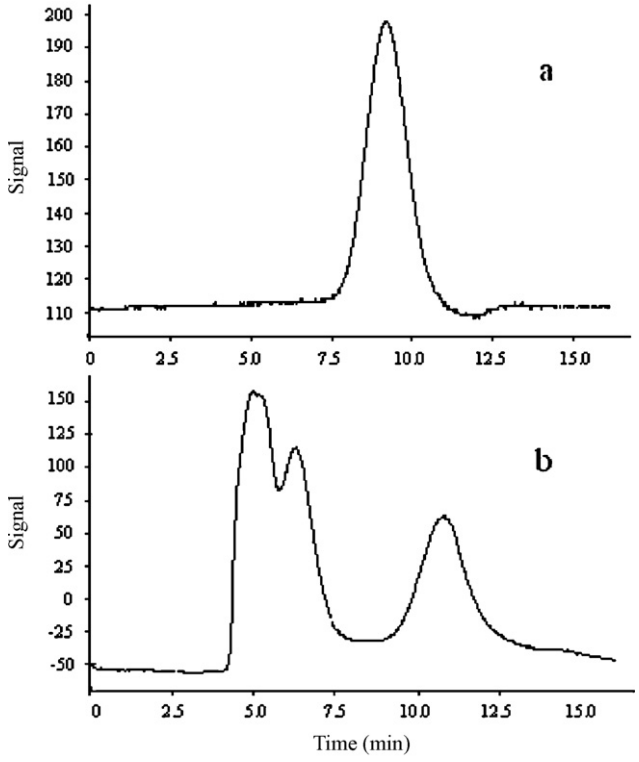

Fig. 2. GPC chromatogram of the mixed standard solution (a) and liver sample (b) at 254 nm.

Preliminary trials were performed by analyzing both standard solutions and sample extracts in RP partitioning mode using different C18 and C8 columns and an elution made up of 0.1% (v/v) acetic acid aqueous solution and acetonitrile or methanol with a percentage of aqueous phase varying between 40% and 60%. Optimal conditions were obtained in correspondence to the use of Inertsil ODS-3 column and the mobile phase composed of acetonitrile – 0.1% (v/v) acetic acid (55:45, v/v). Under optimized LC conditions good separation of the four analytes was achieved within 15 min with high repeatability. The retention time and the analyzing segments are shown in Table 2.

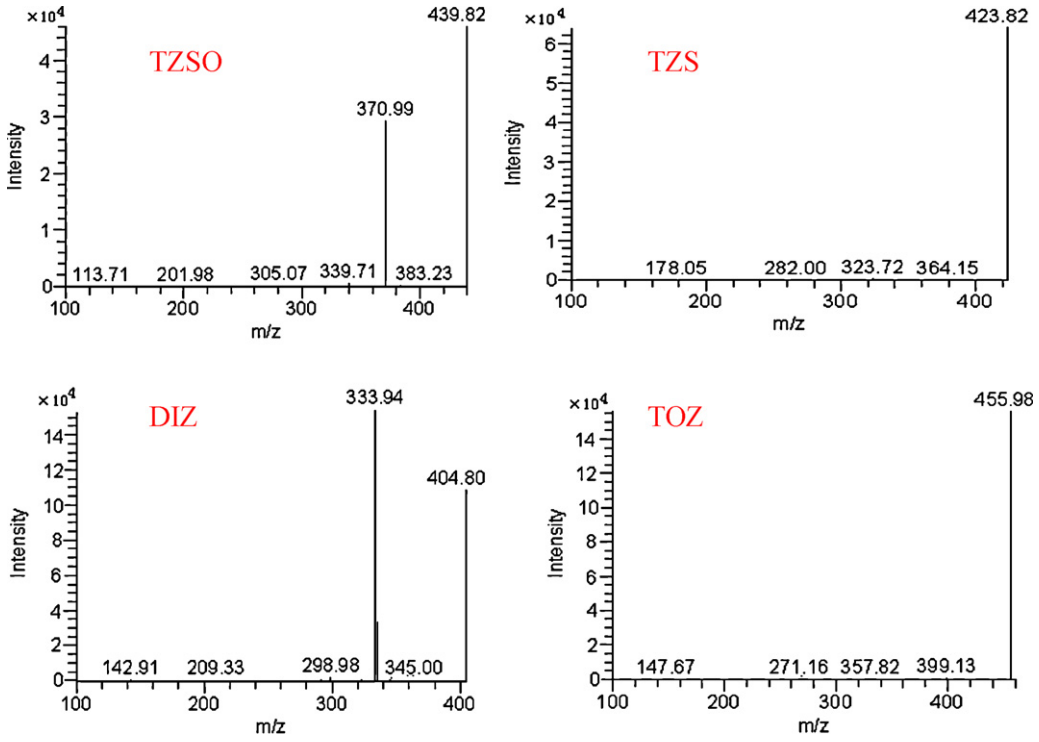

Fig. 3. ESI(−)-MS/MS spectra of TZSO, TZS, DIZ and TOZ obtained after direct injection of a mix standard of 1 μg/mL at 10 μL/min with mobile phase at 200 μL/min (at product ion scan mode and collision energy = 25 eV).

3.4. Method validation

3.4.1. Selectivity

Selectivity was checked by injecting extracts of 24 non-spiked different samples including egg, muscle, liver and kidney. No interferences were observed in corresponding retention times of target compounds by comparing chromatograms of spiked samples and blank samples. The LC–MS/MS chromatograms of blank samples and spiked samples of egg and liver are shown in Fig. 4, from which it is indicated that the present method has high selectivity for the analytes.

3.4.2. Linearity, matrix effect and detection limit

The matrix effects can greatly affect the method reproducibility and accuracy. Linearity and matrix effects were investigated using solvent and matrix-matched calibration curves. The matrix-matched calibration curves were constructed by spiking aliquots of the corresponding matrices with increasing amounts of standards, and the solvent calibration curves were constructed in the same way but without the addition of matrix aliquots. The matrix of liver, kidney, muscle and egg were examined. The calibration curves were prepared at levels of 1, 5, 10, 50, 100, 200 and 500 μg/L for all analytes and 50 μg/L for IS. All standards, in matrix as well

Table 3
Linear equations, correlation coefficients, and concentration range of all analytes studied in different matrix.

Analytes	Matrix	Calibration curve($Y = a + bX$)	Coefficient (R^2)	Concentration range (μg/L)
TZSO	Solution	$Y = -0.00891 + 0.0103X$	0.998	1–500
	Egg	$Y = -0.00972 + 0.0109X$	0.994	
	Muscle	$Y = -0.00911 + 0.00976X$	0.993	
	Liver	$Y = -0.0123 + 0.00946X$	0.992	
	Kidney	$Y = -0.00845 + 0.0104X$	0.993	
TZS	Solution	$Y = -0.0104 + 0.0276X$	0.995	1–500
	Egg	$Y = -0.0113 + 0.0267X$	0.995	
	Muscle	$Y = -0.00208 + 0.0242X$	0.991	
	Liver	$Y = -0.00597 + 0.0243X$	0.991	
	Kidney	$Y = -0.00258 + 0.0260X$	0.997	
DIZ	Solution	$Y = -0.00906 + 0.0309X$	0.999	1–500
	Egg	$Y = -0.00948 + 0.0310X$	0.994	
	Muscle	$Y = -0.00671 + 0.0300X$	0.995	
	Liver	$Y = -0.00639 + 0.0298X$	0.991	
	Kidney	$Y = -0.0181 + 0.0309X$	0.994	
TOZ	Solution	$Y = -0.00114 + 0.0246X$	0.999	1–500
	Egg	$Y = -0.00874 + 0.0224X$	0.996	
	Muscle	$Y = -0.0121 + 0.0222X$	0.994	
	Liver	$Y = -0.0178 + 0.0225X$	0.992	
	Kidney	$Y = 0.00681 + 0.0219X$	0.993	

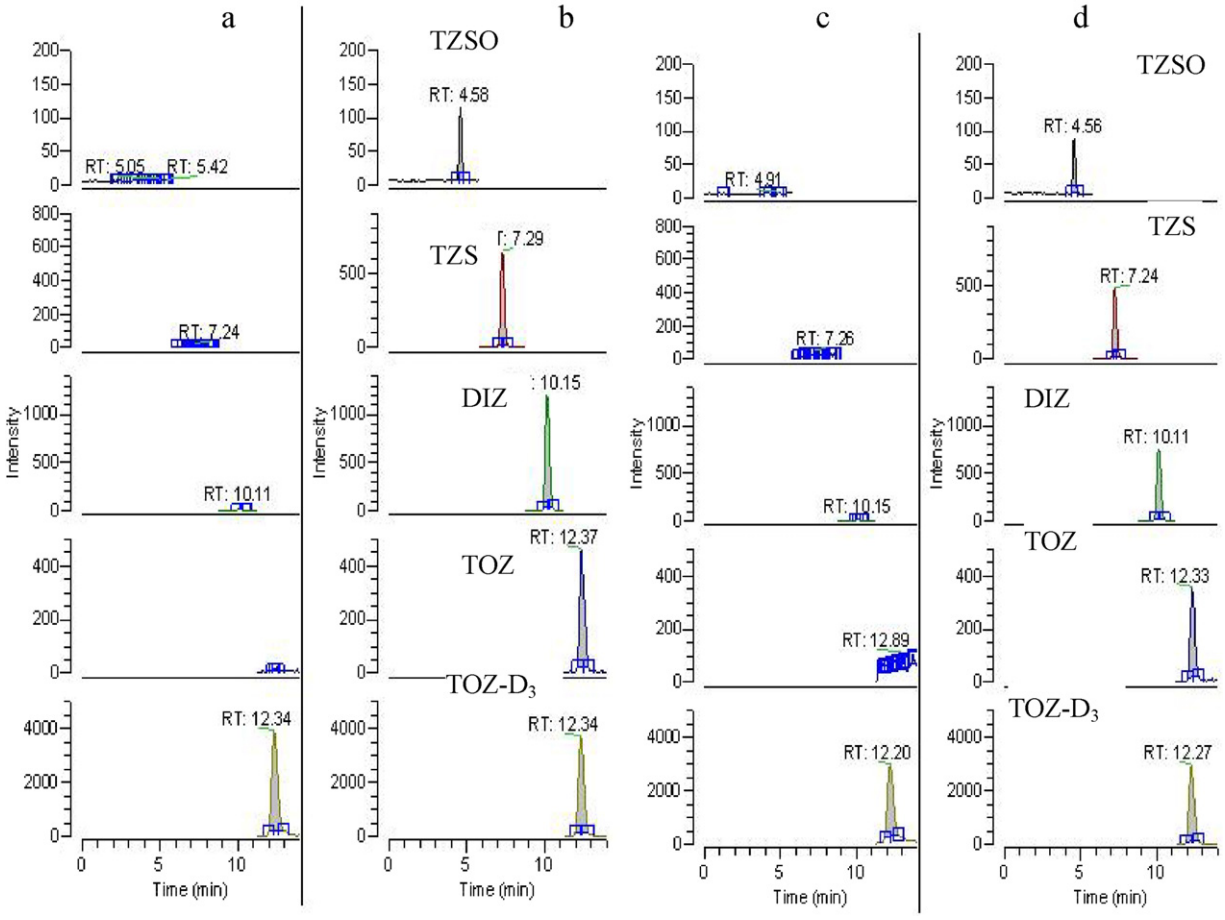

Fig. 4. LC–MS/MS chromatograms obtained from blank samples of liver (a) and egg (c) and blank liver samples spiked at 2.5 μg/kg mix standard of (b) as well as blank egg samples spiked at 2.0 μg/kg mix standard (d).

as in solution, were performed in triplicate and 10 μL of each was injected into the LC–MS/MS system during the same run of analyses.

Data in Table 3 showed the different analytes curves of different matrix and solution, implying that the data of these five sets are not significantly different. It was concluded matrix effect was corrected by IS, and calibration curves in solution could be used to quantify all 4 target analytes. So standard solution calibration curves were used in this work. Excellent linear dynamic range was observed from 1 to 500 μg/L with correlation coefficients (R^2) better than 0.99 for all analytes. It can be seen that the linearity is acceptable based on the criteria ($R^2 \geq 0.98$) described by Green [15].

The limit of detection (LOD) was determined as the sample concentration that produces a peak with a height three times of the level of baseline noise. The limit of quantification (LOQ) was calculated as the sample concentration that produces a peak with an area 10 times the ratio of signal-to-noise. The instrument LOD was 0.2 μg/L for DIZ and TOZ, and 0.3 μg/L for TZSO and TZS. The method LOQ of the four analytes in real samples was 1.2 μg/kg for DIZ and TOZ, and 1.8 μg/kg for TZSO and TZS. The proposed method has high detection ability.

3.4.3. Recovery and precision

Recovery and precision were investigated on the samples spiked with different concentration of analytes (including the near levels of LOQ and MRLs). The detailed data are shown in Table 4. For the four samples, average recovery of four analytes at the spiked three levels was in the range of 84.4–102%, and RSD was in the range of 3.40–8.13%. The results demonstrate that the accuracy of the present LC–ESI-MS method was acceptable for routine monitoring purposes.

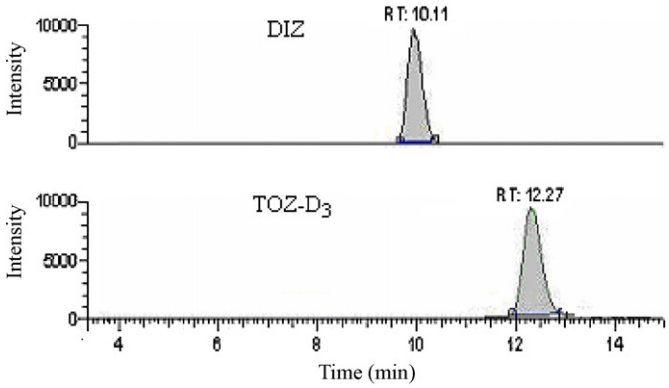

Fig. 5. LC–MS/MS chromatogram of positive rabbit muscle sample with 56 μg/kg of DIZ.

3.5. Application of the method

The more than 500 residue monitoring samples and export samples of different matrices have been analyzed using the presented method in routine work. Among the samples investigated, DIZ in ten rabbit muscle samples was found at the contents in

Table 4
Detailed results of the inter- and intra-day precision and recovery experiments for different matrices ($n = 6$).

Matrix	Analytes	Spiked (μg/kg)	Day 1		Day 2		Day 3		Overall	
			Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Egg	TZSO	2	91.7	5.49	98.3	3.11	93.3	5.06	94.4	5.11
		10	91.8	3.58	94.9	3.44	95.7	7.52	94.1	4.93
		100	99.7	7.31	99.0	3.85	96.7	3.31	98.5	4.69
	TZS	2	95.0	8.62	90.7	1.27	89.3	2.59	91.7	5.45
		10	94.2	8.74	90.6	6.38	98.3	5.88	94.4	7.10
		100	99.7	3.94	94.3	8.50	92.9	3.50	95.6	5.95
	DIZ	2	100	5.00	91.7	5.04	90.7	2.30	94.1	6.04
		10	104	4.92	95.6	5.70	97.7	3.24	99.1	5.63
		100	96.7	7.74	100	5.13	97.4	5.56	98.1	5.64
	TOZ	1	95.0	7.37	92.3	7.37	100	5.11	95.9	6.83
		10	105	3.86	92.0	5.40	96.5	9.74	97.7	8.13
		100	103	3.91	95.4	7.45	101	6.46	100	6.33
Muscle	TZSO	2	86.8	5.18	81.0	3.57	85.4	3.32	84.4	4.75
		10	94.2	5.35	90.3	8.50	89.7	1.84	91.4	5.60
		500	95.5	7.74	84.4	4.27	89.4	6.71	89.8	7.79
	TZS	2	99.7	3.52	99.6	3.93	95.6	1.71	98.3	3.47
		10	95.8	1.36	95.2	6.50	100	1.62	97.0	4.12
		500	99.3	2.44	101	2.39	96.5	12.0	99.0	6.45
	DIZ	2	95.6	5.10	95.3	5.63	103	10.1	97.8	7.40
		10	95.2	4.04	97.8	3.84	101	5.25	97.8	4.49
		500	93.6	2.06	92.3	6.96	93.3	4.97	93.1	4.42
	TOZ	2	97.4	7.45	103	2.44	97.6	7.83	99.5	6.19
		10	101	4.48	102	2.99	103	4.07	102	3.51
		500	93.9	6.23	95.5	6.50	99.0	3.64	96.1	5.35
Liver	TZSO	2	92.0	8.01	82.7	3.54	87.2	3.16	87.3	6.68
		20	94.6	6.20	89.4	9.84	90.1	1.83	91.4	6.44
		600	93.1	7.65	82.6	3.85	87.2	6.78	87.6	7.65
	TZS	2	96.5	9.17	102	4.18	97.8	1.56	98.7	5.56
		20	96.2	1.41	95.4	6.32	101	1.95	97.5	4.23
		600	97.1	2.34	98.9	2.29	92.1	8.30	96.0	5.37
	DIZ	2	95.3	6.68	101	4.23	101	7.55	99.2	6.20
		20	95.7	4.05	98.3	3.91	101	5.26	98.3	4.51
		3000	91.4	1.91	90.2	6.83	91.3	4.98	91.0	4.36
	TOZ	2	98.5	5.72	106	2.17	96.8	3.03	100	5.21
		20	101	4.26	103	2.91	103	4.03	102	3.40
		600	91.8	6.27	93.3	6.28	96.7	3.75	93.9	5.32
Kidney	TZSO	2	89.8	5.58	85.8	5.75	85.3	3.77	87.0	5.08
		20	95.5	8.25	101	7.24	93.5	3.12	96.6	6.67
		400	89.9	3.32	97.3	2.18	95.6	1.49	94.3	4.13
	TZS	20	94.7	5.84	101	2.28	101	3.38	98.7	4.63
		100	95.6	4.89	102	1.72	92.2	9.31	96.4	6.68
		400	95.8	1.66	94.9	2.89	102	5.45	97.4	4.59
	DIZ	2	98.5	7.05	97.3	4.37	98.8	4.81	98.2	4.85
		20	98.5	5.16	97.8	6.58	98.7	3.34	98.4	4.52
		1000	86.8	5.99	91.7	1.76	88.1	1.46	88.9	4.01
	TOZ	2	95.8	7.42	97.8	2.57	99.0	7.16	97.6	5.49
		20	99.5	4.16	97.0	4.23	94.9	2.25	97.1	3.79
		400	89.3	6.29	92.2	4.06	92.9	2.19	91.5	4.25

range 10–300 μg/kg but less than MRL(500 μg/kg), and TZSO, TZS and TOZ have not been detected. The LC–MS/MS chromatogram of a positive rabbit muscle sample with DIZ = 56 μg/kg is showed Fig. 5.

4. Conclusion

A novel GPC–LC–ESI-MS/MS method for quantitative analysis of DIZ, TOZ and two metabolites of TOZ in poultry tissues and egg was developed by using GPC clean-up procedure, isotopically labeled internal standard and ESI(−)-MS/MS detection mode. The use of GPC technique can clean-up samples effectively. The matrix effect can be compensated using IS. The ESI(−)-MS/MS detection can provide accurate determination of selected 4 analytes in complex matrices. The method has a good repeatability and high accuracy with low quantification limits.

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