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Multi-class, Multi-residue LC-MS/MS Method For Veterinary Drug Residues, Mycotoxins And Pesticide In Urine

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Abstract:
In this work, an liquid chromatography- tandem mass spectrometry (LC-MS/MS) methodology is proposed for the multi-class multi-residue screening of veterinary drugs, pesticides and mycotoxins in bovine urine, using an LS-MS/MS both in positive and negative mode.The method currently covers 72 analytes belonging to different families such as antibiotics, steroid hormones, β-agonists, lactones, thyreostatics and contaminants such as pesticides and mycotoxins. After comparing different sample preparation procedures, extraction with sodium acetate and phosphate buffer followed by enzymatic hydrolyze with β-glucuronidase and solid phase extraction with OASIS cartridges was selected as the most appropriate methodology. In the validation study were included linearity, limit of detection, limit of quantification, decision limit, detection capability, accuracy and precision of the method. The method was linear with R^2>0.99. The limit of quantification were established between 0.19 µg/l and 16.7 µg/l, demonstrating the usefulnes of LC-MS/MS as an ideal tool for compliance monitoring in regulatory laboratories. The results for accuracy, expressed as recovery, were with values from 65 – 115%. Intra-day precision (repeatability) and inter-day precision (reproducibility) were expressed thought coefficient of variation. The CV was from 1.26 to 23.31 % for intra-day precision and from 2.29 to 29.42 % for inter-day precision. The results for accuracy and precision fulfill the criteria prescribed in the Commission Decision 2002/657/EC. The method was successfully applied for routine analysis of bovine urine samples. The routine analysis showed that the target components were not detected in the bovine urine samples.

Key words: veterinary drug residues, pesticide residues, mycotoxins, bovine urine,validation study, LC-MS/MS

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1. Introduction

Veterinary drugs such as different class of antibiotics are widely administered in food-producing animals to prevent or treat of diseases. Also, some veterinary drugs like anabolic hormones, β-agonists, thyreostats show growth-promoting effects and are commonly used for these purposes. The residues of veterinary drugs in food from animal origin cause side effects on human health. Due to the side effects, the monitoring of veterinary drug residues in live animals and animal tissues is very important to protect public health (Biselli et al. 2013; Uzunov et al. 2019). The measures to monitor the residue of veterinary drugs in live animals and

food from animal origin are prescribes in Council Directive 96/23/EC (96/23/EC). Also, animals are often simultaneously exposed to mycotoxins mixtures along with other contaminants such as pesticides or heavy metals, making multi-residual and multi-toxin exposure study relevant from a public health perspective. (Agriopoulou et al. 2020; Chinaza et al. 2021).

The development of analytical methods for the determination of residues and contaminants in food of animal origin plays a key role in the protection of public health. Therefore, a large number of analytical method for determination of residues and contaminants separately, but only several multi-residue and multi-class analytical methods have been established for the determination of veterinary drug residues and contaminants such us pesticides and mycotoxins in food matrices (Zhan et al. 2013; Hajrulai-Musliu et al. 2021; Danezis et al, 2016; Gómez-Pérez et al. 2015). On the other hand, methods for the simultaneous determination of residues and contaminants in urine (multi-class and multi-residue methods) are very rare or non-existent. There are a lot of published sensitive and reliable analytical methods, both for screening and confirmation purposes, for determination of residues and contaminants in urine. Most of these methods have been developed for the analysis of each group of residues and contaminants separately. (Ahn et al. 2010; Akre and Mizuno 2016; Escrivá et al. 2017; Uzunov et al. 2019).

The aim of this study is to develop and validate a reliable quantitative method for determination and quantification of a total of 72 residues and contaminants as follow: veterinary drug residues, pesticides and mycotoxins in bovine urine utilizing liquid chromatography-tandem mass spectrometry (LC-MS/MS).

2. Materials and Methods

2.1. Chemicals and reagents

Methanol, acetonitrile and water with LC-MS/MS grade, ethylacetate, dichloromethane, ammonium hydroxide, acetic acid, ammonium acetate (HPLC grade) were purchased from Carlo Erba Reagent S.A.S (Val de Reuil, France); formic acid (LC-MS/MS grade), sodium acetate (p.a.), sodium dihydrogen phosphate hydrate (p.a.), disodium hydrogen phosphate dihydrate (p.a.), sodium chloride (p.a.), β-glucuronidase aryl sulfatase and trichloroacetic acid (≥99.5 %) and Oasis HLB cartridges (500mg/6ml) were from Waters (Milford, MA, USA).

2.2. Analytical standards

Amoxicillin (99.6 %), ampicillin (99.8 %), benzylpenicillin (99.3 %), cloxacillin (98.7 %), oxacillin (98.4 %), lincomycin (100.3 %), tylosin (87.9 %), trimethoprim (99.5 %), tetracyclin (96.8%), cephapirin (98.5%), clenbuterol HCl (99.1 %), isoxsuprine HCl (100 %), salbutamol (99.4 %), zilpaterol HCl (96.0 %), ractopamine HCl (95.5 %), terbutaline hemisulfate salt (100.0 %), taleranol (99.5 %), 19 nortestosterone (99.8 %), clostebol (99.1 %), boldenone (99.1 %), methyltestosterone (99.5 %), testosterone (100.0 %), carbofuran (99.9 %), carbaryl (99.9 %), parathion (99.7 %), malathion (99.2 %), diazinon (98.3 %), dimethoate (99.8 %), atrazine (99.5 %), cypermethrin (98.4 %), permethrin (98.1 %), deltamethrin (99.9 %), coumaphos (99.7 %), dichlorvos (99.8 %), chlorpyrifos (99.8 %), boscalid (99.5 %), fentoate (98.8 %), fenthion (98.5 %), fenvalerate (99.4 %), monocrotophos (99.8 %), malaoxon (99.0 %), methamidophos (98.1 %), metacrifos (96.1 %), amitraz (99.8 %), omethoate (98.4 %), vamidothion (≥98.0 %), phosmet (99.8 %), thiouracil (100 %), propylthiouracil (99.6 %),

methylthiouracil (≥ 98.0 %), tapazol (99.7 %), heptenophos (98.7 %), bifenthrin (99.0 %), methomyl (99.0 %), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Brombuterol (98.0 %), mabuterol HCl (98.0 %), cimbuterol (98.0 %), clenpenterol HCl (98.0 %) were obtained from Witega (Berlin, Germany). Zeranol (99.9 %), stanozolol (99.8 %), ceftiofur (98.01 %), cephalexin (96.6 %), oxytetracycline (96.5 %), enrofloxacin (99.74 %), ciprofloxacin (98.0 %), sulfadimidine (99.6 %), sulfamethoxazole (99.7 %), sulfadiazine (99.8 %), sulfachloropyridazine (99.1 %) and sulfadimethoxine (99.7 %) were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany); ochratoxin A (≥ 98.0%) and zearalenone (99.0%) were obtained from Trylogy Analytical Laboratory, Inc. (Washington, USA).

2.3. Preparation of stock standard solutions, intermediate and working standard solutions

The individual stock standard solutions were prepared in methanol. The concentration of individual stock solutions was in range from 0.5 to 1.0 mg/ml. In the next step, mixed working solutions from standards for construction of calibration curve and fortification of the samples were prepared in methanol. The concentration of these standard solutions was 10 µg/ml.

2.4. Sample preparation

In the first step, 30 ml urine was centrifuged 5 minutes, on 2000 rpm, at room temperature. After centrifugation, 5 ml of urine sample was fortified with the standards. Prior to extraction the samples were left to stand for 10 min at room temperature. In the next step, 5 ml of 0.2 M sodium acetate buffer (pH=5) and 5 ml 0.02 M Phosphate buffer (PBS) (pH=7.2) (1:1, v/v) were added, then the samples were shaken for 1 min on a vortex and 20 µL of β-glucuronidase aryl sulfatase was added. The samples were incubated 17 h at 37°C. After cooling at room temperature, samples were centrifuged 5 minutes, on 2000 rpm, at room temperature. The Oasis HLB cartridges were used for clean-up procedure. The cartridges were activated and conditioned with 5 ml of methanol and 5 ml of water. The whole extract was passed through the cartridges at one drop per second and the cartridge dried, washed with 5 ml of water and dried again. The residues were eluted with two eluent mixtures, first 4 ml of eluent mixture I (48.5:48.5:3, v/v/v, methanol:acetonitrile:ammonium hydroxide) and then with 4 ml of eluent mixtute II (1.5:8.5, v/v, methanol:dichlormethane).
In the next step the solution was evaporated under nitrogen to near dryness at 35°C. The residue was reconstituted with 1 mL of the mobile phase (95:5, v/v, Mobile phase A: Mobile phase B). Prior to LC–MS/MS analysis the extracts were filtered through a 0.45µm membrane filter into 2 mL autosampler vials.

2.5. LC-MS/MS analysis

LC-MS/MS (Waters, Milford, MA, USA) were used for identification and quantification of the target compounds. LC-MS/MS is equipped with a binary pump, vacuum degasser, thermostatted autosampler, thermostatted column manager and triple quadrupole detector. For chromatographic separation was used LC column Kinetex C18 (50 x 2.1 mm, 2.6 µm, Phenomenex, Torrance, CA, USA). For instrument control, data acquisition and processing of results was used software (MassLynx version 4.1, Waters, Milford, MA, USA).
The LC conditions were as follow: flow rate of mobile phase: 0.2 ml/min; column temperature: 40°C, elution program: 0–1 min, 95-80 % A; 1–4 min, 80-60 % A; 4–8 min, 60-95 % A; 8-12 min, 95 % A; mobile phase A contains: water with 5 mMol ammonium acetate, 0.01 % formic acid and 0.01 % trichloroacetic acid; mobile phase B contains: methanol with 0.1% formic acid, temperature in sample chamber: 4°C; injection volume: 5 µL. The MS/MS conditions were optimized as follows: capillary voltage of 3.0 kV; source temperature of 150°C; desolvation temperature of 400°C; cone gas at 100 L/h; desolvation gas at 300 L/h.

3. Results

3.1. MS/MS optimization

For optimization of MS/MS conditions and selection of appropriate diagnostic ions the standard working solution with concentration of 1.0 µg/mL were infused to the MS/MS detector. ESI in both positive and negative ion modes was evaluated for detection of 72 compounds included in the study. The optimal parameters for each compound, such as: polarity, precursor ion, product ions, collision energy, cone voltage and retention time are shown in Table 1. The optimal dwell time which provides suitable signal to noise and good peak shape was 0.025 s.

Table 1. MRM parameters

Standard	Polarity	Precursor ion (m/z)	Product ion (m/z)	Collision energy	Cone voltage	Retention time
Thiouracil TU	+	128.80	112.0 69.86 59.77	20 18 18	30	1.43
Methylthiouracil MTU	+	142.83	125.90 83.85 41.86	18 22 18	30	1.55
Propyltiouracil PTU	+	170.88	154.30 111.91 69.86	20 24 22	32	1.90
Tapazole TAP	+	114.82	110.15 87.83 56.84	16 16	36	0.82
Testosteron TEST	+	289.16	108.99 96.95 178.18	24 28 28	36	6.78
Methyltestosteron MES	+	303.22	96.96 109.0 178.18	28 24 24	36	7.05
Boldenon BOLD	+	287.16	121.03 135.02 171.20	24 16 20	34	6.55
19 Nortestosteron 19 N	+	275.14	80.56 109.0 93.18	34 26 32	38	6.68
Stanozolol STZL	+	329.22	80.95 95.00	46 46	64	7.52

			121.00	42		
Clostebol CLBL	+	323.16	130.98 142.96 157.13	26 26 22	40	7.14
Zeranol ZENL	-	321.03	90.87 40.90 259.2	40 40 36	74	6.34
Taleranol TANL	-	321.03	90.87 40.90 259.2	34 40 42	74	6.70
Clenbuterol CLEN	+	276.97	202.95 131.87 166.77	16 30 30	22	3.84
Brombuterol BROM	+	366.90	292.84 211.42 57.00	20 34 38	26	4.34
Mabuterol MABT	+	310.95	236.99 216.96 57.00	18 26 30	24	4.51
Clenpenterol CLEP	+	291.00	202.92 131.89 167.79	16 30 28	28	4.60
Isoxuprin ISOX	+	302.04	106.96 164.01 120.95	30 16 28	26	3.31
Cimbuterol IMB	+	234.03	159.98 142.94 57.0	16 28 26	22	2.26
Ractopamine RACT	+	302.04	164.01 106.96 120.95	16 28 24	24	3.86
Salbutamol SALB	+	240.03	147.96 165.98 56.94	20 14 24	22	1.99
Zilpaterol HCl ZILP	+	262.03	202.05 185.01 156.98	22 24 32	22	1.95
Terbutalin hemisulfate TERB	+	226.00	152.00 106.97 170.00	14 30 16	26	1.87
Amoxicillin AMOX	+	367.07	159.96	16	28	5.55

			90.89	40		
Ampicillin AMP	+	349.97	105.95 159.94	20 14	34	3.93
Benzylpenicillin BNPC	+	334.99	90.96 80.94	42 52	44	5.52
Lincomycin LINK	+	407.06	126.02 41.75	34 72	22	2.80
Tylosin TYLS	+	916.3	173.99 100.88	46 52	74	6.31
Trimethoprim TRIP	+	290.97	122.94 229.94	28 24	26	2.90
Cephapirin CEPR	+	423.93	291.93 151.89	14 28	42	2.04
Tetracycline TETC	+	445.03	410.01 153.90	20 34	40	5.33
Cloxacillin CLCN	+	435.94	159.97 276.96	18 14	26	6.15
Oxacillin OXIN	+	402.05	159.96 243.03	10 12	24	5.95
Cefalexin CEFA	+	347.97	157.86 173.93	8 14	30	2.75
Ceftiofur CEFT	+	523.96	241.00 125.17	16 58	34	4.90
Enrofloxacin ENRO	+	360.05	245.09 72.02	30 36	36	3.68
Ciprofloxacin CIPR	+	332.01	245.05 230.94	40 28	38	3.56
Oxytetracyclin OXTT	+	462.01	426.02 200.93	38 30	36	3.17
Sulfachloropyridazin SUPZ	+	284.90	155.93 91.93	16 34	28	2.93
Sulfadiazin SUDI	+	250.97	91.93 155.93	30 14	28	1.92
Sulfadimetoxin SUDM	+	310.97	155.93 91.93	20 32	36	4.36
Sulfadimidin SULD	+	278.95	185.93 91.93	18 36	34	2.71
Sulfamethoxazol SULM	+	253.91	92.00 155.94	30 16	28	3.01
Carbofuran CRL	+	222.1	165.0 123.0	12 22	32	5.38

Carbaryl CRB	+	202.0	145.05 127.0	10 32	26	5.74
Parathion PTN	+	292.0	210.0 180.0	12 26	30	6.02
Malathion MTN	+	331.1	98.93 127.0	14 26	30	6.76
Diazinon DNN	+	304.97	168.94 153.00	24 24	44	7.38
Dimethoate DIM	+	229.90	198.83 124.84	10 20	30	3.36
Atrazine ATRZ	+	216.0	174.22 104.14	15 30	32	7.00
Permethrin PEMT	+	390.97	355.02 182.92	6 12	34	8.68
Cypermethrin CIRM	+	433.0	192.80 90.93	20 12	28	8.27
Deltamethrin DELM	+	229.84	198.83 124.85	30 14	30	3.37
Coumaphos COU	+	362.90	226.86 306.86	26 18	52	7.39
Dichlorophos DIRP	+	220.78	108.89 78.83	20 30	44	5.25
Chlorpyrifos CHRS	+	351.78	96.79 199.77	32 16	38	8.11
Fenvalerat FERT	+	419.97	166.89 124.88	14 42	38	8.33
Boskalid BOS	+	342.94	306.94 139.85	20 20	56	6.68
Fentoate FETE	+	320.86	162.87 246.84	12 12	28	7.25
Fenthion FEON	+	278.82	168.87 104.86	18 28	38	7.33
Monocrotophos MOCR	+	223.16	192.87 97.83	8 12	30	2.70
Malaoxon MAON	+	314.94	126.84 98.80	14 26	38	5.60
Methamidophos MEDF	+	141.78	93.80 46.82	14 24	38	1.96
Metacrifos MECF	+	240.93	208.83 124.83	8 20	32	6.40
Amitraz AMRZ	+	294.05	162.96	14	30	7.87

Omethoat OMAT	+	213.84	121.91 182.82 154.84	32 12 18	32	1.78
Vamidothion VAON	+	287.78	145.92 117.87	14 24	30	3.59
Phosmet FOST	+	320.86	246.84 162.87	14 58	32	6.90
Heptenophos HEPH	+	250.78	126.83 89.04	16 34	42	6.26
Bifenitrin BFNT	+	440.03	180.96 165.87	22 42	24	8.65
Methomyl MEML	+	162.84	87.88 105.90	8 10	30	2.35
Zearalenone ZEAN	-	316.97	130.87 174.91	30 26	62	6.85
Ochratoxin A OTAA	+	404.03	238.92 101.8	30 10	46	6.92

3.2. Optimization of mobile phase

During the development of the method due to the differences in the chemical structure between components included in this study six different mobile phases were investigated. The composition of the mobile phases is shown in Table 2.

Table 2. Composition of mobile phases

No.	Mobile phase A	Mobile phase B
1	Water with 5 mM ammonium acetate ar 0.1 % formic acid	Acetonitrile with 0.1% formic acid
2	Water with 5 mM ammonium acetate ar 0.1 % formic acid	Acetonitrile:methanol (50/50; v/v) wi 0.1% formic acid
3	Water with 5 mM ammonium acetate ar 0.1 % formic acid	Methanol with 0.1% formic acid
4	Water with 5 mM ammonium acetate ar 0.01 % formic acid	Methanol with 0.1% formic acid
5	Water with 5 mM ammonium acetate, 0 % formic acid and 0.01 % trichloroacet acid (TCA)	Methanol with 0.1% formic acid
6	Water with 5 mM ammonium acetat 0.01 % formic acid and 0.01 % TCA	Methanol with 0.1% formic acid

The results of the investigation of mobile phases from 1 to 5 (Table 2) showed that some compounds were not detected, moreover, poor separation, bad peak shape, low signal intensity or detection only one daughter ion. The chromatograms for poor separation and bad peak shape are shown in Figure 1 and Figure 2, respectively.

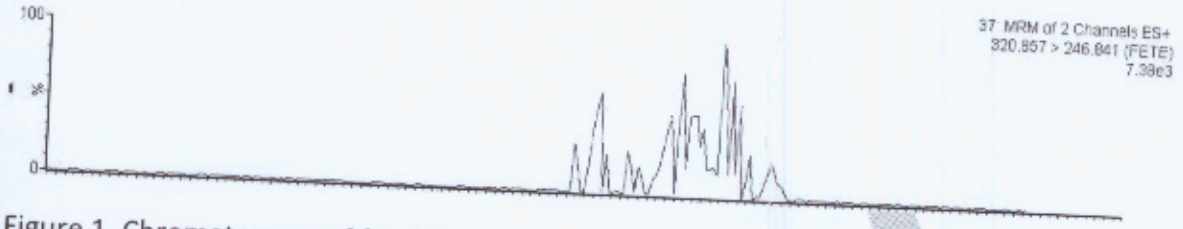

Figure 1. Chromatogram of fentoate – poor separation

Figure 2. Chromatogram of metacrifos – bead peak shape

The optimal mobile phase which provides improved separation, good peak shape, high signal intensity, the best peak symmetry and resolution as well as detection of all target components was water with 5 mM ammonium acetate, 0.01 % formic acid and 0.01 % TCA as mobile phase A and methanol with 0.1% formic acid as mobile phase B. The chromatograms are given in Figure 3 and Figure 4.

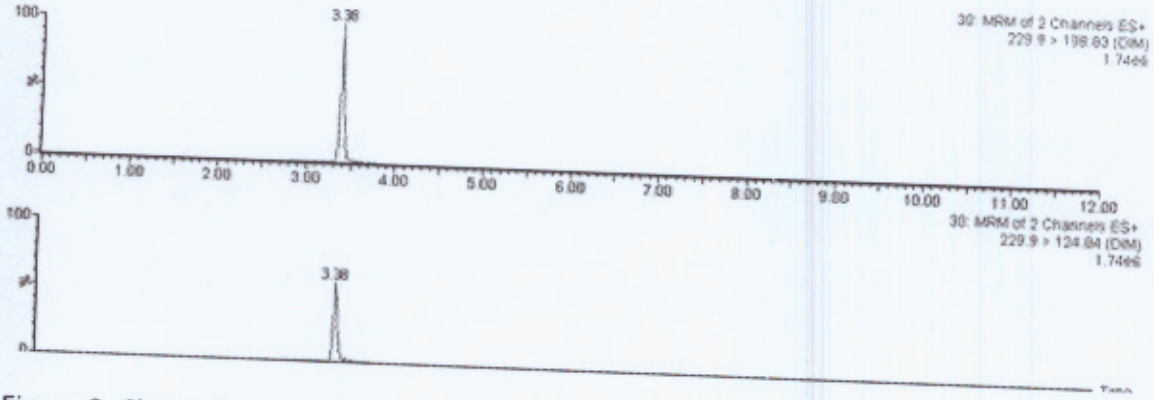

Figure 3. Chromatogram of dimethoate

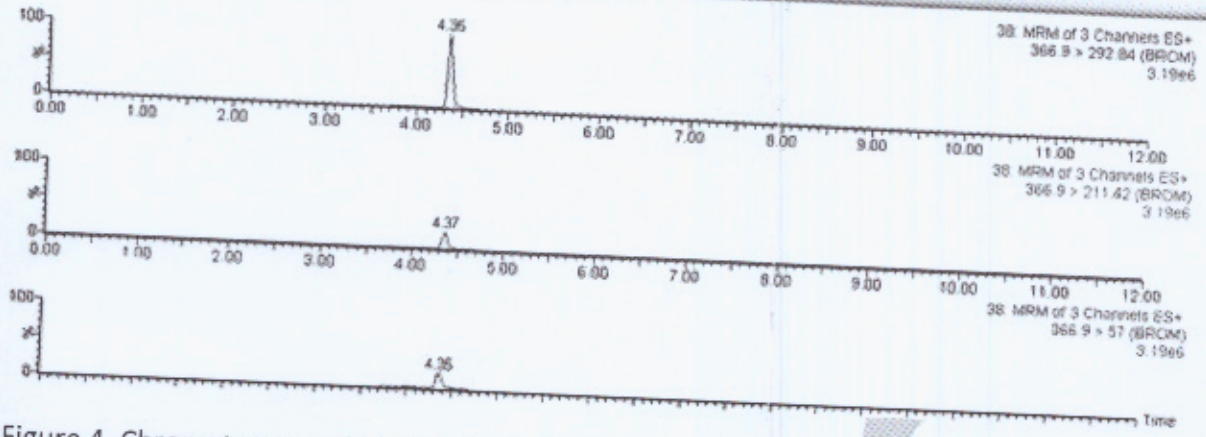

Figure 4. Chromatogram of brombuterol

3.3. Optimisation of the sample preparation

Four extraction protocols were investigated for the extraction of 72 compounds from urine. In the first protocol was used liquid-liquid (LLE) extraction without enzymatic hydrolyze, in the second protocol was used LLE with enzymatic hydrolyze, in third extraction protocols were used solid phase extraction (SPE) without enzymatic hydrolyze, while in fourth protocol was used SPE with enzymatic hydrolyze. The enzymatic hydrolyze was performed with β-glucuronidase aryl sulfatase from Helix pomatia. In the first step, for all protocols, 30 ml urine samples were centrifuged 5 minutes, on 2000 rpm, at room temperature. This step was used to remove the proteins.

In the first and second protocols with LLE, after centrifugation, 5 ml of urine samples was fortified with the standards. Prior to extraction the samples were left to stand for 10 min at room temperature. In the next step, 5 ml of 0.2 M sodium acetate buffer (pH=5) and 5 ml 0.02 M Phosphate buffer (PBS) (pH=7.2) (1:1, v/v) were added, the samples were shaken for 1 min on a vortex. After this step, in the first protocol, samples were centrifuged 5 minutes, on 2000 rpm, at room temperature and the next step was LLE, while in the second protocol for the enzymatic hydrolyze 20 µL of β-glucuronidase aryl sulfatase was added. The samples were incubated 17 h at 37°C. After cooling at room temperature, samples were centrifuged 5 minutes, on 2000 rpm, at room temperature. LLE was the same for both extraction protocols, as follows. In the first step from LLE was used 10 ml methanol:acetonitrile:acetic acid (49:49:2, v/v/v). The samples were shaken for 1 min on a vortex and centrifuged 5 minutes, on 2000 rpm, at room temperature. After that, the supernatant was transferred in test tubes. In the second LLE step 10 ml of ethylacetate:hexane (40:60, v/v) was used. The samples were shaken for 1 min on a vortex and centrifuged 5 minutes, on 2000 rpm, at room temperature. The supernatant was fused to the first supernatant. The samples were evaporated under nitrogen to near dryness at 35°C. The residue was reconstituted with 1 mL of the mobile phase (95:5, v/v, Mobile phase A: Mobile phase B). Prior to LC–MS/MS analysis the extracts were filtered through a 0.45µm membrane filter into 2 mL autosampler vials.

In the third and fourth protocols after centrifugation, 5 ml of urine sample was fortified with the standards. Prior to extraction the samples were left to stand for 10 min, at room temperature. In the next step, 5 ml of 0.2 M sodium acetate buffer (pH=5) and 5 ml 0.02 M Phosphate buffer (PBS) (pH=7.2) (1:1, v/v) were added, the samples were shaken for 1 min on a vortex. After cooling at room temperature, samples were centrifuged

5 minutes, on 2000 rpm, at room temperature. The next step in the third protocol is SPE extraction, while in the fourth protocol the next step is enzymatic hydrolysis. For this purpose, 20 µL of β-glucuronidase aryl sulfatase was added. The samples were incubated 17 h at 37°C. After cooling at room temperature, samples were centrifuged 5 minutes, on 2000 rpm, at room temperature. The SPE step is the same in the both protocols. SPE: Oasis HLB cartridges were activated and conditioned with 5 ml of methanol and 5 ml of water. The reconstituted extract (10 ml) was passed through the cartridges at one drop per second and the cartridge dried, washed with 5 ml of water and dried again. The residues were eluted with two eluent mixtures, first 4 ml of eluent mixture I (48.5:48.5:3, v/v/v, methanol:acetonitrile:ammonium hydroxide) and then with 4 ml of eluent mixture II (1.5:8.5, v/v, methanol:dichlormethane).

After solid phase extraction the eluent was evaporated under nitrogen to near dryness at 35°C. The residue was reconstituted with 1 mL of the mobile phase (95:5, v/v, Mobile phase A: Mobile phase B). Prior to LC–MS/MS analysis the extracts were filtered through a 0.45µm membrane filter into 2 mL autosampler vials.

For optimization of extraction procedure, for all extraction protocols, blank urine samples were spiked with standards at 3 concentration levels.

The thyreostats were not detected with LLE protocols. In the protocols with SPE extraction without enzymatic hydrolysis the results shown low recovery < 55 % for anabolic steroids and zeranol. Stanozolol and taleranol with this protocol were not detected. The optimal recoveries were obtained by SPE extraction with enzymatic hydrolysis and the recoveries were from 65.0 % for mabuterol (spiked at concentration at 0.2 µg/L) to 115.0 % for brombuterol (spiked at concentration at 0.2 and 0.4 µg/L).

3.4. Method validation

3.4.1. Linearity

The linearity of the method was evaluated using matrix-matched calibration curve. The blank urine samples were fortified at six concentration levels. For each concentration levels three replications were performed. The linearity along the research range presented values for coefficient of correlation (R2) from 0.9904 for cypermethrin to 0.9997 for lincomycin and ochratoxin A. The range of calibration curve and R^2 for all compounds are given in Table 3.

Table 3. Linearity of the method

Analytes	Calibration range (µg/L)	R^2
Thiouracil TU	0-100.0	0.9920
Methylthiouracil MTU	0-100.0	0.9942
Propyltiouracil PTU	0-100.0	0.9931
Tapazole TAP	0-100.0	0.9954
Testosteron TEST	0-100.0	0.9968
Methyltestosteron MEST	0-100.0	0.9940
Boldenon BOLD	0-100.0	0.9952
19 Nortestosteron 19 NO	0-100.0	0.9917
Stanozolol STZL	0-100.0	0.9948
Clostebol CLBL	0-100.0	0.9987
Zeranol ZENL	0-100.0	0.9935
Taleranol TANL	0-100.0	0.9972
Clenbuterol CLEN	0-50.0	0.9914
Brombuterol BROM	0-50.0	0.9931
Mabuterol MABT	0-50.0	0.9973
Clenpenterol CLEP	0-50.0	0.9935
Isoxuprin ISOX	0-50.0	0.9995
Cimbuterol CIMB	0-50.0	0.9931
Ractopamine RACT	0-100.0	0.9931
Salbutamol SALB	0-100.0	0.9964
ZilpaterolHCl ZILP	0-100.0	0.9959
TerbutalinTERB	0-100.0	0.9919
Amoxicillin AMOX	0-100.0	0.9965
Ampicillin AMP	0-100.0	0.9913
Benzylpenicillin BNPC	0-100.0	0.9936
Linkomycin LINK	0-100.0	0.9997
Tylosin TYLS	0-100.0	0.9993
Trimetoprim TRIP	0-100.0	0.9963
Cephapirin CEPR	0-100.0	0.9981
Tetracyclin TETC	0-100.0	0.9962
Cloxacillin CLCN	0-100.0	0.9986
Oxacillin OXIN	0-100.0	0.9954
Cefalexin CEFA	0-100.0	0.9996
Ceftiofur CEFT	0-100.0	0.9940
Enrofloxacin ENRO	0-100.0	0.9964
Ciprofloxacin CIPR	0-100.0	0.9932
Oxytetracyclin OXTT	0-100.0	0.9936

Sulfachloropyridazin SUPZ	0-100.0	0.9980
Sulfadiazin SUDI	0-100.0	0.9931
Sulfadimetoxin SUDM	0-100.0	0.9944
Sulfadimidin SULD	0-100.0	0.9910
Sulfamethoxazol SULM	0-100.0	0.9968
Carbofuran CRL	0-100.0	0.9921
Carbaryl CRB	0-100.0	0.9915
Paration PTN	0-100.0	0.9964
Malation MTN	0-100.0	0.9910
Diazinon DNN	0-100.0	0.9914
Dimethoat DIM	0-100.0	0.9975
Atrazine ATRZ	0-100.0	0.9985
Permetrin PEMT	0-100.0	0.9959
Cypermetrin CIRM	0-100.0	0.9904
Deltametrin DELM	0-100.0	0.9946
Coumaphos COU	0-100.0	0.9934
Dichlorophos DIRP	0-100.0	0.9943
Chloropyrifos CHRS	0-100.0	0.9940
Fenvalerat FERT	0-100.0	0.9943
Boskalid BOS	0-100.0	0.9925
Fentoate FETE	0-100.0	0.9913
Fention FEON	0-100.0	0.9963
Monocrotophos MOCR	0-100.0	0.9991
Malaoxon MAON	0-100.0	0.9945
Methamidophos MEDF	0-100.0	0.9941
Metacrifos MECF	0-100.0	0.9956
Amitraz AMRZ	0-100.0	0.9973
Omethoat OMAT	0-100.0	0.9921
Vamidothion VAON	0-100.0	0.9951
Phosmet FOST	0-100.0	0.9925
Heptenophos HEPH	0-100.0	0.9920
Bifenitrin BFNT	0-100.0	0.9959
Methomyl MEML	0-100.0	0.9974
Zearalenone ZEAN	0-100.0	0.9935
Ochratoxin A OTAA	0-100.0	0.9997

3.4.2. LOD, LOQ, CCα and CCβ

The LODs and LOQs were determined as the lowest concentration of the standards which were used for construction of calibration curve (n=20). The LOD was calculated as the mean value plus 3.3 times the

calculated standard deviation (SD), while the LOQ was calculated as the mean value plus 10 times the calculated SD. The LODs were from 0.06 µg/L for clenbuterol to 5.51 µg/L for metacrifos, while the LOQs were from 0.17 µg/L for clenbuterol to 16.70 µg/L to metacrifos. The CCα and CCβ were determined according to the criteria prescribes in the Commission Decision 2002/657/EC. CCα were ranged from 0.11 µg/L for clenbuterol to 10.88 µg/L for cephapirin, while CCβ were ranged from 0.15 µg/L for clenbuterol to 15.23 µg/L for tylosin. The results are shown in Table 4.

Table 4. CCα, CCβ, LOD, LOQ and MRPL

Analytes	CCα (µg/L)	CCβ (µg/L)	LOD (µg/L)	LOQ (µg/L)	MRPL (µg/L
Thiouracil TU	7.17	9.46	2.03	5.08	10
Methylthiouracil MTU	2.26	4.88	1.64	3.56	10
Propylthiouracil PTU	2.23	5.22	2.48	4.75	10
Tapazole TAP	5.03	8.42	4.22	7.15	10
Testosterone TEST	9.86	12.35	2.46	7.45	/
Methyltestosterone MEST	1.36	1.78	0.47	1.45	2
Boldenone BOLD	0.69	0.95	0.31	0.95	1
19 Nortestosterone 19 NO	0.55	0.88	0.21	0.63	1
Stanozolol STZL	1.15	1.64	0.62	1.88	2
Clostebol CLBL	4.36	8.02	2.22	6.80	/
Zeranol ZENL	1.77	1.93	0.65	1.84	2
Taleranol TANL	1.27	1.83	0.42	1.29	2
Clenbuterol CLEN	0.11	0.15	0.06	0.17	0.2
Brombuterol BROM	0.13	0.16	0.07	0.19	0.2
Mabuterol MABT	0.13	0.18	0.07	0.19	0.2
Clenpenterol CLEP	0.32	0.47	0.12	0.36	0.5
Isoxuprin ISOX	0.28	0.38	0.17	0.32	0.5
Cimbuterol CIMB	0.25	0.41	0.13	0.41	0.5
Ractopamine RACT	0.48	0.67	0.18	0.56	1.0
Salbutamol SALB	0.71	0.92	0.22	0.66	1.0
Zilpaterol ZILP	0.56	0.78	0.14	0.40	1.0
Terbutaline	1.77	2.62	0.76	2.30	3.0
Amoxicillin AMOX	7.86	11.54	3.08	9.28	/
Ampicillin AMP	9.22	10.56	2.04	6.15	/
Benzylpenicillin BNPC	9.88	13.54	4.07	12.33	/
Lincomycin LINK	6.64	9.51	4.48	13.28	/
Tylosin TYLS	10.15	15.23	3.28	9.90	/
Trimethoprim TRIP	3.79	6.14	5.08	15.40	/
Cephapirine CEPR	10.88	13.56	2.12	6.51	/
Tetracyclin TETC	4.46	8.78	5.00	8.86	/
Cloxacillin CLCN	8.76	10.15	3.32	10.06	/
Oxacilln OXIN	7.86	9.22	3.12	9.35	/
Cefalexin CEFA	9.13	7.11	4.51	13.70	/
Ceftiofur CEFT	6.54	9.25	3.88	11.65	/
Enrofloxacin ENRO	4.12	8.01	4.52	13.25	/
Ciprofloxacin CIPR	6.57	8.22	2.12	6.35	/
Oxytetracycline OXTT	5.00	7.54	3.36	9.98	/
Sulfachloropyridazin SUPZ	5.36	7.48	2.11	6.48	/
Sulfadiazine SUDI	4.28	8.20	2.88	7.01	/

Sulfadimetoxine SUDM	8.32	11.56	3.51	10.23	/
Sulfadimidine SULD	7.15	10.02	3.13	9.56	/
Sulfamethoxazole SULM	7.28	10.78	4.01	12.15	/
Carbofuran CRL	5.23	8.11	3.35	10.15	/
Carbaryl CRB	5.78	9.01	2.02	6.10	/
Parathion PTN	7.81	9.94	4.38	13.15	/
Malathion MTN	9.14	13.25	1.11	3.40	/
Diazinon DNN	8.64	12.08	3.88	11.65	/
Dimethoate DIM	7.35	10.11	2.31	7.00	/
Atrazine ATRZ	7.22	9.21	2.78	8.45	/
Permethrin PEMT	4.36	6.58	3.56	10.51	/
Cypermethrin CIRM	8.54	10.12	3.11	9.42	/
Deltamethrin DELM	7.12	9.54	4.02	12.18	/
Coumaphos COU	5.48	8.82	2.57	7.65	/
Dichlorvos DIRP	6.54	9.11	3.11	9.39	/
Chlorpyrifos CHRS	6.78	9.82	1.56	4.80	/
Fenvalerate FERT	8.25	11.34	3.11	9.50	/
Boskalid BOS	6.34	8.14	2.78	7.90	/
Fentoate FETE	7.85	10.26	2.33	7.41	/
Fenthion FEON	7.00	9.11	3.56	10.70	/
Monocrotophos MOCR	6.29	9.54	4.01	12.20	/
Malaoxon MAON	8.48	12.08	4.09	12.40	/
Methamidophos MEDF	4.35	7.95	3.37	10.15	/
Metacrifos MECF	6.88	9.02	5.51	16.70	/
Amitraz AMRZ	4.64	7.12	2.92	8.80	/
Omethoat OMAT	6.58	9.04	2.11	6.55	/
Vamidothion VAON	6.12	9.15	2.64	8.02	/
Phosmet FOST	7.32	10.48	3.22	9.70	/
Heptenophos HEPH	10.12	14.11	4.11	12.20	/
Bifenthrin BFNT	8.22	12.56	3.51	10.50	/
Methomyl MEML	6.48	10.14	2.31	7.48	/
Zearalenone ZEAN	5.29	7.78	4.68	14.20	/
Ochratoxin A OTAA	7.79	10.64	4.00	12.10	/

3.4.3. Accuracy and precision

Recovery of the method was used for evaluation of the accuracy. Recovery was studied at three concentration levels obtained by fortification of urine samples by mixed standard solution. The recovery range was from 65 % for mabuterol to 115 % for brombuterol. The intra-day precision (repeatability) and inter-day precision (reproducibility) were studied, as well as recovery, but for inter-day precision the fortified samples at three concentration levels were prepared and tested at three consecutive days. Intra-day and inter-ay precision were expressed thought coefficient of variation (CV, %). The CV for intra-day precision was from 1.26 % for lincomycin to 23.31 % for malathion, while the CV for reproducibility (inter-day precision) was from 2.29 % for lincomycin to 29.42 % for carbaryl. The results are summarized in Table 5.

Table 5. Accuracy and precision of the method

Analytes	Added concentration (µg/L)	Average concentration in the samples (µg/L) (n=6)	Standard deviation (µg/L)	Recovery (%	Repeatability (CVr, %)	Reproducibility (CV_R, %)
Thiouracil TU	10 15 20	9.44 13.27 18.25	1.06 1.78 2.55	94.40 88.47 91.25	11.23 13.41 13.97	16.54 18.28 17.36
Methylthiouracil MTU	10 15 20	8.55 14.02 18.54	0.74 2.56 3.04	85.50 93.47 92.70	8.65 18.27 16.40	13.53 21.00 19.88
Propyltiouracil PTU	10 15 20	8.11 16.42 21.54	0.98 1.15 1.95	81.10 109.47 107.70	12.08 7.00 8.87	16.46 13.08 12.95
Tapazole TAP	10 15 20	8.45 13.80 19.14	1.12 2.04 2.46	84.52 92.00 95.70	13.25 14.78 12.85	17.00 18.54 16.48
Testosteron TEST	10 15 20	10.79 15.90 20.20	2.26 1.80 3.74	107.3 106.0 101.0	20.95 11.32 18.51	22.94 15.38 24.35
Methyltestosteron MEST	2.0 3.0 4.0	1.46 2.74 3.56	0.22 0.41 0.61	73.0 91.3 89.0	15.07 14.96 21.07	20.80 17.46 22.38
Boldenon BOLD	1.0 1.5 2.0	1.01 1.62 2.16	0.14 0.17 0.45	101.0 108.0 108.0	13.86 10.49 17.13	17.45 13.12 21.35
19 Nortestosteron 19 NC	1.0 1.5 2.0	0.84 1.23 1.76	0.11 0.22 0.17	84.0 82.0 88.0	13.10 17.89 9.66	17.10 19.46 12.08
Stanozolol STZL	2.0 3.0 4.0	1.79 3.30 4.31	0.13 0.51 0.28	89.5 110.0 107.8	7.26 15.45 6.50	9.92 19.46 9.12
Clostebol CLBL	10 15 20	10.25 14.60 19.88	1.36 2.17 3.51	102.5 97.3 99.4	13.27 14.86 17.65	16.35 16.87 22.14
Zeranol ZENL	2.0 3.0 4.0	1.55 2.48 3.41	0.08 0.21 0.19	77.5 82.7 85.3	5.16 8.47 5.57	7.17 10.02 6.46
Taleranol TANL	2.0	1.64	0.12	82.0	7.32	9.01

	3.0 4.0	2.78 4.01	0.35 0.40	92.7 100.3	12.59 9.98	15.38 14.46
Clenbuterol CLEN	0.2 0.3 0.4	0.14 0.22 0.34	0.02 0.04 0.06	70.0 73.3 85.0	14.29 18.18 17.65	16.01 21.35 19.12
Brombuterol BROM	0.2 0.3 0.4	0.23 0.33 0.46	0.04 0.03 0.09	115.0 110.0 115.0	17.39 9.09 19.56	20.48 13.04 22.56
Mabuterol MABT	0.2 0.3 0.4	0.13 0.21 0.30	0.01 0.04 0.06	65.0 70.0 75.0	7.69 19.05 20.0	8.12 21.03 22.74
Clenpenterol CLEP	0.5 0.75 1.0	0.56 0.69 0.87	0.04 0.07 0.05	112.0 92.0 87.0	7.14 10.14 5.75	10.46 12.88 7.46
Isoxuprin ISOX	0.5 0.75 1.0	0.40 0.72 0.94	0.02 0.07 0.17	80.0 96.0 94.0	5.00 9.72 18.09	9.25 12.23 21.08
Cimbuterol CIMB	0.5 0.75 1.0	0.48 0.63 0.84	0.05 0.03 0.11	96.0 84.0 84.0	10.42 4.76 13.10	16.35 7.04 16.12
Ractopamine RACT	1.0 1.5 2.0	0.85 1.55 2.10	0.04 0.12 0.14	85.0 103.3 105.0	4.71 7.74 6.67	6.12 8.45 9.12
Salbutamol SALB	1.0 1.5 2.0	0.90 1.50 2.14	0.07 0.21 0.27	90.0 100.0 107.0	7.78 14.0 12.62	10.15 19.23 16.08
ZilpaterolHCl ZILP	1.0 1.5 2.0	0.77 1.22 1.78	0.04 0.13 0.27	77.0 81.3 89.0	5.20 10.70 15.20	7.78 18.14 18.37
TerbutalinTERB	3.0 4.5 6.0	2.90 4.73 5.44	0.22 0.57 0.89	96.7 105.1 90.7	7.58 12.05 16.36	8.24 15.31 17.08
Amoxicillin AMOX	10.0 15.0 20.0	9.77 15.46 20.07	0.52 1.31 1.12	97.7 103.1 100.4	5.32 8.47 5.58	8.46 9.12 7.68
Ampicillin AMP	10.0 15.0 20.0	10.46 16.45 19.56	1.43 2.15 3.78	104.6 109.7 97.8	13.67 13.07 19.33	15.21 16.35 22.18
Benzylpenicillin BNPC	10.0 15.0 20.0	8.22 12.78 17.56	0.76 1.74 1.22	82.2 85.2 87.8	9.25 13.62 6.95	13.35 16.08 8.57

Linkomycin LINK	10.0 15.0 20.0	11,13 16.51 20.85	0.14 1.40 1.78	111.3 110.1 104.3	1.26 8.48 8.54	2.29 13.58 9.88
Tylosin TYLS	10.0 15.0 20.0	11.00 16.38 20.17	0.56 1.12 1.35	110.0 109.2 100.9	5.09 6.84 6.69	8.19 9.38 9.56
Trimetoprim TRIP	10.0 15.0 20.0	11.00 16.25 21.41	2.01 2.64 3.51	110.0 108.3 107.1	18.27 16.24 16.39	22.89 17.23 21.64
Cephapirin CEPR	10.0 15.0 20.0	10.85 16.04 21.52	0.45 1.13 1.46	108.5 106.9 107.6	4.15 7.04 6.78	7.08 10.12 8.68
Tetracyclin TETC	10.0 15.0 20.0	10.14 16.39 19.86	0.28 0.36 0.75	101.4 109.3 99.3	2.76 2.20 3.78	6.02 3.88 7.45
Cloxacillin CLCN	10.0 15.0 20.0	10.01 16.20 21.08	1.46 2.05 2.26	100.1 108.0 105.4	14.59 12.65 10.72	22.14 14.65 14.78
Oxacillin OXIN	10.0 15.0 20.0	8.43 14.48 18.35	0.86 1.33 1.78	84.4 96.5 91.9	10.20 9.19 9.70	14.56 15.02 13.06
Cefalexin CEFA	10.0 15.0 20.0	8.13 12.78 16.90	1.22 2.01 2.26	81.3 85.2 84.5	15.01 15.73 13.37	19.25 18.48 16.30
Ceftiofur CEFT	10.0 15.0 20.0	8.22 12.05 16.11	1.45 2.18 2.48	82.2 80.3 80.6	17.64 18.09 15.39	22.11 22.08 17.66
Enrofloxacin ENRO	10.0 15.0 20.0	9.72 14.05 18.65	0.88 1.12 2.41	97.2 93.7 93.3	9.05 7.97 12.92	14.46 10.12 15.11
Ciprofloxacin CIPR	10.0 15.0 20.0	10.77 15.52 20.90	0.67 0.69 1.25	107.7 103.5 104.5	6.22 4.45 5.98	8.99 9.05 10.12
Oxytetracyclin OXTT	10.0 15.0 20.0	9.46 15.05 17.96	1.45 1.33 2.08	94.6 100.3 89.8	15.33 8.84 11.58	18.14 11.68 15.12
Sulfachloropyridazin SUPZ	10.0 15.0 20.0	10.67 16.07 19.07	1.41 2.28 3.04	106.7 107.1 95.35	13.21 14.19 15.94	16.18 20.08 18.81
Sulfadiazin SUDI	10.0 15.0	9.20 14.23	0.45 1.48	92.0 94.87	4.89 10.40	7.00 13.56

	20.0	20.18	2.03	100.9	10.06	11.12
Sulfadimetoxin SUDM	10.0	10.73	0.44	107.3	4.10	6.48
	15.0	15.21	0.92	101.4	6.05	12.21
	20.0	21.12	2.08	105.6	9.85	14.03
Sulfadimidin SULD	10.0	11.00	1.35	110.0	12.27	14.64
	15.0	15.56	1.78	103.7	11.43	14.92
	20.0	21.64	3.45	108.2	15.94	21.08
Sulfamethoxazol SULM	10.0	10.96	2.04	109.6	18.16	22.46
	15.0	15.79	2.95	105.3	18.68	20.51
	20.0	19.66	3.66	98.3	18.61	21.03
Carbofuran CRL	10.0	9.90	0.48	99.0	4.85	7.01
	15.0	16.02	0.75	106.8	4.68	8.12
	20.0	21.08	2.31	105.4	10.96	15.36
Carbaryl CRB	10.0	8.04	1.74	80.40	21.64	29.42
	15.0	13.80	2.04	92.00	14.78	19.58
	20.0	17.36	1.46	86.80	8.41	12.35
Paration PTN	10.0	8.20	1.35	82.00	16.46	21.02
	15.0	13.46	1.74	87.73	12.93	17.46
	20.0	18.20	2.07	91.00	11.37	16.58
Malation MTN	10.0	8.75	2.04	87.50	23.31	29.11
	15.0	12.64	2.51	82.27	19.86	23.46
	20.0	16.58	4.02	82.90	19.93	21.35
Diazinon DNN	10.0	9.94	1.36	99.4	13.68	17.46
	15.0	15.80	2.08	105.3	13.16	15.21
	20.0	19.10	2.41	95.5	12.62	17.88
Dimethoat DIM	10.0	10.64	1.46	106.4	13.72	16.99
	15.0	14.82	1.02	98.8	6.88	12.08
	20.0	19.43	3.12	97.18	16.06	19.35
Atrazine ATRZ	10.0	9.35	0.66	93.5	7.06	9.78
	15.0	14.47	0.88	94.47	6.08	9.65
	20.0	21.68	2.51	108.4	11.58	15.48
Permetrin PEMT	10.0	8.95	1.11	89.50	12.40	15.35
	15.0	14.88	1.45	99.20	9.74	10.18
	20.0	21.34	2.96	106.70	13.87	17.48
Cypermetrin CIRM	10.0	9.28	0.48	92.80	5.17	8.87
	15.0	13.00	1.92	86.67	14.77	21.23
	20.0	17.48	3.01	87.40	17.22	19.48
Deltametrin DELM	10.0	8.39	0.25	83.9	2.98	5.96
	15.0	14.00	0.61	93.3	4.36	8.11
	20.0	20.43	1.48	102.2	7.24	9.08
Coumaphos COU	10.0	9.23	0.14	92.30	1.52	3.99

	15.0 20.0	16.04 20.14	0.75 1.95	106.93 100.70	4.68 9.68	5.80 14.03
Dichlorophos DIRP	10.0 15.0 20.0	8.01 13.50 18.35	1.15 1.75 3.14	80.10 90.00 91.75	14.36 12.96 17.11	16.08 16.69 21.36
Chloropyrifos CHRS	10.0 15.0 20.0	10.08 14.86 21.80	1.12 2.35 2.78	100.8 99.1 109.0	11.11 15.81 12.75	16.22 22.08 14.35
Fenvalerat FERT	10.0 15.0 20.0	9.23 13.51 18.64	0.21 2.04 3.22	92.30 90.07 93.20	2.28 15.09 17.27	6.02 16.33 22.18
Boskalid BOS	10.0 15.0 20.0	10.45 14.83 18.94	0.92 1.95 2.08	104.5 98.87 94.7	8.80 13.15 10.98	16.35 17.18 14.46
Fentoate FETE	10.0 15.0 20.0	8.88 14.41 17.56	0.65 2.13 3.07	88.80 96.07 87.80	7.32 14.78 17.48	10.56 18.68 22.95
Fention FEON	10.0 15.0 20.0	9.80 14.65 21.32	0.88 1.02 1.95	98.0 97.67 106.60	8.98 6.96 9.15	13.01 11.35 17.12
Monocrotophos MOCR	10.0 15.0 20.0	8.95 15.12 18.46	1.45 1.52 2.03	89.5 100.8 92.3	16.20 10.05 11.00	21.08 12.06 13.88
Malaoxon MAON	10.0 15.0 20.0	10.42 15.78 19.35	1.88 3.15 3.02	104.20 105.20 96.75	18.04 19.96 15.61	22.01 22.96 17.36
Methamidophos MEDF	10.0 15.0 20.0	8.12 12.99 17.84	1.25 2.08 2.14	81.20 86.60 89.20	15.39 16.01 12.00	18.48 20.02 16.11
Metacrifos MECF	10.0 15.0 20.0	9.35 14.48 21.04	0.88 1.36 1.22	93.50 96.53 105.20	9.41 9.39 5.80	12.36 14.08 8.01
Amitraz AMRZ	10.0 15.0 20.0	9.98 15.35 21.85	0.65 0.99 2.04	99.80 102.33 109.25	6.51 6.45 9.34	9.66 7.35 11.08
Omethoat OMAT	10.0 15.0 20.0	8.46 16.35 21.53	1.04 1.22 1.95	84.60 109.00 107.65	12.29 7.46 9.06	13.06 9.54 12.03
Vamidothion VAON	10.0 15.0 20.0	8.98 13.10 19.44	0.48 0.61 0.77	89.8 87.3 97.2	5.35 4.66 3.96	7.08 6.36 5.12

Phosmet FOST	10.0 15.0 20.0	9.24 14.61 17.46	1.23 1.35 3.04	92.40 97.40 87.30	13.31 9.24 17.41	15.64 10.66 21.88
Heptenophos HEPH	10.0 15.0 20.0	8.05 14.46 18.48	0.27 1.54 1.95	80.50 97.40 92.40	3.35 10.65 10.55	5.12 14.45 12.95
Bifenitrin BFNT	10.0 15.0 20.0	9.00 16.43 21.24	0.62 1.13 3.35	90.0 109.5 106.2	6.89 6.88 15.77	9.35 8.18 16.35
Methomyl MEML	10.0 15.0 20.0	9.54 14.23 22.35	1.25 1.17 3.06	95.4 94.9 111.8	13.10 8.22 13.69	15.64 17.82 16.38
Zearalenone ZEAN	10.0 15.0 20.0	10.34 15.58 21.38	0.22 0.95 1.36	103.4 103.9 97.2	2.13 6.10 6.36	3.56 8.81 11.25
Ochratoxin A OTAA	10.0 15.0 20.0	10.63 15.37 18.05	0.45 1.92 2.04	106.3 102.5 90.25	4.23 12.49 11.30	6.64 13.58 14.61

4. Discussion

According to Commission Decision 2002/657/EC for banned substances, thyreostats, anabolic hormones, lactones and β-agonists were selected one precursor ion and three product ions, while for other substances, antibiotics, pesticides and mycotoxins were selected one product and two precursor ions. The most abundant product ion was used for quantification, while the second product ion was used for confirmation.

Sample preparation is the critical step during the application of methods for simultaneous detection of different class of compounds from samples and the crucial steps in achieving the purifying effect and satisfactory recovery simultaneously are extraction procedure and clean up (Hajrulai-Musliu et al., 2021). The preparation of urine samples can be relatively convenient and easy in conditions when aqueous characteristic of urine is combined with LC-MS/MS as one of the advanced separation techniques. Urine is widely used to monitor the illegal use of growth-promoting agents and veterinary drugs, besides that these substances in the urine generally show high clearance rates (Stolker and Th Brinkman 2005; Stolker et al. 2007). The simplest methods for detection of pesticides in urine are direct injection of urine samples or dilute-and-shoot procedures but urinary salts or macromolecules cause major problems such as decrease of the instrument sensitivity, clogging on the injection syringe or clogging on the ESI probe. To avoid adverse effects and to achieve more efficiency are used solid phase extraction (SPE) and liquid-liquid extraction (LLE) for residues from veterinary drugs and contaminants extraction ([a]Kaufmann et al. 2008; [b]Kaufmann et al. 2011; Hu et al. 2005).

In this study four extraction protocols were tested. The optimal recoveries were obtained by SPE extraction with enzymatic hydrolysis. The results are comparable with Kellman et. all (2009) and Makarov et al., (2006) who conclude that the LLE is simpler and easier than SPE, but interferences from urine may remain in the extract and cause a serious matrix effect or lead to low extraction efficiency.

From the validation study can conclude that the results for R^2 showed good linearity for all compounds included in the study. The gained results for LOD, LOQ, CCα and CCβ showed that the method was sensitive, while from the results for recovery and precision can conclude that the analytical method demonstrated good accuracy and precision. The results are in agreement with the criteria described in 2002/657/EC and would be useful for multi-class and multi-residue screening of veterinary drugs, pesticides and mycotoxins in bovine urine.

4.1. Real sample analysis

In order to test the applicability of the developed method, the method was applied to the analysis of real bovine urine samples. A total of 65 local samples from bovine urine were collected and tested. According to gained results can conclude that residues of the target compounds weren't detected in bovine urine samples.

5. Conclusion

The method describes extraction, clean up, identification and quantification of 72 residues of veterinary drugs and other contaminants in bovine urine. In the method development were optimized MS/MS methods and extraction procedure, while in the validation study were evaluated linearity, LOD, LOQ, CCα, CCβ, accuracy and precision of the method. The gained results fulfill the performances prescribed in the Commission Decision 2002/657/EC. Consequently, the method could be used in routine analysis of bovine urine samples for simultaneous detection of veterinary drug residues and contaminants.

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