Multi-residue Analysis of Eight Veterinary Drugs in Buffalo Milk using Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

In this study, we developed an analytical method for veterinary drug residues in buffalo milk using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The multi-residue method was established for the simultaneous identification and quantitation of eight common veterinary drugs, including metronidazole, salbutamol, atropine, trimethoprim, hydrocortisone, kitasamycin, roxithromycin and tylosin. The sample processing method for buffalo milk was compared and optimised. The precision, recovery and matrix effects of the method were validated. The precision and accuracy of all analytes ranged from 1.81% to 12.35% and 1.25% to 14.57%, respectively. The average recovery percentages varied between 93.59% and 114.57%, and the average matrix effect ranged from 87.12% to 103.76%. All eight analytes in buffalo milk exhibited stability under different treatment conditions. The developed method was successfully applied to laboratory analysis and routine sample analysis. The method was demonstrated to be rapid, sensitive and reliable for the rapid monitoring of veterinary drug residues in buffalo milk. The findings of this study contribute to the risk assessment of veterinary drug residues for preventing the human consumption of contaminated buffalo milk and its derivatives.

1. Introduction

Veterinary drugs are used to prevent, treat and diagnose diseases in animals. The misuse or illegal use of these drugs causes allergic reactions (Ortelli, Sporri et al. 2018) and increases the resistance of drug-resistant pathogenic bacterial strains (Stolker and Brinkman 2005, Atta, Atta et al. 2022). Residues accumulate in the bodies of animals through the food chain and pose a major threat to the ecosystem and human health. Research published by the World Health Organization confirmed that the long-term accumulation of pesticide and veterinary drug residues in the human body may lead to various health problems (Damalas and Eleftherohorinos 2011). In milk, which is an important food product consumed daily in several cultures, veterinary drugs should be absent or present at concentrations lesser than the maximum tolerance. To minimise the risk to human health, the European Union has established the regulation of the maximum residue levels (MRLs) of veterinary drugs in milk (Chicoine, Erdely et al. 2020). To enforce the regulation and ensure food safety, it is necessary to develop an effective and sensitive analytical method to monitor drug residues in milk (Kaufmann, Butcher et al. 2014).

Numerous detection methods have been developed to satisfy the requirements of food safety supervision. Common analytical methods for veterinary drug residues include microbiological (Parmar, Chaubey et al. 2021), immunological (Lei, Chen et al. 2006), biosensor-based (Preuß, Reich et al. 2020), capillary electrophoresis-based (Kowalski, Olędzka et al. 2003) and liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based methods (Hou, Xu et al. 2020, Wang, Xie et al. 2021). Among these, LC-MS/MS strategies enable both the qualitative and quantitative analysis of veterinary drugs (Wang, Xie et al. 2021) and are effective for the detection of non-steroidal anti-inflammatory drugs (van Pamel and Daeseleire 2015), chlorinated hydrocarbon pesticides (Jadhav, Pudale et al. 2019), polyethylenes (Zhu, Yang et al. 2016), beta-lactams (Jank, Hoff et al. 2012), macrolides (Jank, Martins et al. 2015), lincomycins (Jank, Martins et al. 2015) and tetracyclines (Moreno-González and García-Campaña 2017).

However, despite considerable advancements in separation and detection techniques, the cost-effectiveness of analytical procedures remains a critical issue for the experimental design in the analysis of drug residues in milk. The goal is to maximise the quantity and type of target analytes that can be determined simultaneously using a single simple procedure. Milk has been extracted using various techniques, such as protein chemical precipitation, solid-phase extraction and the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method (Perestrelo, Silva et al. 2019). Compared with other milk sources, buffalo milk has more complex matrix components and a higher fat and protein content. Drug residues are more difficult to detect, and the analytical methods for drug residues in other milk sources cannot be effectively applied to buffalo milk. Therefore, it is important to develop a sensitive and reliable analytical method with a simple procedure to assay veterinary drug residues in buffalo milk.

The objective of this study was to develop a rapid and reliable multi-residue ultra-high performance (UHP)LC-MS/MS method for identifying eight veterinary drugs, namely metronidazole, salbutamol, atropine, trimethoprim, hydrocortisone, kitasamycin, roxithromycin and tylosin, in buffalo milk. A simple liquid-liquid extraction method with rapid chromatographic separation was developed in the process. The proposed method has been validated per the FDA regulations and applied to simultaneously detect the drug residues in buffalo milk samples.

2 Materials And Methods

2.1 Reagents and chemicals

Acetonitrile (MeCN, LC-MS grade), methanol (MeOH, LC-MS grade) and formic acid (FA, 99%, LC-MS grade) were supplied by Thermo Fisher Scientific (MA, USA). Hexane was purchased from Merck (Darmstadt, Germany). Pure water was obtained from the Milli-Q ultra-pure water system (Millipore, MA, USA). The standards of metronidazole, salbutamol, atropine, trimethoprim, hydrocortisone, kitasamycin, roxithromycin and tylosin were obtained from Vicki (Sichuan, China).

2.2 Preparation of standard solutions

Stock solutions of each veterinary drug standard were prepared at a concentration of 1000 mg/L in MeOH or MeCN and water. The highest concentration of the working solution was prepared by combining the stock mixtures and diluting them with MeCN/H_2O. A thirteen-level standard mix working solution was prepared by combining known volumes of each stock solution with MeCN/H_2O to reach the desired concentration at 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500 and 1000 µg/L. Calibration standards were obtained by gradient dilution of the working solutions at concentrations ranging from 0.1 µg/L to 1000 µg/L. All standard stock and working solutions were stored in the dark at -20 °C.

2.3 Extraction procedure

Ten millilitres of buffalo milk (Buffalo Research Institute, Chinese Academy of Agricultural Sciences, Guangxi) was aliquoted into a centrifuge tube and spiked with 100 µL of a veterinary drug standard

mixture. Subsequently, 10 mL of MeCN was added, and the sample mixture was vortexed for 10 min. The sample mixture was centrifuged at 3800 × *g* for 10 min. The supernatant was transferred to a new centrifuge tube and defatted twice with 3 mL of hexane for ultrasonic treatment for 10 min. Under nitrogen at 45 °C, 3.5 mL of the lower layer supernatant was evaporated. The residue was dissolved in 1 mL of MeCN and subsequently vortexed in an ultrasonic bath for 5 min. The solution was filtered through a 0.22 μm membrane, which was then transferred to a microvial for LC-MS/MS analysis.

2.4 Instrumental conditions

An LC instrument (Ultimate 3000, Thermo Fisher Scientific) was used for chromatographic separation, which was performed using a Symmetry C18 column (2.1×50 mm, 1.7 μm particle size, Waters, USA) at a flow rate of 0.3 mL/min. The column oven was maintained at 30 °C. Gradient elution was performed with solvent A (0.1% FA, H_2O) and solvent B (0.1% FA, MeCN). The gradient procedure was as follows: 5% B for 2 min, 5%–20% B for 0.10 min, 20%–95% B for 9 min (with a linear increase) and 95%–5% B for 1 min. Before the next sample was injected, the column was equilibrated with 5% solvent B for 3 min. The sample temperature was set at 4 °C, and the injection volume was 2 μL.

A triple quadrupole mass spectrometer (TSQ-Endura, Thermo Fisher Scientific) was used for the quantification of the targeted compounds in positive and negative ion modes. For each compound, the MS/MS parameters were optimised by infusing the work solutions directly into the mass spectrometer by connecting a syringe pump (Thermo Fisher Scientific) to the interface for tuning purposes. These analyte-dependent tuning results are presented in Table 1. In the optimised multi-residue MS/MS method, the mass spectrometer was operated under both positive and negative electrospray ionisation (ESI) with nitrogen as the collision gas. ESI-MS/MS was performed with selective reaction monitoring (SRM). The source and desolvation temperatures were set to 120 °C and 350 °C, respectively. In the confirmed program, at least three product ions per precursor ion were measured in each separate measuring window, with the exception of the deuterated internal standards for which only one product ion was measured (Table 1). The data obtained for each sample was analysed using the Xcalibur 4.0 software (Thermo Fisher Scientific).

2.5 Method validation

To evaluate the performance of the developed method, the specificity, linearity, limit of detection (LOQ), sensitivity, accuracy, precision, extraction recovery, matrix effects, residual effects and stability were validated per the FDA Bioanalytical Method Validation Guideline 16. Control buffalo milk samples were collected from a local farm and tested to confirm the absence of the veterinary drugs using the national standard method followed in China (Li, Ren et al. 2018). Of note, the use of internal standards was not necessary for multiclass residue analysis considering the procedural reproducibility (Li, Ren et al. 2018).

3. Results And Discussion

3.1. LC-MS/MS optimisation

The SRM method was employed for the LC-MS/MS analysis of eight veterinary drugs. For these standard analytes, an LC flow of an 80:20 mixture of mobile phases A and B at 0.1 mL/min was combined with the infusion solution using a tee to simulate the conditions during sample analysis and better optimise the MS parameters. The collision voltage (CV) and collision energy (CE) of the three most abundant transitions were optimised in either a positive or negative ion mode. In the positive ion mode, all analytes yielded the protonated molecule $[M + H]^+$. For this method, SRM was used, and the most intense precursor ions and three daughter ions were selected for each analyte. A primary transition was used for analyte quantification, and other transitions were used for confirmation. The optimised MS parameters for eight veterinary drugs are presented in Table 1. These conditions were also used for optimising chromatographic separation.

Once the MS parameters for the eight veterinary drugs were optimised, different mobile phases were optimised to acquire high sensitivity and suitable chromatographic separation. As presented in Fig. 1, the organic phase composed of MeOH and MeCN was evaluated. The results revealed that MeCN produced better peak shapes for most analytes, and the peak areas were significantly greater than those obtained with the MeOH solution, indicating that MeCN is more suitable for the separation of analytes in buffalo milk.

The additives and composition of the mobile phase affected the ionisation efficiency, retention time and peak shape for the analytes, which consequently affected the sensitivity of analyte detection. For this method, some analytes exhibited poor peak shapes. Initially, combinations with different percentages of FA or acetic acid were compared in an aqueous mobile phase. Fig. 2 shows that the peak areas of most analytes were significantly greater when FA was used. The most effective ionisation of each analyte was achieved when 0.15% FA was added to both mobile phases. The final chromatogram conditions were: MeCN as the organic solvent and 0.15% FA as an additive.

To achieve appropriate separation of the eight veterinary drugs with a shorter elution time, the gradient elution procedure was further optimised to acquire optimal chromatographic resolution and peak shapes. At the beginning of the gradient, to increase the retention of analytes with higher polarity, the percentage of the organic solvent of the initial mobile phase was set to 5%. To facilitate the elution of hydrophilic interfering substances, a 95% aqueous phase was set. During an 11 min chromatographic run, the 95% aqueous phase gradually transitioned to a 95% organic phase. Fig. 3 shows that all eight analytes were successfully separated and identified in different retention times using optimised elution programs.

3.2 Sample preparation optimisation

Sample extraction is the most critical step in the development of a multi-residue method. The objective of this study was to develop a simple, reliable and effective sample preparation method that can provide a clean extract with lower ion interference. The success of the extraction procedure depended on effective degreasing and deproteinization. Two different sample extraction solutions (MeCN and MeOH) were

evaluated. MeCN yielded the highest extraction efficiency and produced relatively clean homogeneous extracts, whereas MeOH did not facilitate significant deproteinization and produced a cloudy extract. As presented in Fig. 4, the peak area results for most analytes obtained from the MeCN extract were significantly higher than the results of those obtained from the MeOH extract solution.

3.3 Method validation

3.3.1 Specificity

The chromatograms of the analytes measured in the blank biological sample (I), mixed standard solution (II) and mixed standard with LOD added to the blank biological sample (III) are presented in Fig. 5. The graphs revealed that all analytes achieved baseline separation, with no interference among the chromatographic retention times, indicating that the method had good specificity and was suitable for quantitative analysis.

3.3.2 Linearity and LOD

The detection limits, linear ranges, linear equations and correlation coefficients (r^2) of the eight analytes are presented in Table 2. The standard curves of each analyte presented good linear ranges, and the r^2 values ranged from 0.9995–0.9999. The LODs of each analyte obtained in this study ranged from 0.1–1 μg/, which were different owing to the different sensitivities of the mass spectra to the detection of the analytes.

3.3.3 Accuracy and precision

Table 3 shows the accuracy and precision of the QC (quality control) samples at the three concentrations. The precision and accuracy ranged from 1.81% to 12.35% and 1.25% to 14.57%, respectively. The results indicated the acceptable accuracy and precision of the proposed method. The method for the determination of the eight analytes in buffalo milk samples proved to be accurate, reliable and reproducible.

3.3.4 Recovery and matrix effect

The extraction recoveries and matrix effect data for eight analytes of QC sample species of low, medium and high concentrations are presented in Table 4. The average extraction recoveries for all analytes ranged from 93.59% to 114.57%. This demonstrates the stable extraction efficiency and accuracy of the optimised method. Matrix effects frequently change the ionisation efficiency of analytes. Therefore, the matrix effects were evaluated by comparing the signal intensity of the matrix-matched standard with different concentrations. The findings of the average matrix effect ranged from 87.12% to 103.76%. All analytes exhibited matrix effects within the acceptable range (80%–120%). This indicated the absence of significant matrix effects for analytes, suggesting that the matrix effect could be disregarded during the quantitative analysis of buffalo milk (Dong, Xian et al. 2020).

No significant peaks (peak area ≥20% of the LLOQ peak area) were observed in the blank sample after the injection of the ULOQ mixed control solution, indicating a negligible residual effect of the method.

3.3.5 Stability

Heating of milk samples may affect the stability of analytes. For this method, the stability of the analytes in the milk samples was evaluated after heating at 63 °C and 80 °C for 1 h . The stabilities of the eight analytes in buffalo milk under different treatment conditions are presented in Table 5. The measured concentrations indicated that the analytes remained stable in heated buffalo milk. This indicates that the concentration of residual veterinary drugs in buffalo milk was not significantly affected by the pasteurisation method. The multi-residue analysis method was appropriate for raw buffalo milk, pasteurised buffalo milk and buffalo milk products.

3.4 Applicability of the method to real samples

The applicability of the developed and validated method was evaluated by detecting veterinary drug residues in 30 buffalo milk samples obtained from dairy buffaloes from different sources. Twenty-one samples showed the presence of veterinary drug residues, of which the residues in six samples exceeded the MRLs. As presented in Fig. 6, kitasamycin (20%), salbutamol (6.7%), metronidazole (3.3%) and trimethoprim (3.3%) were detected in six milk samples (20%). Kitasamycin was detected most frequently (20%) in the samples, whereas salbutamol was present at the highest levels (97.5 μg/L and 112.7 μg/L). Specific information of the exceeded samples is presented in Table 6. An exceeded sample means that a veterinary drug exceeds the maximum residue limit set by the EU. The results indicated that the multi-residue analysis of the eight veterinary drugs could be applied to the risk assessment of buffalo milk.

4. Conclusions

In this study, a rapid and reliable UHPLC-MS/MS-based method for multi-residue analysis and sample preparation based on liquid-liquid extraction was developed and validated for the detection of metronidazole, salbutamol, atropine, trimethoprim, hydrocortisone, kitasamycin, roxithromycin and tylosin in buffalo milk samples. A series of optimisations were performed for sample extraction and chromatographic separation, which facilitated the rapid and simultaneous extraction of eight veterinary drugs with a wide range of physicochemical properties. Satisfactory validation was obtained with respect to specificity, linearity, LOD, sensitivity, accuracy, precision, extraction recovery, matrix effects, residual effects and stability. The findings present a practical multi-residue analysis method that can be applied in the monitoring of veterinary drugs in buffalo milk.

Declarations

Author Contribution Xingchen Huang, Pingchuan Zhu: Conceptualization, methodology, validation, investigation, formal analysis, writing-original draft. Qinqiang Sun, Runfeng Liu, Wei Hu, Huiyan Xu:

Investigation, data curation. Xingchen Huang, Yangqing Lu, Qiang Fu:Resources, funding acquisition, supervision, writing-review and editing.

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Data Availability No data available.

Declarations

Xingchen Huang and Pingchuan Zhu This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Informed consent not applicable.

Conflict of Interest Xingchen Huang declares he has not confict of interest; Pingchuan Zhu declares she has not confict of interest; Qingqiang Sun declares he has not confict of interest; Runfeng Liu declares he has not confict of interest; Wei Hu declares she has not confict of interest; Huiyan Xu declares she has not confict of interest;Yangqing Lu declares he has not confict of interest; Qiang Fu declares he has not confict of interest.

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liquid chromatography-tandem mass spectrometry." Talanta **148**: 401-411.

Tables

Table 1

Mass spectrometric parameters for eight veterinary drugs

Analytes	Precursor ions (m/z)	Daughter ions (m/z)			Collision voltage (CV) V	Collision energy (CE) V
Kitasamycin	828.49	600.22	109.18	174.18	20.46; 39.22; 30.57	162.57
Atropine	290.17	124.11	93.11	91.04	21.37; 27.54; 36.04	127.08
Trimethoprim	291.05	229.92	123.04	261.00	21.42; 20.61; 23.14	112.21
Hydrocortisone	363.03	301.25	257.09	120.96	21.17; 21.37; 22.38	98.56
Tylosin	916.16	772.22	174.11	101.16	24.91; 34.92; 39.68	238.42
Salbutamol	239.82	148.14	221.98	165.85	16.17; 10.25; 10.25	65.19
Roxithromycin	837.22	679.32	158.18	557.99	15.36; 31.08; 16.42	140.12
Metronidazole	172.25	128.00	82.11	110.91	12.98; 21.98; 19.61	63.07

Table 2

The linear relationships, correlation coefficients(r) and LODs of eight analytes

Analytes	Linear equations	Linear relationships（μg/L）	Correlation	LOD （μg/L）
Metronidazole	y=43975.2+19360.2×x	2 ~1000	0.9999	2
Salbutamol	y=-38100.7+15010.6×x	10 ~ 1000	0.9999	10
Atropine	y=-141889+24992.1×x	0.2 ~ 1000	0.9995	0.2
Trimethoprim	y=85855.5+23582.2×x	0.2 ~ 1000	0.9995	0.2
Hydrocortisone	y=-4013.64+1158.3×x	10 ~ 1000	0.9999	10
Kitasamycin	y=78.6625+91.3827×x	2 ~ 1000	0.9998	2
Roxithromycin	y=-5300.69+16304×x	0.1 ~ 1000	0.9999	0.1
Tylosin	y=343.756+1487.63×x	10 ~ 1000	0.9999	10

Table 3

The precisions and accuracies of eight analytes in QC samples

(n=3 days, 6 replicates per day)

Analytes	Concentration（μg/L）	Accuracy RE (%)	Precision RSD (%)
Metronidazole	10	14.57	8.02
	100	1.25	1.85
	450	13.11	3.97
Salbutamol	10	12.03	12.35
	100	5.39	3.58
	1000	8.22	5.53
Atropine	10	13.35	4.14
	100	8.56	4.05
	450	2.28	2.58
Trimethoprim	10	14.21	4.82
	100	10.44	2.89
	550	13.96	1.81
Hydrocortisone	10	0.85	9.07
	100	0.65	2.03
	400	14.34	3.24
Kitasamycin	10	12.01	8.64
	100	2.13	5.24
	400	12.18	3.11
Roxithromycin	10	3.71	1.92
	100	6.81	2.62
	400	11.89	3.09
Tylosin	10	4.66	4.10
	100	5.90	2.92
	400	14.43	3.24

Table 4

The extraction recoveries and matrix effects of eight analyte QC samples

(n=3 days, 6 replicates per day)

Analytes	Spiked concentration（μg·L^{-1}）	Extraction recovery Mean±SD (％)	Matrix effects Mean±SD (％)
Metronidazole	10	97.71 ± 3.62	99.07 ± 3.53
	100	99.18 ± 1.55	99.86 ± 2.48
	450	101.01 ± 2.83	100.27 ± 3.62
Salbutamol	10	105.36 ± 13.49	93.87 ± 6.57
	100	98.23 ± 3.88	103.76 ± 4.01
	1000	105.51 ± 6.4	94.41 ± 5.77
Atropine	10	101.26 ± 1.7	96.13 ± 5.88
	100	103.35 ± 1.21	96.55 ± 1.63
	450	99.70 ± 0.56	101.08 ± 0.88
Trimethoprim	10	100.10 ± 3.16	99.65 ± 4.40
	100	99.93 ± 2.30	102.73 ± 3.27
	550	100.58 ± 0.76	99.01 ± 0.91
Hydrocortisone	10	93.59 ± 14.51	106.80 ± 20.51
	100	100.27 ± 3.99	99.75 ± 2.3
	400	100.70 ± 1.75	98.75 ± 1.44
Kitasamycin	10	114.57 ± 7.92	87.12 ± 16.20
	100	98.50 ± 9.41	100.20 ± 7.35
	400	103.15 ± 5.44	95.41 ± 5.05
Roxithromycin	10	100.33 ± 2.05	97.31 ± 2.53
	100	100.01 ± 1.23	100.50 ± 0.89
	400	99.82 ± 0.53	99.99 ± 0.74
Tylosin	10	105.23 ± 6.07	96.34 ± 3.59
	100	98.86 ± 2.26	101.97 ± 1.85
	400	100.54 ± 0.65	97.88 ± 1.61

Table 5

Stability of analytes in QC samples (n=6)

Analytes	Spiked concentration (μg/L)	Measurement concentration (μg/L)	
		63 ℃	80 ℃
Metronidazole	10	8.99±0.82	7.68±0.25
	100	100.59±0.86	101.65±1.88
	450	100.59±0.86	427.76±10.71
Salbutamol	10	12.58±0.93	12.78±0.68
	100	94.12±3.11	92.53±3.25
	1000	962.97±16.15	936.71±47.6
Atropine	10	13.24±0.42	13.45±0.19
	100	110.44±1.44	110.69±0.74
	450	452.11±2.71	448.73±1.97
Trimethoprim	10	6.64±0.19	6.68±0.38
	100	111.44±3.07	108.53±2.20
	550	543.67±5.18	545.92±3.62
Hydrocortisone	10	9.87±0.74	9.63±1.10
	100	101.16±1.99	101.23±1.51
	400	391.58±6.81	393.79±2.11
Kitasamycin	10	8.85±0.20	6.55±1.44
	100	101.25±4.36	103.41±6.11
	400	406.7±14.52	414.43±16.02
Roxithromycin	10	9.54±0.19	9.76±0.12
	100	107.81±0.67	107.28±0.55
	400	405.22±1.25	405.98±2.01
Tylosin	10	9.78±0.29	9.67±0.20
	100	106.21±1.16	105.40±0.95
	400	390.00±5.92	396.32±3.49

Table 6

Veterinary drug residues exceeding the MRLs of buffalo milk

No.	Sample	Veterinary drugs	Concentration (μg/L)
1	F1448	Kitasamycin	13.8
2	F1338	Kitasamycin	33.9
3	F1525	Kitasamycin	33.1
		Salbutamol	97.5
4	F1436	Kitasamycin	31.7
		Metronidazole	53.0
5	F7313	Kitasamycin	36.5
		Trimethoprim	51.7
		Salbutamol	112.7
6	F1537	Kitasamycin	34.7

Figures

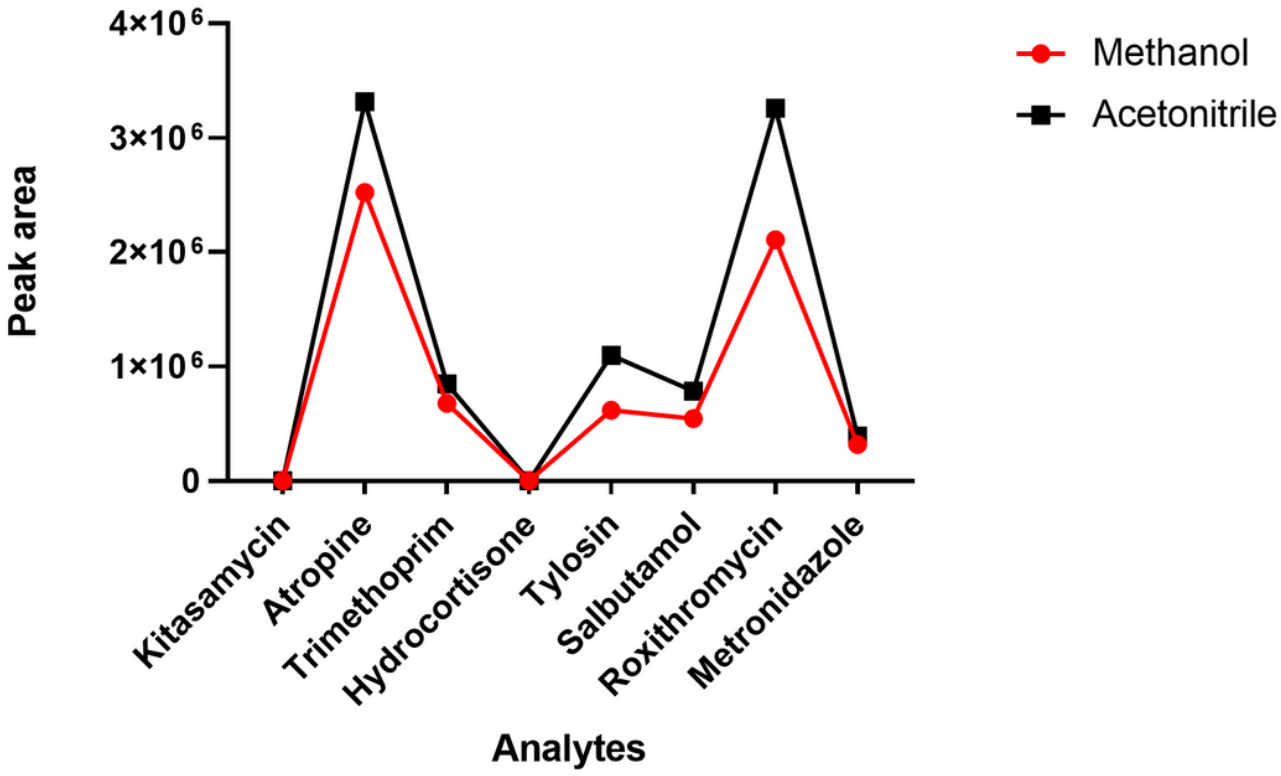

Figure 1

Peak area comparison of different organic solvents

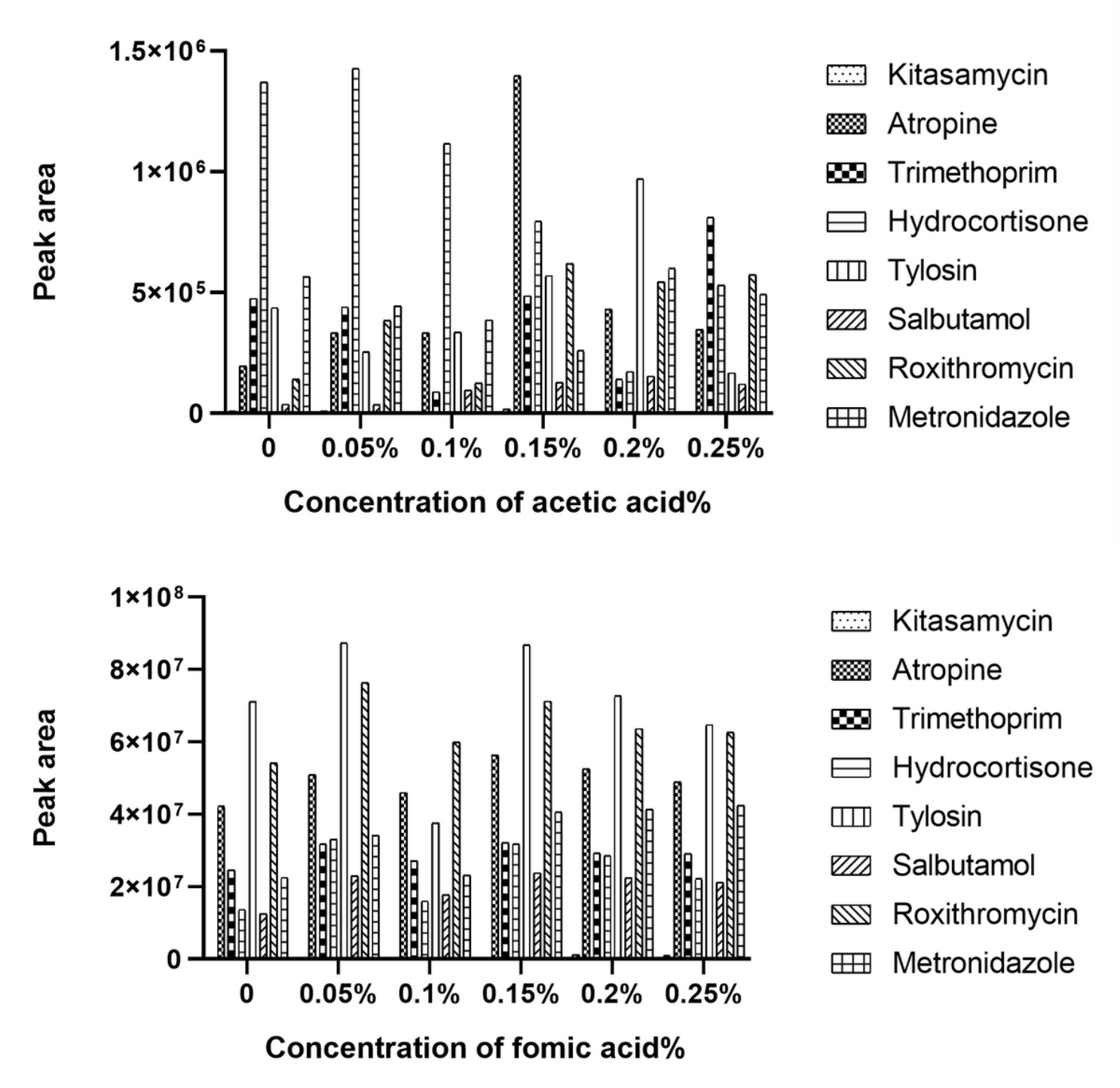

Figure 2

Peak area comparison for analyte combinations with different concentrations of formic acid and acetic acid

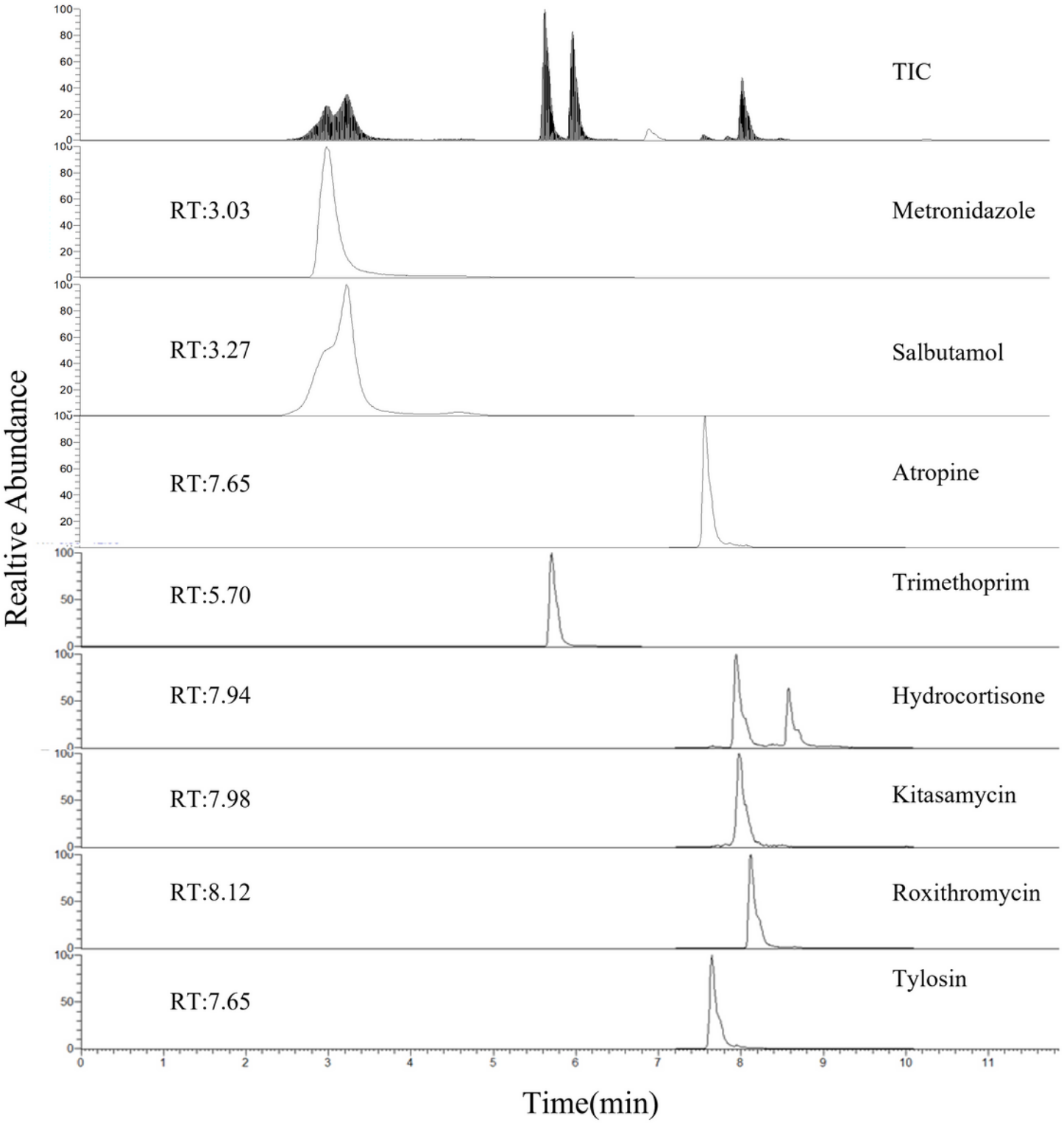

Figure 3

Chromatogram and retention time of eight analyte standards

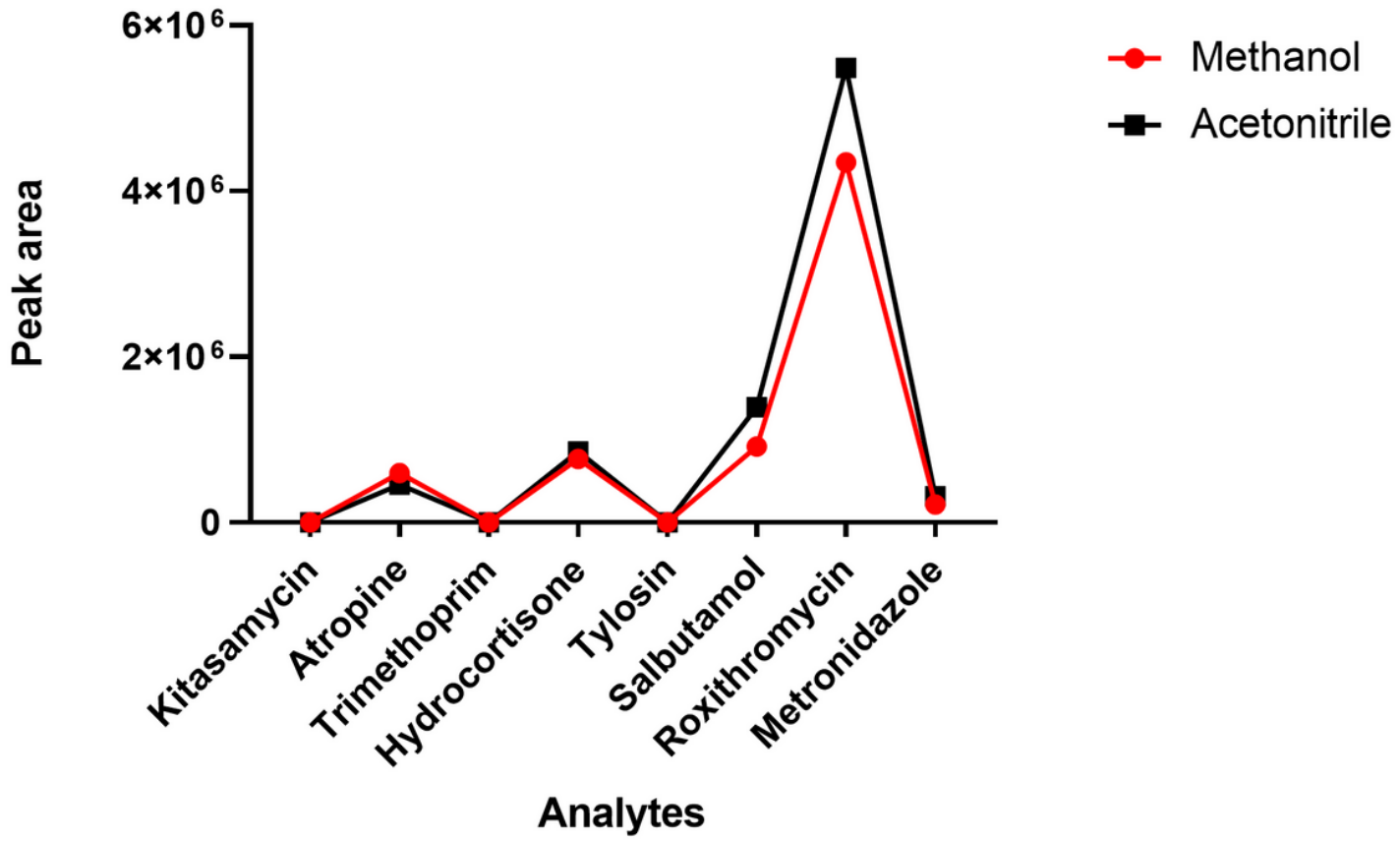

Figure 4

Comparison of peak areas for analytes with different extraction solutions

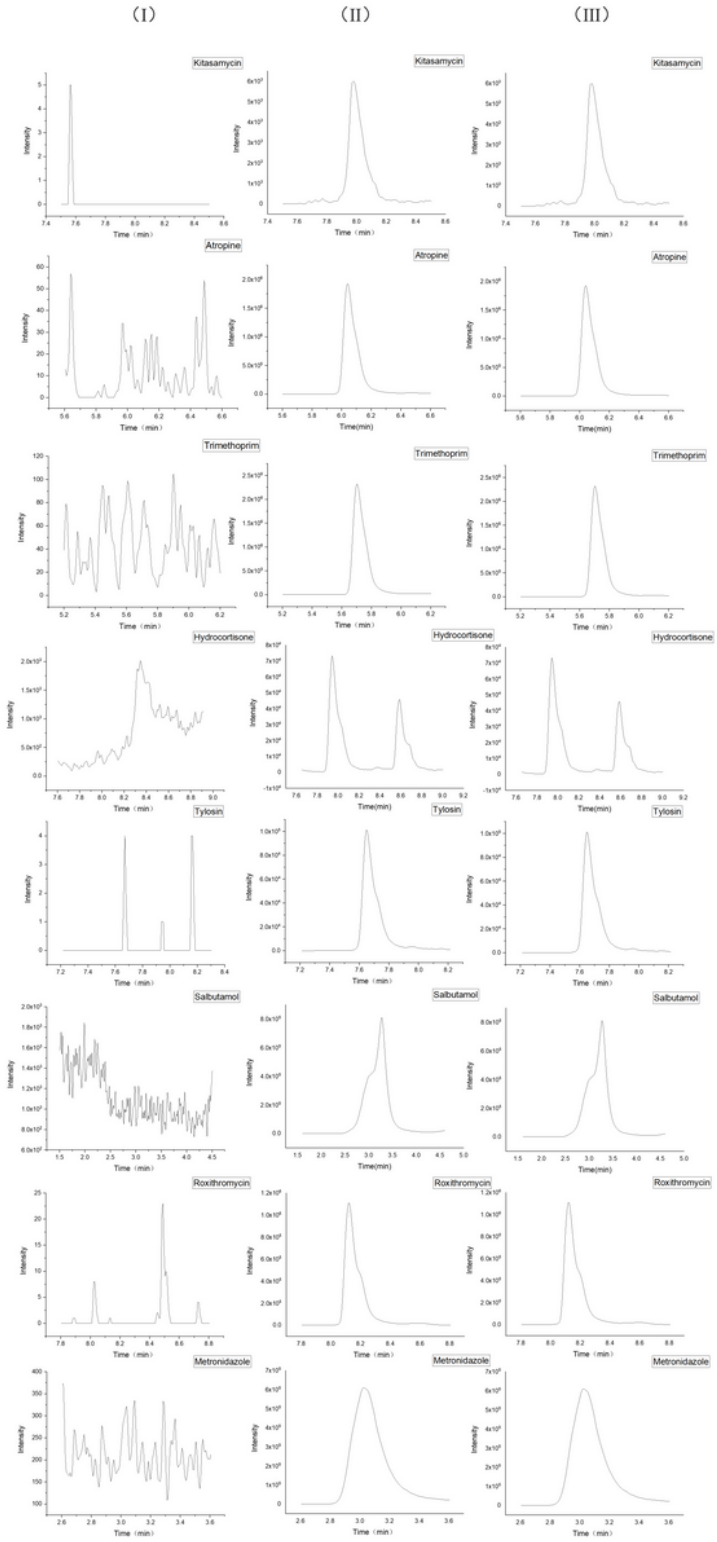

Figure 5

Liquid chromatogram of the eight veterinary drugs in buffalo milk

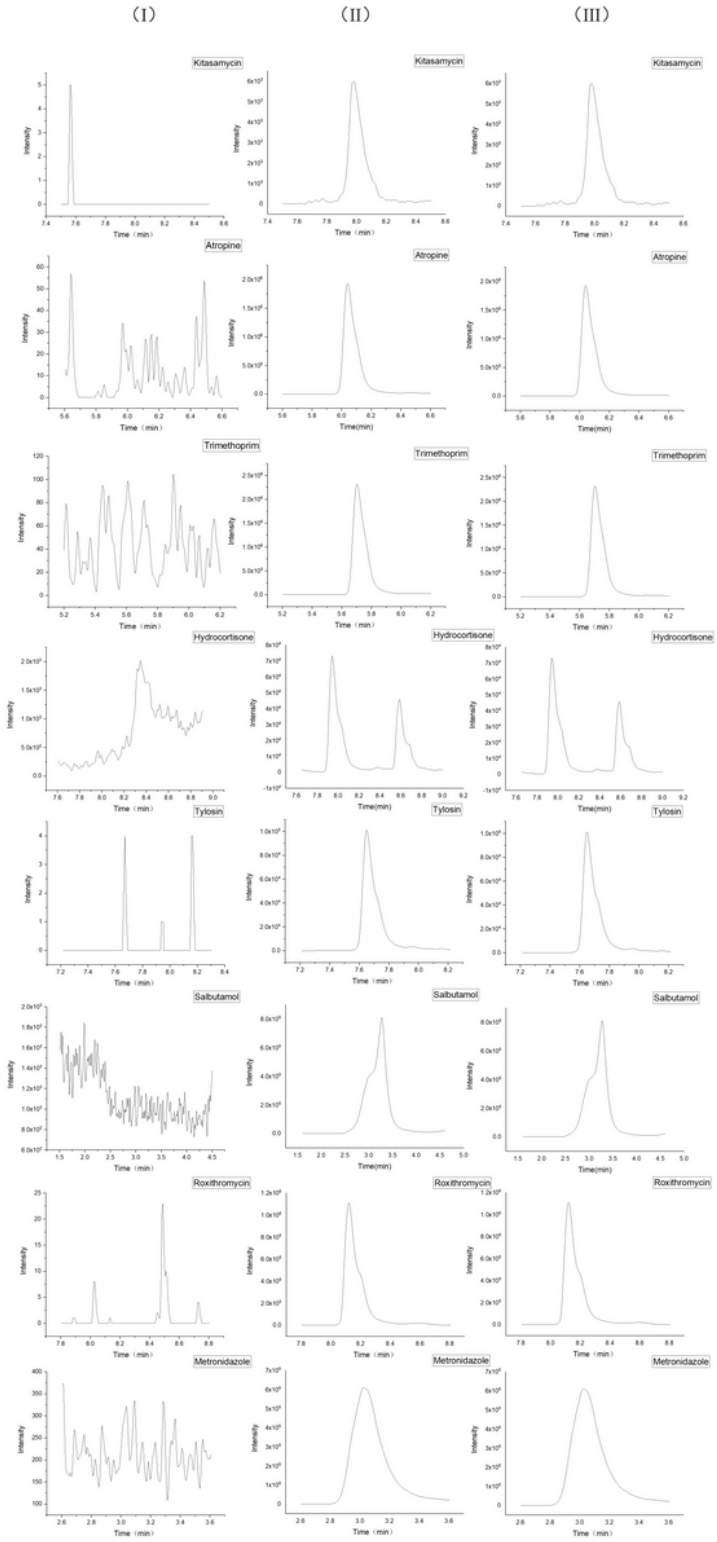

Figure 6

Multi-residue analysis of eight veterinary drugs

Note: The data in the figure are homogenised. The denominator is the MRL value of the drug, the data after processing over this value ≥1, the name at the top of the figure is the content of the test, and the name on the far left is the name of the sample